

**ECOLOGICAL AND BEHAVIORAL STUDIES ON
ASIAN WILD BUFFALOES (*Bubalus bubalis arnee* Linn.)
AT KOSHI TAPPU WILDLIFE RESERVE, NEPAL**

***THESIS SUBMITTED IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN ZOOLOGY (CONSERVATION BIOLOGY)***

By

RAM CHANDRA KANDEL

(Registration No. MZU/PhD/800 of 22.05.2015)



**DEPARTMENT OF ZOOLOGY
MIZORAM UNIVERSITY
AIZAWL-796004, INDIA**

Supervisor

Prof. G. S. Solanki

Head, Department of Zoology

Mizoram University, Aizwal, India

Jt. Supervisor

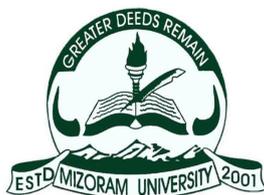
Prof. M. K. Chalise

Central Department of Zoology

Tribhuvan University, Kirtipur, Nepal

13 August 2018

MIZORAM



UNIVERSITY

Aizawl-796004, Mizoram, INDIA

DEPARTMENT OF ZOOLOGY

Ref.:

Date: 13 August 2018



CERTIFICATE

We have a great pleasure in forwarding **Mr. Ram Chandra Kandel's** thesis entitled "**Ecological and Behavioral Studies on Asian Wild Buffaloes (*Bubalus bubalis arnee* Linn.) at Koshi Tappu Wildlife Reserve, Nepal**" for the acceptance of the degree of **Doctor of Philosophy in Zoology (Conservation Biology)**. His thesis carries original piece of research work, unique discoveries and interpretation of facts. This work has not been submitted elsewhere for any degree in the past.

Mr. Ram Chandra Kandel has put forth six terms of research work in the Department of Zoology, Mizoram University. His thesis is submitted as per new University Grant Commission (UGC) regulations, 2009.

Prof. Dr. G. S. Solanki

Supervisor

Head, Department of Zoology

Mizoram University,

Aizawl, INDIA

Prof. Dr. M. K. Chalise

Jt. Supervisor

Central Department of Zoology

Tribhuvan University,

Kirtipur, NEPAL

Mizoram University

August 2018

DECLARATION

I **Mr. Ram Chandra Kandel**, 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 do the best of my knowledge to anybody else and that the thesis has not been submitted by me for any research degree in other University/Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Zoology.

Signature of the Candidate

Head, Department of Zoology

Supervisor

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ACRONYMS

BCC	Biodiversity Conservation Center
BNP	Bardiya National Park
BZ	Buffer Zone
CITES	Convention on International Trade in Endangered Species of Wild Flora & Fauna
CNP	Chitwan National Park
CSP	Concentric Circular Sample Plots
CO1	Cytochrome Oxidase 1
CYT B	Cytochrome B
DBH	Diameter at Breast Height
DNA	Deoxyribonucleic Acid
DNPWC	Department of National Parks and Wildlife Conservation
DoHM	Department of Hydrology and Meteorology
IUCN	International Union for the Conservation of Nature and Natural Resources
IV	Importance Value
KTWR	Koshi Tappu Wildlife Reserve
MFSC	Ministry of Forests and Soil Conservation
MoFE	Ministry of Forests and Environment
MP	Maximum Parsimony
MZU	Mizoram University
NAST	National Academy of Science and Technology
NJ	Neighbor-Joining
NPWCA	National Parks and Wildlife Conservation Act
NPWLC	National Park and Wildlife Conservation
NTNC	National Trust for Nature Conservation
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
PV	Prominence Value
RI	Retention Indices
SE	Standard Error
SSC	Species Survival Commission
TBR	Tree Bisection Reconnection
UGC	University Grant Commission

TABLE OF CONTENTS

<u>CHAPTERS</u>	<u>Page No.</u>
TABLE OF CONTENTS	VII
LIST OF TABLES	IX
LIST OF FIGURES	X
LIST OF ANNEXURES	XI
LIST OF PHOTOPLATES	XII
CHAPTER - I	1
INTRODUCTION	1
CHAPTER - II	7
REVIEW OF LITERATURE	7
CHAPTER - III	14
STUDY AREA	14
CHAPTER - IV	20
HABITAT COMPOSITION OF ASIAN WILD BUFFALO AT KTWR.....	20
CHAPTER - V	29
POPULATION AND DEMOGRAPHY OF ASIAN WILD BUFFALO.....	29
CHAPTER - VI	42
REINTRODUCTION OF ASIAN WILD BUFFALO IN NEPAL: A PROTOCOL.....	42
CHAPTER - VII	61
BEHAVIORAL ECOLOGY &ACTIVITY TIME BUDGET OF WILD BUFFALOES.....	61
CHAPTER - VIII 72
MOLECULAR STUDY OF WILD, FERAL & DOMESTIC BUFFALOES FROM KTWR	72
CHAPTER - IX	92
SUMMARY AND CONCLUSION.....	92
REFERENCES	115
LIST OF PUBLICATIONS.....	130
ANNEXURES (1-12)	132

LIST OF TABLES

Table No.	Legends	Page No.
Table 1	–Number of studied sample plots in KTWR.....	22
Table 2	–Vegetation types and number of sample plots in KTWR.....	23
Table 3	–Tree dominancy in <i>Sissoo-Khair</i> forest.....	23
Table 4	– Prominent shrub and herb in <i>Sissoo-Khair</i> forest.....	24
Table 5	– Prominent grasses/herb in the grassland.....	24
Table 6	– Tree dominancy in mixed riverine forest.....	25
Table 7	– Prominent shrub and herb in mixed riverine forest.....	26
Table 8	– Prominent shrub and herb in shrubland.....	26
Table 9	– Vegetation characteristics and other parameters of the studied forests.....	27
Table 10	– Demographic structure of Asian wild buffalo over different census year.....	33
Table 11	– Exponential growth rate (r_i) of Asian wild buffalo at KTWR over the years.....	34
Table 12	– Composition of male band group observed during the field works at KTWR.....	35
Table 13	–Rate of loss of genetic variation of Asian wild buffalo at KTWR over the years.....	39
Table 14	– Details of the captured wild buffaloes and veterinary interventions	53
Table 15	– Observed herding behavior of translocated wild buffaloes at CNP.....	56
Table 16	– Details of the supplementary diet for one month provided to the wild buffaloes.....	57
Table 17	– Variation of monthly activity time budgets in major activity categories in 2015....	66
Table 18	– Variation of monthly activity time budgets in major activity categories in 2016....	67
Table 19	–Variation of seasonal activity time budgets in major activity categories in 2015....	70
Table 20	–Variation of seasonal activity time budgets in major activity categories in 2016.....	70
Table 21	– Details on types of samples collected from a total of 42 buffaloes from KTWR, Nepal.....	74
Table 22	–Variable nucleotide positions for the partial <i>cytochrome b</i> gene.....	79
Table 23	– Haplotype diversity (h) and nucleotide diversity (π) estimated from partial mitochondrial <i>cytochrome b</i> sequence.....	81

LIST OF FIGURES

Figure No.	Legends	Page No.
Figure 1–	Location of the Study Area, Koshi Tappu Wildlife Reserve, Nepal.....	14
Figure 2–	Location of the Study Area at CNP, Nepal.....	18
Figure 3–	Pattern of male:female & calves: cow ratios of wild buffalo over different years....	35
Figure 4–	Correlation between male to female ratio and Calves to Cows ratio.....	36
Figure 5–	Exponential growth rate (ri) of wild buffalo at KTWR in different census years.....	37
Figure 6–	Structure of Effective population size of wild buffalo in different census years.....	38
Figure 7–	Location map of KTWR Nepal, the site of Donor/Source Population.....	43
Figure 8–	Location map of CNP Nepal, the site of the translocated population (Receiver).....	44
Figure 9–	Layout of soft release enclosure site at CNP, Nepal.....	44
Figure 10–	Behavioral activities of translocated wild buffaloes in the winter season, 2017.....	56
Figure 11–	Behavioral activities of translocated wild buffaloes in summer, 2017.....	58
Figure 12–	Behavioral activities of translocated wild buffaloes in monsoon, 2017.....	59
Figure 13–	Behavioral activities of translocated wild buffaloes in autumn, 2017.....	59
Figure 14–	Seasonal variation in activity time budget during 2015.....	67
Figure 15–	Seasonal variation in activity time budget during 2016.....	68
Figure 16–	Comparison of annual activities time budget of wild buffaloes (2015 & 2016).....	68
Figure 17–	Activity time budget (%) of Asian wild buffalo in 2015.....	69
Figure 18–	Activity time budget (%) of Asian wild buffalo in 2016.....	69
Figure 19–	Map of an extant distribution of Water buffalo at KTWR, Nepal.....	76
Figure 20–	Schematic presentation of <i>Cytochrome b</i> gene region in the mammals (adopted from Adrian and James, 2016).....	77
Figure 21–	Reduced median network constructed using NETWORK 4.1.1.2 program.....	81
Figure 22–	Maximum parsimonious trees constructed using 422 bp partial <i>cytochrome b</i> sequences.....	83
Figure 23–	Neighbour-joining tree for 97 partial <i>cytochrome b</i> sequences of river and swamp water buffaloes.....	84
Figure 24–	Agarose gel electrophoresis showing the size of amplified products by nested PCR using primers targeting mitochondrial cyt-b gene.....	90

LIST OF ANNEXURE

Annexure No.	Legend	Page No.
Annex 1-	Mean Annual Rainfall and Temperature near KTWR and CNP.....	132
Annex 2-	The specific formulae employed for the analysis of vegetation.....	133
Annex 3-	Importance value of tree species in Sissoo-Khair forest	134
Annex 4-	Prominence value of different species in understory layer of Sissoo-Khair forest...	135
Annex 5-	Prominence value of different species in ground vegetation of Sissoo-Khair forest..	136
Annex 6-	Prominence value of different species in the ground vegetation of grassland	137
Annex 7-	Importance value of tree species in mixed riverine forest.....	138
Annex 8-	Prominence value of different species in understory layer of mixed riverine forest..	139
Annex 9-	Prominence value of different species in the ground vegetation of mixed riverine forest	140
Annex 10-	Prominence value of different species in understory layer of shrubland	141
Annex 11-	Prominence value of different species in the ground vegetation of shrubland	141
Annex 12-	Photoplates (1-21)	142

LIST OF PHOTOPLATES

Plate No.	Legend	Page No.
Photoplate 1-	Studying habitat composition in the eastern part of KTWR.....	142
Photoplate 2-	Studying habitat composition in the western part of KTWR.....	143
Photoplate 3-	Researcher talking about the wild buffaloes with Mr. Raghu Baharkher.....	144
Photoplate 4-	Identification of different breeds of buffaloes.....	145
Photoplate 5-	Team of people on wild buffalo count and behavioral studies at KTWR.....	146
Photoplate 6-	Coordination meeting with local leaders at KTWR before translocation.....	147
Photoplate 7-	Field surveys on wild buffaloes along with supervisors at KTWR.....	147
Photoplate 8-	KTWR BZ Community leaders at CNP to share conservation achievements...	148
Photoplate 9-	Design of wild buffalo transportation container and loading the animals.....	148
Photoplate 10-	Blood sample collection from wild, feral and domestic buffaloes.....	149
Photoplate 11-	Darting, Capture, Immobilization, and hand over of wild buffalo at KTWR...	150
Photoplate 12-	Monitoring of soft release enclosure at CNP before the translocation.....	151
Photoplate 13-	Wild buffalo releasing ceremony at CNP, 29 January 2017.....	151
Photoplate 14-	Wild buffaloes during and post-release monitoring at CNP, Feb. 2017.....	152
Photoplate 15-	Post-release monitoring in the soft-release enclosure at CNP, April 2017.....	153
Photoplate 16-	Monitoring of wild buffaloes, its soft-release enclosure, and habitat on a regular basis from the watchtower, on foot or on elephant back at CNP, May 2017.....	153
Photoplate 17-	Habitat management by cutting and burning <i>Mikania macarantha</i> patches inside the enclosure for maintaining grassland at CNP, June 2017.....	154
Photoplate 18-	Outlook of damaged habitats in the grasslands within the enclosure of wild buffaloes just after flash floods, August 2017.....	155
Photoplate 19-	Wild buffaloes on a regular post-release monitoring and habitat management, with supplementation of nutritional diet after August flood, 2017 at CNP, Jan. 2018.....	156
Photoplate 20-	Injured animal under treatment by veterinary team at CNP, March 2018.....	157
Photoplate 21-	Snaps of lab works on molecular studies of wild buffaloes including DNA extraction, PCR, and DNA sequencing.....	158
Photoplate 22-	Reseracher representing in ICCB July 2017 (Cartagena, Columbia)	159
Photoplate 23-	Reseracher representing on CBNSD 2018 Aug. at Nairobi, Kenya.....	160
Photoplate 24-	Reseracher in a pre-submission seminar on April 17, 2018 at MZU, India.....	161

CHAPTER-I

INTRODUCTION

The Asian wild buffalo or wild water buffalo or Asian buffalo (*Bubalus bubalis arnee* Kerr, 1792), henceforth will be called as wild buffalo, belongs to a large ungulate of the family-Bovidae and one of the members of *Bubalus* species (Linnaeus, 1758), and the ancestor of all the domestic water buffalo varieties (Cockrill, 1974; Ashby & Santiapillai, 1987), which was widely distributed in Europe and southern Asia in the Pleistocene period. However as the climate became drier, it was restricted to India, Indo-China and some of the south-east Asian islands (Fahimuddin, 1975; Mason, 1974). Wild cattle includes four genera (IUCN, 2013; IUCN-SSC, 2010) namely *Bubalus* (Asian buffalo), *Syncerus* (African buffalo), *Bos* (Gaur and Cattle), and *Bison* (American and European Bisons). These four genera encompass twelve non-interbreeding species though it is believed to have cross-breeding between European and American Bisons (Melletti and Burton, 2014). Based on morphology, the closest relative of *Bubalus* is the African Buffalo *Syncerus*; both having horn cores which tend to be triangular in cross-section (Mathur *et al.*, 1995; Muley, 2001). Asian wild buffalo falls under the mammalian species of order Artiodactyla, family Bovidae, sub-family Ruminantia/Bovinae, tribe Bovini, genus *Bubalus*, and species *B.bubalis arnee*. In Nepal, it is listed as protected species by the National Parks and Wildlife Conservation Act (NPWCA, 1973 e.g. Heinen and Kandel, 2006; Sah, 1997; Bhandari, 2009; Kandel, 2013; Annon, 2014), which is also included in CITES Appendix III as a result of the known threats to all wild populations (Annon, 1997). Likewise, it is listed as endangered in IUCN Red Data Book (Hedges, 1995; Hedges *et al.*, 2008; Heinen & Srikosamatara, 1996).

The historical distribution of the Asian wild buffalo ranged across south and south-east Asia, from Mesopotamia to Indo-China (Mason, 1974; Cockrill, 1974). But later, Asian wild buffaloes remained confined to three large disconnected areas (Choudhury, 1994, 2010) viz. (1) northern India, Nepal, Bhutan and Bangladesh; (2) Peninsular India; and (3) scattered areas in Indo-China in the early period of the twentieth century. Though the earlier range of Asian wild buffalo is uncertain (Heinen & Kandel, 2006), there is also evidence in the Indus Valley of at least 5000 years ago and the wild form may have occurred from Mesopotamia eastwards (Nowak, 1999; Lenstra & Bradley, 1999). The major populations of Asian wild buffalo are in Assam and Madhya Pradesh, India (Choudhary, 1994), and remnant population in western Thailand, although there is a debate about the genetic integrity of some of those stocks (Diveker & Bhusan,

1988; Hedge, 1995; Mulley, 2001). Microsatellites and mtDNA diversity analyses (Yindee *et al.*, 2010; Groeneveld *et al.*, 2010; Zhang *et al.*, 2011) combined with palaeontological evidence (Higham, 2002) indicated its domestication in south China and Indo-China around 2000 BC and may be even earlier (Clutton-Brock, 1989).

Buffaloes are believed to have originated in South Asia but the wild population of Asian wild buffaloes in Sri Lanka, Borneo and Java are not known whether they have fully or partly domestic origin (Hedge, 1995). Paleontological, anatomical and historical evidence support the contentions that domestic buffalo *B. bubalis* (Linnaeus, 1758) is descended from *B. arnee* (Kerr, 1792). As Buffaloes are classified on morphological and behavioral criteria into two types: river and swamp (MacGregor, 1941), these also differed in chromosome numbers, river buffalo (2n=50) and swamp buffalo (2n=48), based on allozyme and microsatellite genotype frequencies (Fischer & Ulbrich, 1968) and are genetically distinct (Zhang *et al.* 2011). Some researchers refer to them as sub-species, naming river buffalo *B. bubalis bubalis* and swamp buffalo *B. bubalis carabanesis* (Fischer & Ulbrich, 1968).

Asian wild buffaloes (*Bubalus bubalis arnee*, Kerr, 1792), a large ungulate and the ancestor of all the domestic water buffalo varieties, are robust and herd living mega-herbivores profoundly preferring areas around water-logged mainly along the riverine floodplain habitats (Kandel *et al.*, 2014). Water buffaloes are principally found in open grasslands, alluvial plains, and marshes, although they may use woodlands for shelter during heat stress or heavy rainfalls. They are less active and normally territorial.

Asian Wild buffalo is an endangered species (Hedges, 1995; Hedges *et al.*, 2008; Heinen & Srikosamatar, 1996) with the World population around 3300-3400, and it is even possible that no purebred wild animals exist (Scherf, 2000). Present populations of wild buffaloes at Koshi Tappu Wildlife Reserve of Nepal are mainly confined to the grasslands and swampy areas along the Koshi River floodplain and surrounding area. Though the limited numbers of Asian wild buffaloes are survived in the wild, Koshi Tappu Wildlife Reserve (KTWR), which is located in south-eastern Tarai region of Nepal, has a higher chances of maintaining pure wild stock among the current putative wild population (Heinen, 2002) due to strict protection of major wild herds, most preferred suitable habitats and their swampy territories along the Koshi River. The current state of Asian wild buffalo at KTWR entails that the severity of various threats, especially hybridization with the profused domestic water buffaloes and feral buffaloes have posed them in the verge of extinction (Kandel *et al.*, 2014). In addition, competition for food and space with

cattle, domestic and feral buffaloes; threats created due to poaching, poisoning, electrocution, retaliatory killing and other anthropogenic factors have led to the turmoil in its population (Kandel *et al.*, 2014).

Habitat composition of wild buffaloes at KTWR shall represent the pledged for their survival with the presence of ample amount of high quality and varied forage species, and forest composition in terms of proportion and area coverage by different forest types required for this species. Similarly, the updated census report of wild buffaloes at KTWR provides the population status, demographic structures and trend of exponential growth with the rate of effective population size and the percentage rate of loss of variation per generation though this has been affected by various underlying causes that succeed over time. Ultimately it warrants management authorities for trying to maintain it through indispensable actions by also adopting close collaboration with concerned stakeholders by means of strict protection mechanism by deploying the force of Nepal Army and other appropriate interventions.

The behavioral study considers following the performance of animal's behavior in terms of activity time budget such as grazing, moving, resting, wallowing and other activities that would have a significant implication on knowing the ecology of the animals and necessitate its management for their nourishment. The time period that individuals of a species allocate to each of the activities is mainly determined by ecological and social factors. Behavioral ecologists need to quantify animal behavior in order to answer questions pertaining to how behavior contributes to survival, reproduction, and population growth of a species with respect to its surroundings. Recording the time whenever focal animal switches its behaviors from one to another gives a proportion of time budget that an animal performs over a period (Altmann, 1974). Given the transition times, the exact amount of time duration that a target animal devoted to each behavior during the sample can be calculated from the observed data sets.

The behavioral activities of an animal are also influenced by the individuals' physiological states (Li, 2009) which are considered in the active part of the daily activities of wild buffaloes. It is broadly divided into five major categories of maintenance behavior such as feeding (grazing), moving (travelling, walking, running), resting (standing, lying, sleeping), wallowing, and also non-maintenance behavior such as social interactions and territorial activities including playing, fighting, drinking, grooming, sexuality, etc. are kept in a category of other activities. The proportion of time spent in each category of behavior is referred to as the activity budget. However, changes in activity pattern are the first responses of the animal to isolation due to

habitat fragmentation and disturbances in environmental conditions. The key factors recognized as influencing the animal behavior and activity budget (Clutton-Brock and Harvey, 1977a; Passamani, 1988; Passamani and Fernandez 2011) are distribution of the forest types, quality and availability of major food resources or species, requirement of diets, reproductive tactics, seasonality, weather condition, anthropogenic activities or factors, etc. In many species, time spent feeding on fruits was found to be negatively correlated with time and positively related to travel time (Agetsuma, 1995; Agetsuma & Nakagawa, 1998). Sex differences and group sizes can also influence the activity budgets of animal behavior. Such differences in activity budgets are usually credited to the different nutritional and energy requirements of both sexes and social involvements with group members (Schoener, 1971, Clutton-Brock, 1977). Therefore it is very important to note that the proportion of time spent in different behavioral categories of wild buffaloes especially when the distribution pattern and population size of the species are likely to be under any kind of disturbances or threats. Moreover, behavioral flexibility in different land use categories for the same species has found to receive considerable attention of its conservation through appropriate management.

To study behaviors, a present field study was concentrated on focal animals mainly in the herds or solitary/or male-band groups following the technique of Altmann (1974). This study would give the insight on the current status and would happen to ecological basis further to concentrate on the protection of the wild population of Asian wild buffaloes at its prime habitat and also to devise the future conservation strategies. This is also going to be a research technique for any of the students of conservation biology in future.

F₁ progeny of buffaloes that are not found and returned to the domestic herd will become feral and gradually join the wild herds so that the wild herd is likely to contain increasing numbers of animals with varying levels of hybridization over time (Barker, 2001). Genetic quality of feral buffaloes in Koshi Tappu is not known well (Flamand *et al.*, 2003) so there is also doubt about the genetic status of wild buffaloes at KTWR. This research intended to study the habitat composition for the wild buffaloes at Koshi Tappu, its population status, and demographic structures, behavioral activities and analyze the molecular characteristics to compare with the feral and domestic buffaloes sympatric to them. Present research work updates the socio-ecological database, demographic structure and phylogenetic position of Asian wild buffaloes through molecular analysis of the only population of Asian wild buffalo at KTWR. In addition, the research work attempted to look at the preparation of protocol to translocate wild water buffaloes to suitable habitats of other protected areas of Nepal to create an alternative population

in order to safeguard it from any kind of redundant risk of diseases transmission and floods. Most importantly, this study would try to provide the status of genetic purity of wild stocks and differentiates it with that of feral and domestic breeds using DNA marker. It is also expected to generate updated information and to recommend further conservation and survival/or translocation strategy for safeguarding the wild buffalo by setting up the viable population in other suitable alternative habitats. Additionally, we present the molecular-based report on ungulate malaria parasites from water buffalo of Nepal. Prevalence as well as possible economic impacts of water buffalo malaria should be determined using samples of buffalo from wider areas, probably across Nepal would give us significant result.

Moreover, this research would recommend management authority of Koshi Tappu Wildlife Reserve to focus on and increase its management efforts at the critical sites of wild buffalo habitats. The result of this study would be of immense assistance for the DNPWC/Government of Nepal to prepare translocation plan and species survival/or conservation action plan in future due considering the current alarming status of the remnant population at KTWR. The findings of this research work would ultimately support the decision makers to think of ensuring the long-term survival and intervention for appropriate management of wild buffalo in the existing and alternative habitats. Similarly, the result of this study would contribute to updating the database on the status of habitat condition and floral-diversity at KTWR, and that would also contribute to monitor changes over time.

Asian wild buffalo population has been in a big threat as it has been confined to only KTWR as a remnant population and has been posed to various levels of threats since decades. There has been a poor level of enforcement actions despite the regular implementation of existing laws and bylaws to protect the population of the wild stocks in the Wildlife Reserve area through various means of interventions. It has been requiring serious attention for the comprehensive and effective management of the Reserve and endangered species like wild buffaloes. There has also been a lack of ample biological information on wild buffalo such as habitat available for this and other species, information on ecological and social behavior, information on the trend of population growth over time, differences on molecular characteristics of wild, feral and domestic breeds, etc. This raises the pertinent question regarding streamlining basic information for the effective management in future. In this connection, this study is intended to recommend conservation measures of the wild breed of Asian wild buffaloes in the wild habitat of KTWR. The research works on intensive studies carried out regularly and at least at a certain interval could enhance management capability with significant implication to properly distinguish wild

stocks from feral ones. This study would also contribute to know the simultaneous conservation strategies required for the establishment of the alternative population to other suitable habitats by means of translocation and to focus on protection of the wild population, which has been undermined and lacking due to various problems at the field level. Most prominently noticed problems are the lack of human resource for protection duty and to mobilize the local communities in the conservation schemes, and lack of effective program packages as a sustainable alternative for the dependent communities.

OBJECTIVES OF THE STUDY

The principal objective of this study was the assessment of ecological aspects of Asian wild buffaloes including their habitat, population, behavior and genetic studies in Koshi Tappu Wildlife Reserve (KTWR), Nepal.

The specific objectives of the study are:

- a) To know the habitat composition of Asian wild buffalo at KTWR.
- b) To study population and demography of Asian wild buffalo at KTWR and prepare a protocol of wild buffalo translocation from KTWR to CNP.
- c) To assess the behavioral activity time budget of Asian wild buffalo.
- d) To analyse the molecular structure of *Bubalus bubalis arnee* population in KTWR and establish phylogenetic relation among wild buffaloes of Nepal.

CHAPTER-II

REVIEW OF LITERATURES

Archeomeryx of the Eocene period is thought to be the first ancestor of Bovids (Mathur *et al.*, 1995). It was a small generalized animal about the size of the existing chevrotains. It had no horns and whether it was a true ruminant is uncertain (Pilgrim, 1947; Corbin & Uzzel, 1970). *Eotragus* appears to be the first true bovid living some 15-17 million years ago in Miocene (Pilgrim, 1947). In Bovini, *Bos* and *Bubalus* were already distinct some 3 million years ago. *Leptobos* in Europe and India 2-3 million years ago in upper Pliocene was the ancestor of *Bos* with round horn cores. The wild buffalo was once widely distributed over the tracts of tall grasslands and riverain forests in India and Nepal (Mathur *et al.*, 1995). Cockrill (1974) illustrated that there is a substantial free-living population in south-east Asian countries such as Burma, Malaysia and Indonesia, and Australia which was derived from domestic swamp buffaloes; and as regards wild buffaloes in Srilanka, they are thought to be feral and not truly wild. Daniel & Grubh (1966) mentioned the distribution of wild buffaloes in few surviving grasslands of Brahmaputra valley, pockets of Orrisa and Madhya Pradesh in India, and Koshi Tappu area in eastern Tarai of Nepal by the middle of 20th century.

Morphological data, including horn size and shape, shows that river type *B. arnee* and the swamp type *B. carabanesis* are phenotypically similar. That is, following domestication, the swamp type has not changed phenotypically, except for some reduction in body size and they are mostly phenotypically homogenous throughout their distribution, while the river type has been subject to human-mediated selection and the development of a number of distinct breeds. The known distribution of *B. arnee*, including areas where it may still exist, overlaps that of the river buffalo distribution in India, Nepal and Bhutan, and overlaps that of the swamp buffalo in Myanmar, Thailand and Cambodia. Thus conservation of true wild *B. arnee* depends on skill and strategy to distinguish it from feral river buffalo or hybrids with river buffalo in the former three countries (India, Nepal, and Bhutan), and from feral swamp buffalo and hybrids with swamp buffalo in the later three countries (Myanmar, Thailand and Cambodia). Most of the wild buffaloes are now confined into the Wildlife Reserves and National Parks. Nevertheless, the major threat to their genetic integrity is cross-breeding with domestic animals, as farmers allow their female buffaloes to range into forest areas to mate with wild bulls.

Asian wild buffalo (*Bubalus bubalis arnee* Kerr. 1792) is also called as water buffalo. The buffalo was domesticated very early during the ancient human civilization, but exactly when and where is uncertain. None of the document proclaims about the date when the wild buffaloes were domesticated. Mason (1974), Lenstra & Bradley (1999) and Massicot (2004) explained the representations of tame and hence possibly domesticated buffaloes appear on seals both in the Indus valley (*Mohen-jodaro*) and in Mesopotamian civilizations from about the middle of the third millennium B.C. According to McIntosh (2007), 'buffaloes occur as images on Indus seals, where they appear to be wild'.

General accounts on wild buffaloes are also found in Blanford (1891), Sterndale's Mammals of India from the 1880s (reprinted by Finn, 1929), Lydekker (1900, 1907) and Prater (1948). The *Maharajah of Cooch Behar* (1908), Stockley (1928), Annon (1920) and Hall (1936) provided hunting accounts. Useful information is also found in Forsyth (1889), Stockley (1913) and in Dunbar (1931). Ward (1910) listed the record horns of the wild buffalo from across its range. Phillips (1929) and Mooney (1930) covered the status of buffaloes in Ceylon (now Sri Lanka) and Orissa respectively. In the post-1950 period, the notable publications on wild buffalo or general works with information on it were Noronha (1952, 1954), Daniel & Grubh (1966), Oryx (1967, 1989), Sheshadri (1969), Divekar (1976), Tikader (1983), Divekar & Bhushan (1988) and Ranjitsinh *et al.* (2000).

Choudhury (1994a) provided the first detailed account on the distribution and status of wild buffaloes with mapping of north-east India. Since the 1990s, a status report on several areas of north-east India has been published (Choudhury, 1993a, 1994b, 1998a, 2001a). Muley (2001) and Flamand *et al.* (2003) studied the genetics of buffaloes in Assam's Kaziranga National Park and Nepal's Koshi Tappu Wildlife Reserve respectively. Some information on its behavioral aspects is found in Ashby & Santiapillai (1987), Pandey (1988), Mathur *et al.* (1995), Kotwal *et al.* (2003) and Kotwal & Mishra (2004). The current status in Bhutan has also been assessed (Choudhury, 2008). Santiapillai (1990) and Chaiyarat (2002, 2004) provided information on Thailand and Heinen (1993) on Nepal. Groves (1996) reviewed the taxonomy and also proposed a new sub-species from Thailand. Choudhury (2010) transcribed a comprehensive monograph on the wild buffalo, with maps. General information on wild buffalo is found in several other synoptic works, and there are also many other unpublished census reports on Kaziranga National Park, India and Koshi Tappu Wildlife Reserve, Nepal.

Barker *et al.* (1997) studied the history and process of domestication of river and swamp breeds of buffaloes from wild stocks. Heinen & Srikosamatara (1996) assessed the status of the eight species of wild cattle and buffalo that occur in Asia. In a study on systematic relationships in the Bovini (Artiodactyla, Bovidae), Groves (1981) had concluded that there is little information on the former or current status of mainland buffalo (*Bubalus bubalis* [*B. arnee*]). Heinen's (2002) study prescribed the comprehensive method to identify wild buffalo (*B. arnee*) from feral backcrosses based on phenotypic and behavioral characteristics in Nepal. He opined to the urgent need for setting of criteria that can be applied more broadly to distinguish wild from feral forms due to the highly endangered status of the wild buffalo, whereas Dahmer (1978) devised the use of identification criteria to distinguish wild breeds with that of feral backcrosses and domestic buffaloes while assessing population, that has been convincing basis for biological studies of wild buffaloes. Choudhary (2010) conducted a census in four protected areas of India namely Manas National Park, Laokhowa Wildlife Sanctuary, Burhachapori Wildlife Sanctuary and Dibru-Saikhowa National Park in 2008 and suggested for a periodic census practices to distinguish wild stocks from feral backcrosses.

Several researchers, scientists and authors such as Clemmons and Buchholz (1997); Linklater (2004); Whitehead *et al.* (2004); Ryan (2006); Laiolo and Jovani (2007), have called for attention to the importance of preserving behavioral and cultural diversity related to animal populations during the current period of rapid anthropogenic changes. However, it is difficult to say for most of the species whether their behavioral diversity is declining, increasing or remaining stable in the changing environmental conditions. Despite this fact that there is some non-maintenance behavior such as social interactions and territorial activities in which most of the animal species devote a considerable amount of time.

The concept of activity time budget in social animals, especially of diurnal nature, based on the hypothesis, that the daylight time available is a limiting factor to carry out the maintenance activities as well as social activities (Altmann, 1980; Dunbar, 1988; Janson and Boinski, 1992). As the intense feeding or grazing and travelling or moving in most of the diurnal herbivores in tropics occur early in the morning and later part of the day with a long resting period at the noon (Chivers, 1969). The most important mechanism of maintaining daily activities in buffaloes is to optimize energy intake so as to balance feeding behavior with other behaviors performed in the day (MacArthur & Pianka, 1966). Factors influencing the activity duration also influence the survival strategies of animals.

The turn of the 20th century witnessed a remarkable decrease in the densities of wild buffalo population (Gee, 1959, 1963; Spillet & Tamang, 1966), and also imposed to local extinction from some of the riverine or nearby terrestrial habitats of Tarai region of Nepal, mainly from Chitwan valley. This known evidence of extinctions had happened in relatively short time duration and called for urgent measures to revive the population. The most warranted solution involves active management of the species by reintroduction or replenishing them to vacant suitable habitats in addition to current habitat improvement, habitat connectivity and extension, strict law enforcement, etc. To undertake the integral preservation of wild buffalo breeds at KTWR, the next excellent potential habitat could be CNP to multiply and secure the wild buffalo population. So an attempt of wild buffalo translocation to its historic range at CNP was made in 2017 as a conservation initiative where it had been extirpated during the early 1960s. The IUCN/SSC Guidelines for Reintroduction (IUCN, 1998) formed the basis for these translocations. These guidelines proposed the establishment of “a viable, free-ranging population in the wild of a species, sub-species or race, which has become globally or locally extinct” as the goal for reintroduction programs.

Linnaeus (1758), who generally used very wide genera, (because he did not use the family rank), originally named it as *Bos bubalis*. Kerr (1792) named it as *Bos arnee*. Later, both were assigned to the genus *Bubalus* (H. Smith, 1827). The species name *bubalis* is based on a domestic animal and is also the senior synonym (Choudhury, 2014). However, those workers who do not use specific names based on domestic animals prefer to use *arnee* (Corbett & Hill, 1992; Grove, 1996; Choudhury, 1997a, 2010).

Hedges (1995) discussed the need for genetic studies, both mitochondrial and nuclear, to assess differences among the putative wild, wild-type, feral, semi-feral and domestic buffalo that occur throughout tropical Asia. Heinen (2002) had put forth his view as even if mitochondrial genetic markers that differentiate wild from backcrossed matriarchal lines are isolated, a detailed study should also be conducted at other sites. This view is further supported by J. S. F. Barker (2001) as: of course, obtaining samples for genetic analysis is difficult (Muley, 2001) and the analysis expensive, priority for such work should be given to those populations (like Koshi Tappu) where the potential for hybridization is highest. Koshi Tappu area has an acute problem of Human-Wildlife conflict for several reasons (Heinen, 2002) ultimately require long-term conservation strategies so as to adopt a restoration program (Heinen & Kandel, 2006) for wild water buffalo in Nepal. Hence the translocation action (Kandel *et al.*, 2018) was adopted by DNPWC/GoN Nepal in 2017 January.

This research would follow the approach adopted by Flamand *et al.* (2003) to identify with high probability those animals most likely to be pure-bred wild for which ample amount of blood or dung samples collected and analyzed from required sample size of wild, feral and domestic buffalo in or in the vicinity of KTWR, Nepal.

During the domestication process, both river and swamp types were likely to subject to a bottleneck, while subsequent selection (particularly for the river type) and genetic drift will have led to increasing differentiation from each other and from *B. arnee*. Muley (2001) assayed 17 microsatellite loci in pre-defined wild, hybrid and domestic animals. As misclassification is certainly possible if wild and domestic populations have been in contact for many generations that resulted in hybrids with a high proportion of wild genes due to backcrossing from an F₁ individual to domestic animals. Given such potentially mixed populations, animals can first be classed as wild, hybrid or domestic-based on morphological criteria, then genotyped for a set of markers and a genetic admixture and assignment test done to estimate the proportions of wild and domestic ancestry for each individual. This research has followed the approach adopted by Flamand *et al.* (2003) to identify with high probability those animals most likely to be purebred wild for which blood or dung samples were collected and analyzed from a required sample size of water buffalo from the vicinity of KTWR, Nepal.

Dahmer, 1978; Heinen, 2002 illustrated the Description of Animals to identify the various breeds for their distinction as:

- Wild water buffaloes, Feral and Domestic in and around the KTWR exhibits differences based on phenotypic characteristics.
- Wild buffaloes are distinguished from feral and domestic buffaloes in color pattern, shape, size of body and horns as well as a general behavior patterns.
- Young Calves (<6 months) are buff in color and begin to darken in the first year. They retain lighter coloration in the second year but the coat continues to darken until the females achieve adult coloration by the third year.
- Thus, young, juvenile, and adult females can be distinguished on the basis of coloration, chevron marks on different parts of the body, overall structural differences of body parts, and horn size.
- Young wild males up to four years frequently seen in small groups with other males which could be as much as 6 to 8 animals, sometimes up to 12 animals. Adult males have been frequently seen alone or in pairs or even larger herds occasionally.

- Herd bulls are those that accompany female herds, and no more than one at a time attending any herd, be it mixed wild or backcrossed. Mixed herds are extremely wary of any human approach, so has to observe from more than 150m using good magnified binoculars.
- A herd comprising females often expresses phalanx in behavior to protect calves from human approach prior to fleeing. Phalanx refers to forming a lateral line, each facing the intruder, with calves interspersed between the adults. This is the basis to differentiate feral backcrossed with that of wild herds.

Since the phenotypic assessment is not adequate, only genotypic data can provide definitive identification of true wild *Bubalus arnee* or hybrid animals with a high proportion of *B. arnee* genes that may be worth including in a conservation program (Barker, 2001). Obtaining samples for genetic analysis is difficult and the analysis is expensive, so the priority for such work should be given to those populations (like Koshi Tappu wild buffalo) where the potential for hybridization is highest. Adequate genetic data (microsatellites, SNP or mtDNA) is a prerequisite for any attempt to differentiate wild, hybrid and domestic individuals in a mixed population. In recent years, a number of new methods have been developed for genetic mixture and assignment testing (Anderson & Thompson, 2002; Price *et al.*, 2009; Sohn *et al.*, 2012; Manel *et al.*, 2005), which may provide more accurate discrimination than the method used by Flamand *et al.*, 2003 if genetic data were available for a sample of true *B. arnee*. Similarly, Barker *et al.*, 1997; Lau *et al.*, 1998; Tanaka *et al.*, 1996; Yang *et al.*, 2008; and many others studied DNA analysis and genetic diversity of ancient water buffalo (both wild or domesticated) remains from various parts of the World (Shuhei & Kawamoto, 2006) including Asian and African buffaloes.

Mathur *et al.* (1995) explained that Morphometry and genetic studies at the molecular level are used to resolve the distinction between different categories of buffaloes. Use of molecular genetics would be technically sound to confirm the categorization of a different race of species though the morphometry is found to be a more user-friendly field-based methodology in spite of the fact that morphometry of any animal can be highly environmental resilient.

Most of *Plasmodium bubalis* cases were reported on immune-compromised water buffalo (Kolte *et al.*, 2002). Since then, morphological observations of malaria parasites in water buffalo were sporadically reported until recently. Templeton *et al.* (2016) reported molecular-based surveillances of malaria parasites in water buffalo in Thailand and Vietnam and found up to 45%

of the examined animals were infected with malaria parasites. They found that there were two distinct Plasmodium mitochondrial DNA sequences and provisionally designated as Types I and II. Quantitative PCR analysis revealed that the Type I sequence showed higher parasite density and microscopical images obtained from this type, thus Type I sequence was deposited as "*Plasmodium bubalis* (accession number LC090213)" and type II, for which all samples showed very low parasite density, was deposited as "*Plasmodium* sp. ex *Bubalus bubalis* (accession number LC090214)".

The livestock farming in Nepal is suffering economic losses from different species of protozoan pathogens like **Babesia**, **Theileria** and **Trypanosoma**, those infect to cattle, water buffalo, pigs, goats and sheep. Most of the locally available diagnosis techniques to identify the protozoan parasites are either based on microscopic or serological examination (Maharjan and Mishra, 2006). Microscopic study is not an effective technique, because it lacks sensitivity and specificity (Böse *et al.*, 1995). A serological technique, ELISA, despite being highly sensitive at detecting parasitic infection in animals fails to differentiate between current and prior infections. However, Polymerase Chain Reaction (PCR) presenting high sensitivity and specificity for detecting as well as identifying species of the parasite that infects the animals are not commonly used to identify the parasites in Nepal. Besides human, malaria parasite infects wide range of hosts including non-human primates, rodents, bats, ungulates, birds, and reptiles (Cox, 2010; Garnham, 1966). So, regular diagnostic examinations and active surveillance should be included in the conservation and management strategies of the reserve to ensure sound health of wild buffalos protected inside KTWR.

