HONEYBEE-FLORAL INTERACTIONS BETWEEN INVASIVE AND NATIVE PLANTS: DNA BARCODING APPROACH

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CERTIFICATE

I certify that the thesis entitled "**Honeybee-floral interaction between invasive and native plants: DNA barcoding approach**" submitted to Mizoram University for the award of the degree of Doctor in Philosophy in Zoology by **Abinash Giri** a record of research work carried out during the period of 2021-2024 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this university or any other university or institution of higher learning.

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DELARATION

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I **ABINASH GIRI**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

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ABBREVIATIONS

ABBREVIATIONS	FULL FORM
µl	Microliter
µg	Microgram
mg	Milligram
mL	Millilitre
mM	Millimolar
AMOVA	Analysis of molecular variance
BLAST	Basic Local Alignment Search Tool
COI	Cytochrome Oxidase I
DCA	Detrended correspondence analysis
DNA	Deoxyribonucleic acid
MANOVA	Multivariate analysis of variance
NCBI	National Centre for Biotechnology Information
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
IVI	Important value index
Ha	Hectare
M	Meter
KM	Kilometre
°C	Degree Celsius
RPM	Revolutions per minute
nmol	Nanomole
µM	Micrometre
β	Beta
ANOVA	Analysis of Variance
%	Per cent
g	Gram
mAU	milli-Absorbance Units
mS/cm	milliSiemens per centimeter

mg QE/100g	milligrams of Quercetin Equivalents per 100 grams
mgGAE/100g	milligrams of Gallic Acid Equivalents per 100 grams
log CFU/g	logarithm of Colony-Forming Units per gram
mEq/kg	milliequivalents per kilogram

CHAPTER I

INTRODUCTION

1. Introduction

1.1. Honey

Honey has been essential to human nutrition and culture since ancient times, functioning as one of the earliest naturally occurring sweeteners for Homo sapiens. Since the Stone Age, humans and bees have resided together, with early humans exhibiting considerable interest in this precious resource (Crane, 1983). The search for and extraction of honey involved significant risk. Archaeological evidence, such as ancient rock art, demonstrates that early humans dangerously harvested honey from bees located high in cliffs or trees, reflecting the considerable value they placed on this delightful substance.

The medicinal use of honey is deeply embedded in human history. The first recorded reference to honey as a therapeutic agent appears in Sumerian literature dating from 2100 to 2000 BCE (Crane, 1975), which include honey in prescriptions for the treatment of wounds and various ailments. In this early period, honey became a vital element of ancient therapeutic practices across diverse societies. The ancient Egyptians, Greeks, and Romans used honey for its nutritional advantages and valued it for its medicinal properties. Honey functioned as a topical treatment for wounds, a solution for gastrointestinal ailments, and an ingredient in various religious and ceremonial practices (Jones, 2009; Crane, 1999; Allsop and Miller, 1996).

Throughout much of human history, honey has been the most readily available sweetener, obtained organically from bees with minimal processing. This status persisted until the advent of modern sugar production, which began to progressively supplant honey as the primary sweetener around 1800 CE (Crane, 1975; Bogdanov *et al*., 2008). Historically, honey was an essential component in the diets and medicinal practices of ancient civilisations, valued for its sweetness and purported health benefits. Its significance was further underscored by its symbolic role in various cultural and religious rites, where it was often associated with purity, healing, and prosperity. The persistent reverence for honey across history highlights its varied importance in human civilisation. It served as both sustenance and medicine, illustrating the deep connection between humanity and the natural environment. The

story of honey illustrates humanity's persistent effort to harness the natural environment for nourishment, health, and well-being, preceding the developments in modern agriculture and food processing that transformed the production and use of sweets. Honey is a naturally occurring biological product obtained from nectar and pollen, employed in diverse cultures throughout. The composition consists of roughly 20% water, alongside significant amounts of proteins, enzymes (including catalase, invertase, glucose oxidase, and phosphatases), amino acids, organic acids (notably acetic acid and glutamic acid), lipids, vitamins (such as pyridoxine, niacin, and ascorbic acid), volatile compounds, phenolic acids, flavonoids, carotenoid-like substances, and minerals. The composition of honey is mainly determined by its floral, geographic, and entomological origins; nonetheless, processing and numerous external factors, including seasonal and environmental conditions, significantly contribute as well (Crane and Walker, 1984; Blasa *et al*., 2006; Saxena *et al*., 2010; Khalil *et al*., 2012).

1.2 Honey bee

Honey bees are very diverse insects categorised within the order Hymenoptera, which include ants, wasps, and numerous other bee species. Considering the estimated 100,000 unique species of bees, their evolutionary adaptability and ecological significance are critical. Bees are primarily categorised into two main types: solitary and social species. While honey bees (*Apis* species), distinguished by their highly formed colonies, are the most recognised members of the social group, more than 90% of bee species are solitary. These solitary animals operate independently from their social counterparts, demonstrating autonomous behaviours that ensure the survival of future generations without the cooperative framework of a colony.

There are over twenty-five thousand species of solitary bees globally, each exhibiting unique reproductive and survival strategies. Unlike social bees that collectively construct communal hives and rear their offspring, solitary bees bear full responsibility for their reproduction and nest construction. Female solitary bees independently create nesting sites by excavating tunnels in soil, wood, or plant stems.

These nests serve as breeding chambers for egg deposition and are provisioned with pollen and nectar, carefully gathered by the female to sustain the larvae after hatching.

The reproductive process of solitary bees is both novel and efficient. The female deposits an egg in each chamber of the tunnel, along with a supply of pollen and nectar to nourish the developing larva. The tunnel is subsequently sealed to protect the offspring until they are ready to emerge as mature bees. This reproductive strategy ensures species survival while highlighting the individual effort of these bees, in sharp contrast to the cooperative activities seen in honeybee colonies.

Solitary bees are essential pollinators and play a vital role in ecosystems by promoting the reproduction of flowering plants. Their adaptability to diverse environments, evidenced by their ability to nest in various habitats such as the ground, hollow stems, or decaying wood, illustrates their resilience and evolutionary success. Their autonomy and adaptability allow solitary bees to thrive in various situations, highlighting their ecological importance.

The resourcefulness and adaptability of these bees in establishing a safe environment for their progeny highlight their remarkable evolutionary strategies. They exhibit creativity and autonomy, ensuring the survival of future generations without reliance on communal structures. Their solitary existence contrasts sharply with the social behaviour of species like the honey bee; however, both types of bees are crucial for maintaining biodiversity and bolstering the health of ecosystems worldwide (Abrol, 1997).

Contrary to other bee species that are found on all continents, Apis bees were historically restricted to the Old World, encompassing Asia, Africa, and Europe. This geographical limitation may clarify why the Apis genus emerged after many other bee species. Over time, the genus evolved in these locations, leading to distinct species adapted to varying environmental conditions. The genus Apis has four primary species: *Apis florea*, the small honey bee; *Apis dorsata*, the gigantic honey bee; *Apis cerana*, the eastern honey bee; and *Apis mellifera*, the western honey bee.

Each species exhibits unique behavioural and ecological characteristics, although they all share the common traits of honey production and wax secretion.

The geographical distribution of *Apis* bees demonstrates continuous regional specialisation. Researchers have identified five distinct bee species in the complex ecology of Tanhril: *Apis cerana*, *Apis florea*, *Apis dorsata*, *Amegilla cingulata*, and *Bombus huntii*. This demonstrates the biological richness of the region and its ability to support many bee species. *Apis mellifera*, the western honey bee native to Europe, is notably absent from this environment. The species' disappearance in Tanhril may be ascribed to climatic and environmental fluctuations or to its specific adaptations to European habitats. The presence of many *Apis* species in Tanhril highlights the adaptability and evolutionary triumph of the genus in its historically developed habitats.

The genus *Apis* is important both biologically and as the exclusive group within the tribe Apini, known for its honey bees. These bees are distinguished by their complex social structures and inventive behaviours, enabling them to thrive in many settings. Honey bees are eusocial insects, distinguished by their colony structure and a strict division of labour between reproductive and non-reproductive individuals. This division facilitates the creation and maintenance of large, intricate nests made solely of wax, a material secreted by worker bees. These wax nests have multiple purposes: they provide shelter, store food, and house the brood (eggs, larvae, and pupae) that will eventually mature into the next generation of bees.

The ability of bees to produce and store honey, a concentrated nectar converted into a sugary liquid, is a notable trait that underscores their ecological importance. Honey is a vital energy source for bees, especially during food scarcity such as winter, and has been employed by humans for millennia as a natural sweetener, medicinal remedy, and preservative. The prolific production of honey and wax is facilitated by the social organisation of honey bee colonies, in which thousands of bees cooperate to gather nectar, produce honey, and sustain the colony.

The social and behavioural traits of honey bees make them vital to their ecosystems. Their pollination activities promote the reproduction of flowering plants,

improving the health and sustainability of diverse ecosystems. This promotes biodiversity and the stability of trophic networks. The impact of honey bees extends beyond natural ecosystems, as their role in pollinating agricultural crops makes them essential for global food production. The intricate relationship between honey bees and their environment exemplifies the delicate balance sustaining ecological systems, underscoring the imperative to safeguard and preserve these vital pollinators in the face of environmental challenges such as habitat destruction, climate change, and pesticide use (Kohli, 1958).

Thus, the *Apis* genus, noted for its great diversity and specialised activities, represents the evolutionary success of honey bees. Their ability to adapt to various habitats and form complex social structures has allowed them to thrive across continents and play a vital part in both natural ecosystems and human agricultural practices.

A. cerana, referred to as the Asian cavity-nesting honey bee, has been extensively dispersed across temperate and tropical regions of Asia for almost two thousand years. It is referred to by various names, such as Asian honey bee, Asiatic bee, Indian honey bee, Chinese bee, Eastern honey bee, and Fly Bee (Ruttner, 1988; Crane, 1995). This species serves as a crucial pollinator within its indigenous habitat and has co-evolved alongside other Asian plant species. *A. cerana* is classified within the Kingdom Animalia, Phylum Arthropoda, Class Insecta, Order Hymenoptera, Family Apidae, Genus *Apis*, and Species *A. cerana* (Winston, 1991).

The geographical distribution of *A. cerana* is widespread, encompassing a broad expanse of Asia. It spans from Afghanistan in the west to Japan in the east, extending north to the foothills of the Himalayas and south to Indonesia (Crane, 1999; Hepburn and Radloff, 2011; Ruttner, 2013). This distribution underscores the species' exceptional resilience to diverse climatic conditions, encompassing tropical forests, rainforests, wet deciduous forests, tropical savannahs, grasslands, steppes, coniferous forests, and taigas (Radloff *et al*., 2010). The species' capacity to flourish in varied habitats exemplifies its evolutionary success, enabling it to fulfil a vital function in both natural and agricultural ecosystems across its distribution.

A. cerana is characterised by its unique nesting behaviour. Similar to other species within the Apis genus, *A. cerana* constructs its nests in cavities, providing the colony with protection against predators and environmental extremes. Nests are typically located in tree hollows, rock crevices, caves, and home cavities in urban environments (Oldroyd and Wongsiri, 2009; Koetz, 2013). The cavity-nesting behaviour of *A. cerana* differs from that of species such as *Apis dorsata* and *Apis florea*, which construct exposed nests. The selection of cavities enables *A. cerana* colonies to more effectively regulate temperature and humidity within the hive, which is essential for brood growth and honey production.

Swarming is the principal reproductive method for *A. cerana*, especially in natural environments. Swarming is a natural phenomenon that enables colonies to multiply by dividing into smaller factions, wherein a segment of the bees departs from the original colony with a queen to form a new nest. In optimal conditions, *A. cerana* colonies can generate as many as ten swarms each year, markedly surpassing the swarming frequencies of other *Apis species* (Koetz, 2013). The elevated rate of swarming enhances the species' resilience and facilitates population expansion across many settings.

Multiple variables can induce swarming in *A. cerana*. These encompass reproductive requirements, nest disruptions, insect invasions, illness challenges, and deficiencies in pollen or nectar (Koetz, 2013; Egelie *et al*., 2015). Colonies may swarm due to congestion in the nest or as a natural reaction to seasonal reproductive cycles. Moreover, environmental stressors, such a reduction in available food or the presence of parasites such as the *Varroa destructor* mite, might provoke swarming behaviour as a survival strategy. Notwithstanding the risks linked to swarming, including the possible collapse of the new colony, swarming continues to be a crucial evolutionary strategy that has enabled *A. cerana* to sustain its populations for millennia.

Furthermore, *A. cerana* displays behaviours that distinguish it from other *Apis* species and augment its survival in many habitats. One such behaviour is its capacity to protect itself from predators and parasites. *A. cerana* has evolved distinct

defence strategies against the giant hornet (*Vespa mandarinia*), a significant predator. *A. cerana* bees can eliminate the hornet by creating dense defensive clusters and generating heat through forceful wing movements, employing hyperthermia, a tactic rarely seen in other bee species.

Moreover, *A. cerana* exhibits a certain level of tolerance to the *Varroa destructor* mite, a parasite that has ravaged *Apis mellifera* populations in many regions globally. Although *Varroa* mites can still impact *A. cerana* colonies, this species has developed grooming behaviours and other adaptive measures that enable it to control mite infestations more efficiently than its European equivalent. This resistance underscores the evolutionary benefits that *A. cerana* has acquired in reaction to environmental stresses.

A. cerana is a highly adaptable and generalist bee species that, similar to other members of the genus Apis, forages on a diverse array of flowering plants for nectar and pollen. This generalist foraging behaviour enables *A. cerana* to engage with a wide variety of plant species, encompassing both wild flora and developed crops. *A. cerana* plays a significant ecological role in pollination by facilitating the reproductive success of several plants by the passage of pollen between flowers, therefore enabling fertilisation (Winston, 1987). Pollination is a crucial environmental service rendered by bees, with *A. cerana* being essential for the maintenance of biodiversity and agricultural output. Their pollination services are essential in both natural ecosystems and human-managed environments, such as agriculture, where the health and productivity of numerous crops rely on effective pollinators like honey bees.

A. cerana plays a vital role in pollinating numerous crops in agricultural environments, encompassing fruits, vegetables, and nuts. This has significant ramifications for food security and the livelihoods of farmers, especially in Asian locations where *A. cerana* is indigenous and plays a pivotal role in pollination. Crops include apples, almonds, and citrus fruits derive substantial advantages from the pollination services provided by *A. cerana*, which can markedly improve crop yields. Furthermore, due to *A. cerana*'s superior adaptation to its local environment, it

frequently outperforms non-native species such as *A. mellifera* as a pollinator in Asian settings.

A. cerana possesses considerable commercial potential for apiculture, with its ecological significance. This species is extensively maintained by both small-scale and commercial beekeepers across many regions of Asia, due to its inherent resilience to local diseases and environmental challenges, rendering it a desirable choice for honey production and other apiculture-related endeavours (Theisen-Jones and Bienefeld, 2016). Although *A. cerana* yields less honey per colony than *A. mellifera*, the western honey bee, it possesses numerous advantages that facilitate management, especially for beekeepers in developing areas.

The honey output of *A. cerana* is inferior to that of *A. mellifera*; however, this is compensated by the species' reduced management demands and its adaptability to various environments. In numerous instances, reduced honey production is counterbalanced by simplified maintenance and a diminished occurrence of colony losses attributable to illness or environmental stressors. Moreover, *A. cerana* frequently exhibits superior foraging efficiency in its indigenous habitats, where it has co-evolved with the native flora. This renders it a more sustainable and ecologically integrated option for apiculture in numerous regions of Asia (Theisen-Jones and Bienefeld, 2016).

A. cerana's exceptional resilience to external climatic fluctuations is mostly due to its capacity to flourish in particular habitats marked by intricate topography, varied climates, and abundant floral diversity. These settings create diverse ecological niches for *A. cerana*, facilitating the development and preservation of genetic adaptations that enhance resilience to environmental shocks. The ecological versatility of *A. cerana* is a primary factor contributing to its status as a crucial pollinator species in numerous regions, especially in its native Asia. Its capacity to pollinate a diverse array of flowering plants, both indigenous and cultivated, is essential for maintaining ecosystems and enhancing agricultural productivity.

A. cerana serves as a crucial pollinator, impacting both natural ecosystems and the agricultural economy of the region. Their pollination services are essential

for the reproduction of various flowering crops and plants, like as fruit trees, vegetables, and medicinal plants. Numerous crops hold significant economic value for local communities, especially small-scale farmers who depend on bee pollination to enhance their harvests. The ecological advantages conferred by *A. cerana* also contribute to the preservation of wild plant species' health and diversity, which subsequently sustains the survival of numerous animal species reliant on these plants for sustenance and habitat.

Studies have shown that *A. cerana* exhibits significant genetic plasticity, especially for its functional genes, allowing it to adjust to environmental changes (Evans *et al*., 2013). This genetic adaptability enables the species to address diverse environmental problems, including climate change, habitat fragmentation, and the emergence of invasive species. The genomic malleability of *A. cerana* is considered a contributing element to its efficacy as a pollinator in varied and dynamic ecosystems. Research indicates that *A. cerana* populations display considerable variability in characteristics such as foraging behaviour, thermoregulation, and disease resistance, all of which are crucial for survival under changing environmental conditions.

The exceptional biological variety of *Apis cerana*, along with its capacity for dispersal across multiple locations, renders it an exemplary model organism for phylogeographic and speciation research, especially in the biodiverse environments of Northeast India. The area is recognised for its intricate ecological zones, spanning from lowland tropical forests to high-altitude temperate ecosystems. These varied environments establish a natural laboratory for examining the evolution and diversification of *A. cerana* populations across time. Phylogeographic investigations of *A. cerana* have yielded significant insights into the evolutionary history of honey bees, elucidating the adaptation of various populations to their local surroundings and the genetic diversity that has transpired across geographical barriers (Koetz, 2013).

A. cerana is distinguished as a species of significant ecological, economic, and scientific value. Its capacity to acclimatise to diverse environmental

circumstances, along with its essential function in pollination, renders it a keystone species in the ecosystems and agricultural systems of Asia. The continuous examination of *A. cerana*'s genetic variety and adaptation enriches our comprehension of honey bee biology and offers critical insights for the conservation and sustainable management of pollinator species amid global environmental changes. As pressures on pollinator populations escalate, the resilience of species such as *A. cerana* provides optimism for preserving the biodiversity and ecological services crucial for both natural ecosystems and human welfare (Koetz, 2013).

Pollinators are essential for sustaining ecosystem health and facilitating global food production. They facilitate the transmission of pollen between flowering plants, enabling fertilisation and the generation of fruits and seeds. The complex interdependence between pollinators and plants is essential for agricultural products and the survival of several wild plant species, many of which depend on particular pollinators for reproduction (Koetz, 2013). Bees, butterflies, birds, and bats are prominent pollinators, each playing distinct roles in the pollination process.

The reduction in pollinator populations presents a substantial risk to biodiversity and environmental stability. The decline of pollinators can result in the failure of plants reliant on them for reproduction to produce seeds or fruits, triggering a series of repercussions within the ecosystem. Various species, ranging from herbivores to predators, depend on these plants either directly for sustenance or indirectly via the food web. The decline of pollinators can result in diminished plant diversity, therefore impacting entire ecosystems and potentially upsetting ecological processes and the equilibrium of life forms reliant on these plants for survival (Rath, 1999).

The loss of pollinators is a direct risk to human food security. Approximately 75% of world food crops rely to differing extents on animal pollinators. These comprise fruits, vegetables, nuts, seeds, and oilseeds, which are vital for a balanced human diet. In the absence of pollinators, agricultural yields would significantly diminish, resulting in diminished availability of healthy foods and potentially escalating the prices of these goods. This may have far-reaching implications,

particularly for at-risk populations in developing nations who are already experiencing food shortages (Rath, 1999).

Multiple causes contribute to the concerning decrease in pollinator populations, including habitat destruction, pesticide application, climate change, and the proliferation of illnesses. Habitat loss is arguably the foremost cause of pollinator decline. As human activities proliferate, natural habitats like woods, meadows, and wetlands are transformed into agricultural fields, urban developments, and infrastructure, resulting in diminished favourable settings for pollinators to flourish. Numerous pollinators necessitate certain habitats for nesting, foraging, and reproduction, and the obliteration of these environments can result in both local and worldwide population decreases. The utilisation of pesticides, especially the extensive usage of neonicotinoids, has been associated with decreases in pollinator populations. These pollutants may adversely affect pollinators, even at sublethal concentrations. Exposure to chemicals can hinder the navigation, foraging, and reproductive capabilities of bees and other pollinators, ultimately resulting in population decreases. The enduring presence of these compounds in the environment presents an ongoing risk to pollinators, as they may taint the nectar and pollen of flowering plants (Rath, 1999).

Climate change intensifies the reduction of pollinator populations by modifying the timing of flowering and nutrient availability. Numerous pollinators have adapted to align their life cycles with the blooming phases of the flora they fertilise. Nonetheless, climate change is rapidly disrupting this synchronisation due to alterations in temperature and precipitation patterns. Flora may flower earlier or later than customary, while pollinators may appear during periods of food scarcity, resulting in reductions in both plant and pollinator numbers (Rath, 1999).

A diversified approach is necessary to tackle these difficulties and reverse the decline of pollinators. Safeguarding and rehabilitating natural habitats is crucial for supplying pollinators with the necessary materials for their survival and prosperity. This can be accomplished by creating pollinator-friendly habitats in agricultural environments, including hedgerows, wildflower strips, and cover crops. Minimising

pesticide application and implementing integrated pest control strategies can reduce pollinators' exposure to detrimental chemicals (Rath, 1999). Moreover, initiatives to alleviate climate change and preserve pollinator species via breeding programs and disease management are essential for guaranteeing the enduring survival of these critical organisms (Koetz, 2013).

Moreover, public awareness and policy measures are essential for advancing pollinator protection. Governments, scientists, and conservation organisations must collaborate to establish regulations that save pollinator habitats, restrict pesticide application, and promote sustainable farming practices. The use of pollinator-friendly agricultural practices, including organic farming and agroforestry, can foster environments that enhance both biodiversity and agricultural yield (Koetz, 2013).

In Northeast India, where *A. cerana* is prevalent, researchers have identified considerable genetic variation among various populations, indicative of the region's biological richness. This genetic variety is crucial for comprehending the mechanics of speciation and the influence of environmental conditions on the evolutionary paths of honey bee species. The utilisation of *A. cerana* as a model organism in these studies has ramifications for conservation biology, since it aids in identifying essential genetic features that enhance the species' resilience and adaptation. These findings can be utilised for the conservation and control of honey bee populations against global problems including habitat loss and climate change (Takahashi *et al*., 2007).

Populations of *A. cerana* are geographically separated by natural barriers including mountains, forests, rivers, and seas. This geographic isolation facilitates the evolution of distinct local ecotypes and subspecies. Honey bee ecotypes and subspecies can be differentiated by morphometric and genomic techniques. Morphometric and molecular genetic techniques have been employed to delineate subspecies of the European honey bee *A. cerana* (Evans *et al*., 2013). Research on *A. cerana* also use these strategies. Polymorphisms in two specific regions are frequently utilised to examine genetic variations among the subspecies of *A. cerana*: the microsatellite loci in nuclear DNA and the mitochondrial marker located between

the transfer RNA Leu and cytochrome oxidase subunit 2 genes (tRNAleu COII) (Tan *et al*., 2007). Lee *et al*., (2016) assessed the genetic variation of the *A. cerana* population in Korea by tRNAleu COII locus polymorphism analysis. Xu *et al*., (2013) found that the *A. cerana* population on Damen Island significantly differentiates from mainland populations based on the analysis of five microsatellite loci and mitochondrial tRNAleu COII sequences. A study by Yu *et al*. (2019) indicates that the elongated and constricted alpine valleys of the Qinghai Tibetan Plateau display genetic diversity of *A. cerana*. This was achieved by analysing mitochondrial tRNAleu COII fragments and 31 microsatellite sites. Additionally, *A. cerana* has undergone genome-wide analysis in recent investigations (Koetz, 2013).

1.3 Invasive and native plants

Environmental change is mostly driven by species invasions and climate change, affecting ecosystems globally. Rising temperatures, altered precipitation patterns, and extreme weather disrupt natural equilibrium, complicating species' adaptation efforts. Human activity facilitates the displacement of native species by invading species, disrupts trophic balances, and modifies the terrain of a region. The dynamic interplay between these forces may yield synergistic or antagonistic effects, either amplifying or diminishing their impacts. Understanding these interactions is crucial for predicting and mitigating environmental impacts (Hoover *et al*., 2012; Fox *et al*., 2014). Climate change is affecting the reproductive cycles of native plants, potentially disrupting the fragile ecological equilibrium between these plants and their pollinators. The complex interplay between indigenous flora and their pollinators may be disturbed by the various adaptations of invasive species to climate change. This may impact the ecosystem overall and necessitate a deeper comprehension of how temperature fluctuations influence plant reproductive dynamics (Giejsztowt *et al*., 2020).

An introduced species that inflicts harm on its new environment is classified as an invasive species. Invasive species cause ecological, environmental, and economical damage to habitats and bioregions (Davis and Thompson, 2000). The invasion of alien plant species poses a significant threat to biodiversity, potentially

altering ecosystems and economic services, hence jeopardising livelihoods and instigating essential social changes. The impact of invasive alien plant species (IAPS) on natural ecosystems can lead to competition with native species, limiting resource availability, disrupting ecosystem equilibrium, and adversely affecting agricultural productivity and human health (Vilà and Hulme, 2017).

The threat level of invasion to biodiversity may differ based on the distinct characteristics of each location; nonetheless, the correlation between invasion and other environmental disturbances, such as habitat loss and climate change, is undeniable (Young and Larson 2011). According to the United Nations' Global Assessment Report on Biodiversity and Ecosystem Services, the principal danger to global biodiversity loss is biotic invasion (IPBES 2019). The unintentional or deliberate introduction of Invasive Alien Plant Species (IAPS) as ornamental or commercial flora via human intervention can adversely impact the environment, economy, livelihoods, human health, and the diminishment of ecosystem services, thereby obstructing sustainable development (Rai and Singh 2020a, b). The IAPS adversely impacts the United Nations' Sustainable Development Goals (SDGs) pertaining to human well-being and environmental enhancement by establishing policies or guidelines across various sectors, including sustainable agriculture, groundwater remediation, and public health (United Nations 2015; Haines 2016).

In recent decades, invasive alien plant species (IAPS) have significantly jeopardised the region's ecosystem services, biodiversity, environmental quality (Aerts *et al*., 2018; Stone *et al*., 2018; Jones, 2019), and public health (Pyšek and Richardson, 2010; Jones and McDermott, 2018). The Intergovernmental Platform for Biodiversity and Ecosystem Services (IPBES) of the United Nations (UN) estimated that biological invaders threaten around 25% of the Earth's surface, including global biodiversity hotspots (IPBES, 2019). In this context, high-income nations recorded 30 times higher IAPS than low-income nations (Seebens *et al*., 2018). As a result, IAPS hotspots are generally confined to high-income nations within the European Union, Australasia, and North America, rather than the Asia Pacific or African regions (Seebens *et al*., 2018; IPBES, 2019).

Future alterations in biodiversity are primarily influenced by land-use changes, temperature variations, nitrogen deposition, and biotic interactions during the Anthropocene epoch (Sala *et al*., 2000; Singh 2012). Biotic exchange, defined as the introduction of non-native plant species, is considered a significant threat to human health, agricultural sustainability, biodiversity, and the economy (Rai and Singh 2020a, b). Invasive alien plants (IAPS) have proliferated as a result of human-induced disturbances, a trend not confined to the pristine or vulnerable ecosystems of Antarctica or the Galapagos Islands (Hughes and Convey 2010; Jager *et al*., 2009). It is estimated that 0.5–0.7% of trees and shrubs globally are presently invasive (Rejmanek and Richardson 2013; Lamsal *et al*., 2018). Several deleterious invasive alien plants has the capacity to disrupt the abiotic environment and ecosystem services, potentially triggering a cascade of adverse impacts and hastening the extinction of indigenous flora (Lamsal *et al*., 2018).

Protected forest areas in Mizoram and other Indian Himalayan regions are highly vulnerable to significant alien plant invasions due to considerable human-induced disturbances. Recently, 163 alien plant species have been reported by the state. This encompasses neo-invasives such as Ocimum americanum, Hyptis suaveolens, and Bidens pilosa, alongside harmful invasive alien plant species (IAPS) including Ageratina adenophora, Ageratina riparia, Chrolaena odorata, Mikania micrantha, and Ageratum conyzoides, all of which exert a persistent ecological influence on native flora and ecosystems (Sengupta and Dash, 2020). Mizoram, a secluded state in northeastern India, lacks an effective system for managing plant invasions. Consequently, the imperative for an effective and sustainable management strategy to prevent the incursion of invasive plant species is critical. The primary attribute of alien plants is their ability to proliferate in uncharted or pristine regions (Raghubanshi *et al*., 2005; Rai and Singh, 2020b). The majority of invasive plant species originate from South America. Out of 374,000 known plant species, 6,075 have been designated as invasive globally (Christenhusz and Byng, 2016). One hundred seventy-three plant species, constituting 1% of the Indian flora, are classified as foreign invaders (Reddy, 2008; Rai and Singh 2021). Research on invasive plant species exists, although it is limited and predominantly focused on

aggressive plant invaders, such as Lantana camara and Parthenium hysterophorus (Hiremath and Sundaram 2013).

Indigenous flora play a significant role in carbon conservation and can function as sinks for atmospheric pollutants (Pejchar and Mooney, 2009; Shackleton *et al*., 2019). Consequently, alterations in environmental quality may indirectly affect human health due to the decline of native plant diversity resulting from invasive plant infestations (Jones and McDermott, 2018). Research indicates that certain IAPS can serve as ecological indicators of environmental pollution (Rai, 2016). The invasive emerald ash borer (EAB), a plant pathogen, proliferated across the United States and significantly devastated predominant ash trees, which would have otherwise functioned as effective sinks for air pollutants (Jones and McDermott, 2018). Human populations had cardiovascular and pulmonary complications due to exposure to high levels of hazardous air pollution. Severe pollution stress results in economic loss and mortality (Jones and McDermott, 2018).

The success of the IAPS is affected by numerous biological and environmental factors. The connection among plant invasions, anthropogenic disturbances, climate change, biodiversity, and human health may be complex and intricate (Rai and Kim, 2019). Consequently, invasion ecology has increasingly been recognised as a transdisciplinary domain intricately linked to land-use alteration, global change biology, health sciences, restoration, and conservation biology (Pysˇek and Richardson, 2010; Heshmati *et al*., 2019). Invasive weeds proliferate and produce biomass at a faster pace than native plants. They exhibit significant competitiveness, high reproductive efficiency through prolific seed production, effective dispersal, vegetative reproduction, rapid establishment, and other traits that facilitate adaptation to novel habitats (Sharma *et al*., 2005; Simberloff *et al*., 2005). Invasive exotic plants are associated with the decrease of threatened and endangered species because to their significant numbers and biomass, which transform vegetative structures, alter ecological processes, and displace native species (Denslow, 2007).

The rapid proliferation of vector-borne diseases intensifies the impact of the invasion on human health (Clow *et al*., 2017; Schindler *et al*., 2018). IAPS tend to

impede forest diversity and function as invasive species, hence diminishing global agricultural productivity (Haines, 2016). The Sustainable Development Goals (SDGs), which encompass issues such as sustainable agriculture, water sanitation, food safety and security, poverty, and human health and well-being, have been negatively impacted by the concurrent effects of current environmental disturbances associated with invasion biology (Haines, 2016; Pysˇek and Richardson, 2010). The concept of "planetary health" arises from the global nature of environmental issues and their detrimental effects on public health, including hazardous compounds, allergies, and vectors of emerging diseases (Haines, 2016; Ebi *et al*., 2017).

The global proliferation of Invasive Alien Plant Species (IAPS), particularly in disturbed environments such as landfills and dumps—potential epicentres for these species—can significantly jeopardise human health due to their toxins and pollen (Plaza *et al*., 2018). Global biotic invasions are a significant priority on the Convention on Biological Diversity's (CBD) agenda for mitigating the impacts of invasive alien plant species (IAPS) on ecosystems and public health. The Cartagena Protocol and biosafety underscore this assertion (Pysˇek and Richardson, 2010). The Rio de Janeiro 1992 Earth Summit has further identified IAPS in forestry and agroforestry systems as a key contributor to worldwide environmental degradation, biodiversity loss, and adverse effects on human health. IPBES (2019) particularly identified the threats that Invasive Alien Plant Species (IAPS) bring to ecosystem services, human health, livelihoods, and global biodiversity in its deliverable 3(b)(ii). The establishment of a database of naturalised species will not only serve as the foundational step in the advancement of invasion biology but will also provide more comprehensive research on the biology and impacts of particular species (Wu *et al*., 2004).

Ecosystems are influenced by intricate connections between species invasions and climate change. The phenology and reproductive behaviours of indigenous plant species are affected by these elements alongside plant invasion. This research aims to find molecular pathways that elucidate how these determinants influence biodiversity and environmental equilibrium. The use of an integrative strategy provides valuable

insights for effective conservation and management practices (Giejsztowt *et al*., 2020).

1.4 Important value index (IVI)

The development of ecosystems is influenced by plant variety and is contingent upon the relative abundance and species composition of the ecosystem. In addition to enhancing the aesthetic and biological diversity of any given location, it preserves the fragile balance of the biosphere on Earth. When biodiversity is present, ecosystems are more resilient and long-lasting because it influences processes like pollination, pest control, and nutrient cycling. Monitoring changes in biodiversity can help us better understand environmental pressures such as pollution, habitat loss, and climate change. Because of this, maintaining biodiversity is essential to the wellbeing of the planet and the interdependence of all life on Earth (Solbrig, 1991; Rawat and Chandra, 2014).

Comprehending the composition and structure of plant communities is essential to ecological studies. The characterisation of these communities entails identifying species as well as assessing their roles, dominance, and relative significance within an ecosystem. The Important Value Index (IVI) is a crucial metric for evaluating the ecological significance of individual species within plant communities, as it consolidates various dimensions of a species' ecological function into a singular, measurable index. This metric is extensively utilised in ecological research, especially in investigations of plant ecology, community organisation, biodiversity, and conservation biology (Curtis and McIntosh, 1950; Christopher, 2020).

The IVI provides an extensive instrument for ecologists to ascertain species dominance within a community, elucidate species-environment interactions, and evaluate the overall ecosystem health. This introduction explores the conceptual basis, computation, and ecological importance of IVI, establishing a framework for its use in diverse research settings. The significance of IVI in biodiversity assessment, conservation planning, and ecosystem management is examined,

underscoring its relevance in modern ecological research (Curtis and McIntosh, 1950; Solbrig, 1991; Rawat and Chandra, 2014).

The Importance Value Index (IVI) is a tool used to quantify a species' ecological relevance within a particular environment. When key measures like relative dominance, relative frequency, and relative density are combined, a numerical representation of the species' environmental contribution is obtained. To facilitate informed decision-making on conservation and management, the IVI gauges a species' overall significance within its community. This supports biodiversity and ecosystem health. It clarifies the intricate connections between different species and their roles in the ecosystem (Phillips, 1959).

1.4.1 Historical Context and Evolution of Important value index

The Important Value Index was initially presented by Curtis and McIntosh in 1950 during their research on plant communities in Wisconsin, USA. Their objective was to develop a method capable of statistically evaluating the relative significance of plant species within a certain environment. Historically, the majority of ecological studies concentrated on species richness or diversity indices, such as Shannon or Simpson's diversity index, which offered a limited perspective on community structure by emphasising species quantity or their evenness (Magurran, 2004). Although these measurements were informative, they did not comprehensively consider the ecological impact or predominance of particular species within a community. The IVI was created to overcome this constraint by incorporating three essential parameters: relative density, relative frequency, and relative dominance (Curtis and McIntosh, 1950).

The invention of the IVI by Curtis and McIntosh represented a pivotal moment in plant ecology, offering a more comprehensive perspective on plant communities. Their research enabled ecologists to identify the species existing in an ecosystem and comprehend their relative significance regarding biomass, distribution, and ecological impact. The IVI has evolved into a standard instrument in plant ecology, commonly employed in forest inventories, biodiversity evaluations, and ecological succession research (Odum, 1971).

1.4.2 Conceptual Framework of IVI

The Important Value Index is a composite metric that integrates three distinct measures of species significance: relative density, relative frequency, and relative dominance. Each component provides a distinct viewpoint on a species' interaction with its environment and its role within a community. Relative Density denotes the ratio of members of a species to the overall population within the community. This metric offers insight into the prevalence or abundance of a species within the community. A high relative density signifies that a species is numerically predominant. Relative Frequency quantifies the distribution of a species across sampling plots or its prevalence within the community. A species exhibiting a high relative frequency is prevalent in numerous regions and may signify enhanced adaptability or competitive advantage across diverse microhabitats. Relative Dominance quantifies the extent of space a species occupies, typically represented by biomass, basal area (for trees), or canopy cover. Species that are huge or possess substantial biomass may exert a more pronounced ecological impact, even if they are less prevalent. This measure is especially pertinent in forest ecosystems where huge trees predominate in spatial occupation, light interception, and water utilisation. The three factors are computed separately and subsequently integrated to get the IVI. The IVI is mathematically determined by summing relative density, relative frequency, and relative dominance, represented as a percentage. All three components contribute equally to the final IVI number, which spans from 0 to 300. The formula for computing IVI is as follows. This straightforward yet potent method facilitates effortless comparisons among species within the same community and across diverse environments.

1.4.3 Ecological Importance of IVI

The IVI is an essential instrument in plant community ecology as it offers a thorough assessment of a species' ecological status. In contrast to other indexes that may solely emphasise species richness or variety, the Importance Value Index (IVI) considers both the abundance and the impact of species within a community. This is especially significant in ecosystems where a limited number of species predominate

in biomass or coverage, although a greater variety of species may enhance overall diversity (Rawat and Chandra, 2014).

In ecosystems like tropical rainforests or savannas, where some species frequently dominate, the Importance Value Index (IVI) aids ecologists in identifying keystone species within the ecosystem. Although some species may not consistently be the most prevalent, their considerable size or extensive distribution can render them disproportionately significant to ecosystem functionality (Begon *et al*., 2006). Large canopy trees in a tropical forest significantly contribute to the forest's biomass, affect light availability for understory species, and are essential for nutrient cycling (Solbrig, 1991; Rawat and Chandra, 2014).

The IVI is crucial for comprehending successional dynamics within ecosystems. Ecological succession entails temporal alterations in species composition and dominance. Early-successional species, characterised by rapid growth and a preference for light, may initially prevail in relative density and frequency. As the ecosystem ages, shade-tolerant, slow-growing species may increasingly dominate in biomass and relative prevalence. Monitoring IVI throughout time enables ecologists to understand the successional trajectories of ecosystems and the evolving roles of species throughout this process (Connell and Slatyer, 1977).

1.4.4 Utilisation of IVI in Ecological Research

The IVI has diverse uses in ecological research, encompassing biodiversity assessment, conservation planning, ecosystem management, and restoration. Notable applications encompass: Biodiversity Evaluation: The IVI is frequently employed to evaluate the biodiversity of plant communities in both natural and damaged habitats. Through the calculation of the IVI for various species, ecologists can ascertain which species are most critical for ecosystem functionality. This information is essential for prioritising conservation initiatives, especially in biodiversity hotspots or regions experiencing fast environmental change (Magurran, 2004). Conservation Planning: In conservation biology, the Importance Value Index (IVI) is utilised to identify keystone species that exert disproportionately significant influences on ecosystem

functionality. Conservationists frequently prioritise the protection of these species, as their extinction may result in cascade repercussions within the ecosystem (Noss *et al*., 1999). The IVI can assist in identifying rare or endangered species that, while not predominant in relative density, are crucial for sustaining ecological resilience.

Ecosystem Management and Restoration: In ecosystem management, the IVI serves to direct restoration initiatives by determining which species warrant prioritisation for reintroduction or conservation. In forest restoration initiatives, species exhibiting elevated IVI values may be chosen for planting to guarantee the restored ecosystem recovers its ecological functionality (Hobbs and Harris, 2001). Furthermore, IVI can be employed to assess the efficacy of restoration initiatives by monitoring temporal variations in species composition and dominance. The IVI is valuable for observing ecological changes resulting from natural or human-induced disturbances. In forests affected by logging or fire, the IVI can identify which species are gaining dominance and how the general structure of the plant community is evolving. This knowledge is essential for comprehending the long-term effects of perturbations on ecosystem health and resilience (Turner, 2010).

1.4.5 Critiques and Constraints of IVI

Notwithstanding its extensive application, the IVI possesses certain restrictions. A primary objection is that the IVI assigns equal importance to relative density, relative frequency, and relative dominance, despite the fact that these components may not always possess identical ecological value. In certain environments, biomass or canopy cover may be more critical than individual counts in determining species dominance. The IVI is mostly effective for evaluating plant communities, but its relevance to other community types, such as animal or microbial communities, is restricted (Whittaker, 1972). A further drawback is that the IVI is a relative metric, indicating that it solely conveys information regarding the significance of species within a certain community. This complicates the comparison of species significance across various ecosystems or biomes without supplementary context. The IVI fails to consider species interactions, such

competition, facilitation, or predation, which are crucial in assessing species significance (Tilman, 1982).

1.5 Melissopalynology

Bees are an essential component in the maintenance of both natural and artificial plant ecological systems. This is due to the fact that they are the most important pollinators in the world. In tropical regions, where pollination by insects is critical to the survival of ecosystems and the production of agricultural goods, the contribution that they make is especially important with regard to the importance of the contribution that they make. Melissopalynology has arisen as an important topic of research in this environment, which has led to a considerable increase in the amount of attention that has been paid to the study of bees and their interactions with plants. Melissopalynology will continue to be an important field of research. A scientific field of research known as melismapalynology is concerned with the investigation of pollen that is found in honey. It is possible for bees to intentionally collect pollen, or it is also possible for pollen to be unintentionally incorporated into honey when it is being created. Honey has been thoroughly tested for its purity, as well as its geographical origin and floral source (Winfree 2010; Ollerton *et al*., 2011). The field has been exploited for this purpose in a significant way.

Since the early 1900s, mellissopalynology has been used as an analytical method for honey authentication and quality testing. Early studies by Waters (1915) and later by Nair (1964) provided the foundation for our understanding of the pollen composition in honey. Song *et al*.'s (2012) more recent work has considerably enhanced these methods. Through the identification of specific floral sources and the clarification of the ecological connections between bees and plants, these studies contribute significantly to our understanding of both agricultural and wild ecosystems.

For the purpose of enhancing the dependability of melissopalynological research, researchers have made use of several cutting-edge statistical procedures, particularly ordination methods. Using these methods, it is possible to quantitatively describe honey by revealing patterns in the composition of pollen that are

representative of the geographical and botanical origins of the samples. According to this definition, honey can be described. There have been studies carried out by Herrero *et al*. (2002) and Aronne and De Micco (2010) that have demonstrated that ordination is an effective strategy for discriminating between different kinds of honey based on the melissopalynological profiles of the honey. It is possible to establish a more accurate categorisation through the utilisation of this statistical method, which in turn helps to support efforts to trace the origin of honey, defend against adulteration, and maintain high standards of quality control in the production of honey.

1.6 DNA barcoding

DNA barcoding is an innovative tool in taxonomy, acclaimed for its accuracy and efficacy in species identification and classification. This molecular approach use short, standardised DNA sequences from a specific genomic area, commonly known as a "barcode," to identify and distinguish species. This method has demonstrated significant use in classifying both entire organisms and fragmented or incomplete specimens, rendering it an essential tool for systematic study and biodiversity investigations (Hebert *et al*., 2003a, 2003b)

Before the establishment of DNA barcoding, molecular data, especially DNA sequences, had been employed for specimen identification since the 1980s. Nonetheless, the capacity of DNA sequencing to function as a comprehensive natural history instrument was not completely acknowledged until 2003. In that crucial year, the principle of DNA barcoding was established, laying the groundwork for the creation of a worldwide framework for its implementation. This program was initiated by three fundamental organisational meetings at the Banbury Centre at Cold Spring Harbour, facilitated by the Alfred P. Sloan Foundation, which advanced DNA barcoding as a widely recognised instrument for taxonomic and ecological study. Innovative articles by Stoeckle (2003) and Hebert *et al*. (2003a, 2003b) established the scientific foundation for the approach, showcasing its effectiveness and applicability in species identification across many taxa.

DNA barcoding fundamentally depends on the notion that a brief and standardised segment of the genome serves as a universal identifier for species. The mitochondrial cytochrome c oxidase I (COI) gene in mammals has been designated as the standard barcode region because of its rapid evolutionary pace and interspecies variance, while maintaining conservation within species. This area offers adequate genetic difference for species differentiation while maintaining consistency among species to function as a dependable identifier. In plants, certain gene sections, including the chloroplast genes rbcL and matK, are frequently utilised because of the reduced variety of mitochondrial DNA in these organisms (Hebert *et al*., 2003a, 2003b).

The creation of the Barcode of Life Data System (BOLD) has been a major advancement in the international DNA barcoding endeavour. BOLD is an open-access internet repository enabling researchers to upload, store, and analyse barcode sequences. The system enables the worldwide sharing of barcoding data and functions as a centralised repository for species identification. The expanding database augments the efficacy and accuracy of DNA barcoding as a resource for taxonomists and ecologists globally. BOLD has been crucial in broadening the scope of barcoding initiatives, ranging from regional assessments to global biodiversity inventories, and has significantly contributed to the democratisation of access to genetic taxonomy tools.

Molecular identification techniques, such as DNA barcoding, can substitute for morphological identification. Barcodes facilitate the rapid, precise, and cost-effective identification of information. Organisms are classified using DNA barcodes, which consist of a brief DNA sequence endorsed by the scientific community. The mitochondrial cytochrome oxidase subunit I gene is recognised as the essential barcode region for animal survival (Hebert *et al*., 2003; Ratnasingham and Hebert, 2007). Diverse markers are utilised for plants, fungi, and microorganisms. COI is a mitochondrial sequence, indicating a greater abundance of copies compared to nuclear DNA sequences. In addition to offering unique energy and possessing well-protected areas suitable for concealment, COI varies significantly among species. The small length of 658 bp facilitates sequencing.

Minibar codes, which are even shorter segments, were equally efficient in identification (Hajibabaei *et al*., 2006; Meusnier *et al*., 2008).

One advantage of short amplicons is their compatibility with the latest next-generation sequencing technologies. DNA barcoding facilitates identification by contrasting the query sequence with a repository of previously catalogued sequences. Although incomplete, contemporary barcode libraries are progressing. Certain genes, such as the 16S and 28S ribosomal genes, are not considered barcoding genes due to the absence of extensive databases for identification; yet, they are valuable for higher taxonomy and are commonly utilised in phylogenetic development (Yao *et al*., 2010; Dupuis *et al*., 2012). Research by Rubinoff *et al*. (2006), Trewick (2008), Calvignac *et al*. (2011), and Klopfstein *et al*. (2016) identifies hybridisation, Wolbachia-mediated infiltration, and potential conflation with mitochondrial nuclear traits as limitations in employing COI for barcoding purposes. COI is the optimal selection for DNA barcoding, notwithstanding its limitations, due to its extensive and continually growing sequence libraries and the previously mentioned benefits. DNA barcoding has been utilised for the assessment and examination of several species (Hajibabaei *et al*., 2006; Meier *et al*., 2006; Raupach *et al*., 2014; Hebert *et al*., 2016; Schmidt *et al*., 2017; Sikes *et al*., 2017).

Discrepancies between DNA barcoding and morphological assessments frequently result in flaws in taxonomic assumptions, rather than faults in barcoding for completely separated species (Hebert *et al*., 2004; Smith *et al*., 2008). Why is the same concept inapplicable to the description of novel species? The International Code of Zoological Nomenclature contains no provision that prohibits or dissuades DNA-based descriptions. The Ichneumon concept, in particular, holds significant potential for DNA characterisation. This category is very diversified, contains numerous rare species, is often perplexing, and specimens are occasionally collected with minimal comprehension of the surrounding ecosystem. Additionally, DNA barcoding has been employed to identify some enigmatic braconids and ichneumonids (Smith *et al*., 2008; Veijalainen *et al*., 2011; Quicke *et al*., 2012; Schwarzfeld and Sperling, 2015).

1.7 Physicochemical properties of honey

Honey is a naturally occurring sweet substance produced through honeybees (*Apis mellifera*) from floral nectar or plant secretions. It is among the most ancient foods ingested by humans, esteemed for both its sweetness and its nutritional and therapeutic attributes. Throughout the years, honey has been integral to traditional medicine, food preservation, and religious ceremonies in diverse societies. In contemporary times, honey has garnered heightened interest in scientific inquiry owing to its intricate composition and extensive array of health advantages (Solayman *et al*., 2016).

The distinctive qualities of honey arise from its intricate chemical composition, shaped by elements including flower source, geographical origin, processing techniques, and storage conditions. The physicochemical qualities of honey, such as moisture content, pH, sugar composition, electrical conductivity, and viscosity, are essential for assessing its quality, stability, and appropriateness for various applications. Comprehending these qualities is crucial for both consumers and industries, as they affect the nutritional content, flavour, medicinal attributes, and shelf life of honey. This introduction will examine the physicochemical properties of honey, emphasising their biological importance, the variables that affect them, and their impact on honey quality and classification. The introduction will emphasise the significance of these features for food safety, international trade, and honey adulteration (Solayman *et al*., 2016).

1.7.1 Historical Context and Significance of Honey

Honey has been utilised by humans for more than 8,000 years, as demonstrated by cave paintings in Spain that illustrate early humans collecting honey from wild bees (Crane, 1999). In ancient Egypt, honey served as both sustenance and a valuable resource utilised in embalming and religious rituals (Bogdanov, 2011). Ancient civilisations, such as the Greeks and Romans, acknowledged the therapeutic benefits of honey, employing it to address wounds, digestive disorders, and respiratory conditions. The importance of honey in traditional medicine persists, particularly in Ayurvedic and Chinese medical systems. Nonetheless, the emergence

of contemporary scientific study has directed attention to the physicochemical properties of honey, especially regarding its antibacterial, antioxidant, and anti-inflammatory effects (Alvarez-Suarez *et al*., 2010). The biological activities are directly associated with honey's chemical makeup, which is affected by its physicochemical qualities.

1.7.2 Constituents of Honey

Honey is a supersaturated sugar solution, containing a water content that often varies from 15% to 20%. The principal components are carbohydrates (70-80%), predominantly fructose and glucose, which constitute the majority of its energy content. In addition to carbohydrates, honey comprises several secondary constituents, such as organic acids, amino acids, proteins, vitamins, minerals, enzymes, and polyphenols, which enhance its distinctive flavour, aroma, and health advantages (White, 1978). The composition of honey is affected by various elements, including as the floral source, environmental conditions, and processing techniques. Honey obtained from various nectar sources can change markedly in its sugar composition, moisture content, and pH (Persano Oddo and Piro, 2004). The differences in composition subsequently influence the physicochemical qualities of honey, which are essential for evaluating its quality.

1.7.3 Essential Physicochemical Characteristics of Honey

The quality of honey is mostly influenced by its physicochemical characteristics. Numerous international standards, including those set by the Codex Alimentarius and the International Honey Commission (IHC), delineate the permissible ranges for these characteristics to guarantee the purity and safety of honey. The following are the key physicochemical parameters of honey that are commonly evaluated to determine its quality:

1.7.3.1 Moisture Content

The moisture level of honey is a crucial determinant of its stability and shelf life. Honey is hygroscopic, indicating its ability to absorb moisture from the surrounding environment. Elevated moisture levels in honey, typically over 20%,

heighten the likelihood of fermentation caused by osmophilic yeasts (Krell, 1996). Fermentation influences both the flavour and fragrance of honey while also diminishing its shelf life. The moisture level of honey is influenced by various elements, including as the floral source, meteorological circumstances, and the extraction and storage methods (Acquarone *et al*., 2007). The moisture content of honey can be assessed by refractometry, which evaluates the refractive index of the substance. The moisture content is inversely related to the refractive index; increased moisture content results in a diminished refractive index. Regulating moisture content in honey is crucial for ensuring its stability and preventing deterioration during storage (Bogdanov, 2009).

1.7.3.2 pH and Acidity

Honey possesses a natural acidity, exhibiting a pH range of 3.2 to 4.5, contingent upon its floral source and geographical provenance. The acidity of honey mostly results from organic acids, particularly gluconic acid, generated during the enzymatic conversion of glucose by glucose oxidase. The acidic composition of honey enhances its antibacterial capabilities by establishing an inhospitable environment for bacterial and fungal proliferation (Mundo *et al*., 2004).

Total acidity frequently serves as a criterion for assessing honey quality. It can be categorised as free acidity (attributable to organic acids) and lactone acidity (resulting from lactones that convert to acids upon hydrolysis). Elevated acidity in honey is typically linked to fermentation, which may arise from incorrect storage or excessive moisture content (White, 1975).

1.7.3.3 Composition of Sugar

The sweetness and caloric value of honey originate from its sugar composition, mostly comprising fructose (38%) and glucose (31%), along with lesser quantities of sucrose, maltose, and other oligosaccharides. The fructose-to-glucose ratio is a critical determinant of honey's crystallisation behaviour. Honey with elevated glucose levels crystallises more rapidly, while honey with increased fructose concentration, such acacia honey, retains its liquid state for extended durations

(Bhandari *et al*., 1999). The sugar composition of honey is determined by its floral source and can serve to verify its botanical origin. Manuka honey from New Zealand is recognised for its elevated levels of methylglyoxal (MGO), a chemical that imparts distinctive antibacterial characteristics (Adams *et al*., 2008). The sugars in honey may be quantified accurately by high-performance liquid chromatography (HPLC), enabling the detailed analysis of individual sugars.

1.7.3.4 Electrical Conductivity

Electrical conductivity quantifies the quantity of mineral salts, organic acids, and proteins in honey. This characteristic is crucial for differentiating various honey kinds, especially nectar honeys from honeydew honeys. Honeydew honey, generated by bees gathering exudates from plant-sucking insects instead of nectar, typically exhibits elevated electrical conductivity owing to its increased mineral content (Persano Oddo *et al*., 1999). Conductivity is assessed with a conductivity meter, and readings exceeding 0.8 mS/cm generally signify honeydew honey or honeys derived from particular floral sources, such as chestnut or eucalyptus. The mineral composition of honey is affected by the soil characteristics of its production area, rendering electrical conductivity a valuable metric for determining the geographical origin of honey (Anklam, 1998).

1.7.3.5 Viscosity

The viscosity of honey quantifies its flow resistance and is affected by various parameters, such as moisture content, temperature, and sugar composition. Honey with elevated glucose levels generally exhibits increased viscosity, whereas honey with higher fructose concentration is more fluid. Temperature significantly influences viscosity, since honey exhibits reduced viscosity at elevated temperatures (Tosi *et al*., 2004). Viscosity is crucial for the processing, packaging, and management of honey. It also influences honey's sensory attributes, including mouthfeel and spreadability. Viscosity can be quantified with a viscometer, which evaluates the flow properties of honey.

1.7.3.6 Hue

The hue of honey significantly differs based on its botanical source, spanning from almost colourless to deep amber. The hue of honey is chiefly influenced by its mineral composition, polyphenols, and other pigments. Darker honeys, like buckwheat and heather honey, generally exhibit elevated antioxidant activity attributable to their increased polyphenol content (Beretta *et al*., 2005). The colour of honey is typically assessed using the Pfund scale, which classifies honey according to its optical density. The hue of honey may alter throughout storage, especially when subjected to heat and light, as a result of the Maillard process and the caramelisation of sugars. Although colour does not directly affect honey quality, it is a significant factor in consumer preference and marketability.

1.7.3.7 Enzymatic Activity

Enzymes are essential in the synthesis and biological function of honey. Key enzymes present in honey comprise invertase, diastase (amylase), and glucose oxidase. Invertase catalyses the conversion of sucrose into glucose and fructose, whereas diastase hydrolyses starch into maltose and glucose. Glucose oxidase facilitates the transformation of glucose into gluconic acid and hydrogen peroxide, enhancing honey's antibacterial characteristics (White, 1975). The enzymatic activity serves as a benchmark for the freshness and quality of honey. Enzyme activity diminishes with time owing to natural deterioration, especially when honey is subjected to heat or inadequate storage conditions. Diastase activity is utilised as a quality criteria for honey, and its measurement is mandated by numerous international standards (Bogdanov *et al*., 1999).

1.7.4 Determinants of the Physicochemical Characteristics of Honey

A multitude of factors affects the physicochemical characteristics of honey, including: the botanical source of honey influences its composition, especially for sugar content, acidity, and antioxidant activity (Persano Oddo and Piro, 2004). The geographical location, including soil composition, climate, and altitude, influences the mineral content, electrical conductivity, and flavour profile of honey (Anklam,

1998). The techniques employed for the extraction, filtration, and storage of honey can influence its moisture levels, enzymatic activity, and flavour profile. Heating honey at elevated temperatures can impair enzymes and diminish its antioxidant capabilities (Tosi *et al.*, 2002). Environmental Factors: Climatic circumstances, including precipitation and temperature, can affect the accessibility of nectar and the moisture content of honey. Honey generated in humid conditions typically possesses elevated moisture levels, hence augmenting the likelihood of fermentation (Acquarone *et al*., 2007).

1.7.5 Significance of Physicochemical Properties in Honey Authentication and Quality Assurance

The physicochemical characteristics of honey are crucial for its verification and quality assessment. Given the elevated market value of honey, it frequently undergoes adulteration, wherein less expensive ingredients such as sugar syrups are used to augment volume. The adulteration of honey undermines its nutritional and therapeutic properties and endangers consumer health (Siddiqui *et al*., 2017). Analytical techniques that evaluate honey's physicochemical characteristics, including sugar content, enzyme activity, and electrical conductivity, are frequently employed to identify adulteration and verify adherence to international quality standards.

Besides adulteration detection, the physicochemical qualities of honey are crucial for ascertaining its geographical and botanical provenance. This is especially significant for monofloral honeys, such manuka, acacia, and lavender honey, which fetch high prices in the market. Researchers can authenticate speciality honeys and safeguard customers from counterfeit products by examining characteristics such as sugar composition, acidity, and electrical conductivity (Anklam, 1998). The physicochemical characteristics of honey are essential for comprehending its quality, authenticity, and biological efficacy. The moisture content, sugar composition, pH, and enzyme activity affect the stability, flavour, and health advantages of honey. With the increasing global demand for honey, it is essential to comprehend the factors influencing its physicochemical qualities to ensure product quality, prevent

adulteration, and promote the sustainable cultivation of this vital natural resource. This PhD thesis will analyse the physicochemical properties of honey to investigate their correlation with botanical origin, geographical location, and potential health advantages of honey from particular region or floral source.

1.8 Major Royal Jelly Protein 2

Royal jelly, generated by worker bees, functions as a vital nutrition supply for both larvae and the queen in honeybee colonies. Research on royal jelly has predominantly concentrated on *A. mellifera*, the Western honeybee, but there is a growing interest in its counterpart, *A. cerana*, the Eastern honeybee. These two species are extensively dispersed throughout Asia and hold significant ecological and agricultural value. Major Royal Jelly Proteins (MRJPs) are essential components of royal jelly, significant for their function in larval feeding and caste determination. Among these, Major Royal Jelly Protein 2 (MRJP2) has attracted considerable attention for its diverse functions in development, immunology, and social regulation in *A. cerana*. (Srisuparbh *et al*., 2003)

MRJP2 is a significant protein found in the royal jelly of *A. cerana*, belonging to a family of proteins essential for the separation of queen bees from worker bees. Royal jelly is exclusively administered to queen larvae during their entire developmental phase, whilst worker and drone larvae are provided with it solely during the initial days of their lives. The prolonged exposure to royal jelly, abundant in MRJPs, induces the epigenetic modifications requisite for the queen phenotype, characterised by enhanced size, longevity, and reproductive capacity. MRJP2 is considered to be crucial in this process, akin to its involvement in *A. mellifera* (Buttstedt, Moritz, and Erler, 2014). *A. cerana* demonstrates species-specific variations in protein composition and functionality, rendering it a distinctive subject for investigation.