CHAPTER-III

STUDY AREA

KOSHI TAPPU WILDLIFE RESERVE

LOCATION AND BOUNDARIES

Koshi Tappu Wildlife Reserve (KTWR) lies between 26°33’-26°45’ N and 86°54’-87°04’E with 176 km², on the floodplain of the Sapta Koshi River (Sah & Suselo, 1996) near the Nepal-India border, in the Tarai physiographic region of south-eastern Nepal (Fig. 1). The area experiences mainly sub-tropical monsoonal climate where the average annual rainfall ranges between 1,300 mm (Fatehpur) and 2,051 mm. The reserve was established in 1976 to preserve habitat for the only remaining population of wild buffalo, Arna (*Bubalus arnee*). In 1987, Koshi Tappu has been recognized as Ramsar site, a wetland of international significance. The government of Nepal has declared the buffer zone (173.5 km²) surrounding the Reserve in 2004. The Reserve covers parts of Sunsari District of the Koshi Zone and Saptari and Udayapur Districts of Sagarmatha Zone. The area is almost flat and roughly rectangular in shape with a length of about 16.3 km and width of 9.3 km. The elevation ranges from 75 to 100 m above mean sea level. It is drained by the Sapta Koshi River, a major tributary of the Ganges, and the Trijuga River which joins the Koshi in the western border.

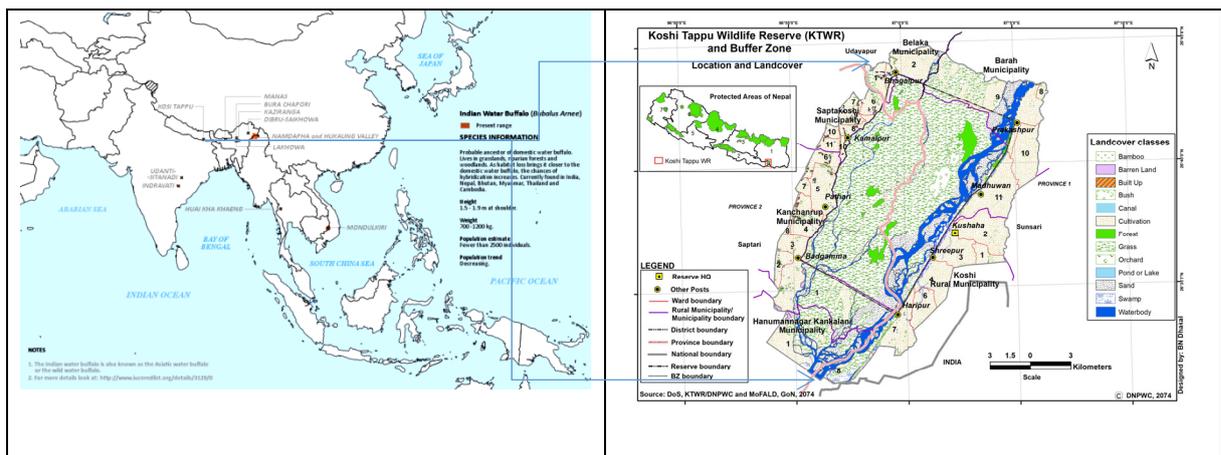


Figure 1. Location map of the study area, Koshi Tappu Wildlife Reserve, Nepal

The southern boundary of the Reserve runs parallel to the Koshi Barrage (the Barrage is almost 6.5 km away from the southern boundary of the wildlife Reserve). The Barrage was constructed on the river at the Indo-Nepal border between 1958 and 1962 by the Indian Government under

an agreement with Government of Nepal for the purposes of flood control, irrigation and generation of electricity. The area between the Barrage and the southern boundary of the wildlife reserve is submerged and leased to the Indian Government for the purpose of an irrigation project in the Sapta Koshi River. The eastern and western boundaries of the reserve run along the 5-7 m high eastern and western embankments almost parallel. Both embankments bound the river floodplain, thereby preventing water from flooding agricultural fields during the monsoon. The northern boundary runs through the floodplain from the eastern embankment near Prakashpur to the village of Tapeshwori, north of Trijuga River in the west.

Koshi Tappu is rich in biological resources mainly related to wetlands which support a large number of plant and animal species, among which water birds and fishes are more prominent. Vegetation is structural and functional backbones of a wetland ecosystem as it provides habitat, food, and energy to its faunal and microbial communities. Altogether, 236 angiospermic plant species have been recorded from Koshi Tappu (Sah, 1997).

CLIMATE AND SEASONS

The average daily maximum temperature of Koshi Tappu area ranges between 23⁰C to 34⁰C, and the minimum between 16⁰C and 29⁰C (Annex 1). The rise in temperature starts from pre-monsoon (April) and lasts until May, and lowers from October to January. The area has the sub-tropical monsoonal climate, the average annual rainfall ranges between 1,300 mm and 2,051 mm and 80-85% rainfall occurs during the monsoon period from mid-June to late-September. The humidity of the Koshi Tappu region remains higher almost all the year round varying between 75% and 95% (Sah, 1997).

The reserve experiences clearly three main distinct seasons. Summer (February through May) is intensely hot (Sah, 1997) with minimum precipitation. Ambient temperatures sometimes can reach up to 40⁰ C. The monsoon starts in late May/early June and lasts until September bringing heavy and frequent rainfalls. The rainfall is highest during July but high humidity and temperatures are experienced throughout the season. Winter (October through January) is characterized by clear skies and moderate temperature, but can still get quite cold.

SUMMER SEASON

Summer season ranges between February and May. The average daytime temperature of this season is 36.4⁰C. The average maximum and minimum temperature (during 2000 – 2010) of summer season recorded is 32.2⁰C and 16.5⁰C respectively with the average rainfall of 82.6 mm per month. Relative humidity of the area is lowest in March. Sporadic storms associated with strong wind, hail and rains occur from April to May. Some years scattered rain with the storm and some years with intense shower indicating the forthcoming monsoon that starts from May (Sah, 1997).

MONSOON SEASON

Monsoon season ranges between June and September. The south-easterly winds carry intense rainfall resulting in heavy flooding in the plain area including streams and rivers. The average maximum rainfall receives in July while it peaks up from June and slows down in September. There is sometimes westerly scattered winter rain with 25.3 mm rainfall per month. However, average rainfall occurred at 445.1 mm per month. By the end of September frequency of rain begin to decline so as of temperature.

WINTER SEASON

Cool and dry winter period often prevails during October to January. The average day and night time temperatures reduced to 26.8⁰C and 13⁰C respectively. Night time is damp and cold having heavy fog that ends up in the late morning. There is not or only very few rainfalls in this season.

TOPOGRAPHY AND LAND USES

Koshi River is a perennial water source for all the animals and water birds. The KTWR is rich in geo-morphological settings and a wide range of wetlands. Ponds and wetlands, in and around the reserve, serve as a source of water for wetland birds and others wildlife, making KTWR ideally suitable habitat for a variety of wild animals.

FLORA AND FAUNA

Koshi Tappu is rich in biological resources mainly related to wetlands which support a large number of plant and animal species, among which water birds and fishes are more prominent. Vegetation is structural and functional backbones of a wetland ecosystem as it provides habitat,

food, and energy to its faunal and microbial communities. Altogether, 236 angiospermic plant species have been recorded from Koshi Tappu.

The vegetation of KTWR is mainly composed of tall grasses, and there are also small patches of Khair-Sisoo scrub forest and deciduous mixed riverine forest. The reserve has important habitat for a variety of wildlife. The reserve is also home to around 20 other mammalian species such as spotted deer, barking deer, hog deer, wild boar, blue bull, small cats; and rock python is also pervasively-sighted.

Around 485 species of birds (14 endemic species) have been recorded, including 20 duck species, 2 Ibis species, White-tailed stonechat, striated marsh warbler, 30 shorebirds, 114 water birds, and the endangered swamp partridge and Bengal Florian. The Koshi Barrage is an extremely important resting place for many migratory birds, containing 87 winter and Trans-Himalayan migratory species. The Koshi River is home to 80 species of fish. The endangered Gharial crocodile and Gangetic Dolphin have been recorded in the river as well. During winter, many of the migratory birds can be seen on the Koshi barrage and on the river channel. Migration is evident at peaks in mid-March (DNPWC, 2018).

FOREST AND GRASSLAND

The vegetation of KTWR is mainly composed of tall grasses, and there are also small patches of Khair-Sisoo forests and deciduous mixed riverine forests. The main vegetation types found in the study area include tall and short grasses such as *Saccharum spontaneum*, *Imperata cylindrica*, *Phragmites karka*, and Sedges, and tree species such as *Dalbergia sissoo*, *Acacia catechu*, *Trewia nudiflora*, and *Bombax ceiba* (Sah, 1997). The reserve has important habitat for a variety of wildlife. Various types of vegetation communities provided a suitable home to several herbivores including wild buffaloes (*Bubalus arnee*), spotted deer (*Axis axis*), barking deer (*Muntiacus muntjak*), hog deer (*Axis porcinus*), blue bull (*Boselaphus tragocamelus*), small cat species, and reptiles such as Rock Python (*Python* sps), turtles species, etc which are pervasively sighted over the floodplain of KTWR (DNPWC, 2018).

For the convenience of research work, the whole Reserve area was divided into two parts: one in the east and the other in the west of the Sapta Koshi River, then again each of them was divided into north and south blocks as follows:

Eastern Blocks: East to Sapta Koshi River that includes South-East Block (Haripur-Kusaha area); and North-East Block (Madhuwan-Prakashpur Side including Sri Lanka Tappu); and

Western Blocks: West to Sapta Koshi River that includes South-West Block (Badgama-Jagatpur area down to Pathari Range Post) and North-West Block (Pathari side-Kamalpur-Bhagalpur area) above Pathari Range Post.

CHITWAN NATIONAL PARK

CNP (952.23 sq km), established in 1973 to preserve unique habitat of Tarai ecosystem and to protect some of the most endangered wild animals such as rhino, tiger, elephant, gaur, gharial, lies between N 27°20'19' and 27°43'16' longitude and between E 83°44'50' and 84°45'03' latitude on the floodplain of the confluence of Rapti and Narayani rivers in the lowland of central Nepal (Fig. 2). The area is located in the tropical monsoonal climate with average annual rainfall between 2600mm. In 1987, Bees Hazari Lake in its buffer Zone has been declared as Ramsar Site, a wetland of international significance so it's been special for a habitat of waterfowl and aquatic animals. The government of Nepal has declared the buffer zone (728 km²) surrounding the Reserve in 1996. The area is extended from east to west from Harda stream in north-east and Amuwa stream in the south-east to Daunne and Tribeni beyond Narayani River in the west, and the elevation ranges from 150 m to 815 m amsl. It is drained by Rapti, Reu and the Narayani River, a major tributary of the Ganges. The average daily maximum temperature of CNP ranges between 25°C to 35°C, and the minimum between 15°C and 25°C. The temperature rises from pre-monsoon and lasts until May, and lowers from October to January (Annex 1). Almost >80% of rainfall occurs during the monsoon period from June through September. Humidity of the area remains higher almost all the year round varying between 75% and 100%.

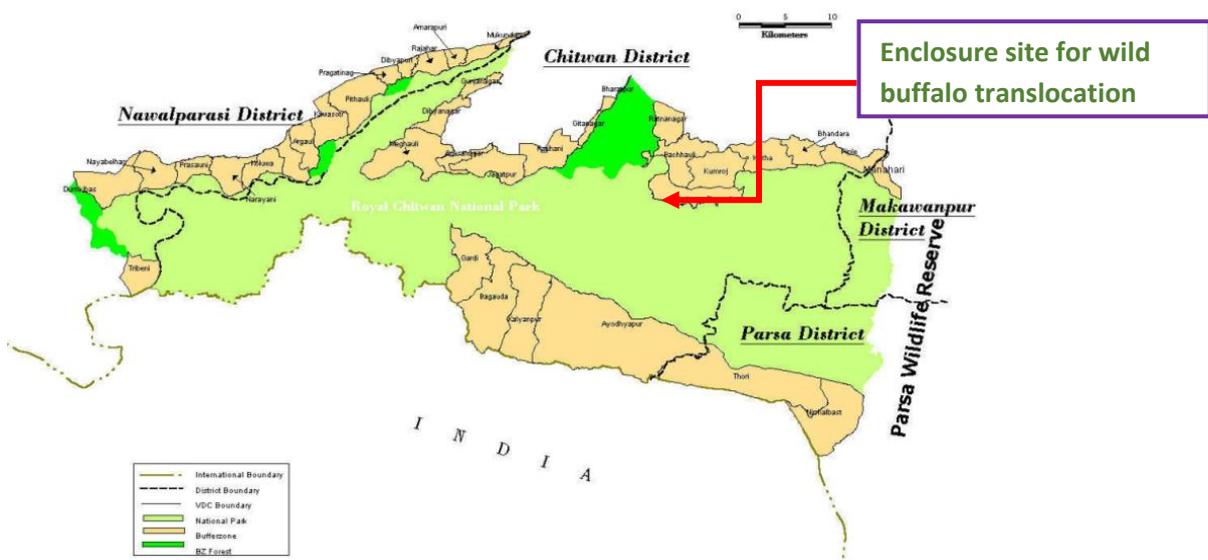


Figure 2. Location map of the Study Area at CNP, Nepal

The vegetation of CNP is mainly composed of almost 70% of Sal forests including huge stretches along the Chure hill, short to dominant tall grasses (Lehmkuhl, 1993; Peet *et al.*, 1999) mainly composed of *Saccharum spontaneum*, *Imperata cylindrica*, *Narenga porphyrocoma*, *Fragmites karka*, *Saccharum bengalensis*, *Chrysopogan aciculatus*, *Themeda arundinacea*, *Arundo donax*, there are riverine mixed forests and deciduous mixed forest along the river belts, and Chure forests mainly comprised of Pine forest. The Park has important habitat for a variety of wildlife. There is home to many of the important wildlife such as greater one-horned rhinoceros (*Rhinoceros unicornis*), tiger (*Panthera tigris*), Asian wild elephants (*Elephas maximus*), sloth bear (*Melurus ursinus*), gaur (*Bos gaurus*), sambar deer (*Cervus unicolor*), spotted deer (*Axis axis*), barking deer (*Muntiacus muntjak*), hog deer (*Axis porcinus*), wild boar (*Sus scrofa*), blue bull (*Boselaphus tragocamelus*), python (*Python* species), gharial (*Gavialis gangeticus*), mugger crocodile (*Crocodylus palustris*), Gangetic dolphin (*Platinista gangetica*), etc (CNP, 2015). The Narayani River is also home to 120 species of fish and around 576 species of birds including 22 globally threatened species recorded so far from CNP.

Padampur area of an eastern sector of CNP was selected for the reintroduction of a small population of Asian wild buffalo from KTWR. This area was encroached and had become a permanent human habitation for almost more than 30-years and it was accomplished to evacuate by 2003.

CHAPTER-IV

HABITAT COMPOSITION OF ASIAN WILD BUFFALO (*Bubalus bubalis arnee*) IN KOSHI TAPPU WILDLIFE RESERVE, NEPAL

Asian wild buffalo competes with cattle, domestic and feral buffaloes for food and space (Heinen & Srikosamatara, 1996; Kandel *et al.*, 2014). This issue is acute in KTWR, and the habitat of wild buffalo has severely encroached for several reasons in recent time (Heinen, 1993; Heinen & Kandel, 2006). But how its original habitat has been altered over time due to natural and human-induced factors is not well understood. In this contextual circumstance, a detailed study of wild buffalo habitat with a special focus on habitat composition (Kandel & Jhala, 2008) for the existing population is indispensably important. Therefore, this study was conducted in Koshi Tappu Wildlife Reserve, Nepal which covers the floodplain of the Koshi River, to examine or characterize habitats available for Asian wild buffalo. The aim of this study was to explore the habitat composition of Asian wild buffalo (*Bubalus bubalis arnee* Kerr, 1792) at KTWR. A detailed habitat study would also provide forward motion to a long-term conservation strategy of this species, including the urgency of translocation to alternative habitats. KTWR also has a severe problem of human-wildlife conflict for several reasons (Heinen, 2002), that requiring a long-term conservation strategy to adopt the restoration program for Asian wild buffalo in Nepal.

MATERIALS AND METHODS

VEGETATION SAMPLING AND ANALYSIS

Three parallel transects traversing north and south with the approximate distance of 2-3 km each were laid on the ground to represent all the segments divided by river courses throughout the whole reserve area including parts of buffer zone area. Plots were laid for the first and random-systematically allocated along each transect. Vegetation sampling was carried out in circular concentric sample plots (CSP) (Yadav, 1988; Yadav & Chalise, 2013). The whole area of KTWR was surveyed for identifying the habitat composition and vegetation analysis. Three north-south parallel transects extending up to 16 km long with 2-3 km apart representing each habitat type available in the reserve such as – trees, understory vegetation (shrubs/saplings), and grasses/herbs – that were laid in alongside of the Sapta Koshi River. Circular sample plots of 10m radius for trees were laid out along each transect at every 500m interval. Collectively, a total of 76 sample plots were allocated following a systematic random sampling on each of these

transects. The total time (hours) invested for the field survey to fulfill the objective of vegetation study was 96 hours (3 Transects*4 Days per transect*8 hrs per day).

The areas of the circular plots were measured with the help of measuring tape and DBH of the trees with the help of diameter tape. Information on tree characteristics (local name, scientific name, DBH, height, etc.) was recorded. Plots with 5m radius were laid out to collect information on shrubs/saplings in each CSP. Square plots of 1m² sizes were laid out at the north and south cardinal directions of each CSP for grass and other herbaceous plants (Shrestha *et al.*, 2015). For the shrubs/sapling, information such as local name, and scientific name for each species were collected, and for herbaceous species and grass, information such as local name, scientific name, and area covered by each species were collected. However, if no tree species is recorded in a CSP, only one 1m² square plot was used to collect information on herbs and grasses.

VEGETATION ANALYSIS

Vegetation data sampled in the circular plots and quadrates were analyzed for vegetation types using general ecological principles as frequency, density, dominance, important value (IV), and other analysis where appropriate. Habitat conditions based on the canopy cover and ground cover was also studied, and habitat was categorized based on the ecological principles and biophysical occurrences. The frequency of each species was analyzed by dividing the number of quadrats in which the specific species occurred by a total number of quadrates. Similarly, the percentage ratio of the frequency of particular species with the sum of frequencies of all species was presented as relative frequency is calculated as given in Annex 2.

Similarly, the density which represents a number of individuals per unit area is calculated by dividing the number of individuals of particular species by total area (ha) of the studied quadrates. Relative density was the percentage ratio of the density of one species with the sum of densities of all species. Dominance, amount of ground covered by the tree trunk, was the ratio of the total basal area of particular species with the total area (ha) of the studied quadrates. The relative amount of ground covered by the tree trunk called as relative dominance was the percentage ratio of the dominance of particular species with the total dominance of all species. Importance values of each species of trees were analyzed by adding relative frequency, relative density, and relative dominance. Name of each forest type was determined from the sequential order of the large Importance Values (IV) of tree species (Zobel *et al.*, 1987). Prominence value (PV) was calculated for shrubs and ground flora such as grasses and herbaceous species. Prominence values were calculated by the multiplication of mean percentage covers of particular

species with the square roots of its frequency. These values were used for classification of the shrub and ground vegetation type from the sequential order of large PV (Sharma *et al.*, 2012). The specific formulae that are employed for the vegetation analysis are mentioned in Annex 2.

RESULTS AND DISCUSSIONS

VEGETATION ASSESSMENT

A total of 181 sample plots of various sizes covering vegetations of different life forms, such as circular plots of 10 m radius for sampling trees, 5 m radius plot within the tree plots for sampling shrubs, and 1m² quadrates to survey herbaceous species were used to collect vegetation data. The detailed analysis of the vegetation recorded in circular and square plots are illustrated in the Annexures 3-11. The maximum total numbers of sample plots were recorded in *Sissoo-Khair* forest (n=100) and lowest in shrubland (n=8) (Table 1).

Table 1. Number of studied sample plots in KTWR

Vegetation types	Number of sample plots and quadrat			
	Trees (in 10m radius circular plots)	Understory (shrubs/sapling) (5m radius)	Ground vegetation (grasses/herbs) (1m ² quadrat)	Total no. of sample plots
<i>Sissoo-Khair</i> forest	25	25	50	100
Mixed riverine forest	8	8	16	32
Shrub land	2	2	4	8
Grassland			41*	41
Total	35*	35*	111	181

* Out of 76 circular sample plots surveyed for trees and shrubs/saplings, only 35 sample plots have trees and shrubs/saplings, but 41 sample plots only have grasses/herbs on them. In each of the 41 sample plots, we assessed the composition of grasses/herbs based on only one 1 m² quadrat.

VEGETATION TYPES

Forest (*Sissoo-Khair* forest and mixed riverine forest), shrubland and grassland are the major vegetation types recorded from the whole area of KTWR. From the phyto-sociological analysis, four major vegetation communities are categorized in this study. They are *Sissoo-Khair* forest, mixed riverine forest, shrubland, and grassland. On the basis of plot location, a large proportion of KTWR area is covered by grassland (both tall and short) followed by *Sissoo-Khair* forest and only the smallest proportion is covered by shrubland (Table 2).

Table 2. Vegetation types and number of sample plots in KTWR

Types of vegetation	Number of plots (in percentage)
Sissoo-Khair forest	25 (33%)
Mixed riverine forest	8 (11%)
Shrubland	02 (03%)
Grassland	41 (54%)
Total	76

Less assessed types of vegetation with two broad categories called riverine forest and grassland/Savannah was described by the previous study in KTWR (Sah, 1997). Based on the floristic associations following Sah (1997), vegetation types have been categorically classified into four major types in this study is as follows:

a. Sissoo-Khair Forest

Among the sample plots, *Sissoo-Khair* forest was observed in 40.7% (35 out of 76) of sample plots which are predominant in the eastern part of the Reserve. This forest included a total of 444 trees stems per hectore. Twelve species of tree mainly comprises this forest type, in which *Dalbergia sissoo* is more important species with the highest importance value (IV = 179.1) (which shows the ecological importance) followed by *Trewia nudiflora* (IV = 42.8) (Table 3).

Table 3. Tree dominancy in *Sissoo-Khair* forest (in percentage)

Species	Relative Frequency	Relative Density	Relative Dominance	Importance Value
<i>Dalbergia sissoo</i>	42.3	61.0	75.77	179.1
<i>Trewia nudiflora</i>	21.2	11.5	10.19	42.8
<i>Acacia catechu</i>	13.5	20.3	6.04	39.8
<i>Bombax ceiba</i>	3.8	1.1	6.76	11.7
<i>Strablus asper</i>	3.8	1.7	0.41	6.0

This forest was further categorized into three different vegetation types such as *Sissoo-Khair* forest, Riverine forest, and Grassland. In terms of tree composition, both current and previous studies (Sah, 1997) found similar results in which *A. catechu* was of higher IV (112.4) followed by *D. Sissoo* (108.18). It is examined that *Acacia catechu*, *Bombax ceiba*, *Dalbergia sissoo* and

Trewia nudiflora were the most common and dominant tree species recorded from both the studies. The presence of the other two species viz. *Bombax ceiba* and *Strablus asper* are associated with a change in soil type (Puri, 1960).

There are 33 species in the understory layer of this type of forest. Among these species, *Clerodendron viscosum* is the most prominent one (PV = 25.79) followed by *Lantana camara* (PV = 12.85). The ground vegetation of this forest indicates the high diversity of species (n = 34), with invasive alien *Mikania macarantha* being the most prominent species (PV = 51.7) followed by *Imperata cylindrica* (PV = 41.5) (Table 4).

Table 4. Prominent shrub and herb in Sissoo-Khair forest

Shrubs	Prominence value
<i>Clerodendron viscosum</i>	25.79
<i>Lantana camara</i>	12.85
<i>Pogostemon bengalensis</i>	3.20
Herbs/grass	Prominence value
<i>Mikania macarantha</i>	51.70
<i>Imperata cylindrica</i>	41.50
<i>Dryopteris cochleata</i>	16.11

b. Grassland

Grassland is found to occupy the highest portion (54%) of the study area in KTWR. A total of 32 species of plants were identified, of which *Saccharum spontaneum* (PV = 282.57) and *Imperata cylindrica* (PV = 168.79) were the most prominent grasses in the grassland community of the Koshi Tappu region (Table 5).

Table 5. Prominent grasses/herb in the grassland

Species	Prominence value
<i>Saccharum spontaneum</i>	282.57
<i>Imperata cylindrica</i>	168.79
<i>Dryopteris cochleata</i>	14.41
<i>Cyanodon dactylon</i>	8.82
<i>Citronella species</i>	6.93

In the dry and heavily grazed areas of KTWR, *Citronella* species is the co-dominant, whereas *Cyanodon dactylon* is the co-dominant grass species in the moist southern floodplain area. This condition was also observed by the previous study (Sah, 1997) and found that *Phragmites karka* was the co-dominant, while *Cymbopogon* becomes the co-dominant in some dry area. Similar situations have been observed in Chitwan and Bardiya National Parks (Dinerstein, 1979). The changing trend might have been the effect of river dynamics of Sapta Koshi (Sah & Suselo, 1996), excessive grazing and high floods.

c. Mixed riverine forest

This forest occupies 11% area of the Koshi Tappu Wildlife Reserve. It is mostly disbursed in the southern and western part of the reserve. The forest included 274 stems of tree per hectare. Among 11 reported tree species of this forest, *Trewia nudiflora* is more dominant (IV = 140.6) followed by *Bombax ceiba* (IV = 50.2) (Table 6).

Table 6. Tree dominancy in the mixed riverine forest (in percentage)

Species	Relative Frequency	Relative Density	Relative Dominance	Importance Value
<i>Trewia nudiflora</i>	17.6	68.1	54.8	140.6
<i>Bombax ceiba</i>	17.6	4.3	28.2	50.2
<i>Dalbergia sissoo</i>	17.6	4.3	7.2	29.2
<i>Kydia calycina</i>	5.9	2.9	5.1	13.9
<i>Acacia catechu</i>	5.9	4.3	1.4	11.6

Total Area of Mixed riverine forest was found to increase from 2.9% in 1997 (Sah, 1997) to 11% in 2015 from the current study. The dominancy of the *Trewia nudiflora* is found to be the highest (PV=140.6) in this study compared to *Bombax ceiba* as the dominant (IV=98.72) tree species found in the earlier study (Sah, 1997). It is likely that the deposition of silt materials on river bed (Sah & Suselo, 1996) led to the formation of humus along the river bank over a time, and resulted to a higher rate of moisture retention. As a result, the composition of Riverine mixed forest might have changed during the past decades, leading to the dominance of *Trewia nudiflora* over *Bombax ceiba*.

Understory layer of mixed riverine forest included 16 plant species. Among all the recorded species, invasive alien *Lantana camara* (PV = 7.3) and *Clerodendron viscosum* (PV = 3.3) are the two prominent species in the understory layer. The ground vegetation of mixed forest comprised 23 plant species, primarily composed of *Imperata cylindrica* (PV = 224.3) and *Dryopteris cochleata* (PV = 127.7) (Table 7).

Table 7. Prominent shrub and herb in mixed riverine forest

Shrubs	Prominence value
<i>Lantana camara</i>	7.3
<i>Clerodendron viscosum</i>	3.3
<i>Urena lobata</i>	1.3
Herbs	Prominence value
<i>Imperata cylindrica</i>	224.3
<i>Dryopteris cochleata</i>	127.7
<i>Saccharum spontaneum</i>	96.8

d. Shrubland

Shrubland, as one of the vegetation types in this study, is an additional category compared to the previous study (Sah, 1997). It was observed in 2 out of 76 sample plots in the Reserve. In this habitat type, 6 plant species are recorded in the understory, of which *Zizyphus jujuba* (PV = 37.5) and *Clerodendron viscosum* (PV = 1.1) are the more prominent species (Table 8). There are 11 ground vegetation species found in the shrubland with *Imperata cylindrica* (PV = 38.97) and *Dryopteris cochleata* (PV = 17.71) being the 2 most prominent species (Table 8).

Table 8. Prominent shrub and herb in shrubland

Shrubs	Prominence value
<i>Zizyphus jujuba</i>	37.5
<i>Clerodendron viscosum</i>	1.1
<i>Solanum xanthocarpum</i>	0.2
Herbs	Prominence value
<i>Imperata cylindrica</i>	38.97
<i>Dryopteris cochleata</i>	17.71
<i>Cynadon dactylon</i>	5.00

Based on the inventory data, 12 and 11 tree species were recorded from the Sissoo-Khair and mixed riverine forest of KTWR respectively. The density of tree in Sissoo-Khair and mixed riverine forests were 444 and 274 tree/ha respectively. In understory vegetation, the number of recorded species was higher in Sissoo-Khair forest (n = 33) than mixed riverine forest (n = 16) and shrubland (n = 6). The number of species in the ground vegetation is higher in Sissoo-Khair forest (n = 34) followed by grassland (n = 32) which implies that the forest area is more

productive shelter to grow understory vegetations. This has indicated that Sissoo-Khair forest is the most diverse than other vegetation types in term of species richness of trees, understory and ground flora.

The average tree height of Sissoo-Khair forest was 12.0 ± 8.6 m with 15.4 ± 10.9 cm of average DBH. Similarly, average tree height of mixed riverine forest was 14.1 ± 11.1 m with 22.3 ± 19.2 cm of average DBH (Table 9).

Table 9. Vegetation characteristics and other parameters of the studied forests

Characteristics/ parameters	Sissoo-Khair forest	Mixed riverine forest	Grassland	Shrubland
<i>Structural categories of vegetation</i>				
Tree	12	11	--	--
Understory	33	16	--	6
Ground cover	34	23	32	11
<i>Measured parameters</i>				
Average height of tree (m)	12.0 ± 8.6	14.1 ± 11.1	--	--
Average DBH (cm)	15.4 ± 10.9	22.3 ± 19.2	--	--
Area covered (%)	33	11	54	3
Tree density (number/ha)	444	274	--	--

FOREST COMPOSITION

Floral species richness with their community mosaics resulted in KTWR as prime habitat for the wild Buffaloes. Floral associations provided four vegetation types with grassland communities being the most prominent habitat in KTWR. The Sissoo-Khair and mixed riverine forest associated with the ecotonal habitats are most preferably used by this wildlife. For conserving overall biodiversity of KTWR, the presence of large ungulates such as Asian wild buffalo is considered to play the most important role to increase ecological functional values of KTWR by being optimally and architecturally used. The currently available structural pattern of vegetation community in the Koshi Tappu region suggests that this area is very appropriate biological ground to provide a best primarily adaptive environment for carrying out various activities by wild buffaloes such as foraging, ranging, resting, wallowing, etc. Moreover, the existence of different vegetation communities including the mixed types of young forests, increased proliferation of *Trewia nudiflora* and a vast range of grassland predominantly *Saccharum spontaneum* shows the signs of habitat suitability for other megaherbivores including rhinoceros, elephant, and swamp deer. So the Koshi Tappu area could be one of a preferable alternative site

for creating rhino population if the area could be extended towards foothills of Churiya range (Lower Young Mountain) in the north of KTWR and upto Koshi barrage in the south, and through the strict law enforcement along with community incentives to encourage participatory conservation and local stewardship. The potential area of extension could be free from monsoon floods and would become safe habitat during the flood season though the area needs immediate actions for the active management and to avoid invasion by the alien species, wherein grasses are more prominent with higher species richness due to being associated with the habitats of the core area. The area would be the resultant of the extended prime habitat for wild buffaloes and potential seasonal ground for other migratory species from mountains and vice versa. Asian wild buffalo, the pride for the local communities, has the potential role to back up themselves for their social, economic and biological benefits which would be crucial for the survival of this beast as well.

Koshi Tappu has also the potential value of developing a site as a cushion of adjusting possible impacts of climate change in the Tarai region since the huge areas of this range have been deteriorated with the onset of extended settlements and hilly migration since after the Malaria eradication in early 1960s. So the blanket approach of habitat extension for the wildlife corridor and connectivity (Kandel, 2012) would be instrumental for the improvement of multi-dimensional ecological importance and socio-economic potentials. The value of PV for various plant species supports the importance of biological values of the vegetation communities. Due to the frequency of river dynamics every year, vegetation composition and structure have been so fragile which is unpredictable for satisfying the requirement of wild buffaloes. On the other hand, there are thousands of cattle and buffaloes that are abandoned or taken to the Wildlife Reserve and fringes every day have radically slowed down the survival and growth of the wild buffalo population in terms of space and forage because heavy cattle grazing reverts the succession (Bakker, 1985). This is the resilient to consider why the close monitoring of habitat, impacts of management interventions, and translocation to the best alternative habitats of other protected areas could only support the population of wild buffaloes to survive in a long run.

CHAPTER-V

POPULATION AND DEMOGRAPHY OF ASIAN WILD BUFFALO (*Bubalus bubalis arnee* Kerr, 1792) IN KOSHI TAPPU WILDLIFE RESERVE, NEPAL

Genus *Bubalus* was widely distributed in Europe and Southern Asia during Pleistocene period, but later restricted to Indo-China and south-east Asian islands (Fahimuddin, 1975). Buffalo was reintroduced in Europe from the east in its domesticated form (Cockrill, 1974). Water buffaloes are broadly classified (MacGregor, 1941) into two types: swamp buffaloes and river buffaloes; whereas swamp buffaloes are distributed in south-east Asia extending northward to Yangtze valley in China and westward to Assam in India; these are more closure to wild-type. Swamp types prefer swampy grasslands or marshlands in contrast to the river type which (river type) had natural habitat of river valley and clean river water, hence, called as River buffaloes. These buffaloes are distributed in the countries mostly to the west of Assam.

Asian wild buffalo normally maintains stable home range but is found to exhibit seasonal movement within different habitat ranges. They are a social animal and live in a female-dominated herd of varying sizes. Generally, each herd includes one or two adult males with 30-40 females with calves and many of the sud-adults. Largest group size of the wild buffaloes counted as high as 90-100 animals (Heinen, 2002) with an alpha male. Bachelor males are also evident in the herd. Physical structures of Asian wild buffaloes are morphologically robust with strong musculature and typical horns; bulls are even larger and stronger. Adult bulls are mostly solitary except temporal coalition of sub-adults in many incidences.

In Koshi Tappu Wildlife Reserve (KTWR), a prime habitat of Asian wild buffalo, census of this species was started from 1976 and repeated by 1987 and 1988 followed by periodic censuses after 2000. Koshi Tappu is highly prone to anthropogenic and natural threats such as habitat invasion by feral and domestic buffaloes and cattle, annual flood, invasion by alien weeds, etc. The overlapping habitats in the habitats of KTWR with feral and domestic buffaloes provided chances of cross-breeding that increases risk of genetic erosion of wild breed. The data collected during these censuses have been considered to understand the status and trend of population growth, demographic structures, effective population size, and rate of loss of genetic variation to evaluate the biological basis for the sustenance of this endangered species and to conceive future conservation plans. Therefore, the present study would likely to contribute for devising the

thoughts of long-term conservation and management of this endangered species due considering scientific principles and its critical habitat through assessment of population size, demographic structure and growth trend of the species over a period (1976-2016).

MATERIALS AND METHODS

CENSUS TECHNIQUES

Wild buffaloes are primarily restricted in their occurrence to floodplain grasslands, savannah, and riverine mixed forest; and rarely venture away from water sources (Dinerstein, 1991; 2003). To study the population of wild buffalo, the whole area of the reserve was divided into different blocks and used sweeping with detail count method (Kandel *et al.*, 2014) in order to avoid the likelihood of missing out any of the animals. Chances of sighting wild buffaloes over all the habitat types at KTWR were fair. For carrying out the wild buffalo census using detail count method, the total area of KTWR was broadly divided into two segments: east and west from the main course of Sapta Koshi River; each segment was further divided into two separate blocks, north and south blocks. The division of blocks in KTWR for the census work was as follows:

a. Eastern Blocks: East to Sapta Koshi River:

South-East Block: Haripur-Kusaha area

North-East Block: Madhuwan-Prakashpur side including Sri Lanka Tappu

b. Western Blocks: West to Sapta Koshi River:

South-West Block: Badgama-Jagatpur area- south from Pathari Range Post

North-West Block: Kamalpur-Bhagalpur area north from Pathari Range Post

During the census, the animals were closely observed to distinguish and clear identification of their ages and sex. Tracking the animals from four-wheel drives, elephant back and on-foot observation were applied for the census in order to maximize animal sightings. Elephants were especially used in habitats predominated by tall grasses and swamps to improve animal detection in case of dense vegetation. In each block, team members were assigned at a visible distance of about 50-100 meters. Experienced and trained field technicians were involved for the census work. GPS units, binoculars of 7x35 magnification, and digital cameras were used to locate and identify each herd and solitary males. Direct observation was used to count and observe each individual. Field census was carried out in two sessions: 0700-1100 hours in the morning and from 1500 hours until visibility permitted. Morphometric and phenotypic characteristics were referred to distinguish wild buffaloes with feral backcross and domestic buffaloes following

Cockrill (1974), and Heinen (2002) methods. The time (hours) spent in the field to fulfill the objective of census work of wild buffaloes and to studying population and demography was 80 hours (5 days for each of the 2 censuses - in 2014 and 2016 (8 hours a day for each census i.e. 40 hours*2 times).

AGE AND SEX IDENTIFICATION

The criteria for identification of the wild buffaloes, their age and sex were used as described by Dahmer (1978), Heinen (2002), Heinen and Singh (2004) and Heinen & Kandel (2006). Major criteria considered during the census were skin and pelage color, change pattern in coloration in male and female individuals, horn size and shape, herding behavior, etc. The animal age was determined using various criteria as explained below:

- **Young calves/Infants (<1 year):** buff in color.
- **Juvenile Calf (1-2 years):** darken buff color in the first year that gradually turns lighter in color in the second year.
- **Young Adult/Adult (>3 years):** the coat color changes to dark to very dark with noticeable whitish markings in different parts of the body. There are one or two white chevron marks on the underside of the neck. They have white hocks, white tail tips, and fine white markings around the eyes and along the sides of the nose and mouth.
- **White markings are more apparent in an adult age** which gradually fades out with age. Young females are smaller and the pelage retains the light coloration. Adult males are dark black, except for whitish markings as described above at prime breeding age (>4 years). Adult females have longer upwardly extended horns than males, but males have much thicker horns. A number of young calves <1 year of age and number of females in a population were taken into account for calculating calf to cow ratio. It is an index of the growing population.

POPULATION GROWTH RATE

Population growth rate of wild buffalo population in KTWR was calculated using exponential growth rate formula as adopted from Dahmer (1978), Heinen (1993a), Heinen (2002), and Heinen & Kandel (2006). Demographic structure and calves to cows' ratio were also calculated from the same data sets.

Exponential growth rate, $r_i = \frac{\ln(N_j/N_k)}{t}$

Where, r_i = Growth rate calculated for that time interval
 N_j = Population size at the end of the interval
 N_k = Population size at the beginning of the interval
 t = Number of years in the interval

EFFECTIVE POPULATION

The concept of the effective population provides an understanding of the potential population to grow further and consequences of isolated and small population size. The effective population size of Asian wild buffaloes in KTWR is calculated from original population data as described by Stiling (2002).

Effective population size (N_e) = $4(N_m \times N_f) / (N_m + N_f)$

Where, N_e = Effective population size,

N_f = No. of female individuals,

N_m = No. of male individuals

RATE OF LOSS OF GENETIC VARIATIONS

If an individual fails to mate to an individual possesses a rare gene, and the genetic information encoded in that gene may not pass on to the next generation that would ultimately result in loss of genetic variation from the population (Berger, 1990). This normally happens in the small and fragmented populations as of wild buffalo population in KTWR. The percentage ratio of population loss per generation (Stiling, 2002) of wild buffalo of KTWR is calculated using the following formula:

Percentage loss of genetic variation (per generation) = $(1/2N) \times 100$

Where N is the number of individuals in the population (population size)

RESULTS AND DISCUSSION

POPULATION STATUS AND DEMOGRAPHY

The last natural habitat of the Asian Wild Buffalo (*Bubalus arnee*) in Nepal is Koshi Tappu, the riverine floodplains of the Sapta Koshi River lie in the Eastern Tarai. The range of the wild buffalo in Nepal was once much more extensive all over the *Tarai* region. Asian wild water buffaloes were also reported to occur in Chitwan National Park (CNP), Nepal until the early 1960s (Spillett & Tamang, 1966; Seidensticker, 1975). They were exterminated from CNP as a result of diseases transmitted by cattle and buffaloes; while barring 20 to 30 individuals in the floodplain along the Koshi River in the eastern part of the country at that time (Spillett & Tamang, 1966).

Finding shelter in the riverine forests and forage, that is available in the floodplains, even while the monsoon water subsides, is the most preferable and ideal habitat for wild buffaloes in KTWR. Now, the range of the wild buffalo has been much restricted by the construction of the Koshi barrage along the Indo-Nepal border (Kandel *et al.*, 2014), and they are confined to the strips of Koshi Tappu due to the settlements developed all around the reserve area alongwith the establishment of KTWR. Similarly, other reasons are a yearly unpredictable shifting of the main course of the Koshi river, and also internal migrant (Chalise, 2016). Sometimes, the local domesticated buffaloes also mixed up with the wild breeds as feral buffaloes intermix with wild stocks in many instances but the study was precisely focused on the pure herds of wild population using precise identification criteria and by segregating them (wild and feral) into their separate natal herds.

The first census of Asian wild buffalo in KTWR was done in the year 1976 and counted were 76 animals in total, out of which there were 12 adult males, 18 adult females, 22 juveniles and 11 infants (Dahmer, 1978), and the population of this species found to increase gradually from the first census (Table 10). Two subsequent censuses of wild buffaloes were done in 1987 and in 1988 (Heinen, 1993b) and recorded were 91 and 93 individuals respectively. The census program of Asian wild buffaloes at KTWR after 2000 has been undertaken almost in every 2-5 years. The increasing population trend over the years suggests that there is exponential growth except in 2010 census; in which total animal counted were 215. In the year 2008, devastating flood in the Koshi River took a heavy toll of animals of all age groups. The population size in the year 2011 and onwards indicated a gradual increase in population (Table 10).