Structurally, MRJP2 from *A. cerana* resembles that of *A. mellifera*, comprising approximately 420 amino acids and undergoing post-translational changes, including glycosylation (Schmitzova *et al*., 1998). These alterations are crucial for the protein's stability and functionality. Recent proteomic analyses have

established that MRJP2 is one of the predominant proteins in *A. cerana* royal jelly, and its structure indicates evolutionary conservation with MRJPs from other honeybee species. Nonetheless, nuanced variations in amino acid sequences between *A. cerana* and *A. mellifera* MRJPs have been noted, potentially indicating the adaptation of *A. cerana* to distinct environmental circumstances and floral resources in its indigenous habitat. MRJP2 in *A. cerana* royal jelly is crucial for the nutritional regulation of larvae. Research indicates that MRJPs, particularly MRJP2, facilitate the transfer of vital nutrients, including proteins, lipids, vitamins, and trace minerals, to developing larvae (Han *et al*., 2011). This function is essential for larval development and differentiation into queens or workers. Moreover, MRJP2 may influence gene expression in *A. cerana* larvae, affecting developmental pathways that result in the caste differentiation observed in honeybee colonies (Kucharski *et al*., 2008). The elevated levels of MRJP2 in larvae destined to become queens corroborate its function in promoting their distinct developmental pathway.

Besides its involvement in larval development, MRJP2 has been associated with immunological functions in *A. cerana*. Due to the high population density of beehive colonies and their exposure to several diseases, proteins such as MRJP2 are believed to offer antimicrobial protection. MRJP2 demonstrates antibacterial capabilities that safeguard both the queen and the brood against bacterial illnesses (Albert and Klaudiny, 2004). The immunological function is notably important in *A. cerana*, which has adapted to habitats with distinct microbial stresses relative to *A. mellifera*. Consequently, *A. cerana* MRJP2 may exhibit distinctive antibacterial capabilities that enhance the colony's general health and resilience.

Moreover, MRJP2 in *A. cerana* is thought to affect social behaviour and pheromonal communication. Studies have shown that MRJPs have a role in the synthesis of pheromones that govern colony dynamics, especially in queen-worker interactions (Kucharski *et al*., 2008). While the precise processes are yet to be completely clarified, MRJP2 probably plays a role in regulating reproductive hierarchies and social cohesiveness within *A. cerana* colonies, similar to its function in *A. mellifera*. This PhD thesis will analyse the physicochemical properties and MRJP2 of honey to investigate their correlation with botanical origin, geographical

location, and potential health advantages of honey from particular region or floral source (Han *et al*., 2011).

CHAPTER II

REVIEW OF LITERATURE

2. Review of literature

2.1. Native plants of India

India possesses a tremendous diversity of indigenous plant species, influenced by its diverse climatic zones, geography, and cultural traditions. The flora of the Indian subcontinent includes tropical, temperate, alpine, and desert vegetation, contributing to a diverse botanical legacy. Native plants, or indigenous species, are those that have naturally evolved in a certain place without human influence. In India, these plants possess ecological, economic, and cultural importance, enhancing biodiversity, food security, traditional medicine, and environmental sustainability. Furthermore, indigenous flora is essential for sustaining local fauna, preserving soil and water, and ensuring ecological equilibrium.

This literature review examines the relevance of native plants in India, their ecological functions, their application in ancient medicinal systems like Ayurveda, their cultural and religious importance, and their conservation status. Furthermore, it tackles the obstacles presented by habitat degradation, climate change, and invasive species to the preservation of India's indigenous flora.

2.1.1. Ecological Significance of Indigenous Flora in India

The indigenous flora of India constitutes the foundation of its many ecosystems, ranging from the tropical rainforests of the Western Ghats to the alpine meadows of the Himalayas. Indigenous species are exceptionally suited to local environmental circumstances, enhancing ecosystem stability and resilience. They offer habitat and sustenance for many animals, including pollinators, herbivores, and seed dispersers, hence preserving biodiversity (Singh *et al*., 2003).

The Western Ghats, acknowledged as a biodiversity hotspot, host various endemic plant species, such as Syzygium travancoricum, Kingiodendron pinnatum, and Pandanus furcatus. These plants not only enhance the region's biodiversity but also contribute significantly to carbon sequestration and soil stabilisation (Myers *et al*., 2000). The Sundarbans mangroves in eastern India, primarily composed of indigenous species such as Rhizophora and Avicennia, safeguard coastal areas from

erosion and serve as a protective barrier against natural calamities, including cyclones and tsunamis (Giri *et al*., 2011).

Indigenous flora enhances soil fertility via nitrogen fixation and nutrient cycling. Species such as Prosopis cineraria (Khejri) and Acacia nilotica (Babul) in arid and semi-arid parts of India improve soil fertility, hence facilitating agricultural practices in these areas (Jha *et al*., 2015).

2.1.2. Indigenous Flora in Indian Agriculture and Nutritional Frameworks

India's agricultural systems have historically depended on indigenous plant species for sustenance, livestock feed, and various applications. Indigenous crops and wild edible plants are essential for food security, especially in rural and tribal regions. Conventional agricultural methods in India, including shifting cultivation (jhum) in the Northeastern states and agroforestry systems in the Western Ghats, utilise indigenous plant species that are optimally suited to local environmental conditions (Ramakrishnan, 2001). Eleusine coracana, often known as finger millet or ragi, is a drought-resistant indigenous cereal grown in southern India. Finger millet serves as a fundamental food source for numerous societies and offers vital nutrients, including calcium, iron, and fibre. Likewise, *Vigna mungo* (black gramme) and *Cajanus cajan* (pigeon pea) are significant pulses that enhance protein-rich meals in the nation (Dewan, 2014). Alongside food crops, indigenous fruit-bearing plants such as *Mangifera indica* (mango), *Tamarindus indica* (tamarind), and *Phoenix dactylifera* (date palm) have been extensively planted in India for their nutritional and economic significance. These species are essential to the rural economy and support sustainable lifestyles, particularly in marginal agricultural regions (Reddy, 2016).

2.1.3. Conventional Medicine and Ethnobotany

India's indigenous flora has been utilised for thousands of years in traditional medicinal systems including Ayurveda, Siddha, and Unani. Ayurvedic scriptures, like the Charaka Samhita and the Sushruta Samhita, record the medicinal characteristics of several indigenous plant species, many of which are still utilised in

contemporary herbal therapy and pharmaceutical research (Sharma *et al*., 2013). Notable medicinal plants indigenous to India comprise: *Azadirachta indica* (Neem): Esteemed for its antibacterial, anti-inflammatory, and immunomodulatory attributes, neem is extensively utilised in the management of dermatological ailments, infections, and inflammatory diseases (Subapriya and Nagini, 2005). *Withania somnifera* (Ashwagandha): Recognised for its adaptogenic characteristics, ashwagandha is utilised to alleviate stress, improve physical performance, and promote mental well-being. It is an essential herb in Ayurvedic medicine (Singh *et al*., 2011).

Tinospora cordifolia (Guduchi) is acknowledged for its immunomodulatory and anti-diabetic properties and is utilised in the treatment of fever, respiratory infections, and metabolic diseases (Upadhyay *et al*., 2010). *Terminalia chebula* (Haritaki) is utilised in Ayurveda for its restorative and laxative attributes, and it constitutes one of the three elements of the renowned Ayurvedic preparation, Triphala (Baliga, 2010). Ethnobotanical research throughout India has recorded the utilisation of obscure indigenous plants by tribal populations for medicinal purposes. The Bhil and Gond tribes of central India utilise species such as *Butea monosperma* (Palash) and *Holarrhena antidysenterica* for the treatment of gastrointestinal ailments and fever (Pattanaik *et al*., 2009).

2.1.4. Indigenous Flora in Indian Culture and Religion

The indigenous flora of India possesses substantial cultural and religious importance. Numerous species are intricately woven within Hindu, Buddhist, and Jain traditions, where they are venerated as sacred symbols, utilised in religious rites, or linked to deities. The Peepal tree (*Ficus religiosa*) is regarded as sacred in Hinduism, Buddhism, and Jainism. It is thought to symbolise the cosmic realm and is linked to Lord Vishnu and Lord Buddha. The Peepal tree is frequently cultivated in temple courtyards and venerated for its alleged capacity to purify the surroundings and confer spiritual enlightenment (Dwivedi, 1993). Tulsi (*Ocimum sanctum*), sometimes referred to as holy basil, is a highly esteemed herb in Hindu families. Tulsi is thought to have therapeutic characteristics and is utilised in religious rituals,

Ayurvedic medicine, and home treatments for respiratory conditions, fever, and digestive disorders (Prakash and Gupta, 2005). Sandalwood (Santalum album) has been utilised for millennia in religious ceremonies, fragrance production, and traditional medicine. It is esteemed for its soothing and refreshing attributes, and its timber is frequently sculpted into effigies and utilised in religious ceremonies (Rohit *et al*., 2014).

2.1.5 Dangers to Indigenous Flora in India

Notwithstanding the ecological and cultural significance of indigenous flora in India, numerous species are endangered due to various human-induced reasons. Habitat destruction, deforestation, urban development, and agricultural proliferation have resulted in the degradation of essential ecosystems, jeopardising the existence of indigenous plant species (Kumar and Rao, 2006). The Western Ghats and Himalayan biodiversity hotspots are experiencing significant deforestation and habitat fragmentation, jeopardising many rare plant species (Reddy *et al*., 2007).

Invasive species present a considerable threat to the sustainability of indigenous flora. Species such as Lantana camara, Parthenium hysterophorus, and Prosopis juliflora have proliferated extensively throughout India, outcompeting indigenous plants and destabilising local ecosystems (Dogra *et al*., 2010). Invasive species frequently establish dense thickets, diminishing biodiversity and modifying soil chemistry, so inhibiting the establishment of native plants. Climate change poses a significant threat to India's indigenous vegetation. Increasing temperatures, alterations in precipitation patterns, and a rise in the frequency of extreme weather events are expected to modify the distribution of plant species and disrupt their phenology. This may result in the extinction of animals that cannot acclimatise to altered environmental conditions (Chaturvedi *et al*., 2011).

Conservation Initiatives for Indigenous Flora in India: Preserving indigenous flora is crucial for maintaining ecological equilibrium, safeguarding biodiversity, and supporting traditional livelihoods. Numerous governmental and non-governmental organisations in India are engaged in the conservation of indigenous plant species through programs like protected area networks, seed banks, and community-based

conservation projects. The Protected Area Network of India, comprising national parks, animal sanctuaries, and biosphere reserves, is essential for the conservation of indigenous plant species. The Nilgiri Biosphere Reserve in southern India, recognised by UNESCO, hosts a variety of indigenous species, including uncommon and endangered flora such as Nilgirianthus ciliatus (blue kurinji) (Nayar *et al*., 2009).

2.2. Invasive plants in India

Invasive flora presents a considerable risk to the biodiversity, economy, and ecological equilibrium of numerous nations, including India. These non-indigenous species, introduced either deliberately or inadvertently, thrive in novel ecosystems and frequently surpass native species in the competition for resources such as sunlight, water, and nutrients. Biological invasion is an escalating global concern and is seen as a primary contributor to biodiversity loss (Sharma *et al*., 2005). In India, invasive plant species (IPS) have disrupted ecosystems, ruined agricultural fields, and presented hurdles for biodiversity conservation initiatives. This literature review examines the historical context of invasive plant introduction in India, identifies the most problematic invasive species, assesses their ecological and economic repercussions, and evaluates existing management and policy frameworks. The review analyses the socio-economic issues presented by invasive species and outlines prospective avenues for research and management inside the country.

2.2.1 Historical Overview of Invasive Plant Introductions in India

The introduction of invasive plant species to India has transpired via multiple channels, both deliberate and inadvertent. Throughout the colonial era, numerous plants were imported for ornamental, agricultural, or afforestation purposes. Species such as *Lantana camara* and *Eucalyptus* were intentionally planted for decorative purposes and lumber production, but they rapidly became invasive (Love *et al*., 2009). Additional plants were unintentionally introduced via commerce and travel. As India augmented its agricultural and economic endeavours, seeds and plant materials were disseminated worldwide, frequently resulting in the inadvertent introduction of non-indigenous species to the nation. Upon establishment, these

species proliferate swiftly, aided by conducive weather conditions and disrupted ecosystems (Khuroo *et al*., 2007).

2.2.2 Notable Invasive Plant Species in India

Numerous invasive plant species in India have had significant ecological and economic repercussions. Among the most troublesome invasive plant species are: *Lantana camara*, indigenous to Central and South America, is among the most infamous invasive species in India. Initially introduced in the early 19th century as an ornamental species, it has subsequently proliferated aggressively across diverse environments, especially in forested regions. *Lantana* encroaches upon agricultural territories, displaces indigenous flora, and diminishes the regenerative potential of forest ecosystems (Sharma *et al*., 2005). *Parthenium hysterophorus*, commonly referred to as Congress grass, is indigenous to tropical America and was brought to India in the 1950s via infected grain imports. This very invasive plant inflicts significant harm on agricultural fields, pastures, and open spaces. It presents health hazards to humans and livestock, resulting in skin allergies, respiratory problems, and dermatitis (Rao *et al*., 2003). *Prosopis juliflora*, indigenous to Central and South America, was brought to India in the late 19th century for the purpose of soil stabilisation in arid and semi-arid areas. Nonetheless, it has since grown invasive in other environments, especially in Rajasthan, Gujarat, and Tamil Nadu. The species establishes dense thickets, surpasses local flora, and exhausts groundwater resources (Pasiecznik *et al*., 2001). *Eichhornia crassipes*, commonly referred to as water hyacinth, was brought from South America as a beautiful aquatic plant. This plant has emerged as a significant invasive aquatic species in India, obstructing streams, hindering water flow, and diminishing biodiversity in freshwater habitats. It obstructs agriculture by impeding irrigation channels and facilitates the proliferation of waterborne infections (Gopal, 1987). *Chromolaena odorata*, indigenous to Central and South America, has proliferated throughout tropical areas of India, especially in the Northeast and the Western Ghats. It encroaches upon agricultural fields, woods, and grasslands, diminishing agricultural output and biodiversity (Muniappan *et al*., 2005).

2.2.3 Ecological Consequences of Invasive Flora

Invasive plant species in India have inflicted significant ecological disturbances by outcompeting indigenous species, modifying habitat structure, and impacting ecosystem processes. The repercussions can be classified as biodiversity loss, modification of ecological functions, and alterations in species relationships.

2.2.4 Decline in Biodiversity

Invasive flora diminishes native biodiversity by monopolising resources including sunshine, water, and soil nutrients. Lantana camara creates dense thickets in forest understories, inhibiting the regeneration of native tree species and modifying the structure of forest ecosystems (Sundaram and Hiremath, 2012). Parthenium hysterophorus invasively occupies grasslands and agricultural areas, displacing indigenous plant species and diminishing fodder availability for cattle (Tiwari *et al*., 2005).

2.2.5 Modification of Ecosystem Functions

Invasive plants can modify essential ecological activities, including nutrient cycling, water control, and fire regimes. Prosopis juliflora depletes soil nutrients and groundwater, hindering the survival of native species (Shiferaw *et al*., 2004). Invasive aquatic flora such as Eichhornia crassipes diminish oxygen concentrations in aquatic ecosystems, adversely impacting ichthyofauna and aquatic biodiversity (Patel, 2012).

2.2.6 Disruption of Species Interactions

Invasive species frequently disturb established species relationships, including pollination, herbivory, and seed distribution. The proliferation of Chromolaena odorata in Northeast India has been associated with the deterioration of native plant-pollinator interactions, as this invasive species draws a reduced number of pollinators relative to indigenous flora (Murali and Setty, 2001).

2.2.7 Economic Consequences of Invasive Flora

The economic ramifications of invasive plant species in India are extensive. They impact agriculture, forestry, fisheries, and public health, resulting in substantial economic losses.

2.2.8 Agricultural Losses

Invasive species such as Parthenium hysterophorus and Lantana camara diminish agricultural output by competing with crops for resources and depleting soil fertility. These species elevate agricultural production costs as farmers must invest in weed management strategies, including manual weeding, chemical herbicides, and biological control agents (Kohli *et al*., 2006).

2.2.9 Forestry and Livelihoods

In wooded areas, invasive flora impact the livelihoods of communities reliant on forest resources. The proliferation of Lantana camara in the Western Ghats has diminished the accessibility of non-timber forest products (NTFPs), including medicinal plants, wild fruits, and fuelwood (Sharma *et al*., 2016). The decline of biodiversity in these areas has significantly impacted rural livelihoods and traditional knowledge systems.

2.2.10 Health and Societal Expenditures

Some invasive plant species present concerns to public health. Parthenium hysterophorus is infamous for inducing allergies, rashes, and respiratory issues in people. This plant also impacts livestock, since its intake can result in toxicity and diminished milk production in cattle (Kanchan and Jayachandra, 1980). The economic burdens related to illness, weed management, and diminished animal productivity are substantial (Rao *et al*., 2003).

2.3. Important value index (IVI)

2.3.1. Origins and Theoretical Framework of IVI

The Important Value Index was initially proposed by Curtis and McIntosh (1951) in their foundational study of plant communities in temperate forests. Before the introduction of the IVI, ecologists generally concentrated on individual characteristics, such as species abundance or dominance, to assess the significance of species within ecosystems. Nonetheless, these individual measurements inadequately represented the intricacies of species relationships or their comprehensive impact on community structure (Whittaker, 1965). Curtis and McIntosh advocated for a more comprehensive approach, resulting in the creation of the IVI, which integrates three essential components of plant communities relative density, frequency, and dominance into a unified metric (Curtis and McIntosh, 1951).

The three components of the IVI offer distinct insights into the roles of species within their communities. Relative Density quantifies the abundance of a species in relation to the overall population within the community. This measure indicates the prevalence of a species within the environment. Relative Frequency denotes the distribution of a species within sampling units, such as quadrats or plots. It illustrates the spatial distribution and the frequency of a species' occurrence within a research area. Relative Dominance is generally computed as the basal area (for trees) or biomass (for herbaceous plants) of a species in relation to the total basal area or biomass of all species within the community. Dominance may reflect the influence of larger or more competitive species on the community's composition and resource utilisation (Kent and Coker, 1992). The ultimate IVI score is derived by aggregating these three relative measurements, each spanning from 0 to 100, resulting in a potential IVI range of 0 to 300. This extensive measure is especially significant as it provides insights into species that may hold ecological importance despite their scarcity, alongside species that prevail in the environment regarding both population density and spatial distribution (Magurran, 2004).

2.3.2. Computational and Methodological Strategies

The computation of the IVI entails a methodical approach to data acquisition and analysis. The study area is initially segmented into quadrats or plots, within which researchers document the presence and abundance of several species. The basal area, commonly employed as an indicator of tree species dominance, is determined by the diameter at breast height (DBH) of individual trees (Muller-Dombois and Ellenberg, 1974). In herbaceous plants, dominance can be quantified by biomass or coverage. The relative values for density, frequency, and dominance are calculated as percentages and subsequently totalled to derive the Importance Value Index (IVI) for each species. The IVI offers a comparative assessment of species significance within a community, rendering it very valuable in plant ecological research. It is essential to recognise that the IVI is affected by both the size of individuals and their population density. Dominant, large trees can exhibit a high Importance Value Index (IVI) despite their relative scarcity in the community, although smaller, more prevalent species may also attain elevated scores on the index owing to their density and frequency (Magurran, 2004). Consequently, the interpretation of IVI necessitates meticulous evaluation of the biological environment and the distinct attributes of the species under examination.

2.3.3 Forested Ecosystems

In forest ecosystems, the Importance Value Index (IVI) has proved crucial for assessing species composition, dominance, and community dynamics. It is commonly employed to ascertain the most significant species within forest communities, regarding their ecological functions and their contributions to forest structure. Curtis (1959) utilised the Importance Value Index (IVI) in temperate forests of Wisconsin, USA, demonstrating that prominent species like Acer saccharum (sugar maple) and Quercus rubra (red oak) had elevated IVI values attributable to their prevalence and spatial distribution. Research in tropical forests, characterised by significant species variety, has demonstrated that the Importance Value Index (IVI) may effectively identify keystone species essential for ecosystem functionality (Oliveira-Filho *et al*., 2006). In the Brazilian Atlantic Forest, the

Importance Value Index (IVI) has been employed to differentiate between pioneer species prevalent in early successional stages and climax species that dominate later phases of forest development (Condit *et al*., 2002).

Logging and other disturbances can substantially modify species' Importance Value Index (IVI), frequently resulting in alterations to community structure. Hamzah *et al*. (2015) employed the IVI to investigate the impacts of selective logging in dipterocarp forests of Southeast Asia. Their findings indicated that logging diminished the Importance Value Index (IVI) of dominant tree species, such as Dipterocarpus spp., while augmenting the IVI of rapidly growing pioneer species.

2.3.4 Grassland Ecosystems

In grasslands, the Importance Value Index (IVI) is frequently employed to assess species composition and their reactions to environmental influences, including grazing, fire, and climate fluctuations. Grasslands exhibit significant species turnover and dynamic interspecies interactions, rendering the IVI an essential instrument for tracking ecological changes over time. Singh *et al*. (2014) utilised the IVI in dry tropical grasslands of India, finding major grass species, including Cenchrus ciliaris and Dichanthium annulatum, as significant contributors to grassland structure. Their research indicated that overgrazing might result in alterations in species composition, as severely grazed regions exhibited an increase in the Importance Value Index (IVI) of less appealing, frequently invasive species, to the detriment of more acceptable forage species. In savanna ecosystems, where interactions between trees and grasses are crucial, the Importance Value Index (IVI) has been employed to evaluate the relative significance of woody and herbaceous species. Shackleton and Scholes (2000) employed the Importance Value Index (IVI) to examine savanna vegetation in southern Africa, discovering that certain dominant tree species, notably Acacia spp., exhibited elevated IVI values, whereas other species contributed to the ecosystem in more nuanced manners, including nitrogen fixation and habitat provision for wildlife.

2.3.5 Wetland Ecosystems

Wetland ecosystems are defined by their distinct hydrological regimes, which significantly influence species composition and dominance. The IVI is utilised in wetland research to evaluate species significance concerning water availability, nutrient conditions, and many environmental aspects. Tamang and Barik (2006) utilised the IVI to assess wetlands in northeastern India, discovering species with elevated IVI values, including Eichhornia crassipes (water hyacinth) and Cyperus rotundus (nutgrass), which are adept at adapting to the region's variable water levels. Their research emphasised the significant correlation between hydrology and plant community composition, as species with elevated IVI values typically prevailed in particular moisture regimes within the marsh. In wetland restoration initiatives, the IVI has been employed to assess alterations in species composition and community structure over time. Middleton (1999) employed the IVI to evaluate the efficacy of wetland restoration initiatives in the Mississippi River Valley, USA. His results indicated that restored wetlands frequently exhibited a predominance of invasive species, including Typha spp. Cattails had elevated IVI values, signifying that restoration initiatives should prioritise the facilitation of native species establishment.

2.3.6 Agroforestry and Agricultural Practices

In agroforestry systems, the Importance Value Index (IVI) is utilised to evaluate the significance of tree species based on their contributions to ecosystem services, including soil fertility, carbon sequestration, and biodiversity conservation. Agroforestry systems, which incorporate trees into agricultural environments, pose distinct issues for species management and conservation. Kumar *et al*. (2012) performed a study in southern India, employing the IVI to evaluate the relative significance of various tree species in agroforestry systems. Their findings indicated that species such as *Azadirachta indica* (neem) and *Leucaena leucocephala* (lead tree) exhibited high IVI values, signifying their substantial ecological contributions in nitrogen fixation and offering shade and shelter for crops. The IVI demonstrated its utility in evaluating the sustainability of agroforestry systems by identifying plants that enhance long-term soil fertility and carbon sequestration.

2.3.7 The Function of IVI in Biodiversity and Conservation Research

The implementation of IVI in biodiversity protection is essential, especially regarding global environmental change. Biodiversity faces escalating threats from habitat degradation, climate change, and anthropogenic activity, necessitating the development of techniques to evaluate and prioritise species for conservation initiatives. The IVI is especially beneficial in this situation since it aids in identifying not only the most prevalent or dominating species but also those that hold ecological significance, regardless of their abundance (Gaston and Spicer, 2004). Conservation planning frequently necessitates compromises between safeguarding biodiversity and fulfilling human need, including agriculture and development. The IVI offers a method to prioritise species for conservation based on their ecological functions rather than solely their population size. This is especially crucial in regions with constrained conservation resources, necessitating decisions regarding which species to prioritise for protection (Margules and Pressey, 2000). In forest ecosystems, for instance, IVI has been employed to identify species essential for sustaining ecosystem services like carbon sequestration and hydrological management. These species may not consistently be the most conspicuous or numerous, although they are crucial for sustaining ecological equilibrium (Lindenmayer *et al*., 2000). By concentrating on species with elevated IVI values, conservation initiatives can prioritise the preservation of ecosystem functionality over merely maintaining species diversity.

2.4. Melissopalynology

Melissopalynology, the examination of pollen in honey, is a specialised field within palynology that has attracted considerable attention for its utility in identifying the botanical and geographical sources of honey. This domain offers insights into the interactions between bees and their surroundings, encompassing the plant species they forage, the prevailing climatic conditions, and potential markers of climate change. Consequently, melissopalynology has been widely employed to verify honey authenticity, investigate ecological systems, and evaluate agricultural landscapes. This review examines the historical evolution, methodology, and

applications of melissopalynology, as well as its relevance in contemporary research on pollination biology, honey quality, and climate change.

2.4.1 Chronological Progression of Melissopalynology

Melissopalynology originated in the early 20th century when researchers commenced examining the pollen composition in honey to ascertain the flora utilised by bees. Pons' work in 1910 and, more significantly, Waters' research in 1915 established the groundwork for the scientific examination of pollen in honey. Waters (1915) established that pollen grains in honey can identify its floral origins, so founding melissopalynology as a technique for authenticating honey. Over the decades, pollen analysis techniques in honey have advanced, notably due to Nair's (1964) refinement of identification and quantification procedures. Improvements in microscopy and the establishment of pollen databases facilitated more accurate identification of pollen grains. By the mid-20th century, melissopalynology emerged as an essential method for evaluating honey quality and authenticity, particularly in areas prone to honey adulteration (Louveaux *et al*., 1978). The establishment of standardised protocols for pollen analysis enhanced the dependability of this technology.

2.4.2 Methodological Approaches in Melissopalynology

The fundamental methodology in melissopalynology entails the microscopic examination of honey to ascertain the pollen grains present. The procedure commences with the dissolution of honey samples in water, succeeded by centrifugation to isolate the pollen grains. The grains are subsequently affixed to slides and examined under a light microscope for identification according to their morphological traits, including size, shape, and ornamentation (Erdtman, 1969). Identifying pollen grains necessitates an extensive reference collection of local pollen types and an understanding of the physical traits of pollen from many plant species. Louveaux *et al*. (1978) established protocols for melissopalynological study, encompassing slide preparation, pollen identification, and quantification techniques. These guidelines underscore the necessity of standardising methodologies to guarantee reproducibility and precision across research investigations.

Recent technological breakthroughs have facilitated the integration of molecular techniques with conventional microscopy procedures. DNA barcoding facilitates the identification of plant species in honey samples by the analysis of genetic material derived from pollen. Valentini *et al*. (2010) illustrated the effectiveness of DNA barcoding in identifying plant species within honey, offering a supplementary method to conventional microscopy. This technique provides enhanced accuracy, particularly in instances where pollen grains are challenging to distinguish just by shape.

2.4.3 Utilisation of Melissopalynology for Honey Authentication

Melissopalynology is mostly utilised for the verification of honey according to its floral and geographical origins. The pollen composition of honey acts as a botanical identifier, indicating the plant species from which bees collected nectar during honey manufacturing. This is especially crucial for the labelling of monofloral honeys, which are predominantly derived from a single plant species. Clover, acacia, and lavender honeys are frequently promoted as premium products, with melissopalynological examination employed to authenticate them by proving the predominant presence of pollen from the corresponding plants (Von Der Ohe *et al*., 2004). Melissopalynology aids in detecting instances of honey adulteration, a major issue in the international honey business. Adulterated honey, frequently combined with syrups or derived from undisclosed floral sources, can be identified using melissopalynological examination, which exposes pollen spectra that are incongruous with the stated origins of the honey (Moar, 1985). A honey designated as “acacia honey” must possess a substantial proportion of acacia pollen; departures from this standard may suggest adulteration or mislabeling (Siddiqui *et al*., 2017).

Furthermore, melissopalynology can detect impurities in honey. Wind-pollinated plant species, which generate substantial amounts of pollen, can adulterate honey. This phenomenon frequently occurs in regions with dense populations of plants such as pine or grasses, whose pollen may be found in honey even though they do not serve as nectar sources for bees (Jones and Bryant, 2004).

2.4.4 Ecological and Agricultural Perspectives Derived from Melissopalynology

In addition to honey authentication, melissopalynology offers significant ecological insights, especially about plant-pollinator interactions. Researchers can deduce the foraging behaviour of bees and determine the plant species crucial for sustaining bee populations in a specific location by analysing the pollen composition of honey. This knowledge is essential for comprehending the dynamics of pollination networks, particularly in areas experiencing fast environmental change (Winfree, 2010). Melissopalynology has been employed to evaluate the health and biodiversity of ecosystems. Herrera (1995) and Ollerton *et al*. (2011) highlighted that the variety of pollen present in honey can indicate the floral richness of the adjacent environment. An increased variety of pollen signifies a robust and diverse ecosystem, whereas a restricted assortment of pollen species may indicate habitat destruction or monoculture agricultural practices. This information is crucial for conservation initiatives focused on safeguarding pollinator habitats and sustaining biodiversity in agricultural environments. In agricultural settings, melissopalynology is employed to assess the accessibility of forage plants for bees. By analysing the pollen types found in honey, researchers can ascertain the crops or wild plants that bees exploit over particular seasons. This is beneficial for apiarists and agriculturists who depend on bee pollination for agricultural yield. Jato *et al*. (1994) investigated the pollen content of honey from agricultural areas to evaluate the impact of crops such as sunflower and clover on bee foraging.

2.4.5 Climatic and Environmental Associations in Melissopalynology

Melissopalynology has become an essential method for examining the influence of climatic variables on pollination. The pollen composition in honey can reveal insights about plant phenology and flowering timing, which are affected by climate factors including temperature and precipitation. Bilisik *et al*. (2008) examined the correlation between pollen spectra in honey and meteorological parameters in Turkey, identifying associations between pollen diversity and variations in temperature and precipitation. The results indicate that melissopalynology may serve as a tool to assess the impact of climate change on

plant-pollinator interactions. Besides examining contemporary environmental circumstances, melissopalynology has been employed to evaluate historical alterations in plant communities and pollinator behaviour. Fossilised honey carrying pollen offers insights into historical ecosystems, enabling researchers to reconstruct ancient plant-pollinator interactions and monitor vegetative changes throughout time (Carrión *et al*., 2010). These investigations are crucial for comprehending the responses of pollination networks to historical climatic alterations and their potential impacts from forthcoming environmental modifications.

2.4.6 Statistical Methodologies in Melissopalynological Research

The quantitative study of pollen in honey is a crucial component of melissopalynology, offering insights into the relative abundance of various plant species within the bees' foraging territory. Ordination techniques, including principal component analysis (PCA) and cluster analysis, are frequently employed to examine melissopalynological data to discern trends in pollen composition. These statistical methods assist researchers in distinguishing between monofloral and polyfloral honeys and identifying geographical or seasonal differences in pollen spectra (Herrero *et al*., 2002). Herrero *et al*. (2002) illustrated the application of ordination techniques to categorise honey samples according to their botanical and geographical origins. Through the analysis of pollen composition in honeys from various places, they successfully categorised samples with analogous pollen spectra and deduced the predominant plant species in each area. Such investigations are crucial in characterising honeys from various landscapes and ensuring that honey labels appropriately represent their floral and geographical origins. Alongside PCA, additional multivariate methodologies, like discriminant analysis and cluster analysis, have been utilised in melissopalynological research. Aronne and De Micco (2010) utilised these methods to differentiate honeys produced in various seasons and to detect temporal variations in pollen content. Their research emphasised the significance of statistical analyses in improving the accuracy and reliability of melissopalynological results.

2.4.7 Obstacles and Constraints in Melissopalynology

Notwithstanding its extensive uses, melissopalynology encounters numerous problems and restrictions. A primary problem is the identification of pollen grains from closely related plant species, which may exhibit physical similarities that complicate differentiation under a microscope. This poses significant challenges for monofloral honeys, as precise identification of the predominant pollen type is essential for authenticity verification (Von Der Ohe *et al*., 2004). In these instances, DNA barcoding and molecular techniques are proposed as supplementary methods to enhance species-level identification (Valentini *et al*., 2010). A further disadvantage of melissopalynology is its dependence on pollen as the exclusive indicator of floral origins. Pollen research offers significant insights into the flora frequented by bees, although it fails to consider the nectar's role in honey production. Certain plant species generate nectar but produce minimal or no pollen, thus leading to their under-representation in melissopalynological studies. Researchers propose integrating melissopalynology with additional chemical investigations, including stable isotope analysis and metabolomics, to achieve a more thorough comprehension of the floral and geographical origins of honey (Siddiqui *et al*., 2017).

2.5. DNA Barcoding

Comparative morphology has historically been fundamental in taxonomy and systematics, significantly contributing to species delineation and taxonomic revisions. This conventional method depends on the analysis and comparison of physical characteristics among organisms to determine evolutionary links and identify species. Morphological traits, including dimensions, form, hue and composition, have traditionally been employed to distinguish species and classify taxonomic groups. This strategy may be constrained by the plasticity of morphological characteristics, which can fluctuate due to environmental influences, developmental phases, or genetic diversity within species (Gilbert *et al*., 2007).

Recent breakthroughs in molecular techniques have opened new opportunities for taxonomic inquiry, especially through DNA analysis. A notable benefit of molecular techniques is their capacity to extract and sequence DNA from

museum specimens and other historical materials without compromising the morphological characteristics essential for taxonomy identification. This is crucial as numerous museum specimens have unique genetic information that has been conserved over time, offering significant insights into species variety and evolutionary history. Research indicates that even deteriorated or old materials can provide viable genetic material, enabling researchers to do phylogenetic studies and reveal concealed biodiversity (Gilbert *et al*., 2007; Hernández Triana *et al*., 2014; Burrell *et al*., 2015).

Nonetheless, despite the promise of DNA analysis to improve taxonomic comprehension, numerous problems accompany its application. A principal challenge is the requirement for specialised facilities and equipment to guarantee that samples are processed in a sterile laboratory setting. The hazards of contamination are considerable when handling historical materials, as environmental DNA can readily undermine conclusions. Furthermore, the financial expenditures related to modern sequencing technologies and laboratory resources can be exorbitant, rendering this study paradigm less attainable for several researchers (Gilbert *et al*., 2007). Thus, although the incorporation of molecular techniques into taxonomic research has significant potential for enhancing species classifications and revealing genetic diversity, it remains an impractical method globally. The integration of comparative morphology and DNA data can yield a more thorough comprehension of species relationships and improve the precision of taxonomic revisions. Nevertheless, for numerous researchers, particularly in resource-constrained environments, conventional morphological techniques continue to be the most feasible approach for executing taxonomic investigations (Burrell *et al*., 2015).

Comparative morphology has traditionally been essential in taxonomic revisions and species delineation; however, the emergence of molecular tools, such as DNA extraction from museum specimens, presents promising potential to deepen our comprehension of biodiversity. Despite ongoing obstacles of contamination, expense, and laboratory accessibility, the amalgamation of genetic analysis with conventional morphological techniques can yield a more refined and precise taxonomy of species. As research procedures progress, it is crucial to investigate

creative approaches that integrate the advantages of both morphological and genetic data to enhance taxonomy and enrich our comprehension of the complex interactions among species (Hernández Triana *et al*., 2014).

Regardless of the species concept utilized frequently unspecified in scientific literature comparative morphology has traditionally been fundamental in taxonomic revisions and species differentiation. This method has enabled taxonomists to categorise creatures according to their physical traits, establishing a foundation for comprehending evolutionary relationships and variety in the natural world. Nonetheless, a notable constraint of this conventional methodology has been the difficulty in analysing genetic material from museum specimens, especially those that are antiquated or deteriorated. Historically, the extraction and analysis of DNA from these specimens frequently jeopardised the morphological characteristics essential for species identification (Assing, 2014; Shi and Liang, 2015; Gilbert *et al*., 2007; Hernández Triana *et al*., 2014; Burrell *et al*., 2015). Presently, the genetic examination of historical samples needs a sterile laboratory environment to avert contamination, presenting practical challenges for researchers. The extraction of DNA sequences from historical museum records can be technically challenging and expensive, rendering it an unrealistic study tool for many. Furthermore, the extraction of only a few brief DNA sequences—particularly in instances involving closely related species—complicates data processing due to the restricted informational content of these sequences (Sogin *et al*., 2006). These limits underscore the difficulty involved in incorporating molecular data into taxonomic frameworks, especially with historical specimens.

Approximately ten years ago, it was proposed that taxonomy should mostly, if not only, depend on sequence data. This viewpoint signifies an increasing agreement among scientists about the significance of molecular methods in precisely evaluating biodiversity and defining species boundaries. As the incorporation of genetic data into taxonomic methods broadens, it is crucial to evaluate how new methodologies can enhance traditional morphological evaluations. This integrated approach may improve our comprehension of species diversity, enable more precise classifications, and eventually result in more successful conservation policies. In

conclusion, whereas comparative morphology has historically been essential in taxonomy, the advancement of genetic analysis introduces novel opportunities and problems in the discipline. With advancements in laboratory procedures and the increased accessibility of sequencing technologies, the integration of DNA data into taxonomic study is expected to rise, offering a more thorough comprehension of biodiversity's intricacies (Sogin *et al*., 2006). This paradigm shift highlights the necessity of integrating both morphological and molecular techniques in taxonomy, promoting a comprehensive approach to the examination of species and their evolutionary connections.

Furthermore, the identification of exceedingly rare species presents further challenges. Lim *et al*. (2012) emphasise that methodologies necessitating substantial sampling across loci and individuals may be impractical when dealing with populations that are rarely encountered or when the specimens available for research are scarce. The intrinsic scarcity of such species requires a more adaptable and flexible methodology for species identification, capable of addressing the distinct issues posed by rare taxa.

The reliability of clustering algorithms for species identification in large-scale datasets remains a considerable difficulty. Notwithstanding progress in genetic analysis techniques, the efficacy of categorisation algorithms remains constrained. Certain methodologies, including those suggested by Pons *et al*. (2006) and Zhang (2013), are intended for use with data obtained from a singular location. Although these methods may provide valuable insights, they frequently inadequately handle the intricacies of species boundaries, particularly in biodiversity studies that span numerous ecosystems or geographical areas.

A significant disadvantage of single-site data is the intrinsic variance in genomic distances both intra- and inter-specifically. These changes may be affected by several factors, including the evolutionary history of the examined loci and the present condition of the species (Ferguson, 2002; Meier *et al*., 2008). Thus, approaches that depend exclusively on distance measurements, such the Automatic Barcode Gap Discovery (ABGD) algorithm (Puillandre *et al*., 2012), although

computationally efficient and simpler to apply to large datasets, frequently fall short in precisely ascertaining species status. The difficulty of determining a credible distance criterion for species classification exacerbates the dependence on these methods. Although these advanced methodologies have promise, it is crucial to acknowledge that data from a single location, while beneficial, are frequently inadequate for thorough taxonomic investigation. Nevertheless, when integrated with morphological data or disparate sources, these datasets can considerably accelerate the identification process, resulting in species classifications that may otherwise be difficult to ascertain. The amalgamation of various data types not only strengthens taxonomic evaluations but also offers a more comprehensive perspective on biodiversity (Lim *et al*., 2012).

In this context, it is crucial to employ terminology that encapsulates the intricacies of species identification derived from single-site data. The designation "active taxon" or Operational Taxonomic Unit (OTU) is frequently more suitable than "species" for characteristics identified using restricted datasets (Blaxter *et al*., 2005). This distinction recognises the temporary nature of classifications obtained from single-site analyses and underscores the necessity for caution in interpreting results. This thesis will utilise the word OTU to denote such traits (Muthanen *et al*., 2013; Riedel *et al*., 2013).

The intrinsic variety in morphology, along with the constraints of conventional identification techniques, highlights the necessity for novel methodologies in species categorisation. DNA-based identification serves as an effective solution to these issues, offering a dependable method for connecting juvenile and adult life phases and aiding in the identification of species from hard-to-classify remnants. The ongoing advancements in the discipline will facilitate the incorporation of genetic data into taxonomy study, thereby augmenting our comprehension of species variety and making substantial contributions to ecological studies and conservation initiatives (Sperling *et al*., 1995). Hebert *et al*. (2003) emphasised that the notion of a taxonomy fundamentally reliant on DNA, as well as DNA-based identification methods, is not a recent concept. Earlier proposals, such those by Sperling *et al*. (1995) and Wells and Sperling (2001), established the

foundation for incorporating molecular data into taxonomy methodologies. Hebert *et al*. (2003) presented the concept of "DNA barcoding," likening it to the barcodes used in grocery stores. Their primary argument asserts that analogous barcodes are present in the animal realm, functioning as a dependable method of identification.

While the word "barcode" was previously utilised in certain research contexts, including soil nematodes (Floyd *et al*., 2002) and Plasmodium strains (Arnot *et al*., 1993), Hebert *et al*. (2003) expanded its application to the wider field of animal identification. The optimal genetic marker for DNA barcoding is a ~650 bp segment obtained from the 5'-end of the mitochondrial cytochrome oxidase I (COI) gene. This marker has demonstrated efficacy in species differentiation, as indicated by the extensive database curated by Life Data Systems, which presently houses over 4.4 million animal COI barcodes (Ratnasingham and Hebert, 2007). Concerns have been expressed about the methodology and efficacy of DNA barcoding, especially regarding its application in phylogenetic research. A significant concern is the susceptibility of distance-based approaches, such Neighbour Joining (NJ), to variations in species lineage and the sequence in which species are incorporated into the data matrix. Farris *et al*. (1996) and Felsenstein and Felenstein (2004) emphasised that these factors might profoundly affect the resultant phylogenetic trees, potentially resulting in erroneous interpretations of species relationships.

Notwithstanding these critiques, the rapidity of NJ and like techniques persists as a principal justification for their ongoing application in barcoding research. In extensive datasets comprising thousands of sequences, the efficacy of these techniques frequently surpasses that of the more computationally demanding maximum likelihood methods. Barcoding research generally does not endeavour to establish extensive evolutionary links; instead, it focusses on aiding species identification. The diminutive, swiftly changing barcode loci, although beneficial for species differentiation, offer restricted phylogenetic signal, especially at more profound evolutionary tiers (Hajibabaei *et al*., 2006). Consequently, trees derived from barcode data are predominantly utilised as visual representations to depict sequence-based classifications rather than to deduce evolutionary relationships.

Kimura's two-parameter model of nucleotide substitution is a prevalent framework employed in distance-based investigations and has achieved considerable recognition. This approach is sometimes implemented without comprehensive model testing, raising questions regarding the stability of the conclusions. Model selection is crucial for the correctness of phylogenetic reconstructions, and overlooking this factor can compromise the validity of the results (Srivathsan and Meier, 2012). Furthermore, a study by Srivathsan and Meier (2012) suggests that "barcode spacing," the genetic distance between species indicated by barcodes, may not substantially influence the results of distance-based analyses, implying that dependence on such metrics could be deceptive.

Another aspect of DNA barcoding is the possibility of character-based analysis, which emphasise nucleotide alterations instead of solely examining the gaps between sequences. Nonetheless, distance-based identification approaches possess intrinsic limitations. The "best match" for any query sequence is frequently located in the barcode master database, potentially resulting in misidentifications if closely related species are insufficiently distinguished by their barcodes. This dependence on matching may undermine the precision of species identification, especially when barcode sequences exhibit similarity due to recent divergence or common ancestry (DeSalle *et al*., 2005). Although DNA barcoding is an effective instrument for species identification, it is crucial to acknowledge its limitations and the critiques regarding its techniques. The susceptibility of distance-based approaches to variations in pedigree and species inclusion sequence, dependence on inadequately validated models, and the risk of misidentification stemming from "best match" criteria highlight the necessity for careful interpretation of findings (DeSalle *et al*., 2005). As the discipline advances, integrating more rigorous procedures and resolving these issues will be essential for improving the reliability and utility of DNA barcoding in biodiversity research and conservation initiatives. The differentiation between DNA barcoding as an identification method and the overarching notion of DNA categorisation is essential for comprehending its uses and constraints. DeSalle *et al*. (2005) assert that DNA barcoding is essentially a pattern recognition method that utilises distinct combinations of diagnostic features

included in barcode sequences. This method enables researchers to identify the presence or absence of these characters, thus enhancing the reliability of recognition result interpretations.

In practice, DNA barcoding is frequently utilised as a preliminary screening instrument for delineating species boundaries rather than as a conclusive classification system. This initial role has ignited considerable discourse within the scientific community, with both advocates and detractors participating in discussions regarding the implications and effectiveness of barcoding. Goldstein and DeSalle (2011) emphasise that much of the controversy stems from misunderstandings regarding the aims of DNA barcoding in contrast to traditional classification techniques. This misconception may result in erroneous interpretations of the outcomes and expectations concerning the efficacy of barcoding. Moreover, researchers have employed DNA barcoding to investigate species boundaries, a process necessitating meticulous interpretation of genetic data. Research conducted by Mutanen *et al*. (2013), Huemer *et al*. (2014), and Kekkonen and Hebert (2014) demonstrates the efficacy of barcoding as an initial method for defining species boundaries. These studies indicate that although barcoding offers significant insights into genetic diversity and species differentiation, it is not an exhaustive method for classification.

2.6. Physicochemical properties of honey

Honey, a natural sweetener generated by honey bees (*A. mellifera*) from floral nectar, is both a widely consumed food item and a topic of significant scientific inquiry owing to its distinctive physicochemical characteristics and potential health advantages (Mandal and Mandal, 2011). The composition and characteristics of honey can differ markedly due to factors including floral origin, geographic region, climatic conditions, and processing techniques (Bogdanov, 2013). In India, renowned for its diverse flora, honey production serves as a crucial agricultural practice and holds substantial cultural and economic significance. This literature review examines the physicochemical properties of honey produced in India, encompassing its

chemical composition, moisture content, acidity, pH, electrical conductivity, and antioxidant activity, as well as their implications for quality and health benefits.

2.6.1 Chemical Composition of Honey

Honey's chemical composition is intricate, comprising carbohydrates, water, vitamins, minerals, enzymes, amino acids, and phenolic compounds (Molan, 2001). The primary sugar in honey is fructose, closely followed by glucose, both of which contribute to its sweetness and caloric value. Bhat *et al*. (2012) conducted a study examining the sugar composition of diverse honey types from various regions in India, demonstrating that the fructose-to-glucose ratio significantly differs according to the floral source. Honey sourced from floral origins like eucalyptus and mustard exhibited distinct sugar profiles in contrast to wildflower honey. Besides sugars, honey comprises minor quantities of proteins and amino acids, enhancing its nutritional value. Indian honey contains essential amino acids, such as proline, suggesting its potential as a dietary supplement (Kumar *et al*., 2010).

2.6.1.1 Humidity Level

The moisture content is a critical determinant of honey's quality and shelf-life. Elevated moisture levels may result in fermentation and deterioration, whereas reduced moisture levels promote stability (White, 1978). The ideal moisture content for honey is normally around 17-18% (Bogdanov, 2013). In India, various investigations have revealed variable moisture levels in honey samples. For example, Patil *et al*. (2017) observed that honey samples obtained from different Indian states displayed moisture content varying from 16% to 23%. This difference can be linked to environmental conditions, such as humidity and floral sources, as well as harvesting and processing procedures.

2.5.1.2 Acidity and pH

The acidity of honey is mostly attributed to the presence of organic acids, such as gluconic acid, which is generated during the enzymatic conversion of glucose (White, 1978). The pH of honey normally ranges from 3.2 to 4.5, making it somewhat acidic. This acidity is vital for limiting the growth of spoilage bacteria and

adds to honey's long shelf-life (Mandal and Mandal, 2011). Research conducted on Indian honey has showed variable amounts of acidity and pH. A study by Kaur *et al*. (2016) indicated that the pH of honey samples from Punjab ranged from 3.5 to 4.2, matching with the general pH range of honey documented in the literature. The acidity and pH values of honey can vary based on the floral source, indicating the importance of botanical sources in determining honey quality.

2.6.1.3 Electrical Conductivity

Electrical conductivity is a significant physicochemical property of honey that correlates with its mineral composition, particularly the presence of ash and organic acids (Bogdanov, 2013). High electrical conductivity values may indicate the presence of a substantial number of minerals or adulteration with other compounds. Studies conducted in India have demonstrated that the electrical conductivity of honey can vary greatly. Saini *et al*. (2018) reported that honey samples from Himachal Pradesh displayed electrical conductivity values between 0.3 and 1.2 mS/cm, contingent upon the floral source. These findings underscore the necessity for additional research on the ramifications of electrical conductivity in evaluating honey quality.

2.6.1.4 Antioxidant Efficacy

Honey possesses antioxidant capabilities due to the presence of phenolic chemicals, flavonoids, and ascorbic acid (Liu *et al*., 2018). Antioxidants are essential in safeguarding the body against oxidative stress, associated with numerous chronic diseases. The antioxidant properties of honey fluctuate based on flower origins, processing methods, and storage conditions. Numerous research in India have examined the antioxidant properties of indigenous honey. Kumar *et al*. (2010) conducted a study indicating that honey from several floral sources in Uttarakhand demonstrated differing amounts of antioxidant activity, with wildflower honey exhibiting the highest activity. This variance highlights the significance of local flora in influencing the health benefits linked to honey consumption.

2.6.1.5 Physicochemical Characteristics

In addition to chemical composition, the physical qualities of honey, including viscosity, colour, and crystallisation behaviour, are critical markers of its quality. Viscosity, regulated by sugar concentration and moisture content, impacts honey's flow properties and processing (Vargas *et al*., 2008). Colour is a significant attribute, frequently influenced by the floral origin and the existence of pigments. Indian honey exhibits a spectrum of colours, from light amber to dark brown, contingent upon its source. Crystallisation is a natural phenomenon that transpires in honey, affected by variables such as glucose concentration, temperature, and storage conditions (White, 1978). Honey derived from specific floral sources, such mustard and sunflower, exhibits a propensity to crystallise more rapidly than others. Comprehending the crystallisation behaviour of honey is essential for both producers and consumers in assessing honey quality and applicability.

2.6.2 Health Advantages and Therapeutic Attributes

The physicochemical characteristics of honey enhance its health advantages, acknowledged in traditional medicinal systems such as Ayurveda and Unani. Honey is utilised for its antibacterial, anti-inflammatory, and wound-healing attributes (Mandal and Mandal, 2011). Antioxidants in honey have been associated with potential cardiovascular advantages, anti-cancer effects, and enhanced immunological function (Liu *et al*., 2018). Recent research in India has underscored honey's medicinal potential across several health issues. Maiti *et al*. (2020) conducted a study examining the impact of honey on diabetic rats, revealing its efficacy in reducing blood glucose levels and enhancing lipid profiles. These findings underscore the necessity for additional research into the health advantages of honey, especially for traditional medicinal practices in India. The physicochemical characteristics of honey produced in India are affected by several factors, including flower origin, environmental circumstances, and processing techniques. Comprehending these qualities is crucial for evaluating honey quality and its possible health advantages. Notwithstanding the extensive knowledge available, additional research is vital to investigate the variability in honey characteristics across several

areas and floral sources in India. Moreover, standardised methodologies for evaluating honey quality are necessary to enhance consumer knowledge and bolster the local honey economy.

2.7 Major Royal Jelly Protein 2

Royal jelly, a nutrient-dense secretion generated by worker honeybees, has been acknowledged for its critical function in the growth of honeybee larvae and its impact on the health and longevity of the queen. The major royal jelly proteins (MRJPs), especially MRJP2, have attracted considerable interest owing to their multifaceted functions within the honeybee colony. Although extensive study has been performed on the MRJPs of *A. mellifera*, there is increasing interest in elucidating the biological functions of these proteins in *A. cerana*, a species prevalent throughout Asia that is vital for pollination and local agriculture (Zhang *et al*., 2020).

MRJP2 is a pivotal protein present in royal jelly, recognised for its significant involvement in larval development and caste distinction. Zhang *et al*. (2020) observed that MRJP2, in conjunction with other MRJPs, participates in epigenetic alterations that facilitate the distinction of queen bees from worker bees in *A. cerana*. The elevated levels of MRJP2 in royal jelly provided to queen-destined larvae indicate its essential involvement in queen development, encompassing enhanced size, reproductive ability, and lifespan, akin to its role in *A. mellifera* (Buttstedt, Moritz, and Erler, 2014). Nonetheless, there may be nuanced variations in the functionality of MRJP2 in *A. cerana* attributable to species-specific adaptations influenced by environmental conditions and foraging behaviours (Yu *et al*., 2018).

Regarding protein structure, MRJP2 in *A. cerana* has significant homology to its equivalent in *A. mellifera*. Schmitzova *et al*. (1998) performed an early investigation into the structural characterisation of MRJPs, observing that these proteins comprise roughly 420 amino acids and are glycosylated, a characteristic that improves their stability and biological function. Recent proteomic analyses by Yu *et al*. (2018) validated the presence of MRJP2 as a predominant protein in *A. cerana* royal jelly, exhibiting a structure akin to *A. mellifera* MRJP2, albeit with variations

in amino acid composition that may influence the species-specific functions of this protein in diverse environmental contexts.

The functional attributes of MRJP2 surpass its involvement in larval nourishment. MRJP2 is posited to possess immune-modulatory functions in *A. cerana*, offering protection against pathogens in the densely populated and pathogen-laden hive environment. Albert and Klaudiny (2004) discovered that MRJP2 in *A. mellifera* demonstrated antimicrobial properties, suggesting that MRJP2 in *A. cerana* may possess a comparable function. Considering that *A. cerana* frequently resides in environments with distinct microbial pressures relative to *A. mellifera*, the antimicrobial attributes of MRJP2 may be especially crucial for the health of colonies in this species (Han *et al*., 2011). Besides its functions in growth and immunity, MRJP2 may also play a role in social communication inside the hive. Pheromonal communication is essential for sustaining colony organisation and operation, and MRJP2 has been associated with the regulation of reproductive and social hierarchies. Kucharski *et al*. (2008) established that MRJPs, particularly MRJP2, may contribute to pheromonal signalling that affects queen-worker interactions and sustains the social hierarchy within the colony. Although these studies predominantly examine *A. mellifera*, it is probable that analogous mechanisms are present in *A. cerana*; nonetheless, additional research is required to elucidate the specific functions of MRJP2 in the social dynamics of this species.

Recent research has investigated the possible applications of MRJP2 beyond the realm of honeybee biology. Tian *et al*. (2018) examined the interaction of MRJP2 with lipids, emphasising its function in nutrition transport and metabolism. This research highlights the multifunctionality of MRJP2 and indicates that it may have wider biological implications, potentially affecting nutrient delivery to developing larvae and the storage and utilisation of energy within the colony. The existing literature underscores the significance of MRJP2 in *A. cerana* as a multifunctional protein involved in development, immunity, and social regulation. Nevertheless, there is a necessity for more targeted research on *A. cerana*, as the majority of current understanding regarding MRJP2 has been obtained from investigations on *A. mellifera*. Subsequent research will probably uncover further insights into the

species-specific roles of MRJP2 and its contribution to the distinctive biology of *A. cerana* (Kucharski *et al*., 2008).

CHAPTER III

OBJECTIVES

1. Inventory and Monitoring of bee polliniferous native and invasive plant species and evaluation of bee-floral interactions in the Tanhril forest, Mizoram, India.

2. A DNA Barcoding approach to characterize pollen collected by *Apis cerana* colonies.

3. Study the effects of land use change on the quality of *Apis cerana* honey.

CHAPTER IV

MATERIALS AND METHODOLOGY

4. MATERIALS AND METHODOLOGY

4.1 Inventory and Monitoring of bee polliniferous native and invasive plant species

4.1.1 Study area and design

The study was carried out in the vast 365ha Tanhril Tropical Forest, which is situated in the picturesque Aizawl, Mizoram, at the geocoordinates 23° 44' 32.50"N latitude and 92° 40' 38.78"E longitude. The study area spans an elevation gradient of 500–1000 meters above sea level and is rich in biodiversity, offering many chances to explore different ecological niches. A sampling area of 25–50 ha was taken from the 365ha total forest cover in order to examine the bee flower interactions between native and invasive plant species. The quadrat method was used to sample the vegetation. Forty 10-by-10-meter quadrats were randomly sampled in each research stand to measure and record the trees (>10 cm GBH). Herbs were documented by inserting two 1 m × 1 m quadrats into each 10 m x 10 quadrat, whereas shrubs were recorded within the same 10 m x 10 m quadrat. All native and invasive plant species' specimens were gathered, herbariums were set up, and taxonomists from the Botanical Survey of India, Shillong, assisted with the identification process (Curtis and McIntosh, 1950).

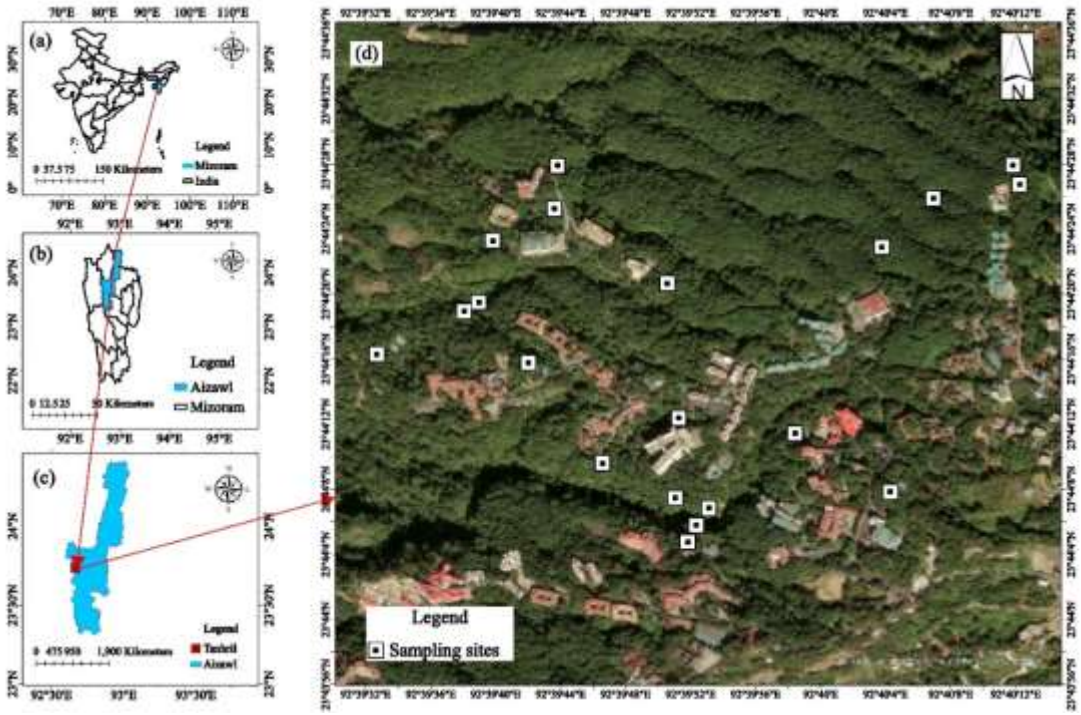

Figure 1. Map showing the sampled localities in the Tanhril forest, Mizoram, Northeast India.

In this study, the Tanhril forest area was selected purposively based on the availability of *A. cerana* bee colonies. A total of 45 sites were selected in the east (10), west (12), north (13) and south (11) zones of Tanhril forest, Aizawl, Mizoram. Flowering plant anthers were gathered, the pollen was extracted, and the acetolysis procedure was used to treat the pollen to create a reference pollen collection for palynological examination and reference data base. Fresh honey samples (20 nos.) were collected from the four zones of Tanhril forest, Aizawl, Mizoram, at four different seasons (winter, fall, rainy and spring) for laboratory analysis (Mueller-Dombois, 1974).

4.1.2 Floral sampling of native and invasive

Important community metrics were determined by following Misra (1968) and Muller–Dombois and Ellenberg (1974), including frequency, density, abundance, basal area, and significance value index (IVI) of all plant species. For tree species, the IVI was computed by adding the relative values of basal area,

density, and frequency; on the other hand, the relative values of frequency and density were added to obtain the IVI for shrubs and herbs.

4.1.3 Important Value Index

A number of collection sites were carefully chosen in order to undertake a comprehensive study of the forest, and a methodical procedure was employed to sample the various vegetation. To survey the plants, shrubs, and trees, precisely measured transects measuring 1 × 1 m for herbs, 3 × 3 m for shrubs, and 25 × 25 m for trees were employed. Capturing the nuances of the vegetation structure at different scales allowed this spatially variable sampling methodology to aim for a more nuanced understanding of the ecological dynamics at play. A thorough assessment of the composition and organisation of the forest ecosystem was made possible by the inclusion of transect sizes that varied, ensuring a representative sampling of the entire plant community (Curtis, and Mcintosh, 1950; Ellenberg, and Mueller Dombois, 1974).

$$\text{Density} = \frac{\text{Total no. of individual of a species}}{\text{Total number of quadrats studied}}$$

$$\text{Frequency} = \frac{\text{No. of quadrate occurrence of a species}}{\text{Total number of quadrats studied}}$$

$$\text{Abundance} = \frac{\text{Total no. of individual of a species}}{\text{Total number of quadrats occurrence a Species}}$$

$$\text{Relative density}(\%) = \frac{\text{Density of a species}}{\text{Total density of all the species}} \times 100$$

$$\text{Relative frequency}(\%) = \frac{\text{Frequency of a species}}{\text{Total frequency of all the species}} \times 100$$

$$\text{Relative dominance}(\%) = \frac{\text{Basal area of a species}}{\text{Total basal area of all the species}} \times 100$$

Important value index (IVI) = Relative frequency + Relative density + Relative dominance (Curtis, and Mcintosh, 1950; Ellenberg, and Mueller Dombois, 1974).

4.1.4 Honey bee floral interaction studies

The specimens were sampled twice, over three days each, between September 2021 and August 2023, one for each of the four seasons (rainy, winter, autumn, and spring). We established two 180 × 20 m transects along a path within the forest. Only blossoming plants were sampled at each transect. Every transect's samples were limited to blooming plants. Since most bees feed during the day, especially in the early morning, the collection time was from 6:00 am to 6:00 pm (Kelber *et al*., 2005). Each season required 108 hours of sampling (12 hours × 3 days × 3 months), with two people gathering bees and one person gathering pollen and plant samples. All native and invasive plant species' specimens were gathered, herbariums were set up, and taxonomists from the Botanical Survey of India, Shillong, assisted with the identification process. Bees from flowering plants were collected using insect nets, with five minutes allotted to each plant. Both transects underwent continuous sampling, with about five counts per plant each day (Flórez-Gómez *et al*., 2020).

4.2 DNA barcoding of pollen in honey

4.2.1. Melissopalynology

In order to prepare a reference pollen collection for palynological examination, blooming plant anthers were collected, the pollen was extracted, and the pollen was treated using the acetolysis procedure. The pollen were placed on each micro-slide, which was used to directly mount the pollen samples. We inspected and noted the pollen grains using an LEICA DM500 magnifying lens, capable of magnifying up to 100 times. Additionally, these served as reference pollen for palynological collections, which helped to distinguish between the many types of pollen in honey. Honey pollen analysis was carried out following the procedure adopted by Louveaux *et al*. 1978 for the determination of the botanical composition and frequency of pollen types in honey. The pollen isolated from honey were placed on each micro-slide, which were used to directly mount the pollen samples. A comparison was done between the reference slides made from the specified plants and the pollen grains that were extracted from the honey samples to identify the plant

species. Pollen sample were categorized and counted on the basis of native and invasive plant species (Tulu *et al*., 2023).

4.2.2 Reference DNA barcoding database

A specialised DNA barcoding reference library of 95 plant taxa was put together for this work. Plant entries were chosen from the floristic list pertaining to the Tanhril Natural forest. DNA barcoding sequences from all the most frequent species found in regions where honey is produced and pollinated by bees. Rare and endemic species were also mentioned. As supporting information, a comprehensive list of the species chosen as references for DNA barcoding analysis was given.

DNA barcode sequences for *matK* and *rbcL* were taken into consideration for each taxon. Young leaves or buds were taken from each individual and kept in storage at -20°C. Following the guidelines provided by the Global Registry of Biodiversity Repositories (http://grbio.org/) and the data requirements for BARCODE Records, all samples were vouchered as "MZU-TNRL" ((Hanner, 2009). Table 1 contains a list of specimen and voucher codes.

4.2.3 DNA extraction and purification

. Each honey sample was heated to 45 °C for five minutes after being diluted with 25 millilitres of distilled water, resulting in a total volume of 50 millilitres. Following centrifugation for 20 minutes at 13,000 rpm, the pellet was resuspended in 20 millilitres of distilled water and agitated to achieve dissolution. The supernatant was discarded. After an additional 20 minutes of centrifugation at 13,000 rpm, the pellet (about 120 mg) was resuspended in 200 µl of 1 x TE buffer. Utilising a DNeasy Isolation and Purification kit (Qiagen, Hilden, Germany), 100 microlitres of each processed sample were employed for DNA extraction. To check the purity of the extracted DNA, we analyse the genomic DNA using 1% agarose gel electrophoresis. Following gel electrophoresis, the DNA samples were quantified using the NanoDropTM 1000 Spectrophotometer (Thermo Scientific, U.S.A.), with concentrations exceeding 10 ng/µl selected for subsequent analysis. Using the same commercial kit, 100 mg of fresh plant materials (young leaves or buds) were utilised

for the DNA extraction of the local species that were used to create the reference DNA database. With a NanoDropTM 1000 Spectrophotometer (Thermo Scientific, U.S.A.), the purified DNA concentration of each sample was determined both fluorometrically by measuring the absorbance (Abs) at 260 nm, above the concentration of 10 ng/µl were taken for further analysis. For more confirmation whether good quality of DNA was extracted or not we track the genomic DNA in agarose gel (1%). The DNA were utilised as templates for DNA barcoding investigations.

4.2.4. Amplification, cloning and sequencing of barcode regions

Using the plastidial *rbcL* region and the *matK* intergenic spacer, DNA barcoding study was carried out. The primer combination used for rbcL PCR amplification and sequencing was *rbcLa*: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' and *rbcLb*: 5'-GTAAAATCAAGTCCACCRCG-3' (Fay, Bayer, Alverson, de Bruijn and Chase, 1998). For *matK*, the primer combination was *matK-F*: 5'-3' and *matK-R*: 5'-3'. Following the manufacturer's recommendations, PCRs were carried out using Bio-Rad T100^{TM} Thermal Cycler PCR in a 25 µL reaction, commencing with 10 ng of DNA. A first denaturation stage lasting three minutes at 94 °C was followed by 35 cycles of denaturation (40 s at 94 °C), annealing (40 s at 50 °C for *rbcL* and 40 s at 52 °C for *matK*), extension (40 s at 72 °C), and a final extension lasting seven minutes at 72 °C.

Sequencing was done immediately on PCR results that came from the reference species. By using 1.2% (w ⁄ v) agarose electrophoresis, the amplification products from the honey samples were examined. Using the pGEM-T Easy Vector System (Promega Corporation, Madison, WI, USA), the PCR products were cloned. The Miniprep kit from Applied Biosystems in Foster City, CA was used to isolate recombinant plasmids. The DNA content and insert size were determined using gel electrophoresis on 2.0% (w ⁄ v) agarose that had been stained with ethidium bromide. To move further with the insert sequencing, 100 clones were randomly chosen for each of the five honey samples.

The identical primer pairs that were utilised in the PCR stage were used to conduct bidirectional sequencing on all samples (clones and reference species) for every barcode region. An automated ABI 155 3730XL sequencer at Macrogen Inc. in Korea was used to obtain the sequences. Our method of correcting sequencing problems involved manually altering raw traces and then aligning the forward and reverse sequences. For each taxon, consensus sequences were created by trimming the 3~ and 5~ terminals. Whitlock, Hale, and Groff (2010) reported that the EMBOSS Software program (Rice, Longden, and Bleasby, 2000) was utilised to identify short inverted repeat areas in the trnH-psbA spacer. The occurrence of inversions in the trnH-psbA area was detected using the EINVERTED algorithm (Guindon and Gascuel, 2003) with default parameters.

First, the 100 sequences were aligned using Clustal W XI (Larkin *et al*., 2007) and then MEGA XI (Tamura *et al*., 2011) was used to analyse the data and construct MOTUs (Molecular Operational Taxonomic Units). This process helped identify the composition of honey. The obtained sequences were run through a BLAST analysis (Altschul, Gish, Miller, Myers and Lipman, 1990) on the specific DNA barcoding reference database (Table S1, Supporting information) in order to determine the botanical composition of the four honeys. According to each MOTU, the species displaying the closest matches (maximum identity) were designated (Bruni *et al*., 2012; De Mattia *et al*., 2012). The MOTU was deemed "unidentifiable" if the identity match value was less than 99%. To identify the plant species in honey samples, the two examined markers underwent separate analyses, with the results merged.

Accumulation curves with 1,000 iterations were performed for each sampling site and for both DNA barcoding markers using EstimateS 8.2.0 in order to determine whether the number of clones sequenced per sampling site was sufficient to detect the vast majority of pollen sequences. To exclude any effect from variations in plant phenology between sites, the cloned sequences acquired for each marker and sampling location throughout the three collecting periods were combined (Colwell, 2006)).

4.3 Effects of land use change on the quality of *Apis cerana* honey

4.3.1 Study Area

In Aizawl, Mizoram, field work was conducted on 365 hectares of natural woodland and 90 H of shifting agriculture habitat, which are located at (23° 30'–24° and 92° 30'–93° 00' E). Twenty locations in all were selected for natural habitat gathering and another twenty for changing agriculture habitat. Every site was one to three kilometres apart. Honey samples were collected four times (winter, spring, rainy and fall season) at the maturity phase (5 per site) during **September 2020** to **August 2023** from natural forest habitat and shifting cultivation habitat. Thus, eighty different honey samples were collected aseptically for this study. Once collected, samples were kept in sterile vials, sealed, labelled, dated, and stored at room temperature (25–30 °C) until analysis.

4.3.2 Physicochemical Characterization of Honey Samples

4.3.2.1 Moisture content

Refractometry was used to determine the water content. A standard model Abbetype refractometer was used to measure the refrac-tive index (RI) at 20 °C in accordance with the Harmonised procedures of the International Honey Commission procedures (2009). The refractive index was measured and noted. After measuring each sample twice, the average value was determined.

4.3.2.2. pH

The crude honey's pH level was extremely unstable and difficult to evaluate. Consequently, honey was diluted with a 10% (w/v) solution. Prior to usage, the pH meter was calibrated at pH 3.7 (4.0), 7.0, and 9.0. A sample of 10 g honey was dissolved in 75 ml of distilled water without carbon dioxide. A magnetic stirrer was used to combine this solution. The solution was submerged beneath the pH electrodes. The honey solution's pH was measured to two decimal places.

4.3.2.3 Electrical conductivity

Electrical conductivity (EC) measurements were first made a long time ago (Vorwohl, 1964). Currently, it is the most helpful quality criterion for categorising unifloral honeys, which may be ascertained using comparatively cheap equipment. The data presented in this issue (Persano Oddo and Piro, 2004) has corroborated this. On the basis of an extensive survey of EC values on honeys originating from different parts of the world (Bogdanov *et al*., 1999), this parameter was included recently in the new international standards for honey (Codex Alimentarius, 2001; European Commission, 2002), replacing the determination of ash content. Indeed, EC correlates well with the mineral content of honey, (Accorti *et al*., 1987). In these standards maximal EC values for blossom honeys (except chestnut honey) are introduced for differentiation between honeydew and blossom honeys.

4.3.2.4 HMF content

This technique was developed in the Honey Research Lab, Department of Molecular Medicine, University of Malaya Faculty of Medicine, drawing inspiration from White's 1979 original research. 5-(hydroxymethyl-) furan-2-carbaldehyde (HMF) concentration was ascertained using this technique. Milligrams per kilogramme (mg/kg) is the standard unit of measurement for the HMF level result. The HMF level was measured by precisely weighing 5.0g of each honey sample into a beaker. Once the 5.0g honey sample was fully diluted and dissolved, 25.0 ml of distilled water was added and thoroughly mixed. Next, the blended mixture was put into a 50 ml volumetric flask.

The volumetric flask was filled with 0.5 ml of Carrez solution I, and it was well mixed by vortexing. After adding Carrez solution II to the combined solution, it was thoroughly mixed using a vortex. After that, the volumetric flask was filled to the brim with distilled water. It may be necessary to add a drop of ethanol to reduce the froth that forms when mixing. After then, filter paper was used to filter this combination. After filtering, the first 10 ml of the solution were discarded, and the remaining solution was saved. The filtrated solution was pipetted into two different test tubes, each holding 1.0 ml of volume.

The test tube containing the honey solution was filled with 1.0 ml of distilled water for the sample solution. After thoroughly mixing the solution with a vortex, 1.0 ml of 0.2% sodium bisulphate was added and thoroughly mixed as a reference. Using a 1cm quartz cell, the absorbance of the sample solution at 284 nm and 336 nm was measured against the reference solution in less than an hour. In order to get a sample absorbance low enough for accurate results, distilled water and 0.2% bisulphite were added to the reference if the absorbance at 284 nm was more than the value of 0.6. Every sample of honey was prepared and put through three tests. Outcomes were noted as Mean ± Standard Error Mean (S.E.M).

4.3.2.5 Hydrogen peroxide

3 g of honey were weighed and then diluted with 10 mL of distilled water to yield a 30 % (w/v) concentration. The mixture was then allowed to incubate for 30 minutes at 37 ºC in a waterbath. The test strips from the kit were dipped into the mixture, and the concentrations of the H_2O_2 produced were determined by comparing the colour created with the colour code. To ensure accuracy, each sample was examined three times, and the outcomes were noted.

4.3.2.6 Proline

The proline content was assessed following the official TS 13357 procedure of Akgün *et al*. 2021. The process entails the creation of a coloured complex due to the reaction involving proline and ninhydrin, along with spectrophotometric assessment of colour intensity at a wavelength of 510 nm. The proline concentration is quantified as mg proline per kilogramme of honey.

4.3.2.7 Total phenolic content

The total phenolic content of the honey samples was assessed using the Folin–Ciocalteu method as outlined by Gülçin *et al*. 2004. A 1 mL aliquot of honey stock solution was diluted with 46 mL of distilled water, followed by the addition and thorough mixing of 1 mL of Folin-Ciocalteu reagent. After three minutes, 3 mL of 2% Na_2CO_3 was introduced, and the mixture was diluted to a final volume of 50 mL, then incubated at room temperature in the dark for 2 hours with periodic shaking.

Absorbance measurements at 760 nm were documented. A standard curve was constructed utilising gallic acid to determine the concentration of total phenolic compounds in honey (mg gallic acid equivalent (GAE)/g honey).

4.3.2.8 Total flavonoid content

Total flavonoid content of honey samples were assessed by aluminium chloride ($AlCl_3$) colorimetric method (Hoerudin, 2004). Five grammes of each honey sample were dissolved in 25 mL of 80% methanol. One millilitre of each honey solution was put to a test tube. Thereafter, analogous processes employed for the construction of the standard curve were utilised to assay 1 mL of each standard solution. A corresponding blank was created for each honey sample, substituting the identical volume of 10% AlCl3 solution (0.2 mL) with distilled water. Absorbance measurements were obtained using a UV/Visible spectrophotometer at 415 nm.

4.3.2.9 Total acidity

Titrimetric analysis was conducted to ascertain total acidity according to the method outlined by Bogdanov, 2011. The acidity was determined by measuring the volume of 0.1 M NaOH required to titrate a diluted honey sample (10 g: 75 mL distilled water) to a pH of 8.3, with findings reported in milliequivalents per kilogramme of honey.

4.3.2.10 Bacterial load

Honey samples were stored in a dark environment at between 23 and 25 °C. Honey samples were utilized at 75, 50, and 25% dilutions in addition to being used undiluted to assess antibacterial potential. Every bacterial isolate underwent testing for antibacterial activity. It was done in accordance with the National Committee for Clinical Laboratory Standards' disc diffusion method (Bauer and Kirby 1966). A mixture of 25 liters of each honey dilution and streptomycin (positive control) was used to create filter paper discs. Using a sterile cotton swab, the test organism cultures were streaked onto Nutrient agar plates. After being put on the plates, the discs were incubated for 24 hours at 37˚C. The measurements made in accordance

with Patel *et al*. (2017) yielded the widths of the zones of inhibition (Iqbal *et al*., 2015).

4.3.2.11 Fructose/glucose ratio

Using glucose oxidase reagent (Randox Laboratories Ltd., UK), the glucose content of the honey samples was determined by enzymatic oxidation. Twenty microlitres (20 µL) of the standard or sample were combined with 2.0 millilitres (mL) of the reagent, thoroughly mixed, and incubated at 37°C for ten minutes. In less than 60 minutes, the absorbance of the sample (Asample) and standard (Astandard) was measured against a reagent blank. This is how the glucose concentration was determined (Buba *et al*., 201)

Glucose content (mg/dL) = $(A_{sample}/A_{standard})$ x Conc. of standard

$$= (A_{sample}/A_{standard}) \times 100 \text{ (mg/dL)}$$

AOAC (2000) employed the resorcinol reagent method to assess the fructose content. After thoroughly mixing 1.0 mL of resorcinol reagent into a honey sample solution, 1.0 mL of diluted HCl was added. Standard solutions with concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL that were diluted to a volume of 2 mL using distilled water were likewise subjected to the same 1.0 mL resorcinol reagent and 1.0 mL diluted HCl treatment. Along with the standard, a blank solution was also developed and handled similarly. After heating the test solution, standard, and blank solutions in a water bath for approximately ten minutes at 80°C, the solutions were taken out and allowed to cool for five minutes under running tap water. After that, the absorbance of the test and standard solutions was measured at 520 nm within thirty minutes, compared to the blank solution. Next, utilising a standard curve created with the standard's absorbance, the fructose contents of the honey samples were extrapolated (Buba *et al*., 2011). The fructose to glucose ratio was computed after the acquisition of the values at each site.

4.3.2.12 Colour intensity

The CIE L*a*b* approach was used to evaluate the colour of honey samples. In this method, brightness is represented by L*, and a* and b* are two colour coordinates. The colour was determined by Spectrophotometer CM2600d (Konica inolta, Japan). The measured honey's hue, brightness, and saturation are represented by the values of L*a*b*. We used standard illumination D65, 10 ° observer SCE measurements (the measurement of specular reflection is not included in SCE mode). Additionally, colour difference was calculated using reflectance measurements in spectra with wavelengths between 360 and 740 nm. After heating honey samples to 40 °C to dissolve sugar crystals, they were put into a white plastic container with a diameter of 29 mm, a honey layer height of 17 mm, and a capacity of 10 ml. The glass plate was then placed on top of the honey container to prevent air bubbles from forming on the surface.

4.3.3 Major royal jelly protein

4.3.3.1 DNA extraction

Each honey sample was heated to 45 °C for five minutes after being diluted with 25 millilitres of distilled water, resulting in a total volume of 50 millilitres. Following centrifugation for 20 minutes at 13,000 rpm, the pellet was reconstituted in 20 millilitres of distilled water and agitated to achieve dissolution. The supernatant was discarded. The particle, approximately 120 mg, was resuspended in 200 µl of 1 x TE buffer following a further 20 minutes of centrifugation at 13,000 rpm. Utilising a DNeasy Isolation and Purification kit (Qiagen, Hilden, Germany), 100 microlitres of each processed sample were employed for DNA extraction. To verify the purity of the collected DNA, we conduct a genomic DNA analysis using 1% agarose gel electrophoresis. Following gel electrophoresis, DNA samples were quantified using the NanoDropTM 1000 Spectrophotometer (Thermo Scientific, U.S.A.), with concentrations exceeding 10 ng/µl selected for subsequent analysis.

4.3.3.2 Polymerase chain reaction

The polymerase chain reaction (PCR) amplification was performed in a 20 µL reaction volume comprising 10 µL of 2× Taq PCR StarMix with Loading Dye (GenStar BioSolutions Co., Ltd., Beijing, China), 10 µM of each oligonucleotide primer, 2 µL of template DNA (C-F 5'-TTTAACAATAAAAATAATCAGAAGA-3' and C-R 5'-TTACATCCTAATTGATTTTAATGCG-3'), and 6 µL of double-distilled water. In duplex PCR, two pairs of primers were incorporated, and the volume of distilled water was decreased to 4 µL, but the other components remained constant. The thermal cycling protocol included an initial pre-denaturation at 94 °C for 2 minutes, succeeded by 35 cycles including denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, and concluding with a final extension at 72 °C for 5 minutes. The PCR amplification was conducted using a T-Gradient Thermoblock (Biometra, Göttingen, Germany). The PCR results (7 µL each reaction) were subjected to electrophoresis in a 2% Tris-acetate-EDTA agarose gel (Biowest, Shanghai, China) supplemented with 0.01% Gelview (BioTeke, Beijing, China). The agarose gel was visualised and captured under UV light with an automatic gel image analyser (JS-6800, Shanghai Peiqing Science and Technology Co., Ltd., Shanghai, China). The favourable products were recycled and subsequently sequenced from both termini by an ABI 3730XL automated sequencing machine (TSINGKE, Hangzhou, China). Sequence specificity analyses for the MRJP2 segment were conducted utilising BLAST (http://blast.ncbi.nlm.nih.gov/) (National Library of Medicine, Bethesda, MD, USA) (Zhang *et al*., 2019).

4.3.3.3 Agarose gel analysis using ImageJ

The 'Gel Analysis' tool in ImageJ was employed to quantify band intensities (Schneider *et al*., 2012). The procedure encompassed the subsequent steps:

- Access the gel image using ImageJ.
- Utilise the 'Rectangular' tool to delineate a rectangle encompassing the initial lane.
- Utilise 'Analyse > Gels > Select First Lane' to designate the lane.

- Shift the rectangular selection to the subsequent lane and replicate for all lanes.
- After selecting all lanes, the command 'Analyse > Gels > Plot Lanes' was employed to produce a densitometric profile for each lane.
- The peak regions associated with DNA bands were discovered and quantified using the 'Wand (tracing) tool' to determine band intensity (area under the curve) in arbitrary units (a.u.).

4.3.3.4 Statistical analysis

The data were subjected to statistical analysis utilising instat, Excel, Prism. The intensities were presented as mean values ± standard deviation derived from 3 replicates. The statistical significance between samples was assessed using ANOVA, succeeded by Tukey's multiple comparison tests.

CHAPTER V

RESULTS

5. Results

5.1 Honeybee-floral interactions in the Tanhril forest

Table 1. List of specimen and voucher codes

SN	Plant name	VOUCHER NUMBER
1	*Mangifera indica*	TMZ001
2	*Rhus chinensis*	TMZ002
3	*Impatiens balsamina*	TMZ003
4	*Bombax ceiba*	TMZ004
5	*Terminalia crenulata*	TMZ005
6	*Terminalia bellirica*	TMZ006
7	*Cucumis sativus*	TMZ007
8	*Thladiantha cordifolia*	TMZ008
9	*Momordica charantia*	TMZ009
10	*Cyperus rotundus*	TMZ010
11	*Tetrameles nudiflora*	TMZ011
12	*Elaeocarpus lanceifolius*	TMZ012
13	*Emblica officinalis*	TMZ013
14	*Mallotus paniculatus*	TMZ014
15	*Acacia pruinescens*	TMZ015
16	*Bauhinia variegate*	TMZ016
17	*Derris robusta*	TMZ017
18	*Indigofera zollingeriana*	TMZ018
19	*Parkia timoriana*	TMZ019
20	*Castanopsis tribuloides*	TMZ020
21	*Holmskioldia sanguine*	TMZ021
22	*Leucosceptrum canum*	TMZ022
23	*Punica granatum*	TMZ023
24	*Colona floribunda*	TMZ024
25	*Moringa oleifera*	TMZ025
26	*Musa paradisiaca*	TMZ026
27	*Eucalyptus tereticornis*	TMZ027
28	*Averrhoa carambola*	TMZ028
29	*Rosa macrophylla*	TMZ029
30	*Ixora coccinea*	TMZ030
31	*Citrus limon*	TMZ031
32	*Acronychia pedunculata*	TMZ032
33	*Schima wallichii*	TMZ033
34	*Callicarpa arborea*	TMZ034
35	*Amaranthus spp.*	TMZ035
36	*Coriandrum sativum*	TMZ036
37	*Asclepias curassavica*	TMZ037
38	*Ageratum conyzoides*	TMZ038

39	*Bidens pilosa*	TMZ039
40	*Cosmos sulphureus*	TMZ040
41	*Matricaria chamomilla*	TMZ041
42	*Mikania micrantha*	TMZ042
43	*Spilanthes acmella*	TMZ043
44	*Tagetes erecta*	TMZ044
45	*Tithonia diversifolia*	TMZ045
46	*Zinnia elegans*	TMZ046
47	*Crassocephalum crepidioides*	TMZ047
48	*Alnus nitida*	TMZ048
49	*Tecoma stans*	TMZ049
50	*Bixa orellana*	TMZ050
51	*Brassica camprestris*	TMZ051
52	*Raphanus sativus*	TMZ052
53	*Carica papaya*	TMZ053
54	*Sechium edule*	TMZ054
55	*Croton joufra*	TMZ055
56	*Euphorbia pulcherrima*	TMZ056
57	*Riccinus communis*	TMZ057
58	*Macaranga peltata*	TMZ058
59	*Caesalpinia pulcherrima*	TMZ059
60	*Cassia javanica*	TMZ060
61	*Mimosa pudica*	TMZ061
62	*Leucaena leucocephala*	TMZ062
63	*Phaseolus vulgaris*	TMZ063
64	*Pisum sativum*	TMZ064
65	*Senna spectabilis*	TMZ065
66	*Tamarindus indica*	TMZ066
67	*Crotalaria pallida*	TMZ067
68	*Delonix regia*	TMZ068
69	*Coleus scutellarioides*	TMZ069
70	*Lagerstromia speciosa*	TMZ070
71	*Althaea rosea*	TMZ071
72	*Anthurium andreanum*	TMZ072
73	*Hibiscus rosa sinensis*	TMZ073
74	*Ipomoea batatas*	TMZ074
75	*Malvaviscus arboreus*	TMZ075
76	*Urena lobata*	TMZ076
77	*Triumfetta rhomboidea*	TMZ077
78	*Callistemon lanceolatus*	TMZ078
79	*Psidium guajava*	TMZ079

80	*Syzygium cumini*	TMZ080
81	*Syzygium jambos*	TMZ081
82	*Oryza sativa*	TMZ082
83	*Zea mays*	TMZ083
84	*Antigonon leptopus*	TMZ084
85	*Portuluca oleracea*	TMZ085
86	*Prunus persica*	TMZ086
87	*Coffee Arabica*	TMZ087
88	*Jatropha curcus*	TMZ088
89	*Nicotianum tobaccum*	TMZ089
90	*Solanum melongena*	TMZ090
91	*Solanum torvum*	TMZ091
92	*Tropaelum majus*	TMZ092
93	*Lantana camara*	TMZ093
94	*Vitis vinifera*	TMZ094
95	*Mussaenda erythrophylla*	TMZ095

List of plants samples collected from Tanhril forest, Mizoram. Voucher number started from TMZ01-TMZ095, where T- stands for Tanhril forest and MZ- stands for Mizoram.

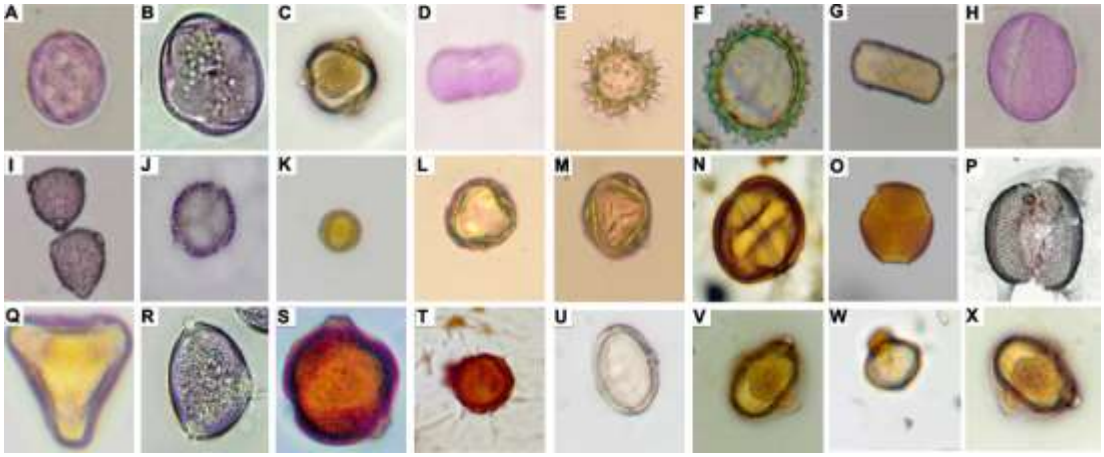

Figure 2. Bee polliniferous native and invasive plants observed in the sampling sites of Tanhril forest area (East Zone). Plant photograph A) *Amaranthus sps.*, B) *Mangifera indica*, C) *Rhus chinensis*, D) *Coriandrum sativum*, E) *Asclepias curassavica*, F) *Ageratum conyzoides*, G) *Bidens pilosa*, H) *Cosmos sulphureus*, I) *Matricaria chamomilla*, J) *Mikania micrantha*, K) *Spilanthes acmella*, L) *Tagetes erecta*, M) *Tithonia diversifolia*, N) *Zinnia elegans*, O) *Crassocephalum crepidioides*, P) *Impatiens balsamina*, Q) *Alnus nitida*, R) *Tecoma stans*, S) *Bixa orellana*, T) *Bombax ceiba*, U) *Brassica camprestris*, V*) Raphanus sativus*, W) *Carica papaya* and X) *Terminalia crenulata*.

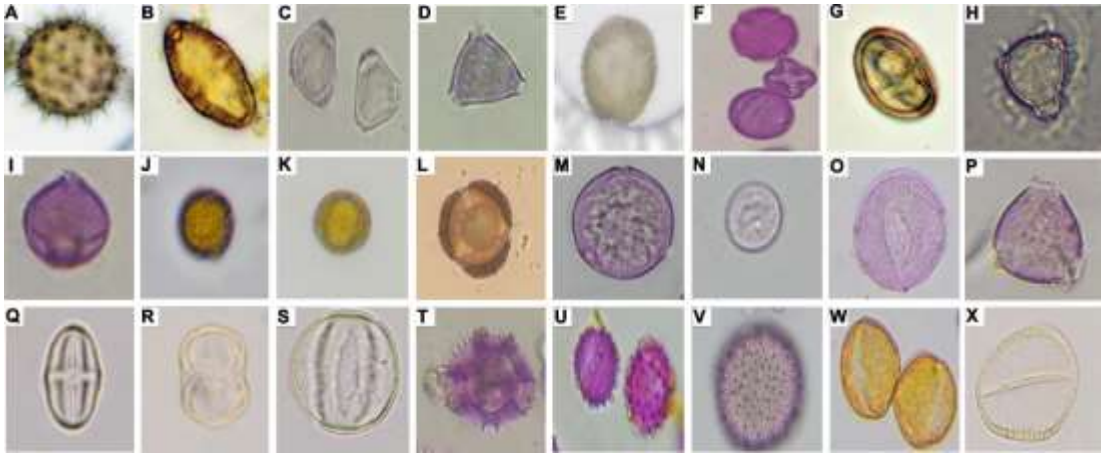

Figure: 3. Bee polliniferous native and invasive plants observed in the sampling sites of Tanhril forest area (West Zone). A) *Terminalia bellirica*, B) *Cucumis sativus*, C) *Thladiantha cordifolia*, D) *Momordica charantia*, E) *Sechium edule*, F) *Cyperus rotundus*, G) *Tetrameles nudiflora*, H) *Elaeocarpus lanceifolius*, I) *Croton joufra*, J) *Emblica officinalis*, K) *Euphorbia pulcherrima*, L) *Riccinus communis*, M) *Mallotus paniculatus*, N) *Macaranga peltate*, O) *Acacia pruinescens*, P) *Bauhinia variegate*, Q) *Caesalpinia pulcherrima,* R) *Cassia javanica*, S) *Derris robusta*, T) *Mimosa pudica*, U) *Indigofera zollingeriana*, V*) Leucaena leucocephala*, W) *Parkia timorian* and X) *Phaseolus vulgaris*.

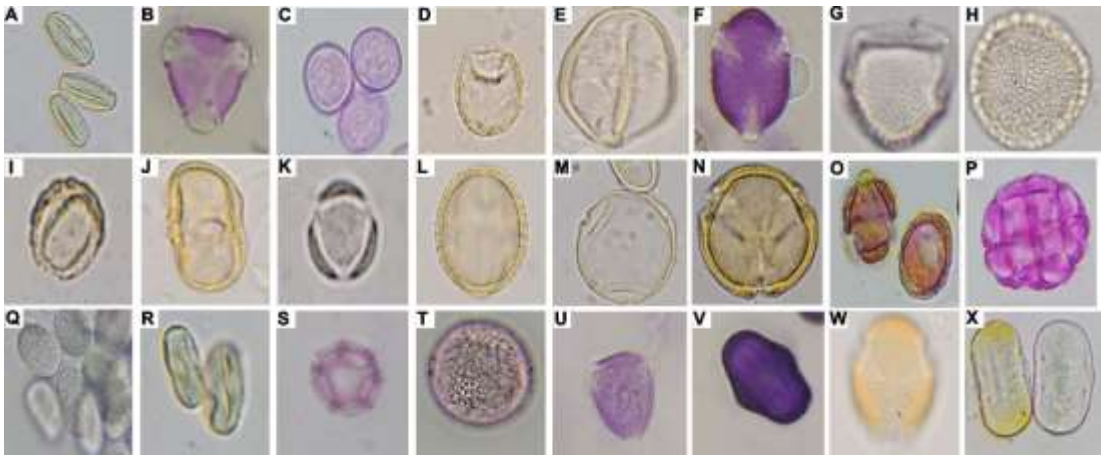

Figure: 4. Bee polliniferous native and invasive plants observed in the sampling sites of Tanhril forest area (North and South Zone). A) *Pisum sativum*, B) *Senna spectabilis*, C) *Tamarindus indica*, D) *Crotalaria pallida*, E) *Delonix regia*, F) *Castanopsis tribuloides*, G) *Holmskioldia sanguine*, H) *Leucosceptrum canum*, I) *Coleus scutellarioides*, J) *Lagerstromia speciosa*, K) *Punica granatum*, L) *Althaea rosea*, M) *Anthurium andreanum*, N) *Colona floribunda*, O) *Hibiscus rosa sinensis*, P) *Ipomoea batatas*, Q) *Malvaviscus arboreus,* R) *Urena lobata*, S) *Triumfetta rhomboidei*, T) *Moringa oleifera*, U) *Musa paradisiaca*, V*) Callistemon lanceolatus*, W) *Eucalyptus tereticornis* and X) *Psidium guajava*

Figure: 5. Bee polliniferous native and invasive plants observed in the sampling sites of Tanhril forest area (South Zone). A) *Syzygium cumini*, B) *Syzygium jambos*, C) *Tamarindus indica*, D) *Crotalaria pallida*, E) *Delonix regia*, F) *Castanopsis tribuloides*, G) *Holmskioldia sanguine*, H) *Leucosceptrum canum*, I) *Coleus scutellarioides*, J) *Lagerstromia speciosa*, K) *Punica granatum*, L) *Althaea rosea*, M) *Anthurium andreanum*, N) *Colona floribunda*, O) Hibiscus rosa sinensis, P) *Ipomoea batatas*, Q) *Malvaviscus arboreus,* R) *Urena lobata*, S) *Triumfetta rhomboidei*, T) *Moringa oleifera*, U) *Musa paradisiaca*, V*) Callistemon lanceolatus* and
W) *Eucalyptus tereticornis.*

Table 2. Floral calendar of native plants found in Tanhril forest.

SN	Plant name	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1	*Mangifera indica*			✓	✓								
2	*Rhus chinensis*								✓	✓			
3	*Impatiens balsamina*					✓	✓						
4	*Bombax ceiba*	✓	✓	✓									
5	*Terminalia crenulata*										✓	✓	
6	*Terminalia bellirica*	✓	✓								✓	✓	✓
7	*Cucumis sativus*					✓	✓	✓					
8	*Thladiantha cordifolia*				✓	✓	✓						
9	*Momordica charantia*						✓	✓	✓				
10	*Cyperus rotundus*	✓	✓	✓					✓	✓	✓	✓	✓
11	*Tetrameles nudiflora*		✓	✓	✓								
12	*Elaeocarpus lanceifolius*					✓	✓						
13	*Emblica officinalis*												
14	*Mallotus paniculatus*	✓	✓					✓	✓	✓	✓		
15	*Acacia pruinescens*				✓	✓							
16	*Bauhinia variegate*		✓	✓	✓								
17	*Derris robusta*				✓	✓	✓						
18	*Indigofera zollingeriana*					✓	✓	✓	✓	✓			
19	*Parkia timoriana*										✓	✓	
20	*Castanopsis tribuloides*								✓	✓	✓	✓	
21	*Holmskioldia sanguine*										✓	✓	
22	*Leucosceptrum canum*	✓	✓										
23	*Punica granatum*					✓	✓						

24	*Colona floribunda*				✓	✓	✓	✓	✓	✓	✓		
25	*Moringa oleifera*	✓	✓	✓	✓	✓							
26	*Musa paradisiaca*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
27	*Eucalyptus tereticornis*	✓	✓	✓									
28	*Averrhoa carambola*						✓						
29	*Rosa macrophylla*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
30	*Ixora coccinea*				✓								
31	*Citrus limon*			✓	✓	✓							
32	*Acronychia pedunculata*		✓	✓	✓	✓	✓	✓	✓				
33	*Schima wallichii*				✓	✓							
34	*Callicarpa arborea*					✓	✓	✓					

Table 3. Floral calendar of invasive plants found in Tanhril forest.

SN	Plant name	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1	*Amaranthus spp.*			✓	✓	✓							
2	*Coriandrum sativum*						✓	✓	✓				
3	*Asclepias curassavica*							✓	✓	✓	✓		
4	*Ageratum conyzoides*					✓	✓	✓	✓				
5	*Bidens pilosa*						✓	✓					
6	*Cosmos sulphureus*											✓	✓
7	*Matricaria chamomilla*						✓	✓	✓				
8	*Mikania micrantha*								✓	✓	✓	✓	✓
9	*Spilanthes acmella*						✓	✓	✓	✓	✓		
10	*Tagetes erecta*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
11	*Tithonia diversifolia*									✓	✓	✓	

12	*Zinnia elegans*						✓	✓	✓				
13	*Crassocephalum crepidioides*						✓	✓	✓	✓	✓	✓	
14	*Alnus nitida*									✓	✓	✓	
15	*Tecoma stans*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
16	*Bixa orellana*										✓	✓	✓
17	*Brassica camprestris*		✓	✓	✓	✓	✓						
18	*Raphanus sativus*			✓	✓								
19	*Carica papaya*							✓	✓				
20	*Sechium edule*							✓	✓				
21	*Croton joufra*							✓	✓	✓	✓		
22	*Euphorbia pulcherrima*	✓	✓										✓
23	*Riccinus communis*	✓	✓	✓									
24	*Macaranga peltata*	✓	✓										
25	*Caesalpinia pulcherrima*			✓	✓	✓	✓	✓					
26	*Cassia javanica*			✓	✓	✓	✓	✓	✓	✓			
27	*Mimosa pudica*				✓	✓							
28	*Leucaena leucocephala*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
29	*Phaseolus vulgaris*					✓	✓						
30	*Pisum sativum*	✓	✓	✓									
31	*Senna spectabilis*			✓	✓	✓							
32	*Tamarindus indica*						✓						
33	*Crotalaria pallida*	✓	✓							✓	✓	✓	✓
34	*Delonix regia*					✓	✓	✓					
35	*Coleus scutellarioides*				✓	✓	✓	✓	✓	✓			
36	*Lagerstromia speciosa*				✓	✓	✓						
37	*Althaea rosea*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

38	*Anthurium andreanum*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
39	*Hibiscus rosa sinensis*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
40	*Ipomoea batatas*											✓	✓
41	*Malvaviscus arboreus*					✓	✓	✓	✓				
42	*Urena lobata*				✓	✓	✓	✓	✓	✓			
43	*Triumfetta rhomboidea*									✓	✓	✓	
44	*Callistemon lanceolatus*		✓	✓	✓	✓	✓						
45	*Psidium guajava*						✓	✓	✓				
46	*Syzygium cumini*			✓	✓	✓							
47	*Syzygium jambos*			✓	✓								
48	*Oryza sativa*									✓			
49	*Zea mays*							✓					
50	*Antigonon leptopus*					✓	✓	✓					
51	*Portuluca oleracea*							✓	✓	✓			
52	*Prunus persica*		✓	✓	✓	✓							
53	*Coffee Arabica*									✓			
54	*Jatropha curcus*			✓	✓								
55	*Nicotianum tobaccum*			✓	✓								
56	*Solanum melongena*						✓						
57	*Solanum torvum*		✓	✓	✓				✓	✓	✓	✓	
58	*Tropaelum majus*						✓	✓	✓	✓			
59	*Lantana camara*			✓	✓	✓	✓	✓	✓	✓			
60	*Vitis vinifera*				✓	✓							
61	*Mussaenda erythrophylla*			✓	✓	✓	✓						

Table 4. Availability of floral resources (native and invasive plant species) to bee pollinators at different months.

Month	**Availability of native plant species**	**Availability of invasive plant species**
Winter		
December	4	14
January	9	12
February	12	16
Total	**25**	**42**
Spring		
March	11	23
April	14	26
May	16	26
Total	**41**	**75**
Rainy		
June	14	30
July	9	26
August	10	25
Total	**33**	**81**
Fall		
September	8	20
October	10	19
November	8	14
Total	**26**	**53**

The data collected on the presence of native and invasive plant species across four seasons Winter, Spring, Rainy, and Fall (**Table 4**) demonstrates clear patterns in the prevalence of both plant categories throughout the year. This section delineates the principal observations and trends for each season. 1. Winter (December to February) - The overall number of native species available in winter is 25, increasing from 4 in December to 12 in February. This indicates a consistent rebound from the coldest season as indigenous species increase in abundance. Invasive species exhibit significantly greater prevalence during winter, up to 42. The prevalence of invasive species begins at 14 in December and rises to 16 in February, indicating their persistence and capacity to flourish during colder months. 2. Spring (March to May) - Native Species: The abundance of native plant species reaches its zenith in Spring, totalling 41. This season witnesses the peak abundance of native species, increasing from 11 in March to 16 in May. The increase is ascribed to advantageous

environmental circumstances and vigorous regeneration throughout this period. Invasive species peak in Spring, reaching a total of 75. Their availability rises significantly from 23 in March to 26 in April and May. This indicates that invading species may potentially capitalise on advantageous spring conditions, occasionally surpassing native species. 3. Monsoon Period (June–August) - Indigenous Species: The wet season experiences a little reduction in the availability of native plants, totalling 33 species. Availability decreases in July (9 species) but recovers in August (10 species). Notwithstanding the precipitation, several indigenous flora contend with competition from non-native species. Invasive species proliferate during the wet season, amounting to 81. Their presence continuously remains elevated, with 30 species in June, 26 in July, and 25 in August. The inclement weather appears to facilitate the spread of exotic species. 4. Autumn (September–November) - Native Species: In the autumn, the availability of native species decreases once more, culminating in a total of 26. The figures vary marginally, with 8 species recorded in September and November, and 10 in October. This decrease corresponds with the shift to cooler conditions. Invasive Species: Invasive species continue to prevail throughout the autumn, amounting to 53. Despite a decrease in numbers during the rainy season, invasive species remain prevalent, with counts declining from 20 in September to 14 in November.

5.1.2. Important value index (IVI)

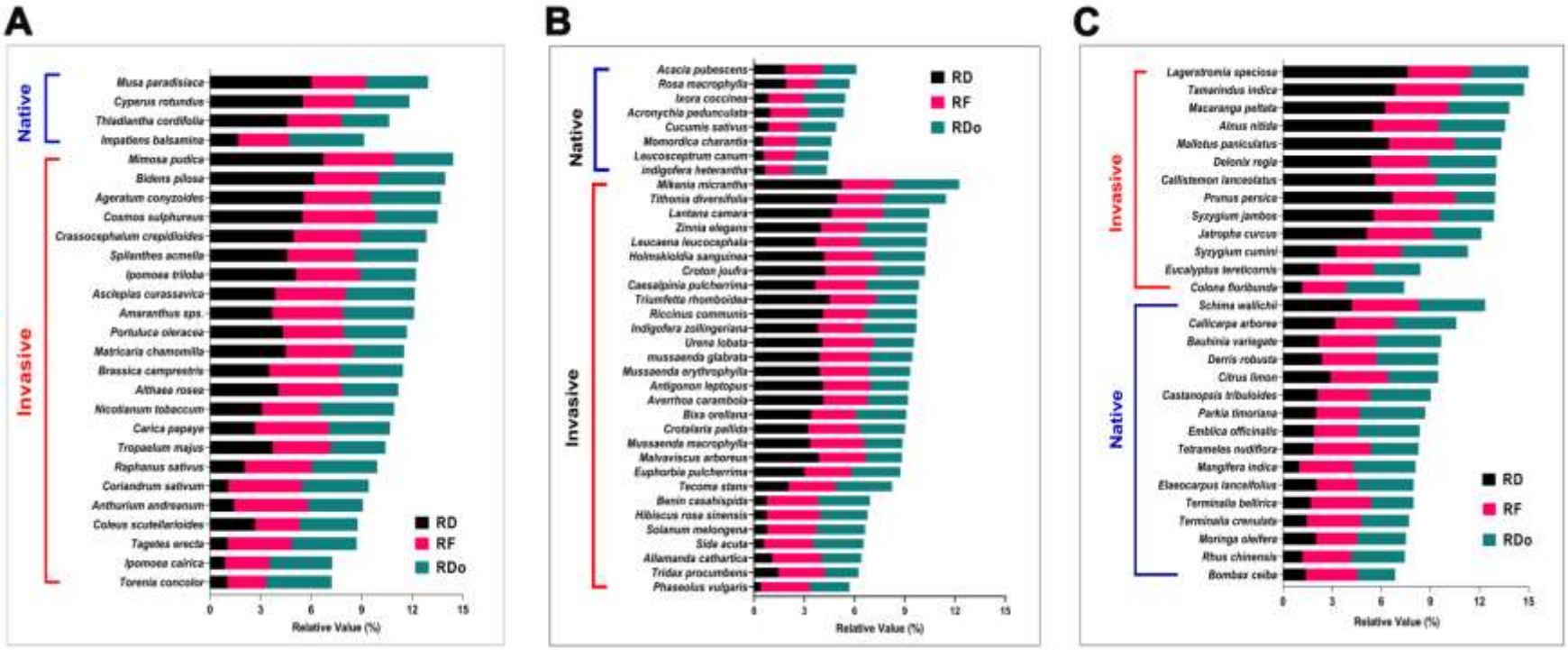

Figure 6. Relative density (RD), Relative frequency (RF), Relative dominance coverage (RDo) of bee polliniferous native and invasive of (A) herbs, (B) shrub and (C) tree in the Tanhril tropical forest, Mizoram, Northeast India.

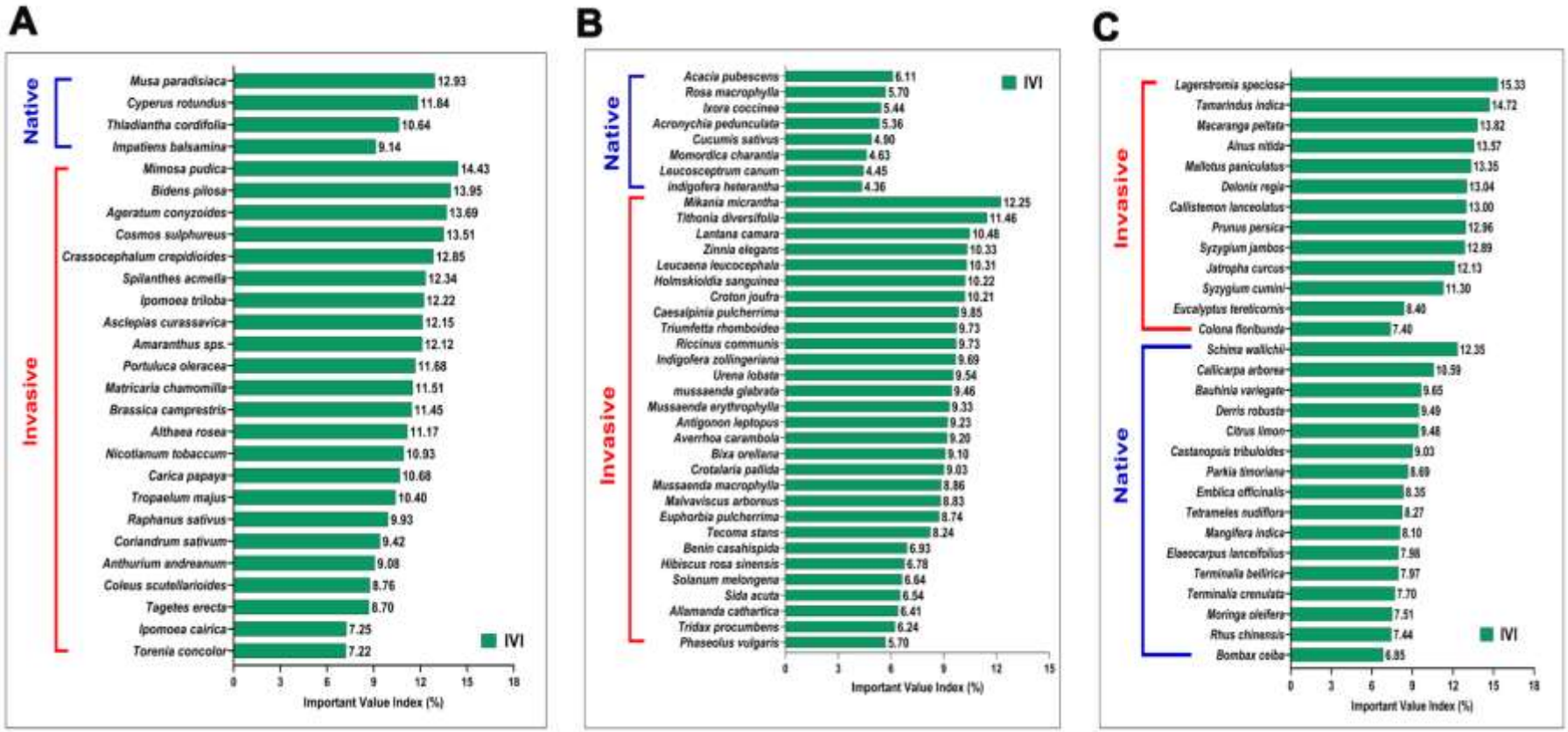

Figure 7. Important value Index (IVI) of bee polliniferous native and invasive of (A) herbs, (B) shrub and (C) tree in the Tanhril tropical forest, Mizoram, Northeast India.

The bar graphs designated as A, B, and C illustrates the comparative values of species significance across three distinct ecosystems or sampling locations. The bar chart illustrates a comparison of Relative Density (RD), Relative Frequency (RF), and Relative Dominance (RDo) between native and invasive plant species. In all three Fig (6A, 6B, and 6C), the invasive species (marked in red) exhibit higher combined RD, RF, and RDo values when compared to the native species (marked in blue). The subsequent trends can be noted in each panel: **Fig 6A**: The invasive species including *Musa paradisiaca*, *Imperata cylindrica*, and Amaranthus cruentus demonstrate notably high values of RD, RF, and RDo, suggesting that these species are firmly established regarding density, frequency, and dominance within this specific ecosystem. Indigenous species like *Thalassia cordifolia* and *Oryzae rondales* exhibit reduced values in general. **Fig 6B**: Invasive species such as *Acacia pubescens*, *Leucaena leucocephala*, and *Phaseolus vulgaris* exhibit dominance in RD, RF, and RDo values, highlighting their significant presence in this ecosystem. In contrast, native species, although still found, typically show reduced values in these metrics. **Fig 6C**: Invasive species like *Lagerstroemia speciosa* and *Tamarindus indica* continue to prevail in RD, RF, and RDo, indicating their extensive distribution, increased frequency, and dominance in the region. Indigenous species, although present, exhibit relatively diminished values.

The bar graphs **Fig 7** (**A**, **B**, and **C**) illustrate the Importance Value Index (IVI) for native (blue) and invasive (red) plant species across three distinct ecosystems or sample sites. The IVI serves as a composite index that combines the relative frequency, relative density, and relative dominance of species, offering a thorough perspective on their ecological significance within a specific area. **Fig 7A**: The graph depicts the IVI of species within a specific ecosystem. Both invasive and native species are present; however, invasive species such as *Bidens pilosa* (13.39), *Amaranthus spp*. (12.46), and *Tagetes erecta* (7.25) exhibit significant values, highlighting their substantial ecological presence. Species like *Musa paradisiaca* (12.93) and *Cyperus rotundus* (11.84) exhibit elevated IVI, indicating a balanced competition between native and invasive species at this specific location. **Fig 7B**: Within this ecosystem, invasive species such as *Acacia pubescens* (6.11), *Zinnia*

elegans (10.14), and *Leucaena leucocephala* (5.70) lead the IVI rankings, highlighting their significant ecological impact. Species such as *Rosa macrophylla* (5.70) and *Curcuma aromatic* (4.85) exhibit lower IVI values, indicating a greater dominance of invasive species in this location. **Fig 7C**: Within this ecosystem, the invasive species *Lagerstroemia speciosa* (15.33), *Tamarindus indica* (14.72), and *Macaranga peltata* (13.82) exhibit the highest IVI values, indicating their significant establishment and ecological dominance. Indigenous species like *Parkia timoriana* (8.53) and *Terminalia alata* (7.87) are present, yet they show reduced IVI values, suggesting a lesser degree of dominance relative to the invasive species.

Important Value Index (IVI)- Significant differences were found between IVI of invasive and IVI of native plant species. The IVI values for invasive plants were significantly higher, with percentages ranging from 7.22% to 14.43% for herbaceous species, 5.70% to 12.25% for shrubs, and 7.40% to 15.33% for trees. Nonetheless, native plant species exhibited comparatively lower IVI values, with tree species ranging from 6.85% to 12.35%, shrub species from 4.36% to 6.11%, and herb species from 9.14% to 12.93%. Invasive plant species and biodiversity- These findings draw attention to the detrimental ecological effects of invasive plants and increase the likelihood that native vegetation may become outcompeted or dispersed. Higher IVI values for invasive species point to their increased dominance and raise concerns about potential impacts on biodiversity and environmental stability.

Table 5. Abundance of Bee species in the native and invasive plant species at winter and fall seasons in the Tanhril tropical forest, Aizawl, Mizoram

Winter Season			Fall Season		
SN	Species	Bee abundance	SN	Species	Bee abundance
1	*Cosmos sulphureus*	58	1	*Asclepias curassavica*	34
2	*Mikania micrantha*	76	2	*Cosmos sulphureus*	50
3	*Tagetes erecta*	12	3	*Mikania micrantha*	70
4	*Tecoma stans*	15	4	*Spilanthes acmella*	32
5	*Bixa orellana*	17	5	*Tagetes erecta*	14
6	*Brassica camprestris*	14	6	*Tithonia diversifolia*	25
7	*Euphorbia pulcherrima*	5	7	*Crassocephalum crepidioides*	30
8	*Macaranga peltata*	30	8	*Alnus nitida*	35
9	*Leucaena leucocephala*	45	9	*Tecoma stans*	25
10	*Pisum sativum*	19	10	*Bixa orellana*	34
11	*Crotalaria pallida*	25	11	*Croton joufra*	26
12	*Althaea rosea*	18	12	*Cassia javanica*	39
13	*Anthurium andreanum*	6	13	*Leucaena leucocephala*	34
14	*Hibiscus rosa sinensis*	8	14	*Crotalaria pallida*	25
15	*Ipomoea batatas*	14	15	*Coleus scutellarioides*	34
16	*Callistemon lanceolatus*	6	16	*Althaea rosea*	36
17	*Prunus persica*	10	17	*Anthurium andreanum*	45
18	*Solanum torvum*	40	18	*Hibiscus rosa sinensis*	9
19	*Terminalia crenulata*	25	19	*Urena lobata*	16
20	*Terminalia bellirica*	19	20	*Triumfetta rhomboidea*	18
21	*Tetrameles nudiflora*	16	21	*Oryza sativa*	16
22	*Bauhinia variegate*	35	22	*Portuluca oleracea*	14
23	*Leucosceptrum canum*	26	23	*Coffee Arabica*	21
24	*Moringa oleifera*	11	24	*Solanum torvum*	23
25	*Musa paradisiaca*	38	25	*Tropaelum majus*	24
26	*Rosa macrophylla*	14	26	*Lantana camara*	27

27	*Acronychia pedunculata*	20	27	*Rhus chinensis*	45
28	*Musa paradisiaca*	31	28	*Terminalia crenulata*	31
			29	*Terminalia bellirica*	21
			30	*Cyperus rotundus*	39
			31	*Mallotus paniculatus*	49
			32	*Indigofera zollingeriana*	34
			33	*Parkia timoriana*	41
			34	*Castanopsis tribuloides*	46
			35	*Holmskioldia sanguine*	30
			36	*Colona floribunda*	35
			37	*Musa paradisiaca*	31
			38	*Rosa macrophylla*	21

The table shows abundance of Bee species in the native and invasive plant species winter and fall season. In winter season *Mikania micrantha* species was having highest abundance of Bee species species that is 72 while *Anthurium andreanum* was least that was 6. Similarly in fall same *Mikania micrantha* was highest bee abundance species carrying score 70 and *Tagetes erecta* was the species that carried score 9.

Table 6. Abundance of Bee species in the native and invasive plant species at spring and rainy seasons in the Tanhril tropical forest, Aizawl, Mizoram

Spring Season			Rainy Season		
SN	Species	Bee abundance	SN	Species	Bee abundance
1	*Mangifera indica*	31	1	*Coriandrum sativum*	21
2	*Ageratum conyzoides*	24	2	*Asclepias curassavica*	31
3	*Tagetes erecta*	9	3	*Ageratum conyzoides*	12
4	*Impatiens balsamina.*	35	4	*Bidens pilosa*	62
5	*Tecoma stans*	36	5	*Matricaria chamomilla*	24
6	*Bombax ceiba*	34	6	*Mikania micrantha*	55
7	*Brassica camprestris*	33	7	*Spilanthes acmella*	22

8	*Raphanus sativus*	26	8	*Tagetes erecta*	21
9	*Thladiantha cordifolia*	25	9	*Zinnia elegans*	36
10	*Tetrameles nudiflora*	34	10	*Crassocephalum crepidioides*	45
11	*Riccinus communis*	35	11	*Impatiens balsamina*	36
12	*Acacia pruinescens*	24	12	*Tecoma stans*	54
13	*Bauhinia variegate*	26	13	*Brassica camprestris*	31
14	*Caesalpinia pulcherrima*	35	14	*Carica papaya*	21
15	*Cassia javanica*	36	15	*Cucumis sativus*	25
16	*Derris robusta*	34	16	*Thladiantha cordifolia*	32
17	*Mimosa pudica*	74	17	*Momordica charantia*	41
18	*Indigofera zollingeriana*	32	18	*Sechium edule*	26
19	*Leucaena leucocephala*	36	19	*Cyperus rotundus*	21
20	*Phaseolus vulgaris*	29	20	*Elaeocarpus lanceifolius*	20
21	*Pisum sativum*	24	21	*Croton joufra*	14
22	*Senna spectabilis*	26	22	*Mallotus paniculatus*	6
23	*Delonix regia*	35	23	*Caesalpinia pulcherrima*	17
24	*Coleus scutellarioides*	41	24	*Cassia javanica*	19
25	*Lagerstromia speciosa*	38	25	*Derris robusta*	31
26	*Punica granatum*	25	26	*Mimosa pudica*	25
27	*Althaea rosea*	45	27	**Indigofera zollingeriana*	24
28	*Anthurium andreanum*	12	28	*Leucaena leucocephala*	23
29	*Colona floribunda*	12	29	*Phaseolus vulgaris*	26
30	*Hibiscus rosa sinensis*	10	30	*Tamarindus indica*	21
31	*Malvaviscus arboreus*	32	31	*Delonix regia*	32
32	*Urena lobata*	31	32	**Indigofera zollingeriana*	12
33	*Moringa oleifera*	12	33	*Leucaena*	36

				leucocephala	
34	**Musa paradisiaca*	31	34	*Castanopsis tribuloides*	26
35	*Callistemon lanceolatus*	21	35	*Coleus scutellarioides*	27
36	**Eucalyptus tereticornis*	12	36	*Lagerstromia speciosa*	31
37	*Syzygium cumini*	14	37	*Punica granatum*	34
38	*Syzygium jambos*	22	38	*Althaea rosea*	36
39	*Antigonon leptopus*	35	39	*Anthurium andreanum*	19
40	*Prunus persica*	42	40	*Colona floribunda*	9
41	**Rosa macrophylla*	26	41	*Hibiscus rosa sinensis*	10
42	**Ixora coccinea*	21	42	*Malvaviscus arboreus*	17
43	*Jatropha curcus*	33	43	*Urena lobata*	13
44	*Citrus limon*	21	44	*Musa paradisiaca*	11
45	**Acronychia pedunculata*	15	45	*Callistemon lanceolatus*	42
46	*Nicotianum tabaccum*	25	46	*Psidium guajava*	23
47	*Solanum torvum*	26	47	*Averrhoa carambola*	26
48	**Schima wallichii*	31	48	*Zea mays*	32
49	*Lantana camara*	22	49	*Antigonon leptopus*	22
50	**Callicarpa arborea*	31	50	*Portuluca oleracea*	14
51	*Musa paradisiaca*	32	51	*Rosa macrophylla*	32
52	*Rosa macrophylla*	37	52	*Acronychia pedunculata*	15
			53	*Solanum melongena*	42
			54	*Tropaelum majus*	25
			55	*Lantana camara*	24
			56	*Callicarpa arborea*	35

The table shows abundance of Bee species in the native and invasive plant species spring and rainy season. In spring season *Mimosa pudica* species was having highest abundance of Bee species species that is 74 while *Colona floribunda* was least that was 8. Similarly in fall same *Bidens pilosa* was highest bee abundance species carrying score 70 and *Musa paradisiaca* was the species that carried score 9.