Table 10. Demographic structure of Asian wild buffalo over different census years

Years of Census	Adult Male	Adults Female	Sub-Adult Male	Young Female	Juvenile (2-yr)	Infant (1-yr)	Total	Calves/Cow ratios	M/F ratio
1976	12	18	NA	NA	22	11	63	0.611	0.67
1987	32	29	NA	NA	14	16	91	0.552	1.10
1988	37	33	NA	NA	8	15	93	0.455	1.12
2000	56	53	NA	NA	17	19	145	0.358	1.06
2004	54	63	NA	NA	24	18	159	0.286	0.86
2009	55	119	NA	NA	22	23	219	0.193	0.46
2010	57	108	NA	NA	24	26	215	0.241	0.53
2011	66	117	NA	NA	15	39	237	0.333	0.56
2014	47	125	64	44	32	38	350	0.304	0.38
2016	114	180	26	20	27	66	433	0.367	0.63

SEX RATIO AND CALVES TO COWS' RATIO

The latest census conducted during March-April 2016 indicated the increase of wild buffaloes to 433 individuals; where male and female ratio was 0.63 and calf to cow ratio was 0.36 (Table 10). Whereas in censuses in 2014 and 1988, male to female ratio was the lowest, in which 0.38 and highest was 1.12 in respective census years. This ratio has been gradually decreasing after 2000 and 2004 censuses (Kandel, 2004). Male to female ratio focuses on the fecundity rate of the wild buffalo population in KTWR, the sizes of the females need to be higher to adapt the overall population biologically more viable. This needs to have a long-term study on changing pattern of male to female ratio. Higher the number of female more would be the fecundity rate of the population which could be analyzed and predicted by evaluating sex ratio at different census episodes. High fecundity rate affects the calves to cow ratio; as higher as the ratio more would be the viability of the population. Calves to cow ratio were highest in 1976 (0.61) which was gradually declined thereafter and the lowest was 0.19 in 2009 (Table 10, Fig. 3) because the huge flood in 2008 affected population dreadfully. The consequence of flood has led to severe effect on the number of calves and young animals. Impact of that massive flood was also seen in the growth of the population. The overall trend of wild buffalo population growth over time is depicted in Table 11. Such downfalls of population sizes are highly prone to natural disasters such as flood, diseases, and other anthropogenic factors like hunting as well.

Table 11. Exponential growth rate (ri) of Asian wild buffalo at KTWR over the years

Years of Census	Total Population	Growth interval in Consecutive Years of Census	Exponential growth rate (ri)	Growth interval between first to each census	Exponential growth rate (ri)
1976	63	1976 to 1987 (r1)	0.033		
1987	91	1987 to 1988 (r2)	0.022	1976 to 1987 (r1)	0.033
1988	93	1988 to 2000 (r3)	0.037	1976 to 1988 (r2)	0.032
2000	145	2000 to 2004 (r4)	0.023	1976 to 2000 (r3)	0.035
2004	159	2004 to 2009 (r5)	0.064	1976 to 2004 (r4)	0.033
2009	219	2009 to 2010 (r6)	-0.018	1976 to 2009 (r5)	0.038
2010	215	2010 to 2011 (r7)	0.097	1976 to 2010 (r6)	0.036
2011	237	2011 to 2014 (r8)	0.018	1976 to 2011 (r7)	0.038
2014	350	2014 to 2016 (r10)	0.106	1976 to 2014 (r8)	0.045
2016	433	1976 to 2016 (r tot)	0.048	1976 to 2016	0.048

During the latest census of 2016, males group of single to many male band group of various sizes were noticed. The composition of the male band group and number of event/occasion they appeared during the field work are presented in table 12.

Table 12. The composition of the male band group observed at KTWR

Age Group of Males	Sizes of Male band	No. of events
Adult Male	1	37
	2	9
	3	2
Sub-adult Male	2	2
	3	6
	4	2
	5	3
	6	3
	7	1
	9	1
	10	1

Male to female ratio in wild buffalo population in KTWR was also inconsistent. There was a rise in male to female ratio in 1987 which was gradually declined till 2009 and again regained but looks like 'with zigzag turns' of escalation.

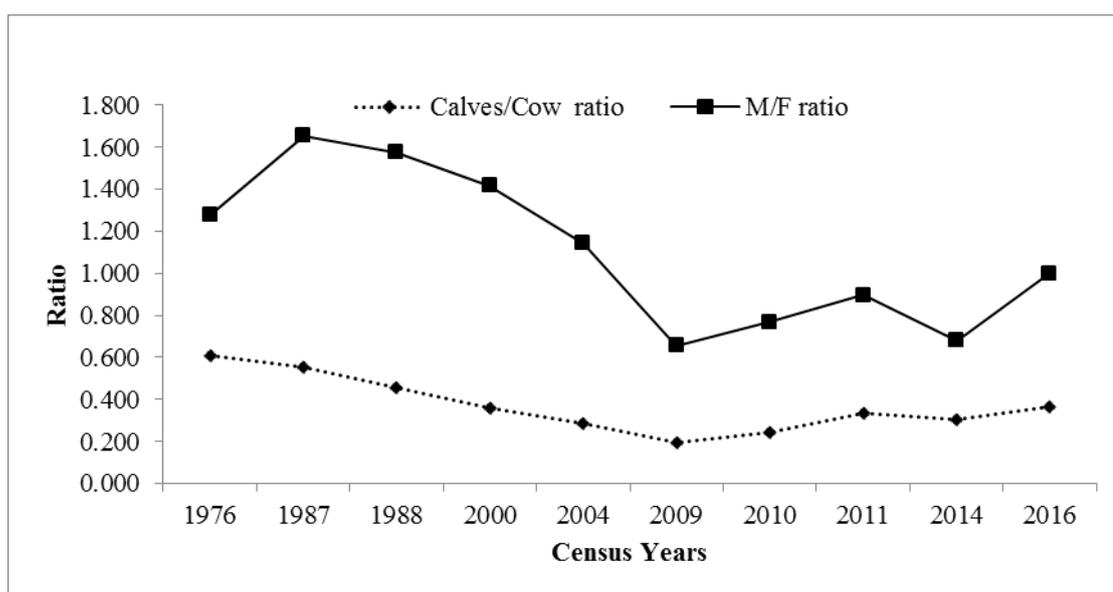


Figure 3. The pattern of male to female and calves to cow ratios of Asian wild buffalo in different census years

Flood had conferred uniform impact on both males: female and cow: calves ratios. There are some other factors in the area which had a longer impact on male: female ratio in spite of the overall trend of increase in population size. Gradual downfall of the calves to cows' ratio till 2009 was also evident which gradually increases in a similar manner as the male to female ratio. There is a strong correlation between male to female ratio and calves to cows' ratio as is

illustrated in Fig. 3. Correlation between male to female ratio and calves to cows ratio (Fig. 4) shows that they are closely related to each other (R^2 Linear = 0.099), which implies that the calves to cows ratio increases with increase in male to female ratio. That would have biological significance to maintain the viable as well as effective population.

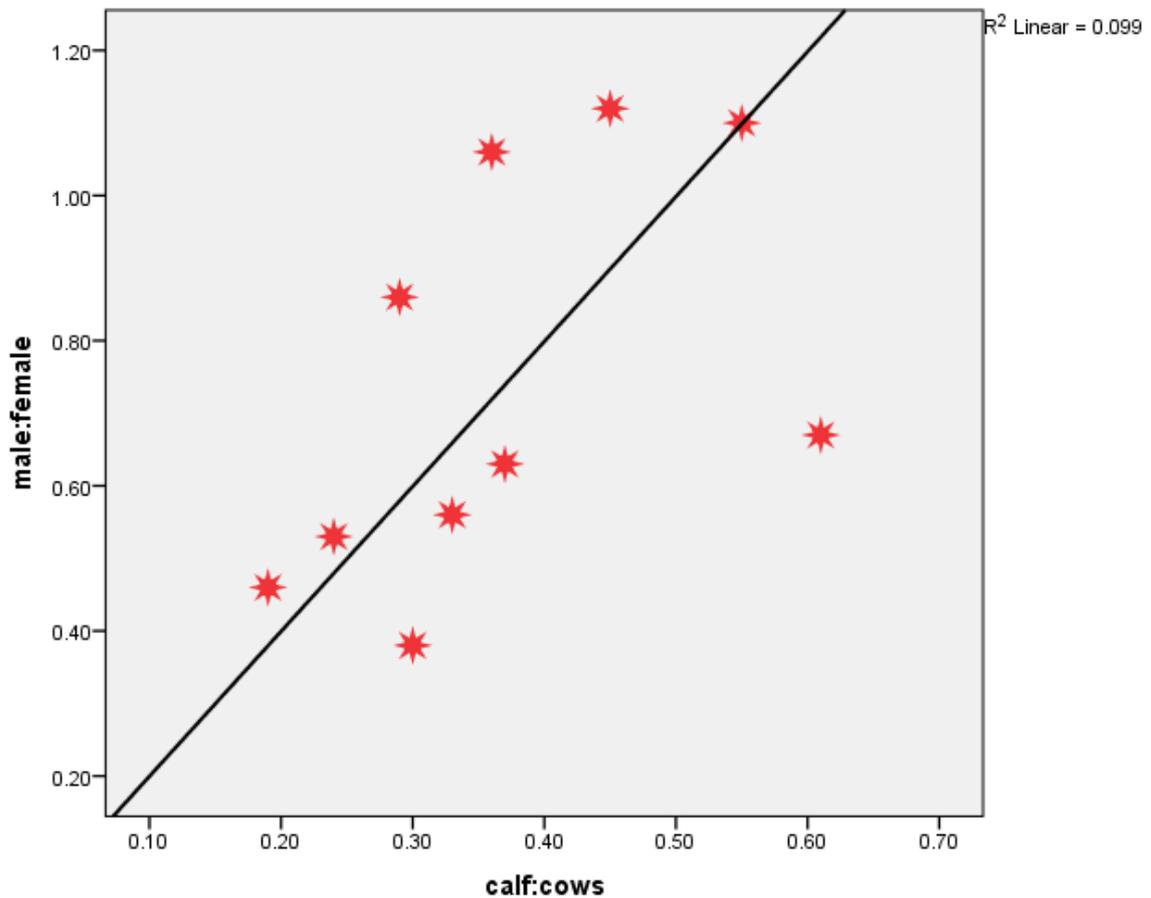


Figure 4. Correlation between male to female ratio and calves to cows' ratio

It is quite probable that some male individuals might be leaving the herd and joining male band population. These individuals could be sub-adult males which are a failure of triumph to mate females of the natal herds. Therefore frequencies of male band size in the sub-adult category is large (Table 12). It reflects that there are strong hierarchy and pre-dominance of alpha male amongst the herds of wild buffalo population. The main course of River Koshi is found to be very dynamic and always learning of change almost every year affect the occupancy of home range by wild buffaloes resulting to solitary or drive the males to leave and be scattered from the natal herd (Dahmer, 1978) after entering the age of sub-adulthood. Extensively large natal herd also gets split due to such a dynamics of Koshi River. Splitted herds are reported to occupy different territories other than natal herds - such as they are likely to abandon the herd of the south block at Haripur Island and shift to south-west part across the Koshi River to small herds.

POPULATION GROWTH

Population size and its composition would help to determine the growth rate. The current population size of the Asian wild buffalo at KTWR is highest in comparison to previous censuses. The overall population growth (ri) over past 40-years (1976 to 2016) is 0.048, while growth rate between 1976 and 1987 was 0.033 and between 1988 and 2000 was 0.037. The population growth rate was the highest from 2011 to 2014 (0.106). It is striking to mention that there was negative growth (-0.018) recorded in 2009 (Table 11, Fig. 5) as the populations of wild buffalo bounced back after decimating impact of flood in 2008 which was discouraging; while the average rate of growth (0.048) during 1976 and 2016 shows an imperative inclination towards the sustenance of wild buffalo population in future. However, there is a reciprocal relationship between the growth rate and size of the population i.e. growth rate varies with changes in population size. Presence of a number of calves in the population at present is an indication of the prospective status of wild buffalo population in KTWR.

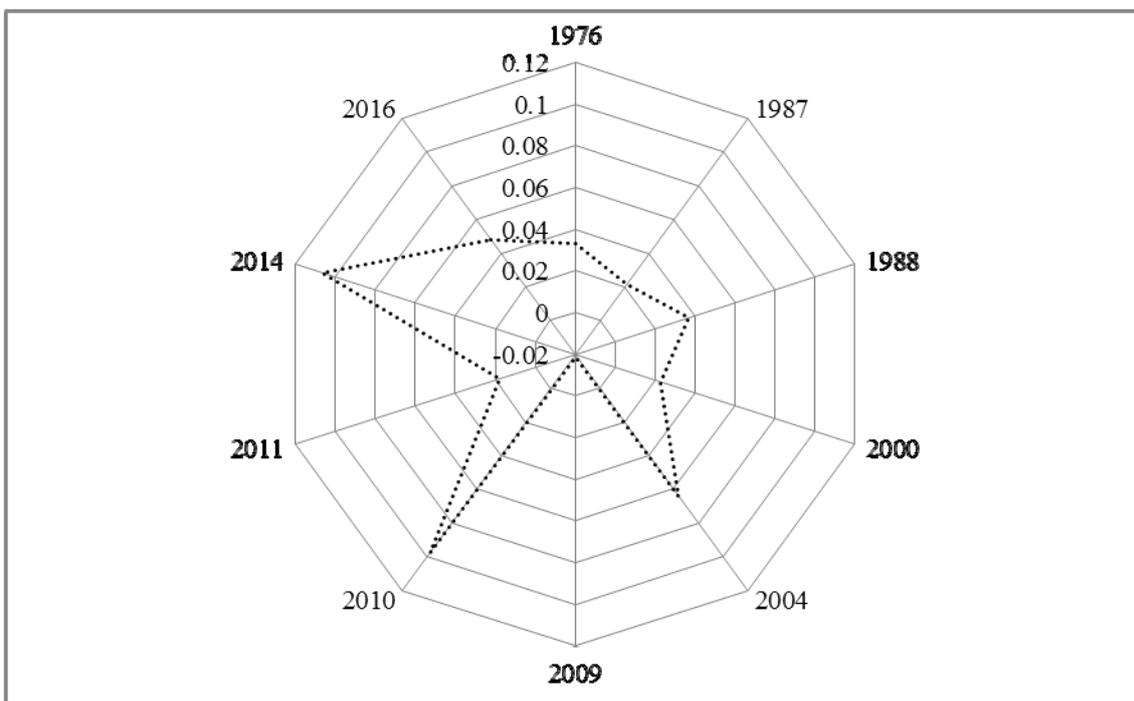


Figure 5. Exponential growth rate (ri) of Asian wild buffalo at KTWR in different census years

EFFECTIVE POPULATION SIZE

Effective population size is the number of adult males and females of a species in a given area or habitat. If individuals of a species mate only within a neighborhood, the effective population size may actually be smaller than the number of individuals in the population (Stiling, 2002) even

though the population size is larger. Knowledge of effective population size is vital to upscale the phenomenon of the success of conservation of species and their interactions. Allendorf (1994) indicated in case of grizzlies that population as large as hundreds would likely to lose genetic variation because effective population sizes are only about 25 percent of actual size. That is even fairly large isolated population becomes vulnerable to the harmful effects of loss of genetic variation. This situation truly applies to wild buffalo population of KTWR. Structure of effective population size of Asian wild buffalo in KTWR over different census years is represented in Figure 6, and percentage rate of loss of variation per generation as well as loss of variation of 10 generations is calculated as presented in Table 13. The likelihood of an allele being represented in just one or a few individuals is higher in small than in large populations. Small and isolated populations face challenges in transferring of rare genes which lead to loss of variations. Such isolated populations will lose a percentage of their original variation over the time.

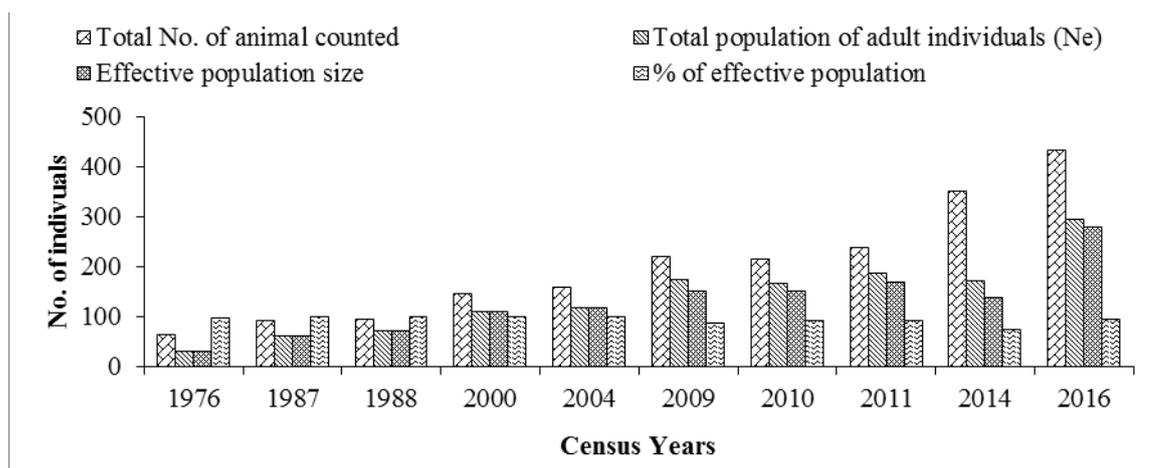


Figure 6. Structure of Effective population size of Asian wild buffalo at KTWR over different census years

If population size goes less than 50 individuals, then the likelihood of excessive inbreeding need to be prevented to stop loss of genetic variation. Calculated percentage loss of variation of wild buffalo of KTWR per generation and also 10 generations is presented in table 13. If population loss goes beyond the 500 individuals, it becomes critical size to prevent genetic drift (Simberloff, 1988). The Koshi Tappu population is increasing towards a fairly effective size; and likewise, the reintroduced population shall need to increase in future. Lacey (1987) showed that the effects of genetic drift that could be countered by the immigration of individuals into a population. That situation is not applicable for this isolated population at KTWR.

Table 13. The rate of loss of genetic variation of Asian wild buffalo at KTWR over the years

Year of Census	Population Size	Effective population size	% rate of loss of variation per generation	loss of variation of 10 generations
1976	63	30	1.67	16.7
1987	91	61	0.82	8.2
1988	93	70	0.71	7.1
2000	145	109	0.46	4.6
2004	159	117	0.43	4.3
2009	219	174	0.29	2.9
2010	215	165	0.30	3.0
2011	237	185	0.27	2.7
2014	350	172	0.29	2.9
2016	433	294	0.17	1.7

MANAGEMENT IMPLICATIONS

As the size of the wild buffalo population at KTWR is increasing every year, the population is likely to split into several groups; and those split herds would likely to be intermixed with feral or backcrossed herds. So there is a danger of genetic erosion of putative wild stocks due to increased chances of cross breed animals in the wildlife reserve and around. Now it is urgently necessitated to ensure that abandoned or private feral/or backcrossed buffaloes are immediately relocated from the Wildlife Reserve so that the wild stocks would remain safeguarded. However, a few concerns regarding the long-term conservation and effective management of wild buffalo in KTWR must be evaluated and seriously be taken up immediately. On the other hand, as genetic variation is reduced by inbreeding, genetic drift or influence of neighborhoods of feral or domestic buffaloes, the effect of all these factors are likely to increase in severity towards triggering declined population size.

Knowledge of genetics and breeding behavior or breeding potentials of wild buffaloes is vital to know for safeguarding its population in the larger landscapes. Genetic conservation of the wild populations must be used in conjunction with preservation of the organism's most significant habitat. The effects of inbreeding are more extreme in small populations (Stilling, 2002). The wild buffalo population at KTWR is still not considered viable and secure in this habitat due to various reasons such as floods in the Koshi River, the risk of disease transmission from domestic cattle and buffaloes, genetic degradation due to cross-breeding with feral buffaloes, tough competition for food and space, etc. However the total population size (433) and effective population size (294) at present is convincing for its sustenance, and percentage rate of loss of variation (0.17) in 2016 indicated optimistic inclination for a viable population. Reintroduction

of species into the new habitat at CNP of central Nepal has been successfully taken up during January-February of 2017 as a part of species conservation, and the DNPWC has planned to establish an effective population size in future.

There are the factors of impediments for having a normal course of being intermixed between the different herds or individual of wild buffalo populations in KTWR due to the extreme interference by thousands of cattle and domestic buffaloes as well as feral buffaloes. Such issues shall need to be seriously handled by enforcing the National Parks and Wildlife Conservation (NPWLC) Act 1973 that would help to render the country a leader among developing countries in conservation practices (Lehmkuhl, 1993; Yonzon & Hunter, 1991) for improving the management of protected area and offering wildlife protection (Heinen, 1993) effectively for a long run.

Wild buffaloes, the forest-living free ranging mega-herbivore and one of the bovines, have key roles in their natural ecosystems. They have the ability to modify landscapes at quite an unprecedented scale (Prins, 1996; Prins & Oeveren, 2014), most important functioning to interact with the forest mainly as seed dispersers, but they also change the forest structure by affecting the interspecific differential mortality rates of the different woody species (Prins, 1996; Prins & Oeveren, 2014). Considering this significant role of wild buffaloes in the riverine ecosystem, immediate actions are prescribed for the long-term management of the habitat and survival of the Asian wild buffaloes at KTWR and increase alternative viable population at new habitat such as in CNP, Nepal is recommended as below:

- Conduct official surveys of wild buffaloes and update the status very regularly;
- Monitor population, distribution and habitat conditions of wild buffaloes and other associated sympatric species every year;
- Intervene in the active management of habitat uniformly over the Reserve for palatability and accessibility to grasslands and wetlands regularly;
- Take effective and appropriate measures to stop grazing by domestic animals and to limit contacts with wild buffaloes;
- Restrict the domestic buffaloes to breed with wild bulls;
- Develop community animal health-care facilities to Saptari and Udayapur sides as was provided at Madhuban, Sunsari District from 2005, and encourage stall feeding for the livestock;

- Carry out intensive molecular study of wild, feral and domestic buffaloes to characterize them genetically;
- Encourage to carry out intensive studies on basic ecology including behavioral and other ecological aspects of the wild buffalo population at KTWR that should have long-term implication for the conservation;
- Enhance the level of motivation and awareness amongst the local communities on the significance of wild buffalo conservation in Nepal;
- Provide increasing support from the Government of Nepal, the international community, and donors for conservation, management, research and promotion of eco-tourism;
- Further, plan to translocate some more animals to create a viable and effective population size into Chitwan National Park.

CHAPTER-VI

REINTRODUCTION OF ASIAN WILD BUFFALO (*Bubalus bubalis arnee* Kerr, 1792) IN NEPAL: A PROTOCOL FOR WILD BUFFALO TRANSLOCATION

Wild buffaloes have once ranged across south and south-east Asia from Mesopotamia to Indo-China (Mason, 1974; Cockrill, 1974). It is considered the ancestors of today's domesticated buffaloes (Scherf, 2000). Wild buffalo is mystified with feral, domesticated and their contemporaneous hybrids; hence conservation of the species become a challenging task. Some scientists suspect that there might not be the pure breed of wild buffalo fundamentally due to increased interbreeding with domestic buffalo; this is why IUCN Red list of threatened species rescheduling its category as Endangered in 2008.

Asian wild buffalo *Bubalus arnee* (Kerr, 1792) population status is progressively decreased in its global distribution and confined to certain pockets, so its population is in jeopardy due to various reasons. The result of which is the global status of the species is endangered in the IUCN Red List (IUCN, 2013). It has also been legally protected in Bhutan, India, and Thailand with focussed conservation programs on protecting them and their natural habitats. In Nepal, it is restricted only to Koshi Tappu Wildlife Reserve (KTWR). Wild buffalo has been legally protected wild mammal of Nepal by National Parks and Wildlife Conservation Act (NPWCA), 1973 by enlisting it as Schedule I species, and the CITES has listed it in Appendix-II.

Wild buffalo population of KTWR is facing various levels of threats for maintaining pure genetic superiority such as inbreeding, cross-breeding with domestic buffalo which are leading to genetic erosion. Other prevalent threats are disease transmission from domestic livestock, competition with domestic and feral cattle and buffaloes for food resources and spaces. However, there still persevere of some pure breed of wild buffaloes which are clearly distinguished from highly backcrossed, semi-feral population and domestic buffaloes based on morphometric, social and behavioral characteristics. Even though hybrids are better adapted to local conditions, this would be undesirable if one of the parental population is a taxon, despite it has great value on its own, but would be no longer discernible after hybridization (Schonewald-cox *et al.*, 1983). To prevent genetic and phenotypic erosion and extinction, the species need to have a secluded habitat. When conventional conservation efforts are inadequate (Hunter, 2007) for endangered species like wild buffaloes, then creating alternative population on an alternative

habitat could be an appropriate and key strategy for species conservation in a long-run. So the main objective of the translocation project was to ensure the long-term survival of the wild buffaloes in Nepal by creating next population; to maintain and/or restore natural biodiversity of CNP, and also to provide value addition of CNP through long-term economic benefits to the local and/or national economy to a possible extent, and to augment the ecosystem functions. In total, 16 wild buffaloes (12 females and 4 males) were translocated from KTWR to CNP (Fig. 7, 8, 9) during January-February 2017. The sources of translocated animals (n = 16) were from KTWR (n = 13; 10 females and 3 males) and Central Zoo of Nepal (n = 3; 2 female and 1 male) (Table 14) to establish an alternative population in CNP. Out of total captured wild buffaloes, 1 male from KTWR was translocated to Central Zoo to maintain genetic variability amongst the wild buffaloes of the Zoo. The animals were transported from road networks (>400 km) and took about 10 hours to reach CNP, and 16 hours to Central Zoo. This was the first event of mass capture and relocation of wild water buffaloes in Nepal and probably even in the World.

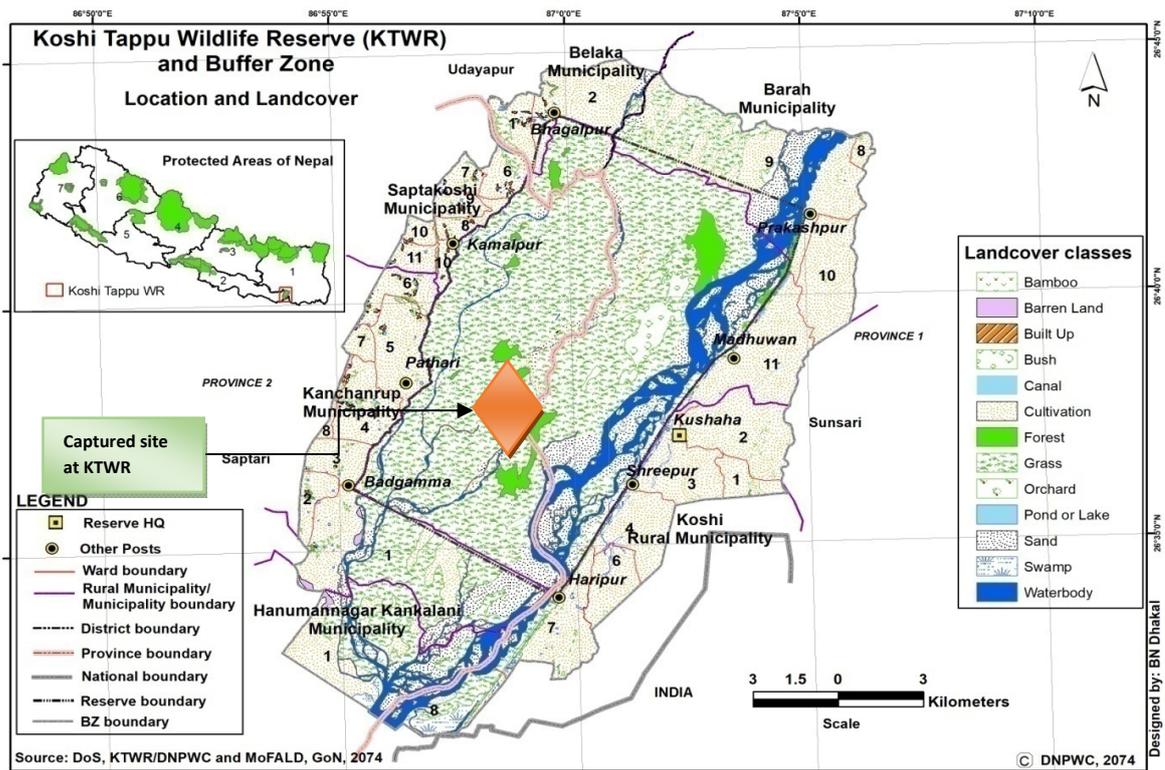


Figure 7. Location map of KTWR, Nepal indicating the site of Donor/Source Population

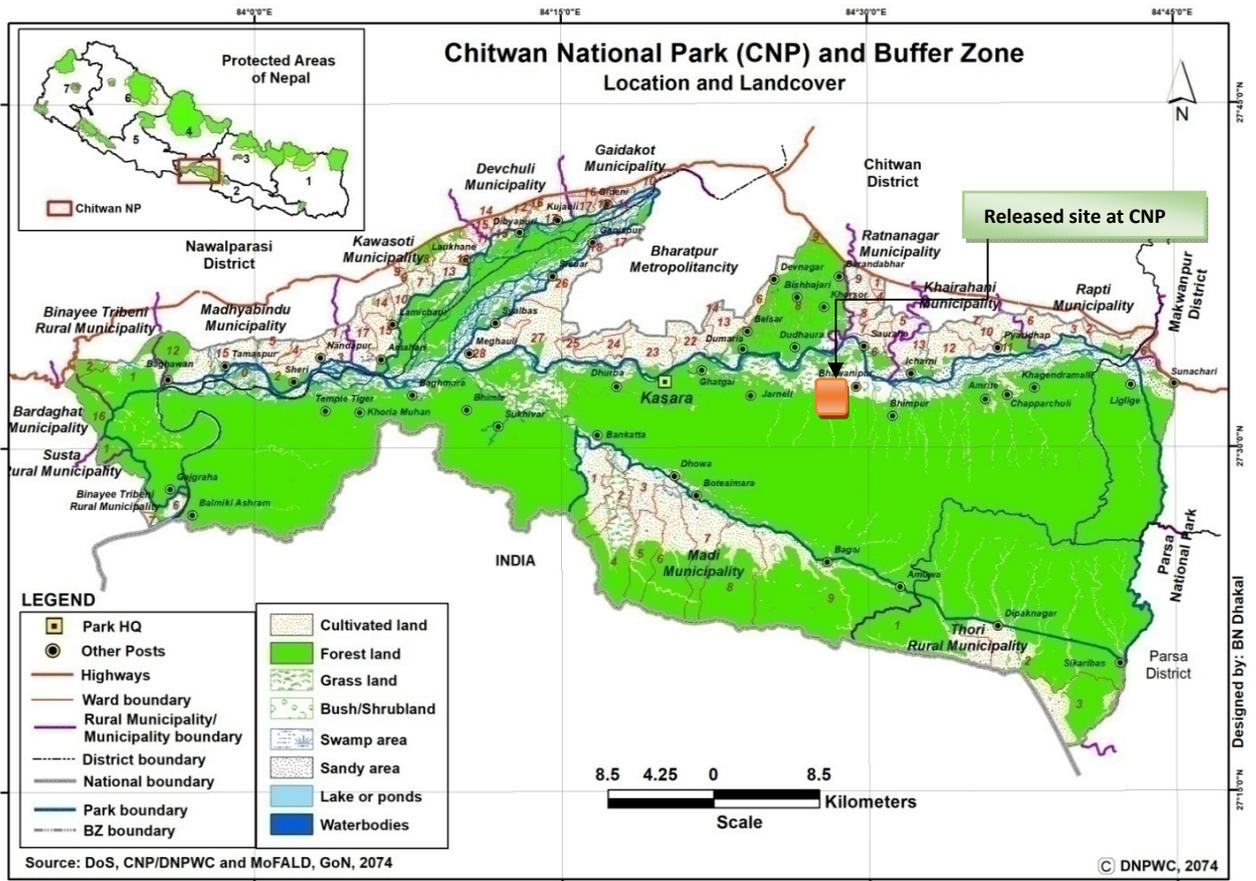


Figure 8. Location map of CNP, Nepal showing the site of translocated Population of Wild buffaloes (Receiver)

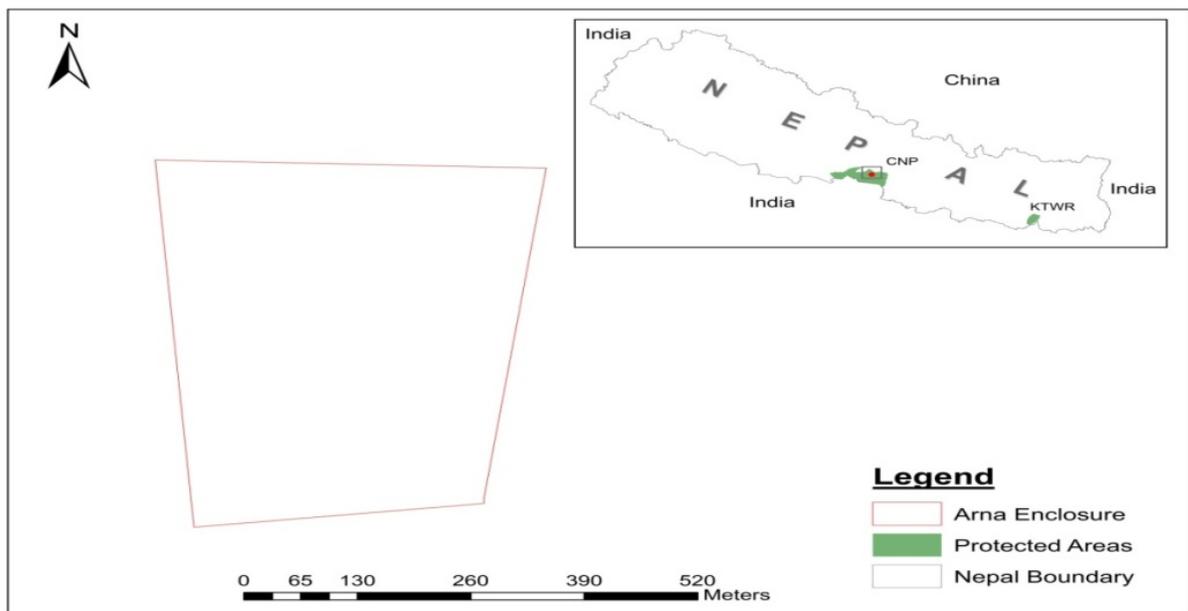


Figure 9. The layout of soft release enclosure site at CNP, Nepal

METHODS AND MATERIALS: PROTOCOLS OF TRANSLOCATION

Total time (hours) taken for the whole operation of translocation of wild buffaloes from KTWR to CNP: In total at least 110 hours used while performing the capture operation, transportation and releasing of wild buffaloes, this was held during 23 January to 09 February 2017. Translocated wild buffaloes were monitored for 286 hours over four seasons to study behavioral activities in CNP.

PRE-REQUISITE

With the policy decision of Government of Nepal, the translocation project was initiated from the end of 2016 that included carrying out a feasibility study to consider biophysical requirements for the wild buffaloes. The conjured up set-up of the preliminary study indicated to reintroduce a maximum of 20 individuals in the first phase that was to be captured from founder population of KTWR with a male to female ratio of at least 1:3 to relocate them in the recently evacuated human settlement at Padampur area of the CNP. The recommendation for reintroducing number was made on the basis of requirement of basic size of the herd and herding behavior of wild buffaloes together with defensive behavior towards the large predator like tigers. To implement the decision, standard protocol was developed and planned to implement the decision.

Grasslands of CNP possesses the mosaics of short and tall grasslands including a patch of woody vegetation with less tiger density which has scope of having availability of wider range of Rapti River bank with hills of fresh water sources. Captured individuals were to be reclamationized into a pre-release or soft-release holding enclosure prepared in the north-east part of CNP near Rapti River at evacuated Padampur area.

PREPARATORY PHASE

Species Translocation Advisory Committee conducted the feasibility study including preconditions and requirements for translocation operations; understanding of ecological requirements for wild buffaloes mainly the habitat components like availability of food, space, water, cover; evaluation of protection measures and strategies against anthropogenic and biological factors; identifying source animals at KTWR; and other specific factors relevant for translocation. The action plan was developed; standard veterinary protocols (Pabla *et al.*, 2011) for wild buffalo capture, immobilization and translocation were prepared; and activities were planned to translocate 20 individual wild buffaloes from KTWR and Central Zoo to CNP.

CONTAINER DESIGNING

The containers for transportation of wild water buffalo were designed based on the size of the target animals. The container made up of iron plates and wood frames that were prepared to transport wild buffalo with the ease of comfort. Animal holding and transportation cages of size 2.5mx2mx2.5m were prepared in Sauraha, CNP. Frontal and back doors were designed with cross locking system.

HOLDING ENCLOSURE

For the soft release of the translocated wild buffalo, an enclosure with mesh wire fence was prepared at Padampur area inside the CNP. It covers approximately 30 hectares of area. An elongated water body (400 m X 15m and 3m deep) which trespasses the enclosure with regular water source from Churiya range was considered as a permanent water source. A pond was constructed for having an extra source of water. To avoid damage of enclosure by the animals, mesh wire fence of the enclosure was backed up with solar fences from inside and outside.

This soft-release enclosure was constructed temporarily to keep them for close monitoring until the animals were permanently released for free-ranging to the new habitat in CNP. Holding the animal inside the soft-release enclosures after transportation to CNP is important for the following reasons:

- a) To let them take complete rest from their long trip and become used to a new environment;
- b) It allows for social bonding and the development of a social hierarchy amongst the translocated wild buffaloes that may come from different herds among which they may not be well familiar with each other;
- c) To provide sufficient time for disease and genetic test results to become adaptive to a new environment;
- d) To create climate setting for the rumen micro-flora to adapt to the new forage species available in Chitwan, an important consideration for ruminants;
- e) To initiate quarantine measures as required;
- f) It allows the animals to become accustomed to the new odors of predators with which they are unaccustomed in the former range of its habitat or environment.

The holding enclosure was spacious enough for free movement and to acquire sufficient food sources, water and performing day to day activities along with the assurance of tiger-proof solar fence all along the gabion-wired fence.

TIMING FOR CAPTURING AND TRANSLOCATION

Wild buffaloes do not tolerate the high stress of temperature. So, the winter months which provide optimum temperature conditions were chosen for translocation (Dinerstein *et al.*, 1990; Caulkett, 2001). To avoid heat-related complications, capture and translocations were timed to coincide with the cooler hours, morning sessions (below 25⁰ C) for capture and night hours for the translocation. Hence the most suitable months well thought-out were November to March. The ambient temperature of that area rises significantly thereafter, and it is not advisable to conduct translocation after March.

COORDINATION TEAM

A field-level coordination committee under the supervision of Chief Warden of CNP was formulated to coordinate and carry out all the management and technical arrangements related to the capture and translocation activity. Accordingly, necessary arrangement for the construction of soft release enclosure at CNP, protection of the area from wildlife and human intrusion by means of security provisions, technical and physical preparation for transportation, and handling other necessary actions, were managed through coordination committee. Before undertaking operation for translocation of wild buffalo, coordination meetings, interactions, and dialogues for local consensus were organized with communities at local levels at KTWR as well as at CNP. The stakeholders showed their willingness for wild buffalo conservation in KTWR and concomitantly were keen to improve the status of conservation of the species.

ANIMAL CAPTURE OPERATION

VETERINARY PROTOCOLS, SUPPLIES, AND EQUIPMENT

A joint team of DNPWC, CNP, KTWR and NTNC-BCC (a conservation partner) including a team of veterinarians and technicians of CNP and NTNC-BCC detailed out a technical plan of operation to capture and translocation from KTWR and reintroduction to soft release enclosure site at CNP. The essential veterinary supplies, dart equipment, drugs and veterinary requirements were also revisited before getting into the operation.

FIELD TEAM AND ORIENTATION

A field team with 206 personnel including park staff, cattle herders, local community members, and 6 trained domestic elephants were formed for the capture operation during the translocation. Orientation training was organized for the field staff due focus on field requirements for

translocation, monitoring; locating wild buffalo herd; arrangement for darting, capture, and immobilization; loading to a tractor, shifting immobilized animals to a cage and to push into the truck; emergency management and logistics in Koshi Tappu; actions for transportation from Koshi Tappu to Chitwan; unloading/release and post-release monitoring in Chitwan. Specific roles and responsibilities were also assigned to each team members and staff.

CRITERIA USED TO SELECT ANIMAL FOR TRANSLOCATION

Wild water buffaloes of KTWR are distinctly categorized based on the morphometric and phenotypic characteristics to distinguish between wild, feral, and domestic breeds. Largely, phenotypic characteristics (Cockrail, 1974; Hall & Raune, 1993; Heinen, 2002) and behavioral characteristics (Tulloch, 1970, 1978, 1979; Heinen, 1993a; Caro, 1998; Heinen, 2002) were applied to distinguish wild buffaloes with feral backcrossed and domestic ones through the close observation. Experienced observers were involved to distinguish wild from domestic and feral. Backcrossed are thought to be derived from domestic females that have interbred with wild bulls for at least 6 generations (Heinen & Kandel, 2006), herds of which are mostly composed of females accompanied by their calves that share most but not all phenotypic and behavioral characteristics of wild buffalo (Heinen, 2002). Muley (2001) also used morphometric and genetic data to differentiate wild, hybrid and domestic swamp buffalo in Kaziranga National Park, Assam, India. Animals shall need to first classify as wild, hybrid or domestic on morphological criteria—chevron markings, stockings, horn size and shape, tail length, and herding behavior. For each animal, morphometric information was taken from frontal photographs and subjected to identify from various angles of the body and various markings on their body parts.