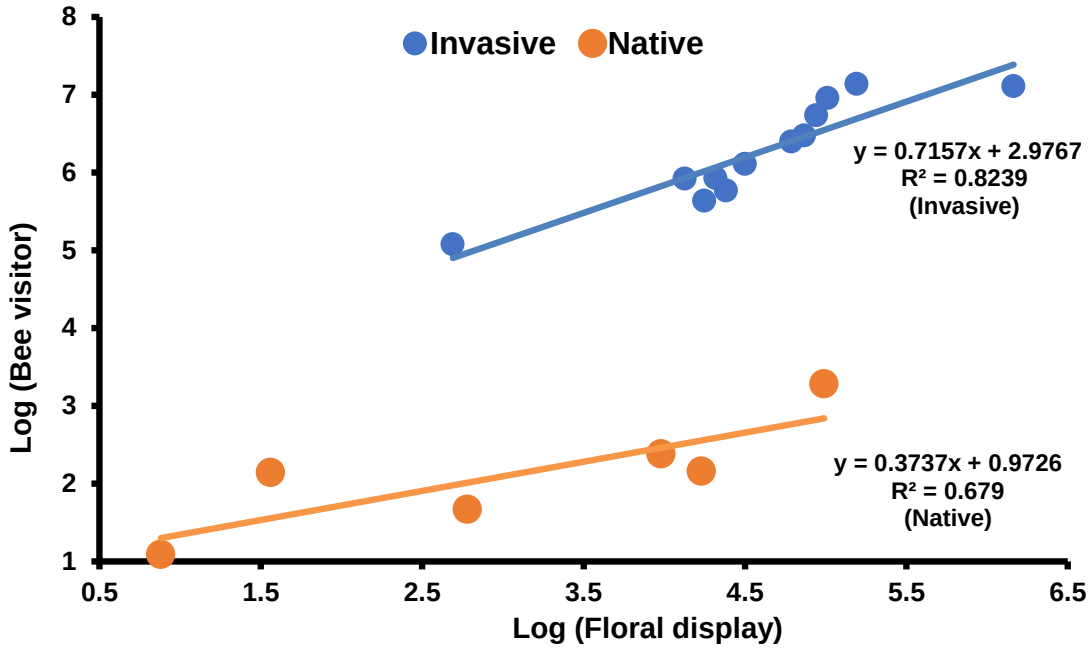

Figure 8. Correlation and regression analysis between floral display and bee visitors of native and invasive plant species at spring and rainy seasons in the Tanhril tropical forest, Aizawl, Mizoram.

The scatter plot illustrates the relationship between the quantity of bee visitors and the floral display for both invasive (blue) and native (orange) plant types. The floral display and bee visitation data are log-transformed, facilitating interpretation of the relationship. The graph (**Fig 8)** contains the linear regression equations for each group, along with their corresponding R^2 values. Floral display was found to be high in the invasive plant species (2.7-6.4) than natives (0.7-5.0). Correlation and regression analysis revealed the fact that floral display is positively correlated with bee visit. For invasive species, the R^2 value is 0.8239, indicating a robust positive association between floral display and bee visitation. Larger flower displays of invasive species are particularly effective at attracting bee visitors, with a considerable increase in the number of visitors as the size of the floral display increases. For indigenous species: The R^2 score is 0.679, signifying a moderate positive connection between flower display and bee visitation. Although a positive

correlation exists, it is less pronounced than that for invasive species, indicating that the quantity of bee visitors to native plants escalates less significantly with greater flower displays.

5.2.1 Melissopalynology

Figure 9. Light microscopy photographs of pollen grains observed in bee polliniferous native and invasive plants observed in the sampling sites of Tanhril forest area (East Zone). A) *Amaranthus sps*., B) *Mangifera indica*, C) *Rhus chinensis*, D) *Coriandrum sativum*, E) *Asclepias curassavica*, F) *Ageratum conyzoides*, G) *Bidens pilosa*, H) *Cosmos sulphurous*, I) *Matricaria chamomilla*, J) *Mikania micrantha*, K) *Spilanthes acmella*, L) *Tagetes erecta*, M) *Tithonia diversifolia*, N) *Zinnia elegans*, O) *Crassocephalum crepidioides*, P) *Impatiens balsamina*, Q) *Alnus nitida*, R) *Tecoma stans*, S) *Bixa orellana*, T) *Bombax ceiba*, U) *Brassica camprestris*, V) *Raphanus sativus*, W) *Carica papaya*, and X) *Terminalia crenulata*.

Figure 10. Light microscopy photoghraphs of pollen grains observed in bee polliniferous native and invasive plants observed in the sampling sites of Tanhril forest area (West Zone). A) *Terminalia bellirica*, B) *Cucumis sativus*, C) *Thladiantha cordifolia*, D) *Momordica charantia*, E) *Sechium edule*, F) *Cyperus rotundus*, G) *Tetrameles nudiflora*, H) *Elaeocarpus lanceifolius*, I) *Croton joufra*, J) *Emblica officinalis*, K) *Euphorbia pulcherrima*, L) *Riccinus communis*, M) *Mallotus paniculatus*, N) *Macaranga peltate*, O) *Acacia pruinescens*, P) *Bauhinia variegate*, Q) *Caesalpinia pulcherrima*, R) *Cassia javanica*, S) *Derris robusta*, T) *Mimosa pudica*, U) *Indigofera zollingeriana*, V) *Leucaena leucocephala*, W) *Parkia timorian*, and X) *Phaseolus vulgaris*.

Figure 11. Light microscopy photographs of pollen grains observed in bee polliniferous native and invasive plants observed in Tanhril forest area (North and South Zone). A) *Pisum sativum*, B) *Senna spectabilis*, C) *Tamarindus indica*, D) *Crotalaria pallida*, E) *Delonix regia*, F) *Castanopsis tribuloides*, G) *Holmskioldia sanguine*, H) *Leucosceptrum canum*, I) *Coleus scutellarioides*, J) *Lagerstromia speciosa*, K) *Punica granatum*, L) *Althaea rosea*, M) *Anthurium andreanum*, N) *Colona floribunda*, O) *Hibiscus rosa sinensis*, P) *Ipomoea batatas*, Q) *Malvaviscus arboreus*, R) *Urena lobata*, S) *Triumfetta rhomboidei*, T) *Moringa oleifera*, U) *Musa paradisiaca*, V) *Callistemon lanceolatus*, W) *Eucalyptus tereticornis and* X) *Psidium guajava*

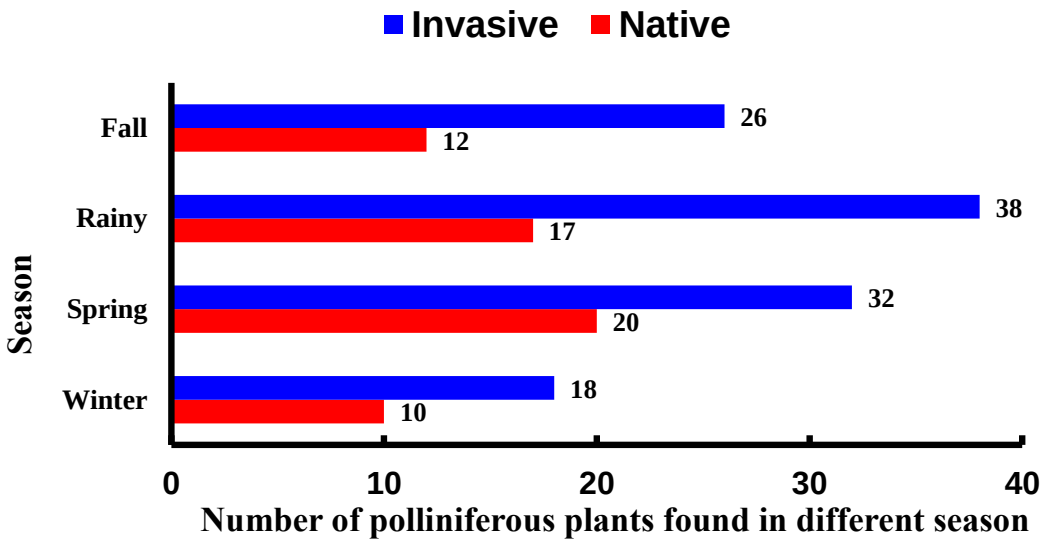

Figure 12. Graph showing number of polliniferous plants found in different season

The bar graph compares the presence of invasive and native plant species across four seasons: Fall, Rainy, Spring, and Winter (**Fig 12**). The data highlights the following key trends: Fall Season- Invasive species dominate, with a count of 26, while native species have a much lower count of 12. The proportion of invasive species is more than double that of native species during this season. Rainy Season- Invasive species reached their peak sum in the rainy season with 38 species. Native species show a

much lower count of 17, indicating a substantial dominance of invasive species in this period as well. Spring Season- Invasive species are again significantly higher, with a count of 32 compared to 20 native species. While native species are relatively higher in spring compared to other seasons, invasive species still maintain a considerable lead. Winter Season- Both invasive and native species show lower counts compared to other seasons, but invasive species (18) still outnumber native species (10). This shows that even in less favorable conditions, invasive species retain dominance over native plants.

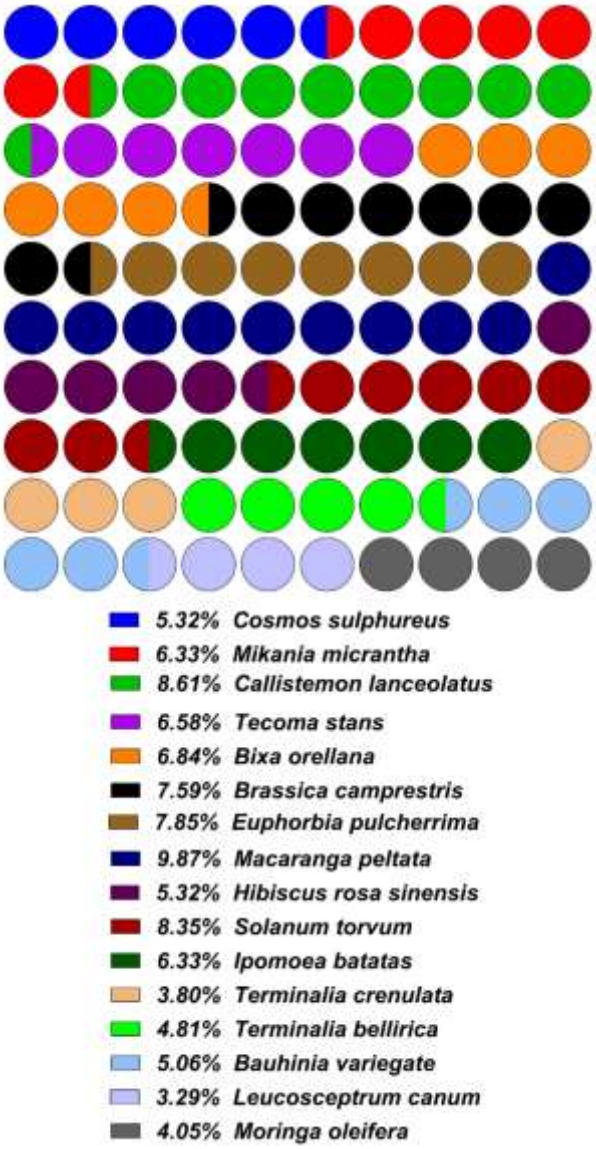

Figure 13. Parts hole graph showing plants present in honey samples during winter season.

The "Parts hole graph" (**Fig 13**) representing the plant species present in honey samples during winter seasons. Winter: The graph represents various plant species based on percentages, with colors indicating specific plants. The plants with the highest percentages include highest: *Macaranga peltata* (9.87%) and lowest: *Leucosceptrum canum* (3.29%).

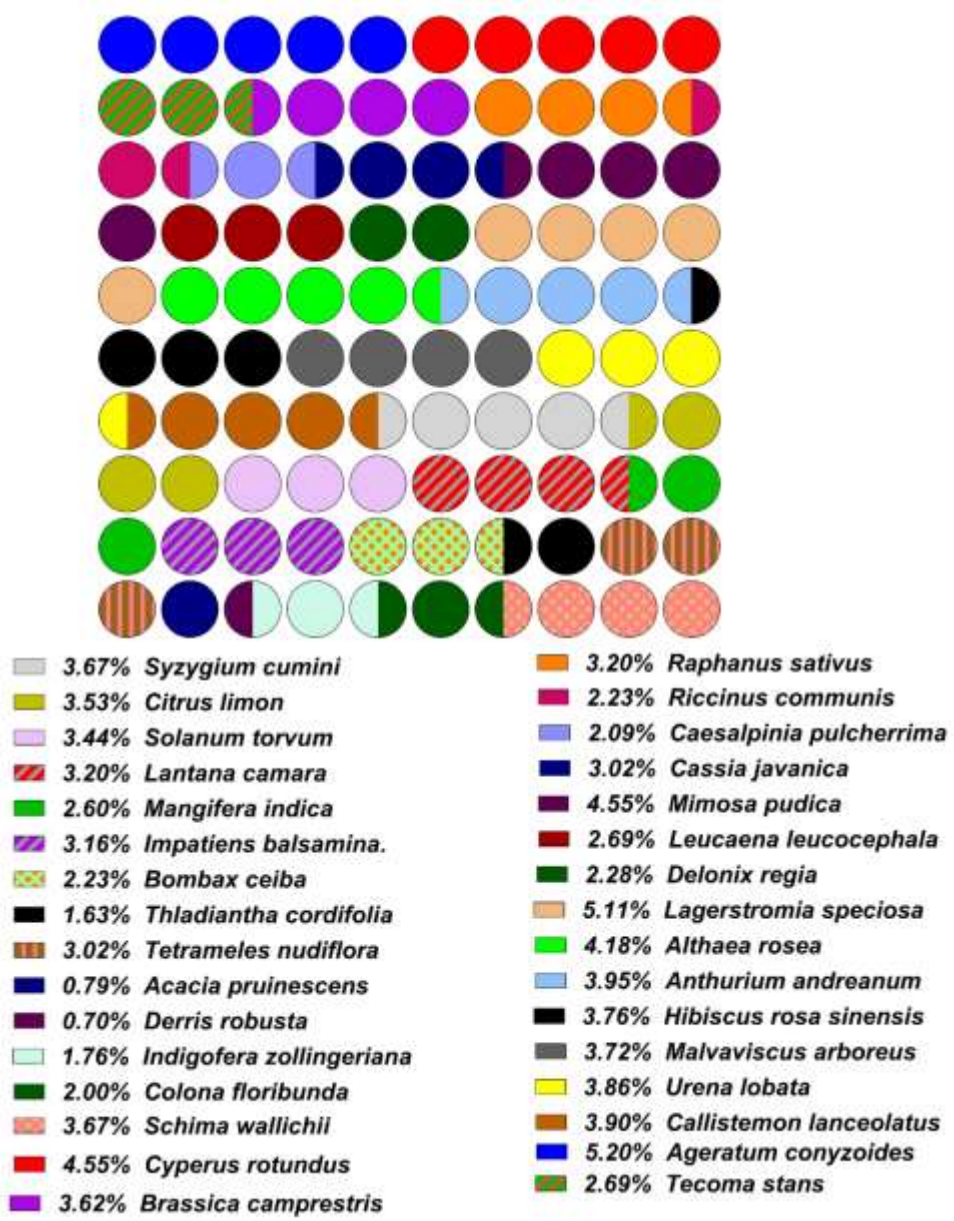

Figure 14. Parts hole graph showing plants present in honey samples during spring season.

The "Parts hole graph" (**Fig 14**) representing the plant species present in honey samples during spring seasons. The graph represents various plant species based on percentages, with colors indicating specific plants. The plants with the highest percentages include highest: *Ageratum conyzoides* (5.20%) and lowest: *Derris robusta* (0.79%).

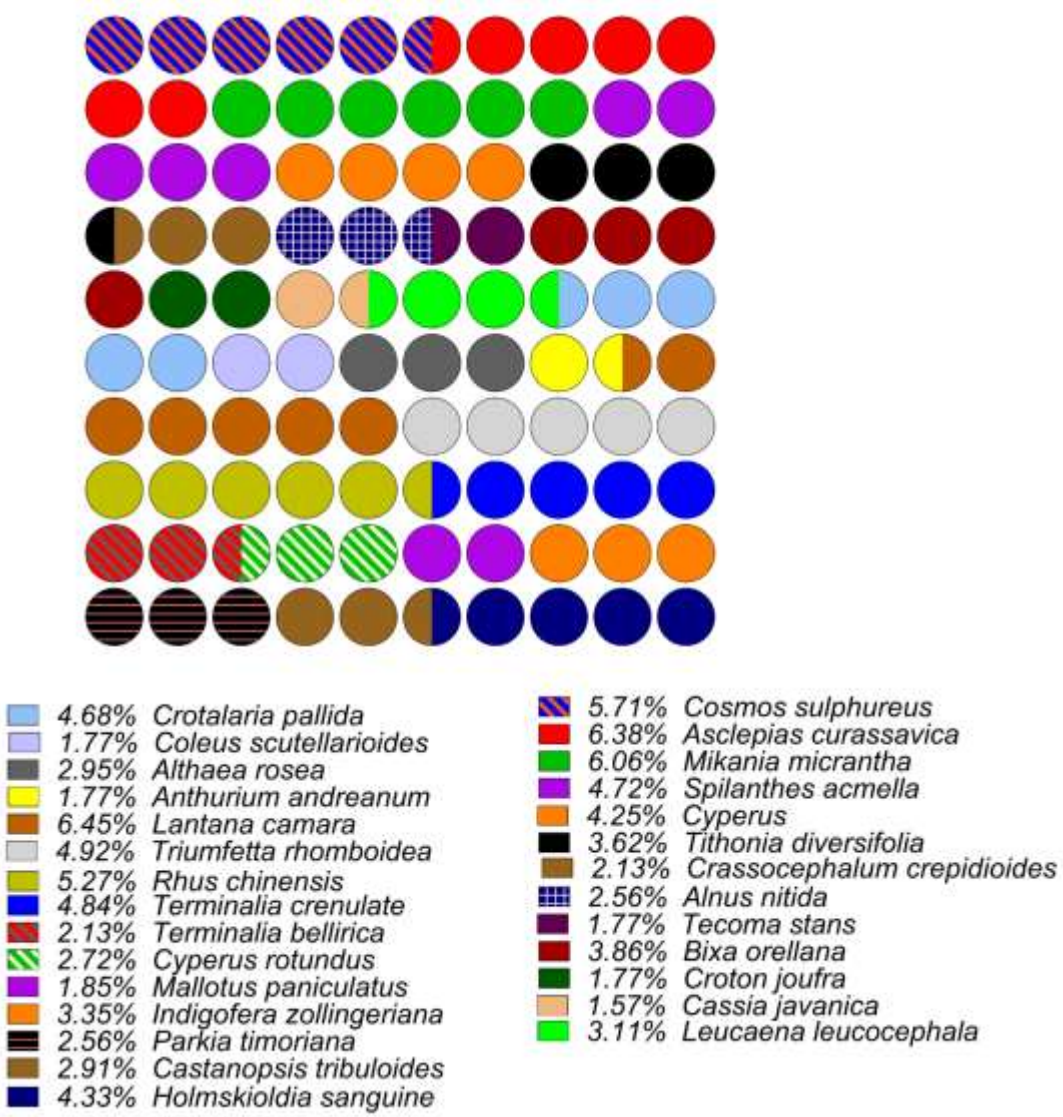

Figure 15. Parts hole graph showing plants present in honey samples during fall season.

The "Parts hole graph" (**Fig 15**) representing the plant species present in honey samples during fall seasons. The graph represents various plant species based on percentages, with colors indicating specific plants. The plants with the highest percentages include highest: *Lantana camara* (6.45%) and lowest: *Cassia javanica* (0.79%).

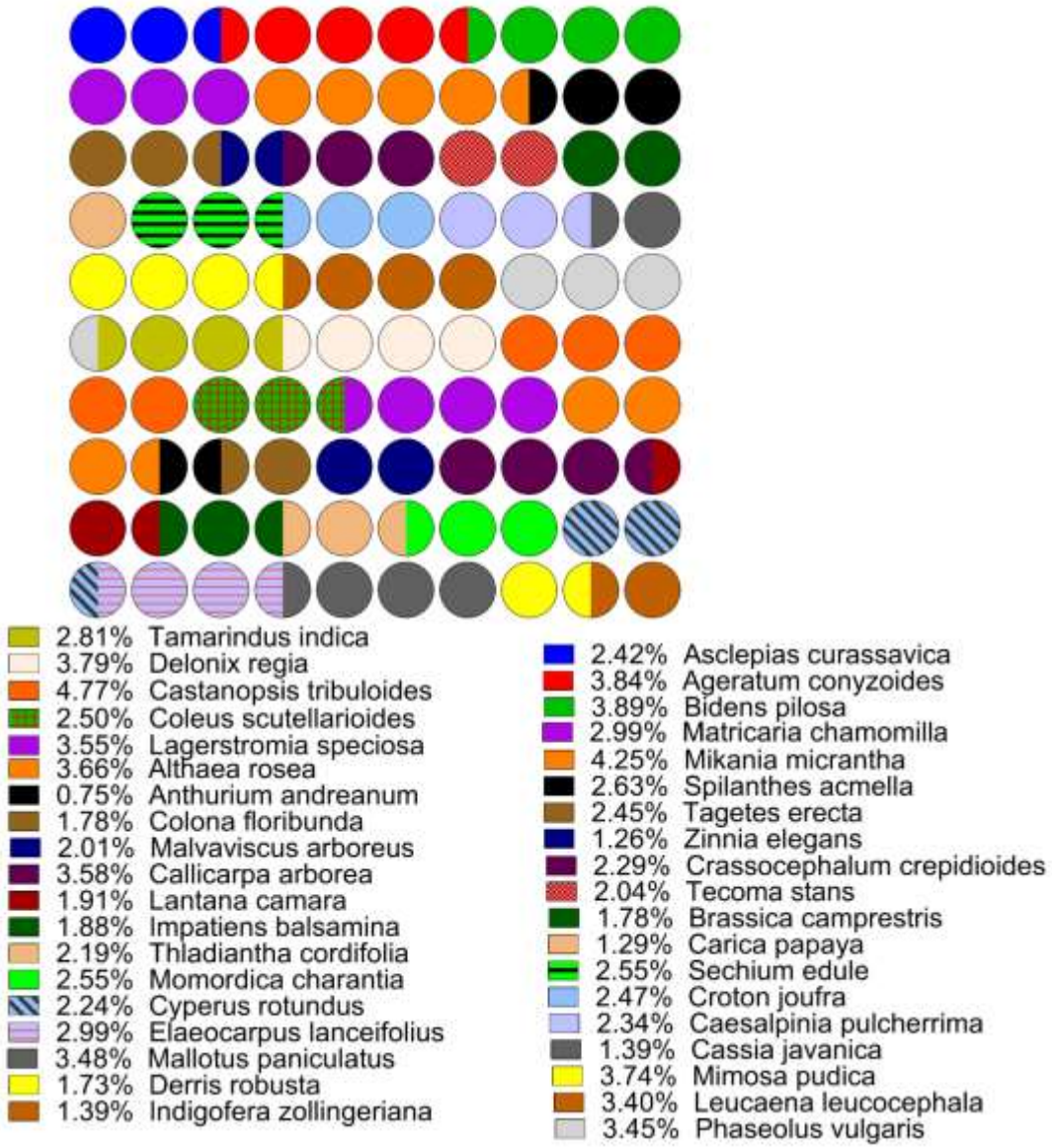

Figure 16. Parts hole graph showing plants present in honey samples during (D) rainy season.

The "Parts hole graph" (**Fig 16**) representing the plant species present in honey samples during rainy seasons. The graph represents various plant species based on

percentages, with colors indicating specific plants. The plants with the highest percentages include highest: *Castanopsis tribuloides* (4.77%) and lowest: *Anthurium andreanum*(0.75%).

5.2.2 DNA barcoding

Table 7: Molecular identification of plant species detected in honey in Winter season. The number of clones for each MOTU, the species match in reference database (RD) and the related identity values (ID%) obtained with the BLAST search are reported for the two barcode regions.

SN	Identified plant	*matK*			*rbcL*		
		MOTUs (no. clones)	**Species match in RD**	**ID %**	**MOTUs (no. clones)**	**Species match in RD**	**ID %**
1	*Cosmos sulphureus*	7	*Cosmos sulphureus* *Cosmos atrosanguineus* *Cosmos caudatus*	100 99.8 99.5	7	*Cosmos sulphureus* *Cosmos atrosanguineus* *Cosmos caudatus*	100 99.7 99.8
2	*Mikania micrantha*	16	*Mikania micrantha*	100 99.6	16	*Mikania micrantha* *Mikania andrei*	100 99.8
3	*Tagetes erecta*	17	*Tagetes erecta* *Tagetes elongata*	100	17	*Tagetes erecta* *Tagetes elongate* *Tagetes epapposa*	100 99.8 99.5
4	*Tecoma stans*	13	*Tecoma stans*	100	13	*Tecoma stans*	100
5	*Bixa orellana*	17	*Bixa Orellana* *Bixa acuminata*	100 99.5	16	*Bixa Orellana* *Bixa acuminata* *Bixa arborea*	100 99.7 99.0
6	*Brassica camprestris*	5	*Brassica camprestris*	100	6	*Brassica camprestris* *Raphanus sativus*	100 99.6
7	*Euphorbia pulcherrima*	13	*Euphorbia pulcherrima*	100	12	*Euphorbia pulcherrima*	100 99.

						Euphorbia esula Euphorbia cyparissias	8 99.6
8	*Macaranga peltata*	9	*Macaranga peltata Macaranga capensis*	100 99.2	9	*Macaranga peltate Macaranga capensis Macaranga bancana*	100 99
9	*Leucaena leucocephala*	7	*Leucaena leucocephala Leucaena collinsii*	100 99.0	12	*Leucaena leucocephala Leucaena collinsii Leucaena cuspidata*	100 99.5 99.2
10	*Pisum sativum*	15	*Pisum sativum*	100	18	*Pisum sativum Pisum abyssinicum*	100 99.5
11	*Crotalaria pallida*	14	*Crotalaria pallida*	100	14	*Crotalaria pallida Crotalaria abscondita*	100 99.2
12	*Althaea rosea*	0	----		7	*Althaea rosea Althaea officinalis*	199. 99.4
13	*Anthurium andreanum*	3	*Anthurium andreanum*	100	5	*Anthurium andreanum*	100
14	*Hibiscus rosa sinensis*	5	*Hibiscus rosa sinensis*	100	3	*Hibiscus rosa sinensis Hibiscus moscheutos*	100 99.5
15	*Ipomoea batatas*	9	*Ipomoea batatas Ipomoea purpurea*	100 99.7	5	*Ipomoea batatas*	100
16	*Callistemon lanceolatus*	5	*Callistemon lanceolatus*	100	6	*Callistemon lanceolatus Callistemon acuminatus Callistemon citrinus*	100 99.2 99.0

17	*Prunus persica*	12	*Prunus persica*	100	19	*Prunus persica* *Rosa macrophylla*	100 99.7
18	*Solanum torvum*	8	*Solanum torvum* *Solanum melongena*	100 99.6	13	*Solanum torvum* *Solanum melongena* *Solanum nigrum*	100 99.9 99.5
19	*Senna spectabilis*	6	*Senna spectabilis*	100	5	*Senna spectabilis*	100
20	**Terminalia crenulate*	7	**Terminalia crenulate* **Terminalia bellirica*	100 99.1	10	**Terminalia crenulate* **Terminalia bellirica*	100 99.0
21	**Terminalia bellirica*	15	**Terminalia bellirica*	100	14	**Terminalia bellirica* **Terminalia arjuna*	100 99.0
22	**Tetrameles nudiflora*	13	----		13	**Tetrameles nudiflora*	100
23	**Bauhinia variegate*	5	**Bauhinia variegate*	100	18	**Bauhinia variegate* *Bauhinia acuminata* *Bauhinia augusti*	100 99.8 99.3
24	**Leucosceptrum canum*	6	**Leucosceptrum canum*	100	5	**Leucosceptrum canum*	100
25	**Moringa oleifera*	13	**Moringa oleifera*	100	9	**Moringa oleifera* *Moringa ovalifolia*	100 99.1
26	**Musa paradisiaca*	4	**Musa paradisiaca*	100	4	**Musa paradisiaca* *Musa aurantiaca*	100 99.0
27	**Rosa macrophylla*	3	**Rosa macrophylla*	100	8	**Rosa macrophylla* *Rosa canina*	100 99.6
28	**Acronychia pedunculata*	17	**Acronychia pedunculata* *Acronychia*	100 99.8	19	**Acronychia pedunculata* *Acronychia*	100 99.1

			aberrans			*aberrans Acronychia acuminata*	99.0
29	Unidentified	10			10		

* =Native species. Molecular identification of the pollen collected from at Tanhril natural forest. The number of clones for each MOTU, the species match in reference database and the related identity values higher than 99% (ID%) obtained with the BLAST search are reported for the two barcode regions; From the floral calender we can can see there are 28 plants blooms winter and though barcoding we identified 28 plants. So from 28 plants matK and rbcL identified 26 and 28 plants respectively.

Table 8. Molecular identification of plant species detected in honey in Spring season. The number of clones for each MOTU, the species match in reference database (RD) and the related identity values (ID%) obtained with the BLAST search are reported for the two barcode regions.

SN	**Identified plant**	***matK***			***rbcL***		
		MOTUs (no. clones)	**Species match in RD**	**ID %**	**MOTUs (no. clones)**	**Species match in RD**	**ID %**
1	**Mangifera indica*	13	**Mangifera indica*	100	16	**Mangifera indica Mangifera altissima*	100 99.5
2	*Ageratum conyzoides*	2	----		7	*Ageratum conyzoides Ageratum chortianum*	100 99.7
3	*Tagetes erecta*	5	*Tagetes erecta*	100	4	*Tagetes erecta Tagetes elongata*	100 99.0
4	**Impatiens balsamina.*	4	**Impatiens balsamina*	100	6	**Impatiens balsamina Impatiens capensis*	100 99.2
5	*Tecoma stans*	3			5	*Tecoma stans*	100
6	**Bombax ceiba*	4	*Bombax ceiba*	100	8	*Bombax ceiba Bombax anceps*	100 99.4
7	*Brassica*	5	*Brassica*	100	4	*Brassica*	100

	camprestris		*camprestris*			*camprestris Brassica oleracea*	99.2
8	*Raphanus sativus*	4	*Raphanus sativus*	100	3	*Raphanus sativus*	100
9	**Thladiantha cordifolia*	13	**Thladiantha cordifolia*	100	16	**Thladiantha cordifolia Thladiantha cordifolia*	100 99.3
10	**Tetrameles nudiflora*	12	**Tetrameles nudiflora Tetrameles horsfieldii*	100 99.4	5	**Tetrameles nudiflora*	100
11	*Riccinus communis*	2	*Riccinus communis*	100	5	*Riccinus communis*	100
12	**Acacia pruinescens*	6	----		16	*Acacia pruinescens Bauhinia variegate*	100 99.5
13	**Bauhinia variegate*	8	**Bauhinia variegate*	100	18	**Bauhinia variegate Caesalpinia pulcherrima*	100 99.8
14	*Caesalpinia pulcherrima*	3	*Caesalpinia pulcherrima*	100	3	*Caesalpinia pulcherrima*	100
15	*Cassia javanica*	3	*Cassia javanica*	100	4	*Cassia javanica*	100
16	**Derris robusta*	2	**Derris robusta*	100	3	**Derris robusta*	100
17	*Mimosa pudica*	5	*Mimosa pudica*	100	9	*Mimosa pudica Mimosa andina*	100 99.6
18	**Indigofera zollingeriana*	4	*Indigofera zollingeriana*	100	5	*Indigofera zollingeriana*	100
19	*Leucaena leucocephala*	5	*Leucaena leucocephala*	100	6	*Leucaena leucocephala Leucaena collinsii*	100 99.0
20	*Phaseolus vulgaris*	9	*Phaseolus vulgaris*	100	12	*Phaseolus vulgaris Phaseolus angustissimu*	100 99.6 99.

						s Phaseolus filiformis	2
21	*Pisum sativum*	10	*Pisum sativum Pisum fulvum*	100 99.1	4	*Pisum sativum*	100
22	*Senna spectabilis*	5	*Senna spectabilis*	100	14	*Senna spectabilis Senna alata Senna auriculata*	100 99.7 99.2
23	*Delonix regia*	3	*Delonix regia*	100	4	*Delonix regia*	100
24	*Coleus scutellarioides*	5	*Coleus scutellarioides*	100	10	*Coleus scutellarioides Coleus amboinicus*	100 99.4
25	*Lagerstromia speciosa*	11	*Lagerstromia speciosa* Lagerstroemia anisoptera	100 99.7	13	*Lagerstromia speciosa Lagerstroemia anisoptera*	100 99.1
26	**Punica granatum*	5	**Punica granatum*	100	5	**Punica granatum*	100
27	*Althaea rosea*	16	*Althaea rosea Althaea officinalis*	100 99.1	11	*Althaea rosea Althaea officinalis*	100 99.5
28	*Anthurium andreanum*	6	*Anthurium andreanum*	100	5	*Anthurium andreanum*	100
29	**Colona floribunda*	5	**Colona floribunda*	100	12	**Colona floribunda Colona auriculata*	100 99.0
30	*Hibiscus rosa sinensis*	18	*Hibiscus rosa sinensis Hibiscus moscheutos*	100 99.4	4	*Hibiscus rosa sinensis*	100
31	*Malvaviscus arboreus*	4	*Malvaviscus arboreus*	100	6	*Malvaviscus arboreus*	100
32	*Urena lobata*	6	*Urena lobata*	100	7	*Urena lobata*	100
33	**Moringa oleifera*	5	**Moringa oleifera*	100	5	**Moringa oleifera*	100
34	**Musa*	15	**Musa*	100 99.	12	**Musa*	100 99.

	paradisiaca		*paradisiaca Musa acuminata*	2		*paradisiaca Musa acuminata*	5
35	*Callistemon lanceolatus*	6	*Callistemon lanceolatus*	100	6	*Callistemon lanceolatus*	100
36	**Eucalyptus tereticornis*	5	**Eucalyptus tereticornis*	100	4	**Eucalyptus tereticornis*	100
37	*Syzygium cumini*	6	*Syzygium cumini*	100	10	*Syzygium cumini Syzygium jambos*	100 99.2
38	*Syzygium jambos*	5	*Syzygium jambos*	100	14	*Syzygium jambos* Averrhoa carambola	100 99.6
39	*Antigonon leptopus*	6	----	100	6	*Antigonon leptopus*	100
40	*Prunus persica*	3	*Prunus persica*	100	8	*Prunus persica Prunus amygdalus*	100 99.0
41	**Rosa macrophylla*	5	**Rosa macrophylla*	100	9	**Rosa macrophylla*	100
42	**Ixora coccinea*	16	**Ixora coccinea Ixora chinensis*	100 99.1	10	**Ixora coccinea Ixora chinensis*	100 99.0
43	*Jatropha curcus*	6	*Jatropha curcus*	100	6	*Jatropha curcus*	100
44	*Citrus limon*	5	*Citrus limon*	100	4	*Citrus limon*	100
45	**Acronychia pedunculata*	4	**Acronychia pedunculata*	100	13	**Acronychia pedunculata Acronychia acidula*	100 99.0
46	*Nicotianum tabaccum*	6	*Nicotianum tabaccum*	100	5	*Nicotianum tabaccum Nicotiana glauca*	100 99.3
47	*Solanum torvum*	12	*Solanum torvum Solanum melongena*	100 99.3	12	*Solanum torvum Solanum melongena*	100 99.5
48	**Schima*	6	**Schima*	100	6	**Schima*	100

	wallichii		*wallichii*			*wallichii*	
49	*Lantana camara*	6	*Lantana camara*	100	4	*Lantana camara*	100
50	**Callicarpa arborea*	2	----		5	**Callicarpa arborea*	100
51	Unidentified	18			19		

* =Native species. Molecular identification of the pollen collected from at Tanhril natural forest. The number of clones for each MOTU, the species match in reference database and the related identity values higher than 99% (ID%) obtained with the BLAST search are reported for the two barcode regions; From the floral calender we can can see there are 52 plants blooms spring but though barcoding we identified 50 plants. So from 50 plants matK and rbcL identified 46 and 50 plants respectively.

Table 9. Molecular identification of plant species detected in honey in Rainy season. The number of clones for each MOTU, the species match in reference database (RD) and the related identity values (ID%) obtained with the BLAST search are reported for the two barcode regions

SN	**Identified plant**	***matK***			***rbcL***		
		MOTUs (no. clones)	**Species match in RD**	**ID %**	**MOTUs (no. clones)**	**Species match in RD**	**ID %**
1	*Coriandrum sativum*	11	*Coriandrum sativum Mangifera indica*	100 99.6	3	*Coriandrum sativum*	100
2	*Asclepias curassavica*	6	*Asclepias curassavica*	100	11	*Asclepias curassavica Ageratum conyzoides*	100 99.2
3	*Ageratum conyzoides*	5	*Ageratum conyzoides*	100	12	*Ageratum conyzoides Bidens pilosa*	100 99.7
4	*Bidens pilosa*	5	*Bidens pilosa*	100	6	*Bidens pilosa*	100
5	*Matricaria chamomilla*	12	*Matricaria chamomilla Mikania micrantha*	100	13	*Matricaria chamomilla Mikania micrantha*	100 99.1
6	*Mikania micrantha*	5	*Mikania micrantha*	100	6	*Mikania micrantha*	100
7	*Spilanthes acmella*	3	*Spilanthes acmella*	100	11	*Spilanthes acmella Spilanthes*	100 99.8

						anactina	
8	*Tagetes erecta*	5	*Tagetes erecta*	100	5	*Tagetes erecta*	100
9	*Zinnia elegans*	5	*Zinnia elegans*	100	15	*Zinnia elegans* *Zinnia acerosa* *Zinnia anomala*	100 99.6 99.1
10	*Crassocephalum crepidioides*	6	*Crassocephalum crepidioides*	100	5	*Crassocephalum crepidioides*	100
11	**Impatiens balsamina*	5	**Impatiens balsamina*	100	6	**Impatiens balsamina*	100
12	*Tecoma stans*	5	*Tecoma stans*	100	9	*Tecoma stans* *Tecoma tenuiflora*	100 99.5
13	*Brassica camprestris*	5	*Brassica camprestris*	100	6	*Brassica camprestris*	100
14	*Carica papaya*	4	----		7	*Carica papaya*	100
15	**Cucumis sativus*	6	**Cucumis sativus*	100	5	**Cucumis sativus*	100
16	**Thladiantha cordifolia*	5	**Thladiantha cordifolia*	100	6	**Thladiantha cordifolia*	100
17	**Momordica charantia*	6	**Momordica charantia*	100	3	**Momordica charantia*	100
18	*Sechium edule*	3	*Sechium edule*	100	4	*Sechium edule*	100
19	**Cyperus rotundus*	11	**Cyperus rotundus* *Cyperus laxus*	100 99.8	6	**Cyperus rotundus*	100
20	**Elaeocarpus lanceifolius*	5	**Elaeocarpus lanceifolius*	100	7	**Elaeocarpus lanceifolius*	100
21	*Croton joufra*	4	*Croton joufra*	100	4	*Croton joufra*	100
22	**Mallotus paniculatus*	7	**Mallotus paniculatus*	100	9	**Mallotus paniculatus* *Mallotus barbatus*	100 99.7
23	*Caesalpinia pulcherrima*	4	----		7	*Caesalpinia pulcherrima*	100
24	*Cassia javanica*	13	*Cassia javanica* **Derris robusta*	100 99.2	11	*Cassia javanica* *Cassia abbreviata*	100 99.1
25	**Derris robusta*	6	**Derris robusta*	100	6	**Derris robusta*	100

26	*Mimosa pudica*	12	*Mimosa pudica Mimosa pigra*	100 99.2	13	*Mimosa pudica Mimosa pigra*	100 99.4
27	**Indigofera zollingeriana*	7	**Indigofera zollingeriana*	100	5	**Indigofera zollingeriana*	100
28	*Leucaena leucocephala*	5	*Leucaena leucocephala*	100	6	*Leucaena leucocephala*	100
29	*Phaseolus vulgaris*	6	*Phaseolus vulgaris*	100	10	*Phaseolus vulgaris Phaseolus acutifolius*	100 99.0
30	*Tamarindus indica*	3	----		12	*Tamarindus indica*	100
31	*Delonix regia*	6	*Delonix regia*	100	3	*Delonix regia*	100
32	**Indigofera zollingeriana*	7	**Indigofera zollingeriana*	100	5	**Indigofera zollingeriana*	100
33	*Leucaena leucocephala*	5	*Leucaena leucocephala*	100	6	*Leucaena leucocephala*	100
34	*Castanopsis tribuloides*	5	*Castanopsis tribuloides*	100	7	*Castanopsis tribuloides*	100
35	*Coleus scutellarioides*	3	*Coleus scutellarioides*	100	4	----	100
36	*Lagerstromia speciosa*	3	*Lagerstromia speciosa*	100	4	*Lagerstromia speciosa*	100
37	*Punica granatum*	13	*Punica granatum Punica protopunica*	100 99.5	4	*Punica granatum*	100
38	*Althaea rosea*	3	*Althaea rosea*	100	3	*Althaea rosea*	100
39	*Anthurium andreanum*	3	*Anthurium andreanum*	100	4	*Anthurium andreanum*	100
40	*Colona floribunda*	6	*Colona floribunda*	100	12	*Colona floribunda Colona isodiametrica*	100 99.2
41	*Hibiscus rosa sinensis*	5	*Hibiscus rosa sinensis*	100	5	*Hibiscus rosa sinensis*	100
42	*Malvaviscus arboreus*	2	*Malvaviscus arboreus*	100	8	*Malvaviscus arboreus Malvaviscus achanioides*	100 99.1
43	*Urena lobata*	2	*Urena lobata*	100	2	*Urena lobata*	100
44	*Musa paradisiaca*	3	*Musa paradisiaca*	100	3	*Musa paradisiaca*	100 99.

			Musa acumita			*Musa acumita*	3
45	*Callistemon lanceolatus*	14	*Callistemon lanceolatus* *Callistemon speciosus*	100 99.1	19	*Callistemon lanceolatus* *Callistemon speciosus* *Callistemon citrinus*	100 99.7 99.2
46	*Psidium guajava*	2	*Psidium guajava*	100	5	*Psidium guajava* *Psidium acunae*	100 99.6
47	*Averrhoa carambola*	4	*Averrhoa carambola*	100	4	*Averrhoa carambola*	100
48	*Zea mays*	3	*Zea mays*	100	5	*Zea mays*	100
49	*Antigonon leptopus*	12	*Antigonon leptopus* *Antigonon flavescens*	100 99.2	15	*Antigonon leptopus* *Antigonon flavescens* *Antigonon guatimalense*	100 99.5 99.0
50	*Portuluca oleracea*	3	*Portuluca oleracea*	100	2	*Portuluca oleracea*	100
51	*Rosa macrophylla*	4	*Rosa macrophylla*	100	3	*Rosa macrophylla*	100
52	*Acronychia pedunculata*	4	----		5	*Acronychia pedunculata*	100
53	*Solanum melongena*	6	*Solanum melongena*	100	4	*Solanum melongena*	100
54	*Tropaelum majus*	3	*Tropaelum majus*	100	4	*Tropaelum majus*	100
55	*Lantana camara*	2	*Lantana camara*	100	5	*Lantana camara*	100
56	*Callicarpa arborea*	6	*Callicarpa arborea*	100	7	*Callicarpa arborea*	100
57	Unidentified	14			16		

* =Native species. Molecular identification of the pollen collected from at Tanhril natural forest. The number of clones for each MOTU, the species match in reference database and the related identity values higher than 99% (ID%) obtained with the BLAST search are reported for the two barcode regions; Out of 56 plants matK and rbcL identified 53 and 55 plants respectively.

Table 10. Molecular identification of plant species detected in honey in Fall season. The number of clones for each MOTU, the species match in reference database (RD) and the related identity values (ID%) obtained with the BLAST search are reported for the two barcode regions.

SN	Identified plant	*matK*			*rbcL*		
		MOTUs (no. clones)	**Species match in RD**	**ID %**	**MOTUs (no. clones)**	**Species match in RD**	**ID %**
1	**Rhus chinensis*	6	*Rhus chinensis*	100	5	*Rhus chinensis*	100
2	*Asclepias curassavica*	5	*Asclepias curassavica*	100	10	*Asclepias curassavica* *Asclepias tuberosa*	100 99.2
3	*Cosmos sulphureus*	3	*Cosmos sulphureus*	100	6	*Cosmos sulphureus*	100
4	*Mikania micrantha*	2	*Mikania micrantha*	100	12	*Mikania micrantha* *Mikania cordifolia*	100 99.2
5	*Spilanthes acmella*	3	----	100	4	*Spilanthes acmella*	100
6	*Tagetes erecta*	12	*Tagetes erecta* *Tagetes lucida*	100	12	*Tagetes erecta* *Tagetes lucida*	100 99.4
7	*Tithonia diversifolia*	4	*Tithonia diversifolia*	100	3	*Tithonia diversifolia*	100
8	*Crassocephalum crepidioides*	14	*Crassocephalum crepidioides* *Crassocephalum baoulense*	100 99.3	2	*Crassocephalum crepidioides*	100
9	*Alnus nitida*	2	*Alnus nitida*	100	3	*Alnus nitida*	100
10	*Tecoma stans*	3	*Tecoma stans*	100	10	*Tecoma stans* *Tecoma tenuiflora*	100 99.2
11	*Bixa orellana*	4	*Bixa orellana*	100	2	*Bixa orellana*	100
12	**Terminalia crenulate*	6	*Terminalia crenulate*	100	11	*Terminalia crenulate* *Terminalia bellirica*	100 99.4
13	**Terminalia bellirica*	2	*Terminalia bellirica*	100	4	*Terminalia bellirica*	100

14	*Cyperus rotundus	10	Cyperus rotundus Cyperus laxus	100	10	Cyperus rotundus Cyperus laxus	100 99.4
15	Croton joufra	2	----	99.4	4	Croton joufra	100
16	*Mallotus paniculatus	3	Mallotus paniculatus	100	6	Mallotus paniculatus	100
17	Cassia javanica	4	Cassia javanica	100	17	Cassia javanica Cassia abbreviate Cassia afrofistula	100 99.5 99.1
18	*Indigofera zollingeriana	13	Indigofera zollingeriana Indigofera tinctoria	100 99.5	12	Indigofera zollingeriana Indigofera tinctoria	100 99.4
19	Leucaena leucocephala	3	Leucaena leucocephala	100	4	Leucaena leucocephala	100
20	*Parkia timoriana	10	Parkia timoriana Crotalaria pallida	100 99.8	9	Parkia timoriana Parkia biglobosa	100 99.4
21	Crotalaria pallida	3	Crotalaria pallida	100	2	Crotalaria pallida	100
22	*Castanopsis tribuloides	11	Castanopsis tribuloides Castanopsis chinensis	100 99.5	15	Castanopsis tribuloides Castanopsis chinensis Castanopsis fissa	100 99.2 99.0
23	*Holmskioldia sanguine	6	----		4	Holmskioldia sanguine	100
24	Coleus scutellarioides	12	Coleus scutellarioides Coleus caninus	100	3	Coleus scutellarioides	100
25	Althaea rosea	3	Althaea rosea	99.5	4	Althaea rosea	100
26	Anthurium andreanum	4	Anthurium andreanum	100	3	Anthurium andreanum	100
27	*Colona floribunda	3	Colona floribunda	100	7	Colona floribunda Colona	100 99.5

						javanica	
28	*Hibiscus rosa sinensis*	3	*Hibiscus rosa sinensis*	100	3	*Hibiscus rosa sinensis*	100
29	*Urena lobata*	3	*Urena lobata*	100	6	*Urena lobata*	99.5
30	*Triumfetta rhomboidea*	3	*Triumfetta rhomboidea*	100	2	*Triumfetta rhomboidea*	100
31	**Musa paradisiaca*	14	*Musa paradisiaca Musa acuminata*	100 99.4	9	*Musa paradisiaca Musa acuminata*	100 99.4
32	*Oryza sativa*	5	*Oryza sativa*	100	4	*Oryza sativa*	100
33	*Portuluca oleracea*	4	*Portuluca oleracea*	100	3	*Portuluca oleracea*	100
34	**Rosa macrophylla*	3	*Rosa macrophylla*	100	7	*Rosa macrophylla Rosa gallica*	100 99.7
35	*Coffee Arabica*	4	*Coffee Arabica*	100	3	*Coffee Arabica*	100
36	*Solanum torvum*	6	*Solanum torvum*	100	2	*Solanum torvum*	100
37	*Tropaelum majus*	7	*Tropaelum majus*	100	4	*Tropaelum majus*	100
38	*Lantana camara*	4	*Lantana camara*	100	6	*Lantana camara*	100
39	Unidentified	17			18		

* =Native species. Molecular identification of the pollen collected from at Tanhril natural forest. The number of clones for each MOTU, the species match in reference database and the related identity values higher than 99% (ID%) obtained with the BLAST search are reported for the two barcode regions; Out of 38 plants *matK* and *rbcL* identified 35 and 38 plants respectively.

5.3.1 Physicochemical analysis

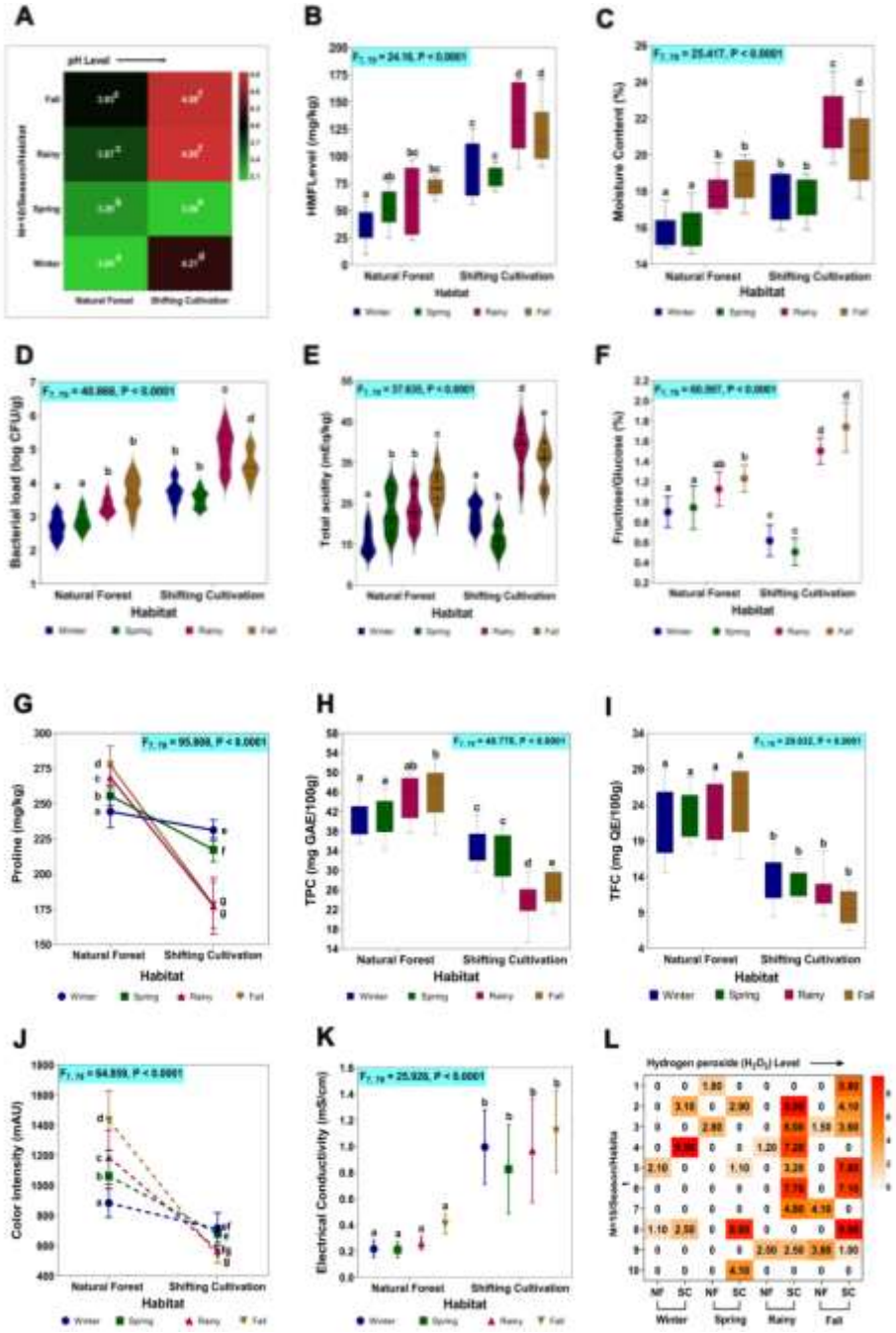

Figure 17. Assessment of *Apis cerana* honey quality at different seasons from natural and shifting cultivation habitats. A) pH level, B) Hydroxymethylfurfural (HMF) level (mg/kg), C) Moisture content (%), D) Bacterial load (log CFU/g), E) Total acidity (mEq/kg), F) Fructose/Glucose (%),G) Proline (mg/kg), H) Total phenolic content (TPC) (mgGAE/100g) I) Total flavonoid content (TFC) (mg QE/100g), J) Color intensity (mAU), K) Electrical conductivity (mS/cm), L) Hydrogen peroxide(H2O2) level

pH Level (**Fig 17A**): The pH varies significantly between habitats and seasons. Highest pH is observed in Shifting Cultivation during Fall (4.88). Lowest pH is seen in Natural Forest during Winter (3.04). HMF Level (**Fig 17B**): HMF (Hydroxymethylfurfural) content shows habitat and seasonal variability. Shifting Cultivation during Fall has the highest HMF levels (~160 mg/kg). Natural Forest during Winter shows the lowest HMF levels (~30 mg/kg). Moisture Content (**Fig 17C**): Moisture content is higher in Shifting Cultivation compared to Natural Forest. Shifting Cultivation in Fall has the highest moisture (~23%). Natural Forest during Winter has the lowest moisture (~15%). Bacterial Load (**Fig 17D**): Bacterial load varies significantly, with higher counts in Shifting Cultivation. Shifting Cultivation during Fall exhibits the highest bacterial load. Natural Forest during Winter shows the lowest bacterial count. Total Acidity (**Fig 17E**): Total acidity increases in Shifting Cultivation, especially in Fall. Shifting Cultivation during Fall shows the highest total acidity (~50 mEq/kg). Natural Forest during Winter has the lowest acidity (~25 mEq/kg). Fructose/Glucose Ratio (**Fig 17F**): Shifting Cultivation in Fall has the highest fructose/glucose ratio (~2.1%). Natural Forest during Winter shows the lowest ratio (~1.1%). Proline (**Fig 17G**): Proline levels show significant seasonal and habitat variation. Natural Forest during Winter has the highest proline content (~325 mg/kg). Shifting Cultivation during Fall has the lowest proline content (~150 mg/kg). Total Phenolic Content (TPC) (**Fig 17H**): TPC levels are higher in Natural Forest compared to Shifting Cultivation. Natural Forest during Spring has the highest TPC (~53 mg GAE/100g). Shifting Cultivation during Fall shows the lowest TPC (~15 mg GAE/100g). Total Flavonoid Content (TFC) (**Fig 17I**): TFC levels are higher in Natural Forest for most seasons. Natural Forest during Winter shows the highest TFC (~32 mg QE/100g). Shifting Cultivation during Fall exhibits the lowest TFC (~12 mg QE/100g). Color Intensity (**Fig 17J**): Color intensity is highest in Natural Forest, especially during Spring (~1500 mAU). Shifting Cultivation during Fall has the lowest color intensity (~250 mAU). Electrical Conductivity (**Fig 17K**): Electrical conductivity is higher in Shifting Cultivation for most seasons. Shifting Cultivation during Fall exhibits the highest conductivity (~1.25 mS/cm). Natural Forest during Winter shows the lowest conductivity (~0.4 mS/cm). Hydrogen Peroxide (H_2O_2) Levels (**Fig 17L**): The heatmap shows the H_2O_2

levels across different habitats and seasons. Shifting Cultivation during Fall has the highest H_2O_2 levels (~8.0). Natural Forest during Winter shows the lowest H_2O_2 levels (~0.30).

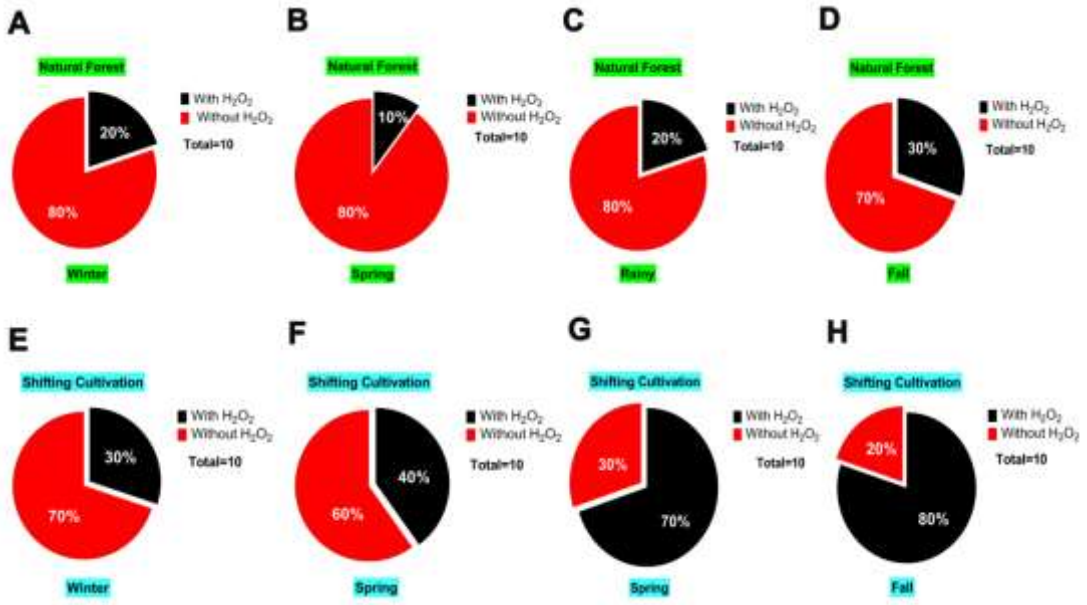

Figure 18. Percentage of the honey samples from the natural forest and shifting cultivation that contains hydrogen peroxide and does not. Total honey sample values (n=10/Season/Habitat) are used to calculate percentages.