SELECTION OF CAPTURE SITE AND ANIMAL

The capture operation, transportation and releasing activities were conducted from 23 January 2017 to 09 February 2017. Trained elephants of KTWR, 4-wheeled vehicles and local herders (citizen scientists) were consigned to search the animal herd and to target the suitable animals with the convenience of capture and immobilization. A preliminary field visit of the prospective location of wild water buffalo herd was carried out jointly by capture team, veterinary unit, and local herders to better understand the habitat and terrain for a successful capture. Once the herd size, composition, distance from the area, ease of the capture site, were reported, then the veterinary, capture and darting teams dealt with to move to the designated location. The terrain,

visibility of animal and distance from the target points were taken into contemplation before darting. If the herd or target animal was not in approachable distance or terrain or visibility condition, either herd or target animal was pushed to a suitable place. The selection of capture animal was decided based on its physical condition at the site; however, sex of animal to capture was decided in advance before going to the field and for further planning.

IMMOBILIZATION

Darting of the selected animal was done from an approachable distance by foot or from a vehicle or from the tree trunk. Body health condition, physiological status, age and estimated weight were considered while determining the drug dosages to immobilize the animal (Harthoorn & Luck 1962; Pienaar & Van Niekerk, 1963; Blane *et al.*, 1967; Wallah *et al.*, 1967; Yusof, 1990). The weight of the selected animal was estimated based on published information and past experiences that approximately ranged between 250kg and 700kg for sub-adults and adults (Curro, 2007). When the target animal was selected from the herd; they were approached from a close distance for efficiently capturing through the drug delivery. The capture of wild buffalo was done in two ways: 1) by darting, and 2) by traditional chase method. For darting the target animals, remote drug delivery was facilitated using all-plastic darts delivered through an air-pressurized Dan-Inject (Model JM) projector from close distance preferably using branched trees or elephant back or on foot from a mount.

Etorphine Hydrochloride mixed with *Azaperone* at approximate doses was remotely injected with a dart gun. The animal was left undisturbed until it becomes safe for handling. Antidote *M 50-50* was used for *Etorphine Hydrochloride* and *Antisedan* for *Xylazine*. The animals were to be transported more than 400km from Koshi Tappu to Chitwan, so *Haloperidol* was injected into each captured animal for relaxation during transportation and to minimize travel stress. Other medications used were antibiotics, Avil as anti-histaminic and antibiotic ointments to prevent infection on the body parts. The details of the drug used for capturing is given in table 14. Altogether, 16 Wild buffalo were captured using prescribed methods. Out of them, 06 were captured using traditional method and others were captured by darting phenomena. Out of 16 animals, 13 were captured from Koshi Tappu and 3 were from Central Zoo, Nepal. Among 13 captured from Koshi Tappu, 12 (10 F & 02 M) were translocated to Chitwan and one (01 M) was translocated to Central Zoo; and, three captured from Central Zoo (2 F and 1 M) were also translocated to Chitwan.

CARE OF IMMOBILIZED ANIMALS

Once the darted buffalo is down, then the team succeeds the necessary arrangement to make the wild buffalo safe and comfortable. The buffalo first to be placed safely in sterna recumbence and its vital signs such as respiration, pulse, rectal temperature, etc. are monitored.

Each animal needs to be administered with:

- a) An injection of long-acting broad-spectrum antibiotic (e.g. long-acting *oxytetracycline*) to deal with hidden bacterial diseases, which the buffalo may be harboring, and to prevent dart wounds from becoming infected; and injectable *ivermectin* (Ivomec) at a dose rate of 1ml per 50kg to eliminate nematodes;
- b) A suitable liver fluke remedy;
- c) Animals are also prepared for their new period of confinement with a suitable tranquilizer;
- d) *Trilafon* LA 200mg intra-muscularly to calm the animals for a period of about 10 days effect beginning about 24 hours after capture.

The following items must be on hand with the team in case of emergency treatment required:

- a) *Narcan, naloxone hydrochloride*, in case of human accidental injection;
- b) *Dopramdoxapram hydrochloride*, in case of respiratory distress of the buffalo, to be administered at a dose of 1 mg/kg intravenously;
- c) Eye drops and few pieces of clothes to cover the eyes need to be taken in hand. Cold water shall be taken priorly to cool the darted buffalo in case it suffers from heat-stress. Water is also useful to wash spillage of drugs. A 20 lit container to be had is adequate;
- d) A number of cotton thread ropes need to be taken in hand to tie the legs of the darted buffalo if it is not fully relaxed from the effect of the drug.

COLLECTION OF BLOOD SAMPLE

Blood samples of captured buffaloes were collected for the purpose of disease screening and genetic studies. Two 10ml un-heparinized tubes of blood were collected from a superficial vein of each of the captured wild buffaloes, and those were recorded on the tubes. The samples of blood and also the hair follicles were collected at capture and taken to Molecular Lab at NTNC-BCC, Chitwan to test the genetic purity for further analysis. The blood was allowed to clot and stand for 24 hours at ambient temperature before the serum is decanted for freezing.

ANIMAL LOADING

After darting or capture by chase method, the animals were first pulled down to a tractor site by using sledge from the captured site and loaded to the tractor. A loading ramp was dug out to make loading process comfort. Loading truck was not taken up to darting site because of difficult terrain as were sandy and swampy areas; instead immobilized animals were first loaded into container which was fixed on a tractor; and once the animal was shifted to tractor, it was provided with antidote supplemented with *haloperidol* and other antibiotics; then transported to appropriate site to shift the animal with loaded container into the truck with the help of JCB where truck was kept in a ready position for transportation. However, in many of the locations animal captured sites were approachable so were loaded directly onto the truck.

TRANSPORTATION OF CAPTURED ANIMALS

Orientation training was given to transportation crew including truck staffs on safety measures while transporting animals. The truck staffs were strictly advised not to overtake any vehicle in order to maintain a speed limit, and also to keep up the distance at least 50m from the front vehicle. The distance of transportation from Koshi Tappu to Chitwan was more than 400 km road distance and almost 10 hours of drive. The transport team moved in the evening to avoid heat stress, vehicular disturbance and travel strain to animals and to reach early morning to a releasing site. The transport crews were to keep on regular monitoring of the animals throughout the journey with a constant speed of 40-50 km/hr according to the road condition.

RELEASE CEREMONY

A release ceremony was organized on the first day approx. 5km away from the exact release site to avoid the disturbance in the proposed site within the core area. At the release site, the truck was placed properly at unloading ramp (within the enclosure at Padampur). A core team of translocation operation and some other limited people were only present while releasing them. All translocated animals were released with proper care after substantiated check-up of physical fitness of the animal. All animals were released safely and none of the translocated animals came across any unusual incidences during transportation or release procedures.

LESSONS LEARNT AND CONCLUSION

There are many forms of conditions likely to prevail in the field while implementing the translocation process, so there need to take many subsequent precautionary measures for the successful mission and safety.

- While doing field operations, female wild buffaloes always had to dart twice due to their cohesive herding behavior. Therefore, the team should always be ready in a very careful position to target focal animal and take quick action in the field.
- No such dangerous situation arose except the presence of wild elephants that happened to appear to obstruct the movement of the capture operation during the field work.
- This must be the first mass translocation of wild buffalo ever done in the World although few wild buffaloes were already translocated to the central zoo, Nepal from Koshi Tappu in the past.
- Anaesthetic medication includes a combination of *Etorphine HCl*+*Azaperone* was applied to capture the first animal, but other animals were applied with a combination of *Etorphine HCl*+*Xylazine*; which worked very well. Traditional chase and capture method was also used with the help of local herders/citizen scientists, was a new experience of translocation.
- During the translocation, a total of 225 personals from different institutions were directly and indirectly involved during the whole period of translocation. A teamwork was an effective and efficient means of a successful mission in wild buffalo translocation.
- Translocation of Wild Buffalo to a new habitat, at CNP, established a milestone for the conservation of one of the endangered species.
- This has played a vital role to increase and further to improve the biological values of CNP, the World Heritage Site, added value for the tourism and opportunities for improving socio-economy of the local communities.
- It has also created a challenge for the management and conservation which shall be tackled with appropriate management prescriptions and sincerity.
- The experiences and standard protocol developed from this operation of wild buffalo translocation would be an instrument for the new users to handle the same operation in the future for other species too.

Table 14. Details of the captured wild buffaloes and veterinary interventions during translocation operation, 2017 Jan/Feb

Date	Site	Wt/ kg	Age/ Tooth	Sex	Capture Drug (mg)			Temp.	Resp/m in	Call Y/N	Drugs Used								Comments	Dart Time		
					M99	Xyl	Azp				M50-50 (12mg/ml)	Antisedan (5mg/ml)	Yoh mg	Halo peridol 5mg/ml	Pro LA ml	Atropine (0.6mg/ml)	Avil ml	Lim LA ml			Dex mg	Tylox tube
073.10.12	Moriyadhar	150		F	4	0	150	106	8	Y	1	0	0	5mg each iv,im and sc	0	4	10	15	0	1	Captured by Chemical immobilization	13: 10
073.10.13	South Duban	300		M	5	60	0	108	18	Y	1	1.2	0	5mg iv and 5mg im	0	5	15	15	0	1	Captured by Chase	16: 00
073.10.14	Moriyadhar	150		F	0	0	0	101.4	0	Y	0	0	0	5mg iv and 5mg im	20	0	10	0	0	0	Captured by Chase	11: 40
073.10.16	Bouchi Area	150		F	3	50	0	102	NA	N	0.5	1	0	5mg iv and 2.5mg im	0	2	10	15	0	1	Mild Salivation noticed	16: 52
073.10.17	Garia Tapu	300		F	3.5	60	0	105	12	Y	0.8	1.2	0	5mg iv and 2.5mg im	15	4	10	0	0	1	Hyperbreathing and trembling after rise and at Dhalke	13: 30
073.10.18	Moriyadhar	250		F	0	0	0	102.4	NA	N	0	0	0	5mg iv and 2.5mg im	12	0	10	0	0	0	Captured by Chase	15:30
073.10.19	Gariya Tapu	300		F	4	60	0	100.8	12	Y	0.8	1.2	0	5mg iv and 5 mg im	15	4	8	0	0	1		12:03
073.10.19	Gariya Tapu	150		F	3.5	60	0	101.2	NA	N	1	1.2	0	5mg iv	12	3	7	0	0	1	Fall in 10 minutes	14:30
073.10.21	South Duban	250		F	0	0	0	104.2	NA	N	0	0	0	5mg iv, 2.5mg im	15	0	10	0	0	0	Drug did not pass. Captured by Chase	11:45
073.10.21	South Duban	200		M	0	0	0	108.1	NA	Y	0	0	0	5mg iv, 5mg im	10	0	7	0	20	0	Hyperbreathing , Captured by chase	14:00
073.10.22	?	400		M	4.5	60	0	104.7	NA	Y	1	1.2	0	5mg iv	15	4	10	0	0	1 tube	Arose in 4 minutes	14 :20
073.10.24	Gariya Tapu	400		F	4	60	0	105.4	NA	Y	1.5	2	0	5mg iv and 5 mg im	15		15	0	0	1 tube	1 st dart came out instantly after hit. 2 nd dart only slowed down the animal. 2 nd dart composed of 3 mg m99 and 50 mg xylazine.	11:00
073.10.24	Moriyadhar	150		F	0	0	0	NA	NA	N	0	0	0	2mg iv	5	0	5	0	0	0	Captured by Chase at Upper Moriyadhar area across Koshi	15:00
073.10.23	Central Zoo	500	8	M	6	50	0	NA	NA	N	1.2	0	25	15 mg im	0	0	0	0	0	0	No salivation. Additional M99 2 mg and Xyl 20mg	15:30
073.10.26	Central Zoo	400	6	F	4	40	0	NA	NA	N	1	0	20	12mg im	0	0	0	0	0	0	No salivation.	14:30
073.10.26	Central Zoo	300	2.5	F	4.5	30	0	NA	NA	N	1	0	15	9mg im	0	0	0	0	0	0	Additional M99 2 mg and Xyl 30mg	15: 20

POST RELEASE MONITORING

Regular monitoring of re-introduced animals is being carried out intensively with the joint efforts of CNP and NTNC-BCC to understand their foraging, movement, ranging pattern and herding behavior. Two staffs are assigned for taking care of animals and maintenance of enclosure as required with the provision of a daily update to CNP through NTNC-BCC. Protection Unit of Nepal Army at CNP has taken the responsibility of security arrangement on site at Padampur.

COMPILED REPORT ON POST RELEASED MONITORING

After the vanishing of wild buffalo from CNP in the 1960s, the park has attempted to revive this species by re-introducing in the grassland of Padampur. An area of almost 30 hectare with 643m length in east-west of either side, 336m in North and 300m in the south is enclosed with PCC foundation and Mesh wire supplemented with solar fencing all around the enclosure. The translocation phase took almost 2 weeks during January-February of 2017. A total of 15 wild water buffaloes were translocated to CNP with 3 male and 12 female individuals. In a post-translocation phase, the park started monitoring them regularly with a great sincerity. In the meantime, all the 3 animals (1 Male, 2 Female) as translocated from the Zoo died as they were unable to adapt to the wild habitat.

METHODS USED FOR MONITORING

Different methods are adapted to monitor the re-introduced wild buffalo in Padampur Area of Chitwan National Park. The monitoring is carried out by park officials with the security support of Nepal army (a protection unit of CNP) and technical support of NTNC-BCC staffs.

Some techniques applied for monitoring are as follows:

1. Sweeping Operation: This includes sweeping within the enclosure by means of elephants drives. In this method, four to five elephants are moved from north to south covering length part of the enclosure. Various herds with numerous dung piles with trampled grasses can be observed within the enclosure.

2. Observing from Tower: It includes monitoring from a tower (Dhedauli machan) which is adjacent to a western section of the enclosure. During the daytime, two supervisors keep on their eyes to the enclosure, and in the night time Ambush teams of Nepal Army, CNP Protection Unit are deployed on rotation basis to monitor and secure the enclosure.

3. Surveillance around the enclosure: Two Supervisors and army staffs regularly carry out the patrolling and monitoring of the enclosure from outside. They keep the wild buffalo record in the given format and regularly check conditions of the fence, get them repaired as needed, and report to the Park authorities on daily basis.

INTERVENTIONS WITHIN THE ENCLOSURE

A number of interventions have been carried out for the management of soft-release enclosure area of wild buffaloes at Padampur, CNP after 2017 April along with the regular monitoring. Major interventions are as follows:

1. Grassland Management:

Grassland inside the enclosure has been managed in two ways: (a) Manual cut, and (b) controlled burning. The manual cut of unpalatable reeds and invasive species are done in the western edge (in front of Dhedauli Machan) of the enclosure. Invasive species and dry bushes are burned by controlled burning inside the enclosure.

2. Construction of watch-tower

Three additional temporary towers have been constructed alongside the enclosure. They are located in north-east, east and south directions of the enclosure in the periphery. These towers are temporary and built with *Sal* tree poles and thatch grasses.

3. Cleaning up and maintenance of water source

Water sources originating from the Chure hills supplying water to the ponds and channels inside the enclosure for wild buffalo are cleaned up from time to time. With the onset of the dry season, water channels originating from *Chure* are blocked frequently due to the accumulation of huge amount of leaf litters. Considering this fact, cleaning and managing these streams had been carried out to facilitate for availing the ample amount of water within the enclosure.

4. Removal of weeds and grasses, and cleaning small trench around the enclosure

The grass around the enclosure was cut to make the solar fence free from being in touch with grasses, weeds, and shrubs in order to operate effectively, and *Mikania micrantha* patch inside the enclosure was chopped down and burnt as a part of grassland management. Along with the enclosure, a trench of 0.3m deep and 0.5m width has been constructed to prevent the mega wild animals to strike the fence.

MONITORING THE HERDING AND BEHAVIORAL ACTIVITIES OF TRANSLOCATED WILD BUFFALOES

Translocated wild buffaloes have formed an average herd size of as much as 5 to 7 individuals (Table 15). At the same time, it has also been noticed that one male and few females sometimes showed preference of roaming around freely either single or a small herd. They can be observed frequently from the north-east tower of the enclosure. Most of the time, they are found grazing in herds and wallowing individually or in two numbers. Data shows that they prefer north-east area of the enclosure where abundant green grasses mainly *Saccharum spontaneum* are available abundantly than those in opposite region of the enclosure, which is dominated by *Eupatorium* bushes along with open grassland. Further, the north-east region is larger than another region. Record also reveals that they prefer to graze in the morning time from 0700 to 1100 hrs, and wallow, rest or loaf after 1300-1400 hrs. Physically, it is observed that the wild buffalo individuals are slowly gaining weight compared to the released time.

Table 15. Observed herding behavior of translocated wild buffaloes at CNP, 2017.

SN	Group sizes of animals	Frequency of observation
1	1	1
2	3	1
3	4	2
4	5	3
5	6	1
6	7	1
7	8	1
8	10	1
9	15	1

OBSERVATION OF ANIMALS, HABITATS AND NUTRIENT SUPPLEMENTATION

By observing the behaviors, wild buffaloes within the enclosure in CNP were mostly seen while grazing, wallowing and resting together in the herd. Meanwhile, some young males were observed wandering solely. The herds were seen to use the small patch of trees inside the enclosure during the rainy moment. They had also created a small pond in the middle part of the grassland to wallow and rest as a part of thermoregulation, so are called habitat architects. The dominant female was observed to leading the herd at most of the time. They showed an aggressive response to the elephants while observing them during the sweeping operation as a part of regular monitoring at the post-translocation phase.

Minor damage was occurred to the fencing due to the flood of August 12, 2017. The *Chure* flood causes spontaneous overflow in the water canal that passes over the grasslands within the enclosure. The water canal was formed from the streams of *Chure* hill. Due to persistent rainfall, the water volume in the water canal increases resulting burst at the north end (exit or outlet point) of the enclosure which was actually dammed to reserve water to the translocated animals; the nearly 25m gap was created due to its effect. Alongside, the solar fencing did not work properly due to damaged poles, wires, and short daylight.

Wild buffalo monitoring has been carried out regularly according to the annual monitoring plan of CNP. Special attention has been given to health condition of the translocated animals and its habitat inside the enclosure. Staffs are engaged for regular maintenance of enclosure and testing the strength of solar fence to ensure solar power supply along with its effective operation. Frequent monitoring by veterinary personnel has ensured the health condition of wild buffaloes. All wild buffaloes were observed in healthy condition. After the huge flood of August 2017, health conditions of some animals were badly affected; and 3 animals were died (1M, 2F). So the medication and diet supplementation have been started. Supplementary diet, medicine, and frequent medical test were carried out as prescribed by veterinary officials (Table 16). Supplementary diet was provided to Arna from Mid September 2017 by providing iodine salt in plastic plates. Diet was provided on alternative days. Later it was followed by *Choker/Dhuto* (Husk of corn/paddy), Hay, and solid salt.

Table 16. Details of the supplementary diet provided to the wild buffaloes at CNP

S.N	Diet Name	Quantity	Remarks
1.	Solid salt	30 kg	
2.	<i>Choker/Dhuto (paddy dust)</i>	500 kg	Approx. 30kg/day: 3 kg per wild buffaloes
3.	Chickpea (<i>Chana</i>)	300kg	Approx. 20kg/day: 2 kg per wild buffaloes
4.	Minerals	As per prescription	

Note: Calculations indicating per day accounts of diets on alternate days. However, as prescribed by the veterinary doctor providing daily diet have been started from January 2018

POST TRANSLOCATION BEHAVIORAL STUDIES OF WILD BUFFALOES

Behaviors of translocated wild buffaloes have been observed almost similar to the behaviors of free-ranging wild buffaloes of KTWR. Maximum time (mean value in minutes) spent by translocated animals in the winter season was in resting (40.28 ± 6.94) followed by grazing (38.89 ± 12.70), moving (26.67 ± 5.39), wallowing (15.83 ± 5.94) and other activities (6.67 ± 2.45) (Fig. 10).

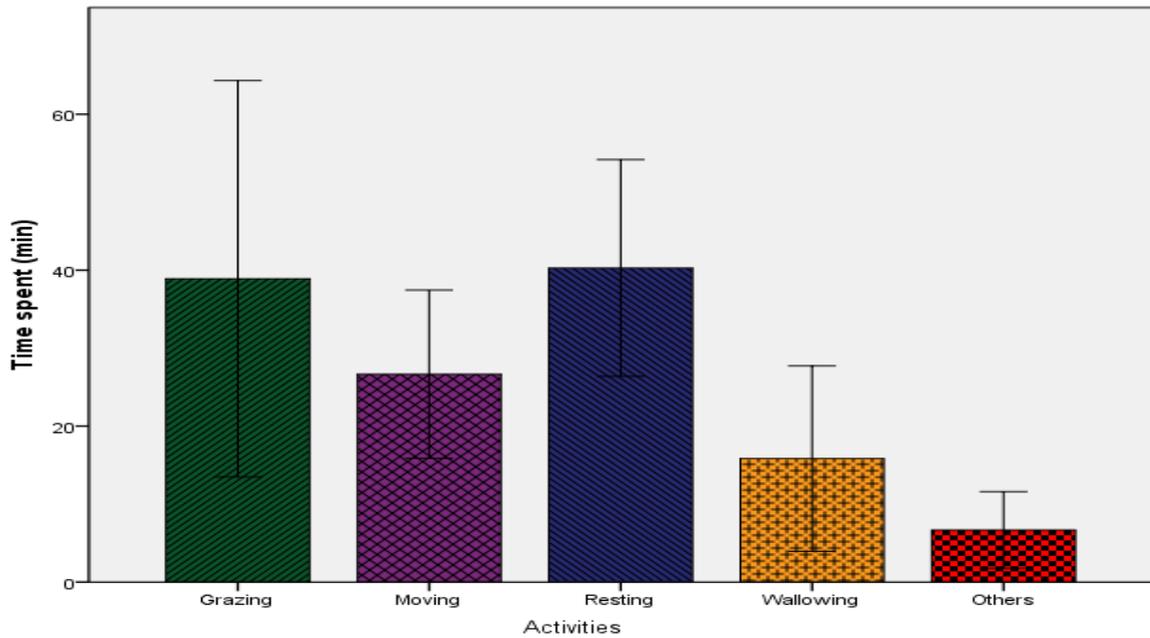


Figure 10. Summary of behavioral activities of translocated wild buffaloes within the enclosure at CNP during Winter season, 2017

On the other hand in the summer season, maximum time (mean value in minutes) spent on grazing (60.95 ± 9.59) followed by resting (38.57 ± 5.92), moving (28.33 ± 3.05), wallowing (20.24 ± 5.28) and other activities (5.24 ± 2.11) (Fig. 11).

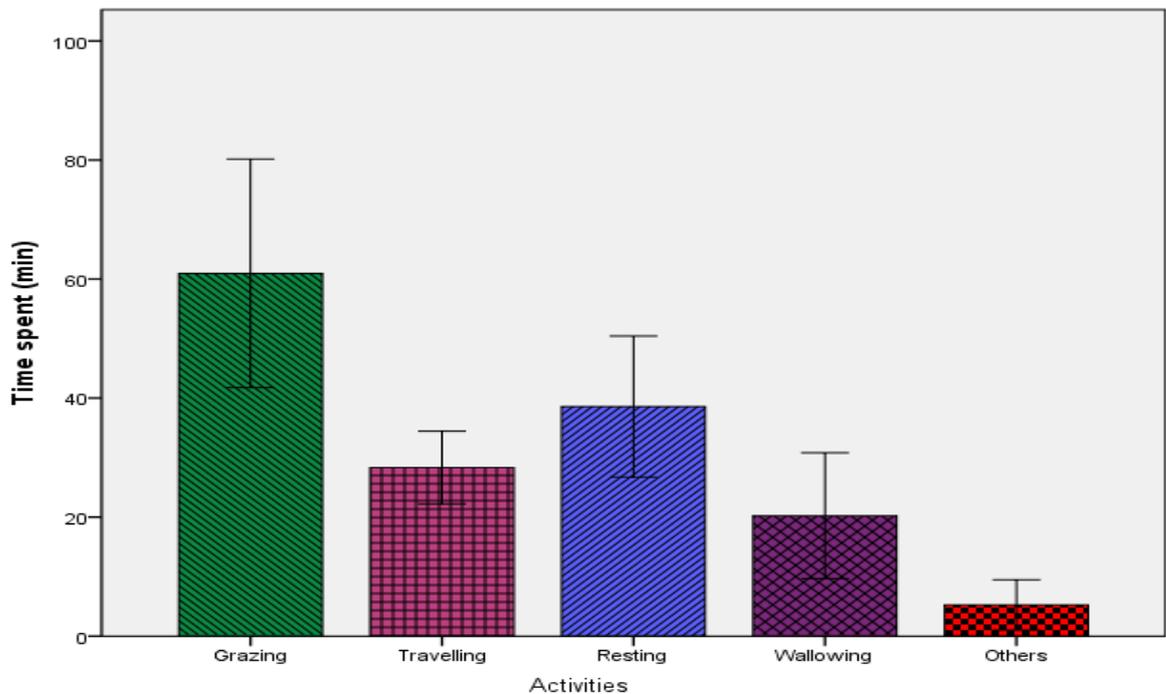


Fig. 11. Summary of behavioral activities of translocated wild buffaloes within the enclosure at CNP during Summer season, 2017

Similarly, in the monsoon season, maximum time (mean value in minutes) spent on grazing (107.17 ± 8.25) followed by wallowing (27.17 ± 4.33), moving (21.74 ± 2.66), resting (17.83 ± 2.32), and other activities (2.39 ± 1.71) (Fig. 12).

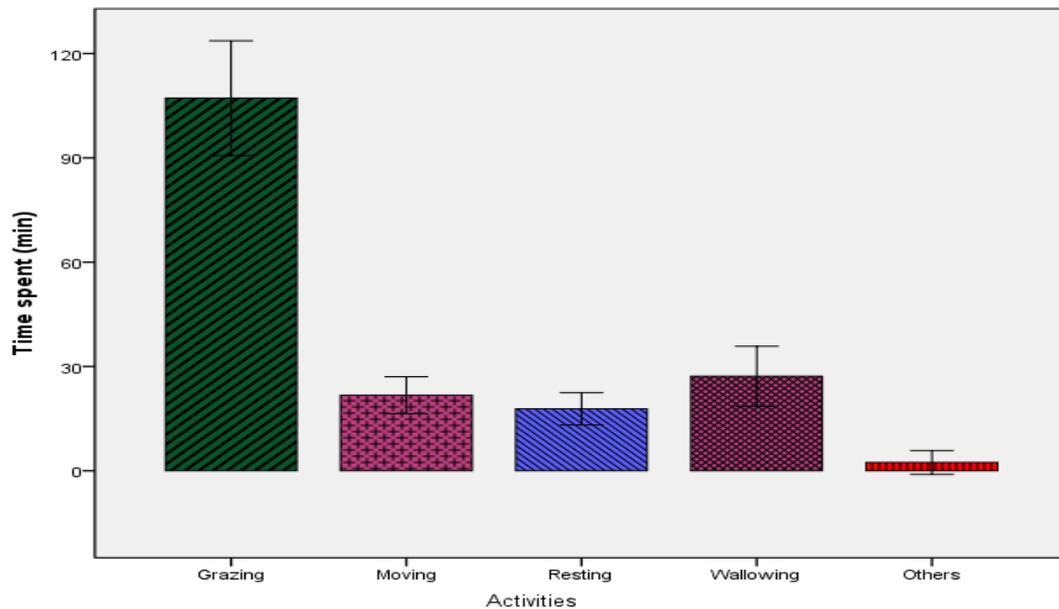


Figure 12. Summary of behavioral activities of translocated wild buffaloes within the enclosure at CNP during Monsoon season, 2017

In the autumn season, maximum time (mean value in minutes) spent on grazing (175.42 ± 11.82) followed by moving (42.92 ± 3.67), wallowing (31.88 ± 3.63), resting (31.04 ± 3.02) and other activities (13.33 ± 1.26) (Fig. 13).

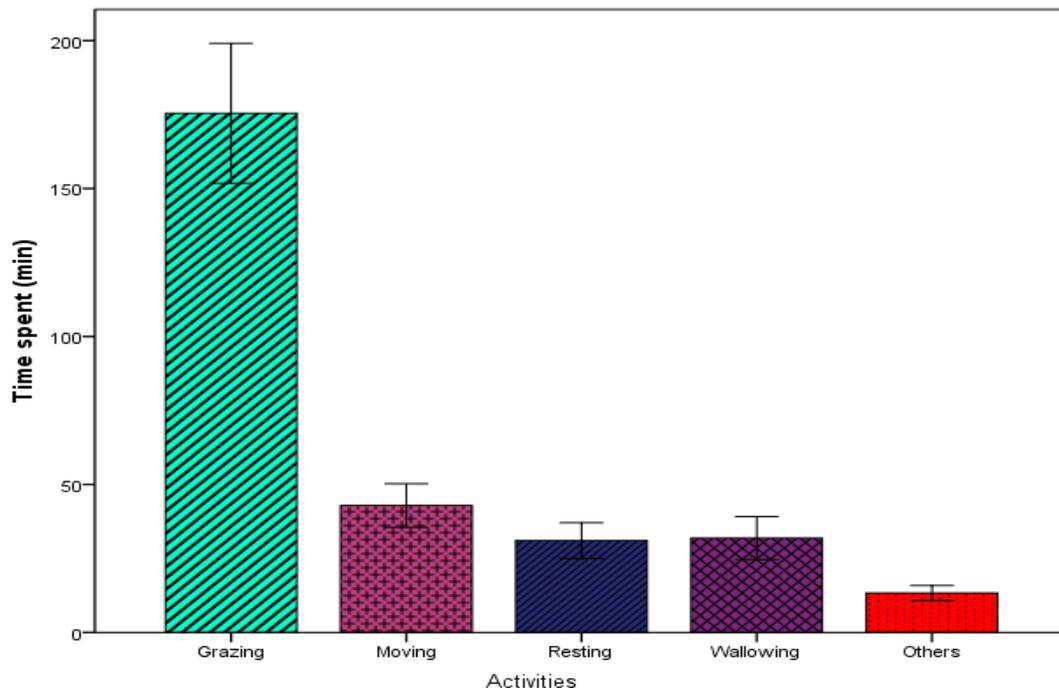


Figure 13. Summary of behavioral activities of translocated wild buffaloes within the enclosure at CNP during Autumn season, 2017

Comparing with the behavioral study of wild buffaloes at KTWR, there is diminutive variation in activity budgets among the different individuals irrespective of months or seasons across two different years; similar is the result of the post-translocation behavior of wild buffaloes at CNP. Time spent on other activities of wild buffaloes in all of the seasons was negligible, which are only the usual parts of their daily activities. As there have not been any significant variations in behavioral time budget across different years with respect to the same seasons, these can be inferred that the animals are triggered as per the survival need except coincidences of performing slightly differences probably due to weather, terrain or availability of palatable forage in the given habitat. Sometimes it has been observed to have slight differences based on the resources available which are more important as an ecological requirements as well as the physiological phenomenon of the wild buffaloes.

ADAPTATION TO THE NEW HABITAT BY WILD BUFFALOES

The translocated small population of wild buffaloes at CNP is doing well though some are forfeited in the course of adaptation at the initial stage. They are kept inside the soft release enclosure under the close observation to see if there would be any effect on them regarding their feeding and other activities along with the health conditions. The behavioral activities are found in a similar trend as like in KTWR, and herding behavior is clearly shown by forming varied sizes right from the very beginning of post-translocation. Since the earlier population of this beast was wiped out from Chitwan due to some reasons probably outbreak of diseases or low quality of habitat variables after the established human settlements inside the CNP in Padampur, long-term monitoring is highly necessitated by the Government's management authority. So these are to be minutely evaluated to witness if there has been any effect on their natural behavior and health condition along with regular intervention on habitat management (Kandel *et al.*, 2018) including maintenance of grassland, cleaning and improving quality and quantity of water sources, removal of weed species from within the enclosure, regular health check-up as per necessary, nutrient supplementation as have been practiced since after the August flood 2018. To increase the number of animals and size of the enclosure is highly advisable to create a viable population of wild buffalo in future at the earliest possible. We prescribe here to increase size of wild buffalo population in CNP with at least more than 50 animals and release to wilderness condition almost within 6 months or so after they make aggregated herd size.

CHAPTER-VII

BEHAVIORAL ECOLOGY AND ACTIVITY TIME BUDGET OF ASIAN WILD BUFFALO (*Bubalus bubalis arnee* Kerr, 1792)

Activity budget of an animal essentially depicts the behavioral diversity in terms of the periodicity of activities that have been defined by Caro and Sherman (2012) as the total number of species or population-specific behavioral and life history variants in the wild. The conceptual reasons behind the patterns of behavioral diversity have also been illustrated, and it is considered as a worthy conservation-oriented goal (Ryan, 2006; Caro and Sherman, 2012). Caro and Sherman (2012) explained three main reasons for studying activity budget or behavioral diversity as (1) Loss of behavioral diversity may prevent a population from adapting to future environmental changes, and loss of behavioral flexibility within an individual to sudden habitat alterations; (2) When behavior disappears that may indicate the loss of mechanisms due to altered allelic frequencies, hormonal pathways, and so on that predisposes individuals to behave in a certain way, or absence of environmental conditions that allow a particular behavior to develop or to be manifested; and (3) Behavioral diversity provides a window in which social and ecological challenges were important enough in a population's evolutionary history that leads to observable behavioral responses.

Behavioral studies focus on how an organism responds to their physical, social and biological environments and the reactions they realized to shape up their survival strategy through the action of natural selection, kin selection, and sexual selection. Behaviors are variable between individuals in terms of forms and frequencies. According to which behavioral ecologists shall be interested in how this variability affects an individual's survivorship and mating success as a part of their existence along with adaptation to their environment, ultimately leading towards the evolution. Several researchers, scientists, and authors have given attention on the importance of preserving behavioral and cultural diversity related to animal populations during the current period of rapid anthropogenic change (Clemmons and Buchholz, 1997; Linklater, 2004; Whitehead *et al.*, 2004; Ryan, 2006; Laiolo and Jovani, 2007). However, it is difficult to say for most of the species whether their behavioral diversity is declining, increasing or remaining stable in the changing environmental conditions. Despite this fact that there is some non-maintenance behaviors such as social interactions and territorial activities in which most of the animal species devote a considerable amount of time. To study the behavior of Asian wild buffaloes at present,

parts of islands of KTWR at either side, that is extended north to south along the Koshi River, were almost well covered up. It is well understood that the shift in Koshi river course (Sah, 1993b) has been a crucial factor to shift the behavioral and habitat use pattern by wild buffaloes as like other sympatric species since there are frequent changes in habitat composition almost every couple of years due to river dynamics. Detail studies on habitat status and composition were carried out quite a long back (Sah, 1993a; WMI/IUCN-Nepal, 1994, Shrestha, 1996) but present-day updated studies on it are lacking. Similarly, the intensive study on this species as a long-term research is not carried out so far till date. So the daily, monthly and seasonal activities, and time-activity budget performed by the Asian wild buffaloes as well as habitat composition for wild buffaloes is vital to document to know the biology of the animals and focus on its conservation in future.

Animals exhibit interspecific to intraspecific interactions, and with also the changes in environmental conditions. The exploration of their relationship and the process of interaction to their physical environment and to other organisms provided the basis for the behavioral study. The causes of behavior include both the external stimuli and the internal mechanisms. The functions of behavior includes its immediate effects on animals and its adaptive value in helping animals to survive or reproduce successfully in a particular environment. The environments which mainly influence the behaviors of wildlife are habitat quality and climate (Gopal, 1992).

How a species allocate it's time for various activities is a part of its lifestyles and behavior (Struhsaker and Leland, 1979). Animals adjust to variations in habitats in order to optimize utilization of resources for survival and reproduction (Dunbar, 1988). The concept of time budget in social animals such as diurnal herbivores, assuming that the daylight time available is a limiting factor in that animal, and that has to carry out the maintenance activities as well as social activities (Altmann 1980; Dunbar, 1988; Janson and Boinski, 1992). These constraints may exert a more general pressure to time budget allocations for various activities (Chapple, *et al.* 1963; Altmann, 1980). The key factors which influence the animal behavior and activity budget (Clutton-Brock and Harvey, 1977a; Passamani, 1988; Passmani and Fernandez 2011) are spatial distribution, quality and seasonality of food resources, requirement of diets (Agetsuma, 1995; Agetsuma and Nakagawa, 1998), reproductive tactics (Schoener, 1971, Clutton-Brock, 1977), seasonality, weather condition, anthropogenic pressures, other mineral supplements, etc.

Males and females of wild buffalo as like other mammalian species have different energetic demands which are sometimes described by its different body size in most of the herbivore

species (Rodman, 1977; Ghiglieri, 1984). There were reports of differences in feeding and resting time in sexually dimorphic species i.e. males generally feed less and rest more than females in such species (Clutton-Brock, 1977) as also attained from the general observation of wild buffalo. Another explanation may be lactating and pregnant females spend more proportion of time in feeding to compensate for the additional energy required (Altmann, 1980; Strier, 1987). In this study, it appeared that some of the females were engaged in more feeding than the males which may be because of having been a lactating stage of the females. Wild buffaloes spent long time (almost 6-8 hours) for wallowing, especially during hot seasons; they contemplate in water sources such as along the major river courses like Saptakoshi, Mariya and Trijuga rivers, and other water sources formed due to these sources like ponds, oxbow lakes, puddles, mudflows, etc. all over the areas of wildlife reserve. Therefore waterholes observations are used for locating the major herds of wild buffaloes as per our research objective. They mostly rely on tall grasses and also short grassland especially during dry winter to hot weather while there remains scarcity of greenery and low palatability in the vegetation communities.

MATERIALS AND METHODS

The time (hours) consumed to fulfill the objective of behavioral studies of wild buffaloes with continuous monitoring by focal animal sampling was 288 full days and 1728 hours (2 years-12 hours/day*6 full days each month*for 24 months= 864 hours in a year).

FOCAL ANIMAL SAMPLING

Wild buffaloes are passive movers and normally territorial that undertake behavioral activities mainly during foraging are feeding, resting, moving, wallowing, and social interactions within its territory. They live in groups of varying sizes, however, some male individuals also stay solitary especially adult ones. Despite the wider geographic distribution of the wild buffaloes at either side of the main course of Koshi River, focal animals were monitored mainly at an eastern block of the wildlife reserve since the major herds are ordinarily confined to the eastern side of Koshi Tappu (Heinen, 1993; Heinen & Kandel, 2006). The major fragment of the river is reported to shift at the eastern side wherein the very first censuses had also reported major herds of wild buffaloes (Dahmer, 1976; Heinen, 1987, 1988).

Focal animal sampling technique was performed using the protocol described by Altmann (1974) and also followed by Chalise (1999, 2003) to record the social and ecological behavior of the

wild buffaloes. Selection of focal animal was random for each session of observation. Approximate time of each session for focal animal monitoring was 6 hours in each sampling session day, and a number of days every month were 12 covering half day for a session and another half of the session covered on the next day to complete a full session of a whole day. Each of the sessions was between 0600-1200 and 1200-1800 hrs every day. Adult animals were preferred for sampling as a focal animal since it has influences upon others in performing their activities. During the observations, the activity of a focal animal was recorded in every 5-minutes interval using focal animal sampling technique (Altmann, 1974). Observations were recorded for two years, 2015 and 2016. In an average, 12 animals were monitored every month and 144 animals were chosen as focal animals in a year. Total duration monitored every month was 72 hours, and 864 hours in a year; hence the total number of days covered during two years of field study was 288 that correspond to 1728 hours. Focal animals were either solitary sub-adult to adult males or one of a sub-adult to an adult male from male band groups or a sub-adult to adult male or female from the major herds. Those were calculated as monthly, seasonal or yearly proportional variations of time spent on different activities as explained above. Total numbers of wild buffaloes associated with focal animals in different groups during the monitoring in 2-years' study were 4753.

BEHAVIORAL ACTIVITIES

The behavioral activities are also influenced by the individuals' own physiological state (Li, 2009) which is considered in the active part of the daily activities of Asian wild buffaloes. It is broadly divided and further regrouped into five major categories of maintenance behavior such as feeding (grazing), moving (travelling, running), resting (standing, sitting, lying, sleeping), wallowing, and also non-maintenance behavior (other activities) such as social interactions and territorial activities including aggression, social play, sexuality, fighting, drinking, grooming (both auto-grooming and allo-grooming), etc encompassed a category of other activities. The proportion of time spent in each category of behavior is referred to as the activity budget.

Major activities and time spent on each activity are defined as follows:

- 1) Grazing: foraging the food plant or parts of available food.
- 2) Moving: travel to different feeding ground while performing other social behaviors.
- 3) Resting: motionless time of the animals including standing, sitting, lying, sleeping.
- 4) Running: sudden quick movement of the animals with specific purpose or reactions.

- 5) Rumination: swallowing the food content from the mouth with regurgitation.
- 6) Wallowing: thermoregulatory activity for cooling its body surface on swamp or river.
- 7) Social interaction: playing, aggression, sexuality, sympatric, territorial encounters.

DATA ANALYSIS

Data collected on various activities and time spent on those activities on daily, monthly, seasonal and annual basis were analyzed. Variations between different months and seasons, and years was reflected while calculating the mean and standard error (mean±S.E) of each of the activities. To compute the seasonal variations in time budget during a year is divided into 4 main seasons as follows: winter (Jan-Mar); summer (Apr-June); monsoon (July-Sept); and autumn (Oct-Dec). The Krushkal-Wallis one-way ANOVA was also applied to evaluate the activity budgets of the different focal animals irrespective of whether they are in a natal herd or male band group or solitary. Different categories of behavioral activities were tested for significance of time budget with the percentage of time devoted to each of the activities. Significance was set at $p < 0.05$ for all statistical tests.

RESULTS AND DISCUSSION

TIME BUDGET ACTIVITY PATTERN

The total duration of focal animal sampling was 72 hours in every month and 864 hours in a year. During 2 years of field study, a total of 288 full days and 1728 hours were used to observe the focal animals. The data on time spent on different activities such as grazing, moving, resting, wallowing and other activities across the months, seasons and years during 2015 and 2016 is elaborated in table 17 and 18.

MONTHLY ACTIVITY PATTERNS

General observations in this study indicated that wild buffaloes spent their time mostly in resting followed by grazing. However, grazing was higher during May, June, July, and October whereas wallowing occurred only during March through September in both the years. Year-wise variations in the time spent in different activities are further described below in details.

In the year 2015, the resting time (in a minute) was maximum in the month of February (243.3 ± 5.3 SE) followed by January (236.7 ± 6.5 SE) and December (185.0 ± 13.3 SE) (Table 17). While the time spent in grazing was maximum in June (218.3 ± 8.9 SE) followed by May

(165.4±8.4 SE) and October (158.8±6.8 SE), and the wallowing was performed more during August (29.2±5.9 SE) followed by July (28.8±4.0 SE) and no wallows were recorded during October through February. On the other hand, animals were found moving more during March (91.3±4.3 SE) followed by April (83.3±2.5 SE) and least during February (33.1±3.1 SE). They were seen to moving in all the months of both the study years in an equal proportion.

Table 17. Monthly variations in activity budgets (mean value in minutes with SE) in five major activity categories in 2015

Months	Observed no. of days total	Grazing		Moving		Resting		Wallowing		Other Activities	
		Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
January	12	82.5	5.9	37.1	3.1	236.7	6.5	0.0	0.0	5.0	1.9
February	12	77.1	5.3	33.1	3.1	243.3	5.3	0.0	0.0	4.2	1.7
March	12	125.8	6.5	91.3	4.3	126.7	7.3	3.8	2.7	12.5	1.2
April	12	125.4	10.2	83.3	2.5	130.4	11.5	12.1	3.5	8.3	2.0
May	12	165.4	8.4	40.8	1.6	130.4	7.9	18.8	5.2	4.6	1.6
June	12	218.3	8.9	37.9	3.1	81.3	6.5	19.6	5.7	2.9	1.6
July	12	140.8	11.1	49.2	2.9	136.7	10.3	28.8	4.0	4.6	1.8
August	12	130.4	8.7	62.1	3.5	132.9	9.3	29.2	5.9	5.4	1.7
September	12	133.3	8.8	48.8	3.0	159.6	8.9	15.4	4.1	2.9	1.3
October	12	158.8	6.8	58.3	2.2	135.0	7.7	0.0	0.0	7.9	1.6
November	12	138.8	9.2	51.3	3.1	165.0	11.4	0.0	0.0	5.0	1.5
December	12	118.3	9.2	50.0	4.2	185.0	13.3	0.0	0.0	6.7	1.9

In the year 2016, monthly activities budgets of wild buffaloes in Koshi Tappu shows that the major activity categories like resting time in January (244.6±5.1 SE) was highest followed by February (225.6±15.7 SE) and least during June (72.08±8.08 SE) (Table 18). The total time spent in grazing was maximum in June (221.7±7.6 SE) followed by May (162.9±9.05 SE) and October (157.5±8.6 SE), and least during January (76.67±4.4 SE) and February (74.58±2.9 SE), while the wallowing was performed higher during June (22.92±4.9 SE) followed by July (21.25±5.9 SE) and no wallows, was recorded from October through February which was same as in the year 2015.

Table 18. Monthly variations in activity budgets (mean value in minutes with SE) in five major activity categories in 2016

Months	Observed no. of days total	Grazing		Moving		Resting		Wallowing		Other Activities	
		Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
January	12	76.67	4.41	37.08	2.851	244.6	5.129	0	0	1.667	0.94
February	12	74.58	2.917	38.75	3.085	225.6	15.74	0	0	2.083	0.965
March	12	122.9	7.869	85.83	3.735	133.3	6.918	6.25	4.314	11.67	0.94
April	12	122.1	8.604	81.25	2.895	139.2	7.997	13.33	4.741	4.167	1.486
May	12	162.9	9.055	40.42	2.172	134.2	7.173	16.25	4.812	6.25	2.143
June	12	221.7	7.621	38.75	2.313	72.08	8.082	22.92	4.941	4.583	1.438
July	12	145	12.31	50	2.683	136.7	12.86	21.25	5.907	7.083	2.258
August	12	124.6	10.86	64.17	2.809	149.6	10.56	18.33	4.701	3.333	1.281
September	12	135	10.66	45.42	2.172	162.5	9.663	10	3.198	7.083	1.564
October	12	157.5	8.627	59.17	2.741	135.4	6.976	0	0	7.917	1.3
November	12	127.5	11.93	48.75	2.471	177.1	14.62	0	0	6.667	1.978
December	12	120.4	9.444	51.25	4.225	183.8	11.32	0	0	6.667	1.281

SEASONAL ACTIVITY PATTERNS

The data on time spent on different activities across the seasons in different years were analyzed for seasonal variations. The detail of the result is presented in figure 14. Kruskal-Wallis test was performed to know seasonal influence on activity patterns in the year 2015 and 2016. Based on the seasonal observation (Fig. 14) of the activities budgets in the year 2015, the analysis showed that each category of activities significantly varies across the seasons ($H= 16.59$, $HC: 16.59$, $p = 0.002326$).

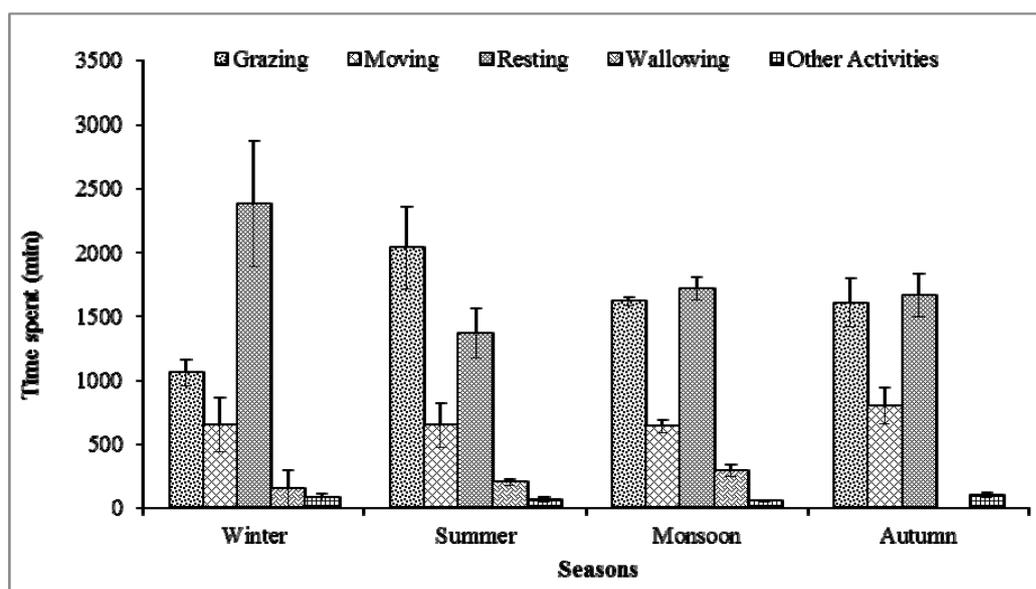


Figure 14. Seasonal variation in time budget activities during 2015

To understand seasonal variation, the observations were repeated in 2016, activity pattern was found similar and consistent to the previous year, 2105 (Fig. 15) across the seasons ($H= 16.59$, $HC: 16.59$, $p= 0.002326$). This indicates that seasonal influences on time spent on each activity remain mostly the same in both 2015 and 2016.

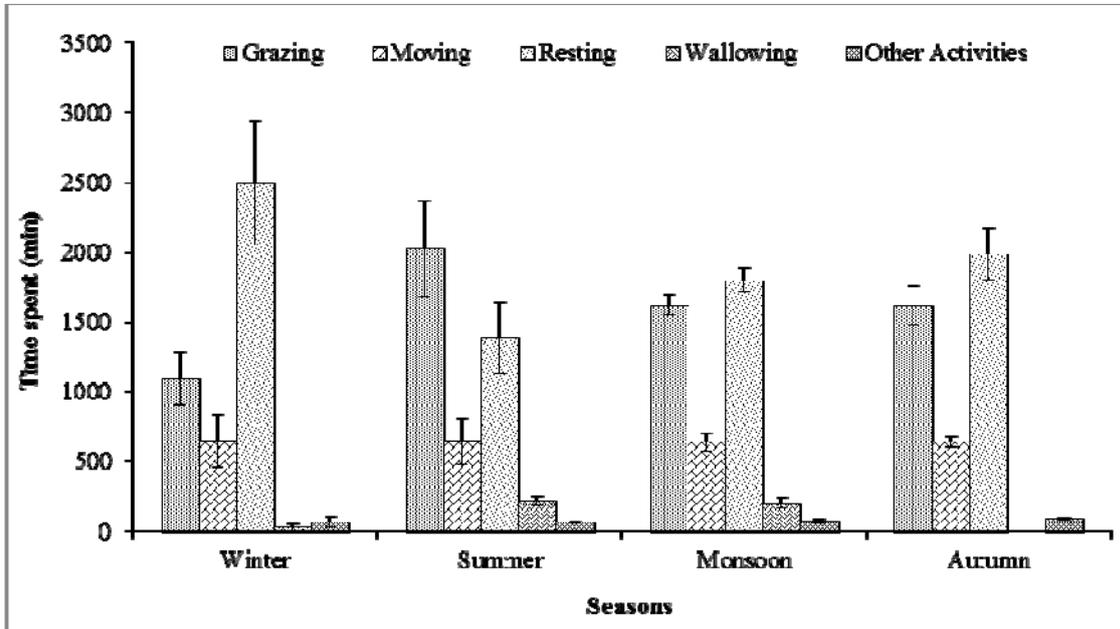


Figure 15. Seasonal variation in time budget activities during 2016

ANNUAL ACTIVITY PATTERNS

Time spent on different activities during 2015 and 2016 shows no significance variation (Figure 16) across the years ($H= 8.727$, $HC= 8.727$, $p= 0.06829$). The time on different activities was influenced by the weather and season of the year. They were involved in grazing more time during winter and more wallowing in summer and monsoon in order to keep them cool by means of thermoregulation.

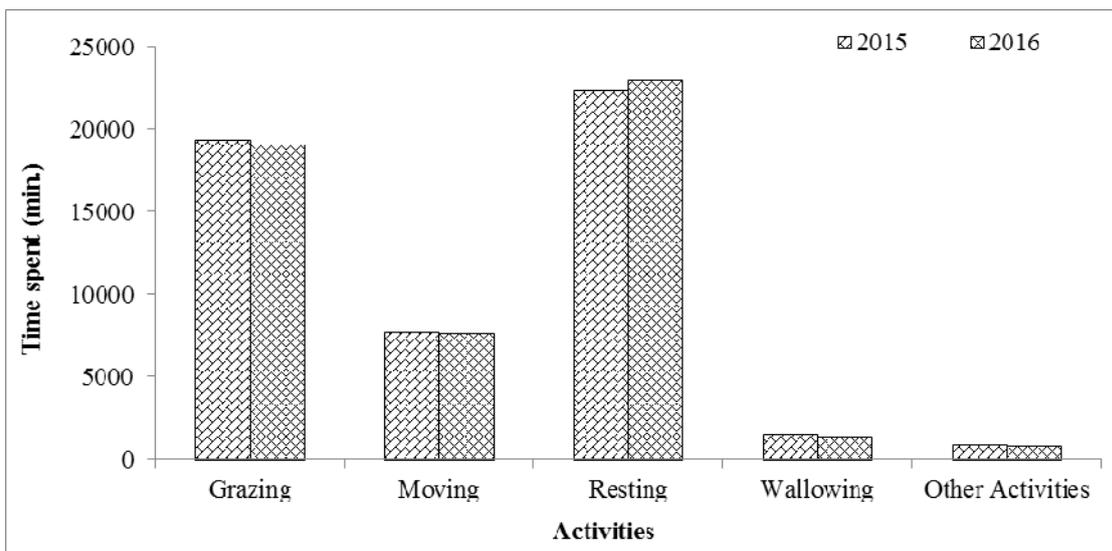


Figure 16. Comparison of annual activities time budget of wild buffaloes

The activities distribution pattern of time allocated to them in different year are described below and presented in figures 17 & 18. Time budget for activities during 2015 and 2016 showed that there is a similar pattern of activities budget across the different years ($H= 8.727$, $HC= 8.727$, $p= 0.06829$).

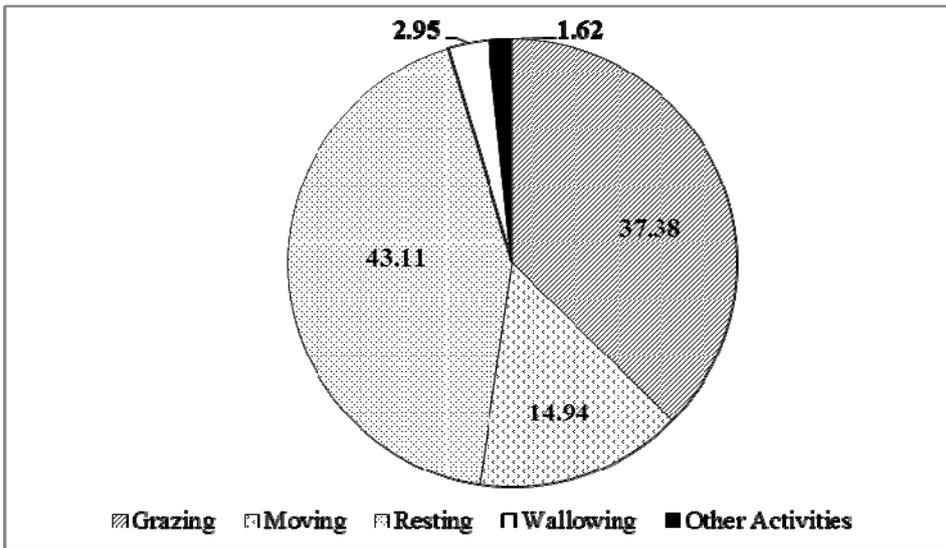


Figure 17. Activity time budget (%) of Asian wild buffalo in 2015

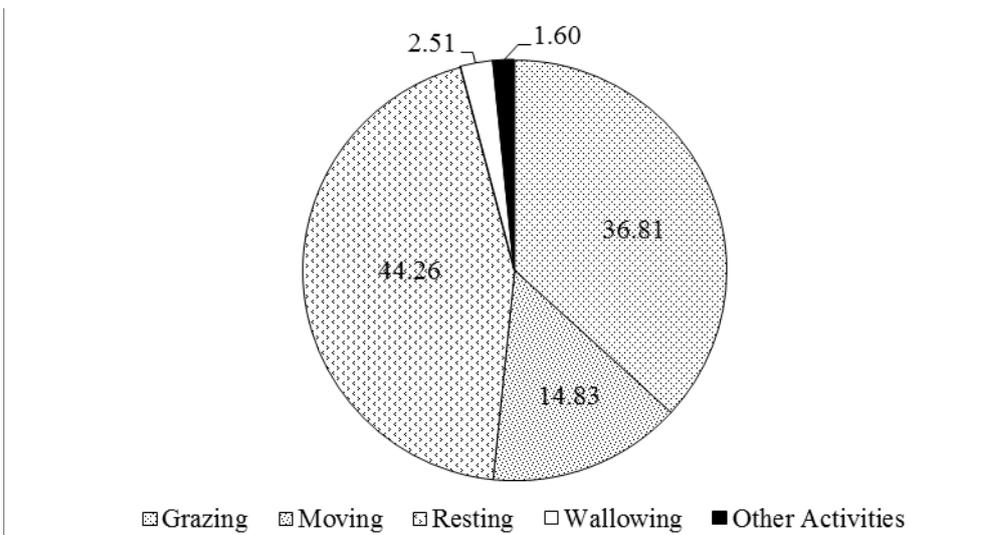


Figure 18. Activity time budget (%) of Asian wild buffalo in 2016

Major activities related to their survival such as grazing and wallowing are performed during the summer season followed by monsoon season since these seasons are more preferable for foraging and required cooling themselves through wallowing in the river. While moving and resting including other minor activities are performed during the winter season as they require heat energy from the sunlight in order to protect themselves from the severity of cold during winter (Table 19, 20).

Table 19: Seasonal variations in activity budgets (mean value with SE) in five major activity categories in 2015

2015 Seasons	Grazing		Moving		Resting		Wallowing		Other activities	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
Winter	1141.7	185.1	655.0	220.1	2426.7	453.9	15.0	15.0	86.7	31.79
Summer	2038.3	321.5	648.3	176.1	1368.3	196.7	201.7	28.5	63.3	19.22
Monsoon	1618.3	37.2	640.0	52.5	1716.7	100.0	293.3	54.2	51.7	8.82
Autumn	1663.3	140.0	638.3	31.1	1940.0	174.4	0.0	0.0	78.3	10.14

Summer months are highly preferred for grazing by wild buffaloes while winter season mainly for more rest, while and more time for wallowing during monsoon hours as depicted by table 19 and 20 in both the observation years.

Table 20: Seasonal variations in activity budgets (mean value with SE) in five major activity categories in 2016

2016 Seasons	Grazing		Moving		Resting		Wallowing		Other Activities	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
Winter	1096.7	189.3	646.7	191.8	2490.0	445.0	25.0	25.0	61.7	39.19
Summer	2026.67	346.82	641.67	166.76	1381.67	258.9	210	34.03	60	7.64
Monsoon	1618.33	70.73	638.33	67.72	1795	89.49	198.33	40.45	70	15
Autumn	1621.67	136.39	636.67	37.67	1985	181.5	0	0	85	5

The primary factor in a given set of environmental conditions that influences time allocation for various activities by animals is the pattern of adjustment of their lifestyle. This factor is taken into considerations as one of the adaptive strategies (Ryan, 2006; Caro and Sherman, 2012) for the survival of their life. So, it is very important to note that the proportion of time spent in different behavioral categories, especially when a population is under threats, is paramount. Allocating time to different activities (Martin and Bateson, 1997) during the day is also important to understand how animals adjust to the various type of habitats for optimum utilization of food for survival and reproduction because the time is a limiting factor (Dunbar, 1988). They are found to perform daily activities such as feeding, resting, standing, moving, wallowing, social interactions around the territories mainly at open areas with tall and short grasslands mostly in proximity to water-logged sites, and scrub or savannah forests.

Habitat condition is also a factor for variations in behavioral time budget in months and seasons which was noted to differ with the opening of the dense grassland with the onset of the winter season. Similarly, the findings of the current studies on activity budget would have strong

implication to habitat management with respect to animal behavior. There were some observations of the daily active period of wild buffalo that was affected by other factors such as human-induced disturbances and sometimes the presence of predators, so are the wild buffaloes become highly sensitive and change the activity spontaneously. Besides that, the presence of other species such as domestic buffaloes, cattle, and feral buffaloes affect on behavioral activities pattern in long run through the sympatry because sympatric species share the diet, space and intervene in social activities. since buffalo's day hours consists of portions of time to spend in feeding, resting, traveling between food sources and essentially the social interactions, this is also true for many of the diurnal animals in performing such behaviors.

This suggests that month to month variation in their activity budgets represents a reaction to the socio-ecological factors, rather than being an artifact of the sampling regime or sample size. On the other hand, the proportion of time spent in different activities remains almost similar during the same season over two years irrespective of whether they are a solitary or small herd of males or a small herd of female or larger mixed herds. While carrying out the behavioral studies of wild buffaloes, based on the total number of animal observation (N=134), it was also noted that the patterns of habitats use at KTWR was found that the grassland and ecotone habitat the most (58.21%) followed by Sissoo, Sissoo Savannah and Sissoo mix forest (32.09%). An additional area of *Typha* dominated grassland comprises 6.72%.

Feeding as a major behavioral category significantly differs between months and seasons, same was the situation of resting and other behavioral activities across seasons. However, the species' activity pattern is found to be more or less with the same tone with respect to different habitats, months, seasons in the study area despite the different disturbance gradients. For overall feeding behavior of the large herbivores, the current finding resembles the earlier results of Hutchins and Kreger (2006). They reported that the feeding time of the wildlife depends on nutritional requirement as per their body size. Being mainly a diurnal animal, behavioral activities of wild buffaloes are also reported to perform over the wide range of grasslands and savannah forests. The impacts of the habitat management (Kandel *et al.*, 2018) have the importance in preserving their behavior through which wild buffalo population would also be automatically preserved. Behavioral activities of the wild buffaloes are directly related to the nature and frequency of the anthropogenic activities which affect the natural behavior of wild buffaloes as like in other herbivorous animals.

CHAPTER - VIII

MOLECULAR STUDY OF WILD, FERAL AND DOMESTICATED BUFFALOES OF KTWR, NEPAL USING *CYTOCHROME-B* MARKER

The domesticated water buffalo (*Bubalus bubalis*) is one of the most important animals in South Asia. Buffaloes are traditionally raised for milk, meat, draught, hides, and manure. This livestock also acts as a reserve of capital for each family in the rural economy. This animal is enormously useful for ploughing agriculture fields. Buffaloes are broadly categorized into two types namely River buffalo (*Bubalus bubalis bubalis*) and Swamp buffalo (*Bubalus bubalis carabanesis*) (MacGregor, 1939). These two types can be distinguished based on their morphology, karyotypes, and behavior (Flamand *et al.*, 2003). The swamp type has $2n = 48$ chromosomes, relatively straight pale colored horns, white chevron, socks, and the tip of the tail. On the other hand, the river type is recognized with a black body and curved horns which has $2n = 50$ chromosomes (Ulbricht & Fischer, 1967). The river buffalo is found from Indian sub-continent to the west of Italy, and the swamp buffalo is restricted mainly from south-east Assam towards east up to Yangtze valley of China. Studies have shown that the genus *Bubalus* was distributed from South East Asia to Europe in the Pleistocene age but later its population has been restricted from Indian continent to South East Asia (Mason, 1974). From these remaining populations, river and swamp types have been estimated to diverge from 15000 to 1.7 million years (Tanaka *et al.*, 1996; Barker *et al.*, 1997; Lau *et al.*, 1998). However, the domestication history of Buffalo in India and China is suggested to be 5000 years and 4000 - 7000 years old respectively (Cockrill, 1974; Chen and Li, 1989). Despite the differences between river and swamp buffalo types, they interbreed easily and produce progeny with intermediate chromosomes (Mishra *et al.* 2015). The swamp type is more similar to the wild-type Asian buffalo (*Bubalus arnee*) (Lau *et al.* 1998, Kumar *et al.* 2007, Mishra *et al.* 2015).

The government of Nepal has declared Koshi Tappu Wildlife Reserve in the year 1976 primarily to conserve the probable wild population of buffalo (*Bubalus arnee*) which is commonly known as Arna. This species is considered as highly endangered in this region and is thus getting legal protection in an isolated population of Koshi Tappu Wildlife Reserve of Nepal, Manas Wildlife Sanctuary and Raipur and Bastar districts of India, Royal Manas National Park of Bhutan and Huai Kha Kaeng Wildlife Sanctuary of Thailand (Groves, 1996). In 2016, a total of 433 individuals of wild buffaloes were counted from Koshi Tappu Wildlife Reserve, Nepal (Kandel *et al.*, 2018). This figure probably includes both potentially pure wild buffaloes and highly

backcrossed or semi-feral individuals. In this context, it is very challenging to protect purebred wild animals. Some biologists even suspected that there could be no purebred animals in the herds of KTWR (Scherf, 2000). Despite the possibility of interbreeding between wild and domesticated buffaloes inside the reserve, it is very important to assign extant individuals to wild and other types to broaden our understanding on the genetic structure of different types and also to maintain genetic fingerprinting for the conservation of pure wild breed. Microsatellite genotyping for ten microsatellite loci of 45 individuals (domestic 28, wild 10, and hybrid 7) carried out by Flamand *et al.* (2003) found very small genetic distances among the studied three groups. Zhang *et al.* (2011) has analyzed three previously published datasets including that of Flamand *et al.* (2003) and showed that wild buffalo of Nepal, the phenotypical swamp type but genetically like river type, were significantly different from all the domestic river populations. They further concluded that wild breed of Koshi Tappu may represent the ancestral *Bubalus arnee*. Given the introgression from domestic to wild population is female-mediated, mitochondrial DNA (mtDNA) sequencing would help us to find out group specific mitotypes. Presence of wild specific or domestic-bred specific haplotype in either of the group would allow us to identify hybrids (Flamand *et al.*, 2003; Lau *et al.*, 1998). Furthermore, mtDNA sequence variations have been widely applied in mammals to study inter and intra-species phylogenetic relationships (Su *et al.*, 1999; Kuwayama *et al.*, 2000; Cook *et al.*, 1999; Lau *et al.*, 1998; Thomas *et al.*, 1989; Irwin *et al.*, 1991; Kikkawa *et al.*, 1997; Kumar *et al.*, 2007). We present here results for DNA sequencing variation in partial *cytochrome b* gene among pure wild breed, feral and domesticated individuals of buffaloes. Long-term conservation of wild breed and translocation of potential individuals from Koshi Tappu to other areas of Nepal require detailed knowledge of genetic variability in an extant population. In addition, understanding the genetic make up of wild breed of the buffalo would be advantageous to use them as the basis for genetic improvement as a source of bull.

MATERIALS AND METHODS

Total time consumed on Molecular studies of wild buffaloes was mainly devoted to collect samples from wild buffaloes consists of 16 blood samples, 40 dung samples, and 2 tissues; 23 blood samples from feral buffaloes; and 17 blood samples from domestic buffaloes. During the capture operation, it took 30 minutes for each of the sample collection that consists of 2900 minutes in total equivalent to 49 hours; additional time spent in collecting blood sample and time taken for analysis including extraction of DNA, PCR and DNA sequencing was ca. 50 hours.

SAMPLE COLLECTION

Samples were collected from 42 buffaloes (Table 21) from in and around the Koshi Tappu Wildlife Reserve, eastern Tarai region of Nepal (Figure 19). Animals were divided into 3 classes, classified as wild (n=20), domestic (n=11) and hybrids (n=11). This classification was based on location of sampling (in the wild or near villages), behavioral and phenotypic traits. All animals classified as domestic were river type with black body and curled horns (as in the Murrah breed river buffalo), while those classified as wild had white chevron, socks, and the tip of tail, and larger, relatively straight, pale-colored horns (similar to swamp buffalo) (Heinen, 2002). Hybrids were crossbred of wild and domestics. Blood samples from wild were collected during the translocation of wild buffaloes from Koshi Tappu Wildlife Reserve to Chitwan National Park. Fecal samples from wild buffaloes were also collected fresh with GPS locations from the core area.

Table 21. Details on types of samples collected from a total of 42 buffalos of KTWR, Eastern Nepal

SN	Samples code	Group	Sex, age, date of collection	Remarks
1	BuffH1	Hybrid	Female, 10 months, 02/07/2017	Blood sample
2	BuffH2	Hybrid	Female, 6 years, 29/06/2017	,, ,,
3	BuffH3	Hybrid	Female, 2 years, 29/06/2017	,, ,,
4	BuffH4	Hybrid	Female, 2 years, 02/07/2017	,, ,,
5	BuffH5	Hybrid	Male, 2 years, 02/07/2017	,, ,,
6	BuffH6	Hybrid	Male, 2 years, 02/07/2017	,, ,,
7	BuffH7	Hybrid	Male, 2 years, 29/06/2017	,, ,,
8	BuffH8	Hybrid	Female, 2 years, 29/06/2017	,, ,,
9	BuffH9	Hybrid	Male, 10 month, 02/07/2017	,, ,,
10	BuffH10	Hybrid	Male, 1.5 years, 02/07/2017	,, ,,
11	BuffH11	Hybrid	Female, 8 month, 02/07/2017	,, ,,
12	BuffD12	Domestic	Female, 1.5 years, 02/07/2017	,, ,,
13	BuffD13	Domestic	Female, Adult , 27/06/2017	,, ,,
14	BuffD14	Domestic	Female, 10 years, 27/06/2017	,, ,,
15	BuffD15	Domestic	Male, 2 years, 01/07/2017	,, ,,
16	BuffD16	Domestic	Male, 2 years, 01/07/2017	,, ,,
17	BuffD17	Domestic	Male, 1 years, 01/07/2017	,, ,,
18	BuffD18	Domestic	Female, 12 years, 01/07/2017	,, ,,
19	BuffD19	Domestic	Male, 1 year, 29/06/2017	,, ,,
20	BuffD20	Domestic	Male, 1 year, 29/06/2017	,, ,,
21	BuffD21	Domestic	Male, 2.5 years, 27/06/2017	,, ,,
22	BuffD22	Domestic	Female, 1 year, 01/07/2017	,, ,,
23	BuffW23	Wild*	Male, Adult, 04/02/2017	,, ,,
24	BuffW24	Wild*	Male, Adult, 26/01/2017	,, ,,
25	BuffW25	Wild*	Female, Sub-adult, 06/02/2017	,, ,,
26	BuffW26	Wild*	Female, Adult, 01/02/2017	,, ,,
27	BuffW27	Wild	Male, Adult	Fecal Sample
28	BuffW28	Wild**	Male, Adult, 05/02/2017	Blood sample
29	BuffW29	Wild**	Female, Adult, 01/02/2017	,, ,,
30	BuffW30	Wild**	Female, Adult, 01/02/2017	,, ,,
31	BuffW31	Wild**	Female, Adult, 06/02/2017	,, ,,

SN	Samples code	Group	Sex, age, date of collection	Remarks
32	BuffW32	Wild**	Female, Adult, 05/01/2017	, , , ,
33	BuffW33	Wild**	Female/2.5 years, 06/01/2017	, , , ,
34	BuffW34	Wild	Female, Adult	Fecal sample
35	BuffW35	Wild	Male, Adult	Fecal Sample
36	BuffW36	Wild	Male, Adult	Fecal sample
37	BuffW37	Wild	Female, Adult	Fecal Sample
38	BuffW38	Wild*	Female, Adult, 31/01/2017	Blood sample
39	BuffW39	Wild*	Female, Adult, 29/01/2017	, , , ,
40	BuffW40	Wild*	Female, Adult, 27/01/2017	, , , ,
41	BuffW41	Wild	Male, Adult	Fecal sample
42	BuffW42	Wild	Male, Adult	Fecal sample
Total	42	Wild= 20 Hybrid=11 Domestic=11		

*Translocated from KTWR,

** Translocated from Zoo

DNA EXTRACTION

Genomic DNA was extracted from blood and tissue samples using Qiagen DNEasy Blood and Tissue kits according to the prescribed manufacturer protocol. For the fecal samples, Qiagen QIAMP DNA Stool Mini Kit was used following the instructions provided by the manufacturer. The extracted DNA samples were stored at 4 °C until used for molecular analyses. Aliquots of extracted DNA samples were used for upstream analysis like PCR and sequencing.

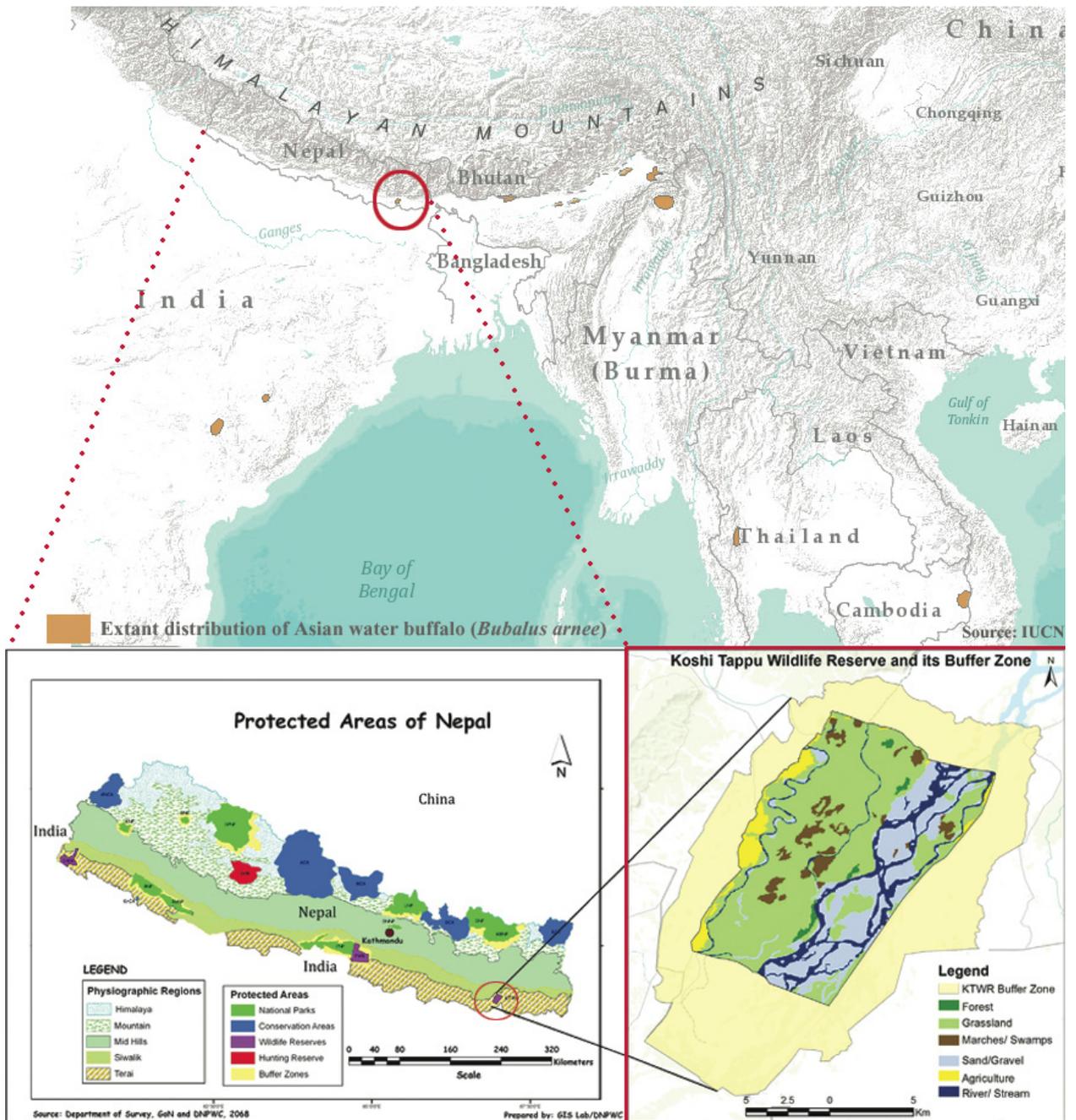


Figure 19. Map of an extant distribution of Asian Water Buffalo (*Bubalus arnee*) and the location of study area, Koshi Tappu Wildlife Reserve, Nepal

POLYMERASE CHAIN REACTION AND SEQUENCING

Small section of *cytochrome b* gene within the mitochondrial genome was amplified using primer pair as described in Kocher *et al.* (1989) (Fig.20).

L14724 CGAAGCTTGATATGAAAAACCATCGTTG and
H15149 AAAGTGCAGCCCCTCAGAATGATATTTGTCCTCA

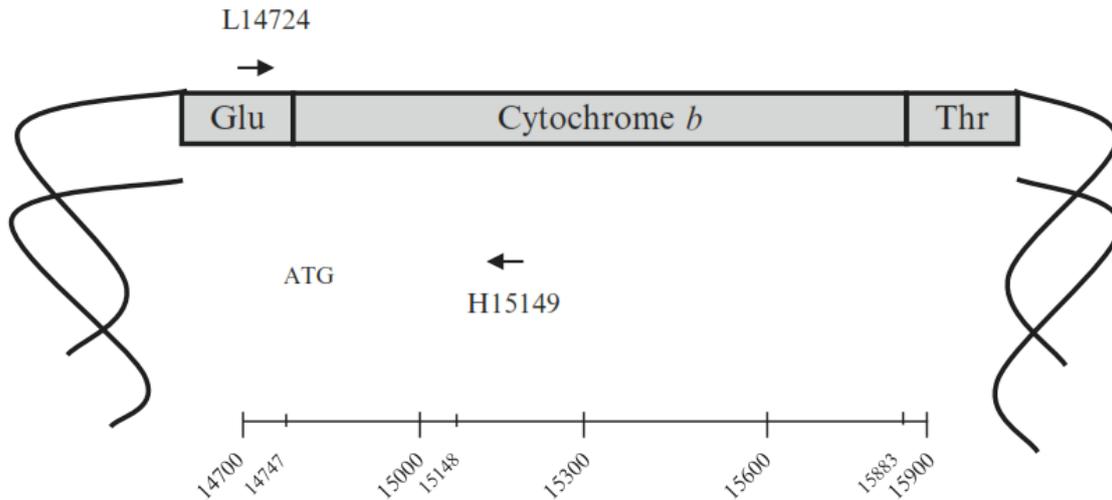


Figure 20. Schematic presentation of *Cytochrome b* gene region in the mammals (adopted from Adrian and James, 2016)

PCR was carried out with 3 μ l template DNA, 15 μ l of Hot Start Taq 2X Mastermix (New England Bio-lab, UK), 1 μ l of each primer and 7 μ l of nuclease-free water in a total reaction volume of 30 μ l using an ABI Veriti™ Thermal Cycler (Model no. 9902). The PCR conditions were an initial denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 45 s and a final extension at 72°C for 10 minutes. The PCR products were electrophoresed at 100 Volts for 30 minutes in 1.5% agarose gels, viewed under UV light after staining with *Ethidium bromide* and photographed. The amplified DNA fragments were purified using ExoSap-IT Express PCR Product Cleanup (Affymetrix Inc. Santa Clara, CA, USA) following a cycle of 37°C for 15 min and 80°C for 1 minute in a thermocycler. Out of 45 selected samples, PCR optimization was amplified successfully only in 36 samples. High quality purified PCR amplicons were subjected to Dideoxy sequencing in a total volume of 10 μ l containing 1 μ l purified PCR product, 1 μ l Big Dye terminator sequencing mixture (V3.1) (BIGDYE Terminator Cycle Sequencing Kit, Applied Biosystems, Foster, CA, USA), 1.5 μ l sequencing buffer and 1.5 μ l primer (10 μ m). Sequencing was done in an ABI 3500 XL automated DNA sequencer (Applied Biosystems, Forster City, CA, USA).

SEQUENCE ALIGNMENT

Raw sequence fragments of 422 bp lengths were assembled, checked and edited with Sequencer 5.0 (Gene Codes Corp., Ann Arbor, MI, USA) and the contigs of both reverse and forward primers were created. Sequences were aligned with ClustalX (Thompson *et al.*, 1997) in BioEdit.

In 36 cytochrome b partial sequences (D=11, H=10, and W=16), we have included 42 additional Nepalese samples (D=20, H=15, and W=7) of Flamand *et al.* (2003) sequenced and analyzed by Zhang *et al.* (2016) (KR009944-85). To understand the possible haplotypic variation among the river buffalos of the neighboring region, we further added four *Cytochrome b* haplotypes identified by Kumar *et al.* (2007) from Indian river buffaloes (EF409939 H1-4) and 15 *cytochrome b* haplotypes defined by Kikkawa *et al.* (1997) representing both river buffaloes from Bangladesh, Srilanka, Italy and Pakistan and swamp buffaloes from Japan, Taiwan, Thailand, Philippines, Indonesia and Bangladesh (D34637-38, D88627-38, D88983). This yielded a total of 97 sequences (36 from the present study, 42 from Zhang *et al.*, 2016; four from Kumar *et al.*, 2008; and 15 sequences from Kikkawa *et al.*, 1997) of buffaloes representing river and swamp types of the region.

SEQUENCE ANALYSIS, HAPLOTYPE IDENTIFICATION, AND PHYLOGENY

All the sequences were aligned in and mitochondrial haplotypes (mitotypes) were defined in DnaSP v5 (Librado and Rozas, 2009). Haplotype diversity (h) and nucleotide diversity (π) for the buffalo samples were estimated using DnaSP 5.0 (Librado and Rozas, 2009). A phylogenetic relationship between the haplotypes was derived through a reduced median-joining network applied to the data set using Network v4.6 (Bandelt *et al.*, 1999). To identify phylogenetic lineages Maximum parsimony (MP) analysis was performed in PAUP 4.0 b10 (Swofford, 2002) using the heuristic search option with 1000 random additions and tree bisection-reconnection (TBR) swapping and MULTrees option on. Branch support was provided by a bootstrap analysis of 10000 replicates of heuristic searches, with a MULTrees option on and TBR swapping off. Consistency indices (CI) and retention indices (RI) were obtained in PAUP. Moreover, the evolutionary history was inferred using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) with 10000 bootstrapping replicates in MEGA7 (Kumar *et al.*, 2016).