Twenty percent of honey samples in their natural environment and thirty percent in their shifting cultivation habitat contained hydrogen peroxide throughout the winter (**Fig 18**). During spring, we discovered that ten percent of honey samples from natural habitats contained hydrogen peroxide, while forty percent of samples from shifting agricultural habitats exhibited the same. In natural habitats, twenty percent of honey samples included hydrogen peroxide, whereas in changing cultivation sites, seventy percent of honey samples exhibited hydrogen peroxide concentration. Thirty percent of honey samples from natural habitats and eighty percent from shifting cultivation sites contained hydrogen peroxide.

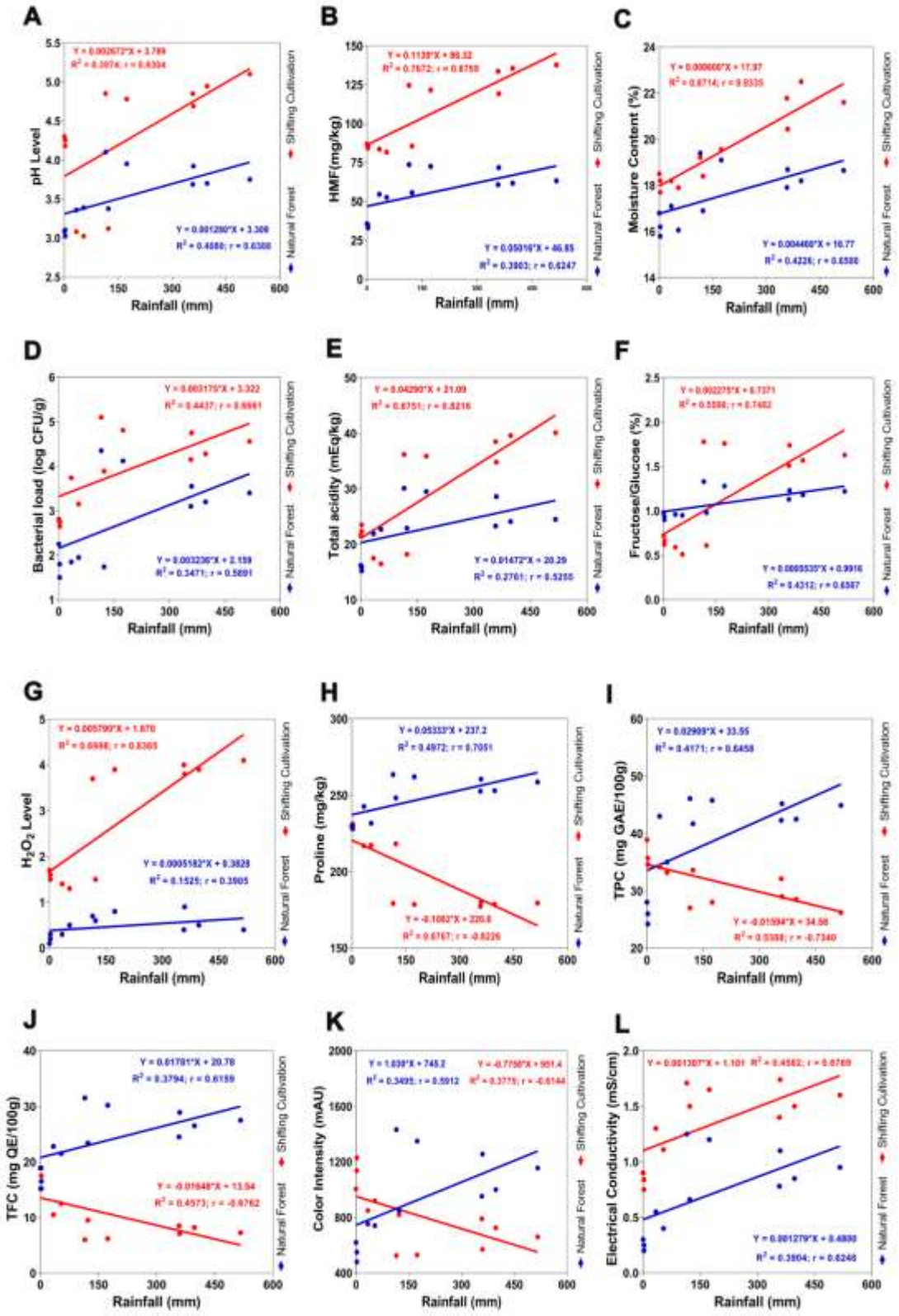

Figure 19. Correlation between rain fall and physicochemical properties. Correlation of A) pH level, B) Hydroxymethylfurfural (HMF) level (mg/kg), C) Moisture content

(%), D) Bacterial load (log CFU/g), E) Total acidity (mEq/kg), F) Fructose/Glucose (%), G) Hydrogen peroxide (H_2O_2 level), H) Proline (mg/kg), I) Total phenolic content (TPC) (mgGAE/100g), J) Total flavonoid content (TFC) (mg QE/100g), K) Color intensity (mAU), L) Electrical conductivity (mS/cm) against rainfall (mm) of the honey sample collected at different seasons from natural forest and shifting cultivation. Values are the mean of ten honey sample (n = 10/Season/Habitat).

Positive Correlations- pH: Natural conditions (R^2 = 0.4080) and shifting conditions (R^2 = 0.3974), rain fall moderately elevates pH levels in both ecosystems (**Fig 19A**). Rain fall can neutralise acidic or basic compounds deposited from atmospheric pollution, consequently modifying the pH of soil and water. HMF (Hydroxymethylfurfural): Natural conditions (R^2 = 0.3903) and Shifting conditions (R^2 = 0.7672) (**Fig 19B)**. The correlation between HMF and rain fall is significantly more pronounced in dynamic situations. HMF is a result of sugar breakdown, frequently linked to environmental stress or substandard organic matter quality. Moisture Content: Natural conditions (R^2 = 0.4226) and Shifting conditions (R^2 = 0.8714) (**Fig 19C)**. As anticipated, rain fall favourably influences moisture content, with the correlation being significantly stronger in dynamic situations. Bacterial Load: Natural conditions (R^2 = 0.3471) and Shifting conditions (R^2 = 0.4437) (**Fig 19D)**. Precipitation exhibits a positive, albeit moderate, connection with bacterial burden in both natural and altered environments. Total Acidity: Natural conditions (R^2 = 0.2761) and Shifting conditions (R^2 = 0.6751) (**Fig 19E)**. Precipitation exerts a more pronounced influence on total acidity in dynamic ecosystems, since rainwater can leach acidic compounds from soil and contaminants, thereby elevating overall acidity Fructose/Glucose Ratio: Natural conditions (R^2 = 0.4312) and Shifting conditions (R^2 = 0.5598) (**Fig 19F)**. Rainfall exhibits a favourable link with the fructose/glucose ratio in both habitats, with a more pronounced correlation in changing environments. H_2O_2 (Hydrogen Peroxide): Natural conditions (R^2 = 0.1525) and Shifting conditions (R^2 = 0.6998) (**Fig 19G)**. Precipitation exerts a negligible influence on H_2O_2 concentrations in natural settings, although demonstrates a significantly greater positive association in altered ecosystems. H_2O_2 serves as an indicator of oxidative stress, with its accumulation likely influenced by

stressors such as pollution or disrupted ecosystems. Electrical Conductivity: Natural conditions (R^2 = 0.3904) and Shifting conditions (R^2 = 0.4582) (**Fig 19H)**. Precipitation exhibits a positive association with electrical conductivity in both settings, indicating elevated ion concentration resulting from the leaching of dissolved salts and minerals by precipitation. Negative Correlations- Proline: Natural conditions (R^2 = 0.4972) and shifting conditions (R^2 = 0.6767) (**Fig 19I)**: Rainfall exhibits a significant negative connection with proline concentrations, especially in variable situations. Total Phenolic Content: Natural conditions (R^2 = 0.4171) and Shifting conditions (R^2 = 0.5388) (**Fig 19J)**. The total phenolic content diminishes as rainfall increases, with a more pronounced correlation in shifting habitats. Total Flavonoid Content: Natural conditions (R^2 = 0.3794) and Shifting conditions (R^2 = 0.4573) (**Fig 19K)**. Similar to phenolics, total flavonoid content exhibits an inverse connection with rainfall, indicating that plants diminish the synthesis of these defensive chemicals when water supply is less constrained. Colour Intensity: Natural conditions (R^2 = 0.3794) and Altered conditions (R^2 = 0.4573) (**Fig 19L)**: Colour intensity, an indicator of pigmentation in plants, diminishes with heightened rainfall, exhibiting a marginally stronger association in dynamic situations. The findings demonstrate that rain fall substantially influences several environmental characteristics, with more pronounced effects noted in dynamic ecosystems.

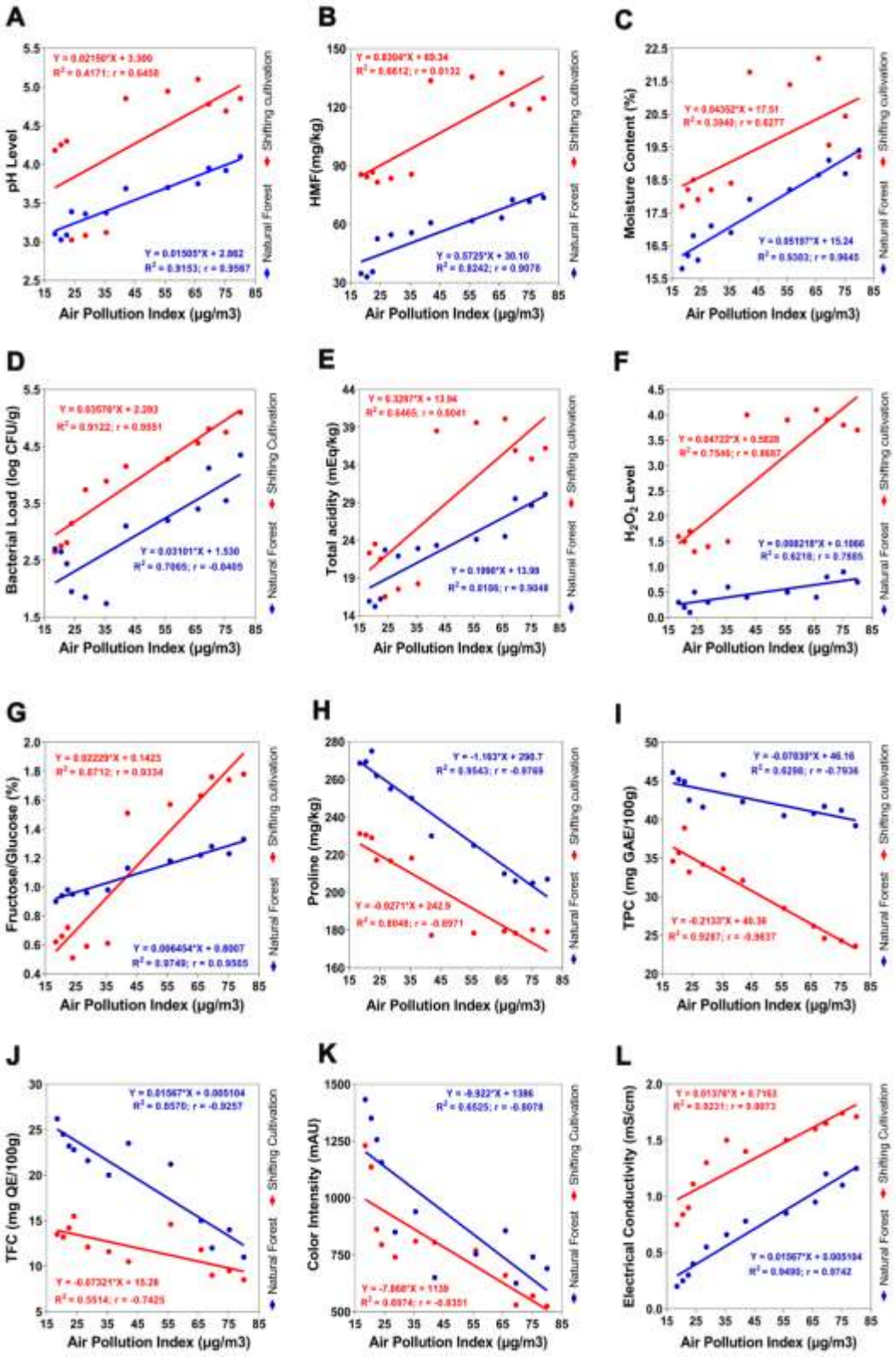

Figure 20. Correlation between air pollution index and physicochemical properties. Correlation of A) pH level, B) Hydroxymethylfurfural (HMF) level (mg/kg), C)

Moisture content (%), D) Bacterial load (log CFU/g), E) Total acidity (mEq/kg), F) Hydrogen peroxide (H_2O_2 level), G) Fructose/Glucose (%), H) Proline (mg/kg), I) Total phenolic content (TPC) (mgGAE/100g), J) Total flavonoid content (TFC) (mg QE/100g), K) Color intensity (mAU), L) Electrical conductivity (mS/cm) against air pollution index of the honey sample collected at different seasons from natural forest and shifting cultivation. Values are the mean of ten honey sample (n = 10/Season/Habitat).

Positive Correlations- pH: Natural Conditions: A robust positive association exists with air pollution levels ($R^2 = 0.9153$) (**Fig 20A**), suggesting that when air pollution escalates, the pH level dramatically increases under natural conditions. Variable settings: The correlation is diminished ($R^2 = 0.4171$), indicating that under variable conditions, pH is less significantly affected by pollution than in natural habitats. HMF (Hydroxymethylfurfural): Natural Conditions: A robust connection ($R^2 = 0.8242$) indicates that elevated air pollution is associated with increased levels of HMF in natural habitats. The correlation diminishes however remains significant ($R^2 = 0.6612$). Moisture Content: Natural Conditions: There is a robust connection ($R^2 = 0.9303$) (**Fig 20B**), indicating that moisture content escalates with increasing air pollution in natural environments. Shifting conditions: A substantially smaller association ($R^2 = 0.3940$) (**Fig 20C**), demonstrating that in shifting surroundings, moisture content is less effected by air pollution. Bacterial Load: Natural conditions: A relatively good association ($R^2 = 0.7065$) (**Fig 20D**) implies that bacterial proliferation increases as pollution levels rise in natural environments. Shifting conditions: This association becomes significantly stronger ($R^2 = 0.9122$) in changing settings, showing that bacteria may respond more strongly to air pollution in transitioning habitats. Total Acidity: Natural conditions: Strong association ($R^2 = 0.8186$) (**Fig 20E**), demonstrating that air pollution tends to increase total acidity under natural conditions. Shifting conditions: This association is somewhat weaker ($R^2 = 0.6465$). H_2O_2 (Hydrogen Peroxide): Natural Conditions: A moderate connection ($R^2 = 0.6218$) indicates that H_2O_2 concentrations increase alongside elevated air pollution in natural settings. The association intensifies ($R^2 = 0.7546$) (**Fig 20F**) in changing circumstances. Fructose/Glucose Ratio: Natural Conditions: A

robust positive connection (R^2 = 0.9749) (**Fig 20G**) demonstrates a considerable rise in the fructose/glucose ratio corresponding with elevated air pollution under natural conditions. Changing circumstances: Despite being weaker, the association persists robustly (R^2 = 0.8712) in fluctuating contexts. Electrical Conductivity: Natural conditions: A robust correlation (R^2 = 0.9490) (**Fig 20H**) indicates that air pollution markedly elevates electrical conductivity in natural settings. The association is robust albeit marginally diminished (R^2 = 0.8231) in fluctuating situations. Negative Correlations- Proline: Natural Conditions: A robust negative correlation (R^2 = 0.9543) (**Fig 20I**) suggests that proline levels diminish when air pollution escalates in natural conditions. Changing circumstances: The association remains robust (R^2 = 0.8048) while being weaker under varying settings. Total Phenolic Content: Under natural conditions, there is a moderate negative association (R^2 = 0.6298) (**Fig 20J**), indicating that total phenolic content tends to diminish as air pollution increases in natural environments. The link intensifies markedly (R^2 = 0.9287) in altered environments, indicating a substantial decline in phenolic content with rising air pollution levels. Total Flavonoid Concentration: Environmental circumstances: Significant negative correlation (R^2 = 0.8570) (**Fig 20K**), demonstrating a reduction in total flavonoid content as air pollution escalates. The association is diminished (R^2 = 0.5514), indicating that the influence of air pollution on flavonoid content is less significant under shifting conditions. Colour Intensity: Natural settings exhibit a moderate negative association (R^2 = 0.6525) (**Fig 20L**), indicating that air pollution diminishes colour intensity under natural circumstances. Shifting conditions: The association is significant (R^2 = 0.6974), indicating that air pollution diminishes colour intensity in varying situations.

5.3.2 Major Royal Jelly Protein 2

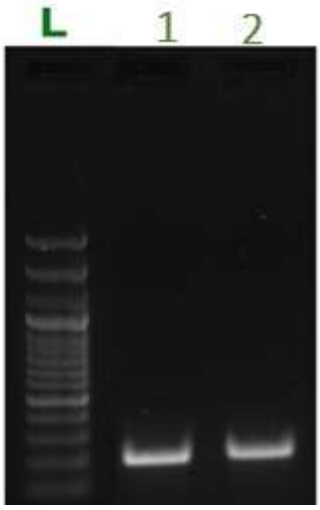

Figure 21: Genomic DNA band of honey samples

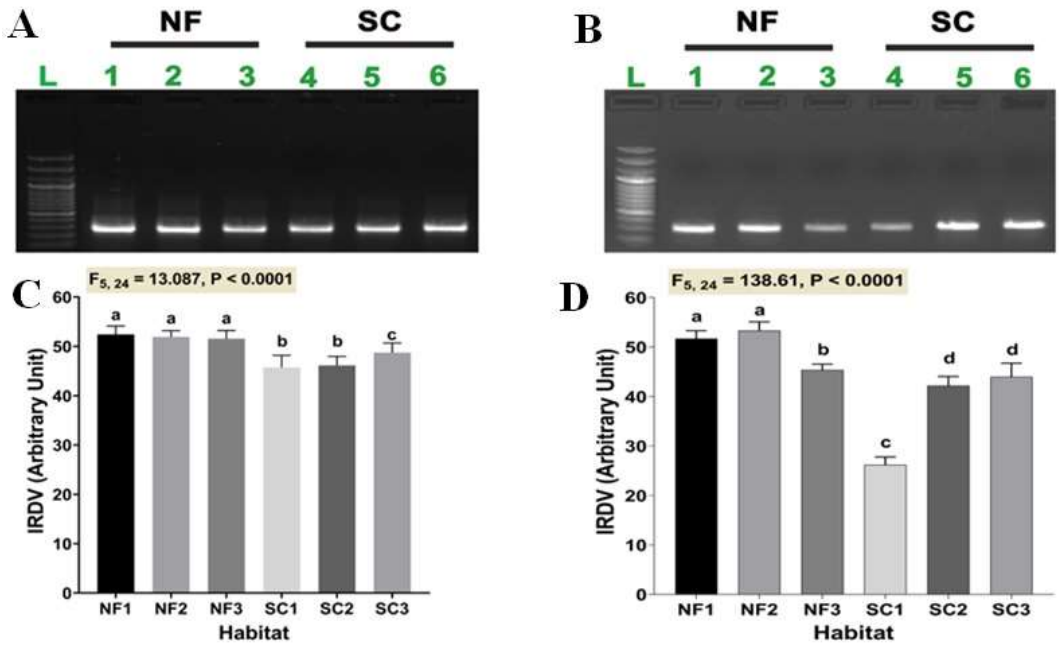

Figure 22: Assessment of MRJP2 gene from *Apis cerana* honey. A and B showing seasonal variation of MRJP2 gene expression in Fall and rainy seasons with respect to Natural forest (NF) and Shifting cultivation (SC). C and D showing IRDV integrated relative density values. Values are the mean of five honey sample (n = 5/habitat site). All values are expressed as the mean ± standard deviation. Statistical

comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests with a different letter means statistical significant difference at $p < 0.0001$ and similar letters are not significant.

Fig 21 illustrates the seasonal fluctuations in the expression of the MRJP2 gene in *A. cerana* honey across distinct habitats: Natural Forest (NF) and Shifting Cultivation (SC) during the Fall and Rainy seasons. **Fig 22A** and **Fig 22B** depict the PCR-based identification of the MRJP2 gene from honey samples obtained in both environments during the Fall (**Fig 22A**) and Rainy (**Fig 22B**) seasons. The bands associated with the MRJP2 gene are distinctly observable in all samples, exhibiting variable intensities across the different habitats. Lanes 1–3 denote samples from the NF habitat, whereas lanes 4–6 denote samples from the SC habitat. The findings indicate a more significant expression of the MRJP2 gene in the NF habitat relative to SC, particularly during the Fall season, implying a habitat-specific effect on gene expression. **Fig 22C** and **Fig 22D** display the Integrated Relative Density Values (IRDV) of MRJP2 gene expression obtained from the densitometric analysis of the bands in **Fig 22A** and **Fig 22B**. The IRDV offers a quantitative assessment of gene expression. During the Fall season (**Fig 22C**), NF1, NF2, and NF3 demonstrated elevated IRDV values, which were statistically distinct from SC1, SC2, and SC3. NF1 exhibited the highest expression of MRJP2, with significant differences noted among the habitats ($p < 0.0001$). The NF samples exhibited elevated expression levels in comparison to the SC samples. During the Rainy season (Graph D), the expression pattern exhibited a modest alteration, with NF1 and NF2 maintaining highly elevated IRDV values, but NF3 encountered a decrease. SC1 exhibited the lowest MRJP2 expression, considerably lower than other environment samples ($p < 0.0001$). The expression levels of MRJP2 in the SC habitat were continuously inferior to those in the NF habitat throughout the seasons.

CHAPTER VI

DISCUSSION

6. Discussion

6.1.1 Honeybee-floral interactions in the Tanhril forest

The results highlight significant seasonal variations in the availability of native and invasive plant species, reflecting how each group responds to environmental conditions and competitive pressures. Seasonal Dynamics: Native species experience peak availability during the spring, while their lowest availability is recorded in winter. This seasonal behavior is consistent with their reliance on specific environmental conditions for growth, such as temperature and precipitation patterns. Invasive species, on the other hand, exhibit less variation across seasons, maintaining a high presence throughout the year (Hussain, 2009). Their ability to thrive in diverse conditions, including colder winter months and wetter rainy seasons, underscores their competitive advantage. Impact of Invasive Species: Invasive species consistently outnumber native species in all seasons, posing a significant threat to the biodiversity of the ecosystem. Their higher availability, particularly in the rainy season when native species decline, suggests that invasive plants may have adaptations that enable them to outcompete native species for resources like water and sunlight. This imbalance could potentially lead to the displacement of native plants, altering the structure and function of local ecosystems. Environmental and Ecological Implications: The dominance of invasive species year-round could have long-term consequences on ecosystem stability, nutrient cycling, and habitat availability for native fauna. Their ability to rapidly colonize and spread in various climatic conditions poses a challenge to the conservation and restoration of native plant species. Management Considerations: The data suggest that targeted management efforts need to focus on controlling invasive species, especially during the spring and rainy seasons when their proliferation is at its highest. Additionally, strategies to support the growth and regeneration of native plants, particularly during seasons when their availability is low, such as winter and fall, are crucial to restoring ecological balance. This study emphasizes the need for continued monitoring and active management of both native and invasive species to ensure the preservation of biodiversity and the resilience of ecosystems against the spread of invasive plants.

6.1.2 Floral Resource Availability and Pollinator Dynamics

Another significant finding is the differential availability of floral resources between invasive and native plant species, which has important implications for pollinator dynamics. The floral calendar indicates that invasive plants provide more substantial floral resources for bee pollinators compared to native species. In particular, honey bees had the highest access to floral resources during the rainy season (June-August) and fall (September-November), followed by spring (March-May) and winter (December-February). This enhanced availability of floral resources by invasive plants may have both positive and negative implications for pollinators and native plant species. On the positive side, invasive species can provide a continuous supply of nectar and pollen, especially in times when native species are not in bloom. This may be particularly beneficial for generalist pollinators such as honey bees, which can exploit a wide variety of floral resources. In ecosystems where native plants are scarce or have been degraded, invasive plants may serve as an important food source for pollinators, helping to sustain populations (Sponsler *et al*., 2023). However, the increased reliance on invasive species for floral resources can also have detrimental effects on native plant-pollinator interactions. Native plants and their associated pollinators have co-evolved over time, often forming specialized relationships that are crucial for the reproduction of certain species (Woodard and Jha 2017). The dominance of invasive species in providing floral resources may disrupt these mutualistic interactions. Pollinators may shift their foraging preferences towards invasive plants, reducing the pollination success of native species and potentially leading to their decline. In the long term, this can result in a reduction of native plant diversity and a loss of specialized pollination networks, which are critical for maintaining ecosystem resilience.

6.1.3 Important Value Index (IVI)

The comparison of Important Value Index (IVI) between invasive and native plant species reveals significant ecological differences that underscore the substantial impact of invasive species on biodiversity and ecosystem stability. IVI is a critical ecological measure, representing the relative importance of a species in a given

habitat by integrating its relative density, dominance, and frequency (Curtis and McIntosh, 1951). The disparities in IVI values between invasive and native species suggest a competitive advantage for invasive species, which may have far-reaching implications for the structure and function of ecosystems. Disparities in IVI Between Invasive and Native Species: The significantly higher IVI values for invasive plant species across different vegetation strata herbs, shrubs, and trees highlight their dominant presence within the ecosystems under study. For herbaceous invasive species, IVI values ranged from 7.22% to 14.43%, while native herbs exhibited lower IVI values ranging from 9.14% to 12.93%. Invasive shrubs had IVI values between 5.70% and 12.25%, significantly higher than native shrubs, which ranged from 4.36% to 6.11%. Likewise, for tree species, invasive plants exhibited IVI values from 7.40% to 15.33%, compared to the lower IVI values of native tree species, which ranged from 6.85% to 12.35%. These findings point to the competitive superiority of invasive species. A higher IVI reflects an increased ability to occupy space, utilize resources, and thrive across different environmental conditions (Sharma *et al*., 2005). The success of invasive species can often be attributed to their robust growth patterns, high reproductive rates, and tolerance of a wide range of environmental factors. In contrast, many native species are adapted to more specific ecological niches, making them vulnerable to competition from invasives that can exploit resources more efficiently (Whittaker, 1972). Implications for Biodiversity and Ecosystem Stability: The significantly higher IVI values of invasive species raise concerns about the impact on biodiversity and ecosystem stability. The dominance of invasive species often results in the suppression or even local extinction of native species, particularly those that are less competitive or specialized. Native plants, which form the foundation of ecosystem processes such as nutrient cycling, habitat provision, and trophic interactions, are critical for maintaining biodiversity (Tilman, 1982). As invasive species displace native plants, the overall biodiversity of ecosystems may decline, leading to a cascade of negative effects on ecosystem services. Invasive plants have the capacity to alter the physical environment, modify fire regimes, change hydrological cycles, and disrupt mutualistic relationships between native species (Vitousek *et al*., 1997). For example, Lantana camara, one of the most widespread invasive species in India, forms dense thickets that reduce light

penetration and limit the growth of native understory vegetation (Sharma and Raghubanshi, 2009). This not only affects the plant community but also impacts herbivores and other organisms that depend on native plants for food and shelter.

The higher IVI values of invasive species suggest that they are better equipped to adapt to disturbances, whether anthropogenic or natural. In disturbed environments, invasive species often have a competitive edge due to their rapid growth rates and ability to colonize quickly. This trait allows them to establish dominance in areas where native species might struggle to recover after disturbances such as deforestation, agriculture, or climate-related events (Catford *et al*., 2012). As a result, invasive species can rapidly alter ecosystem dynamics, reduce habitat quality, and threaten the survival of native species. Competitive Advantages of Invasive Species: The success of invasive species in achieving higher IVI values compared to native species can be attributed to several competitive advantages. Invasive plants often exhibit traits such as high seed production, rapid growth, and the ability to spread vegetatively, allowing them to quickly establish and outcompete native vegetation (Curtis and McIntosh, 1950). Many invasive species also have a broad ecological tolerance, enabling them to thrive in a wide range of environmental conditions. This versatility allows them to colonize both disturbed and undisturbed habitats, further increasing their competitive advantage.

Invasive species may also benefit from a lack of natural enemies in their new environments, a concept known as the "enemy release hypothesis" (Curtis and McIntosh, 1950). In their native habitats, invasive plants are often kept in check by herbivores, pathogens, and other organisms that limit their growth and reproduction. However, in new environments, these control mechanisms are often absent, allowing invasive species to proliferate unchecked. This lack of predation or competition further enhances their ability to dominate ecosystems and displace native species. Additionally, many invasive plants possess allelopathic properties, meaning they can release chemicals into the soil that inhibit the growth of other plants. Parthenium hysterophorus, for example, is known for its allelopathic effects, which can suppress the germination and growth of native species, giving it a competitive edge (Dogra *et*

al., 2010). Such mechanisms further contribute to the ecological success of invasive species and their ability to achieve higher IVI values in comparison to native flora.

Management and Control Implications: The findings from the IVI analysis have important implications for the management and control of invasive species in India. The higher IVI values of invasive plants indicate their increasing dominance, which poses a serious threat to native biodiversity and ecosystem stability. Effective management strategies must be developed to mitigate the spread of invasive species and reduce their impact on native ecosystems. One of the key challenges in managing invasive species is the need for early detection and rapid response (EDRR). Once invasive species become established and achieve high IVI values, they are often difficult to control or eradicate (Phillips, 1959). Therefore, early identification of potential invasive species and proactive measures to prevent their introduction and spread are critical. This may include stricter regulations on the import and movement of plant species, as well as monitoring programs to detect new invasions before they become widespread. In cases where invasive species have already become established, integrated management approaches that combine mechanical, chemical, and biological control methods may be necessary (Rao *et al*., 2015). Mechanical removal of invasive plants can be effective in the short term, but it is often labor-intensive and may not provide a long-term solution. Chemical control using herbicides can help reduce the spread of invasive plants, but care must be taken to minimize the impact on non-target species and the environment. Biological control, which involves the introduction of natural enemies such as herbivores or pathogens, has shown promise in managing certain invasive species. For example, biological control agents have been successfully used to control the spread of Eichhornia crassipes (Water hyacinth) in India (Phillips, 1959). However, the introduction of biological control agents must be carefully studied to avoid unintended consequences, such as the control agents becoming invasive themselves.

6.2. Floral displays

The association graph illustrates significant disparities in bee responses to floral presentations of invasive and native plant species. Invasive plants have a more

pronounced link between floral display size and bee visitation, as seen by the elevated slope (0.7157) and R^2 value (0.8239). This indicates that invasive species, characterised by larger or more conspicuous floral displays, are more effective at attracting bees than native species. The diminished correlation for native species (reduced slope and R^2) suggests that whereas floral display continues to affect bee visits, additional factors may exert a more substantial influence on the appeal of native species to bees. These criteria may encompass floral characteristics such as nectar content, flower morphology, or the temporal availability of blossoms, which could be more specialised or less visually conspicuous than the qualities of invasive species. The results indicate possible ecological ramifications: a) Invasive species may surpass native species in securing pollinator services by drawing an excessive number of bee visitors, potentially affecting the reproductive success and viability of native plants. b) Pollinator networks may become biassed, favouring invasive species and potentially resulting in alterations to ecosystem dynamics, including a decline in biodiversity (Goodell and Parker 2017)). Comprehending the fundamental causes of these disparities in bee attraction is essential for ecosystem management, especially in areas with a high prevalence of invasive species. Initiatives to promote native species should concentrate not only on augmenting floral displays but also on improving other attributes that enhance their appeal to bees, in order to counteract the competitive edge of alien species.

6.2.1. Pollen avalailablility in honey samples

The analysis of honey samples across different seasons reveals a diverse range of plant species contributing to the pollen content, reflecting the seasonal variability of floral resources available to bees. The "Parts hole graph" illustrates these seasonal variations, highlighting key plant species that dominate each season while also showing the presence of less prevalent species. This variability is crucial in understanding the ecology of bee foraging behavior and the role of specific plants in honey production. Winter Season: During the winter season, *Macaranga peltata* stands out as the most dominant species, contributing 9.87% of the pollen content in honey samples. The high prevalence of *Macaranga peltata* during this season suggests that it is a crucial nectar and pollen source for bees when floral availability

might be limited due to colder conditions. On the other hand, *Leucosceptrum canum* represents the least abundant species in winter with a contribution of 3.29%, indicating that while it is present, it may not be a major foraging resource for bees during this period. Spring Season: In spring, a different pattern of plant dominance emerges, with *Ageratum conyzoides* contributing the highest pollen percentage at 5.20%. This shift reflects the seasonal blooming patterns and the increase in floral diversity typically associated with spring. *Ageratum conyzoides*'s abundance during this season suggests its importance in early-season foraging. Conversely, *Derris robusta*, which only contributes 0.79%, shows that some species may be less available or less attractive to bees during this time, possibly due to competition from other flowering plants. Fall Season: The fall season presents *Lantana camara* as the dominant species with a 6.45% contribution, highlighting its significance as a late-season floral resource. The presence of this species in higher quantities indicates that bees rely heavily on it during the transition from summer to cooler months. *Cassia javanica*, however, is the least represented species in the fall samples with only 0.79%, which could reflect its limited bloom during this time or lower attractiveness to bees compared to other available species. Rainy Season: During the rainy season, *Castanopsis tribuloides* emerges as the highest contributing species with 4.77%. The dominance of this species during the rainy period suggests it is a critical resource when other species may be less accessible due to environmental conditions such as heavy rainfall. In contrast, *Anthurium andreanum* shows the lowest contribution at 0.75%, indicating its limited role in honey production during this season. The presence of *Anthurium andreanum* suggests that while bees forage across a wide range of species, some plants provide relatively less pollen or nectar (Kirk, 2018).

Seasonal Influence on Bee Foraging Behavior: The variation in dominant plant species across seasons underscores the adaptability of bees to changing floral landscapes. The highest contributing species in each season *Macaranga peltata* in winter, Ageratum conyzoides in spring, Lantana camara in fall, and *Castanopsis tribuloides* in the rainy season highlight the dynamic foraging strategies of bees in response to the availability of resources. These findings suggest that bees optimize their foraging behavior by targeting the most abundant and accessible plants in each

season. Additionally, the presence of lower-contributing species like *Leucosceptrum canum, Derris robusta, Cassia javanica,* and *Anthurium andreanum* in each season indicates that while bees may prioritize certain plants, they continue to forage from a diverse array of species to maintain a balanced diet. Ecological Implications: The observed seasonal patterns also have broader ecological implications. The reliance on specific plants during particular seasons could influence the pollination success of these species, contributing to their reproductive cycles. Additionally, the availability of diverse floral resources across different seasons may enhance the health and resilience of bee populations by providing a consistent supply of nectar and pollen throughout the year. In conclusion, the "Parts hole graph" analysis provides valuable insights into the seasonal dynamics of honey production and bee foraging behavior. The identification of key plant species in each season not only enhances our understanding of the relationship between bees and their floral environment but also has practical implications for the management of honeybee colonies and the conservation of plant biodiversity (Kirk, 2018). Future research could focus on how environmental changes, such as climate variation and habitat loss, might impact these seasonal foraging patterns and the availability of critical floral resources for bees.

6.2.2 DNA barcoding

The successful isolation and amplification of DNA from plant reference samples and pollen mixtures demonstrate the efficacy of DNA barcoding techniques in identifying and cataloging plant species in ecological research. The concentrations of 20 ng/ml, considered acceptable for such analyses, along with the high-quality DNA features, underscore the robustness of the DNA extraction protocols employed in this study. The high success rate of DNA amplification using standard primer pairs and thermal cycling conditions for two common plant barcoding markers, rbcL and matK, further validates the utility of these markers for plant species identification (Hebert *et al*., 2003a). These molecular markers are widely recognized in the scientific community for their versatility in amplifying a broad range of plant species, which is essential for biodiversity assessments and ecological studies (Hebert *et al*., 2003b).

DNA Barcoding in Plant Identification- The first DNA barcoding entries in GenBank, comprising 95 plant species collected from the Tanhril forest, represent a significant contribution to the global DNA barcode database and found out 90 plants through DNA Barcoding. This collection forms the foundation of a reference library that enhances the ability of researchers to identify and classify plant species with precision. DNA barcoding, a technique that uses short, standardized regions of the genome to differentiate between species, has revolutionized plant taxonomy and ecology (Hebert *et al*., 2003). In particular, the combination of rbcL and matK gene regions is considered the gold standard for plant barcoding due to their high universality and discriminatory power (CBOL Plant Working Group, 2009).

In this study, the construction of a 94-taxon reference database based on the barcoding data from Tanhril forest plants plays a pivotal role in understanding the local flora. The reference database serves as a critical tool for comparing unknown plant species to documented reference sequences, allowing for accurate species identification in environmental samples, such as pollen mixtures. The availability of such databases in public repositories like GenBank ensures that this information is accessible to researchers worldwide, facilitating comparative studies and the monitoring of plant biodiversity in various ecosystems Hajibabaei et al., 2006).

Molecular Operational Taxonomic Units (MOTUs) and Pollen Mixtures- One of the significant outcomes of this research was the identification of between twelve to eighteen Molecular Operational Taxonomic Units (MOTUs) from pollen mixtures collected from beehives at the three research sites across three sampling days. MOTUs, which are defined based on sequence similarity thresholds, are used as proxies for species or taxonomic units in DNA-based biodiversity studies (Blaxter *et al*., 2005). These MOTUs consisted of one to twenty-eight sequences that exhibited little nucleotide divergence, indicating the presence of closely related plant taxa or a lack of significant genetic variation within the sampled taxa. This finding suggests that the plant species represented in the pollen mixtures were either genetically similar or belonged to species that exhibit low nucleotide divergence in the selected barcoding regions (rbcL and matK).

The use of MOTUs in this context is highly informative for understanding plant-pollinator interactions and the diversity of plant species utilized by bees for foraging. Bees play a critical role in the pollination of many plant species, and the pollen they collect provides valuable insights into the floral diversity available to pollinators in a given ecosystem (Keller *et al*., 2015). By analyzing the pollen DNA, researchers can identify the plant species that contribute to the diet of bees and assess the availability of floral resources in different environments. The ability to identify MOTUs from pollen samples thus provides a powerful tool for monitoring plant-pollinator dynamics and assessing the health of ecosystems that rely on pollinators for maintaining biodiversity and ecosystem services (Kremen *et al*., 2007).

Ecological Implications of Pollen Diversity- The identification of a range of MOTUs in the pollen samples suggests a relatively diverse array of plant species contributing to the pollen foraged by bees at the three research locales. The variation in the number of sequences per MOTU, ranging from one to twenty-eight, highlights the unequal contribution of different plant species to the overall pollen pool. Some plant species were represented by numerous sequences, indicating their dominance in the floral landscape and suggesting that they were preferentially foraged by bees. Conversely, other species were represented by fewer sequences, which may reflect their lower abundance in the environment or their limited attractiveness to pollinators (Bruni *et al*., 2015).

This variation in pollen composition across the three sampling sites could be influenced by several factors, including the spatial distribution of plant species, temporal changes in floral availability, and the foraging preferences of bees. The study's observation of similar MOTU patterns across different sampling days points to a degree of temporal consistency in the pollen-plant relationships, suggesting that certain plant species provide stable floral resources throughout the sampling period. However, variations in the number of MOTUs between sites may indicate differences in local plant communities, which could be shaped by factors such as microclimatic conditions, land use patterns, and habitat quality (Bruni *et al*., 2015).

The presence of specific plant species in the pollen mixtures, particularly those that are known to be ecologically important or rare, could have broader implications for conservation efforts. Identifying the key species that support pollinators can inform habitat management strategies aimed at enhancing floral resource availability. Conservation practices that promote the growth of these plant species can contribute to maintaining healthy pollinator populations, which are essential for pollinating crops and wild plants alike (Bruni *et al*., 2015).

Pollen Foraging and Bee Ecology- The collection of pollen from beehives offers valuable insights into the foraging behavior of bees and the types of plants that constitute their diet. Bees are generalist foragers, meaning that they collect pollen from a wide variety of plant species, depending on the availability of floral resources (Bruni *et al*., 2015). However, their foraging behavior can be influenced by a variety of factors, including floral morphology, nectar rewards, and environmental conditions. In this study, the floral calendar revealed that honey bees had the highest access to floral resources during the rainy season (June-August) and fall (September-November), followed by spring (March-May) and winter (December-February). This temporal variation in floral resource availability reflects the phenology of the local plant species and their bloom periods.

The dominance of particular MOTUs in the pollen samples during certain seasons suggests that some plant species play a more prominent role in supporting bee populations at specific times of the year. For instance, the rainy season may coincide with the blooming of key plant species that provide abundant nectar and pollen, making them important for maintaining bee colonies during periods of high foraging activity. The identification of such species can be critical for understanding seasonal shifts in pollinator behavior and planning conservation strategies that ensure the availability of floral resources throughout the year (Bruni *et al*., 2015).

Moreover, the presence of MOTUs with low nucleotide divergence in the pollen samples may reflect the bees' preference for closely related plant species or species with similar floral traits. This could have implications for the genetic diversity of plant populations, as bees may preferentially pollinate plants within certain

taxonomic groups, potentially influencing patterns of gene flow and plant reproduction (Waser and Price, 1991). Understanding these patterns is essential for managing pollinator-dependent ecosystems and ensuring the long-term viability of both plant and pollinator populations.

Future Applications of DNA Barcoding in Ecological Research- The success of DNA barcoding in this study, particularly in identifying plant species from pollen mixtures, highlights its potential for broader ecological applications. DNA barcoding can be used to assess plant diversity, monitor changes in plant communities over time, and track the spread of invasive species. Additionally, barcoding can aid in understanding plant-pollinator networks by providing detailed information on the composition of floral resources used by pollinators. Such data are invaluable for conserving pollinator populations, which are increasingly threatened by habitat loss, pesticide use, and climate change (Bruni *et al*., 2015).

Further research could expand on the current findings by incorporating more sampling sites and additional barcoding markers to capture a broader range of plant species. Integrating environmental DNA (eDNA) techniques, which involve the analysis of genetic material found in environmental samples such as soil and water, could also provide insights into plant diversity in ecosystems where traditional sampling methods are challenging. This approach would allow for a more comprehensive understanding of the interactions between plants and pollinators, as well as the factors influencing these relationships.

In inference, the application of DNA barcoding and the identification of MOTUs from pollen mixtures offer a powerful tool for studying plant-pollinator interactions, assessing biodiversity, and informing conservation strategies. The ability to accurately identify plant species from pollen provides valuable insights into the ecological dynamics of plant-pollinator networks and highlights the importance of maintaining diverse floral resources to support healthy pollinator populations (Bruni *et al*., 2015).

6.3. Physicochemical properties

This study's results reveal substantial ecological and seasonal differences in the physicochemical and microbiological quality of honey. These variations underscore the intricate interaction of environmental variables, plant biodiversity, and management approaches in determining the composition and quality of honey. The data highlight the influence of habitat and seasonality on critical quality markers, including pH, moisture content, bacterial load, proline content, phenolic and flavonoid chemicals, and hydrogen peroxide levels Solayman *et al*., 2016.

1. pH Value: The pH of honey significantly affects its durability, flavour, and microbiological characteristics. This investigation revealed significant variation in pH across habitats and seasons, with the highest pH (4.88) noted in shifting agriculture during autumn and the lowest pH (3.04) documented in wild forest samples during winter. An elevated pH in shifting cultivation honey during autumn may signify reduced acidity, typically linked to honey derived from plants experiencing environmental stress (Persano Oddo and Piro, 2004). The reduced pH in natural forest honey during winter indicates an elevated acidity level, commonly associated with enhanced microbiological stability and honey preservation (Solayman *et al*., 2016). The pH variations indicate habitat-specific and seasonal factors affecting the organic acid concentration in honey.

2. Concentrations of Hydroxymethylfurfural (HMF): HMF is a byproduct of sugar breakdown in honey, and its concentration escalates with the ageing of honey or exposure to heat. The research indicated that HMF concentrations were elevated in shifting cultivation honey during autumn (~160 mg/kg), significantly surpassing the permissible international threshold of 40 mg/kg established by the Codex Alimentarius for fresh honey (Solayman *et al*., 2016). Elevated HMF levels frequently signify inadequate storage or heating conditions, which may have transpired throughout the autumn season in shifting agriculture regions, possibly attributable to post-harvest management or environmental influences. Conversely, the lowest quantities of HMF were seen in natural forest honey during winter (~30 mg/kg), indicating superior preservation conditions. The findings indicate that honey

sourced from natural forests has superior storage stability relative to honey from shifting cultivation, likely attributable to less environmental stress on flora in natural ecosystems (Solayman *et al*., 2016).

3. Moisture content: The moisture level of honey is a crucial determinant of its stability and longevity. Honey derived from shifting agriculture in the autumn exhibited the maximum moisture level (about 23%), whereas honey from natural forests in winter displayed the lowest moisture content (around 15%). A moisture level exceeding 20% is typically regarded as unfavourable due to its elevation of fermentation and microbial development risks (Chirife *et al*., 2006). The elevated moisture content in shifting cultivation honey during autumn may be ascribed to the particular meteorological conditions in these regions, including high humidity levels, or the physiology of the nectar-producing flora. The reduced moisture content in natural forest honey during winter improves its microbiological stability and aligns with research indicating that cooler, drier circumstances yield honey with superior storage capability (Solayman *et al*., 2016).

4. Bacterial Load: The bacterial load serves as a significant microbiological quality indicator in honey. The research indicated that honey sourced from shifting agricultural regions demonstrated a markedly elevated bacterial load, particularly in the autumn season. Conversely, natural forest honey exhibited the lowest bacterial numbers throughout winter. Elevated bacterial levels in honey from shifting production may arise from environmental influences, including soil contamination, plant physiology, or handling methods (Solayman *et al*., 2016). Natural forest honey, subjected to less anthropogenic disturbance, presumably benefits from more pristine environmental conditions, resulting in reduced bacterial levels. The disparities underscore the necessity for enhanced sanitation and handling protocols in shifting agricultural regions to mitigate bacterial contamination in honey.

5. Total Acidity: Total acidity, indicative of organic acid content in honey, serves as a crucial quality parameter associated with flavour and preservation. Shifting cultivation honey exhibited the highest overall acidity in the autumn (50 mEq/kg), whereas wild forest honey demonstrated the lowest acidity in the winter (25

mEq/kg). Increased acidity is frequently linked to enhanced preservation and a more pronounced flavour profile (Solayman *et al*., 2016). The diminished acidity in natural forest honey during winter may signify a lower organic acid concentration or varied flower origins, resulting in a more subdued flavour profile. The findings indicate that ecological and seasonal variables significantly influence the acidity levels of honey.

6. Fructose to Glucose Ratio: The fructose to glucose ratio serves as a metric for honey's sweetness and its propensity to crystallise. This study found that shifting cultivation honey in the autumn exhibited the highest fructose/glucose ratio (2.1%), but wild forest honey in the winter displayed the lowest ratio (1.1%). A higher fructose concentration compared to glucose results in a slower crystallisation rate and an enhanced sweetness (Solayman *et al*., 2016). The diminished ratio of natural forest honey in winter signifies an elevated glucose concentration, potentially leading to expedited crystallisation and reduced sweetness perception. The results align with studies demonstrating that floral sources substantially affect the sugar composition of honey (Solayman *et al*., 2016).

7. Proline Concentration: Proline is an amino acid that acts as a quality indicator in honey, with elevated concentrations indicating enhanced maturity and quality. Natural forest honey in winter exhibited the highest proline concentration (325 mg/kg), whereas shifting cultivation honey in autumn displayed the lowest (150 mg/kg). The elevated proline concentration in natural forest honey indicates that honey from this environment is of exceptional quality, presumably owing to the varied and undisturbed floral resources (Bogdanov *et al*., 1999). Reduced proline concentrations in honey from shifting cultivation may indicate environmental stress or inadequate nectar supplies in these regions.

8. Aggregate Phenolic and Flavonoid Concentration: Total phenolic content (TPC) and total flavonoid content (TFC) serve as significant indications of honey's antioxidant qualities. The findings indicated that total phenolic content (TPC) and total flavonoid content (TFC) were consistently elevated in natural forest honey relative to shifting agriculture honey. Natural forest honey in spring exhibited the highest total phenolic content (TPC) at approximately 53 mg GAE/100g, but the

peak total flavonoid content (TFC) was recorded in winter at around 32 mg QE/100g. The elevated levels are linked to the abundant biodiversity and diverse nectar sources seen in natural forest ecosystems (Alvarez-Suarez *et al*., 2010). Conversely, shifting culture honey harvested in the autumn had the lowest total phenolic content (TPC) at approximately 15 mg GAE/100g and total flavonoid content (TFC) at around 12 mg QE/100g, presumably attributable to the scarcity and stress of floral resources. The results demonstrate that honey sourced from natural forests exhibits superior antioxidant capabilities, advantageous for health and preservation (Solayman *et al*., 2016).

9. Chromatic Intensity: Colour intensity serves as a significant quality indicator in honey, frequently linked to the content of phenolic and flavonoid components. Natural forest honey exhibited the greatest colour intensity in spring (~1500 mAU), correlating with its elevated total phenolic content (TPC) and total flavonoid content (TFC). The minimum colour intensity was seen in shifting cultivation honey during autumn (~250 mAU). Darker honey typically contains higher levels of antioxidants and possesses a more robust flavour, rendering it more appealing to specific consumers (Solayman *et al*., 2016). The findings demonstrate that honey sourced from natural forest ecosystems exhibits superior antioxidant and sensory characteristics.

10. Electrical Conductivity: Electrical conductivity quantifies the mineral content of honey and is affected by the sources of nectar and environmental conditions. Shifting cultivation honey demonstrated the maximum conductivity in autumn (1.25 mS/cm), but natural forest honey exhibited the lowest conductivity in winter (0.4 mS/cm). The elevated conductivity in honey from shifting agriculture may result from the particular plant species and soil composition in these regions, which provide a greater mineral content to the nectar (Bogdanov *et al*., 2004). The reduced conductivity in natural forest honey indicates a more refined mineral content, possibly representative of the intact and varied plant sources in these environments.

11. Concentrations of Hydrogen Peroxide: Hydrogen peroxide (H_2O_2) is a crucial element of honey's antibacterial properties. The research indicated that honey from

shifting agriculture in the fall had the greatest H_2O_2 levels (~8.0), whilst honey from wild forests in the winter displayed the lowest values (~0.30). Elevated H_2O_2 concentrations in honey from shifting cultivation indicate enhanced antibacterial capabilities, potentially as a reaction to environmental stressors (Brudzynski, 2006). The reduced concentrations of natural forest honey indicate the stability and diminished environmental stress in these ecosystems (Solayman *et al*., 2016).

6.3.1 Correlation between rain fall and physicochemical properties

The results of this research indicate substantial connections between rainfall and numerous physicochemical, microbiological, and biochemical parameters, illustrating the influence of rainfall on ecosystem dynamics in both natural and altered contexts. The intensity of these relationships fluctuates based on the particular environmental context, with changing ecosystems exhibiting more pronounced reactions to rainfall in several critical metrics. The varying reactions illustrate the intricate relationship between precipitation and environmental stability, with disrupted ecosystems being more susceptible to fluctuations in water supply and rainfall intensity. We will elaborate on these relationships in more depth, referencing current literature (Solayman *et al*., 2016).

Positive Correlations- pH level: The correlation between rainfall and pH is moderate in both natural (R^2 = 0.4080) and altered ecosystems (R^2 = 0.3974). Precipitation significantly affects pH by diluting acidic or basic compounds in the environment, including those resulting from air pollution (Fang *et al*., 2021). Buffering systems in natural ecosystems, including organic matter and vegetation, presumably mitigate the degree of pH fluctuations. In changing environments, the connection stays consistent, likely attributable to differing pollution loads and diminished buffering ability. The diluting impact of rain may be less effective in extensively disturbed areas, where acidity is more prominent (Solayman *et al*., 2016).

HMF (Hydroxymethylfurfural): The association between rainfall and HMF is significantly more robust in shifting habitats (R^2 = 0.7672) than in natural conditions (R^2 = 0.3903). HMF is a byproduct of sugar breakdown, frequently linked to environmental stress and substandard organic materials (Schaarschmidt *et al*., 2020).

In changing ecosystems, marked by frequent disturbances like deforestation and urbanisation, rainfall may intensify the decomposition of organic matter, resulting in increased mobilisation of HMF. The heightened correlation in these conditions indicates the greater susceptibility of disturbed soils to physical and chemical changes caused by rainfall. Conversely, natural ecosystems exhibit a diminished correlation, perhaps because to slower organic matter decomposition and more stable nutrient cycling (Solayman *et al*., 2016).

Humidity Level: Rainfall markedly elevates moisture content, exhibiting a far greater association in altered ecosystems ($R^2 = 0.8714$) compared to natural conditions ($R^2 = 0.4226$). This disparity is likely attributable to the compromised soil structure and diminished plant cover in shifting habitats, which restrict the soils' capacity to hold water. The swift and significant effect of rainfall on moisture content in disturbed ecosystems indicates a diminished water retention capacity, resulting in variations in moisture levels following precipitation events (Gobessa *et al*., 2012). Conversely, natural settings have a more stable soil structure and vegetation cover, which mitigates the impact of rainfall, leading to a diminished correlation (Gobessa *et al*., 2012). The findings align with research indicating that land-use change and deforestation substantially modify the hydrological responsiveness of ecosystems to precipitation (Gupta *et al*., 2020).

Bacterial Load: The relationship between rainfall and bacterial load is mild in both natural ($R^2 = 0.3471$) and shifting environments ($R^2 = 0.4437$), with a marginally higher influence in shifting ecosystems. Precipitation can promote bacterial proliferation by supplying essential moisture and aiding in the conveyance of organic matter and nutrients utilised by bacteria for growth (Santos *et al*., 2019). The heightened correlation in altered habitats may be ascribed to the augmented presence of organic matter and contaminants in disturbed soils, which foster elevated microbial activity (Gobessa *et al*., 2012)). Bacterial communities in natural habitats tend to exhibit more stability and less sensitivity to sporadic fluctuations in water availability, attributable to enhanced nutrient cycling and diminished exposure to contaminants (Gobessa *et al*., 2012).

Aggregate Acidity: Rainfall exhibits a positive correlation with total acidity, demonstrating a more pronounced effect in altered habitats ($R^2 = 0.6751$) than in natural ecosystems ($R^2 = 0.2761$). In disturbed settings, precipitation can transport acidic compounds, including sulphates and nitrates from pollutants, resulting in increased acidity in soils and aquatic systems (Zhou *et al*., 2021). Intact natural ecosystems possess effective buffering systems that can better neutralise acidic inputs, leading to a diminished correlation (Franchini and Malagoli, 2019). This study corroborates prior research demonstrating that the effects of acid rain and other acidifying pollutants are exacerbated in regions with diminished vegetation and compromised soils (Gobessa *et al*., 2012).

Ratio of Fructose to Glucose: The correlation between rainfall and the fructose/glucose ratio is more pronounced in shifting environments ($R^2 = 0.5598$) compared to natural ecosystems ($R^2 = 0.4312$). Precipitation presumably impacts carbohydrate metabolism in plants, with variations in water availability altering the equilibrium of sugars like fructose and glucose. In disturbed ecosystems, where plants experience heightened stress from several reasons (e.g., pollution, land-use alteration), the impact of rainfall on sugar metabolism may be more significant (Gobessa *et al*., 2012). The diminished correlation in natural habitats indicates that plants in these ecosystems exhibit more stable metabolic processes and are less influenced by transient fluctuations in water supply.

Hydrogen Peroxide (H_2O_2): A robust positive association exists between rainfall and H_2O_2 in shifting settings ($R^2 = 0.6998$), whereas the correlation in natural ecosystems is somewhat less ($R^2 = 0.1525$). Hydrogen peroxide (H_2O_2) serves as an indicator of oxidative stress, with its accumulation being linked to environmental stressors such as pollution and disrupted ecosystems (Nguyen *et al*., 2020). In changing ecosystems, precipitation may intensify oxidative stress by dispersing contaminants and organic debris, resulting in heightened H_2O_2 production. The diminished correlation in natural ecosystems indicates that plants and microbial communities in these settings are more adept at handling oxidative stress, perhaps owing to more resilient antioxidant defence mechanisms (Gobessa *et al*., 2012).

Electrical Conductivity: Precipitation exhibits a positive correlation with electrical conductivity, indicating the leaching of dissolved ions and salts into soils and aquatic environments. The link is more robust in altered settings ($R^2 = 0.4582$) than in native ecosystems ($R^2 = 0.3904$), indicating that disturbed environments are more vulnerable to variations in ion concentrations resulting from precipitation. This is probably attributable to contaminants and disrupted soil structures in dynamic situations, where precipitation can more readily mobilise salts and other soluble chemicals (Gobessa *et al*., 2012). In natural ecosystems, improved soil structure and vegetation cover may reduce ion leaching, resulting in a diminished correlation.

Inverse Correlations with Precipitation- Proline: The inverse relationship between rainfall and proline levels is more pronounced in shifting environments ($R^2 = 0.6767$) than in natural ecosystems ($R^2 = 0.4972$). Proline is an amino acid linked to stress reactions, especially in relation to drought and oxidative stress (Gobessa *et al*., 2012). Enhanced precipitation presumably diminishes the necessity for proline synthesis, as water availability alleviates some environmental stresses that stimulate its formation. The more robust link in dynamic habitats may indicate the increased sensitivity of plants in these ecosystems to water stress, wherein rainfall offers more fast alleviation from stresses, resulting in a more significant decrease in proline levels.

Total Phenolic Content: Rainfall exhibits a substantial negative connection with total phenolic content, with a more pronounced influence in shifting habitats ($R^2 = 0.5388$) than in natural ecosystems ($R^2 = 0.4171$). Phenolics are secondary metabolites that are crucial for plant defence against oxidative and environmental stresses (Gobessa *et al*., 2012). In disrupted ecosystems, precipitation may alleviate oxidative stress, resulting in a diminished requirement for phenolic chemical synthesis. The diminished correlation in natural settings indicates that phenolic concentrations are less responsive to variations in water supply, likely owing to more stable environmental circumstances.

Aggregate Flavonoid Concentration: Like phenolics, total flavonoid concentration diminishes with heightened rainfall, exhibiting a greater connection in shifting

habitats (R^2 = 0.4573) compared to natural environments (R^2 = 0.3794). Flavonoids, similar to phenolics, play a role in plant defence against stress and oxidative damage (Gobessa *et al*., 2012). In disrupted ecosystems, characterised by elevated stress levels, rainfall may diminish the necessity for these protective substances, resulting in a more significant reduction in flavonoid concentrations. In natural habitats, the association is less pronounced, indicating that plants in these ecosystems sustain more stable flavonoid levels despite short-term variations in rainfall.

Colour Intensity: Rainfall exhibits a negative link with colour intensity, an indicator of pigmentation in plants, demonstrating a more pronounced correlation in altered habitats (R^2 = 0.4573) compared to natural ecosystems (R^2 = 0.3794). Anthocyanins, a type of pigment, are frequently synthesised in reaction to stress, and their concentrations may diminish when precipitation mitigates certain environmental stressors impacting plants (Gobessa *et al*., 2012). The heightened correlation in altered habitats indicates that plants in disturbed ecosystems exhibit more responsiveness to rainfall concerning the reduction of pigmentation. The diminished association in natural situations suggests that pigmentation levels exhibit greater stability and are less influenced by transient fluctuations in water supply.

Summary; This study emphasises the intricate relationships between precipitation and environmental characteristics in both natural and transitioning ecosystems. The heightened correlations seen in altered settings indicate that disturbed ecosystems exhibit more sensitivity to variations in rainfall, resulting in more significant impacts on factors such as moisture content, HMF levels, and bacterial load. In contrast, natural ecosystems demonstrate more steady reactions to rainfall, probably owing to superior buffering capacity and tolerance to climatic variations. These findings highlight the necessity of accounting for land-use alterations and ecosystem stability when evaluating the influence of climate-related variables, such as precipitation, on ecosystem dynamics (Gobessa *et al*., 2012).

6.3.2 Correlation between air pollution and physicochemical properties

The results of this research offer considerable evidence of the significant relationships between air pollution and different physicochemical, microbiological,

and biochemical parameters in both natural and altered environments. The findings highlight the varying effects of air pollution contingent upon ecosystem stability, exhibiting more pronounced and predictable consequences in natural settings and intricate interactions within dynamic ecosystems. These findings enhance the existing literature on the environmental impacts of air pollution and offer significant insights for forthcoming study and policy formulation (Gobessa *et al*., 2012).

Positive Correlations: A number of physicochemical parameters, including pH, moisture content, fructose/glucose ratio, and electrical conductivity, exhibited significant positive relationships with air pollution levels, especially in natural settings. The elevated coefficients of determination (R^2) for pH ($R^2 = 0.9153$) and moisture content ($R^2 = 0.9303$) demonstrate that air pollution substantially affects these characteristics in natural ecosystems. This aligns with other research indicating alterations in soil and water chemistry resulting from the deposition of air pollution, encompassing both acidic and alkaline substances (Gobessa *et al*., 2012). The elevation in pH associated with escalating air pollution may be attributed to the deposition of alkaline dust or ammonia from industrial emissions, which can neutralise acidic constituents in the environment (Kim *et al*., 2019). In changing ecosystems, the relationship between air pollution and pH is less robust ($R^2 = 0.4171$), likely attributable to the impact of various pollution sources and alterations in land use, resulting in more intricate interactions (Gobessa *et al*., 2012).

The robust connection between air pollution and the fructose/glucose ratio in natural settings ($R^2 = 0.9749$) indicates that air pollution may modify the carbohydrate metabolism of plants or organisms. This outcome corresponds with research on plant stress responses, which has documented alterations in sugar metabolism due to oxidative stress induced by air pollution (Singh *et al*., 2020). In changing environments, the correlation remains substantial ($R^2 = 0.8712$), signifying that even in disrupted ecosystems, the fructose/glucose ratio is responsive to variations in air pollution levels.

The electrical conductivity, a significant measure, exhibited a robust positive connection with air pollution in natural habitats ($R^2 = 0.9490$). This may result from

the heightened solubility of ions in contaminated environments, where hygroscopic contaminants draw moisture and dissolve ionic compounds (Liu *et al*., 2020). The diminished correlation in fluctuating environments (R^2 = 0.8231) may be attributed to the increased fluctuation in pollution sources and ecosystem disturbances, which could attenuate the direct impacts of air pollution on conductivity (Gobessa *et al*., 2012).

Microbiological and biochemical reactions: The correlation between air pollution and bacterial load illustrates a notable distinction between natural and changing settings. The bacterial load exhibits a larger positive connection with air pollution in shifting environments (R^2 = 0.9122) than in natural conditions (R^2 = 0.7065). This discovery indicates that microbial communities in disturbed or transitional ecosystems exhibit heightened sensitivity to alterations in air quality, presumably owing to less biodiversity and resilience in these habitats (Gobessa *et al*., 2012). The escalation of bacterial load associated with heightened air pollution may be attributed to the deposition of organic and inorganic particles, which offer supplementary nutrients or surfaces conducive to microbial proliferation.

The notable relationships between air pollution and biochemical substances, including HMF (hydroxymethylfurfural) and H_2O_2 (hydrogen peroxide), underscore the metabolic stress caused by air pollution. HMF, a consequence of sugar breakdown, frequently serves as an indication of environmental and food quality (Ravindran *et al*., 2021). The robust correlation with air pollution under natural conditions (R^2 = 0.8242) indicates that increased pollution levels may expedite sugar degradation processes, potentially affecting the quality of plant-derived products (Zhou *et al*., 2020). The more robust correlation in altered environments for H_2O_2 (R^2 = 0.7546 compared to R^2 = 0.6218 in natural environments) suggests that oxidative stress may be more significant in disrupted ecosystems, characterised by variable pollution sources and environmental conditions (Gobessa *et al*., 2012).

Negative Relationships: The inverse relationships between air pollution and compounds like proline, total phenolic content, total flavonoid content, and colour intensity indicate that air pollution hampers the synthesis or accumulation of these

protective substances. Proline, an amino acid associated with stress tolerance, demonstrated a robust negative correlation with air pollution in natural settings (R^2 = 0.9543). This finding aligns with prior research indicating that under extreme pollution stress, plants may find it challenging to accumulate adequate proline to mitigate the damage inflicted by oxidative pollutants (Verma and Mishra, 2020). The notable yet diminished negative correlation in altered habitats (R^2 = 0.8048) suggests that proline synthesis is less effective in disrupted ecosystems, maybe owing to competition with alternative stress mechanisms (Gobessa *et al*., 2012).

The decline in total phenolic and flavonoid content correlates with heightened air pollution, aligning with the recognised function of these compounds as antioxidants that neutralise reactive oxygen species (ROS) produced by pollution (Gill and Tuteja, 2019). The pronounced negative correlation identified in shifting environments for total phenolic content (R^2 = 0.9287) indicates that plants in these settings may be especially susceptible to oxidative stress, likely resulting from a confluence of air pollution and additional environmental pressures, including soil degradation or water scarcity (Fini *et al*., 2021). The reduction of phenolics and flavonoids may have considerable ecological and agricultural consequences, as these compounds are essential for plant defence, growth, and reproduction (Gobessa *et al*., 2012).

Comparative Examination of Natural and Transitional Environments: The divergent reactions noted between natural and altered environments illustrate the distinct ecological dynamics inherent in these systems. In natural ecosystems, air pollution's impacts are more immediate and frequently exhibit higher relationships with physicochemical variables, such as pH, moisture content, and the fructose/glucose ratio. This is probably due to the fact that natural ecosystems are comparatively undisturbed and therefore more susceptible to external pollutants (Ghazoul, 2020). The robust relationships among air pollution, bacterial load, H_2O_2, and the reduction of antioxidants such as phenolics in changing settings indicate that these ecosystems are increasingly susceptible to the cumulative impacts of pollution and ecological disruptions. The drop in antioxidant levels, in particular, indicates that plants and organisms in altering habitats may have a diminished capacity to cope with oxidative

stress, which could have long-term ramifications for ecosystem health and resilience (Gobessa *et al*., 2012).

Ecological and Health Implications: The differential impacts of air pollution on natural and changing habitats have substantial ecological and public health implications. In natural habitats, the strong relationships between air pollution and physicochemical qualities, such as pH and moisture content, could lead to major changes in soil and water chemistry, thereby harming plant development and biodiversity (Ciais *et al*., 2020). In shifting ecosystems, the dramatic rise in bacterial load with growing pollution levels raises worries about the possibility for microbial contamination, which could have ramifications for food safety, water quality, and human health (Gobessa *et al*., 2012).

The reduction of antioxidant chemicals, such as phenolics and flavonoids, is particularly alarming, as these molecules are not only necessary for plant defense but also add to the nutritional content of plant-based meals (Scalbert *et al*., 2019). The lowering of these chemicals in polluted environments could have a severe influence on food quality and human health, particularly in urban or industrialized areas where pollution levels are high. This study illustrates the complex and variable effects of air pollution on environmental properties, with natural habitats showing more predictable changes in physicochemical parameters and changing ecosystems demonstrating higher microbiological and biochemical reactions. The differential responses found show that air pollution impacts are context-dependent and shaped by the stability and resilience of the ecosystem. These findings underscore the necessity for tailored pollution mitigation measures that address the specific vulnerabilities of various ecosystems and take into account both direct and indirect impacts on environmental health.

6.3. Major royal jelly protein 2

The results demonstrate that MRJP2 gene expression in *A. cerana* honey shows considerable seasonal and habitat-specific fluctuation. Honey samples from the natural forest (NF) habitat consistently exhibited elevated MRJP2 gene expression compared to those from the shifting cultivation (SC) habitat. This pattern

was particularly pronounced during the autumn season, as NF1 and NF2 habitats exhibited the highest expression levels (Li *et al*., 2010). The reduced gene expression in SC environments may indicate variations in floral diversity, environmental conditions, and resource availability between the two habitats (Li *et al*., 2010).

The seasonal fluctuation in gene expression is also significant. The elevated expression of the MRJP2 gene in the Fall season relative to the Rainy season in NF environments may be attributed to variations in the availability of particular floral resources throughout these periods (Li *et al*., 2010). Conversely, the SC habitat exhibited more consistent yet diminished MRJP2 expression levels throughout both seasons, indicating that environmental stress or worse fodder quality in these regions may constrain the bees' capacity to generate MRJP2-rich honey. These results underscore the significance of habitat and seasonal factors in determining the quality and content of *A. cerana* honey, with NF environments providing more conducive conditions for elevated MRJP2 gene expression. This may affect the preservation of natural ecosystems and the sustainable production of premium honey. Subsequent studies could examine the particular floral sources or environmental variables that enhance MRJP2 expression in NF environments (Li *et al*., 2010)

CHAPTER VII

SUMMARY

7.1. Honeybee-floral interactions in the Tanhril forest

The research indicates notable seasonal fluctuations in the presence of indigenous and invasive plant species, emphasising their reactions to environmental factors and competitive influences. Native species are most prevalent in spring and winter, whereas invasive species sustain a significant presence year-round. This imbalance may result in the displacement of indigenous flora and modify ecosystem architecture. The prevalence of invasive species may result in enduring repercussions for ecosystem stability, nitrogen cycling, and habitat accessibility for native fauna. Management initiatives should prioritise the regulation of invasive species and the promotion of native plant proliferation and restoration.

7.2. Floral Resource Availability and Pollinator Dynamics

The presence of floral resources among invasive and native plant species significantly affects pollinator dynamics. Invasive plants offer increased supplies for bee pollinators, especially during periods of precipitation and autumn. This can be advantageous for generalist pollinators such as honey bees, which can utilise diverse floral resources. The heightened dependence on invading species may disturb native plant-pollinator relationships, potentially resulting in the demise of native species and a decrease in native plant variety. This may lead to specialised pollination networks, essential for ecosystem resilience.

7.3. Important value index

The Important Value Index (IVI) is a vital ecological metric that signifies the relative significance of a species within a specific ecosystem. Invasive species exhibit markedly elevated IVI values compared to native species, signifying their predominance in ecosystems. This indicates a competitive edge for invasive species, potentially affecting the structure and function of ecosystems significantly.

Invasive species frequently demonstrate vigorous growth patterns, elevated reproductive rates, and adaptability to many environmental conditions, rendering them more susceptible to competition from native species. This dominance may result in the suppression or local extinction of native species, which are essential for

preserving biodiversity. Invasive species displace native flora, potentially diminishing overall biodiversity and adversely affecting ecosystem functions.

Invasive species can transform the physical environment, alter fire regimes, shift hydrological cycles, and disrupt mutualistic connections among native species. Their swift growth rates and capacity to rapidly colonise damaged settings confer a competitive advantage. They also gain advantages from the absence of natural predators in their new habitats, referred to as the "enemy release hypothesis."

The results of the IVI research bear significant significance for the management and regulation of invasive species in India. Elevated IVI values signify growing dominance, which presents a significant danger to native biodiversity and environmental stability. It is essential to formulate effective management measures to curtail the proliferation of invasive species and diminish their effects on indigenous ecosystems. Timely identification and swift intervention (EDRR) are essential, as once invasive species get established and attain elevated IVI values, they are frequently challenging to manage or eliminate.

7.4. Floral display and pollen availaibility

The research indicates notable disparities in bee reactions to invasive versus native plant species, with invasive plants exhibiting a more pronounced association with flower display size. Nonetheless, the allure of local species may be affected by several factors. This may impact the reproductive success and vitality of native flora, potentially resulting in skewed pollination networks that favour alien species. The research emphasises the seasonal fluctuations in floral resources accessible to bees, which may affect pollination efficacy and reproductive cycles. Comprehending these inequalities is essential for ecological management.

7.5. DNA barcoding

DNA barcoding methods have demonstrated efficacy in identifying and cataloguing plant species within ecological studies. The research employed 20 ng/ml concentrations and high-quality DNA characteristics to examine plant reference samples and pollen mixes. The elevated success rate of DNA amplification

employing standard primer pairs and thermal cycling parameters for two prevalent plant barcoding markers, rbcL and matK, substantiates their efficacy in plant species identification. The initial DNA barcoding entries in GenBank, consisting of 95 plant species from the Tanhril forest, establish a reference library that improves researchers' capacity to accurately identify and classify plant species and found out 90 plants from the honey samples. The establishment of a 94-taxon reference database utilising barcoding data from Tanhril forest plants is essential for comprehending local flora and enabling comparative studies and monitoring of plant biodiversity across many habitats. The identification of Molecular Operational Taxonomic Units (MOTUs) from pollen mixes in beehives offers significant insights into plant-pollinator interactions and the diversity of plant species foraged by bees.

The study identifies a variety of plant species that provide pollen foraged by bees at three research sites. The fluctuation in the quantity of sequences per MOTU indicates that specific plant species offer consistent floral resources during the sampling duration. This may be affected by factors including the spatial distribution of plant species, temporal variations in floral availability, and the foraging habits of bees. The inclusion of particular plant species in pollen combinations may have significant ramifications for conservation initiatives.

DNA barcoding possesses potential for extensive ecological applications, including evaluating plant diversity, monitoring temporal changes in plant communities, and tracking the proliferation of invasive species. It can also facilitate the comprehension of plant-pollinator networks by offering specific information regarding the composition of floral resources utilised by pollinators. Subsequent research could enhance the existing findings by integrating additional sampling locations and other barcoding markers.

7.6. Physicochemical properties

This study examines the physicochemical and microbiological quality of honey, emphasising the influence of environmental conditions, plant biodiversity, and management practices. Essential indications encompass pH value, Hydroxymethylfurfural (HMF) concentrations, moisture content, bacterial load, total

acidity, fructose-to-glucose ratio, proline concentration, phenolic and flavonoid components, and hydrogen peroxide levels. The research underscores the significance of comprehending these elements in assessing honey quality.

The research underscores the significance of precipitation in ecosystem dynamics, indicating that compromised ecosystems exhibit heightened susceptibility to fluctuations in water supply and rainfall intensity. The correlation between precipitation and bacterial load is moderate, with a more pronounced effect in modified environments resulting from land-use changes and deforestation. The correlation between precipitation and total acidity is more pronounced in altered habitats, exhibiting a greater influence on disturbed ecosystems.

The research emphasises the relationships between air pollution and several physicochemical, microbiological, and biochemical indicators in both natural and altered environments. Positive correlations were seen for pH, moisture content, fructose/glucose ratio, electrical conductivity, and bacterial load. The correlation between HMF and H_2O_2 underscores metabolic stress caused by air pollution, potentially affecting the quality of plant-derived products. In contrast, negative relationships were seen between air pollution and molecules including proline, total phenolic content, total flavonoid content, and colour intensity, indicating that air pollution hinders the generation or accumulation of protective compounds.

The study suggests that natural ecosystems demonstrate more rapid responses and greater associations with physicochemical parameters, whereas dynamic ecosystems are more susceptible to environmental disruptions resulting from urbanisation, land-use alterations, and industrial activity.

7.7. Major royal jelly protein 2

The expression of the MRJP2 gene in *A. cerana* honey exhibits significant seasonal and habitat-specific variation. Honey samples from natural forest settings exhibit elevated expression levels in fall, likely attributable to fluctuations in floral diversity, environmental circumstances, and resource availability. In contrast, shifting cultivation (SC) environments exhibit stable yet reduced expression levels, maybe

attributable to environmental stress or suboptimal fodder quality. These findings underscore the significance of environmental and seasonal variables in influencing honey quality and composition.

CHAPTER VIII

CONCLUSION

CONCLUSION

- The Tanhril forest, Mizoram is home to a large number of bee-pollinating species.
- A higher prevalence of Invasive plant species were observed in the Tanhril forest in Mizoram, than the native plant species, which could have an impact on interactions amongst bee pollinators.
- Due to the prevalence of invasive plant species and their floral displays, Bee Pollinators become more attracted to invasive plants than native ones.
- This is the first comprehensive report that uses a DNA barcoding technique on honey samples to identify bee-pollinated plants in the Tanhril forest, Mizoram.
- matK and rbcL biomarkers have been validated to identify the plant species from the pollen content in the *A. cerana* honey samples
- The utilisation of pollen barcoding technique has proven to be more effective than traditional melissopalynological methods to identify *A. cerana* polliniferous plants within honey samples.
- Shifting cultivation practices significantly affecting the *Apis cerana* honey quality in terms of pH, HMF and Total acidity.
- Seasonal fluctuations in *A. cerana* honey quality were detected in both natural and shifting cultivation habitats; these variations exhibited a significant effect on the physicochemical characteristics of the honey samples that were obtained from the shifting cultivation habitat

CHAPTER IX

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CONFERENCE/WORKSHOP/SEMINAR ATTENDED

- Presented research paper “DNA barcoding approach to characterize pollen collected by Apis cerana” in the National Seminar on Sustainable Development with Women Empowerment. March 2022
- Participated in the DST-sponsored one-week training program on “Instruments in Biotechnology: Theories and Practices” organized by the Department of Biotechnology, Mizoram University. May-2022
- Participated in the International Workshop on “The Value and Interconnections of Human, Animal, Plant, and Microorganisms: Metataxonomics and Metagenomics Approach” organized by the Departmentof Zoology, Mizoram University. March 2023
- Participated in the International workshop on “Computational Genomics With R: A Hands-On Course on NGS Data Analysis” organized by the Department of Zoology, Mizoram University. March 2023
- Participated in the International Workshop on “Nanopore Sequencing and Data Analysis: Opportunities for Rapid Biodiversity and Biosurveillance Programs and Local Capacity Building” organized by the Department of Zoology, Mizoram University. March 2023

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- Murthy, M. K., Khandayataray, P., Tara, M., Buragohain, P., **Giri, A**., & Gurusubramanian, G. (2022). **Optimisation of DNA isolation and PCR techniques for beetle (Order: Coleoptera) specimens**. International Journal of Tropical Insect Science, 42(3), 2761-2771.
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A Review

Pharmacological and therapeutic potential of honey bee antimicrobial peptides

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Honey bees (Apidae: Apini) and stingless bees (Apidae: Meliponini) act as the main pollinators for many wild and cultivated tropical plants, playing a vital role in the ecology, economy, and culture. Honey bees and stingless bees are one of the major sources of antimicrobial peptides/proteins (AMPs) synthesized in fat bodies and blood cells of bees. Bee AMPs are a class of small peptides having amino acid residues between 9 and 340, classified based on source, activity, structural characteristics, and amino acid-rich species. AMP's have a wide range of inhibitory effects against bacteria, fungi, parasites, and viruses. Four antimicrobial peptide families, *i.e*., apidaecins (proline-rich), abaecin (proline-rich), hymenoptaecin (glycine-rich), and defensin (cystine-rich) are synthesized in the haemolymph, signifying a broad spectrum of antimicrobial activity. Jelleines (I-IV), royalisin, apisimin (serine-valine-rich peptide), 10 HDA, apalbumin, and apisin, which are present in royal jelly, have antimicrobial, mast cell degranulating, and hemolysis activity. Bee venom also contains several bioactive peptides, such as apamin (leucine-cystine-rich), melittin (leucine-alanine-rich), melectin (lysine-rich), adolapin, secapin (proline-rich), and tertiapin (cystine-lysine-rich). Currently, AMPs databases are displaying an essential role in exploration, identification, characterization, and annotation. Several AMPs databases (CAMP, DRAMP, APD, InverPep, LAMP, ADAPTABLE, ADAM, AntiBP, AMPer, AVPpred, EFC-FCBF, and class AMP) are open-access resources that have been developed to enhance research on antimicrobial peptides. Bee immune responses are composed of a multifaceted group of individual immune mechanisms and special types of behavioral adaptations. Given the importance of drug discovery from honey bee AMPs, this review is aimed at providing an exhaustive screening of the AMPs detected in honey and honeybee products and their classification, databases, computational tools, physicochemical properties, signaling pathways, pharmaceutical and clinical uses, application status, prospects, and problems to be solved.

Keywords: AMPs, Databases, Honey bees, Signaling pathways, Therapeutic applications

Introduction

Honey bees and stingless bees, act as one of the major pollinators in wild forest and agriculture systems, are indispensable for conserving ecological biodiversity, global ecological stability, productivity and economy[1]. Besides, both honey bees and stingless bees are capable to produce different types of honey, royal jelly, bee wax, propolis, bee bread and bee venom based on diverse floral resources visit and can yield significant contributions to human society (Fig. 1). Nevertheless, several factors seemingly lead to bee depopulation and colony/brood loss events, including pathogens (parasites, fungi, bacteria and viruses), ecosystem alteration or loss, and the use of pesticides and antibiotics intimidate wild plant diversity, terrestrial ecosystem stability, crop production, global food supply, and human welfare[2]. The immune system of bees is capable of changing its defence mechanisms, so it is essential to understand how it works in order to examine how it reacts to various pathogenic and stressful situations that affect the health and behaviour of bee colonies (Fig. 1).

Bee immunity serves as an example of the superorganism theory since it relies on both individual and colony-level defence mechanisms to protect bees from infections and stressful situations[3]. Additionally, bees' social immunity—a network of behavioural, physiological, and organizational responses that prevents the admission, establishment, expansion, and transmission of parasites and microbial diseases in the colony—supplements their physiological immune systems. Bee immune responses are comprised of distinctive categories of behavioral adaptations, evolutionary conserved defense strategies (cellular and humoral responses) and assemblage of multifaceted discrete immune mechanisms that afford

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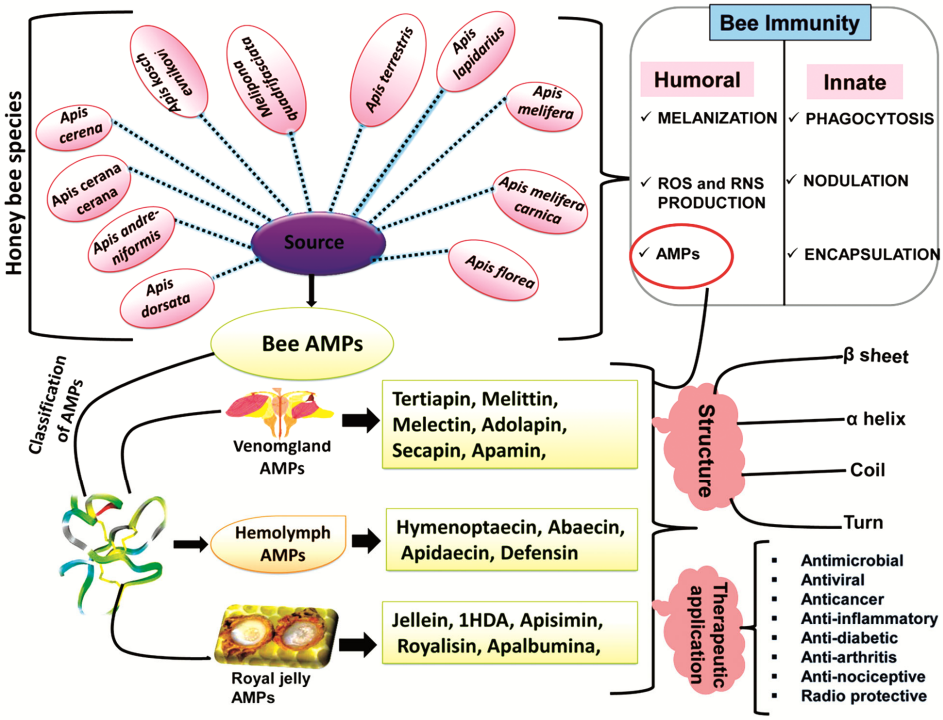

Fig. 1 — Sources of antimicrobial peptides from honey bee species, classification and their role in bee health

immediate responses against different pathogens and biotic and abiotic stressors[4]. Defense machinery in bees is entirely built on an effective innate immune system, which permits a broad and speedy response to different invading pathogenic and parasitic organisms[5]. Bees' immunity consists of the three levels of defense strategies, *i.e.*, 1) first line of defense-physical barriers (exoskeleton, cuticle, tracheal tubes, mucous membranes, intestinal wall, peritrophic membrane *etc.*), 2) cell-mediated immunity (phagocytes and haemocytes), and 3) cell-free humoral immunity, an intricate grid of intracellular signaling pathways precede to instigation of diverse of humoral effectors as supplement system fractions, acute phase proteins, thioester linkage proteins, melanization, coagulation proteins, antimicrobial peptides (AMPs), natural antibodies, and the various cytokines that modulate immune response[6] (Fig. 1). The immune system of bees is clustered into 17 families and analogous to dipteran insects. Bees' immune genes are shared by *Drosophila* and *Anopheles* to the tune of 33.33%. For odour recognition and particular genes for floral resource sensing, social behaviour, and organization, bees have more protein-coding genes than other insects. The significance of social defences (social behaviour and organization) in the bee colony and their propensity to disease from a specific set of pathogens, which have evolved to make up for less expenditure in physiological immunity and reduction of selection pressure for a stronger individual immune defence, may be revealed by the inherent decrease in the number of immune genes in bees[6].Humoral effectors are playing a major role in cellular immune responses in bees. Fat body is the major resource for effectors synthesis followed by hemocytes, epithelial cells and salivary glands. Haemocytes (prohemocytes, clot haemocytes, granular cells and oenocytoids, pro-leukocytes, picnonucleocytes, adipoleukocytes, spherukocytes, granulocytes, macronucleocytes, microleukocytes, and spindle-type cells) are not only modulating the process of nodulation, encapsulation and phagocytosis to combat the invading pathogens but also involved in the signal transduction pathways and aided for the production of AMPs, regulatory role in activation hemolymph coagulation and melanization, and reactive oxygen and reactive nitrogen species (ROS and RNS) production along with the fat body cells[7,8] (Fig. 1).

Innate immune system *vs* social immunity

Innate immunity is playing a prospective role in sting and stingless bees to modulate mortality as well as pathogen transmission among colony members.

Immunity in bees characterizes the "superorganism concept" signifying the presence individual and colony level immune defense system to support survival of both the individual bee as well as the whole bee colony. The decrease in immune genes in bees demonstrates the impact of colony-level defenses than individual immune defense[3]. Once defense mechanisms depend on collective activities to check, minimize or eliminate pathogens and parasites are known as 'social immunity'. The social immunity in bees is categorized into two gradients based on their functional mechanisms as the types of immune activation (constitutive *vs* inducible) and the levels of defense (individual *vs* colony) (Fig. 1). Constitutive defenses are the first line of defense, prophylactic, always exist, and no alteration when individuals or colonies are exposed to abiotic and biotic stresses while inducible defenses are synchronized or activated by pathogens/parasites exposure. Constitutive group behavioral defenses of colony level immunity include the traits of task allocation, external use of glandular compounds, pharmacophory (propolis and resin), trophallaxis of antimicrobials and self-produced/environmentally collected compounds (defensin 1 and glucose oxidase), whereas phenoloxidase cascade is in constitutive defenses of individual immunity. Hygienic behavior, social fever, social exclusion and self-removal, brood cannibalism and allo-grooming traits are highlighted as inducible defenses of social immunity, while self-grooming, AMPs and pharmacophagy are inducible defenses of individual immunity[7,8] (Fig. 1).

Antimicrobial peptides (AMPs)

AMPs are fundamental components of bee innate and cellular immune system and synthesized in response to immune challenge by pathogens and parasites in the fat body or haemocytes and secreted successively into the hemolymph. AMPs are playing a significant role as host defense peptides in honey bees and stingless bees (Fig. 1). It is one of the ancient evolutionary mechanisms of bees which can be produced and transported to the site of infection within the short span of interval[8].

Bee AMPs are small cationic (positively charged), amphiphilic (hydrophilic and hydrophobic) and coiled and α-helical peptide molecules comprising of 9-340 amino acids, that have been identified in honey bees (*Apis cerana*, *A. cerana cerana*, *A. cerana japonica*, *A. mellifera*, *A. mellifera carnica*, *A. terrestris*, *A. koschevnikovi*, *A. andreniformis*, *A. lapidaria*, and *A. dorsata*) and stingless bees (*Melipona quadrifasciata* and *Heterotrigona itama*) (Fig. 1 & Table 1). A total of 3257 AMPs have been recognized till date across phyla as per the APD3 database[9] including bacteria (365), archaea (5), protists (8), fungi (22), plants (360), and animal (2414) origin (Table 2). Until now, the APD3 database is describing 323 insect-derived AMPs[9] wherein only 15 AMPs (apidaecin, abaecin, hymenoptaecin, defensin, royalisin, jelleine, apisimin, 10 HDA, apalbumina, apamin, melittin, melectin, adolapin, secapin and tertiapin) reported for honey bees and stingless bees (Figs 1, 2 and Tables 1-4). Four antimicrobial peptide families, *i.e*., apidaecins (proline-rich), abaecin (proline-rich), hymenoptaecin (glycine-rich) and defensin (cystine-rich) are synthesized in the haemolymph signifying a broad spectrum of antimicrobial activity while jelleines (I-IV), royalisin, 10 HDA, apalbumina and apisimin (serine-valine-rich peptide) present in royal jelly having antimicrobial, mast cell degranulating and hemolysis activity. Bee venom also contains several bioactive peptides like apamin (leucine-cystine-rich), melittin (leucine-alanine-rich), melectin (lysine-rich), adolapin, secapin (proline-rich), and tertiapin (cystine-lysine-rich) (Figs 1,2 and Tables 1-4). Details about honey bee antimicrobial peptides, their physico-chemical properties and available databases[10] are shown in (Tables 1 and 2).

Apidaecin (apidaecin-type peptides)

The smallest and most abundant class of proline-rich peptides (18–20 amino acid residues) is known as apidaecin, and it is generated by stingless bees and honey bees. Apidaecin-like peptides from insects are often shorter than those from mammals[11]. Four isoforms of apidaecins [HbIa (**GNNRP**VYIPQ **PRPPHPR**I), HbIb (**GNNRP**VYIPQ**PRPPHPR**L), HbII (**GNNRP**IYIPQ**PRPPHPR**L) and HbIII (**GNNRP**IYISQ**PRPPHPR**L)] were characterized from the haemolymph of *Escherichia coli* infected *A. mellifera*. It contains conserved and variable regions accountable for antimicrobial activity and conferring microbial specificity, respectively. Apidaecin is protective against gram-negative bacteria by two different mechanisms: 1) inhibition of the DnaK protein (bacterial chaperone network), which blocks ATPase activity and eliminates its capacity to promote chaperone-assisted protein folding and ribosomal biogenesis; and 2) stop codon-dependent

Table 1 — Details about honey bee antimicrobial peptides and their physico-chemical, pharmacological and therapeutic properties

Physico-chemical properties of honey bee antimicrobial peptides

Sl. No	Peptide name	Antimicrobial Peptide	Length of AMP	Molecular Weight (kDa)	References
		Hemolymph			
1	Abaecin	MKVVIFIFALLATICAAFAYVPLPNVPQPGRRPFPTFPGQGPFNPKI KWPQGY	53	5903.09	(10)
2	Apidaecin	MKNFALAILVVTFVVAVFGNTNLDPPTRPTRLRREAEPEAEPGNN RPVYIPQPRPPHPRLRREAEPEAEPGNNRPVYIPQPRPPHPRLRREA EPEAEPGNNRPVYIPQPRPPHPRLRREAEPEAEPGNNRPVYIPQPRP PHPRI	144	16538.80	
3	Defensin	MKIYFIVGLLFMAMVAIMAAPVEDEFEPLEHFENEERADRHRRVT CDLLSFKGQVNDSACAANCLSLGKAGGHCEKGVCICRKTSFKDL WDKRFG	95	10717.45	
4	Hymenoptaecin	MKFIVLVLFCAVAYVSAQAELEPEDTMDYIPTRFRRQERGSIVIQG TKEGKSRPSLDIDYKQRVYDKNGMTGDAYGGLNIRPGQPSRQHA GFEFGKEYKNGFIKGQSEVQRGPGGRLSPYFGINGGFRF	129	14492.42	
		Major Royal Jelly			
1	Jellin	EPFKISIHL	9	1083.30	(10)
2	Apisimin	MSKIVAVVVLAAFCVAMLVSDVSAKTSISVKGESNVDVVSQINSL VSSIVSGANVSAVLLAQTLVNILQILIDANVFA	78	7946.39	
3	Royalisin/ Defensin	MKIYFIVGLLFMAMVAIMAAPVEDEFEPLEHFENEERADRHRRVT CDLLSFKGQVNDSACAANCLSLGKAGGHCEKGVCICRKTSFKDL WDKRFG	78	7946.39	
		Bee Venom			
1	Tertiapin	ALCNCNRIIIPHMCWKKCGKK	21	2460.09	(10)
2	Melectin	GFLSILKKVLPKVMAHMK	18	2040.64	
4	Apamin	MISMLRCIYLFLSVILITSYFVTPVMPCNCKAPETALCARRCQQHG	46	5223.37	
5	Melittin	MKFLVNVALVFMVVYISYIYAAPEPEPAPEPEAEADAEADPEAGI GAVLKVLTTGLPALISWIKRKRQQG	70	7584.86	
6	Secapin	MKNYSKNATHLITVLLFSFVVILLIIPSKCEAVSNDMQPLEARSAD LVPEPRYIIDVPPRCPPGSKFIKNRCRVIVP	77	8664.35	

Pharmacological and therapeutic properties of antimicrobial peptides derived from honey bees

Sl. No	Antimicrobial peptides	Pharmacological and therapeutic properties	References
1	Defensin-1	Antibacterial activity	(27)
2	Apalbumin	inhibition of ROS release, NO production, phagocytosis, and the production of TNF-α	(28)
3	Melittin	Anticancer, anti-tumor, anti-angiogenesis, anti-fungal, anti-parasitic, anti-microbial, anti-arthritic, anti-inflammatory, anti-psychotic, anti-atherosclerosis, anti-bacterial, anti-viral, neuroprotective and anti-multidrug resistant	(20, 30, 32)
4	Apamin	Blood-brain barrier drug-delivery shuttles, anti-fungal, anti-viral, anti-inflammatory, analgesic and neurodegenerative disorders	(19, 22, 23)
5	Jelleins	Antimicrobial activity	(16)
6	Royalactin	Growth and development, and Antiaging	(35)
7	Royalisin	Antibacterial, antifungal	(36)
8	10-HDA	Immunomodulatory activities, antiulcer, anti-inflammatory, and neurodegenerative disorders	(37 – 40)
9	Adolapin	Anti-arthritic, analgesic, anti-inflammatory, antipyretic, anti-nociceptive	(22)
10	MCD peptide	Anti-inflammatory	(34)

inhibition of translation by apidaecin[11,12] (Figs 1 & 2, and Tables 1-4).

Abaecin

It is one of the largest proline-enriched cationic broad-spectrum antibacterial peptide (34 amino acids and no cysteine residues) having 29% of proline residues (10 nos.) which distributed uniformly causing no α-helical conformation[11] (Figs 1 & 2, and Tables 1-4).

Apisimin

Apisimin is a small, serine-valine-rich peptide isolated from the royal jelly of *A. mellifera*. It is an acidic peptide containing 54 amino acids and devoid

Table 2 — Databases available for antimicrobial peptides

Sl. No.	AMPs database	Websites	AIM and objective of the databases	References
1.	NCBI	The National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/	NCBI consists of 769131 AMPs, of which 93213 from *Streptococcus pneumoniae*, 66764 from *Escherichia coli*, 42212 from *Salmonella enterica*, 29225 from *Staphylococcus aureus*, 25851 from *Klebsiella pneumoniae*, and 511866 from all other taxa.	(10)
2.	Uniprot	Universal Protein Resource is a comprehensive resource for protein sequence and annotation data. https://www.uniprot.org/	Uniprot database having 53357 no of antimicrobial peptide across the flora and fauna. Sequence length ranged as 1 - 200 (16,184 numbers), 201 - 400 (17,547 numbers), 401 - 600 (11,646 numbers), 601 - 800 (3,630 numbers) and >= 801 (4,350 numbers)	
3.	$CAMP_{R3}$	Collection of Anti-Microbial Peptides http://www.camp3.bicnirrh.res.in/	This database contain 8164 AMPs sequences, 757 structure, 2083 patented AMPs.	
4.	DRAMP	Data repository of antimicrobial peptides http://dramp.cpu-bioinfor.org/	AMPs sequences, structures, activities, physicochemical, patent, clinical and reference information. 22209 entries, 5841 of which are natural and synthetic AMPs, 16110 patent AMPs and 77 AMPs in drug development (preclinical or clinical stage).	
5.	APD3	Antimicrobial Peptide Database https://wangapd3.com/main.php	Contains 3257 antimicrobial peptides from six different kingdoms 365 from bacteria, 5 from archaea, 8 from protists, 22 from fungi, 360 from plants, and 2414 from animals.	
6.	InverPep	Database for AMPs from invertebrates https://ciencias.medellin.unal.edu.co/gruposdeinvestigacion/prospeccionydisenobiomoleculas/InverPep/public/home_en	702 peptides with identification code, source (phylum and species), name, sequence, length, secondary structure, molar mass, charge, isoelectric point, hydrophobicity, Boman index, aliphatic index, percentage of hydrophobic amino acids, target organisms, experimental verification and external literature.	
7.	LAMP2	A database linking antimicrobial peptides http://biotechlab.fudan.edu.cn/database/lamp/	LAMP2 currently has 23253 AMP sequences, including 7824 natural and 15429 synthetic peptides.	
8.	DBAASP	The Database of Antimicrobial Activity and Structure of Peptides http://dbaasp.org.	DBAASP containing information about 20523 peptides.	

of cysteine, methionine, proline, arginine, histidine, tyrosine, and tryptophan residues. It contains 18.5% of valine and 16.7% of serine with only one phenylalanine residue. Molecular mass of mature apisimin is 5540.4 Da and was found in the high molecular weight fraction of royal jelly. The primary structure of apisimin is KTSISVKGESNVDVVSQIN SLVSSIVSGANVSAVLLAQTLVNILQI LIDANVFA-NH_2. The major secondary structural elements in apisimin is α-helical (34%) followed by β-sheet (20%), β-turn (11%) and random coils (30%). High amount of apisimin mRNA and apalbumin-mRNA (MRJP-1) was found in the heads of honeybees and are synthesized during the whole life span of honeybees. This indicates that both the AMPs are associated with larval development and learning and memory of both nurse and forager honeybees. Protein sequence of apisimin not showing any homology with the other known honeybee peptides such as royalisin, apideacin, hymenoptacin and abaecin, or with the honeybee venom peptides as melittin, apamin and MCD-peptide. The proliferation of human monocytes is regulated by Apisimin. It is demonstrated that the presence of apisimin and arabinogalactan AMPs contributing to immune active quality of honey[11] (Figs 1 & 2, and Tables 1-4).

Apalbumins (major royal jelly proteins)

The vital and reliable honeybee proteins in honey are major royal jelly proteins labelled as apalbumins (Apa). They are members of a protein family with a molecular weight ranged between 49 and 87 kDa. Approximately ninety per cent of the royal jelly protein contents include Apa 1, Apa 2 and Apa 3. Apalbumins (1-3) presenting sequence homology to the tune of 72 % and minor royal jelly proteins are predominantly the homologues of apalbumins, antimicrobial peptides, and enzymes. It is one of the most abundant acidic peptides in royal jelly and existing in royal jelly as monomer (55 kDa) and oligomer (420 kDa). Apisimin and apalbumin form an oligomeric stable complex to form gel. This peptide is

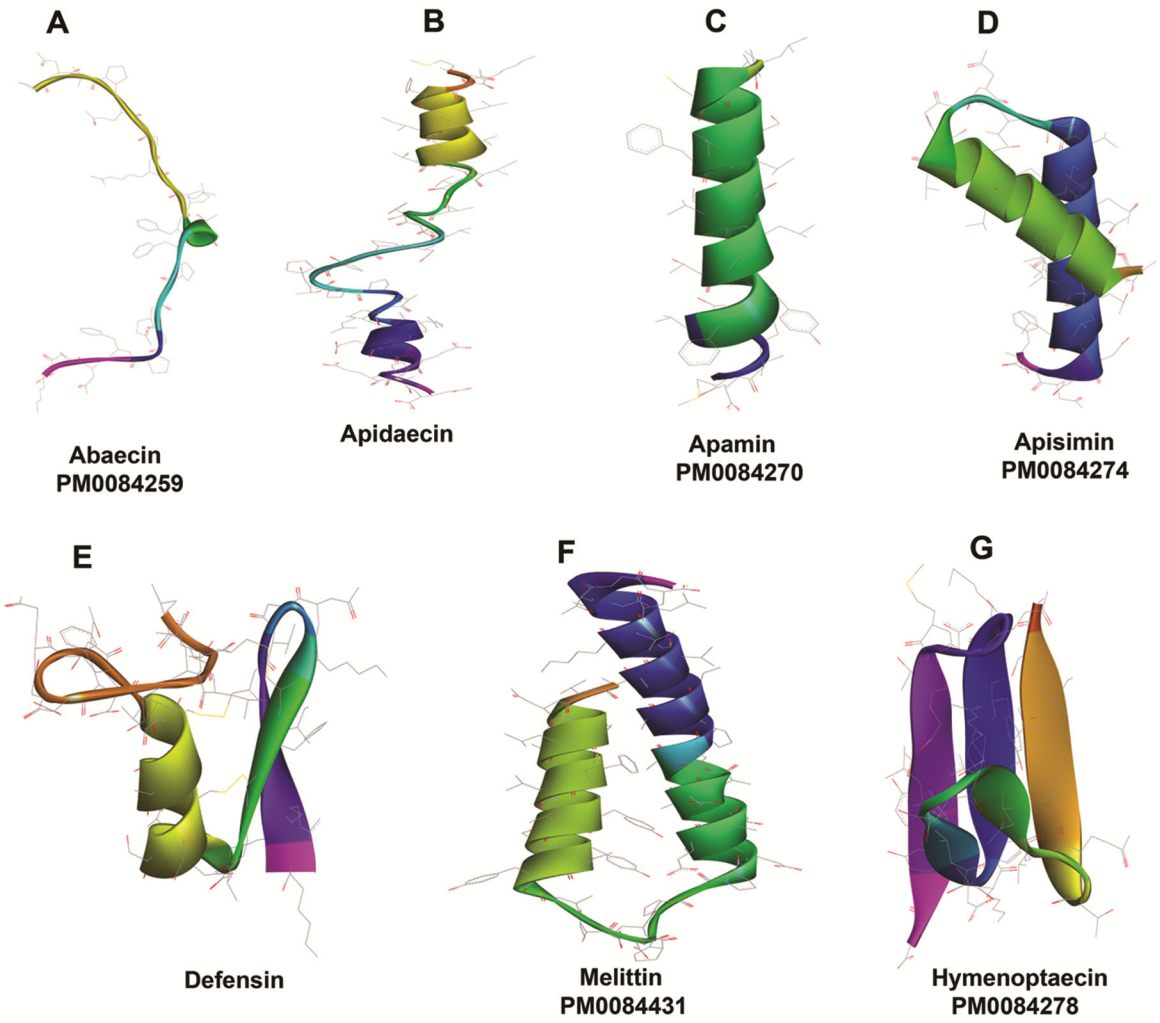

Fig. 2 — Three dimensional structure of antimicrobial peptides structured via homology modeling (SWISS model) and validated the structure by Ramachandran plot using SAVES server tool.

generated throughout the entire life period of nurse and foraging honeybees and involved in many cellular processes and cell proliferation activation alone and in complex with apisimin. Apalbumin has multifunctional roles as apa1 and apa2 induce the release of TNF-α and apa3 regulates immune response[13] (Figs 1 & 2, and Tables 1-4).

Apalbumina/Apalbumin 1

The major unique and authentic components of honey bee proteins in honey are enzymes associated with carbohydrate metabolism and royal jelly proteins. Apalbumina or apalbumin 1 is one of the most prevailing royal jelly proteins of honey. It is a versatile and multifaceted glycoprotein stimulating the TNF-α cytokine via murine macrophages as well as augmenting the hepatocytes proliferation. C-terminal portion of apalbumina is a precursor form of jelleines (I-IV). In royal jelly, apalbumina exists as a monomer (55 kDa) or in oligomeric form (420 kDa). It is actively participating (self-assembled membranes) in improving moisture content during processing of nectar to ripen honey (18% moisture content) in contrast forming filamentous spider web-like networks upon immobilization of pollen grains as pollen pellet. The ratio of apalbumin 1/apalbumina to total proteins of honey can be measured as supportive benchmark for adulteration of honey. The quantity of apa1 in honey is < 50 µg/g would be suggestive of the presence of contamination. Hence, apalbumina can be used as a quality marker to validate honey and honey bee products[11]. In addition, the apalbumina demonstrates the antimicrobial properties toward *B. subtilis* and *E. coli* (Figs 1 & 2, and Tables 1-4).

Table 3 — Prediction of antimicrobial activity and toxicity of honey bee antimicrobial peptides using CAMP database and ToxIBTL server tools

Sl. No.	Peptide name	Uniprot ID[†]	Antimicrobial activity prediction[#]				Toxicity prediction[$]	
			SVM score	RF score	ANN prediction	DA score	Prediction	Score
			Abaecin					
1	*Apis mellifera*	P15450	0.928	0.972	AMP	0.904	non-toxic	3.963
2	*Apis cerana cerana*	A0A2A3E7A0	0.911	0.947	AMP	0.901	non-toxic	5.668
3	*Apis mellifera*	D2YZ38	0.691	0.692	AMP	0.504	non-toxic	2.919
4	*Apis cerana japonica*	Q8WSY9	0.763	0.787	AMP	0.620	non-toxic	2.312
5	*Melipona quadrifasciata*	A0A0N0BGD9	0.971	0.732	AMP	0.807	non-toxic	1.788
			Apamin					
6	*Apis mellifera*	P01500	0.909	0.921	AMP	0.907	non-toxic	1.345
7	*Apis cerana cerana*	Q86QT2	0.811	0.879	AMP	0.618	non-toxic	1.410
8	*Apis mellifera*	A0A2A3EK62	1.000	0.913	AMP	1.00	non-toxic	1.295
			Melittin					
9	*Apis mellifera*	P01501	0.917	0.917	AMP	0.718	non-toxic	2.295
10	*Apis cerana*	Q8LW54	0.814	0.951	AMP	0.820	non-toxic	6.445
11	*Apis florea*	P0DPR9	0.994	0.999	AMP	0.999	non-toxic	2.625
12	*Apis dorsata*	P01504	0.977	0.997	AMP	0.992	non-toxic	1.479
13	*Apis cerana cerana*	P68407	0.825	0.833	AMP	0.651	non-toxic	1.678
14	*Apis mellifera carnica*	I3RJI9	0.917	0.917	AMP	0.718	non-toxic	2.569
Major royal jelly protein 1 and Jellein								
15	*Apis mellifera*	O18330	1.000	0.959	AMP	1.000	non-toxic	8.965
16	*Apis mellifera*	P84759	0.902	0.930	AMP	0.869	non-toxic	2.225
			Apisimin					
17	*Apis mellifera*	Q8ISL8	0.716	0.921	AMP	0.885	non-toxic	7.926
18	*Apis cerana cerana*	A0A2A3EKN9	0.902	0.994	AMP	0.863	non-toxic	2.004
Apidaecins								
19	*Apis mellifera*	Q06602	1.000	0.871	AMP	1.000	non-toxic	5.426
20	*Apis cerana cerana*	B9UKB7	1.000	0.886	AMP	1.000	non-toxic	1.150
21	*Melipona quadrifasciata*	A0A0N0U6Q6	1.000	0.941	AMP	1.000	non-toxic	2.007
			Hymenoptaecin					
22	*Apis mellifera*	Q10416	1.000	0.986	AMP	1.000	non-toxic	3.412
23	*Apis cerana cerana*	B9UKG2	1.000	0.987	AMP	1.000	non-toxic	2.051
24	*Apis dorsata*	C7AHW4	1.000	0.989	AMP	1.000	non-toxic	6.332
25	*Apis andreniformis*	C7AHW3	1.000	0.913	AMP	1.000	non-toxic	1.714
26	*Apis cerana*	V9IC30	1.000	0.913	AMP	1.00	non-toxic	1.399
			Defensin					
27	*Apis mellifera carnica*	Q5J8R1	0.997	0.979	AMP	0.994	non-toxic	1.200
28	*Apis cerana cerana*	A7L3U8	1.000	0.977	AMP	0.999	non-toxic	1.998
29	*Apis cerana*	C7AHS9	0.922	0.958	AMP	0.716	non-toxic	2.354
30	*Apis mellifera*	A0A088A8D5	0.997	0.989	AMP	0.982	non-toxic	5.833
31	*Apis dorsata*	A0A387IGF2	0.750	0.730	AMP	0.641	non-toxic	1.002
32	*Apis cerana japonica*	D3KYH2	0.747	0.806	AMP	0.929	non-toxic	2.720
33	*Melipona quadrifasciata*	A0A0N0U626	1.000	0.979	AMP	0.999	non-toxic	1.999

[†]Antimicrobial peptide sequences were retrieved from Uniprot database (https://www.uniprot.org/help/uniprotkb)

[#]Antimicrobial activity prediction of antimicrobial peptides was predicted through the CAMP database (http://www.camp3.bicnirrh.res.in) using the support vector machine (SVM), random forest (RF), artifcial neural network (ANN) and discriminant analysis (DA) algorithms for scoring. The threshold score to be considered as antimicrobial peptide is 0.5.

[$]Toxicity prediction was done using online ToxIBTL server (https://server.wei-group.net/ToxIBTL). The score >1 is considered as non-toxic and ≤1 is considered as toxic.

Apisin

Apisin is a hetero-oligomer comprising of major royal jelly protein 1 and apisimin. It is a unique protein that constitutes the larger portion of the royal jelly proteins. Apisin is known as 350 kDa major royal jelly glycoprotein-1 multimer (MRJP-1 multimer). It is containing an N-glycan with a distinctive high-mannose type oligosaccharide (Man9–5GlcNAc2). A

Table 4 — Details of antiviral, antifungal and anticancer activity prediction results of antimicrobial peptide from honey bee species using AVPpred, Antifp and iACP tools

A Sl. No	Peptide name	Uniprot ID	Antiviral activity prediction (AVP) #					Antifingal activity$		Anticancer*		
			AVP motif	Alignment model	Composition model (%)	Physio-chemical model (%)	Overall prediction	Score	Prediction	Prediction	Anticancer score	NAC score
Abaecin												
1	*Apis mellifera*	P15450	-	Non-AVP	51.23	64.08	Y	0.559	Antifungal	Anticancer	0.959	0.040
2	*Apis cerana cerana*	A0A2A3E7A0	-	Non-AVP	49.25	64.09	N	-0.295	NAF	Anticancer	0.959	0.040
3	*Apis mellifera*	D2YZ38	-	Non-AVP	49.76	64.08	N	0.067	Antifungal	Anticancer	0.9593	0.040
4	*Apis cerana japonica*	Q8WSY9	-	Non-AVP	49.99	64.08	N	-0.295	NAF	Anticancer	0.959	0.040
5	*Melipona quadrifasciata*	A0A0N0BGD9		Non-AVP	61.21	64.14	Y	0.552	Antifungal	Anticancer	0.959	0.040
Apamin												
6	*Apis mellifera*	P01500	-	Non-AVP	59.57	64.14	Y	0.535	Antifungal	Anticancer	0.959	0.040
7	*Apis cerana cerana*	Q86QT2	-	Non-AVP	61.21	64.14	Y	0.595	Antifungal	Anticancer	0.959	0.040
8	*Apis mellifera*	A0A2A3EK62	-	Non-AVP	59.24	64.14	Y	0.571	Antifungal	Anticancer	0.959	0.040
Melittin												
9	*Apis mellifera*	P01501	-	Non-AVP	47.75	64.08	N	-0.674	NAF	Anticancer	0.609	0.390
10	*Apis cerana*	Q8LW54	-	Non-AVP	59.54	64.08	Y	0.582	Antifungal	Anticancer	0.952	0.047
11	*Apis florea*	P0DPR9	-	Non-AVP	47.08	69.15	N	-0.069	NAF	Anticancer	0.959	0.040
12	*Apis dorsata*	P01504	-	Non-AVP	57.50	66.21	Y	0.528	Antifungal	Anticancer	0.959	0.040
13	*Apis cerana cerana*	P68407	-	Non-AVP	53.82	64.08	Y	0.595	Antifungal	Anticancer	0.609	0.390
14	*Apis mellifera carnica*	I3RJI9	-	Non-AVP	47.75	64.08	N	-0.674	NAF	Anticancer	0.609	0.390
Major royal jelly protein												
15	*Apis mellifera*	O18330	-	Non-AVP	35.64	64.08	N	-0.028	NAF	NAC	0.233	0.766
Jellein												
16	*Apis mellifera*	P84759	-	Non-AVP	45.18	42.90	N	-0.264	NAF	Anticancer	0.959	0.040
Apisimin												
17	*Apis mellifera*	Q8ISL8	-	Non-AVP	45.58	64.08	Y	0.528	Antifungal	NAC	0.157	0.842
18	*Apis cerana cerana*	A0A2A3EKN9	-	Non-AVP	42.79	64.08	N	-1.3901	NAF	NAC	0.038	0.961
Apidaecin												
19	*Apis mellifera*	Q06602	-	Non-AVP	28.35	64.08	N	-1.065	NAF	NAC	0.009	0.990
20	*Apis cerana cerana*	B9UKB7	-	Non-AVP	47.28	64.05	N	-0.186	NAF	Anticancer	0.551	0.448
21	*Melipona quadrifasciata*	A0A0N0U6Q6	-	Non-AVP	43.42	64.08	N	-0.028	NAF	NAC	0.354	0.645
Hymenoptaecin												

(*contd.*)

Table 4 — Details of antiviral, antifungal and anticancer activity prediction results of antimicrobial peptide from honey bee species using AVPpred, Antifp and iACP tools (*contd.*)

A

Sl. No	Peptide name	Uniprot ID	Antiviral activity prediction (AVP) [#]					Antifingal activity[$]		Anticancer[*]		
			AVP motif	Alignment model	Composition model (%)	Physio-chemical model (%)	Overall prediction	Score	Prediction	Prediction	Anticancer score	NAC score
22	*Apis mellifera*	Q10416	-	Non-AVP	35.69	64.03	N	-0.341	NAF	NAC	0.030	0.969
23	*Apis cerana cerana*	B9UKG2	-	Non-AVP	43.13	64.04	Y	0.591	Antifungal	Anticancer	0.809	0.190
24	*Apis dorsata*	C7AHW4	-	Non-AVP	32.54	64.02	N	-0.019	NAF	Anticancer	0.539	0.460
25	*Apis andreniformis*	C7AHW3	-	Non-AVP	28.64	64.04	N	-0.236	NAF	Anticancer	0.954	0.045
26	*Apis cerana*	V9IC30	-	Non-AVP	47.28	64.05	N	-0.486	NAF	Anticancer	0.614	0.386
Defensin												
27	*Apis mellifera carnica*	Q5J8R1	-	Non-AVP	44.24	64.08	N	-0.019	NAF	Anticancer	0.569	0.430
28	*Apis cerana cerana*	A7L3U8	-	Non-AVP	39.03	64.08	N	0.183	Antifungal	Anticancer	0.704	0.295
29	*Apis cerana*	C7AHS9	-	Non-AVP	41.45	64.08	Y	0.536	Antifungal	NAC	0.378	0.621
30	*Apis mellifera*	A0A088A8D5	-	Non-AVP	43.49	64.07	Y	0.507	Antifungal	NAC	0.108	0.891
31	*Apis dorsata*	A0A387IGF2	-	Non-AVP	46.06	64.07	Y	0.536	Antifungal	Anticancer	0.689	0.310
32	*Apis cerana japonica*	D3KYH2	-	Non-AVP	24.64	64.05	N	-0.295	NAF	NAC	0.085	0.914
33	*Melipona quadrifasciata*	A0A0N0U626		Non-AVP	43.13	64.04	Y	0.591	Antifungal	Anticancer	0.809	0.190

[#]Antiviral activity prediction was performed by using the online server AVPpred (http://crdd.osdd.net/servers/avppred/). It exploits four diferent models: (1) the AVP motif, which returns the result as YES or NO; (2) the Alignment model, which gives the result in the form AVP or Non-AVP; (3) the Composition model and the (4) the Physico-chemical model, which return their results in a numerical form (percentage). The overall result is expressed with a YES, if the peptide results have a putative antiviral activity, and with a NO, if otherwise.

[$]Antifungal activity prediction was performed using the Antifp server (https://webs.iiitd.edu.in/raghava/antifp/disp.php), which gives the result as a numerical score, ≥0.5 score is considered as threshold.

[*]Anticancer activity prediction was performed by iACP tool (http://lin-group.cn/server/iACP), providing the results in a numerical form. ≥ 0.5 score considered as anticancer.

Abbreviations: NAC – Non-anticancer; NAF – Non-antifungal; Y – Yes; N – No;

B

Sl. No.	Peptide name	Uniprot ID	#Antimicrobial activity prediction				$Toxicity prediction	
			SVM	RF	ANN	DA	Result	Score
Abaecin								
1	*Apis mellifera*	P15450	0.928	0.972	AMP	0.904	non-toxic	3.963
2	*Apis cerana cerana*	A0A2A3E7A0	0.911	0.947	AMP	0.901	non-toxic	5.668
3	*Apis mellifera*	D2YZ38	0.291	0.692	NAMP	0.004	non-toxic	2.919
4	*Apis cerana japonica*	Q8WSY9	0.263	0.487	NAMP	0.020	non-toxic	2.312
5	*Melipona quadrifasciata*	A0A0N0BGD9	0.971	0.432	AMP	0.807	non-toxic	0.000
Apamin								
6	*Apis mellifera*	P01500	0.909	0.921	AMP	0.907	toxic	0.999
7	*Apis cerana cerana*	Q86QT2	0.211	0.279	NAMP	0.018	toxic	0.999
8	*Apis mellifera*	A0A2A3EK62	1.000	0.913	AMP	1.00	toxic	0.999
Melittin								
9	*Apis mellifera*	P01501	0.017	0.017	NAMP	0.018	non-toxic	2.295

(*contd.*)

Table 4 — Details of antiviral, antifungal and anticancer activity prediction results of antimicrobial peptide from honey bee species using AVPpred, Antifp and iACP tools (*contd.*)

10	*Apis cerana*	Q8LW54	0.114	0.351	NAMP	0.120	non-toxic	6.445
11	*Apis florea*	P0DPR9	0.994	0.999	AMP	0.999	non-toxic	2.625
12	*Apis dorsata*	P01504	0.977	0.997	AMP	0.992	toxic	0.999
13	*Apis cerana cerana*	P68407	0.025	0.033	NAMP	0.051	toxic	0.999
14	*Apis mellifera carnica*	I3RJI9	0.017	0.017	NAMP	0.018	non-toxic	2.569
Major royal jelly protein 1 and Jellein								
15	*Apis mellifera*	O18330	1.000	0.959	NAMP	1.000	non-toxic	8.965
16	*Apis mellifera*	P84759	0.102	0.330	AMP	0.169		
Apisimin								
17	*Apis mellifera*	Q8ISL8	0.516	0.321	NAMP	0.885		
18	*Apis cerana cerana*	Q8ISL8	0.602	0.394	AMP	0.863	non-toxic	2.004
Apidaecins								
19	*Apis mellifera*	Q06602	1.000	0.871	AMP	1.000	non-toxic	5.426
20	*Apis cerana cerana*	B9UKB7	1.000	0.886	NAMP	1.000	non-toxic	1.150
21	*Melipona quadrifasciata*	A0A0N0U6Q6						
Hymenoptaecin								
22	*Apis mellifera*	Q10416	1.000	0.986	AMP	1.000	non-toxic	3.412
23	*Apis cerana cerana*	B9UKG2	1.000	0.987	AMP	1.000	toxic	0.999
24	*Apis dorsata*	C7AHW4	1.000	0.989	AMP	1.000	non-toxic	6.332
25	*Apis andreniformis*	C7AHW3	1.000	0.913	AMP	1.00	toxic	0.999
26	*Apis cerana*	V9IC30	1.000	0.913	AMP	1.00	toxic	0.999
Defensin								
27	*Apis mellifera carnica*	Q5J8R1	0.997	0.979	AMP	0.994	toxic	1.000
28	*Apis cerana cerana*	A7L3U8	1	0.977	AMP	0.999	toxic	0.998
29	*Apis cerana*	C7AHS9	0.922	0.658	AMP	0.516	non-toxic	2.354
30	*Apis mellifera*	A0A088A8D5	0.997	0.989	NAMP	0.982	non-toxic	5.833
31	*Apis dorsata*	A0A387IGF2	0.750	0.430	AMP	0.641	non-toxic	1.002
32	*Apis cerana japonica*	D3KYH2	0.747	0.406	AMP	0.229	non-toxic	2.720
33	*Melipona quadrifasciata*	A0A0N0U626	1.000	0.979	AMP	0.999	toxic	0.999

#Antimicrobial activity prediction was predicted through the CAMP database. CAMP gives four score *i.e.* (i) Support Vector Machine (SVM) score, (ii) Random Forest (RF) score, (iii) Artifcial Neural Network (ANN) result and (iv) Discriminant Analysis (DA) score. The threshold score to be considered as antimicrobial peptide is 0.5; $Toxicity prediction was done by using ToxIBTL server. The score >1 is considered as non-toxic and ≤1 is considered as toxic

galactosyl residue in N-glycans is a natural bee glycoproteins linked to royal jelly glycoprotein mixture. Apisin content was estimated to be more than 1,500 mAU in natural royal jelly samples from Japan. Japan royal jelly INC conducted a study and found that bee larvae fed with royal jelly containing higher content of apisin (1500 mAU) grew to a size several times greater at 24 hours after feeding, and grew incredibly fast into queen bees. These observations evidenced that the growth of bee larvae is affected by apisin and is a key substance in the growth of larvae into queen bees[14] (Figs 1 & 2, and Tables 1-4).