RESULTS AND DISCUSSIONS

SEQUENCE VARIATION AND DIVERGENCE

The lengths of the 36 partial *cytochrome b* sequences of all the three types of buffaloes of this study are of 422 bp. The aligned matrix was 422 characters and contained 2 variable sites (Table 22). However, the 42 Nepalese complete *cytochrome b* sequences of Zhang *et al.* (2016) have 1140 characters and revealed 4 variable sites among the three groups of river buffaloes. The partial *cytochrome b* sequence used in our study is shorter than that described by Zhang *et al.* (2016) and thus has fewer variable sites. However, when our data set was compared with the accession of Kumar *et al.* (2007) and Kikkawa *et al.* (1997), base substitutions at 13 nucleotide positions (variable sites) were obtained and among them three nucleotide positions were specific for river buffaloes and ten positions were specific for swamp buffaloes. The sequence divergence within buffaloes of Nepal and India was 0.24-0.49 %; however, when compared with river buffaloes sequence of Kikkawa *et al.* (1997), the divergence was slightly higher 0.24-0.74 %. Sequence divergence within swamp buffaloes was 0.24-0.98%. The sequence divergence between swamp and river buffaloes was calculated to be 1.49-2.49%.

Table 22. Variable nucleotide positions for the partial cytochrome b gene of the 36 accessions of the present study, 42 accessions of Zhang *et al.* (2016), 4 accessions of Kumar *et al.* (2007) and 15 accessions of Kikkawa *et al.* (1997).

Haplo- types	Nucleotide positions												Frequency	Remarks
	61	79	81	87	99	147	234	240	375	379	411	417		
H1	C	G	T	T	C	T	A	G	T	T	A	A	64	Nepal sample, Zhang <i>et al.</i> , 2016; Kumar <i>et al.</i> , 2007 Kikkawa <i>et al.</i> , 1997
H2	C	G	T	T	T	T	A	G	T	T	A	A	19	„
H3	C	G	T	T	C	T	A	C	T	T	A	A	4	„
H4	C	G	T	T	C	T	A	A	T	T	A	A	1	Kumar <i>et al.</i> , 2007
H5	C	G	C	T	C	C	G	G	C	C	A	G	2	Kikkawa <i>et al.</i> , 1997
H6	C	G	T	T	C	T	A	G	T	T	A	T	1	„
H7	C	A	T	T	T	T	A	G	T	T	A	A	1	„
H8	C	G	C	T	C	C	G	G	C	C	A	A	4	„
H9	G	G	C	A	C	C	G	G	C	C	G	A	1	„
Total													97	

HAPLOTYPES IDENTIFICATION, DISTRIBUTION, AND THEIR ASSIGNMENTS

Analysis of a total of 97 *cytochrome b* partial sequences of 422 bp length of the river buffaloes (from Nepal, India, Bangladesh, Pakistan, Srilanka) and swamp buffaloes (Japan, Taiwan, Thailand, Philippines, Indonesia and Bangladesh) showed 13 variable sites defining 9 haplotypes (H1-H9) (Fig.21). Among the nine observed haplotypes, Nepalese buffaloes both in this study and that of Zhang *et al.* (2016) were represented by just the three common haplotypes (H1, H2, and H3). These three haplotypes are identified by Kumar *et al.* (2007) as well. The most common haplotype (H2) is widely distributed among groups and represented by 66 percent of sequences (Nepal: D=23, W=18, and H=17; one of Kumar *et al.* (2007) and five of Kikkawa *et al.* (1997). The second common haplotype (H1) was represented by 20 percent of the samples (D=7, W=2, and H=8); and one each of Kumar *et al.* (2007 and Kikkawa *et al.* (1997). Three sequences of Nepalese samples (two from present study and one from Zhang *et al.* (2016) and one from Kumar *et al.* (2007) were restricted to the third haplotype (H3). Of the remaining six haplotypes, one was reported by Kumar *et al.* (2007) (H4) while the remaining five (H5-H9) were defined by Kikkawa *et al.* (1997). Haplotype and nucleotide diversities of Nepalese samples (this study and Zhang *et al.*, 2016) are given in table 23. Three common haplotypes were present in three of the two groups wild and domestics. Within groups, haplotype diversity was highest in hybrid groups followed by domesticated groups and slightly lower in wild breeds. Overall haplotypes and nucleotide diversities were 0.403 and 0.00105 respectively. Interestingly, haplotypes were divided into two branches corresponding to river and swamp buffaloes by six nucleotide mutations. We could not find specific haplotypes for wild, hybrid and domesticated groups of buffaloes. In terms of genetic diversity, river buffaloes were found to be less diverse than swamp buffaloes.

Table 23. Haplotype diversity (h) and nucleotide diversity (π) estimated from partial mitochondrial *cytochrome b* sequence for 78 Nepalese buffaloes of three different groups (wild, domesticated and hybrid).

	Buffalo	Mitotypes (H)	Sample size	h (Haplotype diversity)	SD	π (nucleotide diversity)	SD
Wild	This study	3	15	0.362	0.145	0.00095	0.00041
	Zhang <i>et al.</i> , 2016	2	7	0.286	0.196	0.00071	0.00049
	Total	3	22	0.329	0.121	0.00086	0.00034
Hybrid	This study	2	10	0.467	0.132	0.000116	0.00033
	Zhang <i>et al.</i> , 2016	2	15	0.476	0.092	0.00119	0.00023
	Total	2	25	0.453	0.072	0.00113	0.00018
Domesticated	This study	2	11	0.509	0.101	0.00127	0.00025
	Zhang <i>et al.</i> , 2016	3	20	0.353	0.123	0.00092	0.00034
	Total	3	31	0.411	0.087	0.00106	0.00024
All this study		3	36	0.438	0.082	0.00116	0.00024
All Zhanget <i>al.</i> , 2016		3	42	0.382	0.076	0.00098	0.00021
Total		3	78	0.403	0.055	0.00105	0.00016

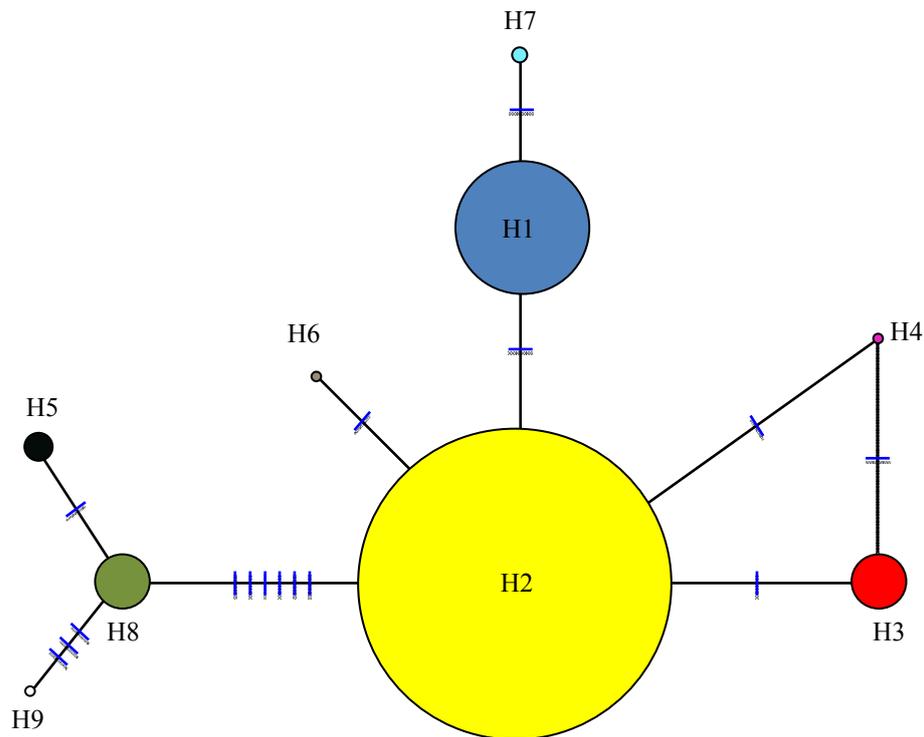


Figure 21. Reduced median network constructed using NETWORK 4.1.1.2 programme with 422 bp sequences of 9 *cytochrome b* haplotypes of the buffaloes from Nepal, India, Pakistan, Bangladesh, Sri Lanka, Japan, Indonesia, Philippines, and Italy. Circle sizes in the Network are proportional to the sequence frequencies over the data sets; colors of circles indicate different haplotype, largest circles are the most abundant haplotypes, Blue slashes across the branches represent mutational steps.

PHYLOGENETIC RELATIONSHIPS OF DIFFERENT BUFFALO TYPES

Maximum parsimonious analysis of 97 partial sequences of *cytochrome b* performed under heuristic search and 1000 bootstraps revealed four distinct clades (Clade A-D) with moderate to high bootstrap values (Fig. 22). Among four clades, one clade with high bootstrap values (99%) showed strong differences between swamp and river buffaloes. Surprisingly relationship among haplotypes revealed by median-joining network corroborates with the four lineages observed in MP tree. Lineage A comprises all sequences of haplotype H2 except Hap3 India. Lineage B comprises 20 sequences of haplotype H1 but accession H4 India, lineage C comprises all four sequences of Haplotype H3 and finally all haplotypes of swamp buffaloes (H5, H8, and H9). In MP analysis, a total of 3 most parsimonious trees (CI = 1.00, RI = 1.00, Length = 15 steps) were recovered (Fig. 22). Phylogenetic analysis and evolutionary history of 97 partial sequences of *cytochrome b* inferred using the Neighbor-Joining (NJ) with 10000 bootstrapping replicates also depicted identical topology of the phylogenetic tree (Fig. 23) as reported in MP analysis.

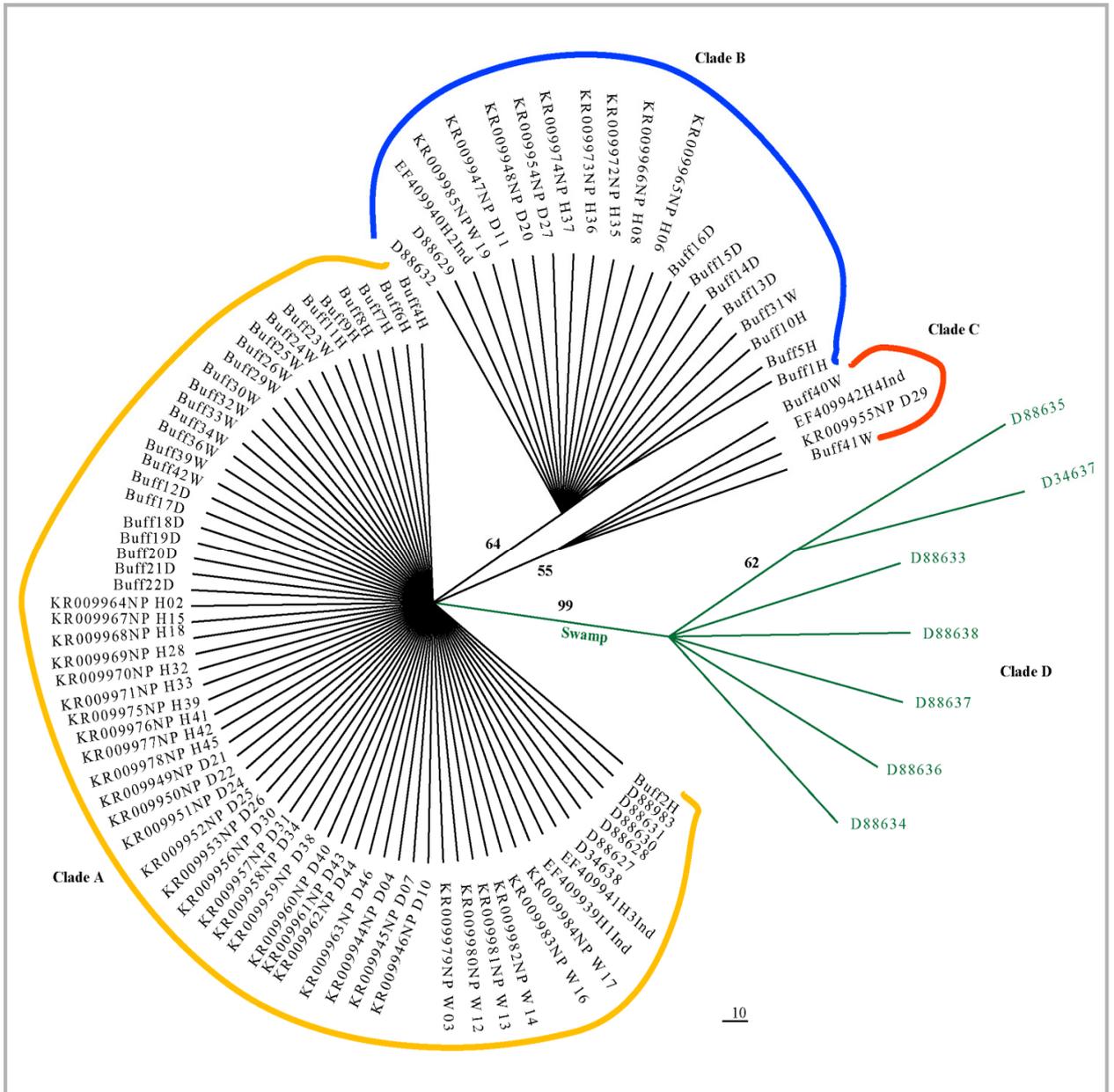


Figure 22. Maximum parsimonious trees constructed using 422 bp partial *cytochrome b* sequences of 36 buffaloes of Nepal (this study), 42 additional Nepalese sequences of Zhang *et al.* (2016), four sequences of Kumar *et al.* (2007) and 13 sequences of both river and swamp buffaloes from Kikkawa *et al.* (1997). Numbers in bold above the branches corresponds figures in the middle of the branch show the bootstrap values for the branch.

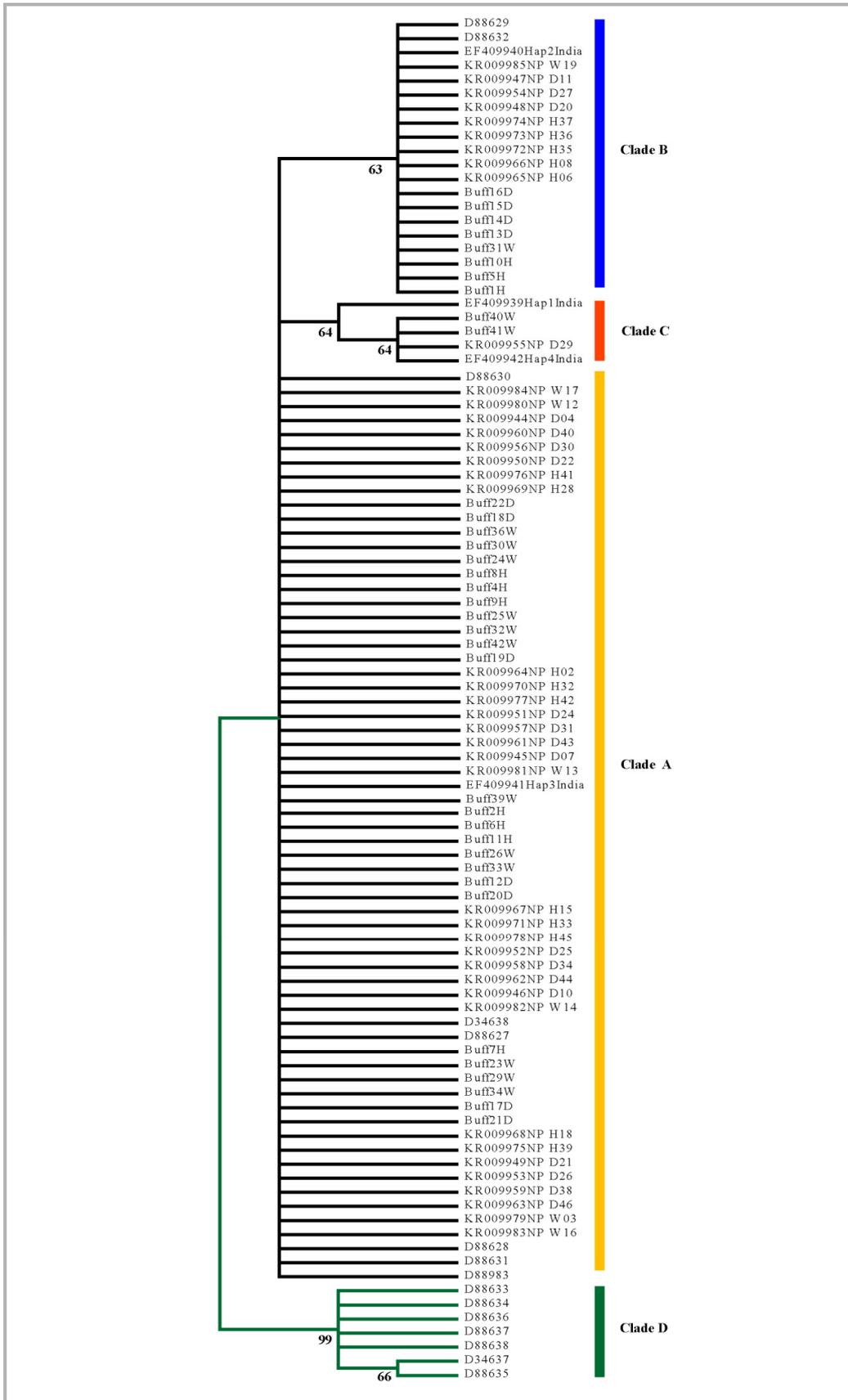


Figure 23. Neighbor-joining tree for 97 partial *cytochrome b* sequences of river and swamp water buffaloes. Numbers on the nodes are percentage bootstrap values from 10000 replications

CONSERVATION CHALLENGES OF WILD BUFFALOES AT KTWR

Nepal is a part of the home to endangered Asiatic wild buffalo (*Bubalus arnee*), the progenitor of domesticated water buffalo (Lei *et al.*, 2007). Currently, the Asiatic wild water buffaloes are distributed only in the isolated localities of south-eastern Nepal, western Thailand, east and central India, southern Bhutan (Corbet & Hill, 1992). A recent census of wild buffalo in Koshi Tappu Wildlife Reserve (KTWR) revealed that the population increased to 433 individuals (KTWR, 2016). In the latest count, a total of 105 individuals increased compared to an earlier count in the year 2014. There was only 63 individual at the time of establishment of KTWR in 1976. The historic range of this species extends further west to Chitwan, however, they are now restricted only in the eastern part of Nepal. In Nepal, KTWR being the only habitat of remnant wild buffalo population so this species is under constant threats of being extinct from the wild area due to natural calamities such as flood, epidemics, fire, habitat deterioration, high anthropogenic pressures, hybridization with domestic buffalo and disease and parasites transmitted by domestic livestock. The wild progenitor had been substantially reduced numerically and eliminated from the greater part of its former range. Nepalese population of wild water buffaloes in Koshi Tappu Wildlife Reserve (KTWR), regarded as the second highest population of wild buffalo in the world is considered as important as other species like Rhino, Tigers, Elephant, Snow leopard, etc.

Given the existence of the last Nepalese population of wild buffalo within a restricted area encompassing less than 200 sq km in KTWR, several wildlife conservationists have consistently emphasized the need of translocation of small populations in their historic distribution ranges like Chitwan to better secure the species in the country. Chitwan National Park had this species at least until the 1960s (Spillett & Tamang, 1966; Seidensticker, 1975; Aryal *et al.*, 2011). Furthermore, Chitwan National Park has extensive grassland areas, much larger abundant riverine habitats and sufficient upland areas that are not prone to flooding (Heinen and Paudel, 2015). To maintain the ecologically viable populations of *Bubalus arnee* in Nepal, recently 15 wild individuals have been translocated to Chitwan National Park (12 from KTWR and 3 from Central Zoo) and 1 male to Central Zoo from KTWR (Kandel *et al.*, 2018).

Adequate morphological and genetic studies need to be carried out to distinguish putative wild from feral backcrossed for translocation programs and to ensure the conservation of truly wild stock of water buffaloes. A sufficient number of animals must be moved to assure genetic variation in the founding population. For this purpose, genetic analyses based on evidence to

distinguish putative wild from feral backcrossed buffalo is highly advised (Heinen, 2001). Although the recent selection of 16 individuals for translocation was based on phenotypic and behavioral characteristics widely recommended by Dahmer (2000); Heinen and Singh (2002); Heinen and Kandel (2006) and Heinen and Paudel (2015), many individuals of mixed wild-domestic ancestry may not be correctly distinguished from wild animals (Flamand *et al.*, 2003). Genetic study based assignment tests only can provide a convincing basis for conservation management plans and conservation decisions. Flamand *et al.* (2003) have used 10 loci of microsatellite markers to distinguish 45 individuals phenotypically classified into three groups—seven hybrids, ten wild and 28 domestic and found that two of the predefined wild animals and seven of the predefined domestic animals showed an evidence of mixed ancestry. Although the sample size of Flamand *et al.*, 2003 was small, his study provides empirical support to select genetically pure wild individuals to be used in the conservation management plans. Genotyping of wild water buffaloes using a substantial number of microsatellite loci and undertaking of mitochondrial DNA sequencing would help to accurately discriminate domestic, hybrid and wild individuals. Introgression from domestic to wild buffalo is taking place through domestic female buffaloes those usually graze in and around KTWR. Since animal mitochondrial DNA is inherited only from the mother, identification of wild and domestic type specific haplotypes (mitotypes) will help to identify the hybrid individuals.

On the basis of the partial *cytochrome b* sequences of 422 bp length, we were able to define very few (only three) haplotypes in the Nepalese buffaloes. None of the haplotypes were wild, domestic and hybrid specific. These haplotypes were already identified by Zhang *et al.* (2016), Kumar *et al.* (2007) and Kikkawa *et al.* (1997) in their Nepalese, Indian and wider samples (Pakistan, Bangladesh, Thailand, and Srilanka). Although we have used the partial sequence (422 bp) of *cytochrome b* gene, the complete length (1120 bp) of this gene sequence reported from other studies (Kumar *et al.*, 2007; and Kikkawa *et al.*, 1997) including 42 Nepalese samples of Zhang *et al.* (2016) could not even reveal distinction between wild, hybrid and domestic river buffaloes. NJ and MP analysis performed (result not shown) with complete length (1120 bp) *cytochrome b* sequences of Zhang *et al.* (2016) (42 Nepalese sequences), Kumar *et al.* (2007) (four river buffalo haplotypes) and Kikkawa *et al.* (1997) (seven river and eight swamp haplotypes) provided essentially same topology of the *tree butan* addition of one more haplotype represented by GenBank accession KR009945NP_D07 alone.

WARRANTING STRATEGY OF THE CONSERVATION SCHEME

Lau *et al.* (1998) using partial *cytochrome b* sequence (303 bp) and D-loop (158 bp) sequence has suggested that wild Asian buffalo (*Bubalus arnee*) of Assam, Nepal, and Indo-China is the possible ancestor of river and swamp buffaloes. Tanaka *et al.* (1996) also support Lau *et al.* (1998) hypothesis. Nepal's wild buffaloes show swamp type phenotypic characteristics thus discriminated as a swamp type, however, Zhang *et al.* (2011) found them genetically close to river type. Our study is consistent with Zhang *et al.* (2011) as in the MP, NJ and Network analysis swamp buffalo showed distinct variation with almost 100% bootstrap value and six nucleotide differences between these groups. A similar result was obtained by Mishra *et al.* (2015) on upper Assamese and Chilika populations (in India) which showed phenotypic similarity to swamp type but genetically closer to wild-type buffaloes in the region. Our multi-lineages MP and NJ trees and several previous studies (Tanaka *et al.*, 1996; Lau *et al.*, 1998; Zhang *et al.*, 2011; Mishra *et al.*, 2015; Zhang *et al.*, 2016) inferred an ancestral nature of wild water buffalo including the remnant population of KTWR. In this regard, genetic distinctness, ancestral nature and existing genetic variability of buffaloes protected in KTWR warrant the need of more genetic studies based on an adequate number of polymorphic markers and mitochondrial DNA sequencing of the entire putatively wild individual. Microsatellite genotyping with sufficient number of loci along with mitochondrial DNA sequences database on the measure of the magnitude of genetic differentiation and genetic relationships among the populations of all the recently counted wild individuals of KTWR will have large implications on rational decision making for the conservation of wild buffaloes in KTWR and translocation of potential individuals in the other suitable areas in addition to CNP. Translocation of some more animals to CNP is also strongly advised to create a viable and defensive population to ensure an alternative population. Furthermore, if the wild Asiatic buffaloes were protected properly with due attention, several important genetic traits (growth, draft, meat, milk, climate adaptation, disease resistance, etc.) presumably present in these populations would be advantageous to use for the genetic improvement programs in the future or a source of bull for use in other populations.

EXAMINING THE PRESENCE OF MALARIAL PARASITE IN BUFFALOES OF NEPAL

The livestock farming in Nepal is suffering economic losses from different species of protozoan pathogens like **Babesia**, **Theileria** and **Trypanosoma**, those infect to cattle, water buffalo, pigs, goats and sheep. Most of the locally available diagnosis techniques to identify the protozoan parasites are either based on microscopic or serological examination (Maharjan and Mishra, 2006). Microscopic study is not an effective technique, because it lacks sensitivity and specificity (Böse *et al.*, 1995). A serological technique, ELISA, despite being highly sensitive at detecting parasitic infection in animals fails to differentiate between current and prior infections. However, Polymerase Chain Reaction (PCR) presenting high sensitivity and specificity for detecting as well as identifying species of the parasite that infects the animals are not commonly used to identify the parasites in Nepal.

Malaria is a vector-borne disease caused by *Plasmodium* parasite. It is an intracellular protozoan parasite transmitted from one individual to others from the bite of mosquitoes. In human, malaria is caused by five different species which causes the greatest burden of morbidity and mortality in the World. Besides human beings, malaria parasite infects wide range of hosts including non-human primates, rodents, bats, ungulates, birds, and reptiles (Cox, 2010; Garnham, 1966). The earliest report of ungulate malarial parasite in domestic water buffalo was from India, and this parasite identified through microscopic observation was named as *Plasmodium bubalis* (Sheather, 1919). Most of *P. bubalis* cases were reported on immune-compromised water buffalo (Kolte *et al.*, 2002). Since then, morphological observations of malarial parasites in water buffalo were sporadically reported until recently. Templeton *et al.* (2016) reported molecular-based surveillances of malaria parasites in water buffalo in Thailand and Vietnam, and found up to 45% of the examined animals infected with malaria parasites. They found that there were two distinct *Plasmodium* mitochondrial DNA sequences and provisionally designated as Types I and II. Quantitative PCR analysis revealed that the Type I sequence showed higher parasite density and microscopical images obtained from this type; thus Type I sequence was deposited as "*Plasmodium bubalis* (accession number LC090213)" and type II, for which all samples showed very low parasite density, was deposited as "*Plasmodium* sp. ex *Bubalus bubalis* (accession number LC090214)". In this study, we performed nested PCR assay to detect presence of malaria parasite in water buffaloes (wild, feral and domestic) in Nepal using oligonucleotide primers specific for the *Plasmodium* mitochondrial cytochrome b (cyt-b) sequence and found two cases.

For the general overview of health status and disease prevalence in buffaloes of Nepal, we tried to investigate the possibility of disease prevalence while doing molecular analysis. The most prominent malarial parasite in buffaloes we recorded is the *Plasmodium bubalis* in domestic and feral individuals. The main objective of this analysis was Molecular detection of malaria parasites in water buffaloes of Nepal.

The Materials and methods used were DNA extraction, PCR, and sequencing, from which Genomic DNA was extracted from blood samples using commercial kit (QIAGEN DNeasy Blood and Tissue kits, QIAGEN, Germany), according to the manufacturer's protocol. The extracted DNA samples were stored at 4°C until used for this analysis. PCR assay using *Plasmodium cytb* gene specific primers followed a previous study (Martinsen et al., 2006). Briefly, the *cytb* gene of *Plasmodium* was amplified by nested PCR using the universal primers for *Plasmodium* DW2 (TAATGCCTAGACGTATTCCTGATTATCCAG) and DW4 (TGTTTGCTTGGGAGCTGTAATCATAATGTG) for the first round PCR. The nested PCR was performed using primers NCYBINF (TAAGAGAATTATGGAGTGGATGGTG) and NCYBINR (CTTGTGGTAATTGACATCCAATCC) as described (Abkallo et al., 2014). Both PCR cycle were performed for 40 cycles with denaturation at 94°C for 20 sec followed by annealing and extension at 62°C for 3 min. All reactions were performed using negative controls, and no contamination was detected.

The quality of the PCR product was confirmed by viewing under InGenius bio-imaging system (Syngene UK) after the agarose gel electrophoresis (Fig. 24). For purification, 5 µL of PCR product and 2 µL of ExoSap-IT Express PCR Product Cleanup (Affymetrix Inc. Santa Clara, CA, USA) were taken. The tubes were then incubated at 37°C for 4 min followed by 80°C for 1 min. The purified products were sequenced with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) using NCYBINF and NCYBINR primers according to the manufacturer's protocol. These sequencing reactions were purified by using Big Dye Xterminator™ Kit (Applied Biosystems) and analyzed in an automated 3500XL Genetic Analyzer (Applied Biosystems).

RESULT AND DISCUSSION

Out of 56 blood samples; 16 (28.6%), 23 (41.1%) and 17 (30.3%) were of wild, feral and domestic buffalo, respectively. Out of 16 wild buffalo, 13 and 3 samples were obtained from KTWR and Central Zoo, respectively. Among these buffalo, 32 (57.1%) are female and 24 (42.9%) are male. The age of buffalo ranged from 0.5 months to 15 years (for only 38 samples).

PCR detected 2 positive cases (3.5%); one was a feral buffalo (2 years old female) and the other was a domestic buffalo (2 years old male). Both positive samples were collected during rainy season from KTWR and its surrounding village. None among 16 wild buffalo samples collected during winter season from KTWR and Zoo were positive however further tests must be done on all existing individuals of the reserve for confirmation. Previously, 16% (15/95) and 65% (65/144) of water buffaloes were PCR positive for malaria parasites in Thailand in 2015 and 2014, respectively and 6% (3/49) and 5% (243) in Vietnam in 2013, and 2010 (Templeton *et al*, 2016). The low frequency of the positivity in this study may be attributed to the difference in the vectorial capacity of each area or seasonality. To clarify this point, more samples from different seasons needs to be analyzed.

Sequences of two positive PCR products (773 bp) from water buffalo were identical, and identical to the Type II sequence except one nonsynonymous substitution at nucleotide position 5344 (number is after LC090214) from A to T, a nucleotide type seen in Type I sequence. Because this *cytb* region in 39 Type II sequences of water buffalo in Thailand were identical (Templeton *et al.*, 2016), identified A5344T single nucleotide polymorphism in Nepal may serve as a unique region-specific marker. In the previous study, Type II sequence was detected from 57/80 (71%) and 2/5 (40%) *Plasmodium* positive cases in Thailand and Vietnam, respectively and Type I was from 43/80 (54%) and 3/5 (60%) positive cases in Thailand and Vietnam, respectively. Thus, we consider that Type I water buffalo malaria parasite likely exists in Nepal, too. More samples are required to evaluate the existence of the type I water buffalo malaria parasite in Nepal.

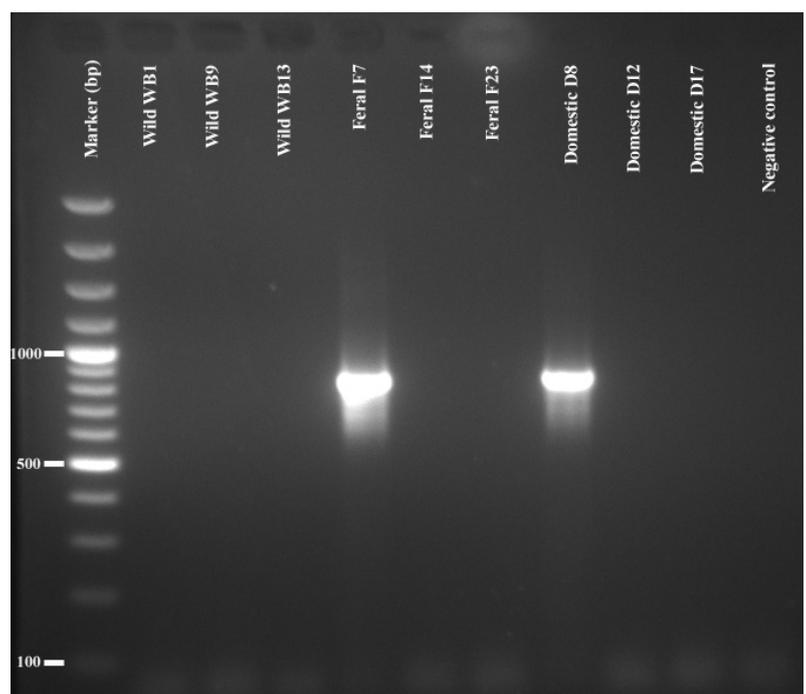


Figure 24. Agarose gel electrophoresis showing the size of amplified products by nested PCR using primers targeting mitochondrial cytochrome b (*cytb*) gene. Lane 1, DNA marker; lane 2-4, samples from wild buffalo; lane 5-7, samples from feral buffalo; lane 8-10, samples from domestic buffalo, lane 11, negative control

Since the first water buffalo malaria parasite reported from India, the prevalence of this pathogen in different areas of India has been reported by many succeeding studies; such as Bareilly/Muktesar (Sheather, 1919), Nagpur (Kolte *et al.*, 2002), and Maharashtra (Shastri *et al.*, 1985; Shinde *et al.*, 2005). In this context, it is important to quarantine the water buffalo across the border between Nepal and India to prevent the spreading of ungulate malaria parasites. Although the early study concluded that the this pathogen causes only mild symptoms and animals (*P. bubalis*) were self-cured (Riaz-Ul-Hassan, 1953), one mortality case has been observed (Templeton *et al.*, 2016). It was not determined the buffalo (*B. bubalis*) died due to malaria infection alone or due to malaria was an opportunistic parasitic infection (Templeton *et al.*, 2016). Nonetheless, the hidden pathogenesis of this pathogen as an opportunistic agent is not evaluated so far. The pathogenesis against the buffalo with different genetic background (wild, feral or domestic buffalo) is also not clearly understood. The sole objective of KTWR is to conserve the last remaining Nepalese population of an endangered Asiatic wild buffalo. Therefore diagnostic assays are strongly recommended to be employed by the reserve to confirm possible health impacts of malaria in wild individuals.

In conclusion, this is the first PCR-based surveillance of water buffalo malaria parasite in Nepal. The presence of malaria parasites in feral and domestic animals may have greater consequences in terms of ecological impact as well as economy of the farmers depending upon livestock farming. Furthermore, active surveillance is necessary for malaria parasite infection in Asiatic wild buffalo to obtain information regarding health status and mortality rates. These steps are important to develop strategies for the management and control of malaria infection especially in wild buffalos in Nepal.

CHAPTER- IX

SUMMARY AND CONCLUSION

The Himalayan country Nepal covers an area of 147,181 Km² with an average east-west lengthwise distance of 800 km and north-south width of 193 Km between 80°40'–88°12' East to 26°22'–30°27' North. The spatial location of the country is in the southern slope of the greater Himalayas with mosaics of different physiographic and climatic conditions. In such short stretch of about 150 Kms, the altitude varies from 8,848m (Mt. Everest) height to 60m (*Kechanakalan*) down in east Tarai. Hence, it encompasses different arrays of habitats within the short vertical distance from north to south resulting the highest level of biological diversity. Such that the floral and faunal species of Nepal represents a mixture of two large global bio-geographic realms: Oriental and Palaearctic.

KTWR is located between 26°33'–26°45' N and 86°54'–87°04' E on the floodplain of the Sapta Koshi River near the Nepalese-Indian border, in the Tarai of south-eastern Nepal. Sapta Koshi River is a major tributary of the Ganges, and Trijuga River joins the Koshi in the western border of the Reserve. The area experiences mainly sub-tropical monsoonal climate where the average annual rainfall ranges between 1300 mm and 2051 mm. The elevation ranges from 75 to 100 m amsl. The average daily maximum temperature ranges between 23°C to 34°C, and minimum between 16°C and 29°C. The rise in temperature starts from pre-monsoon (April) and lasts until May, and lowers from October to January. The average maximum temperature rises up to 39°C and the minimum is as low as 6°C. The reserve experiences clearly three main distinct seasons viz summer, monsoon and winter. The vegetation of KTWR is mainly composed of tall grasses, and there are also small patches of Khair-Sisoo scrub forests and deciduous mixed riverine forests.

CNP (952.23 sq km), where the small population of wild buffalo from KTWR is reintroduced during January-February 2017, lies between N 27°20'19' and 27°43'16' longitude and between E 83°44'50' and 84°45'03' latitude on the floodplain of the confluence of Rapti and Narayani rivers in the lowland of central Nepal. The elevation of CNP ranges from 150m to 815 m amsl. The average daily maximum temperature of CNP ranges between 25°C to 35°C, and the minimum between 15°C and 25°C. The temperature rises from pre-monsoon and lasts until May, and lowers from October to January. The vegetation of CNP is mainly composed of almost 70% of *Sal* forests including huge stretches of short to dominant tall grasses which consist maximum 12% between the *Chure* hill and river banks.

The link between wild cattle and humans has existed for thousands of years, amongst which five species of wild cattle have been domesticated in the last 7000 years, including the wild water buffalo and the yak (Melletti & Burton, 2014). Bovids have key roles in their natural ecosystems; they are not only an important prey species for predators but also play an interesting role in maintaining ecosystems by grazing and wallowing (Melletti & Burton, 2014) so are called the habitat architects of nature. On the other hand, the role of biodiversity to the human society is vital to enrich socio-economy, even this is more special to the local communities living close to nature. This is the reason that the traditional and derived beliefs, religions and myths of the Indian sub-continent emphasized conservation and wise uses of natural resources because the people are attached to the locally available resources as a part of the traditional culture necessitated to their day to day life.

The human society is interlinked physically, psychologically, and spiritually with the plants and animals and there is the close relationship between human society and natural resources since the time immemorial. Such conception and beliefs also supported for reducing the destruction and excessive usage of natural resources and to control over other miscalculation over the natural resources. The consequences of which is the indigenous people are living in a close harmony with nature through the wise use of the available natural resources. This is why there shall need to be congenial harmony between the incredibly important nature gifted resources and human beings. The anthropocentric thought shall focus on utilization of natural resources for human wellbeing without leading to over-exploitation or harm to nature, and also ensure the conservation of the natural resources with the view of eco-centric thought that emphasizes the complete preservation with restrictions. The eco-centric perspective is more protection oriented and does not allow the disproportionate use of the available resources. So there shall be a delicate balance between these two antagonistic views for the sustenance of the natural resources for the human welfare.

The present study intended to look at the various ecological aspects of Asian wild buffaloes at KTWR, Nepal such as habitat composition, population and demography, behaviors, the reintroduction of this species for creating alternative population to a new habitat, and molecular studies. The principal objective of this study was an assessment of ecological aspects of Asian wild buffaloes including genetic studies of wild buffaloes in Koshi Tappu Wildlife Reserve (KTWR), Nepal. Specific objectives of the research were: 1) To determine the habitat composition of Asian wild buffalo at KTWR; 2) To study the current population status and

demographic structure of Asian wild buffalo at KTWR; 3) To plan and set to reintroduction of wild buffaloes and document translocation protocol; 4) To study the pattern of activity time budget in general behavior of Asian wild buffalo, and 5) To study the molecular structure of *B. bubalis arnee* population in KTWR using mitochondrial C01 (*Cytochrome Oxidase I*).

HABITAT COMPOSITION OF WILD BUFFALOES

For studying habitat composition of wild buffalo at KTWR, vegetation sampling was carried out in concentric circular sample plots (CSP) all along the three north-south parallel transects representing each habitat type available in the reserve. Collectively, a total of 76 sample plots were laid following a systematic random sampling on those transects. Plots with 10m radius were used for trees, the 5 m radius was laid out to collect information on shrubs/saplings in each CSP, and square plots of 1m² size were laid out at north and south cardinal directions of each CSP for grass and other herbaceous plants. However, if no tree species is recorded in a CSP, only one 1m² square plot was considered to collect information on herbs and grasses due to homogeneity in habitat. Vegetation data were analyzed using general ecological principles to calculate frequency, density, dominance, cover density, importance value (IV), and prominence value. A total of 181 sample plots of various sizes for trees, shrubs, and herbs were surveyed.

From the phyto-sociological analysis, four vegetation communities are categorized are *Sissoo-Khair* forest, mixed riverine forest, scrubland, and grassland. On the basis of plot location, a large proportion of KTWR area is covered by grassland followed by *Sissoo-Khair* forest and the smallest proportion is comprised of shrubland. Less assessed type of vegetation with two broad categories called as riverine forest and grassland/savannah were described by the previous study in KTWR (Sah, 1997). Among the sample plots, *Sissoo-Khair* forest was observed in 40.7% (35 plots out of 76) of sample plots predominantly in the eastern part of the Reserve which included 444 trees stems per hectore. Out of twelve species of trees of this type, *Dalbergia sissoo* is ecologically more important species with the highest importance value (IV = 179.1) followed by *Trewia nudiflora* (IV = 42.8). There are 33 species in the understory layer of this forest. Among these species, *Clerodendron viscosum* is the most prominent one (PV = 25.79) followed by *Lantana camara* (PV = 12.85).