Royalisin

This is a member of the insect defensin family (5.5 kDa, six cysteine/three disulfide bridge pattern) derived from the royal jelly of *A. mellifera*. It consists of 51 amino acids, a characteristic disulfide-rich structure (40 amino acids), a distinct amphipathic α-helix, an amidated carboxyl-terminal tail (11 amino acids) and having compact globular structure because of forming three intramolecular disulfide linkages via six cysteine residues[15]. This bee AMP demonstrates widespread sequence homology to antibacterial proteins as sapecin and phormicins. The presence of intramolecular disulfide linkages and the 11 amino acids at the C-terminus of royalisin ensures their higher antimicrobial against gram-positive bacteria (*Clostridium*, *Corynebacterium*, *Leuconostoc*, *Staphylococcus*, *Bacillus subtilis*, *Sarcina lutea* and *Streptococcus*), gram-negative bacteria, American foulbrood disease (*Paenibacillus larvae*) and fungi (*Botrytis cinerea* and *Alternaria brassica*) than the royalisin-D (shortened royalisin)[15]. Royalisin (180 μg/mL) inhibits *B. subtilis* significantly in comparison with tetracyclin (50 μg/mL). Royalisin can be used as a food preservative and a potent drug against diseases because of its stable nature at low pH and high temperature (Figs 1 & 2, and Tables 1-4).

Jelleines

The jelleines are hydrophobic, cationic antimicrobial peptides shorter than the most AMPs. They are containing 8 to 9 amino acid residues with a net charge of 1+ or 2+ while the other AMPs having 12 to 50 amino acids with a net positive charge between 2+ and 7+. Four forms of jelleines were isolated from royal jelly of honeybees, *i.e*., Jelleine-I (PFKLSLHL-NH2), Jelleine-II (TPFKLSLHL-NH2), Jelleine-III (EPFKLSLHL-NH2), and Jelleine-IV (TPFKLSLH-NH2). Jelleines encompass surplus of basic amino acid residues and hydrophobic residues (> 50%) which are having direct interactions with bacterial membranes. Jelleine-I, is an amphiphilic AMP (953.17 kDa) showing potent *in vitro*, *in vivo* antimicrobial (neutralize lipopolysaccharide, interrupt the integrity of bacterial cell membrane, intermingle with bacterial genomic DNA and cause the release of ATP) and antifungal activity[16]. The Jelleines (I–III) are showing antimicrobial and anti-fungal activities against Gram-positive bacteria (*Styphylococcus aureus*, *S. saprophyticus* and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and yeast (*Candida albicans*). No cytolytic activity and induction of rat peritoneal mast cell degranulation were observed in the Jelleines (I–IV). The jelleine-IV lacks antibacterial, antifungal, cytolytic activity as well as stimulation of rat peritoneal mast cell degranulation[16] (Figs 1 & 2, and Tables 1-4).

10 HDA and 10 H2DA

10-hydroxy-2-decenoic acid (10HDA, queen bee acid) and 10-Hydroxy-trans-2-decenoic acid (10H2DA, royal jelly acid) are the distinctive major lipid components of royal jelly and present only in royal jelly. 10H2DA (> 100 mM concentration) presents only in royal jelly comprising 50% of the total fatty acid content followed by 10HDA (1.5 – 2.0%) which is a saturated lipid form of 10H2DA. Out of 3-8% lipids of royal jelly, more than 60-80% contains both 10H2DA and 10HDA. Hence, these host defense peptides could be employed as biomarkers as well as internal standards to authenticate quality of royal jelly and straightaway decides the value of royal jelly on the international market. 10HDA and 10H2DA have been reported to have biological activities as immunomodulatory, anti-bacterial (*E. coli*, *B. subtilis*, *Micrococcus pyogenes* and *Staphylococcus aureus*), anti-fungal (*Neurospora sitophila*), anti-rheumatoid arthritis, anti-cancer, anti-angiogenesis, promotion of collagen production, T-lymphocytes and IL-2, epigenetic regulation, modulation of ion channels, estrogenic and neurogenic modulation[16] (Figs 1 & 2, and Tables 1-4).

Hymenoptaecin

The *A. mellifera* hymenoptaecin is a long (93 amino acids) glycine rich cationic AMP, including a 2-pyrrolidone-5-caboxylic acid at the N-terminus. The precursor of bee hymenoptaecins comprises a signal peptide, pro-sequence and a mature peptide. The propeptide of bee hymenoptaecins is showing homology with the wasp *Nasonia vitripennis* and encoding multipeptide precursors. In *A. cerana* adult workers, thirteen different hymenoptaecin peptides were identified. In the wild *A. mellifera* exposed to mild pathogen infection, only the genes for apidaecin and hymenoptaecin were expressed, whereas abaecin and defensin were absent[11] (Figs 1 & 2, and Tables 1-4).

Defensin

It is a family of cystein-rich cationic AMPs (36-51 amino acids) consisting of an α-helix, two antiparallel β-strands and a loop at the amino end which are stabilized by three disulfide bridges. Defensin is the prime defense system in bees and consists of two types namely defensin 1 (5.5 KDa) and 2 (4.8 KDa) modulated by Toll and Imd signal pathways. Defensin 1 is produced in salivary glands and also exists in the royal jelly and honey associated with social immunity while defensin 2 is synthesized in fat body and haemolymph of bee linked with individual immunity. *A. mellifera* defensin AMP comprises 43 amino acids (VTCDVLSWQSKWLSINHSACAIRCLAQRRKGG SCRNGVCICRK), whereas other bee species contains 51 amino acids because of additional amino acid replacements amongst bee defensin peptides. The open reading frame of *A. cerana* defensin contains an additional amidated amino acid, glycine at the carboxy terminal compared with *A. mellifera* defensin. The structure of defensin is showing similarity with *Phormia terranovae* and homology between the defensin 1 and 2 is found to be 55.8%[18]. Three isoforms of Defensin 1 were isolated in honey bee, *i.e*., 1 isoform from hemolymph and 2 isoforms from royal jelly [royalisin – Ro-F (5.525 KDa) and Ro-K (5.515 KDa,)]. The haemolymph isoform 1 defensin variant is varied from the royalysin defensin variants by two amino acids substitutions. The genes

defensin 1 and 2 differ significantly by the length (2012 and 1950 bp), intron-exon structure [2 introns (between 773-1345 and 1525-180 and 1 intron (between 947 and 1283)], pre-pro-peptide region (defensin 2 longer than defensin 1) and mature peptide (51 and 43 amino acids and absence of C-terminal elongation in defensin 1), respectively[18]. proved the close phylogenetic relationship (93%) between *Apis cerana japonica* defensin (AcjDef2) and *Apis mellifera* defensin (AmDef). Defensin showing selective antipathogenic activity against *Paenibacillus larvae*, *Melissococcus pluton*, *Ascosphaera apis*, *Nosema apis*, *N. ceranae*, *Aspergillus flavus*, *A. niger*, *Candida albicans*, *Aurobasidium pullulans*, *Varroa destructor*, *V. jacabsoni*, *Acarapis woodi*, and *Tropilaelaps clareae*[18]. Modulations in antipathogenic activity of the honeybee defensins are found to be observed because of alterations in processing of defensin precursors that lead to generation of molecular variants (29 varied defensin cDNA genes coding 7 diverse defensin peptides)[18] (Figs 1 & 2, and Tables 1-4).

Apamin

Apamin (H-Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH_2) is one of the AMPs found in bee venom (2-3%). It is a globular neurotoxin peptide having 18 amino acid residues and compactly networked with two disulfide bonds. It is an inflexible polypeptide due to the presence of two disulfide bridges between Cys_1- Cys_{11} and Cys_3 - Cys_{15} position and seven hydrogen bonds[19]. It possesses many pharmacological activities as blocking calcium-activated potassium channels, regulation of cell growth via signal transduction pathways, alleviating neurodegeneration, neurofibromatosis, acute pancreatitis, liver fibrosis, atopic dermatitis, atherosclerosis, rheumatoid arthritis, multiple sclerosis, cognitive deficit and dementia and inhibition of Th2-related cytokines, TNF-α- and IFN-γ[19]. (Figs 1 & 2, and Tables 1-4).

Melittin

It is a main AMP of bee venom (40-60%) contains 26 amphipathic amino acids (**H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2**), no disulfide bridge, 6 positive charges located in C-terminal responsible for α-helix stability and no negative charges. It is a small polypeptide denatured in aqueous solutions and folding spontaneously in non-polar media[20]. The amino-terminal portion of melittin is principally hydrophobic with lack of lytic activity whereas the carboxyl-terminal region is hydrophilic, strongly basic and accountable for the lytic action. Melittin (monomeric and tetrameric forms) is soluble in water because of its amphipathic nature. The mode of action of melittin is disrupting the prokaryotic and eukaryotic cell membranes through pore formation and lysis. It is forming transient and stable pore formation when cell membrane orients and permeable to ions and large molecules (glucose), respectively. Melittin is being involved in diverse biological activities as the release of pro-inflammatory cytokines, activates G-protein-coupled receptor-mediated opening of transient receptor potential channels, inhibits protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, myosin light chain kinase, Na+/K+-ATPase (synaptosomal membrane) and blocks transport pumps (Na^+-K^+-ATPase and the H^+-K^+-ATPase), anti-nociceptive and haemolytic, activator of PLA2, anticancer, antitumor, antidiabetic, anti-biofilm, anti-inflammatory, antibacterial, antifungal, antiviral, anti-arthritic and the immune response of bees to infectious diseases[21,22] (Figs 1 & 2, and Tables 1-4).

Melectin

This AMP contains 18 amino acids, cationic (net charge, +5), amphipathic and α-helical structure with a molecular weight of 2038.3 kDa. It has hydrophobic and basic amino acid residues and a proline (H-**GFLSILKKVLPKVMAHMK-NH_2**). Despite short amino acid sequence, low cytotoxicity in mammalian cells, broad-spectrum potency, cell selectivity, salt-resistant properties and rapid bactericidal action than melittin (26 amino acids) melectin exhibits significant antimicrobial action against gram positive (*Staphylococcus aureus*) and gram negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *E. coli*) including drug-resistant bacteria (*S. aureus* MRSA 254/366/660, *P. aeruginosa* 1034/3543/5018 and *E. coli* CCARM 1229/1238). Melectin possesses the biological activities as selectivity of bacterial cells via pro kink in the α-helical structure, degranulation of peritoneal mast cells in rats, low haemolytic activity and anti-tumor[11] (Figs 1 & 2, and Tables 1-4).

Adolapin

Adolapin is one of the components the honey bee venom and constitutes about 0.1-1% in dry venom[19]. It contains 2-5% of the AMPs in bee venom and a

basic AMP (11.5 kDa) comprises 103 amino acids and is showing marked pharmacological properties such as anti-inflammatory, anti-nociceptive, analgesic, antipyretic, inhibition of COX1, prostaglandin synthase and lipoxygenase systems in human platelets, and upsurge c-GMP level in the rat spleen and brain and declines the c-AMP level in the rat spleen[19] (Figs 1 & 2, and Tables 1-4).

Mast Cell Degranulating (MCD) Peptide

MCD or peptide 401 (2587.2 kDa) is one of the cationic (net charge +8), bicyclic peptide components of bee venom (1-3% of dry venom)[21]. It is composed of 22 amino acids (H-**IKCNCKRHVIKPHIC RKICGKN-NH_2**) with two disulfide associations between Cys_3 - Cys_{15} and Cys_5 - Cys_{19}. MCD secondary structure is analogous with apamin AMP. It has several biological functions as immunological and pharmacological activities as analgesic and nociceptive effects and associated with allergy, releasing low concentrations of histamine, potent natural histamine secretagogues, inhibiting mast cell degranulation, anti-allergic agent, potent blocker of voltage-sensitive potassium channels, anti-inflammatory and can be used as a vaccine adjuvant. MCD acts as a mediator at low concentrations inducing mast cell degranulation and the release of histamine, resulting in inflammation processes while at high concentration, MCD inhibits mast cell degranulation and therefore, exerts anti-inflammatory effects[23]. It is acting as an epileptogenic neurotoxin, and an inhibitor of potassium channels instigating dropping of blood pressure in rats. At high concentration, it acts as an anti-allergen because of the presence of disulfide linkage which shares between IgE and the MCD peptide on the mast cell receptors to inhibit mast cell degranulation and to prevent the discharge of histamine and consequently, put forth anti-inflammatory effects[23] (Figs 1 & 2, and Tables 1-4).

Secapin

It is a non-toxic polypeptide of bee venom (0.5-2% dry venom) composed of 25 amino acids (YIIDVPPRCPPGSKFIKNRCRVIVP-NH_2) and rich in proline with a disulfide bond between the Cys_9 and Cys_{20} residues[19]. So far, many secapin isoforms [secapin, secapin-1, secapin-2 and secapin-3) were isolated from *A. mellifera* as well as AcSecapin-1 from *A. cerana*[19]. The amino acid sequences of three isoforms of this AMP are as follows: secapin (YIIDVPPRCPPGSKFIKNRCRVIVP-NH_2), secapin-1 (YIINVPPRCPPGSKFVKNKCRVIVP-NH2) and secapin-2 (YIIDVPPRCPPGSKFV HKRCRVIVP-NH2). The differences between secapin-1 and secapin-2 isoforms were substitutions at amino acids 17-19. AcSecapin-1 showed higher sequence similarity with *A. dorsata* secapin, *A. florea* secapin and *A. mellifera* secapin-3 than AcSecapin. AcSecapin-1 acts as a serine protease-like peptide, anti-fibrinolytic, anti-elastolytic, and anti-microbial activities and exhibits inhibitory effects against trypsin, chymotrypsin, microbial serine proteases, elastases, and plasmin. Secapin-2 is not inducing hemolytic activity, mast cell degranulation, or PMNL chemotaxis; however, it influences hyperalgesia and edema mediated by leukotrienes[24] (Figs 1 & 2, and Tables 1-4).

Procamine

It is one of the AMPs in bee venom and blocks the activity of proteases as trypsin, chymotrypsin, plasmin and thrombin, as a consequence lessening inflammation[11] (Figs 1 & 2, and Tables 1-4).

Tertiapin

This AMP contains 21-amino acids, two disulfide bridges and an amidated form of carboxy terminal residue. It occupies a minor constituent of bee venom (0.1% of dry venom) and belongs to neurotoxin peptide family alike apamin. It can be used as a potassium channel modulator. In human, it blocks inward-rectifier potassium channels[11] and prevents atrioventricular transmission disorders (Figs 1 & 2, and Tables 1-4).

Cardiopep

Cardiopep (cardioactive polypeptide, 1940 kDa) includes 0.7% of bee venom and contains low molecular weight peptides to the tune of 50%. Cardiopep is less toxic (LD_{50}:15 mg/kg in mice) than phospholipase A (LD_{50}:1-5 mg/kg), melittin (LD_{50}: 2-5 mg/kg) and whole bee venom (LD_{50}: 3-5 mg/kg). Along with less toxic, it has beta-adrenergic-like stimulant and anti-arrhythmic properties[11] (Figs 1& 2 and Tables 1-4).

Bee Immune interactions

Microbial pathogens recognition by bees

Microorganisms (bacteria, fungi, viruses and parasites) are recognized differentially by the innate and cellular immune systems. The bee innate immune system recognizes PAMPs (Pathogen-Associated Molecular Patterns), which are conserved and dynamic protein structures exist in distinct pathogen

and parasite groups namely lipopolysaccharides (LPS), lipotheicoic acid, zymosan, glycolipids, glycoproteins or dsDNA. The innate immune system of bees also distinguishes DAMPs (Damage-Associated Molecular Patterns) or thermal shock protein, MAMPs (microbe-associated molecular patterns), and VAMPs (virus-associated molecular patterns), which are molecules expressed in immune cells in response to pathogenic damage. PAMPs, DAMPs, MAMPs and VAMPs act as exogenous ligands and are recognized by PRR (Pattern Recognition Receptor), PGRP (Pathogen recognition peptidoglycan recognition proteins), AMPs or peptides, which are exist in soluble form or in immune system cells[11]. PGRP-S2 and PGRP-LC are generated for the Toll and Imd pathway, respectively in response to pathogen infections. GNBP1 (Gram-negative bacteria-binding protein) recognizes 1,3 glucans and LPS present in Gram-negative bacteria, fungi and also certain Gram-positive bacteria. All the pattern recognition proteins together with serine proteases activate the splitting up of Spaetzle and Toll's endogenous ligand which are activated during immune response. In the bee genome, two orthologous genes of the Spaetzle family were identified. Recognition of microbial structures activates two main events, *i.e.*, signaling (takes place once Toll and/or IMD receptors are stimulated), and phagocytosis. The genes DSCAM and Eater are associated with endocytosis in bees. Trans-generational immune priming (transfer of immunity from mother to offspring) is facilitated by vitellogenin, egg-yolk protein in honeybees (Figs 3 & 4).

Immune response regulation in sting and stingless bees

Immune responses against pathogens and parasites include a series of proceedings that can be categorized into three phases: 1) recognition, 2) activation of signaling pathways and 3) cellular and humoral

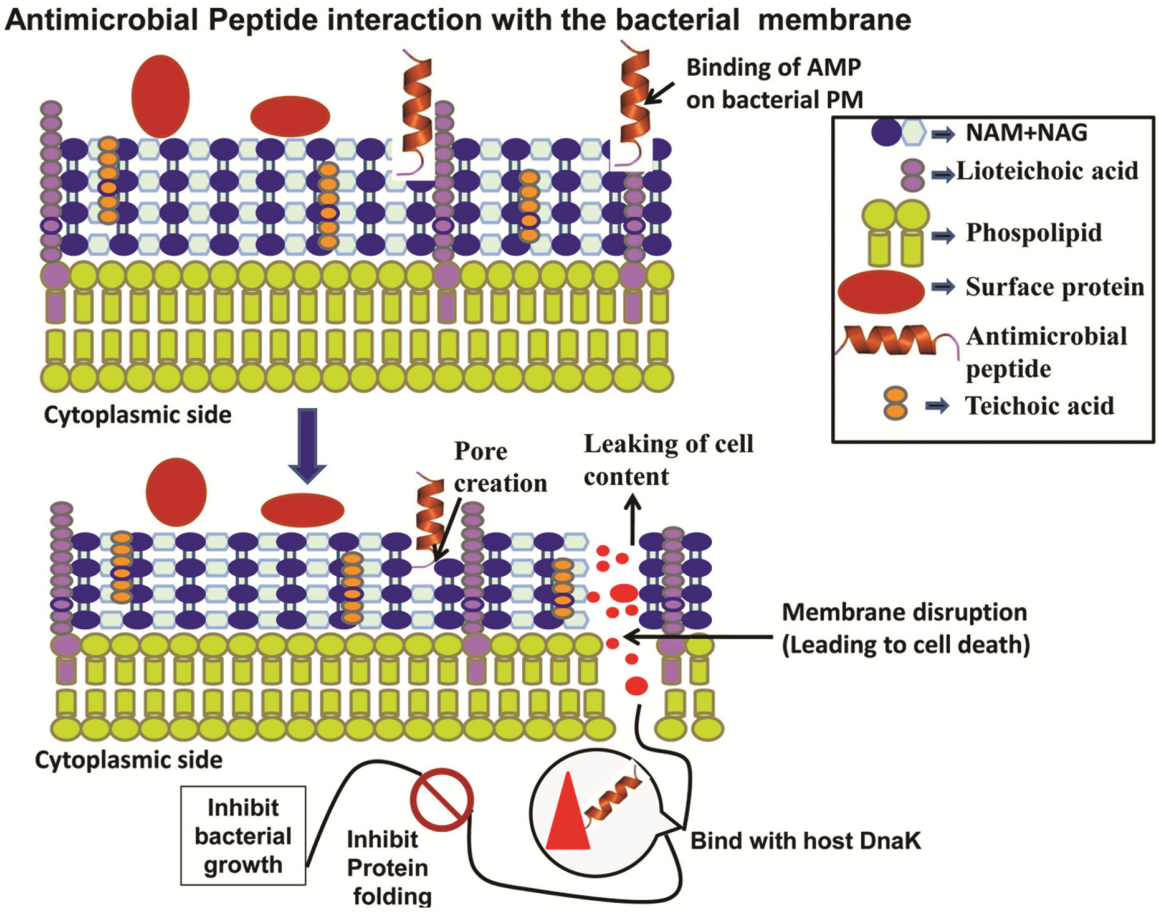

Fig. 3 — Schematic representation of antimicrobial peptides interaction with the bacterial plasma membrane and membranolytic mechanisms begin with adsorption of antimicrobial peptides on target cell membrane

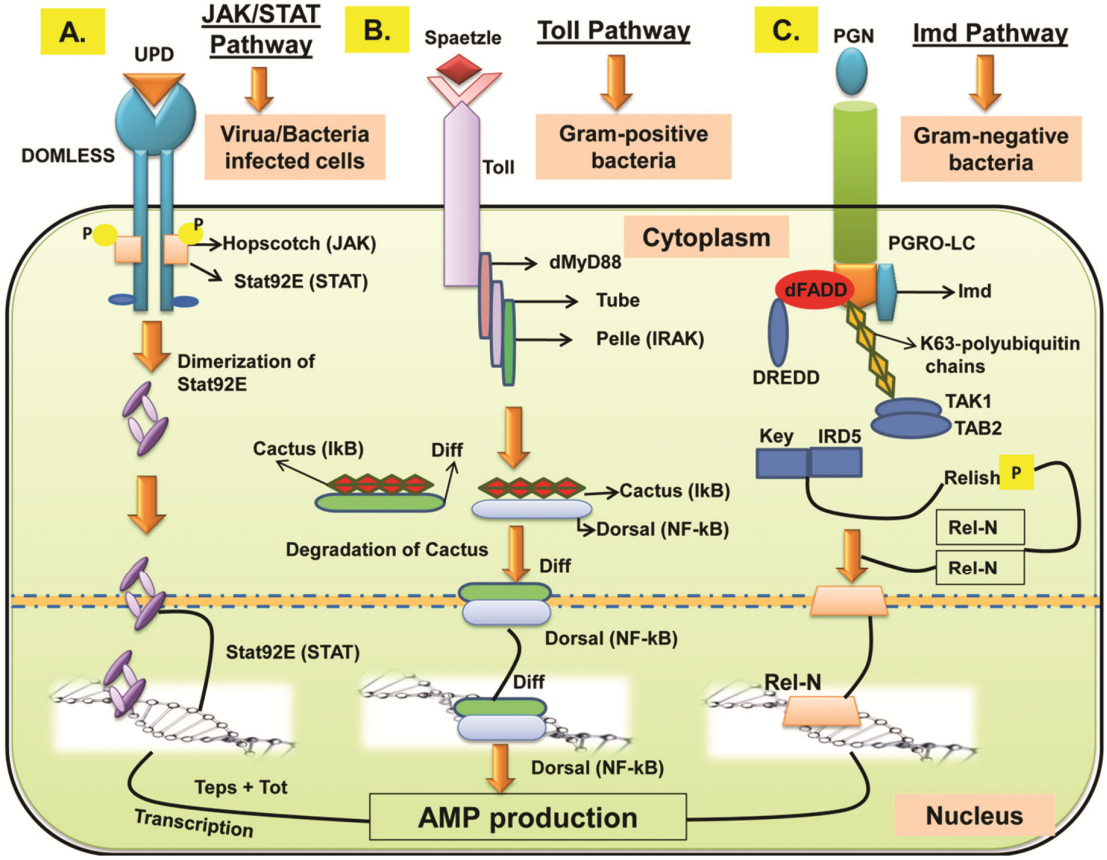

Fig. 4 — Immune pathways (JAK/STAT, Imd, and Toll) and defence mechanisms in honey bees (Manniello et al., 2021). (A) In JAK/STAT pathway, the cytokine receptor, Domeless (Dome) is activated when it binds to Unpaired (Upd) cytokines which induces the JAK tyrosine kinase Hopscotch (Hop) to phosphorylate itself, and the Dome cytoplasmic component. Consecutively, the signal transducer and activator of transcription at 92E (Stat92e) bind to the phosphotyrosines on Dome, and they are phosphorylated by Hop. Phosphorylated Stat92e separates itself from the receptor, dimerize and move into the nucleus, where it induces the transcription of Thioester-containing protein genes (Teps) and Turandot (Tot) genes for the production of AMPs; (B) Schematic representation of Toll signaling pathway. Pathogen recognition peptidoglycan recognition proteins (PGRP) activate a serine proteases cascade, involving ModSP and Grass proteins, which in turn, cleaving the inactive form of Spätzle protein, switch on the molecule. These interactions initiate protease cascades. Spätzle activates the dimer Toll receptor, which, in turn recruits cytoplasmic proteins (dMyD88, Tube, and Pelle) involved in the activation of Cactus signaling. In normal cellular condition, Cactus protein is coupled with the Nuclear Factor kappa B (NF-κB) transcription factors Dorsal-related immunity factor (DIF) and Dorsal, but following the Toll pathway activation, it is phosphorylated, detached from DIF and Dorsal and degraded. Then, both DIF and Dorsal can translocate in the nucleus and induce the transcriptional regulation of specific AMP genes; and (C) The Imd signaling pathway is activated following the binding between PGRP-LC and meso-diaminopimelic acid (DAP)-type peptidoglycan of Gram-negative and some Gram-positive bacteria. The Imd protein is activated following the cleavage by the Fas-associated death domain (FADD) and the death related ced-3/Nedd2-like caspase (DREDD). The K63-polyubiquitin chains help to link this complex with TAK1 and TAB2 proteins that, in turn, act on the IKK complex, which phosphorylates the NF-kB-like nuclear factor Relish. Consequently, TAK1 and TAB2 proteins are activated, that in turn, act on the IKK complex, composed of Immune Response Defcient 5 (IRD5) and Kenny (Key). This activated complex cleaves Relish. In this way, the Rel DNA-binding domain is released from the C-terminal ankyrin-repeat/IκB-like domain, and translocates to the nucleus inducing specific AMP genes transcription

effector mechanisms targeted at destroying pathogens and parasites. The immune response is elicited by the recognition process wherein PAMPs, DAMPs, MAMPs and VAMPs molecules resultant from pathogens and parasites. In response, different signaling pathways are triggered, validating generation of the effectors (AMPs), receptors (PRR) and PGRP involved in the humoral and cellular immune response by bees[11] (Figs 3 & 4).

Activation of AMP genes, Immune Signaling pathways, and mechanisms of action

Numerous immune signaling molecules (AMPs) are triggered in sting and stingless bees after the

immune challenge by different pathogens and parasites. The expression of AMPs are mainly regulated by the three intracellular signaling pathways by which bees showing defense against pests, parasites and pathogens, *i.e*., the Toll pathway, the Immune deficiency (Imd) pathway and the JAK-STAT pathway. Until now, there is an insufficiency and a research gap about the insights into the regulatory mechanisms of sting and stingless bees AMPs[11] (Fig. 4).

Toll pathway

In the bee immune system TLRs (Toll-like receptors) belong to a category of PRR proteins play a significant role in the innate immune system (Fig. 4). TLRs (single-pass membrane-spanning receptors) recognize all the molecular patterns (PAMPs, DAMPs, MAMPs and VAMPs) molecules of bee pathogens. In bees, the Toll pathway is predominantly for the recognition of fungi and Gram-positive bacteria by initiating cellular immune response[25]. Subsequently, humoral immune responses are triggered by the signaling pathways toward the generation of AMPs from the fat body. In response to bacterial or viral antigens, the NF-κB (nuclear factor kappa-light chain enhancer of activated B cells) is synthesized by the Toll pathway stimulus[25]. PGRPs actuate a chain of events of serine proteases along with ModSP and Grass proteins. The inactive "Spatzle protein" (extracellular cytokine like polypeptide) is cleaved and activated by ModSP and Grass proteins and in response the active Spatzle mediates to switch on the transmembrane dimer "Toll" receptor. Hence, Toll signaling pathway is initiated after Spätzle binds with the Toll receptor. The key modulator of the Toll pathway stimulus is facilitated by PGRP-SA, PGRP-SD and GNBP1 for Gram-positive bacteria while GNBP 3 for fungi. Afterward Toll-interleukin receptor domains interact with the two adaptor proteins namely MyD88 (Myeloid differentiation primary response 88) and Tube. Both these adaptor proteins mediate phosphorylation of Pelle (protein kinase) as well as phosphorylation and degradation of Cactus (IκB inhibitor). Recruitment of dMyD88, Tube, and Pelle cytoplasmic adaptor proteins by the Toll receptor is accountable for the commencement of Cactus signaling[25]. In general, Cactus protein is attached with the NF-κB (Nuclear Factor kappa B) transcription factors as DIF (Dorsal-related immunity factor) and Dorsal. Nevertheless succeeding the activation of Toll pathway and phosphorylation of Cactus, both the DIF and Dorsal are detached from the Cactus, translocated into the nucleus to synthesize AMPs[25]. As a whole activation of Toll pathway include detachment of Spaetzle, stimulation of Toll receptors, employment of cytoplasmic adapter proteins (dMyD88, Tube, and Pelle), degradation of CACTUS, activation of kinases and nuclear factors (DIF and Dorsal) and generation of AMPs (Fig. 4).

Imd pathway

Detection and recognition of pathogens in bees are mainly by way of peptide-glucan recognition protein (PGRP-LC) and this process is the primary stage for the onset of immune response through immune-deficiency (Imd) signaling pathway (Fig. 4). In bees, this path way is vital to defend against Gram-negative and some Gram-positive bacteria and modulates expression of most of the AMPs. This pathway is initiated by the assemblage between DAP-type peptidoglycan 2 (meso-diaminopimelic acid in Gram negative and some Gram positive bacteria) and PGRP-LC receptors. Initially binding of FADD (Fas-associated protein with death domain) with Imd protein facilitated the recruitment of DREDD (FADD-death-related ced-3/Nedd2-like protein) caspase like protein to split the Imd protein. After cleavage of Imd protein it is activated by K63-ubiquitination which assists Imd-K63-polyubiquitin complex to recruit TAK1 (transforming growth factor beta (TGF-β)-activated kinase 1) and TAB2 (TGF-β-activated kinase 1 (MAP3K7) Binding Protein 2) proteins. These proteins are involved with IKK complex containing IRD5 (Immune Response Deficient 5) and Key (Kenny) to enable phosphorylation of Relish (NF-kB-like nuclear factor). The triggered TAK1-TAB2 proteins and IKK-IRD5-Key complex cleave the Relish. The Rel DNA-binding domain is free for release from the IκB-like domain after cleavage and phosphorylation of Relish. Afterwards Rel DNA-binding domain is transported to the nucleus, activated and transcribed into specific AMPs. Imd signaling pathway is significantly conserved in bees (presence of orthologues – IRD5, Key, TAK1, TAB2, relish), showing high homogeneity with dipterans with different biological functions[11]. In addition, activation of components of the JNK signaling pathway (Basket, JNK and JNK-protein 1 interaction) in bees is modulated by the Imd pathway also (Fig. 4).

JAK/STAT pathway

In bees, the JAK/STAT {Janus-family tyrosinkinases [JAK]/transcription activator proteins

[STAT])} signaling pathway is mainly activated for the generation of AMP effectors against viruses, bacteria and infected cells (Fig. 4). The involvement of synthesis of AMPs by JAK/STAT pathway is analogous to the complement system, phagocytosis and antiviral feedbacks[11]. The JAK/STAT pathway is indispensable for the synthesis of cytokines in higher vertebrates and is comparatively faster. In this signaling pathway the dimerized transcription factors, STATS are initially phosphorylated for the production of AMPs by means of recruitment of the receptor ligand. JAK /STAT signaling pathway ligand is absent in bees[11]. JAK/STAT pathway in bees is comprised of five components homologous to fruit fly genes namely, a) dome (DOMELESS cytokine receptor), b) Hop (hopscotch, JAK tyrosine kinase), c) STAT92E signal transducer and transcription factor, d) SOCS (suppressors of cytokine signaling proteins – negative pathway regulators), and e) PIAS (protein inhibitor of activated STAT). In JAK-STAT pathway, the Janus kinase and STST molecule are acting as a signal transducer as well as transcription activator. In bees, JAK/STAT pathway is stimulated after binding of the Dome with unknown ligand. This Dome-ligand complex is activated the Hop and is proceeded for phosphorylation of Dome and Hop. Altogether, STAT92E is attached to the specific phosphor-tyrosine residues of Dome and later this complex is phosphorylated by Hop. After phosphorylation these complex residues are integrated with STAT molecules[11]. The integrated complex, STAT92E-STAT is then again phosphorylated by JAKs for the stimulation of immune response genes, split from the receptor to form dimers and transported to the nucleus where it is transcribed into several thioester-carrying proteins (TEPs) and tyrosine phosphatases (Ptp61F and WD40). These proteins are involved in phagocytosis and melanization processes[11] (Fig. 4).

Pharmacological properties and therapeutic potential of AMPs

Honeybees produce honey, royal jelly, propolis, bee venom, bee pollen, and beeswax, which may benefit humans due to the AMPs and other bioactives in them. Clinical standardization of these products is hindered by chemical variability depending on honeybee and botanical sources, but different molecules have been isolated and pharmacologically characterized. In apitherapy, AMPs are commonly used in the treatment of various diseases and alterations of the human being, which are present in various honey bee products, including honey, beeswax, royal jelly, propolis, bee bread, and venom (Table 1).

Honey

Defensin-1 is secreted by honeybees in the hypopharyngeal gland and is eventually transported to honey. This peptide has been identified in Revamil source honey and is responsible for its antibacterial activity against Gram-positive bacteria[26]. Apalbumin-1 isolated from Ziziphus honey inhibited ROS release (zymosan-activated human neutrophils and murine macrophages), NO production, phagocytosis (LPS-activated murine macrophages), and the production of TNF-α (human monocytic cells)[27] (Table 1).

Bee venom

Bee venom has an anti-inflammatory, antibacterial, antiviral, anti-cancer, anti-mutagenic, anti-nociceptive, and radioprotective activity[20] (Table 1). It has been used for the treatment of dermal diseases, blood circulatory system diseases, immune-related defects, tumors, back pain, rheumatism, and arthritis[29]. Bee venom contains mast cell-degranulating peptide (MCDP), melittin, adolapin, melectin, tertiapin, secarpin, minimine, procamine, and apamin[19,30] (Table 1).

Melittin

Melittin, one of the AMPs found in bee venom, has been used against prostate, liver, renal, and cervical cancers[31]. Apitoxin and its components are mostly described to have anti-inflammatory, anticancer, anti-arthritic, and neuroprotective effects[19]. Melittin is known for its anti-inflammatory properties in treating various diseases, such as dermatitis, neuritis, liver inflammation, atherosclerosis, and arthritis[32]. It has also been shown to have strong antimicrobial properties against methicillin-resistant *S. aureus*, anticancer activities, and to induce apoptosis in human ovarian cancer cells (SKOV3 and PA-1). The administration of melittin can induce the body to produce cortisol and acts as a potent anti-inflammatory representative of BV[29] (Table 1).

Apamin

Apamin has the ability to cross the blood-brain barrier, and its exposure to animals alleviates cognitive deficits, signifying that apamin targets (SK channels) would be suitable for the treatment of neurodegenerative disorders. In addition, the possibility of using apamin or less toxic analogs as

blood-brain barrier drug-delivery shuttles has been explored. It possesses anti-fungal, anti-viral, anti-inflammatory, analgesic and neurogenesis action[21,22] (Table 1).

MCD

MCD peptide consists of 22 amino acid residues having 2 disulfide bridges and possesses anti-inflammatory potential[33] (Table 1).

Adolapin

Adolapin possesses anti-arthritic, analgesic, anti-inflammatory, antipyretic, anti-nociceptive and anti-inflammatory properties[21] (Table 1).

Royal jelly

Royalisin, jellein, apisin, 10Hda, apalbumin, royalactin, and apamin are the most abundant antimicrobial peptides (AMPs) in royal jelly, which have antidiabetic, antifungal, antiviral, hypotensive, estrogen-like effects, antitumor, antiaging, antihypercholesterolemic, wound healing, and anti-inflammatory activities (Table 1). The stimulation of larval development into queen bee has been attributed to royalactin (57-kDa major royal jelly proteins) and antiaging[34]. Jellein-I and Jellein-II have broad-spectrum antimicrobial activity, jellein-III is less active, and jellein-IV has no antimicrobial effect (Table 1). Royalisin has antibacterial (gram-positive) activity against *Staphylococcus*, *Streptococcus*, *B. subtilis*, *Micrococcus luteus*, *Sarcina lutea*, *Clostridium*, *Corynebacterium*, *Lactobacillus helveticus*, *Paenibacillus larvae*, and *Leuconostoc*, whereas no inhibition was observed against the gram-negative *E. coli* and *Serratia marcescens*. Antifungal activity against *Botrytis cinerea* was reported for royalisin[35]. In addition, apalbumin 2a (variant of MRJP-2) was found to inhibit the growth of *P. larvae*, *B. subtilis*, and *E. coli* (Table 1).

10-HDA

10-HDA has numerous immunomodulatory activities, such as reduced T cell proliferation, inhibition of interleukin-12 production by spleen dendritic cells, blocking of LPS- and IFN-β-induced NO production in macrophages[36], Th1 stimulation, and Th2 downregulation on human monocyte-derived dendritic cells[37]. 10-HDA was found to protect rats from experimentally induced gastric ulcer[38] and to inhibit LPS-induced NF-κB activation in the murine macrophage cell line RAW264, resulting in an anti-inflammatory effect[16]. 10-HDA has rejuvenated neuron differentiation from rat embryo neural stem cells, probably behaving like the ω-3 docosahexaenoic acid, which is recognized to promote neurogenesis in the central nervous system[39] (Table 1).

Conclusion

AMPs have gained increased attention among researchers, health specialists, and the pharmaceutical industry due to their pharmacological and therapeutic potential. Honey bee AMPs are low molecular weight proteins with a broad range of antimicrobial and immunomodulatory activities against infectious organisms. In addition, honey bee AMPs also exhibit increased efficacy, high specificity, decreased drug interaction, low toxicity, drug delivery shuttles, biological diversity, and direct attacking qualities. Multidrug resistance is multifaceted[40-44], multi-dimensional, and is the second leading cause of death around the world. The urgent need to acquire new antimicrobials has been driving AMPs research. In this regard, AMPs are considered as promising antimicrobial agents for producing a new generation of antimicrobials. Consequently, several pharmaceutical industries are conducting appropriate clinical trials to develop potential therapeutic drugs from AMPs. Over sixty peptide drugs have already been marketed and many novel therapeutic peptides are in preclinical and clinical development. The exploitation of therapeutic AMPs from honey bee sources can also influence novel tactics for the management of human health complications.

Conflict of interest

All authors declare no conflict of interest.

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SHORT COMMUNICATION

Optimisation of DNA isolation and PCR techniques for beetle (Order: Coleoptera) specimens

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Abstract

DNA isolation is critical in many molecular biology studies, including genetics and genomics. Various DNA isolation techniques have been established to extract DNA from beetle species with DNA isolation kits available on the market. The polymerase chain reaction (PCR) success rate depends on the quality of DNA extracted from biological samples. In this study, four DNA isolation methods were compared, Chelex 100, N-cetyl-N, N, N,—trimethyl ammonium bromide (CTAB), Sodium Dodecyl Sulphate (SDS), and Tris, NaCl, EDTA, SDS (TNES), to determine the amount and quality of DNA obtained, the amount of time it took, and the effects of ethanol volume, incubation time, and temperature on DNA precipitation. The Chelex 100 method was the most successful compared to other methods and proved to be the superior DNA isolation method. We obtained the best amplification results for the beetle cytochrome oxidase 1 (CO1) gene at A1 Master Mix concentrations and A5 PCR cycle conditions. This study provides a road map for selecting the optimum DNA isolation methods for beetle species based on DNA quality, quantity, time consumption, cost, protein contamination, and success rate. The DNA isolated by the four DNA isolation techniques was suitable for further molecular studies like conservation genetics and genomics.

Keywords Beetles · Chelex 100 · DNA purity · DNA isolation · PCR

Introduction

DNA isolation is the first step for DNA barcoding, molecular identification, genomics, and the genetic studies of insects (Moritz and Cicero 2004; Allen et al. 2006; Schill 2007; Miller et al. 2009; Lagisz et al. 2010; Dittrich-Schroder et al. 2012; Athanasio et al. 2016). A variety of techniques have been developed and established to isolate DNA from insects to identify the conserved regions in mitochondrial, ribosomal, and nuclear DNA molecules using PCR (Kocher et al. 1989; Milligan 1998; Fitzpatrick et al. 2010; Albers et al. 2013). Commercial insect DNA isolation kits are fast but expensive, with low yields, and may require costly laboratory equipment not readily available everywhere (Gupta and Preet 2012). Laboratory experts and researchers need a relatively fast, inexpensive, and high throughput DNA extraction protocol (Minas et al. 2011). Therefore, an optimised and efficient protocol for DNA extraction from insect tissues is essential for molecular entomology studies (Dittrich-Schroder et al. 2012; Asghar et al. 2015; Kranzfelder et al. 2016). Hence, it is crucial to optimise DNA extraction methods to obtain high quality and quantities of DNA free of contamination, with minimal degradation and no inhibitors, that are quick and cost-effective (Queipo-Ortuno et al. 2008; Chen et al. 2010; Knebelsberger and Stoger 2012; Gupta and Preet 2012).

This study compared the four cost-effective DNA isolation methods: Chelex® 100, CTAB, SDS, and TNES. In the Chelex® 100 DNA isolation method, Chelex 100 resin is made up of styrene divinylbenzene copolymers, which act as a chelating agent with an affinity towards metal ions. This resin prevents DNA degradation at higher temperatures (Singer-Sam et al. 1989; Ip et al. 2015; Josue et al. 2019). In the CTAB DNA isolation method, the CTAB buffer separates DNA from proteins due to its cationic properties (Chen et al. 2010; Muhammad et al. 2013; Josue et al. 2019). Methods like SDS (sodium dodecyl sulphate), TNES, and CTAB DNA are relatively expensive compared to Chelex® 100 method.

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PCR is used to amplify small target genes of interest. Nowadays, numerous PCR related research involves insects (Snounou et al. 1993; Chanteau et al. 1994; Hebert et al. 2003; Crochu et al. 2004; Pentinsaari et al. 2014; Oba et al. 2015; Sire et al. 2019). The successful amplification of PCR depends on DNA quality and quantity, which is affected by the DNA isolation method used (De Armas et al. 2005; Sagar et al. 2014; Dai et al. 2017). Various PCR technique characteristics can influence its accuracy and efficacy, including the concentration of DNA template, magnesium ion concentration, the temperature of the PCR thermal cycling settings, and the presence and concentration of PCR additives (Grunenwald 2003; Roux 2009).

Beetles (Order: Coleoptera) are insects found in all environmental conditions (Banerjee 2014). In addition to aiding reproduction and decomposition, beetles actively devour plant and fungal tissues, which amplify their ecological influence. The overall number of beetle species is estimated to be in the millions (Stork et al. 2015; Adamski et al. 2019). Adult beetles are distinguishable from other insects by their stiffened forewings, which produce elytra. Aerial elytra protect the hind wings and body against mechanical injury and other factors that reduce habitat usage and lifespan in other insects. Beetles primarily feed on dead animals and decayed plant materials and are considered bio-engineers (McKenna et al. 2009, 2016). Beetles are also regarded as pests throughout the world and negatively affect plant nutrition and plant economy (Spochacz et al. 2018; Adamski et al. 2019).

DNA isolation is an essential technique for genetics, genomics, parasite detection, molecular toxicology, and molecular mechanisms of resistance to insecticides and transgenic *Bacillus thuringiensis* (Bt) corn (Miller et al. 2009; Lagisz et al. 2010; Dittrich-Schroder et al. 2012; Athanasio et al. 2016). We observed that both DNA quality and quantity from beetle species varied between DNA isolation techniques, which affected PCR success. Hence, several DNA isolation techniques were evaluated to optimise DNA extraction and PCR techniques.

In the current study, we evaluated the DNA concentration and efficiency of four DNA isolation techniques and compared the time needed to process each sample. Amplification of the beetle cytochrome oxidase subunit 1 (CO1) locus was used to assess the quality of DNA at different PCR cycle conditions and at different Master Mix concentrations. We used 2-propanol for DNA isolation, but ethanol can also be used. Therefore, a separate experiment was performed to determine the impact of ethanol volume, incubation temperature, and incubation time on DNA precipitation. The main objectives of the present study are to assess four DNA isolation methods and optimise the PCR technique for beetle species.

Materials and methods

Ethical Statement

Ethical permission was obtained from the Chief Wildlife Warden, Environment and Forest Department, Sikulpuikawn, Tuikhuahtlang Rd, Aizawl, Mizoram to collect beetle specimens in and around Mizoram (Permission number. A.33011/2/2012-CWLW/Vol.II/79).

Study Sites, collection of beetle species, identification, and storage

In 2020, adult beetles were collected from five study sites, Mission Veng, Kanan, Sakawrtuichhun, Tanhril, and College Veng in Aizawl district, Mizoram, India, using the light trap method (Hebert et al. 2000) (Fig. 1). Geographical coordinates of the study sites (latitude, longitude, and elevation) are shown in Supplementary Table 1. A total of 100 beetle specimens were collected. Twelve families were identified based on morphological identification keys (Bosquet 1990; Bouchard et al. 2017). After morphological identification, the specimens were stored in 70% ethanol (Merck, Darmstadt, Germany) at -20 °C for further analysis.

Evaluation of total genomic DNA isolation protocols from beetle leg tissues

Four different genomic DNA isolation methods were tested to determine the most successful genomic DNA isolation method: CTAB, Chelex®100, SDS, and TNES. In each method, tissue from six individual beetle legs was used. Each beetle leg was placed in a 1.5 ml Eppendorf tube for DNA isolation and quantification. Before DNA isolation, the leg tissue from each beetle was washed twice with 1X PBS (phosphate buffer saline: 8 g of 0.137 M NaCl, 200 mg of 0.0027 M KCl, 1.44 g of 0.01 M of Na_2HPO_4, and 240 mg of 0.0018 M KH_2PO_4, pH 7.4) and stored in 70% ethanol. The beetle specimens with washed and air dried for 30 min to remove the ethanol. All experiments were performed in the barcoding and meta-barcoding laboratory at the Department of Zoology, Mizoram University, Aizawl, Mizoram.

CTAB DNA isolation method

A slightly modified CTAB method was used to extract DNA from beetle leg tissues (Doyle and Doyle 1987, 1990; Cullings 1992; Milligan 1998; Tel-Zur et al. 1999). CTAB (cationic detergent) has the strength to precipitate DNA from homogenate samples. Three per cent (w/v) CTAB extraction buffer was prepared by dissolving 3 g of CTAB (HiMedia Laboratories,

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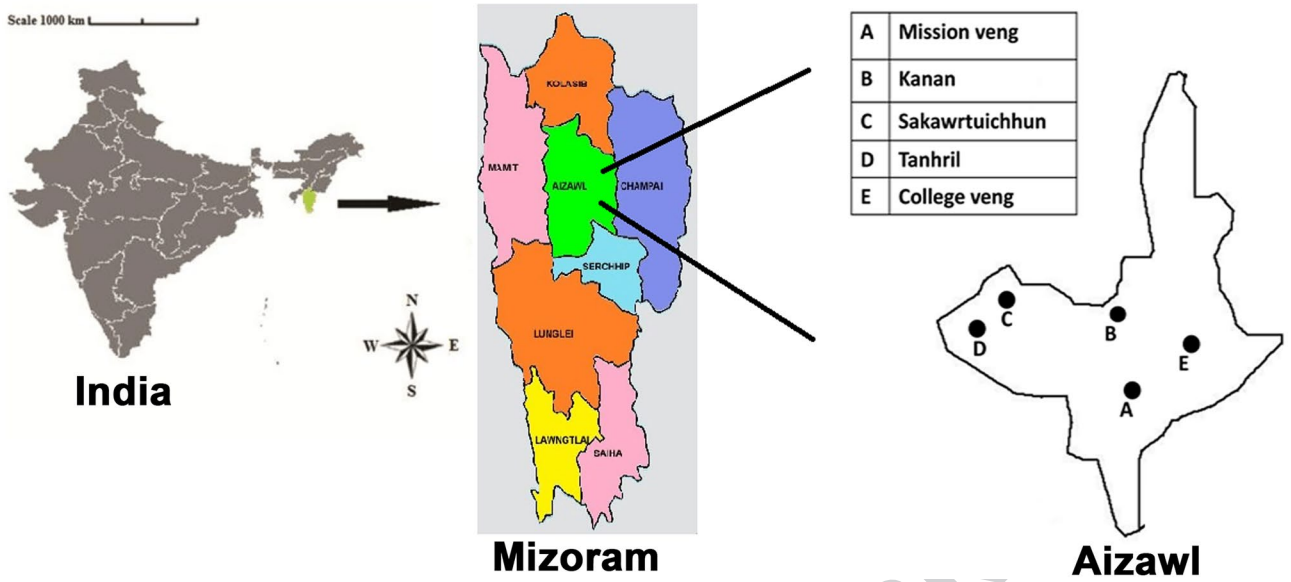

Fig. 1 Sampling sites of beetles in Aizawl, Mizoram, India. The five sampling sites in Aizawl district in Mizoram are marked as black rotundities

Mumbai, India) in 200 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, and 2% (v/v) β- mercaptoethanol ethanol (should be added to the extraction buffer just before use). In this method, the individual beetle legs (50 mg of tissue) were ground in 450 µl of the CTAB extraction buffer using mortar and pestle (single use). The homogenised samples were immediately transferred to labelled 1.5 ml Eppendorf tubes. Then, 20 µl of Proteinase K solution (20 mg/ml, Sisco Research Laboratories Pvt. Ltd, Mumbai, India) was added to the homogenised samples and vortexed for 30 s. After vortexing, the samples were incubated at 56 °C for 90 min in a Thermo Mixer® C (Eppendorf India Pvt. Ltd, Chennai, India). After incubation, the samples were allowed to cool at room temperature for 30 min.

Chelex®100 DNA isolation method

The Chelex®100 DNA isolation protocol was performed with slight modifications (Arrivillaga et al. 2002; Golczer and Arrivillaga 2008; Gang et al. 2009; Nagdev et al. 2010; Musapa et al. 2013). Fifty milligrams of dried beetle leg tissues were ground in 300 µl of buffer H (200 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% SDS, and 250 mM sucrose), 300 µl of TEX buffer (200 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 2% Triton X-100), and 300 µl of 10% Chelex®100 resin (Catalog no. 95577, Merck, Darmstadt, Germany) using mortar and pestle. After grinding, the homogenised samples were transferred to new 1.5 ml Eppendorf tubes with 20 µl of Proteinase K solution (20 mg/ml, Sisco Research Laboratories Pvt. Ltd, Mumbai, India). The samples were incubated at 56 °C for 60 min in a Thermo Mixer®C (Catalog no. 5382000015, Eppendorf India Pvt. Ltd, Tamilnadu, India) then allowed to cooled at room temperature for 30 min.

SDS DNA isolation method

A modified SDS DNA isolation method (Milligan 1998) was performed to extract DNA from individual beetle leg tissues (50 mg). According to this method, the beetle leg tissues were ground in 600 µl of 2% (w/v) SDS extraction buffer, prepared by dissolving 2 g of SDS (HiMedia Laboratories, Mumbai, India) in 200 mM Tris HCl (pH 8.0), 20 mM EDTA (pH–8.0), and 1.4 M NaCl using mortar and pestle. The homogenised samples were immediately transferred to labelled 1.5 ml Eppendorf tubes. Then 20 µl of Proteinase K solution (20 mg/ml, Sisco Research Laboratories Pvt. Ltd, Mumbai, India) was added to the homogenised samples and vortexed for 30 s. After vortexing, the samples were incubated at 56 °C for 2 h in a Thermo Mixer® C (Eppendorf India Pvt. Ltd, Chennai, India). After incubation, the samples were allowed to cool at room temperature for 30 min.

TNES DNA isolation method

The TNES DNA isolation method was performed according to Asahida et al. (1996) with modifications. Fifty milligrams of individual beetle leg tissues were crushed in 500 µl of TNES extraction buffer, which was prepared with 200 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 5 M Urea, 2% SDS, and 2% (v/v) β- mercaptoethanol ethanol (added to the extraction buffer just before use). The homogenised samples were immediately transferred to labelled 1.5 ml Eppendorf tubes. Then 20 µl of Proteinase K solution

(20 mg/ml, Sisco Research Laboratories Pvt. Ltd, Mumbai, India) was added. The samples were vortexed for 30 s. and incubated at 56 °C for 2 h in a Thermo Mixer® C (Eppendorf India Pvt. Ltd, Chennai, India). After incubation, the samples were allowed to cool at room temperature for 30 min.

The rest of the DNA isolation steps were the same for all four DNA isolation methods. After 30 min of cooling, 15 µl of RNase A solution (4 mg/ml, Promega Corporation, Madison, USA) was added. The sample was inverted for 30 s and incubated at 37 °C for 15 min. Then 300 µl of phenol/chloroform/isoamyl alcohol (25:24:1, Sisco Research Laboratories Pvt. Ltd, Mumbai, India) was added to the sample and mixed by inversion for 3 min. The supernatant was separated by centrifugation at 13,000 rpm for 10 min at 4 °C. The clear liquid (supernatant) was transferred to a new 1.5 ml Eppendorf tube, and 300 µl of 2-propanol (Sisco Research Laboratories Pvt. Ltd, Mumbai, India) and 60 µl of 3 M sodium acetate (pH-7.2) (HiMedia Laboratories, Mumbai, India) were added to precipitate the DNA at -20 °C for 30 min. The sample was centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was poured off. The pellet was washed twice with 350 µl of 70% ethanol (Merck, Darmstadt, Germany) at 13,000 rpm for 10 min. The supernatant was removed and airdried at room temperature for 1 h. Finally, the DNA pellet was dissolved in 20 µl of nuclease-free water (Thermo Fisher Scientific, MA, USA) and kept at -20 °C for further analyses.

Measurement of DNA concentration and purity and visualisation of isolated DNA

After genomic DNA isolation, the concentration (ng/µl) was measured as per manufacturer instructions using Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific) and Qubit dsDNA HS assay kit (Invitrogen, Thermo Fisher Scientific). Briefly, in the Qubit assay, 2 µl of genomic DNA was mixed with 198 µl of fluorescent reagent and allowed to incubate for 2 min at room temperature in the dark. After incubation, the DNA concentration/yield was measured in ng/µl at 260 nm. The absorbance values at 230 nm (for RNA), 260 nm (for DNA), and 280 nm (for proteins) were estimated using a NanoDrop spectrophotometer (NanoDrop, Thermo Fisher Scientific). The ratios of 260/280 and 260/230 were also measured to determine the DNA purity obtained. Usually, the 260/280 ratio of pure genomic DNA should range from 1.7–2.0 (Linton et al. 2001; Miller et al. 2009). To compare the efficacy of the DNA isolation methods, 50 ng of each sample was loaded on an 0.8% agarose gel (HiMedia Laboratories, Mumbai, India), stained with 3 µl of ethidium bromide (EtBr, HiMedia Laboratories, Mumbai, India). The electrophoresis was performed at 80 V for 45 min (Ayazi et al. 2013; Yari et al. 2013). After electrophoresis, the agarose gel was visualised and photographed using a ChemiDoc MP imaging system (Bio-Rad, Alfred Hercules, California, USA).

Effect of time, ethanol volume and incubation temperature on DNA precipitation and success rate

To compare the efficacy of the DNA isolation methods, success rate and time required by each method were also assessed (Chen et al. 2010). A separate experiment was performed to evaluate the effect of different ethanol volumes (50 µl, 150 µl, and 250 µl) and different incubation temperatures (room temperature (22 °C), 4 °C, and -20 °C) at different time intervals (30 min and 1 h) on total genomic DNA precipitation (Table 1). This experiment was performed according to Chen et al. (2010) with slight modifications. In this experiment, the Chelex®100 DNA isolation protocol was used to extract DNA from individual beetles. The supernatant from a single beetle was aliquoted into nine parts in 1.5 ml Eppendorf tubes. Each aliquot contained 25 µl of supernatant. We assumed that each aliquot had equal DNA concentrations (Table 1). After DNA isolation, the pellet in each tube was dissolved in 20 µl of nuclease-free water (Thermo Fisher Scientific, MA, USA) and stored at -20 °C for further analysis.

Amplification of barcode region by PCR

The PCR amplification of universal primers (LCO1490 and HCO2198) was performed using a thermal cycler (Mastercycler® nexus gradient, 230 V/50 – 60 Hz, Eppendorf India Private Limited, Ambattur, Chennai, India) with minor alterations (Folmer et al. 1994). Each PCR reaction contained 25 µl of Master Mix and 50 ng of DNA template. PCR cycle conditions (Table 2) and Master Mix concentrations (Table 3) were modified to determine the optimal conditions to achieve optimum results in terms of the band quality (sharpness and intensity), the presence of non-specific bands and primers excess were considered (Echeverria-Fonseca et al. 2015). The forward (LCO1490-5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse

Table 1 Effect of ethanol volume, incubation temperature, and time on total genomic DNA precipitation

Temperature	Ethanol volume		
	50 µl	150 µl	250 µl
At room temperature (22 °C)	30 min	30 min	30 min
At room temperature (22 °C)	1 h	1 h	1 h
4 °C	30 min	30 min	30 min
4 °C	1 h	1 h	1 h
- 20 °C	30 min	30 min	30 min
- 20 °C	1 h	1 h	1 h

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Table 2 Evaluation of primer amplification at different PCR cycle conditions and their qualitative results obtained for beetle DNA samples after 35 PCR cycles

Steps in PCR	A1	A2	A3	A4	A5
Initialization	95 °C for 5 min	95 °C for 2 min	95°C for 3 min	95°C for 3 min	95°C for 3 min
Denaturation	95 °C for 1 min	95 °C for 30 s	95°C for 10 s	95°C for 1 min	95°C for 30 s
Annealing	56.5 °C for 45 s	40 °C for 30 s	45°C for 4 min	56.5°C for 1 min	52°C for 40 s
Extension	72 °C for 1 min	72 °C for 1 min	72°C for 45 s	72°C for 1 min	72°C for 1 min
Elongation	72 °C for 7 min	72°C for 7 min	72°C for 3 min	72°C for 7 min	72°C for 5 min
Bands	-	-	-	-	+ + +
Excess primer	-	-	+ + +	+ + +	+
No amplification	+ + +	+ + +	-	_	_

'−' denotes absent of bands/absent of non-specific bands/absent of primer excess; '+' denotes poor intensity of bands/poor non-specific bands/poor primer excess; '+ +' denotes moderate intensity of bands/moderate non-specific bands/moderate primer excess; and '+ + +' denotes good intensity of bands/more non-specific bands/more primer excess. A1-A5 denotes PCR cycle conditions

(HCO2198-5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primer sequences were used. Thirty-five PCR cycles were used in all PCR cycle and Master Mix conditions. Finally, the PCR amplicons were observed on 1.8% of the agarose gel against 100 bp DNA ladder (Invitrogen, Thermo Fisher Scientific) and photographed using the ChemiDoc MP imaging system (Bio-Rad, Alfred Hercules, California, USA).

Statistical analyses

All statistical data were expressed as the mean ± standard error mean (SEM) of the number of experiments (n = 6). The general linear model (GLM) was used to check the effect of each DNA isolation method on the DNA yield (ng/µl) and absorbance at 230 nm, 260 nm, and 280 nm, as well as the absorbance ratio and success rate (%). One-way analysis of variance (ANOVA) was performed to determine statistical significance. Tukey's pairwise comparisons were used to compare DNA concentration (ng/µl), absorbance at 230 nm, 260 nm, and 280 nm, and the absorbance ratio and success rate (%) between the methods. Values were considered statistically significant when $p < 0.05$. GLM was also used to check the effect of ethanol volume and incubation temperature on DNA precipitation conditions. The statistical data were analysed using SPSS software v16.0 (Norusis 2008).

Results

Quantification of DNA concentration and comparison of cost and time needed for each method

In this study, we compared four DNA isolation methods. The modified Chelex®100 method was the best. (Table 4). The DNA yield was significantly ($F_{(3,23)} = 5.954$; $p < 0.05$) more than that found with the other DNA isolation methods (Table 4). The DNA yield with Chelex®100 was 241 ± 41.25 ng/µl. The yield was 165 ± 38.65 ng/µl with the CTAB method, 124 ± 25.36 ng/µl with the SDS method, and 58 ± 10.26 ng/µl with the TNES method (Table 4). The

Table 3 Final concentrations of the various PCR Master Mixes checked and the qualitative results obtained for beetle DNA samples after 35 PCR cycles

Master mix	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Nuclease free water	-	-	-	-	-	-	-	-	-	-	-	-
Buffer (X)	1	1	1	1	1	1	1	1	1	1	1	1
$MgCl_2$ (mM)	25	15	4	2	1.5	2	2.5	1.8	2.5	1.8	3	2.5
dNTPs (mM)	1.25	4	0.8	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3
LCO 1490 (pMol)	10	20	15	0.5	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6
HCO 2198 (pMol)	10	20	15	0.5	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Taq polymerase (U/µl)	2.5	5	0.4	0.03	0.04	0.04	0.04	0.04	0.05	0.05	0.04	0.04
Bands	+ + +	+ + +	-	-	+	+	+ +	+ +	+ +	+	-	-
Non-specific bands	-	+	-	-	-	-	-	-	+	-	-	-
Excess primer	+	+	-	-	-	-	-	-	+	+ +	+	+ + +

'−' denotes absent of bands/absent of non-specific bands/absent of primer excess; '+' denotes poor intensity of bands/poor non-specific bands/poor primer excess; '+ +' denotes moderate intensity of bands/moderate non-specific bands/moderate primer excess; and '+ + +' denotes good intensity of bands/more non-specific bands/more primer excess. A1-A12 denotes various Master Mix concentrations

Table 4 DNA concentration, absorbance, absorbance ratio, time duration, and recovery rate of genomic DNA isolated by using four different isolation methods, from beetle specimens

Parameters	CTAB	Chelex®100	SDS	TNES
DNA concentration (ng/µl)	165 ± 38.65a	241 ± 41.25a	124 ± 25.36b	58 ± 10.26b
Absorbance at 260 nm	4.63 ± 0.45a	6.01 ± 1.05a	4.41 ± 0.38a	4.24 ± 0.31a
Absorbance at 280 nm	2.83 ± 0.21a	2.95 ± 0.23a	2.78 ± 0.16a	2.59 ± 0.11a
Absorbance at 230 nm	5.93 ± 0.54a	6.85 ± 0.89a	5.85 ± 0.48a	5.41 ± 0.38a
Absorbance ratio (A260/A280)	1.64 ± 0.12a	2.04 ± 0.16a	1.59 ± 0.14a	1.64 ± 0.12a
Absorbance ratio (A260/A230)	0.78 ± 0.08a	0.88 ± 0.09a	0.75 ± 0.06a	0.78 ± 0.08a
Total time (hr) per sample	3.44 h	2.40 h	3.44 h	3.44 h
Estimated cost (USD) per sample	0.6–0.9	0.4–0.5	0.5–0.8	0.5–0.7
Specimens with successful DNA isolation	5	6	4	1
Successful DNA isolation (%)	83.33 ± 15.24a	100 ± 20.54a	66.66 ± 10.25a	16.66 ± 4.25b
Number of samples (N)	6	6	6	6

Data was expressed in Mean ± Standard error (SE). Different alphabet letters show significant variations among DNA isolation methods ($p < 0.05$)

absorbances (at 230 nm, 260 nm, and 280 nm) and absorbance ratio (at A260/A280 and A260/A230) values were highest in the Chelex®100 method (Table 4). The DNA pellet colour and the cost and total time needed for DNA extraction varied with each method (Table 4). The success rate (%) of the Chelex®100 method was 100%, whereas success rates dropped to 83.33%, 66.66%, and 16.66% with the CTAB, SDS, and TNES methods, respectively (Table 4).

Quality of DNA

Gel (0.8%) images for the four DNA extraction methods are shown in Fig. 2. The Chelex®100 method showed a sharp, clear band compared to the other methods (Fig. 2). In the Chelex®100 method, the gel image showed six bands for six samples (Fig. 2d). The TNES method showed one (Fig. 2a), the SDS method showed four (Fig. 2b), and the CTAB method (Fig. 2c) showed five.

Ethanol volume, incubation time, and temperature on DNA precipitation

Ethanol volume and incubation temperatures significantly affected DNA quality and quantity (Fig. 3). The mean DNA yield rate (ng/µl) increased significantly ($p < 0.05$;

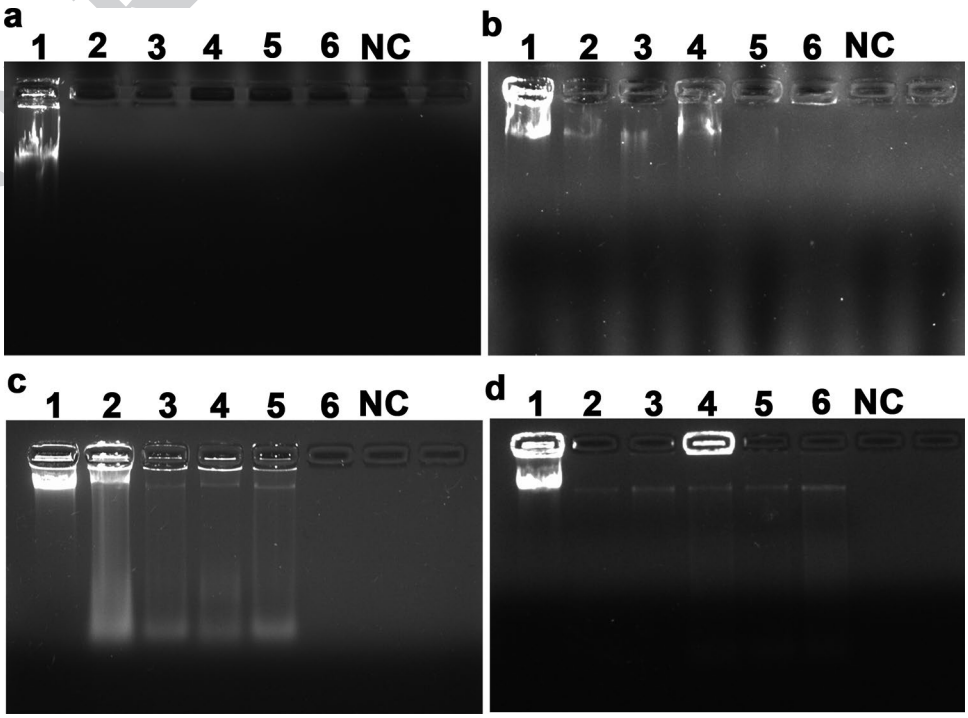

Fig. 2 Agarose gel (0.8%) electrophoresis analysis of extracted total genomic DNA from six adult beetle specimens by using four different DNA extraction methods at 80 V for 45 min. **a**, TNES method; **b**, SDS method; **c**, CTAB method; **d**, Chelex®100 method. 1–6, Beetle species; NC, Negative control

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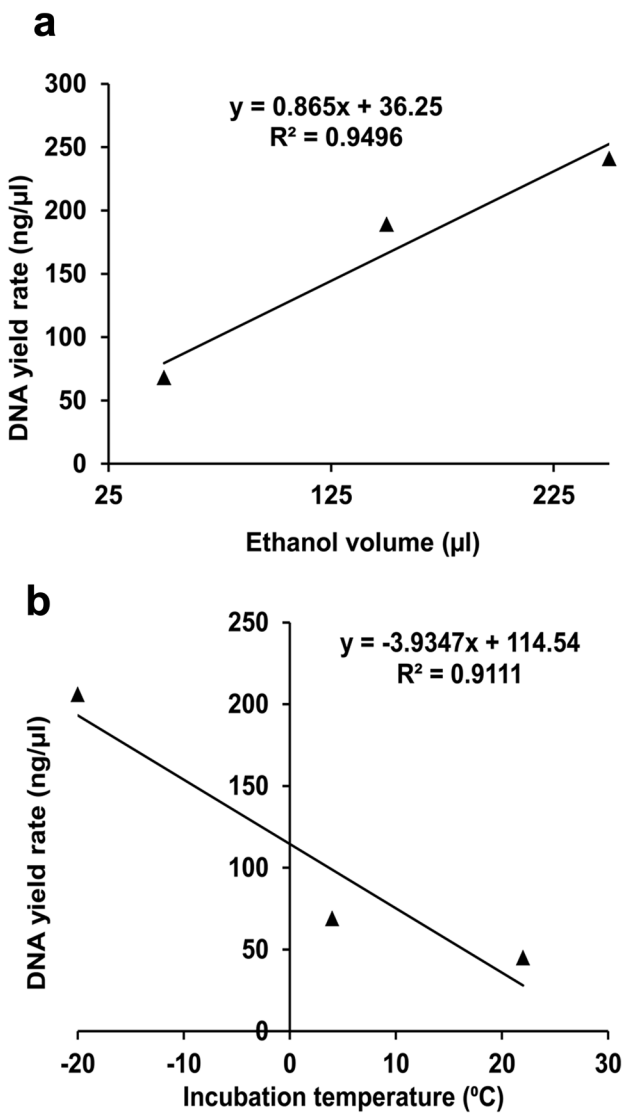

Fig. 3 Correlation analyses of ethanol volume (µl) and temperature (°C) with mean DNA concentration (ng/µl)

$R^2 = 0.9496$) with increases in ethanol volume (50 µl vs 150 µl vs 250 µl), showing a positive relationship between DNA yield and ethanol volume (Fig. 3a). The mean DNA yield (ng/µl) decreased significantly ($p < 0.05$; $R^2 = 0.9111$) with an increase in incubation temperature (-20 °C vs 4 °C vs 22 °C), showing a negative relationship between DNA yield and incubation temperature (Fig. 3b). However, the incubation time (30 min vs 1 h) had negligible effects on DNA and precipitation yields.

Optimisation of PCR cycle conditions

A total of five PCR cycle conditions were tested to amplify the CO1 gene. The A5 cycle showed good amplicon bands (Fig. 4c and Table 2) on a 1.8% agarose gel. PCR cycles A1-A4 showed no bands or only primer dimers (Table 2). Figure 4a and b show the gel visualisations of the A1 and A3 cycles. The size of the CO1 gene was 698 bp against the 100 bp DNA marker (Fig. 4c).

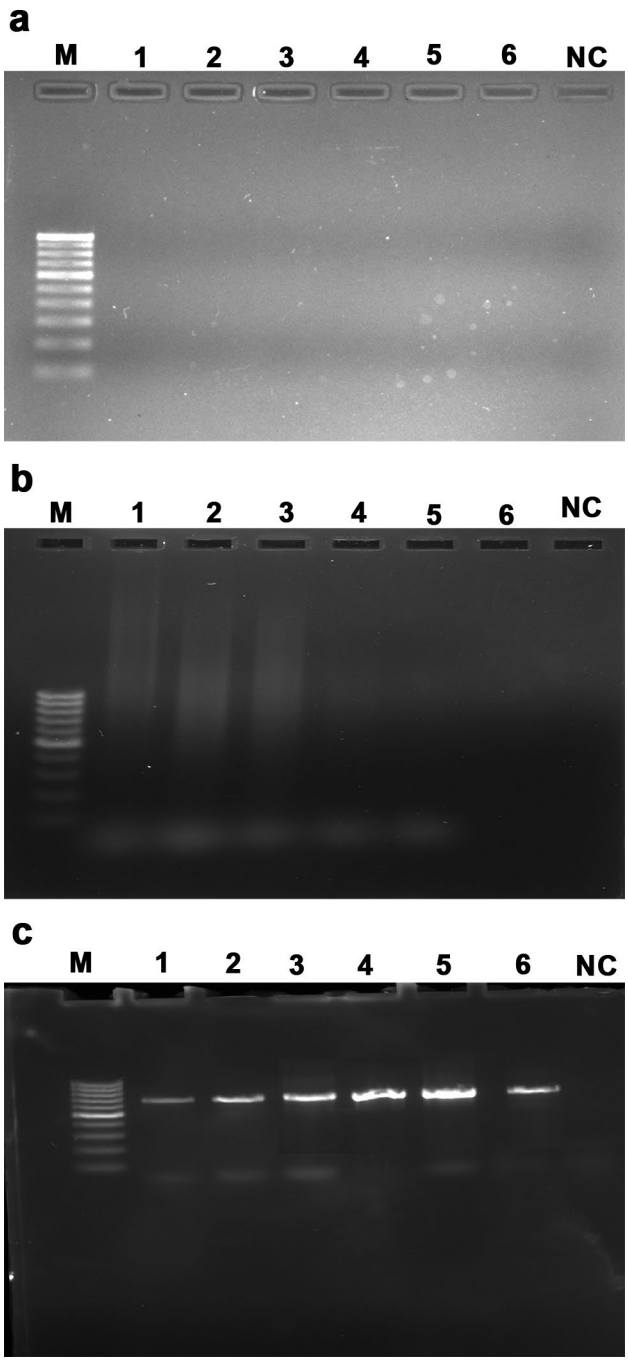

Fig. 4 Illustration of PCR amplicons in 1.8% of the agarose gel, from beetle specimens. **a**, A1 PCR cycle condition; **b**, A3 PCR cycle condition; **c**, A5 PCR cycle condition. Lane M, 100 bp DNA ladder; Lane 1–6, PCR amplicon products of six beetle specimens; Lane NC, Negative control

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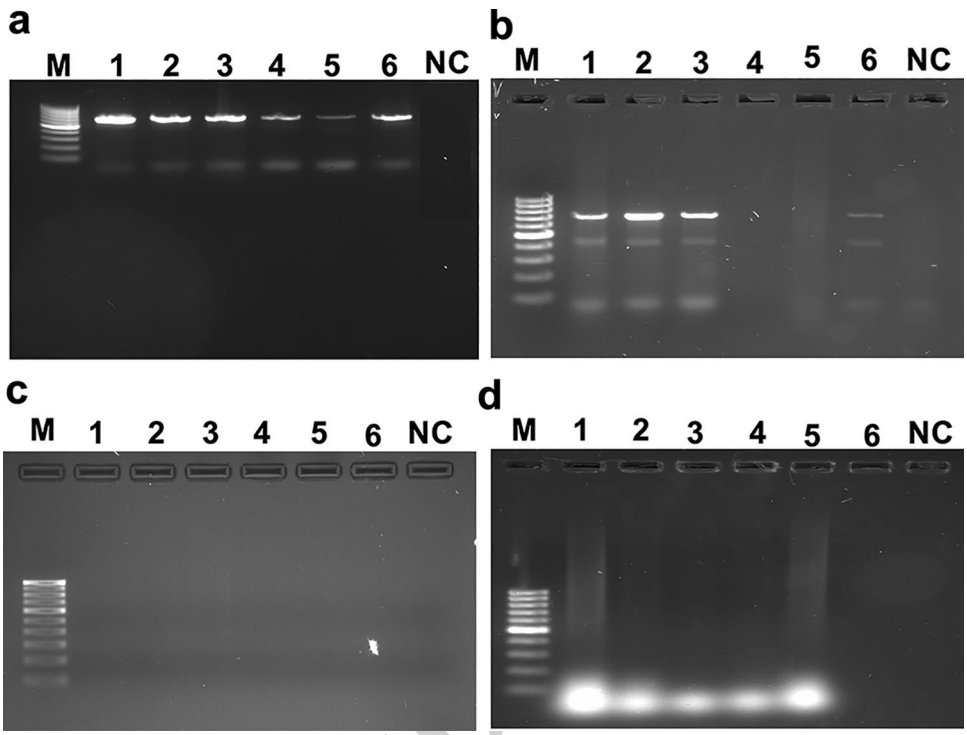

Fig. 5 Illustration of PCR amplicons in 1.8% of the agarose gel, from beetle specimens. **a**, A1 Master Mix concentration; **b**, A2 Master Mix concentration; **c**, A3 Master Mix concentration; **d**, A12 Master Mix concentration. Lane M, 100 bp DNA ladder; Lane 1–6, PCR amplicon products of six beetle specimens; Lane NC, Negative control

Optimisation of PCR Master Mix concentrations

Among 12 master mix concentrations (A1-A12), the A1 Master Mix concentration showed the best results (Table 3 and Fig. 5a). The A2-A12 mixtures showed no bands, primer dimers, non-specific bands, or bands with excess primers, or bands with non-specific bands and excess primers (Table 3). The A2 Master Mix contained non-specific bands and excess primers (Fig. 5b). While the A3 (Fig. 5c) and A12 (Fig. 5d) Master Mix concentrations showed no bands and excess primers, respectively.

Discussion

This study compared four DNA isolation methods to determine the best methods to optimise DNA extraction and PCR for beetle specimens. DNA extraction and quality and the time, effort, and cost needed are important considerations in molecular biology (Allen et al. 2006; Chen et al. 2010; Lagisz et al. 2010; Dittrich-Schroder et al. 2012). In the present study, we confirmed the best DNA isolation method for 70% of alcohol preserved beetle specimens. Acceptable DNA quality and yields were found in all four DNA isolation methods. We used laboratory prepared buffers for the studied DNA isolation methods (Chelex®100, CTAB, SDS, and TNES). Among the four methods, the Chelex®100 method was the most efficient method to obtain good quality and quantity of DNA from insects (Gadau 2009; Lienhard and Schaffer 2019), especially the beetle species. The application of several steps and buffers quickly helped obtain suitable DNA extracts with better PCR amplification. As a result, the reagents cost less than other approaches due to the proper sequence of the various protocol steps.

All four methods were simple to perform. CTAB is a cationic detergent. This method involves lysis, CTAB/chloroform phase segregation, 2-propanol precipitation, two ethanol washing steps, and a dry resuspending step. CTAB is a challenging, toxic, and time-consuming method (Lienhard and Schaffer 2019). Chelex is a chelating resin that prevents the degradation of DNA. Chelex resins are composed of styrene–divinylbenzene copolymers containing paired iminodiacetate ions, which act as chelating groups in binding polyvalent metal ions (Ip et al. 2015; Josue et al. 2019). The SDS DNA extraction method usually uses adult specimens to isolate DNA. However, this is not useful for extracting pure DNA due to contamination of plant digestive debris and phenols in the insect gut (Juen and Traugott 2006; Calderon-Cortes et al. 2010).

The DNA concentration in animal tissues varies from 1000–5000 ng/mg, depending on the type of species, tissue, preservation method, extraction procedure, and precipitation methods used for DNA isolation (Chen et al. 2010). In adult mosquitos, the DNA concentration was between 292.4–459.7 ng/µl (Josue et al. 2019). For mealybugs, the DNA concentrations ranged between 1422 and 12,718 ng/mg (Wang et al. 2019). In this study, the DNA

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concentrations were greater than other methods. Our results correlated with previous studies (Chen et al. 2010; Josue et al. 2019; Lienhard and Schaffer 2019). Possible reasons include manual cell homogenisation and the presence of contaminants like lipids, polysaccharides, and proteins (Josue et al. 2019). Another reason is that chelex resin prevents DNA degradation by binding (chelating) the metal ions (Mg^{2+}) that catalyse the breakdown of DNA (Lienhard and Schaffer 2019). The Chelex®100 DNA extraction method is recommended over other DNA extraction methods when studying arthropods (Casquet et al. 2011; Sahu et al. 2012; Wang and Wang 2012; Josue et al. 2019; Wang et al. 2019; Lienhard and Schaffer 2019). These results were similar to our study results.

As per Beer-Lambert law, the optimum absorbance values of extracted DNA ranged from 0.1 -1 at 260 nm. In this study, we obtained high values because of the presence of DNA and RNA. The 260/280 and 260/230 ratios were higher in the Chelex® 100 method than the other methods in this study. Our results coincide with previous reports (Linton et al. 2001; Chen et al. 2010; Wang et al. 2019). DNA quality and quantity also depend on the incubation time and temperature of lysates. In previous studies, it was reported that high DNA concentrations were obtained at 37 °C. In contrast, very low DNA concentrations were obtained at 19, 65, and 80 °C (Shahjahan et al. 1995). In our study, we used beetle specimens lysate. We incubated at 55 °C for 45 min and obtained good quality and quantity of DNA. Our results were similar to previous studies because the incubation temperatures could vary from species to species and tissue to tissue (Milligan 1998; Gilbert et al. 2007; Chen et al. 2008). Fluctuations in optical density measures indicated intervention/association of the distinct components of the sample (Chen et al. 2010).

To estimate the cost for DNA extraction for a certain number of samples via different methods, one can use the cost for each method in Table 4 multiplied by the number of samples to be extracted. However, the time estimated in Table 4 must be adjusted by the number of incubation and centrifugation steps used to finish all the samples. Studies to create rapid, inexpensive, safe, and optimised protocols for high-quality DNA from insects have increased in recent years (Harvey 2005; Watts et al. 2007; Lagisz et al. 2010). Because of the high concentration of secondary compounds such as polyphenols and polysaccharides in insect tissues, the method for DNA isolation must be optimised for different insect species and even for each insect tissue.

Compared to the other DNA extraction techniques, the Chelex®100 technique used fewer chemicals and reagents. It was also cheaper, faster (Musapa et al. 2013; Josue et al. 2019), and safer while producing more desirable results. According to the comparison of four DNA extraction methods in this study, the Chelex®100 DNA extraction method is recommended because it is the cheapest and fastest, with readily available chemicals and reagents, which produces the greatest quantity of DNA.

To optimise PCR findings, the modification of PCR cycle conditions and the final concentrations of the chemical components in the Master Mix resulted in amplicons with excellent quality according to the criteria tested (quality of bands, non-specific bands, and primer excess). Further research is needed to find potential inhibitors in the sample. In this study, the best results were obtained with the A1 master mix (Table 3 and Fig. 5a) and A5 PCR cyclic conditions (Fig. 4c and Table 2). Our results were similar to previous studies (Echeverría-Fonseca et al. 2015). In this study, good quality PCR amplicons were obtained at higher concentrations of $MgCl_2$ and DNA polymerase and at optimum PCR cycle conditions (Echeverría-Fonseca et al. 2015). If standard PCR settings fail to produce the desired amplicon, PCR optimisation is required to improve results. The specificity of a reaction can be modified by changing variables (e.g., reagent concentrations, cycle settings) that affect the amplicon profile's output (Lorenz 2012). If the reaction is not strict enough, several spurious amplicons of varying lengths will be created. However, no product will be produced if the reaction is too strict. PCR reaction troubleshooting can be a frustrating task. However, careful analysis and knowledge of the chemicals used in a PCR experiment might cut down on the time and trials required to achieve the desired results. Of all the factors that affect PCR stringency, titration of Mg^{2+} and/or adjusting annealing temperatures are likely to fix most issues. However, before making any changes, double-check that an incorrect result was not caused by a human mistake. AQ2

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Author contributions Meesala Krishna Murthy and Guruswami Gurusubramanian contributed to the study conception and design. Rest of the authors including Meesala Krishna Murthy were performed material preparation, data collection and data analysis. The first draft of the manuscript was written by Meesala Krishna Murthy and all authors read and approved the final manuscript.

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Declarations

Conflict of interest Authors declared that there is no conflict.

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ABSTRACT

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Honey

Honey has been essential to human nutrition and culture since ancient times, functioning as one of the earliest naturally occurring sweeteners for Homo sapiens. Since the Stone Age, humans and bees have resided together, with early humans exhibiting considerable interest in this precious resource (Crane, 1983). The search for and extraction of honey involved significant risk. Archaeological evidence, such as ancient rock art, demonstrates that early humans dangerously harvested honey from bees located high in cliffs or trees, reflecting the considerable value they placed on this delightful substance.

The medicinal use of honey is deeply embedded in human history. The first recorded reference to honey as a therapeutic agent appears in Sumerian literature dating from 2100 to 2000 BCE (Crane, 1975), which include honey in prescriptions for the treatment of wounds and various ailments. In this early period, honey became a vital element of ancient therapeutic practices across diverse societies. The ancient Egyptians, Greeks, and Romans used honey for its nutritional advantages and valued it for its medicinal properties. Honey functioned as a topical treatment for wounds, a solution for gastrointestinal ailments, and an ingredient in various religious and ceremonial practices (Jones, 2009; Crane, 1999; Allsop & Miller, 1996).

Throughout much of human history, honey has been the most readily available sweetener, obtained organically from bees with minimal processing. This status persisted until the advent of modern sugar production, which began to progressively supplant honey as the primary sweetener around 1800 CE (Crane, 1975; Bogdanov *et al*., 2008). Historically, honey was an essential component in the diets and medicinal practices of ancient civilisations, valued for its sweetness and purported health benefits. Its significance was further underscored by its symbolic role in various cultural and religious rites, where it was often associated with purity, healing, and prosperity. The persistent reverence for honey across history highlights its varied importance in human civilisation. It served as both sustenance and medicine, illustrating the deep connection between humanity and the natural environment. The story of honey illustrates humanity's persistent effort to harness the natural

environment for nourishment, health, and well-being, preceding the developments in modern agriculture and food processing that transformed the production and use of sweets. Honey is a naturally occurring biological product obtained from nectar and pollen, employed in diverse cultures throughout. The composition consists of roughly 20% water, alongside significant amounts of proteins, enzymes (including catalase, invertase, glucose oxidase, and phosphatases), amino acids, organic acids (notably acetic acid and glutamic acid), lipids, vitamins (such as pyridoxine, niacin, and ascorbic acid), volatile compounds, phenolic acids, flavonoids, carotenoid-like substances, and minerals. The composition of honey is mainly determined by its floral, geographic, and entomological origins; nonetheless, processing and numerous external factors, including seasonal and environmental conditions, significantly contribute as well (Crane and Walker, 1984; Blasa *et al*., 2006; Saxena *et al*., 2010; Khalil *et al*., 2012).

Multiple variables can induce swarming in *A. cerana*. These encompass reproductive requirements, nest disruptions, insect invasions, illness challenges, and deficiencies in pollen or nectar (Koetz, 2013; Egelie *et al*., 2015). Colonies may swarm due to congestion in the nest or as a natural reaction to seasonal reproductive cycles. Moreover, environmental stressors, such a reduction in available food or the presence of parasites such as the *Varroa destructor* mite, might provoke swarming behaviour as a survival strategy. Notwithstanding the risks linked to swarming, including the possible collapse of the new colony, swarming continues to be a crucial evolutionary strategy that has enabled *A. cerana* to sustain its populations for millennia.

A. cerana serves as a crucial pollinator, impacting both natural ecosystems and the agricultural economy of the region. Their pollination services are essential for the reproduction of various flowering crops and plants, like as fruit trees, vegetables, and medicinal plants. Numerous crops hold significant economic value for local communities, especially small-scale farmers who depend on bee pollination to enhance their harvests. The ecological advantages conferred by *A. cerana* also contribute to the preservation of wild plant species' health and diversity, which

subsequently sustains the survival of numerous animal species reliant on these plants for sustenance and habitat.

Studies have shown that *A. cerana* exhibits significant genetic plasticity, especially for its functional genes, allowing it to adjust to environmental changes (Evans *et al*., 2013). This genetic adaptability enables the species to address diverse environmental problems, including climate change, habitat fragmentation, and the emergence of invasive species. The genomic malleability of *A. cerana* is considered a contributing element to its efficacy as a pollinator in varied and dynamic ecosystems. Research indicates that *A. cerana* populations display considerable variability in characteristics such as foraging behaviour, thermoregulation, and disease resistance, all of which are crucial for survival under changing environmental conditions.

A. cerana significantly contributes to ecological and economic systems by enhancing agricultural yield via pollination. In numerous regions of Asia, smallholder farmers rely on the pollination services of *A. cerana* to improve the yields of crops including apples, pears, tea, and coffee. The economic advantages gained from *A. cerana*'s pollination services are significant, especially in areas where agriculture is the foundation of the local economy. *A. cerana* enhances crop yields and maintains plant population health, hence bolstering food security and rural livelihoods. Moreover, the bee's disease resistance and capacity to flourish in many environments render it a dependable and sustainable choice for farmers, diminishing the necessity for artificial pollination methods or the introduction of non-indigenous pollinator species.

In Northeast India, where *A. cerana* is prevalent, researchers have identified considerable genetic variation among various populations, indicative of the region's biological richness. This genetic variety is crucial for comprehending the mechanics of speciation and the influence of environmental conditions on the evolutionary paths of honey bee species. The utilisation of *A. cerana* as a model organism in these studies has ramifications for conservation biology, since it aids in identifying essential genetic features that enhance the species' resilience and adaptation. These

findings can be utilised for the conservation and control of honey bee populations against global problems including habitat loss and climate change.

Apis cerana is distinguished as a species of significant ecological, economic, and scientific value. Its capacity to acclimatise to diverse environmental circumstances, along with its essential function in pollination, renders it a keystone species in the ecosystems and agricultural systems of Asia. The continuous examination of *A. cerana*'s genetic variety and adaptation enriches our comprehension of honey bee biology and offers critical insights for the conservation and sustainable management of pollinator species amid global environmental changes. As pressures on pollinator populations escalate, the resilience of species such as *A. cerana* provides optimism for preserving the biodiversity and ecological services crucial for both natural ecosystems and human welfare.

Pollinators are essential for sustaining ecosystem health and facilitating global food production. They facilitate the transmission of pollen between flowering plants, enabling fertilisation and the generation of fruits and seeds. The complex interdependence between pollinators and plants is essential for agricultural products and the survival of several wild plant species, many of which depend on particular pollinators for reproduction. Bees, butterflies, birds, and bats are prominent pollinators, each playing distinct roles in the pollination process.

The reduction in pollinator populations presents a substantial risk to biodiversity and environmental stability. The decline of pollinators can result in the failure of plants reliant on them for reproduction to produce seeds or fruits, triggering a series of repercussions within the ecosystem. Various species, ranging from herbivores to predators, depend on these plants either directly for sustenance or indirectly via the food web. The decline of pollinators can result in diminished plant diversity, therefore impacting entire ecosystems and potentially upsetting ecological processes and the equilibrium of life forms reliant on these plants for survival.

Besides habitat destruction, pesticide use, and climate change, pollinators are susceptible to illnesses and parasites. Honey bees are impacted by the parasite mite

Varroa destructor, which can diminish colony strength and increase vulnerability to viruses. Additional diseases, including Nosema, and parasites such as small hive beetles, pose extra risks to pollinator health. The dissemination of these viruses and parasites is enabled by international trade and the transboundary movement of pollinator species, resulting in the introduction of novel dangers into habitats where pollinators may be devoid of natural defences.

A diversified approach is necessary to tackle these difficulties and reverse the decline of pollinators. Safeguarding and rehabilitating natural habitats is crucial for supplying pollinators with the necessary materials for their survival and prosperity. This can be accomplished by creating pollinator-friendly habitats in agricultural environments, including hedgerows, wildflower strips, and cover crops. Minimising pesticide application and implementing integrated pest control strategies can reduce pollinators' exposure to detrimental chemicals. Moreover, initiatives to alleviate climate change and preserve pollinator species via breeding programs and disease management are essential for guaranteeing the enduring survival of these critical organisms.

Moreover, public awareness and policy measures are essential for advancing pollinator protection. Governments, scientists, and conservation organisations must collaborate to establish regulations that save pollinator habitats, restrict pesticide application, and promote sustainable farming practices. The use of pollinator-friendly agricultural practices, including organic farming and agroforestry, can foster environments that enhance both biodiversity and agricultural yield.

Populations of *A. cerana* are geographically separated by natural barriers including mountains, forests, rivers, and seas. This geographic isolation facilitates the evolution of distinct local ecotypes and subspecies. Honey bee ecotypes and subspecies can be differentiated by morphometric and genomic techniques. Morphometric and molecular genetic techniques have been employed to delineate subspecies of the European honey bee *A. cerana* (Evans *et al*., 2013). Research on *A. cerana* also use these strategies. Polymorphisms in two specific regions are frequently utilised to examine genetic variations among the subspecies of *A. cerana*:

the microsatellite loci in nuclear DNA and the mitochondrial marker located between the transfer RNA Leu and cytochrome oxidase subunit 2 genes (tRNAleu COII) (Tan *et al*., 2007). Lee *et al*., (2016) assessed the genetic variation of the *A. cerana* population in Korea by tRNAleu COII locus polymorphism analysis. Xu *et al*., (2013) found that the *A. cerana* population on Damen Island significantly differentiates from mainland populations based on the analysis of five microsatellite loci and mitochondrial tRNAleu COII sequences. A study by Yu *et al*. (2019) indicates that the elongated and constricted alpine valleys of the Qinghai Tibetan Plateau display genetic diversity of *A. cerana*. This was achieved by analysing mitochondrial tRNAleu COII fragments and 31 microsatellite sites. Additionally, *A. cerana* has undergone genome-wide analysis in recent investigations.

Invasive and native plants

Environmental change is mostly driven by species invasions and climate change, affecting ecosystems globally. Rising temperatures, altered precipitation patterns, and extreme weather disrupt natural equilibrium, complicating species' adaptation efforts. Human activity facilitates the displacement of native species by invading species, disrupts trophic balances, and modifies the terrain of a region. The dynamic interplay between these forces may yield synergistic or antagonistic effects, either amplifying or diminishing their impacts. Understanding these interactions is crucial for predicting and mitigating environmental impacts (Hoover *et al*., 2012; Fox *et al*., 2014). Climate change is affecting the reproductive cycles of native plants, potentially disrupting the fragile ecological equilibrium between these plants and their pollinators. The complex interplay between indigenous flora and their pollinators may be disturbed by the various adaptations of invasive species to climate change. This may impact the ecosystem overall and necessitate a deeper comprehension of how temperature fluctuations influence plant reproductive dynamics (Giejsztowt *et al*., 2020).

An introduced species that inflicts harm on its new environment is classified as an invasive species. Invasive species cause ecological, environmental, and economical damage to habitats and bioregions (Davis and Thompson, 2000). The

invasion of alien plant species poses a significant threat to biodiversity, potentially altering ecosystems and economic services, hence jeopardising livelihoods and instigating essential social changes. The impact of invasive alien plant species (IAPS) on natural ecosystems can lead to competition with native species, limiting resource availability, disrupting ecosystem equilibrium, and adversely affecting agricultural productivity and human health (Vilà and Hulme, 2017).

The threat level of invasion to biodiversity may differ based on the distinct characteristics of each location; nonetheless, the correlation between invasion and other environmental disturbances, such as habitat loss and climate change, is undeniable (Young and Larson 2011). According to the United Nations' Global Assessment Report on Biodiversity and Ecosystem Services, the principal danger to global biodiversity loss is biotic invasion (IPBES 2019). The unintentional or deliberate introduction of Invasive Alien Plant Species (IAPS) as ornamental or commercial flora via human intervention can adversely impact the environment, economy, livelihoods, human health, and the diminishment of ecosystem services, thereby obstructing sustainable development (Rai and Singh 2020a, b). The IAPS adversely impacts the United Nations' Sustainable Development Goals (SDGs) pertaining to human well-being and environmental enhancement by establishing policies or guidelines across various sectors, including sustainable agriculture, groundwater remediation, and public health (United Nations 2015; Haines 2016).

In recent decades, invasive alien plant species (IAPS) have significantly jeopardised the region's ecosystem services, biodiversity, environmental quality (Aerts *et al*., 2018; Stone *et al*., 2018; Jones, 2019), and public health (Pyšek and Richardson, 2010; Jones and McDermott, 2018). The Intergovernmental Platform for Biodiversity and Ecosystem Services (IPBES) of the United Nations (UN) estimated that biological invaders threaten around 25% of the Earth's surface, including global biodiversity hotspots (IPBES, 2019). In this context, high-income nations recorded 30 times higher IAPS than low-income nations (Seebens *et al*., 2018). As a result, IAPS hotspots are generally confined to high-income nations within the European

Union, Australasia, and North America, rather than the Asia Pacific or African regions (Seebens *et al*., 2018; IPBES, 2019).

Future alterations in biodiversity are primarily influenced by land-use changes, temperature variations, nitrogen deposition, and biotic interactions during the Anthropocene epoch (Sala *et al*., 2000; Singh 2012). Biotic exchange, defined as the introduction of non-native plant species, is considered a significant threat to human health, agricultural sustainability, biodiversity, and the economy (Rai and Singh 2020a, b). Invasive alien plants (IAPS) have proliferated as a result of human-induced disturbances, a trend not confined to the pristine or vulnerable ecosystems of Antarctica or the Galapagos Islands (Hughes and Convey 2010; Jager *et al*., 2009). It is estimated that 0.5–0.7% of trees and shrubs globally are presently invasive (Rejmanek and Richardson 2013; Lamsal *et al*., 2018). Several deleterious invasive alien plants has the capacity to disrupt the abiotic environment and ecosystem services, potentially triggering a cascade of adverse impacts and hastening the extinction of indigenous flora.

Protected forest areas in Mizoram and other Indian Himalayan regions are highly vulnerable to significant alien plant invasions due to considerable human-induced disturbances. Recently, 163 alien plant species have been reported by the state. This encompasses neo-invasives such as Ocimum americanum, Hyptis suaveolens, and Bidens pilosa, alongside harmful invasive alien plant species (IAPS) including Ageratina adenophora, Ageratina riparia, Chrolaena odorata, Mikania micrantha, and Ageratum conyzoides, all of which exert a persistent ecological influence on native flora and ecosystems (Sengupta and Dash, 2020). Mizoram, a secluded state in northeastern India, lacks an effective system for managing plant invasions. Consequently, the imperative for an effective and sustainable management strategy to prevent the incursion of invasive plant species is critical. The primary attribute of alien plants is their ability to proliferate in uncharted or pristine regions (Raghubanshi *et al*., 2005; Rai and Singh, 2020b). The majority of invasive plant species originate from South America. Out of 374,000 known plant species, 6,075 have been designated as invasive globally (Christenhusz and Byng, 2016). One

hundred seventy-three plant species, constituting 1% of the Indian flora, are classified as foreign invaders (Reddy, 2008; Rai and Singh 2021). Research on invasive plant species exists, although it is limited and predominantly focused on aggressive plant invaders, such as Lantana camara and Parthenium hysterophorus (Hiremath and Sundaram 2013).

Indigenous flora play a significant role in carbon conservation and can function as sinks for atmospheric pollutants (Pejchar and Mooney, 2009; Shackleton *et al*., 2019). Consequently, alterations in environmental quality may indirectly affect human health due to the decline of native plant diversity resulting from invasive plant infestations (Jones and McDermott, 2018). Research indicates that certain IAPS can serve as ecological indicators of environmental pollution (Rai, 2016). The invasive emerald ash borer (EAB), a plant pathogen, proliferated across the United States and significantly devastated predominant ash trees, which would have otherwise functioned as effective sinks for air pollutants (Jones and McDermott, 2018). Human populations had cardiovascular and pulmonary complications due to exposure to high levels of hazardous air pollution. Severe pollution stress results in economic loss and mortality (Jones and McDermott, 2018).

The success of the IAPS is affected by numerous biological and environmental factors. The connection among plant invasions, anthropogenic disturbances, climate change, biodiversity, and human health may be complex and intricate (Rai and Kim, 2019). Consequently, invasion ecology has increasingly been recognised as a transdisciplinary domain intricately linked to land-use alteration, global change biology, health sciences, restoration, and conservation biology (Pysˇek and Richardson, 2010; Heshmati *et al*., 2019). Invasive weeds proliferate and produce biomass at a faster pace than native plants. They exhibit significant competitiveness, high reproductive efficiency through prolific seed production, effective dispersal, vegetative reproduction, rapid establishment, and other traits that facilitate adaptation to novel habitats (Sharma *et al*., 2005; Simberloff *et al*., 2005). Invasive exotic plants are associated with the decrease of threatened and endangered

species because to their significant numbers and biomass, which transform vegetative structures, alter ecological processes, and displace native species (Denslow, 2007).

The rapid proliferation of vector-borne diseases intensifies the impact of the invasion on human health (Clow *et al*., 2017; Schindler *et al*., 2018). IAPS tend to impede forest diversity and function as invasive species, hence diminishing global agricultural productivity (Haines, 2016). The Sustainable Development Goals (SDGs), which encompass issues such as sustainable agriculture, water sanitation, food safety and security, poverty, and human health and well-being, have been negatively impacted by the concurrent effects of current environmental disturbances associated with invasion biology (Haines, 2016; Pysˇek and Richardson, 2010). The concept of "planetary health" arises from the global nature of environmental issues and their detrimental effects on public health, including hazardous compounds, allergies, and vectors of emerging diseases (Haines, 2016; Ebi *et al*., 2017).

The global proliferation of Invasive Alien Plant Species (IAPS), particularly in disturbed environments such as landfills and dumps—potential epicentres for these species—can significantly jeopardise human health due to their toxins and pollen (Plaza *et al*., 2018). Global biotic invasions are a significant priority on the Convention on Biological Diversity's (CBD) agenda for mitigating the impacts of invasive alien plant species (IAPS) on ecosystems and public health. The Cartagena Protocol and biosafety underscore this assertion (Pysˇek and Richardson, 2010). The Rio de Janeiro 1992 Earth Summit has further identified IAPS in forestry and agroforestry systems as a key contributor to worldwide environmental degradation, biodiversity loss, and adverse effects on human health. IPBES (2019) particularly identified the threats that Invasive Alien Plant Species (IAPS) bring to ecosystem services, human health, livelihoods, and global biodiversity in its deliverable 3(b)(ii). The establishment of a database of naturalised species will not only serve as the foundational step in the advancement of invasion biology but will also provide more comprehensive research on the biology and impacts of particular species (Wu *et al*., 2004).

Ecosystems are influenced by intricate connections between species invasions and climate change. The phenology and reproductive behaviours of indigenous plant species are affected by these elements alongside plant invasion. This research aims to find molecular pathways that elucidate how these determinants influence biodiversity and environmental equilibrium. The use of an integrative strategy provides valuable insights for effective conservation and management practices (Giejsztowt *et al*., 2020).

Important value index

Processes like migration and adaptation drive the biodiversity of any particular location to alter constantly. Many species are created as a result of interactions between organisms and their environments. Animals that migrate can go across a variety of settings, which means they must adapt in order to live. Individual species are guaranteed to survive thanks to this continuous process that permits the emergence of new traits. A deeper understanding of this intricate web can help us understand the resilience and evolution of ecosystems (Christopher, 2020). Numerous studies on different plant species demonstrate how intricate relationships and interactions among plant communities influence ecological responsibilities. This study recognises the interdependence of plant life and the multitude of factors impacting community makeup. The prevalence of particular plant species is important to consider when characterising the overall composition of a community since it influences aspects such as ecosystem resilience, nitrogen cycling, and biodiversity (Odum, 1971).

The development of ecosystems is influenced by plant variety and is contingent upon the relative abundance and species composition of the ecosystem. In addition to enhancing the aesthetic and biological diversity of any given location, it preserves the fragile balance of the biosphere on Earth. When biodiversity is present, ecosystems are more resilient and long-lasting because it influences processes like pollination, pest control, and nutrient cycling. Monitoring changes in biodiversity can help us better understand environmental pressures such as pollution, habitat loss, and climate change. Because of this, maintaining biodiversity is essential to the wellbeing

of the planet and the interdependence of all life on Earth (Solbrig, 1991; Rawat and Chandra, 2014).

Comprehending the composition and structure of plant communities is essential to ecological studies. The characterisation of these communities entails identifying species as well as assessing their roles, dominance, and relative significance within an ecosystem. The Important Value Index (IVI) is a crucial metric for evaluating the ecological significance of individual species within plant communities, as it consolidates various dimensions of a species' ecological function into a singular, measurable index. This metric is extensively utilised in ecological research, especially in investigations of plant ecology, community organisation, biodiversity, and conservation biology (Curtis & McIntosh, 1950; Christopher, 2020).

The IVI provides an extensive instrument for ecologists to ascertain species dominance within a community, elucidate species-environment interactions, and evaluate the overall ecosystem health. This introduction explores the conceptual basis, computation, and ecological importance of IVI, establishing a framework for its use in diverse research settings. The significance of IVI in biodiversity assessment, conservation planning, and ecosystem management is examined, underscoring its relevance in modern ecological research (Curtis & McIntosh, 1950; Solbrig, 1991; Rawat and Chandra, 2014).

The Importance Value Index (IVI) is a tool used to quantify a species' ecological relevance within a particular environment. When key measures like relative dominance, relative frequency, and relative density are combined, a numerical representation of the species' environmental contribution is obtained. To facilitate informed decision-making on conservation and management, the IVI gauges a species' overall significance within its community. This supports biodiversity and ecosystem health. It clarifies the intricate connections between different species and their roles in the ecosystem (Phillips, 1959).

Melissopalynology

Bees are an essential component in the maintenance of both natural and artificial plant ecological systems. This is due to the fact that they are the most important pollinators in the world. In tropical regions, where pollination by insects is critical to the survival of ecosystems and the production of agricultural goods, the contribution that they make is especially important with regard to the importance of the contribution that they make. Melissopalynology has arisen as an important topic of research in this environment, which has led to a considerable increase in the amount of attention that has been paid to the study of bees and their interactions with plants. Melissopalynology will continue to be an important field of research. A scientific field of research known as Melissopalynology is concerned with the investigation of pollen that is found in honey. It is possible for bees to intentionally collect pollen, or it is also possible for pollen to be unintentionally incorporated into honey when it is being created. Honey has been thoroughly tested for its purity, as well as its geographical origin and floral source (Winfree 2010; Ollerton *et al*., 2011). The field has been exploited for this purpose in a significant way.

Since the early 1900s, Melissopalynology has been used as an analytical method for honey authentication and quality testing. Early studies by Waters (1915) and later by Nair (1964) provided the foundation for our understanding of the pollen composition in honey. Song *et al*.'s (2012) more recent work has considerably enhanced these methods. Through the identification of specific floral sources and the clarification of the ecological connections between bees and plants, these studies contribute significantly to our understanding of both agricultural and wild ecosystems.

Furthermore, melissopalynology can serve as a stand-in for assessing environmental conditions by tying pollen data to in situ meteorological parameters including temperature, humidity, and rainfall (Jato *et al*., 1994; Bilisik *et al*., 2008). Understanding the effects of outside influences, including habitat loss and climate change, on pollinators and the pollination networks they are involved with, depends

on these relationships (Herrera 1995; Jens *et al*., 2008). Because bee numbers around the world are dropping, there is growing worry in this area.

For the purpose of enhancing the dependability of melissopalynological research, researchers have made use of several cutting-edge statistical procedures, particularly ordination methods. Using these methods, it is possible to quantitatively describe honey by revealing patterns in the composition of pollen that are representative of the geographical and botanical origins of the samples. According to this definition, honey can be described. There have been studies carried out by Herrero *et al*. (2002) and Aronne and De Micco (2010) that have demonstrated that ordination is an effective strategy for discriminating between different kinds of honey based on the melissopalynological profiles of the honey. It is possible to establish a more accurate categorisation through the utilisation of this statistical method, which in turn helps to support efforts to trace the origin of honey, defend against adulteration, and maintain high standards of quality control in the production of honey.

DNA barcoding

DNA barcoding is an innovative tool in taxonomy, acclaimed for its accuracy and efficacy in species identification and classification. This molecular approach use short, standardised DNA sequences from a specific genomic area, commonly known as a "barcode," to identify and distinguish species. This method has demonstrated significant use in classifying both entire organisms and fragmented or incomplete specimens, rendering it an essential tool for systematic study and biodiversity investigations.

Before the establishment of DNA barcoding, molecular data, especially DNA sequences, had been employed for specimen identification since the 1980s. Nonetheless, the capacity of DNA sequencing to function as a comprehensive natural history instrument was not completely acknowledged until 2003. In that crucial year, the principle of DNA barcoding was established, laying the groundwork for the creation of a worldwide framework for its implementation. This program was initiated by three fundamental organisational meetings at the Banbury Centre at Cold

Spring Harbour, facilitated by the Alfred P. Sloan Foundation, which advanced DNA barcoding as a widely recognised instrument for taxonomic and ecological study. Innovative articles by Stoeckle (2003) and Hebert et al. (2003a, 2003b) established the scientific foundation for the approach, showcasing its effectiveness and applicability in species identification across many taxa.

DNA barcoding fundamentally depends on the notion that a brief and standardised segment of the genome serves as a universal identifier for species. The mitochondrial cytochrome c oxidase I (COI) gene in mammals has been designated as the standard barcode region because of its rapid evolutionary pace and interspecies variance, while maintaining conservation within species. This area offers adequate genetic difference for species differentiation while maintaining consistency among species to function as a dependable identifier. In plants, certain gene sections, including the chloroplast genes rbcL and matK, are frequently utilised because of the reduced variety of mitochondrial DNA in these organisms.

Besides its utility in species identification, DNA barcoding has proved pivotal in the discovery of novel species, the elucidation of cryptic species complexes, and the clarification of evolutionary relationships among taxa. DNA barcoding has improved the precision of taxonomic categorisation by offering a genetic underpinning for species differentiation, hence enhancing the comprehension of biodiversity patterns. It has also become an essential instrument for ecological monitoring, conservation initiatives, and environmental management. DNA barcoding has been utilised for monitoring invasive species, identifying species in commerce, and evaluating the effects of climate change on biodiversity.

The creation of the Barcode of Life Data System (BOLD) has been a major advancement in the international DNA barcoding endeavour. BOLD is an open-access internet repository enabling researchers to upload, store, and analyse barcode sequences. The system enables the worldwide sharing of barcoding data and functions as a centralised repository for species identification. The expanding database augments the efficacy and accuracy of DNA barcoding as a resource for taxonomists and ecologists globally. BOLD has been crucial in broadening the scope

of barcoding initiatives, ranging from regional assessments to global biodiversity inventories, and has significantly contributed to the democratisation of access to genetic taxonomy tools.

The DNA barcoding endeavour has achieved significant success through its implementation in the International Barcode of Life (iBOL) project, the largest biodiversity genomics endeavour ever conducted. Initiated in 2010, iBOL seeks to sequence and record DNA barcodes from millions of species globally, establishing an extensive repository of Earth's biodiversity. This ambitious initiative has resulted in the discovery of thousands of previously unidentified species and has yielded critical insights into the worldwide distribution of biodiversity. The efficacy of iBOL and analogous projects highlights the revolutionary capacity of DNA barcoding in taxonomy and conservation biology.

GenBank and BOLD are the two most utilised DNA barcoding repositories. Notwithstanding the substantial quantity of sequences, the gene bank is devoid of a research strategy to guarantee precise species identification and offers no valuable information. The Life Data Systems (BOLD) Barcode Database was specifically developed to facilitate DNA barcodes. Finalise the BOLD library record, including all collection details (date, location, collector, etc.), an image of the sampling site, and data on sequence alignments. It also includes Sanger sequencing, coherent sequence data from the DNA library, and unprocessed sequencing data (process trace file). Sequences that exhibit high similarity are classified into BINs, which serve as putative species, analogous to how approximate morphological categories might function as morphological species (Ratnasingham and Hebert 2007). Despite price fluctuations, barcodes can be generated using this technology for a minimum of $3 (Meier *et al*., 2016). Over 4.5 million bolded barcode sequences correspond to more than 440,000 probable species. Many barcoded species not already in the BOLD database can nevertheless be classified by genus, family, or higher taxonomic ranks (Munch *et al*., 2008; Wilson *et al*., 2011). DNA libraries will receive greater acknowledgement as additional species are incorporated; nonetheless, the consideration of collection sites will become increasingly essential in the

identification process (Bergsten *et al*., 2012).Barcoding is an exceptionally effective method, as identification may be performed without specific training or biological expertise, and it has demonstrated accuracy for over 90% of the species examined. No taxonomist has claimed that identification relying exclusively on COI barcodes is as dependable as identification that includes several genes, morphological characteristics, and biological aspects (Hajibabaei *et al*., 2006; Lin *et al*., 2015; Grebennikov *et al*., 2017; Ortiz *et al*., 2017).

Discrepancies between DNA barcoding and morphological assessments frequently result in flaws in taxonomic assumptions, rather than faults in barcoding for completely separated species (Hebert et al., 2004; Smith et al., 2008). Why is the same concept inapplicable to the description of novel species? The International Code of Zoological Nomenclature contains no provision that prohibits or dissuades DNA-based descriptions. The Ichneumon concept, in particular, holds significant potential for DNA characterisation. This category is very diversified, contains numerous rare species, is often perplexing, and specimens are occasionally collected with minimal comprehension of the surrounding ecosystem. Additionally, DNA barcoding has been employed to identify some enigmatic braconids and ichneumonids (Smith et al., 2008; Veijalainen et al., 2011; Quicke et al., 2012; Schwarzfeld and Sperling, 2015).

Physicochemical properties of honey

Honey is a naturally occurring sweet substance produced through honeybees (*Apis mellifera*) from floral nectar or plant secretions. It is among the most ancient foods ingested by humans, esteemed for both its sweetness and its nutritional and therapeutic attributes. Throughout the years, honey has been integral to traditional medicine, food preservation, and religious ceremonies in diverse societies. In contemporary times, honey has garnered heightened interest in scientific inquiry owing to its intricate composition and extensive array of health advantages.

The distinctive qualities of honey arise from its intricate chemical composition, shaped by elements including flower source, geographical origin,

processing techniques, and storage conditions. The physicochemical qualities of honey, such as moisture content, pH, sugar composition, electrical conductivity, and viscosity, are essential for assessing its quality, stability, and appropriateness for various applications. Comprehending these qualities is crucial for both consumers and industries, as they affect the nutritional content, flavour, medicinal attributes, and shelf life of honey.

This introduction will examine the physicochemical properties of honey, emphasising their biological importance, the variables that affect them, and their impact on honey quality and classification. The introduction will emphasise the significance of these features for food safety, international trade, and honey adulteration.

Historical Context and Significance of Honey

Honey has been utilised by humans for more than 8,000 years, as demonstrated by cave paintings in Spain that illustrate early humans collecting honey from wild bees (Crane, 1999). In ancient Egypt, honey served as both sustenance and a valuable resource utilised in embalming and religious rituals (Bogdanov, 2011). Ancient civilisations, such as the Greeks and Romans, acknowledged the therapeutic benefits of honey, employing it to address wounds, digestive disorders, and respiratory conditions.

The importance of honey in traditional medicine persists, particularly in Ayurvedic and Chinese medical systems. Nonetheless, the emergence of contemporary scientific study has directed attention to the physicochemical properties of honey, especially regarding its antibacterial, antioxidant, and anti-inflammatory effects (Alvarez-Suarez et al., 2010). The biological activities are directly associated with honey's chemical makeup, which is affected by its physicochemical qualities.

Constituents of Honey

Honey is a supersaturated sugar solution, containing a water content that often varies from 15% to 20%. The principal components are carbohydrates (70-80%), predominantly fructose and glucose, which constitute the majority of its energy content. In addition to carbohydrates, honey comprises several secondary constituents, such as organic acids, amino acids, proteins, vitamins, minerals, enzymes, and polyphenols, which enhance its distinctive flavour, aroma, and health advantages (White, 1978).

The composition of honey is affected by various elements, including as the floral source, environmental conditions, and processing techniques. Honey obtained from various nectar sources can change markedly in its sugar composition, moisture content, and pH (Persano Oddo & Piro, 2004). The differences in composition subsequently influence the physicochemical qualities of honey, which are essential for evaluating its quality.

Major Royal Jelly 2

Royal jelly, generated by worker bees, functions as a vital nutrition supply for both larvae and the queen in honeybee colonies. Research on royal jelly has predominantly concentrated on *Apis mellifera*, the Western honeybee, but there is a growing interest in its counterpart, *A. cerana*, the Eastern honeybee. These two species are extensively dispersed throughout Asia and hold significant ecological and agricultural value. Major Royal Jelly Proteins (MRJPs) are essential components of royal jelly, significant for their function in larval feeding and caste determination. Among these, Major Royal Jelly Protein 2 (MRJP2) has attracted considerable attention for its diverse functions in development, immunology, and social regulation in *A. cerana*.

MRJP2 is a significant protein found in the royal jelly of *A. cerana*, belonging to a family of proteins essential for the separation of queen bees from worker bees. Royal jelly is exclusively administered to queen larvae during their entire developmental phase, whilst worker and drone larvae are provided with it

solely during the initial days of their lives. The prolonged exposure to royal jelly, abundant in MRJPs, induces the epigenetic modifications requisite for the queen phenotype, characterised by enhanced size, longevity, and reproductive capacity. MRJP2 is considered to be crucial in this process, akin to its involvement in *Apis mellifera* (Buttstedt, Moritz, & Erler, 2014). *A. cerana* demonstrates species-specific variations in protein composition and functionality, rendering it a distinctive subject for investigation.Structurally, MRJP2 from *A. cerana* resembles that of *Apis mellifera*, comprising approximately 420 amino acids and undergoing post-translational changes, including glycosylation (Schmitzova et al., 1998). These alterations are crucial for the protein's stability and functionality. Recent proteomic analyses have established that MRJP2 is one of the predominant proteins in *A. cerana* royal jelly, and its structure indicates evolutionary conservation with MRJPs from other honeybee species. Nonetheless, nuanced variations in amino acid sequences between *A. cerana* and *Apis mellifera* MRJPs have been noted, potentially indicating the adaptation of *A. cerana* to distinct environmental circumstances and floral resources in its indigenous habitat.

MRJP2 in *A. cerana* royal jelly is crucial for the nutritional regulation of larvae. Research indicates that MRJPs, particularly MRJP2, facilitate the transfer of vital nutrients, including proteins, lipids, vitamins, and trace minerals, to developing larvae (Han et al., 2011). This function is essential for larval development and differentiation into queens or workers. Moreover, MRJP2 may influence gene expression in *A. cerana* larvae, affecting developmental pathways that result in the caste differentiation observed in honeybee colonies (Kucharski et al., 2008). The elevated levels of MRJP2 in larvae destined to become queens corroborate its function in promoting their distinct developmental pathway.

Moreover, MRJP2 in *A. cerana* is thought to affect social behaviour and pheromonal communication. Studies have shown that MRJPs have a role in the synthesis of pheromones that govern colony dynamics, especially in queen-worker interactions (Kucharski et al., 2008). While the precise processes are yet to be completely clarified, MRJP2 probably plays a role in regulating reproductive

hierarchies and social cohesiveness within *A. cerana* colonies, similar to its function in *Apis mellifera*. This PhD thesis will analyse the physicochemical properties and MRJP2 of honey to investigate their correlation with botanical origin, geographical location, and potential health advantages of honey from particular region or floral source. The subsequent aims are addressed in the thesis:

1. Inventory and Monitoring of bee polliniferous native and invasive plant species and evaluation of bee-floral interactions in the Tanhril forest, Mizoram, India.
2. A DNA Barcoding approach to characterize pollen collected by *Apis cerana* colonies.
3. Study the effects of land use change on the quality of *Apis cerana* honey.

The Tanhril forest, Mizoram is home to a large number of bee-pollinating species. A higher prevalence of Invasive plant species were observed in the Tanhril forest in Mizoram, than the native plant species, which could have an impact on interactions amongst bee pollinators. Due to the prevalence of invasive plant species and their floral displays, Bee Pollinators become more attracted to invasive plants than native ones. This is the first comprehensive report that uses a DNA barcoding technique on honey samples to identify bee-pollinated plants in the Tanhril forest, Mizoram. matK and rbcL biomarkers have been validated to identify the plant species from the pollen content in the *A. cerana* honey samples. The utilisation of pollen barcoding technique has proven to be more effective than traditional melissopalynological methods to identify *A. cerana* polliniferous plants within honey samples. Shifting cultivation practices significantly affecting the *Apis cerana* honey quality in terms of pH, HMF and Total acidity. Seasonal fluctuations in *A. cerana* honey quality were detected in both natural and shifting cultivation habitats; these variations exhibited a significant effect on the physicochemical characteristics of the honey samples that were obtained from the shifting cultivation habitat