The ground vegetation of this forest indicates high diversity of species (n = 34), with invasive alien *Mikania macarantha* being the most prominent species (PV = 51.7) followed by *Imperata cylindrica* (PV = 41.5). Grassland occupies the highest proportion (54%) of the study area. A

total of 32 grass species of plants were identified, of which the most prominent grasses were *Saccharum spontaneum* (PV = 282.57) and *I. cylindrica* (PV = 168.79) in the grassland community. In the dry and heavily grazed areas, *Citronella* species is the co-dominant, whereas *Cyanodon dactylon* is the co-dominant grass species in the moist southern floodplain area. This condition was also observed by the previous study (Sah, 1997). Mixed riverine forest occupies 11% area of the KTWR. It is mostly grown in the southern and western part of the reserve. The forest included 274 stems of tree per hectare. Among 11 reported tree species of this forest, *Trewia nudiflora* is the more dominant (IV = 140.6) followed by *Bombax ceiba* (IV = 50.2). Forest cover of this area was found to increase from 2.9% in 1997 (Sah, 1997) to 11% in 2015 at the time of the current study. The dominance of the *Trewia nudiflora* is found highest (PV=140.6) in this study compared to *B. ceiba* as the dominant tree species found in the earlier study (Sah, 1997).

It is likely that the deposition of silt materials on river bed led to the formation of humus along the river bank over a time. As a result, the composition of Riverine mixed forest might have changed in the past decades, leading to *Trewia nudiflora*'s dominance over *Bombax ceiba* over many of the floodplains. Understory layer of mixed riverine forest included 16 plant species. Among the recorded species, invasive alien *Lantana camara* (PV = 7.3) and *Clerodendron viscosum* (PV = 3.3) are the two prominent species in the understory layer. The ground vegetation of mixed forest comprised 23 plant species, primarily composed of *I. cylindrica* (PV = 224.3) and *Dryopteris cochleata* (PV = 127.7). In shrubland habitat type, 6 plant species are recorded in the understory, of which *Zizyphus jujuba* (PV = 37.5) and *Clerodendron viscosum* (PV = 1.1) are the more prominent species. There are 11 ground vegetation species recorded in the shrubland with *I. cylindrica* (PV = 38.97) and *D. cochleata* (PV = 17.71) being the 2 most prominent species.

Based on the inventory data, 12 and 11 tree species were recorded from the Sissoo-Khair and mixed riverine forest of KTWR, respectively. The density of tree in Sissoo-Khair and mixed riverine forests were 444 and 274 tree/ha respectively. In understory vegetation, the number of recorded species was higher in Sissoo-Khair forest (n = 33) than mixed riverine forest (n = 16) and shrubland (n = 6). The number of species in the ground vegetation is higher in Sissoo-Khair forest (n = 34) followed by grassland (n = 32). This has indicated that Sissoo-Khair forest is most diverse than other vegetation types in terms of species richness of tree, understory and ground flora. The average tree height of Sissoo-Khair forest was 12.0±8.6 m with 15.4±10.9 cm of

average DBH. Similarly, average tree height of mixed riverine forest was 14.1 ± 11.1 m with 22.3 ± 19.2 cm of average DBH.

Floral species richness with their mosaics community in the Koshi River floodplain resulted in KTWR as prime habitat for the wild buffalo. Floral associations provided four vegetation types with grass communities being the most prominent in KTWR. The Sissoo-Khair and mixed riverine forest associated with the ecotonal habitats preferably used by this wildlife. Conserving overall biodiversity of KTWR, most importantly by being used due to the presence of large ungulates such as Asian wild buffalo plays the key role to increase the ecological/functional value of this protected area. Structural pattern of vegetation community suggests that the KTWR area provide best ground for foraging, ranging, resting as well as wallowing primarily for Asian wild buffalo. Moreover, due to the existence of different vegetation communities including the mixed type of forests and growing trend of *Trewia nudiflora* and a vast range of grassland, *Saccharum spontaneum* predominantly shows the signs of habitat suitability for other mega-herbivores such as rhinoceros, swamp deer, and other sympatric species. So this could be one of a preferable alternative site for creating rhino population by translocation if the area could be extended towards foothills of Churiya range (lower Young Mountain) in the north of KTWR and south upto Koshi Barrage as well.

Asian wild buffalo, the pride for the people of Koshi Tappu region who are residing nearby, has the potential value to back up local people for their social, economic and biological benefits. This is also the most potential and significant biological hotspot for developing a site for a wider range of wildlife through the extension of north and south areas of the wildlife reserve. In paradox, due to the frequency of river dynamics every year, vegetation composition and structure have been so fragile which is unpredictable to meet the requirement of wild buffaloes for their survival so has warranted for its extension. On the other hand, there are thousands of cattle and buffaloes that are abandoned or taken to the fringes and even to the core area of the wildlife reserve that significantly hampers the wild buffaloes in terms of space and sources of forage. The most alarming risk for wild buffalo is the hybridization and disease transmission between domestic animals and wildlife including cattle and wild buffaloes. This is why the intensive monitoring of habitat, adoption of active management, and translocation of some viable population to other most suitable alternative habitats could ensure the long-term survival of wild buffalo population in Nepal.

POPULATION AND DEMOGRAPHY

While studying population and demography of wild buffalo at KTWR, the whole area of the reserve was divided into different blocks: firstly divided into two segments: east and west from the main course of Sapta Koshi River; and each segment was further divided into two separate blocks, north and south blocks into both sides. The animals were closely observed during the census in order to distinguish and for clear identification of the age and sex of the animals. Four-wheel drives, elephant ride and also on-foot tracking were used during census in order to maximize animal sightings. The criteria for identification of the wild breeds of wild buffalo, their age and sex were developed considering skin and pelage color, change pattern in coloration in male and female individuals with the age, horn size, and shape, herding behavior, etc. The age of animals was determined by observing: Young Calves/Infants (<1 year) with buff in color; Juvenile Calf (1-2 years) that darken buff color in the first year and gradually turns lighter in color in the second year; and Young Adult/Adult (>3 years) the coat color changes to dark to very dark with noticeable whitish markings in different parts of the body mainly at joints. There are one or two white chevron marks on the underside of the neck of the adults, and white hocks, white tail tips, and fine white markings around the eyes and along the sides of the nose and mouth. White markings are more apparent in an adult age which gradually fades out with age. Young females are smaller and the pelage retains the light coloration. Adult males are dark black, except for whitish markings as described above at prime breeding age (>4 years). Adult females have longer upwardly extended horns than males, but males have much thicker horns.

A number of young calves <1 year of age and number of females in a population were considered for calculating calf to cow ratio. It is an index of the growing population trend of the animals. Population growth rate of wild buffalo population in KTWR was calculated from exponential growth rate formula as adopted from Dahmer (1978), Heinen (1993a), Heinen (2002), Heinen & Kandel (2006). Demographic structure and calves to cows' ratio Dahmer, 1978), effective population size (Stiling, 2002), percentage ratio of population loss per generation (Stiling, 2002), and rate of loss of genetic variations (Berger, 1990) were calculated from the same data sets.

The first census of Asian wild buffalo at KTWR in 1976 counted 76 animals in total, out of which there were 12 adult male, 18 adult female, 22 juvenile and 11 infants (Dahmer, 1978), and the population of this species found to increase gradually over a period. Two subsequent censuses in 1987 and in 1988 (Heinen, 1993b) recorded 91 and 93 individuals respectively. The

increasing population trend over the years suggests an exponential growth rate except in the 2010 census. The census records from 2011 onwards indicated that there is an increasing rate of population size. The latest census conducted in March-April 2016 increased to 433 individuals; where male and female ratio was 0.63 and calf to cow ratio was 0.36 whereas male to female ratio was lowest (0.38) in 2014 and highest (1.12) in the 1988. As male to female ratio illustrates the fecundity rate of the population, number of females need to be higher to revert the population more viable. Since male to female ratio of wild buffalo population at KTWR was also inconsistent, this needs to have a long-term study on change of male-female structures. High fecundity rate affects the calves to cow ratio i.e. higher is the ratio more is the viability of the population. Calves: cow ratio was highest in 1976 (0.61) which was gradually declined thereafter but the lowest was 0.19 in 2009 because flood in 2008 badly affected population and habitat.

During the census in 2016, single to many male band group of various sizes were noticed. There was a rise in male to female ratio in 1987 which was gradually declined till 2009 and again remarkably regained but found to be in a zigzag manner. Gradual downfall of the calves to cows ratio till 2009 was also evident which gradually increases in a similar way as like male to female ratio. There is a strong correlation (R^2 Linear = 0.099) between male to female ratio and calves to cows ratio. Flood had an unvarying impact on both males: female and cow: calves ratios. There are some other factors in the area which had a long-term impact on male: female in spite of the overall increase. Because of frequent dynamics of River Koshi, it is experienced that the home range of the wild buffaloes have been badly affected resulting to solitary or scattered males to leave the natal herd forcing to entering the groups of sub-adulthood. This is most likely that the large natal herd of wild buffaloes also gets splitted due to such frequent river dynamics.

The current population size of the wild buffalo at KTWR is highest in comparisons to previous censuses. The overall population growth (ri) over past 40-years (1976 to 2016) is 0.048. Growth rate between 1976 and 1987 was 0.033 and between 1988 and 2000 was 0.037. The population growth rate was highest from 2011 to 2014 (0.106). Negative growth (-0.018) was recorded in 2009. Populations of wild buffalo bounced back after decimating impact of the flood in 2008. The average rate of growth (0.048) between 1976 and 2016 shows an imperative development towards the sustenance of this species in the future.

This research has come up with a significant value of policy interventions and management implications as the size of the wild buffalo population at KTWR are increasing every year, and the population is likely to split into several herds; those splitted herds would likely to be

intermixed with feral or backcrossed animal herds. So there is a danger of genetic erosion of putative wild stocks due to increased chances of cross-breeding or hybridization between wild bulls and feral or domestic buffaloes. Now it is urgently necessitated to ensure that abandoned probably private or unattended feral/or backcrossed buffaloes, are immediately removed or relocated from the Wildlife Reserve so that the wild stocks would remain safeguarded. However, a few concerns regarding the long-term conservation and effective management of wild buffalo in KTWR must be evaluated and issues shall seriously be taken up immediately. On the other hand, as genetic variation is reduced by inbreeding, genetic drift or influence of neighborhoods of feral or domestic buffaloes, the effect of all these factors are likely to increase in severity towards triggering declined population size of wild stocks.

The wild buffalo population at KTWR is still not considered viable and secure in this habitat due to various threatening and counteracting reasons such as floods in the Koshi River, the risk of disease transmission from domestic buffaloes and cattle, genetic degradation due to cross-breeding with feral buffaloes, tough competition for food and space, etc. However, the total current population size (433) and effective population size (294) is convincing for its sustenance and only a lower percentage rate of loss of variation (0.17) in 2016 indicated an optimistic trend for the viable population at KTWR. Reintroduction of species into the new habitat at Chitwan National Park of central Nepal has been successfully taken up during January-February of 2017 as a part of species conservation, and the Department of National Parks and Wildlife Conservation (DNPWC), Government of Nepal has planned to establish an effective population size in future.

Considering the significant role of wild buffaloes in the riverine ecosystem, some of the pertinent actions are prescribed for the long-term management of the wildlife reserve and survival of the Asian wild buffaloes at KTWR, and to increase alternative viable population at new habitat at Chitwan National Park, Nepal.

BEHAVIORAL ACTIVITY TIME BUDGET

While studying behaviors of the wild buffaloes, it is realized that preserving behavioral and cultural diversity related to animal populations during the current period of rapid anthropogenic change has an immense significance in the conservation of biodiversity. Daily, monthly and seasonal activities and activity budget performed by the Asian wild buffaloes at KTWR were recorded to know the time budget biology of the animals. Major categories of behavioral

activities that wild buffaloes undertake during foraging are mainly Grazing, Moving, Resting, Running, Chewing, Wallowing, Drinking, Grooming, Social interaction, etc. within its territory. Focal animal sampling technique was carried out to record social and ecological behaviors. Selection of focal animal was random for each session of observation over the areas of the wildlife reserve. Adult animals were selected for sampling as a focal animal since it has influences upon others in performing their activities including ranging behavior. Approximate time of each session for focal animal monitoring was 6 hours in each sampling session day, and a number of days every month was 12 covering half day for every session and another half of the session covered on the next day to complete a full session of a whole day. During the observations, the activity of a focal animal was recorded in every 5-minutes interval. In an average, 12 animals were monitored every month and 144 animals were chosen as focal animals in a year. Total duration monitored every month was 72 hours, and 864 hours in a year; hence the total number of days covered during two years of field study was 288 that cover 1728 hours in total. Those were calculated as monthly, seasonal or yearly proportional variations of time spent for different activities. Total numbers of wild buffaloes associated with focal animals in different groups during the monitoring in 2-years' study were 4753.

Variation between different months and seasons, and years was presented by calculating the mean and standard error (mean \pm S.E) of each of the behaviors. To elaborate the seasonal variations in behavioral time budget for a year is divided into 4 main seasons as winter (Jan-Mar); summer (Apr-June); monsoon (July-Sept); and autumn (Oct-Dec). The Krushkal-Wallis one-way ANOVA was also applied to evaluate the activity budgets of the different focal animals. Different categories of behavioral activities were tested for significance of time budget with the percentage of time devoted to each of the activities. Significance was set at $p < 0.05$ for all statistical tests. The data on time spent on different activities across the seasons during the years 2015 and 2016 were analyzed for seasonal variations among different activities.

The result of the monthly pattern of behavioral time budget (in a minute) in 2015 shows that the resting time was highest in the month of February (243.3 ± 5.3 SE) followed by January (236.7 ± 6.5 SE) and December (185.0 ± 13.3 SE). While the time spent in grazing was maximum in June (218.3 ± 8.9 SE) followed by May (165.4 ± 8.4 SE) and October (158.8 ± 6.8 SE), and the wallowing was performed more during August (29.2 ± 5.9 SE) followed by July (28.8 ± 4.0 SE) and no wallows were recorded during October through February. On the other hand, animals were found moving more during March (91.3 ± 4.3 SE) followed by April (83.3 ± 2.5 SE) and least

during February (33.1 ± 3.1 SE) indicating that they are more focussed on sun basking for thermoregulation. They were seen to moving in each and every months of both the study years in an equal proportion. While in the year 2016, monthly activities budgets of wild buffaloes in Koshi Tappu shows that the major activity categories like resting time in January (244.6 ± 5.1 SE) was highest followed by February (225.6 ± 15.7 SE) and least during June (72.08 ± 8.08 SE). The total time spent in grazing was maximum in June (221.7 ± 7.6 SE) followed by May (162.9 ± 9.05 SE) and October (157.5 ± 8.6 SE), and least during January (76.67 ± 4.4 SE) and February (74.58 ± 2.9 SE), while the wallowing was performed more during June (22.92 ± 4.9 SE) followed by July (21.25 ± 5.9 SE) and no wallows, was recorded from October through February which was same as in the year 2015.

Based on the seasonal observation of the activities budgets in the year 2015, each category of activities significantly varies across the seasons ($H= 16.59$, $HC: 16.59$, $p= 0.002326$). To understand seasonal variation, the observations were repeated in 2016, activity pattern was found almost similar as in 2015 across the seasons ($H= 16.59$, $HC: 16.59$, $p= 0.002326$). This indicates seasonal influences on time spent on each activity remain mostly the same in both 2015 and 2016 provided that the biophysical and environmental conditions remain the same. Time spent on different activities during 2015 and 2016 shows no significance over the years ($H= 8.727$, $HC= 8.727$, $p= 0.06829$). The weather and season of the year have an influence on the allocation of time budget on different activities. They were involved in grazing more during winter and wallowing is more in summer and monsoon. Patterns of habitats used by wild buffaloes at KTWR based on the total observation ($N=144$) of the animal during the behavioral studies, wild buffaloes were found to use grassland and ecotone habitat the most (58.21%) followed by Sissoo, Sissoo Savannah and Sissoo mix forest (32.09%). An additional area of *Typha* dominated grassland stands for 6.72%.

Allocating time to different activities and thus distributed throughout the day is also important to understand how animals adjust to variations in habitats in order to optimize utilization of resources for survival and reproduction. This is primarily because the time is limiting factor (Dunbar, 1988) which may affect all the behavioral aspects of animals. Reasons for studying activity budget (Caro and Sherman, 2012) supports to acquire the knowledge on the loss of behavioral diversity that may prevent a population from adapting to environmental changes and sudden habitat alterations that trigger towards the extinction or altered allelic frequencies of hormonal pathways, disordering the evolutionary history of the species or population. The result

shows that there is a tendency of variations of changes in behavioral time budget in months and seasons which was noted to differ with the management interventions in the dense grassland of KTWR.

The outcome of the current studies on activity budget would have implication to habitat management with respect to animal behavior and food requirements. Intense feeding or grazing and traveling or moving of wild buffaloes as like in most of the diurnal herbivores in tropics occur early in the morning and later part of the day with a long resting period at the noon (Chivers, 1969). Same was the observation in case of wild buffalo at KTWR though the daily active period is affected by various other contemporary factors such as the presence of predators and other human-induced disturbance factors because of which wild buffaloes remains alert and shift their spontaneous activities for instantaneously. Besides that, many of the sympatric species such as domestic buffaloes, cattle, and feral buffaloes affect the prolonged behavioral activities which are resulted in overlapping the diet and space. A buffalo's day consists of portions of time spent in feeding, resting, traveling between food sources and essentially the social interactions which may be true for many of the diurnal animals.

There were reports of differences in feeding and resting time in sexually dimorphic species, i.e. males generally feed less and rest more than females in such species (Clutton-Brock, 1977). Another explanation may be lactating and pregnant females spend more proportion of time in feeding to compensate for the additional energy required (Altmann, 1980; Strier, 1987). Even in this study, it appeared that females were involved in more feeding than the males. This may be because of the lactating stage of the females. Wild buffaloes spend a long time for wallowing (almost 6-8 hours), especially during hot seasons; they contemplate in water sources such as along the major river courses like Saptakoshi, Mariya and Trijuga rivers in KTWR, and other water sources formed due to these sources like ponds, oxbow lakes, puddles, mudflows, etc. all over the areas of wildlife reserve. Therefore waterholes observations were used for locating the major herds of wild buffaloes. They mostly rely on tall grasses during monsoon and also short grassland especially during dry winter to hot weather while there remains scarcity of greenery and low palatability in the vegetation communities.

For overall feeding behavior of the large herbivores, the current finding resembles with the earlier results (Hutchins and Kreger, 2006) that the feeding time of the wildlife depends on their body size and weather condition. Being mainly a diurnal, behavioral activities of wild buffaloes are reported to perform most of the activities that reflect the survival strategies in their lifetime

so were found to carry out over the wide range of grasslands and savannah forests. The impacts of the habitat management have the importance in preserving their behavior through which wild buffalo population would also automatically be preserved. The behavior of the wild buffaloes has also direct relation with the impact of the anthropogenic activities in the natural behavior of wild buffaloes as like other animals, which indicated anthropogenic activities that directly affect the natural behavior of wildlife. Asian wild buffalo *Bubalus arnee* (Kerr, 1792), in its global distribution, is confined to certain pockets, population status is progressively decreased and its population is in jeopardy due to various reasons. Considering this fact, translocation of wild buffaloes was performed at the mean time of this research and the protocol of wild buffalo translocation is developed.

REINTRODUCTION OF WILD BUFFALOES TO CNP

For reintroduction of Asian wild buffaloes on policy decision taken by Government of Nepal, maximum 20 numbers of Asian wild buffaloes to be captured from founder population at KTWR and to translocate to the recently evacuated Padampur area of the CNP in the first phase. The decision is implemented and the standard protocol is developed and devised to be adopted for the future. Grasslands of CNP is containing ample areas of short and tall grassland including a patch of woody vegetation with lower tiger density which has scope of having availability of wider range of Rapti River bank with plenty of fresh water source from Chure hill and also the base of lower Himalayas. Captured individuals were to be rehabilitated into a pre-release or soft-release holding enclosure within the north-east part of CNP near Rapti River. To avoid heat-related complications, capture and translocations were timed to coincide with the cooler hours of the day (below 25⁰C) or night hours. Hence the most suitable months are from February to March.

As a preparatory work, Species Translocation Advisory Committee studied the feasibility of operations and ecological requirements such as availability of food, space, water, cover, evaluation of protection strategies against anthropogenic factors, identifying a source for founder stock. The action plan was developed and standard veterinary protocols for wild buffalo capture and translocation were prepared and activities were undertaken during Jan.-Feb. 2017 to translocate wild buffaloes from KTWR and Central Zoo, Nepal to CNP. A field-level coordination committee in the leadership of Chief Warden of CNP was formed to coordinate and carry out all the management and technical arrangement of the translocation. Arrangement for the construction of soft release enclosure at CNP, protection of the area by security provisions, technical and physical preparation for transportation, and handling all other necessary tasks as

required. Before undertaking operation for translocation of wild buffalo, coordination meetings, interactions and dialogues were organized with the stakeholders, local communities and leaders in KTWR and CNP.

The transport containers were designed according to the size of the target animals. The container used for transportation of rhino during translocation was modified for transporting wild buffalo. For the soft release of the translocated wild buffalo, an enclosure at Padampur area inside the Chitwan National Park was designed. Then holding enclosure at CNP was constructed at the release site, called as a soft-release enclosure to keep them prior to their free movement at new open habitat. For the capture operation, a joint team of DNPWC, CNP, and NTNC-BCC (a conservation partner) including a team of veterinarians and technicians of CNP and NTNC-BCC detailed out a technical plan of operation for capture and translocation from KTWR and reintroduction to soft release site at CNP. The essential veterinary supplies, dart equipment, and veterinary requirement were also reviewed before getting into the operation. At the same time, orientation training to field personnel was accomplished on various aspects of translocations. Considering the field requirement for translocation, team of monitoring and locating animal herd, capture and darting, immobilization, loading to a tractor, shifting immobilized animals to a cage and to push into the truck, emergency management and logistics in Koshi Tappu, transport team between Koshi Tappu and Chitwan; unloading and post-release monitoring team in Chitwan were identified and specific roles were assigned to each team. A total of 206 personnel were involved during the whole process of translocation.

To select appropriate animals for capture and translocation, experienced observers were involved to distinguish wild from domestic and feral. Backcrossed (feral) are thought to be derived from domestic females that have interbred with wild bulls for at least 6 generations, herds of which are mostly composed of females accompanied by their calves that share most but not all phenotypic and behavioral characteristics of wild buffaloes. Animals were first classified as wild, hybrid or domestic on morphological criteria – chevron markings, stockings, horn size and shape, tail length, and herding behavior. For each animal, morphometric information is taken from frontal photographs and subjected to identify from various angles of the body.

Darting of the selected animal was done from an approachable distance by foot or from a vehicle. Body health condition, physiological status, age and estimated weight were considered while determining the drug dosages. Correlation between drug dosage and body weight had been considered carefully. The weight of the selected animal was estimated based on published

information and past experiences that ranged between 250kg and 700kg for sub-adults to adults. *Etorphine Hydrochloride* mixed with *Azaperone* at approximate doses was remotely injected with a dart gun. The animal was left undisturbed until it became safe for immobilization. Other captured animals were darted using *Etorphine Hydrochloride* mixed with *Xylazine*. Antidote M 50-50 was used for *Etorphine Hydrochloride* and Antisedan for *Xylazine*. The animals were to be transported more than 400km from Koshi Tappu to Chitwan, while *Haloperidol* was administered to each captured animal for relaxation to minimize travel stress. When the target animal was selected from the herd, they were approached from a close distance for efficiently capturing through the drug delivery. The capture of wild buffalo was done in two ways: 1) by darting, and 2) by traditional chase method. For darting the target animals, remote drug delivery was facilitated using all-plastic darts delivered through an air-pressurized Dan-Inject (Model JM) projector from close distance preferably using branched trees or elephant back or on foot from a mount. The dose of the drug administered considering the weight of the selected animals ranged between 150kg and 700kg for sub-adults and adults (Curro, 2007) based on published information and past experiences. Altogether, 16 wild buffalo were captured using both of the methods as explained above. Out of them, 06 were captured using traditional method and others were captured by darting. Out of 16 animals, 13 were captured from Koshi Tappu and 3 were from Central Zoo, Nepal. Among 13 captured from Koshi Tappu, 12 (10 F & 02 M) were translocated to Chitwan and one (01 M) was translocated to Central Zoo; and, three captured from Central Zoo (2 F and 1 M) were also translocated to Chitwan. For the sample collection of darted wild buffaloes, two 10ml un-heparinized tubes of blood were collected from a superficial vein of each buffalo and its number recorded on the tubes. Then the samples of blood were taken to Molecular Lab at NTNC, Sauraha, Chitwan to preserve the blood samples for further analysis.

For the appropriate site selection, a preliminary field visit of potential location of wild water buffalo herd was carried out jointly by capture team, veterinary unit and local herders (called as citizen scientists) to better understand the habitat and terrain. Once the herd size, composition, distance from the area and accessibility to capture and transportation was reported, then the veterinary, capture and darting teams managed to move to the herd site. The terrain, visibility of animal and distance from the target points were taken into consideration before darting. Wild water buffalo, which were not feasible to dart due to difficult terrain, poor visibility and unsafe due to deep water pool, were captured by traditional chase method. Local herders (Citizen Scientist) were employed to capture by this method. Selected animals from the herd were pushed away towards sandy bank in one direction until the animal gets tired. As soon as the animals

slowed down their speed, it was allowed to climb sandy bank of the river then to pull downward by catch hold of their tail. The tired animal, while climbing the sandy bank, lose its balance and falls down. Then, a group of cattle herder ties the animal's hind and forelimbs with a strong rope. They also tie its horn and forelimb together so that it cannot run. During wild buffalo translocation, a total of six animals were captured using traditional chase method. Such captured animals were not immobilized with any drugs, but *haloperidol* and other antibiotics were provided to reduce stress in view of the long-distance travel.

After darting or capture by chase method, the animal was first loaded to the tractor. A loading ramp was dugout for making loading process comfort. Loading truck was not taken up to darting site because of difficult terrain and due to sandy and swampy areas; instead immobilized animal were first loaded into a container on the tractor. Then transported to a designated place where the truck was kept ready for transportation and shifted the animal loaded container into the truck with the help of JCB. However, animal captured location was approachable on the truck and loaded directly onto the truck. Once the animal was loaded into a container on tractor, it was provided antidote supplemented with *haloperidol* and other antibiotics. Orientation training to transport crew including truck staffs was given on safety measures to be applied while transporting animals. The truck staffs were strictly advised not to overtake any vehicle and to maintain a limited speed and the distance of at least 50 m from the front vehicle. The transport team moved in the evening to avoid vehicular disturbance and travel stress to the animal and reached early morning to release site which took almost 10 hours. The transport crew monitored the animal throughout the journey and controlled speed of the vehicle for 40-50 km/hr as per road condition.

A release program was organized approximately 5km away from the exact release site to avoid the disturbance in the proposed release site. The animal bearing truck was placed properly at unloading ramp (enclosure at Padampur) at the release site. A core team of translocation operation only went for release work. All translocated animals were released with proper care after inspection of physical condition of the animal. All animals were released safely and none of the translocated animals came across any unusual incidences during transportation or release procedures. Intensive and regular monitoring of re-introduced animals is being carried out jointly by staff of CNP and NTNC-BCC to understand their movement, ranging pattern and herding behavior. Two staffs are assigned for taking care of animals and maintenance of enclosure as required with the provision of a daily update to concerning authorities.

As learning from this mission, the procedures need to take many precautionary measures for the successful translocation and safety, female wild buffaloes always had to dart twice due to herding behavior of the females as they were most likely to be missed. Therefore, the team should be very careful to target focal animal and take quick action in the field. However, no such dangerous situation rose except the presence of wild elephants that happened to come to hinder the movement of the capture operation during the field arrangement. This was probably the first mass translocation of wild buffalo ever done in the world from KTWR to CNP in Nepal, although few wild buffaloes were already translocated to the central zoo from Koshi Tappu in the past. Anaesthetic medication includes a combination of *Etorphine HCl*+*Azaperone* was applied to capture the first animal, but other animals were applied with a combination of *Etorphine HCl*+*Xylazine*; which worked very effectively. Both traditional chase and capture by dart method were applicable and possible with the help of local herders which was a new experience and cheapest method of translocation. During the translocation, a total of 225 persons from different institutions were directly and indirectly involved during the whole period of translocation. A team work was an effective and efficient means of a successful mission for wild buffalo translocation. Translocation of Wild Buffalo to a new habitat, at CNP, established a milestone for the conservation of one of the endangered species. Documentation of the experience and standard protocol developed from this operation would be an instrument for the new users to handle the same operation in future.

Comparing the behavioral activity time budget of wild buffaloes at KTWR with post-translocation behavior at CNP over four seasons, a similar pattern has been observed. But the grazing is more significant due to lack of ample space for doing other free-ranging activities and they are almost like controlled in their behavioral activities.

MOLECULAR STRUCTURES OF WILD, FERAL AND DOMESTIC BUFFALOES

Nepal is a part of the home to endangered Asiatic wild buffalo (*Bubalus arnee*), the progenitor of domesticated water buffalo (Lei *et al.*, 2007). The wild progenitor had been substantially reduced numerically and eliminated from the greater part of its former range. Therefore, Nepal's population of wild water buffaloes in KTWR, regarded as the second largest population of wild buffalo in the World, is considered as important as other species like Rhino, Tiger, Elephant, Snow leopard, Gaur, etc. Given the existence of the last Nepal's population of wild buffalo within a restricted area encompassing only around less than 200 sq km in KTWR, several wildlife conservationist have consigned the need of translocation of small populations in their

historic distribution ranges like Chitwan to better secure the species in the country where there are extensive grassland areas, much larger abundant riverine habitats, fresh water sources, and sufficient upland areas. To maintain the ecological viable population of *Bubalus arnee* in Nepal, recently 15 wild individuals have been translocated to CNP from KTWR. Before the translocation, adequate morphological and genetic studies need to be carried out to distinguish putative wild from feral backcrossed for translocation program and to ensure the conservation of truly wild stocks of water buffaloes. A sufficient number of animals must be moved to assure genetic variation in the founding population. For this purpose, genetic analyses based on evidence to distinguish putative wild from feral backcrossed buffalo for translocation is highly advised (Heinen, 2001). Although the recent selection of 16 individuals for translocation was based on phenotypic and behavioral characteristics widely recommended by Dahmer (2000); Heinen and Kandel (2006); Kandel *et al.* (2014) and Heinen and Paudel (2015), many individuals of mixed wild-domestic ancestry may not be correctly distinguished from wild animals (Flamand *et al.*, 2003). Genetic study based assignment tests only can provide a unconquerable basis for conservation management plans and conservation decisions. Genotyping of wild water buffaloes using a substantial number of microsatellite loci and undertaking of mitochondrial DNA sequencing would help to accurately discriminate domestic, hybrid (backcrossed/feral) and wild individuals. Since animal mitochondrial DNA is inherited only from the mother, identification of wild and domestic type specific haplotypes (mitotypes) will help to identify the hybrid individuals.

Samples were collected from 42 buffaloes from in and around the Koshi Tappu Wildlife Reserve, eastern Tarai region of Nepal. For sample collection, animals were divided into 3 classes, classified as wild (n=20), domestic (n=11) and hybrids (n=11). Blood samples from wild were collected during the translocation of wild buffaloes from KTWR to CNP. Classification of buffaloes was based on location of sampling (in the wild or near villages), behavioral and phenotypic traits. All animals classified as domestic were river type with black body and curled horns (as in the Murrah breed river buffalo), while those classified as wild had white chevron, socks, and the tip of tail, and larger, relatively straight, pale-colored horns (similar to swamp buffalo) (Heinen, 2002). Hybrids were crossbred of wild and domestics. A fecal sample from wild buffaloes was collected fresh with GPS locations from the core area. In addition, 42 (7 wild, 15 hybrid, and 20 domesticated) buffalo samples of Flamand *et al.* (2003) and used by Zhang *et al.* (2016) were also included. Genomic DNA was extracted from blood and tissue samples using Qiagen DNEasy Blood and Tissue kits respectively according to the prescribed manufacturer

protocol. For the fecal samples, Qiagen QIAMP DNA Stool Mini Kit was used following the instructions provided by the manufacturer. The extracted DNA samples were stored at 4°C until used for molecular analyses. *Aliquots* of extracted high-quality DNA samples were used for upstream analysis like PCR and sequencing. Small section of *cytochrome b* gene within the mitochondrial genome was amplified using primer pair as described in Kocher *et al.* (1989).

PCR was carried out and samples were amplified following standard protocols. The PCR products were electrophoresed; the amplified DNA fragments were purified. Out of 45 selected samples, PCR optimization was successful only in 36 samples. High quality purified PCR amplicons were subjected to Dideoxy sequencing was done in an automated DNA sequencer. Raw sequence fragments of 422 bp length were assembled, checked and edited with Sequencer 5.0 and the contigs of both reverse and forward primers were created. Sequences were aligned with ClustalX in BioEdit. In our 36 sequences, we have added 42 reported sequences of Nepalese buffaloes of all the three groups from Zhang *et al.* (2016) (KR009964NPH01-42). To understand the possible haplotypic variation among the river buffalos of the neighboring region, we further added four *Cytochrome b* haplotypes identified by Kumar *et al.* (2007) from Indian river buffaloes (EF409942H1-4) and 15 *cytochrome b* haplotypes defined by Kikkawa *et al.* (1997) representing both river buffaloes from Bangladesh, Srilanka, Italy and Pakistan, and swamp buffaloes from Japan, Taiwan, Thailand, Philippines, Indonesia and Bangladesh (D34637, D88629-38). This yielded a total of 97 sequences } (36 from the present study, 42 from Zhang *et al.* (2016), 4 from Kumar *et al.* (2008) and 15 sequences from Kikkawa *et al.* (1997)) of buffaloes representing river and swamp types.

Sequences were aligned and mitochondrial haplotypes (mitotypes) were defined in DnaSP v5 (Librado and Rozas, 2009). Haplotype diversity (h) and nucleotide diversity (π) for the buffalo samples were estimated using DnaSP 5.0 (Librado and Rozas, 2009). A phylogenetic relationship between the haplotypes was derived through a reduced median network applied to the data-set using Network v4.6. To identify phylogenetic lineages, Maximum parsimony (MP) analysis was performed in PAUP 4.0b10 using the heuristic search option with 1000 random additions and Tree Bisection-Reconnection (TBR) swapping and MULTrees option on. Branch support was provided by a bootstrap analysis of 10000 replicates of heuristic searches, with a MULTrees option on and TBR swapping off. Consistency indices (CI) and Retention Indices (RI) were obtained in PAUP. Moreover, the evolutionary history was inferred using the Neighbor-Joining (NJ) method. The evolutionary distances were computed using the Maximum Composite Likelihood method with 10000 bootstrapping replicates in MEGA7.

The lengths of the 36 partial *cytochrome b* sequences of all the three types of buffaloes of this study have 422 bp. The aligned matrix was 422 characters and contained 2 variable sites. However, the complete *cytochrome b* sequences of the 42 samples of Nepal used in Zhang *et al.*(2016) have 1140 characters and revealed 4 variable sites among the three groups of river buffaloes. The partial *cytochrome b* sequence used in our study is shorter than that described by Zhang *et al.* (2016) and thus has fewer variable sites. However, when our data set was compared with the accession of Kumar *et al.* (2007) and Kikkawa *et al.* (1997), base substitutions at 13 nucleotide positions (variable sites) were obtained and among them three nucleotide positions were specific for river buffaloes and ten positions were specific for swamp buffaloes. The sequence divergence within buffaloes of Nepal and India was 0.24-0.49 % however when compared with river buffaloes sequence of Kikkawa *et al.* (1997), the divergence was slightly higher 0.24-0.74 %. Sequence divergence within swamp buffaloes was 0.24-0.98%. The sequence divergence between swamp and river buffaloes was calculated to be 1.49-2.49%.

Analysis of a total of 97 *cytochrome b* partial sequences of 422 bp length of the river buffaloes (from Nepal, India, Bangladesh, Pakistan, Srilanka) and swamp buffaloes (Japan, Taiwan, Thailand, Philippines, Indonesia and Bangladesh) showed 13 variable sites defining 9 haplotypes (H1-H9). Among the nine observed haplotypes, Nepal's buffaloes both in this study and that of Zhang *et al.* (2016) were represented by just the three common haplotypes (H1, H2, and H3). These three haplotypes are identified by Kumar *et al.* (2007) as well. The most common haplotype (H2) is widely distributed among groups and represented by 66 percent of sequences {(Nepal: D=23, W=18, and H=17; one of Kumar *et al.* (2007) and five of Kikkawa *et al.* (1997)}. The second common haplotype (H1) was represented by 20 percent of the samples (D=7, W=2 and H=8 and one each of Kumar *et al.*, 2007 and Kikkawa *et al.*, 1997). Three sequences of Nepalese samples (two from present study and one from Zhang *et al.*, 2016) and one from Kumar *et al.*, 2007 were restricted to the third haplotype (H3). Of the remaining six haplotypes, one was reported (H4) by Kumar *et al.*, 2007 while the remaining five (H5-H9) were defined by Kikkawa *et al.*, 1997. Three common haplotypes were present in three of the two groups wild and domestics. Within groups, haplotype diversity was highest in hybrid groups followed by domesticated groups and slightly lower in wild breeds. Overall haplotypes and nucleotide diversities were 0.403 and 0.00105 respectively. Interestingly, haplotypes were divided into two branches corresponding to river and swamp buffaloes with six nucleotide mutations. We could not find specific haplotypes for wild, hybrid and domesticated groups of

buffaloes. In terms of genetic diversity, river buffaloes were found to be less diverse than swamp buffaloes.

Maximum parsimonious analysis of 97 partial sequences of *cytochrome b* performed under heuristic search and 1000 bootstraps revealed four distinct clusters or clades (Clade A-D) with moderate to high bootstrap values. Among four sub-clusters, one cluster with high bootstrap values (99%) showed strong differences between swamp and river buffaloes. Surprisingly relationship among haplotypes revealed by median-joining network corroborates with the four lineages observed in MP tree. Lineage A comprises all sequences of haplotype H2 except Hap3 India, Lineage B comprises 20 sequences of haplotype H1 but accession H4 India, lineage C comprises all four sequences of Haplotype H3 and finally all haplotypes of swamp buffaloes (H5, H8, and H9). In MP analysis, a total of 3 most parsimonious trees (CI = 1.00, RI = 1.00, Length = 15 steps) were recovered. Phylogenetic analysis and evolutionary history of 97 partial sequences of *cytochrome b* inferred using the Neighbor-Joining (NJ) with 10000 bootstrapping replicates also depicted identical topology of the phylogenetic tree as reported by MP analysis.

On the basis of the partial *cytochrome b* sequences of 422 bp length, we were able to define very few (only three) haplotypes in the Nepal's buffaloes. None of the haplotypes were wild, domestic and hybrid specific. These haplotypes were already identified by Kumar *et al.* (2007) and Kikkawa *et al.* (1997) in their Indian and wider samples (Pakistan, Bangladesh, Thailand and Srilanka). Although we have used the partial sequence (422 bp) of *cytochrome b* gene, the complete length (1120 bp) of this gene sequence reported from other studies (Kumar *et al.*, 2007 and Kikkawa *et al.*, 1997) including 42 Nepalese samples of Zhang *et al.* (2016) could not even reveal distinction between wild, hybrid and domestic river buffaloes. NJ and MP analysis performed (result not shown) with complete length (1120 bp) *cytochrome b* sequences of Zhang *et al.* (2016) (42 Nepalese sequences), Kumar *et al.* (2007) (four river buffalo haplotypes), Kikkawa *et al.* (1997) (seven river and eight swamp haplotypes) provided essentially the same topography of the tree butan addition of one more haplotype represented by Genbank accession KR009945NP_D07 alone.

Lau *et al.* (1998) using partial *cytochrome b* sequence (303 bp) and D-loop (158 bp) sequence has suggested that Wild Asian buffalo (*Bubulus arnee*) which is in Assam, Nepal, and Indo-China is the possible ancestor of river and Swamp buffaloes. The studies by Tanaka *et al.* (1996) also supports Lau *et al.* (1998) hypothesis. Nepal's wild buffaloes show swamp type phenotypic characteristics thus discriminated as a swamp type, however, Zhang *et al.* (2011) found them

genetically close to river type. Our study is consistent with Zhang *et al.* (2011) as in the MP, NJ and Network analysis swamp buffalo showed distinct variation with almost 100% bootstrap value and six nucleotide differences between these groups. A similar result was obtained by Mishra *et al.* (2015) on upper Assamese and Chilika populations which show phenotypic similarity to swamp type but genetically more closer to wild-type buffaloes in the region. Our multi-lineages MP and NJ trees and several previous studies (Tanaka *et al.*, 1996; Lau *et al.*, 1998; Zhang *et al.*, 2011, 2016; Mishra *et al.*, 2015) inferred an ancestral nature of wild water buffalo including the remnant population of KTWR.

In regard to the results of present research, genetic distinctness, ancestral nature and existing genetic variability of buffaloes protected in KTWR warrants the need of more rigorous genetic studies based on an adequate number of polymorphic markers and mitochondrial DNA sequencing of the entire putatively wild individual. Microsatellite genotyping with sufficient number of loci along with mitochondrial DNA sequences database on the measure of the magnitude of genetic differentiation and genetic relationships among the populations of all the recently counted wild individuals of KTWR will have large implications on rational decision making for the conservation of wild buffaloes in KTWR and translocation of potential individuals to the suitable areas. Furthermore, if the wild Asiatic buffaloes were protected properly with due attention, several important genetic traits (growth, draft, meat, milk, climate adaptation, disease resistance, etc.) presumably present in these populations would be advantageous to use for the genetic improvement programs in the future or a source of bull for use in other populations.

MALARIAL PARASITE IN WATER BUFFALO OF NEPAL

There have been the report of prevalence of *Plasmodium bubalis* cases on immune-compromised water buffalo (Kolte *et al.*, 2002). In this is study, we performed nested PCR assay to investigate the possibility of prevalence of malaria parasite in water buffalo (wild, feral and domestic) in Nepal during our molecular analysis using oligonucleotide primers specific for the *Plasmodium* mitochondrial cytochrome b (*cyt-b*) sequence and found two cases with the main Objective Molecular detection of malaria parasites in water buffaloes in Nepal.

For this study, Genomic DNA was extracted from blood samples using commercial kit (QIAGEN DNeasy Blood and Tissue kits, QIAGEN, Germany) according to the manufacturer's protocol. PCR assay using **Plasmodium** *cyt-b* gene specific primers followed a previous study

(Martinsen *et al.*, 2006). Briefly, the *cyt-b* gene of *Plasmodium* was amplified by nested PCR using the universal primers for *Plasmodium* DW2 (TAATGCCTAGACGTATTCCTGATTATCCAG) and DW4 (TGTTTGCTTGGGAGCTGTAATCATAATGTG) for the first round PCR. The nested PCR was performed using primers NCYBINF (TAAGAGAATTATGGAGTGGATGGTG) and NCYBINR (CTTGTGGTAATTGACATCCAATCC) as described (Abkallo *et al.*, 2014). Both PCR cycle were performed for 40 cycles with denaturation at 94°C for 20 sec followed by annealing and extension at 62°C for 3 min. All reactions were performed using negative controls, and no contamination was detected.

Out of 56 blood samples; 16 (28.6%), 23 (41.1%) and 17 (30.3%) were of wild, feral and domestic buffalo, respectively. Out of 16 wild buffalo, 13 and 3 samples were obtained from KTWR and Central Zoo, respectively. Among these buffalo, 32 (57.1%) are female and 24 (42.9%) are male. The age of buffalo ranged from 0.5 months to 15 years (for only 38 samples). PCR detected 2 positive cases (3.5%); one was a feral buffalo (2 years old female) and the other was a domestic buffalo (2 years old male). Both positive samples were collected during rainy season from KTWR and its surrounding village. None among 16 wild buffalo samples collected during winter season from KTWR and Zoo were positive however further tests must be done on all existing individuals of the reserve for confirmation. Previously, 16% (15/95) and 65% (65/144) of water buffaloes were PCR positive for malaria parasites in Thailand in 2015 and 2014, respectively and 6% (3/49) and 5% (243) in Vietnam in 2013, and 2010 (Templeton *et al.*, 2016). The low frequency of the positivity in this study may be attributed to the difference in the vectorial capacity of each area or seasonality. To clarify this point, more samples from different seasons needs to be analyzed.

Sequences of two positive PCR products (773 bp) from water buffalo were identical to the Type II sequence except one nonsynonymous substitution at nucleotide position 5344 (number is after LC090214) from A to T, a nucleotide type seen in Type I sequence. Because this *cy-tb* region in 39 Type II sequences of water buffalo in Thailand were identical (Templeton *et al.*, 2016), identified A5344T single nucleotide polymorphism in Nepal may serve as a unique region-specific marker. In the previous study, Type II sequence was detected from 57/80 (71%) and 2/5 (40%) *Plasmodium* positive cases in Thailand and Vietnam, respectively and Type I was from 43/80 (54%) and 3/5 (60%) positive cases in Thailand and Vietnam, respectively. Thus, we

consider that Type I water buffalo malaria parasite likely exists in Nepal, too. More samples are required to evaluate the existence of the type I water buffalo malaria parasite in Nepal.

Buffalo farming is one of the major occupations in the lowland and midhills of Nepal. The study area KTWR is stretching northward from the Nepal Indian border where annually buying and selling of buffalo takes place between farmers residing across borders. Since the first water buffalo malaria parasite reported from India, the prevalence of this pathogen in different areas of India has been reported by many succeeding studies; such as Bareilly/Muktesar (Sheather, 1919), Nagpur (Kolte *et al.*, 2002), and Maharashtra (Shastri *et al.*, 1985; Shinde *et al.*, 2005). In this context, it is important to quarantine the water buffalo across the border between Nepal and India to prevent the spreading of ungulate malaria parasites.

Although the early study concluded that the this pathogen causes only mild symptoms and animals (*B. bubalis*) were self-cured (Riaz-Ul-Hassan, 1953), one mortality case has been observed (Templeton *et al.*, 2016). It was not determined the buffalo (*B. bubalis*) died due to malaria infection alone or due to malaria was an opportunistic parasitic infection (Templeton *et al.*, 2016). Nonetheless, the hidden pathogenesis of this pathogen as an opportunistic agent is not evaluated so far. The pathogenesis against the buffalo with different genetic background (wild, feral or domestic buffalo) is also not clearly understood. The sole objective of KTWR is to conserve the last remaining Nepalese population of an endangered Asiatic wild buffalo. Therefore diagnostic assays are strongly recommended to be employed by the reserve to confirm possible health impacts of malaria in wild individuals.

This is the first PCR-based surveillance of water buffalo malaria parasite in Nepal. The presence of malaria parasites in feral and domestic animals may have greater consequences in terms of ecological impact as well as economy of the farmers depending upon livestock farming. Furthermore, active surveillance is necessary for malaria parasite infection in Asiatic wild buffalo to obtain information regarding health status and mortality rates. These steps are important to develop strategies for the management and control of malaria infection especially in wild buffalos in Nepal.

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LIST OF PUBLICATIONS

PAPER ARISING FROM THIS THESIS

A. PAPER PUBLISHED

1. **Kandel, R.C., G.S. Solanki, M.K. Chalise and B.K. Sharma (2018).** Habitat Composition of Asian wild buffalo (*Bubalus bubalis arnee*) at Koshi Tappu Wildlife Reserve, Nepal. In G.S. Solanki (Eds) Biodiversity Conservation Strategies and Application. Pages 151-171. *Scientific Book Centre, Guwahati*. Pp 152-170. ISBN 978-81-287-0035-4.
2. **Kandel, R.C., J. S. F. Barker, and M. Melleti (2014).** Status and Management of the endangered wild water buffalo (*Bubalus arnee*) in the Koshi Tappu Wildlife Reserve, Nepal. In: M. Melleti and J. Burton (Eds) Ecology, Evolution and Behavior of Wild Cattle-*Implications for Conservation*. Pages 403-409. Cambridge University Press, Cambridge, UK. Pp 403-409. ISBN 611-0770-1210-420. Code 59220.
3. **Kandel, R.C., G.S. Solanki, M.K. Chalise and B.K. Sharma (2017).** Population and Demography of Asian Wild Buffalo (*Bubalus bubalis arnee* Kerr, 1792) at Koshi Tappu Wildlife Reserve, Nepal. True Scholar Research Ltd, London. ISBN: 611-0770-1210-420. Code 59220.

B. PAPER PRESENTED

4. **Kandel, R.C., G.S. Solanki, M.K. Chalise, B.K. Sharma (2016).** Habitat Composition of Asian wild buffalo (*Bubalus bubalis arnee*) at Koshi Tappu Wildlife Reserve, Nepal. Presented to National Conference on “*Impact of Climate Change on Biodiversity: Application of Recent Technologies for Conservation of Threatened Species*” held at the Department of Zoology, Mizoram University, India during 22-24 Sept. 2016.
5. **Kandel, R.C., G.S. Solanki, M.K. Chalise, S.R. Jnawali, W. Karl, S. Bhattarai, K.P. Gairhe and C.P. Pokharel (2017).** Reintroduction of Asian Wild Buffalo (*Bubalus bubalis arnee* Kerr, 1792) to Chitwan National Park from Koshi Tappu Wildlife Reserve, Nepal: The historical milestone in conservation. Presented to *International Conference on Conservation Biology (ICCB)* held at Cartagena, Colombia during 23-27 August 2017.

6. **Kandel, R.C., Solanki, G.S., Chalise, M.K. and Sharma, B.K., 2018.** Population and Demography of Asian Wild Buffalo (*Bubalus bubalis arnee* Kerr, 1792) at Koshi Tappu Wildlife Reserve, Nepal. Presented to International Conference on “*Capacity Building for National Sustainable Development (CBNSD 2018)*” held at Nairobi, Kenya during 29-31 August 2018.

C. PAPER ARISING FROM THIS THESIS AND COMMUNICATED

7. **Kandel, R.C., G.S. Solanki, M.K. Chalise and A. Parida (2018).** Behavioral ecology and time budget activities of Asian wild buffalo (*Bubalus arnee*) at KTWR, Nepal. Communicated to *ECOPRINT*.
8. **Kandel, R.C., M.K. Chalise, G.S. Solanki, S.R. Jnawali, S. Bhattarai, and C.P. Pokharel (2017).** Reintroduction of Asian Wild Buffalo (*Bubalus bubalis arnee* Kerr, 1792) to in Nepal: A protocol of wild buffalo translocation. Communicated to *Journal of Natural History Museum*.
9. **Kandel, R.C., R.C. Poudel, A. Sadaula, G.S. Solanki, M.K. Chalise, S. B. Bajracharya and P. Kandel (2018).** Molecular study of Wild, Feral and Domesticated Buffaloes of KTWR, Nepal using *cytochrome b* marker. Communicated to *Journal of Endangered Species*.
10. **Kandel, R.C., Poudel, R.C., Shrestha, M., Sadaula, A., KC, M., Maharjan, J., Asada, M., Kaneko, O., Pandey, K., Solanki, G.S., Chalise, M.K., Pokharel, C.P. 2018.** First report of Ungulate Malaria Parasite in Water buffaloes from Nepal. Communicated to *Journal of IoST (Institute of Science and Technology), TU, Nepal*.

ANNEXURES

Annex 1. Mean Annual Maximum and Minimum Temperatures and Rainfall near KTWR and CNP during 2015-2017 (DoHM/GoN, 2017)

BHARATPUR NEAR CNP {Latitude (deg/min): 27 ^o 40'; Longitude(deg/min): 84 ^o 26'; Elevation (m): 205}												
Rainfall (mm)												
Year	Jan	Feb	Mar	Apr	May	Jun	JUL	AUG	SEP	OCT	NOV	DEC
2016	28.5	2.4	31	0	348.5	356.5	573.6	147.2	726.4	DNA	0	DNA
2017	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Temperature												
Year 2016	Jan	Feb	Mar	Apr	May	Jun	JUL	AUG	SEP	OCT	NOV	DEC
Tmax (°C)	22.7	27.9	32.3	37.8	35.2	34.6	32.7	35	32.8	32.5	29.4	DNA
Tmin (°C)	9	12.1	15.5	20.2	21.4	24	24.2	24.4	22.9	19	12.7	DNA

Year 2017	Jan	Feb	Mar	Apr	May	Jun	JUL	AUG	SEP	OCT	NOV	DEC
Tmax (°C)	NA											
Tmin (°C)	NA											

PHATEPUR NEAR KTWR {Latitude (deg/min): 26 ^o 44'; Longitude(deg/min): 86 ^o 56'; Elevation(m): 100}												
Rainfall (mm)												
Years	Jan	Feb	Mar	Apr	May	Jun	JUL	AUG	SEP	OCT	NOV	DEC
2015	12	28.5	109	90	144.5	154.1	229.9	469.9	241.5	20	0	0
2016	26.5	0.5	24.7	27.8	128.4	533.7	DNA	283	466.4	30.5	0	0
Temperature												
Year 2015	Jan	Feb	Mar	Apr	May	Jun	JUL	AUG	SEP	OCT	NOV	DEC
Tmax (°C)	23.7	27.1	30.7	32.2	33.5	33.1	34.2	33.2	33.6	33.5	30.7	25.9
Tmin (°C)	11	12.7	17.1	20.4	24.2	26	26.3	25.8	25.4	21.6	17	11.1

Year 2016	Jan	Feb	Mar	Apr	May	Jun	JUL	AUG	SEP	OCT	NOV	DEC
Tmax (°C)	24	28.2	33	36	33.7	33.5	DNA	34.8	32.3	33.9	31.7	27.4
Tmin (°C)	10	13	17.3	22.3	23.4	25.3	DNA	25.9	25.2	22.3	16.7	12.3

Annex 2. The specific formulae employed for the analysis of vegetation

<p>a. Frequency (f): Frequency is the proportion of sampling units containing the species.</p> $f_i = \frac{n_i}{N} * 100$ <p>Where, f_i = Frequency of species i n_i = No. of quadrats in which species i occurred N = Total number of quadrats studied</p>	<p>b. Relative frequency (Rf)</p> $Rf_i = \frac{f_i}{\bar{F}} * 100$ <p>Where, Rf_i = Relative frequency f_i = Frequency of species i \bar{F} = Sum of frequencies for all species</p>
<p>c. Density (d): Density is the number of individuals per unit area.</p> $d_i = \frac{n_i}{N * A} * 10000$ <p>Where, d_i = Density (ha^{-1}) of species i n_i = Total number of individuals of species i N = Total number of quadrats studied A = Area of a quadrat</p>	<p>d. Relative density (Rd)</p> $Rd_i = \frac{d_i}{\bar{D}} * 100$ <p>Where, Rd_i = Relative density of species i d_i = Density of species i \bar{D} = Total density of all species</p>
<p>e. Dominance (do): Dominance is the amount of ground covered by the tree trunk.</p> $do_i = \frac{Ba_i}{N * A} * 10000$ <p>Where, do_i = Dominance (ha^{-1}) of species i Ba_i = Total basal/coverage area of species i N = Total number of quadrats studied A = Area of a quadrat</p>	<p>f. Relative Dominance (RDo)</p> $RDo_i = \frac{do_i}{\bar{Do}} * 100$ <p>Where, RDo_i = Relative dominance of species i do_i = Dominance/coverage of species i \bar{Do} = Total dominance/coverage of all species</p>

g. Importance Value Index (IVI): Name of each forest and shrub land type was determined by ordering the Importance Values of each tree species. Importance Value was obtained by summation of the relative frequency, relative density, and relative dominance.

$$IVI_x = RF_x + RD_x + Rdo_x$$

Where,

IVI_x = Importance Value Index of species x

RF_x = Relative Frequency of species x

RD_x = Relative Density of species x

Rdo_x = Relative Dominance of species x

Annex 3. Importance value of tree species in Sissoo-Khair forest (in percentage)

S.N.	Scientific Name	Relative Frequency	Relative Density	Relative Dominance	Importance Value
1	<i>Dalbergia sissoo</i>	42.3	61.0	75.77	179.1
2	<i>Trewia nudiflora</i>	21.2	11.5	10.19	42.8
3	<i>Acacia catechu</i>	13.5	20.3	6.04	39.8
4	<i>Bombax ceiba</i>	3.8	1.1	6.76	11.7
5	<i>Strabulus asper</i>	3.8	1.7	0.41	6.0
6	<i>Ficus species</i>	3.8	1.4	0.26	5.5
7	<i>Zizyphus species</i>	1.9	1.4	0.27	3.6
8	<i>Wedlenda species</i>	1.9	0.3	0.21	2.4
9	<i>Acacia bipinnata</i>	1.9	0.3	0.05	2.3
10	<i>Alstonia schoalaris</i>	1.9	0.3	0.03	2.2
11	<i>Citrus maxima</i>	1.9	0.3	0.01	2.2
12	<i>Cordia dichotoma</i>	1.9	0.3	0.01	2.2
	Total	100.0	100.0	100.00	300.0

Annex 4. Prominence value of different species in understory layer of Sissoo-Khair forest

S.N.	Scientific Names	Prominence Value
1	<i>Clerodendrom viscosum</i>	25.79
2	<i>Lantana camara</i>	12.85
3	<i>Pogostemon benghalensis</i>	3.20
3	<i>Eupatorium adenophorum</i>	2.83
4	<i>Elsholtzia blanda</i>	2.53
5	<i>Solanum</i> species	0.93
6	<i>Ipomea crassipes</i>	0.85
7	<i>Zizyphus</i> species	0.38
8	<i>Triumfetta annua</i>	0.34
9	<i>Callicarpa microphylla</i>	0.25
10	<i>Typha angustifolia</i>	0.20
11	<i>Osyris</i> species	0.15
12	<i>Colebrokia oppositifolia</i>	0.11
13	<i>Cassia oxidentalis</i>	0.10
14	<i>Achyranthes aspera</i>	0.08
15	<i>Artemisia indica</i>	0.07
16	<i>Bombax ceiba</i>	0.06
17	<i>Calotropis</i> species	0.04
18	<i>Pogostemon</i> species	0.03
19	<i>Calatropis gigantean</i>	0.02
20	<i>Ficus</i> species	0.02
21	<i>Trewia nudiflora</i>	0.02
22	<i>Phragmites karka</i>	0.008
23	<i>Acacia catechu</i>	0.004
24	<i>Astrablus asper</i>	0.004
25	<i>Trachelospermum lucidum</i>	0.004
26	<i>Ficus glomerata</i>	0.004
27	<i>Litsea monopetala</i>	0.004
28	<i>Mimosa pudica</i>	0.004
29	<i>Tamarix dioca</i>	0.004
30	<i>Woodfordia fruticosa</i>	0.004
31	<i>Cordia dichotoma</i>	0.00
32	<i>Cassia tora</i>	0.00

Annex 5. Prominence value of different species in the ground vegetation of Sissoo-Khair forest

S.N.	Scientific Name	Prominence Value
1	<i>Mikania sp</i>	51.7
2	<i>Imperata cylindrical</i>	41.5
3	<i>Dryopteris chochleta</i>	16.11
4	<i>Justicia procumbens</i>	7.21
5	<i>Digitaria species</i>	5.54
6	<i>Circium species</i>	4.70
7	<i>Digitaria species</i>	4.26
8	<i>Saccharum spontaneum</i>	2.97
9	<i>Fragaria species</i>	0.57
10	<i>Sonchus arvensis</i>	0.51
11	<i>Chrysopogon acaulis</i>	0.44
12	<i>Elephantopus scaber</i>	0.40
13	<i>Oxalis corniculata</i>	0.35
14	<i>Hemarthria compressa</i>	0.28
15	<i>Typha angustifolia</i>	0.22
16	<i>Phragmites karka</i>	0.21
17	<i>Trichillia species</i>	0.16
18	<i>Equisetum debile</i>	0.15
19	<i>Bidens pilosa</i>	0.14
20	<i>Phylanthus niruri</i>	0.11
21	<i>Stephania elegans</i>	0.11
22	<i>Ageratum conyzoides</i>	0.08
23	<i>Sphaeranthus sp</i>	0.08
24	<i>Cyperus species</i>	0.06
25	<i>Achyranthes species</i>	0.05
26	<i>Scoparia dulcis</i>	0.044
27	<i>Jasminum species</i>	0.04
28	<i>Boehemeria species</i>	0.03
29	<i>Viola species</i>	0.03
30	<i>Achyranthes aspera</i>	0.014
31	<i>Lippia nudiflora</i>	0.01
32	<i>Cyanodon dactylon</i>	0.003
33	<i>Digiteria cialaris</i>	0.003
34	<i>Trachelospermum lucidum</i>	0.003

Annex 6. Prominence value of different species in the ground vegetation of grassland

S.N.	Scientific Name	Prominence Value
1	<i>Saccharum spontaneum</i>	282.57
2	<i>Imperata cylindrical</i>	168.79
3	<i>Dryopteris chochleta</i>	14.41
4	<i>Cyanodon dactylon</i>	8.82
5	<i>Citronella species</i>	6.93
6	<i>Tamarix dioca</i>	3.81
7	<i>Saccharum bengalensis</i>	3.50
8	<i>Typha angustifolia</i>	3.06
9	<i>Cassia tora</i>	1.52
10	<i>Centella asiatica</i>	1.33
11	<i>Scoparia dulcis</i>	1.19
12	<i>Hydrocotyle nepalensis</i>	1.17
13	<i>Hemarthria compressa</i>	1.14
14	<i>Chrysopogon aciculatus</i>	0.76
15	<i>Sonchus species</i>	0.40
16	<i>Flemengia strobilifera</i>	0.38
17	<i>Equisetum debile</i>	0.29
18	<i>Cyperus killing</i>	0.24
19	<i>Phragmites karka</i>	0.19
20	<i>Trichillia species</i>	0.13
21	<i>Lipia nudiflora</i>	0.11
22	<i>Mimosa pudica</i>	0.11
23	<i>Digitaria species</i>	0.10
24	<i>Elusine species</i>	0.08
25	<i>Hemarthria compressa</i>	0.08
26	<i>Mikania species</i>	0.04
27	<i>Persicaria species</i>	0.02
28	<i>Vicia angustifolia</i>	0.02
29	<i>Phyllanthus niruri</i>	0.01
30	<i>Desmodium species</i>	0.004
31	<i>Oxalis corneicolata</i>	0.004
32	<i>Reinwardtia species</i>	0.004

Annex 7. Importance value of tree species in the mixed riverine forest (in percentage)

S.N.	Scientific Names	Relative Frequency	Relative Density	Relative Dominance	Importance Value
1	<i>Trewia nudiflora</i>	17.6	68.1	54.8	140.6
2	<i>Bombax ceiba</i>	17.6	4.3	28.2	50.2
3	<i>Dalbergia sissoo</i>	17.6	4.3	7.2	29.2
4	<i>Cordia dichotoma</i>	5.9	2.9	5.1	13.9
5	<i>Acacia catechu</i>	5.9	4.3	1.4	11.6
6	<i>Vitex negundo</i>	5.9	4.3	0.2	10.4
7	<i>Strablus asper</i>	5.9	2.9	0.7	9.4
8	<i>Zizyphus jujube</i>	5.9	2.9	0.3	9.0
9	<i>Ficus glemmerata</i>	5.9	2.9	0.1	8.8
10	<i>Acacia species</i>	5.9	1.4	1.1	8.5
11	<i>Callicarpa macrophylla</i>	5.9	1.4	1.0	8.3
	Total	100.0	100.0	100.0	300.0

Annex 8. Prominence value of different species in understory layer of mixed riverine forest

S.N.	Scientific Name	Prominence Value
1	<i>Lantana camara</i>	7.3
2	<i>Clerodendrom viscosum</i>	3.3
3	<i>Triumfetta annua</i>	1.33
4	<i>Pogostemon bengalensis</i>	1.25
5	<i>Eupatorium adenophorum</i>	1.12
6	<i>Ardisia solanacea</i>	0.54
7	<i>Osyris species</i>	0.21
8	<i>Coolebrokia oppositifolia</i>	0.16
9	<i>Calatropis gigantean</i>	0.09
10	<i>Callicarpa macrophyllum</i>	0.08
11	<i>Zizyphus jujube</i>	0.04
12	<i>Vitex negundo</i>	0.01
13	<i>Ipomea species</i>	0.004
14	<i>Solanum xanthocarpum</i>	0.004
15	<i>Strablus asper</i>	0.004
16	<i>Trewia nudiflora</i>	0.004

Annex 9. Prominence value of different species in the ground vegetation of the mixed riverine forest

S.N.	Scientific Name	Prominence Value
1	<i>Imperata cylindrica</i>	224.3
2	<i>Dryopteris cochleata</i>	127.7
3	<i>Saccharum spontaneum</i>	96.8
4	<i>Mikania</i> species	71.7
5	<i>Chrysopogon aciculatus</i>	54.7
6	<i>Oxalis corniculata</i>	16.2
7	<i>Ageratum conyzoides</i>	8.5
8	<i>Cynadon dactylon</i>	6.9
9	<i>Eragrostis</i> species	6.3
10	<i>Jasminum</i> sp	4.4
11	<i>Centella asiatica</i>	2.2
12	<i>Digitaria</i> species	1.7
13	<i>Circium</i> species	1.3
14	<i>Digitaria</i> species	1.3
15	<i>Mimosa pudica</i>	0.9
16	<i>Crysopogan</i> sp	0.4
17	<i>Sonchus</i> species	0.4
18	<i>Stephania elegans</i>	0.4
19	<i>Equisetum debile</i>	0.2
20	<i>Hydrocotyle nepalensis</i>	0.09
21	<i>Trichillia</i> species	0.04
22	<i>Cyperus kilingia</i>	0.04
23	<i>Lippia nudiflora</i>	0.04

Annex 10. Prominence value of different species in understory layer of shrubland

S.N.	Scientific Name	Prominence Value
1	<i>Zizyphus</i> species	37.5
2	<i>Clerodendrom viscosum</i>	1.1
3	<i>Solanum xanthocarpum</i>	0.2
4	<i>Lantana camara</i>	0.2
5	<i>Eupatorium adenophorum</i>	0.1
6	<i>Solanum</i> species	0.1

Annex 11. Prominence value of different species in the ground vegetation of shrubland

S.N.	Scientific Name	Prominence Value
1	<i>Imperata cylindrica</i>	38.97
2	<i>Dryopteris cochleata</i>	17.71
3	<i>Cynadon dactylon</i>	5.00
4	<i>Saccharum spontaneum</i>	3.89
5	<i>Saccharum bengalensis</i>	1.25
6	<i>Trichillia</i> species	0.25
7	<i>Stephania elegans</i>	0.03
8	<i>Oxalis corniculata</i>	0.03
9	<i>Hydrocotyle nepalensis</i>	0.03
10	<i>Visia angustifolia</i>	0.03
11	<i>Mimosa pudica</i>	0.03

ANNEX 12
PHOTOPLATES

Photoplate 1: Studying habitat composition of wild buffaloes in the eastern part of KTR



Photoplate 2: Studying habitat composition of wild buffaloes in the western part of KTWR



Photoplate 3: Researcher talking to Mr. Raghu Baharkher (116 years, one of the pioneers of buffalo herder at KTWR) at Barmajhiya, Saptari District about the feral and wild breeds of buffaloes



Photoplate 4: Identification of different breeds of buffaloes based on morphometric and phenotypic characteristics

Wild breeds of wild buffaloes	Feral buffaloes	Domestic buffaloes
		
		
		

Photoplate 5: Team of people on preparation and field visit for wild buffalo count and behavioral studies at KTWR



Photoplate 6: Coordination meeting with local leaders at KTWR before translocation of wild buffalo, January 2017



Photoplate 7: Field surveys on population, behavior and habitats of wild buffaloes along with supervisors at KTWR



Photoplate 8: KTWR BZ Community leaders on tour to CNP to share conservation achievements in Chitwan



Photoplate 9: Design of wild buffalo transporting container and loading devices



Photoplate 10: Blood sample collection of wild, feral and domestic buffaloes from KTWR and around



Photoplate 11: Darting, Capture, Immobilization and hand over of wild buffalo at KTWR, January 2017



Photoplate 12: Monitoring of soft release enclosure at Padampur, CNP before the translocation procedure, December 2016



Photoplate 13: Wild buffalo releasing ceremony at CNP on 29 January 2017



Photo Plates 14: Wild buffaloes during release and post-release monitoring at CNP,
February 2017



Photoplate 15: Post-release monitoring of wild buffaloes in the soft-release enclosure at CNP, April 2017



Photoplate 16: Monitoring of wild buffaloes, its soft-release enclosure, and habitat on a regular basis from the watchtower, on foot or on elephant back at CNP, May 2017



Photoplate 17: Habitat management by cutting and burning invasive *Mikania macarantha* patches inside the enclosure for maintaining grassland at CNP, June 2017



Photoplate 18: Outlook of damaged habitats in the grasslands within the enclosure of translocated wild buffaloes after flash floods, August 2017



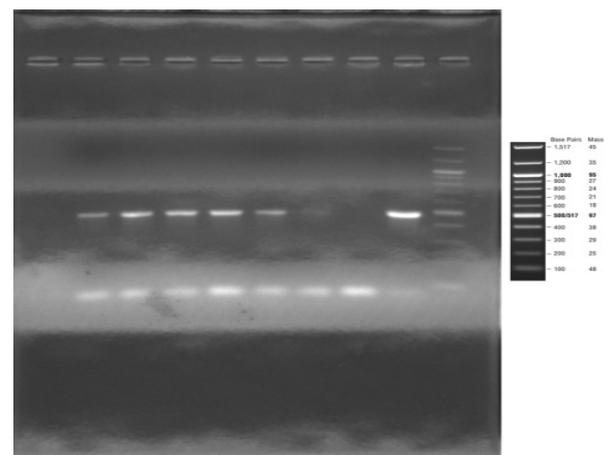
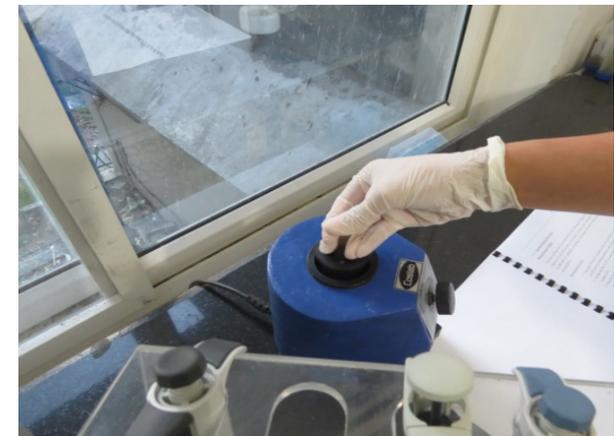
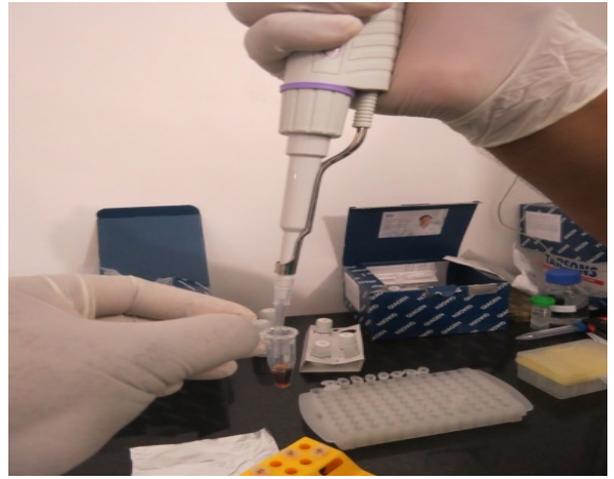
Photoplate 19: Wild buffaloes on a regular post-release monitoring and habitat management on different patches, with supplementation of nutritional diet after a flood at CNP, January 2018



Photoplate 20: Injured animal under treatment by the veterinary team of CNP,
March 2018



Photoplate 21: Snaps of lab works on molecular studies of wild buffaloes including DNA extraction, PCR, and DNA sequencing



CytB Gene of Wild Buffalo
Product Size: 500 bp

Photoplate 22: Snaps of Researcher presenting a paper on “Asian Wild Buffalo Reintroduction in Nepal: a historical milestone in species conservation” in the International Congress for Conservation Biology (ICCB 2017) held at Cartagena, Columbia during 23-27 July 2018

Presentation Slides	Certificate of Participation
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RE-INTRODUCTION OF WILD WATER BUFFALO (*Bubalus arnee*) IN CHITWAN NATIONAL PARK, NEPAL
 Ram Chandra Kandel*, Khambam Sainani*, Mihach Kumar Chalise*, Shant Raj Jhaureli* & Karl Wörner*
 *Chitwan National Park, Nepal; Department of Zoology, Muzoon University, India; Department of Zoology, Tribhuvan University, Nepal; †Harioo Ban Program, WWF Nepal; USAID/Nepal

INTRODUCTION
 The Wild Water Buffalo *Bubalus arnee* (Kerr, 1792) is known as Arna in Nepal. It is a robust animal, believed to be distributed in Europe and southern Asia in the Pleistocene age, but was later restricted to the Indian subcontinent and Southeast Asia (Masson 1974). The remnant population of Arna in Nepal is only found in Koshi Tappu Wildlife Reserve.



PRE-TRANSLOCATION PROCESS IN CNP
 Design of enclosure in Chitwan National Park, Water buffalo management, Habitat suitability assessment

TRANSLOCATION PROCESS
 Capture and immobilization, Site/enclosure preparation, Traditional Capture Method, Holding, Transport, Release

POST-RELEASE MONITORING, CONCLUSION & RECOMMENDATION
 • Arna are thriving well in CNP
 • Source population shall be conserved for survival and future translocation.
 • Post monitoring and research are being carried out and to be continued.

Area herd in Chitwan National Park, Regular Monitoring of enclosure

Acknowledgement: DNPWC, NTNC, USAID- Harioo Ban Program, ZSI, KTWR, CNP & local Communities

ICCB 2017
 CARTAGENA, COLOMBIA, JULY 23-27, 2017
Insight for sustaining life on Earth

Society for Conservation Biology
 A global community of conservation professionals

Certificate of Presentation

We confirm that **Ram Chandra Kandel** presented a poster entitled:
 “Asian Wild Buffalo (*Bubalus bubalis arnee* Linn.) Reintroduction In Nepal: A Historical Milestone In Species Conservation”

Authors: Ram Chandra Kandel, Man Bahadur Khadka, Shant Raj Jhaureli, Karl Wörner, Khambam Sainani, Mihach Kumar Chalise

at the 28th International Congress for Conservation Biology
 “Insight for Sustaining Life on Earth”
 23-27 July 2017, Cartagena, Colombia

O. Chassot
 Olivier Chassot
 President of SCB Latin America and Caribbean Section

Deborah Lake
 Deborah Lake
 SCB Executive Director

Photoplate 23: Snaps of Researcher presenting a paper on “Population and Demography of Asian Wild Buffalo in Koshi Tappu Wildlife Reserve, Nepal” in the International Conference on Capacity Building for National Sustainable Development (CBNSD 2018) held at Nairobi, Kenya during 29-31 August 2018



Photoplate 24: Snaps of Researcher presenting in pre-submission defense at the Department of Zoology, School of Life Sciences, Mizoram University, Aizawl, India on 17 April 2018



**ECOLOGICAL AND BEHAVIORAL STUDIES ON
ASIAN WILD BUFFALOES (*Bubalus bubalis arnee* Linn.)
AT KOSHI TAPPU WILDLIFE RESERVE, NEPAL**

*THESIS SUBMITTED IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN ZOOLOGY (CONSERVATION BIOLOGY)*

ABSTRACT

By

RAM CHANDRA KANDEL

(Registration No. MZU/PhD/800 of 22.05.2015)



**DEPARTMENT OF ZOOLOGY
MIZORAM UNIVERSITY
AIZAWL-796004, INDIA**

Supervisor

**Prof. G. S. Solanki
Head, Department of Zoology
Mizoram University, Aizawl, India**

Jt. Supervisor

**Prof. M. K. Chalise
Central Department of Zoology
Tribhuvan University, Kirtipur, Nepal**

13th August 2018

Ecological and Behavioural study on Asian wild buffaloes (*Bubalus bubalis arnee* Linn.) at Koshi Tappu Wildlife Reserve, Nepal

ABSTRACT

Present study area, Koshi Tappu Wildlife Reserve (KTWR) Nepal, is located between 26°33'-26°45' N and 86°54'-87°04'E on the floodplain of the Sapta Koshi River near the Nepalese-Indian border, in the Tarai of south-eastern Nepal. The area experiences mainly sub-tropical monsoonal climate where the average annual rainfall ranges between 1300 mm and 2051 mm, elevation ranges from 75 to 100 m amsl, average daily maximum and minimum temperature ranges between 23⁰ C to 34⁰ C, between 16⁰C and 29⁰C respectively. The vegetation of KTWR is mainly composed of tall grasses, and there are also small patches of Khair-Sisoo scrub forests and deciduous mixed riverine forests.

Chitwan National Park (CNP, 952.23 sq km), where the small population of wild buffalo from KTWR is reintroduced during January-February 2017, lies between N 27°20'19' and 27°43'16' longitude and between E 83°44'50' and 84°45'03' latitude on the floodplain of the confluence of Rapti and Narayani rivers in the lowland of central Nepal. The elevation of CNP ranges from 150m to 815m amsl, average daily maximum and minimum temperature of CNP ranges between 25 C to 35⁰ C and between 15⁰C and 25⁰C respectively. The vegetation of CNP is mainly composed of almost 70% of *Sal* forests including huge stretches of short to dominant tall grasses which consist around 12% between the *Chure* hill and river banks.

The present study intended to look at an assessment of ecological aspects of Asian wild buffaloes including genetic studies of wild buffaloes in Koshi Tappu Wildlife Reserve (KTWR), Nepal. Specific objectives of the research were: 1) To determine the habitat composition of Asian wild buffalo at KTWR; 2) To study the current population status and demographic structure of Asian wild buffalo at KTWR; 3) to plan reintroduction of wild buffaloes and develop translocation protocol; 4) To study the pattern of activity time budget in general behavior of Asian wild buffalo, and 5) To study the molecular structure of *B. bubalis arnee* population in KTWR using mitochondrial C01 (*Cytochrome Oxidase I*).

Vegetation sampling was carried out in concentric circular sample plots (CSP) all along the three north-south parallel transects representing each habitat type available in the reserve. Collectively, a total of 76 sample plots were laid following a systematic random sampling on those transects. Plots with 10m radius were used for trees, the 5m radius was laid out to collect information on shrubs/saplings in each CSP, and square plots of 1m² size were laid out at north and south cardinal directions of each CSP for grass and other herbaceous plants. Vegetation data were analyzed using general ecological principles to calculate frequency, density, dominance, cover density, importance value (IV), and prominence value. A total of 181 sample plots of various sizes for trees, shrubs, and herbs respectively were surveyed.

On the basis of plot location, a large proportion of KTWR area is covered by grassland followed by *Sissoo-Khair* forest and the smallest proportion is comprised of shrubland. Among the sample plots, *Sissoo-Khair* forest was observed in 40.7% (35 plots out of 76) of sample plots predominantly in the eastern part of the Reserve which included 444 trees stems per hector. Out of twelve species of trees of this type, *Dalbergia sissoo* is ecologically more important species with the highest importance value (IV = 179.1) followed by *Trewia nudiflora* (IV = 42.8). There are 33 species in the understory layer of this forest. Among these species, *Clerodendron viscosum* is the most prominent one (PV = 25.79) followed by *Lantana camara* (PV = 12.85). The ground vegetation of this forest indicates high diversity of species (n = 34), with invasive alien *Mikania macarantha* being the most prominent species (PV = 51.7) followed by *Imperata cylindrica* (PV = 41.5). Grassland occupies the highest proportion (54%) of the study area. A total of 32 grass species of plants were identified, of which the most prominent grasses were *Saccharum spontaneum* (PV = 282.57) and *I. cylindrica* (PV = 168.79) in the grassland community. Among 11 reported tree species of riverine forest, *Trewia nudiflora* is the more dominant (IV = 140.6) followed by *Bombax ceiba* (IV = 50.2). Forest cover of this was found to increase from 2.9% in 1997 (Sah, 1997) to 11% in 2015 at the time of the current study. The dominance of the *Trewia nudiflora* is found highest (PV=140.6) in this study. Among the recorded species, invasive alien *Lantana camara* (PV = 7.3) and *Clerodendron viscosum* (PV = 3.3) are the two prominent species in the understory layer. The ground vegetation of mixed forest comprised 23 plant species, primarily composed of *I. cylindrica* (PV = 224.3) and *Dryopteris cochleata* (PV = 127.7). In shrubland habitat, 6 plant species recorded in the understory, of which *Zizyphus jujuba* (PV = 37.5) and *Clerodendron viscosum* (PV = 1.1) were more prominent

species. 11 ground vegetation species recorded in the shrubland with *I. cylindrica* (PV = 38.97) and *D. cochleata* (PV = 17.71) being the 2 most prominent species.

Based on the inventory data, 12 and 11 tree species were recorded from the Sissoo-Khair and mixed riverine forest of KTWR, respectively. The density of tree in Sissoo-Khair and mixed riverine forests were 444 and 274 tree/ha, respectively. In understory vegetation, the number of recorded species was higher in Sissoo-Khair forest (n = 33) than mixed riverine forest (n = 16) and shrubland (n = 6). The number of species in the ground vegetation is higher in Sissoo-Khair forest (n = 34) followed by grassland (n = 32). The average tree height of Sissoo-Khair forest was 12.0 ± 8.6 m with 15.4 ± 10.9 cm of average DBH. Similarly, average tree height of mixed riverine forest was 14.1 ± 11.1 m with 22.3 ± 19.2 cm of average DBH.

To study population and demography of wild buffalo, the whole area of the reserve was divided into different blocks: firstly divided into two segments: east and west from the main course of Sapta Koshi River; and each segment was further divided into two separate blocks, north and south blocks into both sides. The animals were closely observed during the census in order to distinguish and for clear identification of the age and sex of the animals. Four-wheel drives, elephant ride and also on-foot tracking were used during census in order to maximize animal sightings. The criteria for identification of the wild breeds of wild buffalo, their age and sex were used considering skin and pelage color, change pattern in coloration in male and female individuals with the age, horn size, and shape, herding behavior, etc. The age of animals was determined by observing: Young calves/Infants (<1 year) with buff in color; Juvenile Calf (1-2 years) that darken buff color in the first year and gradually turns lighter in color in the second year; and Young Adult/Adult (>3 years) the coat color changes to dark to very dark with noticeable whitish markings in different parts of the body mainly at joints. A number of young calves <1 year of age and number of females in a population were considered for calculating calf to cow ratio. Population growth rate, demographic structure and calves to cows' ratio, effective population size, percentage ratio of population loss per generation, and rate of loss of genetic variations of wild buffalo population in KTWR was calculated from exponential growth rate formula (Dahmer, 1978; Heinen, 1993a, Heinen, 2002); Heinen & Kandel, 2006).

The census records from 2011 onwards indicated that there is an increasing rate of population size. The latest census conducted in March-April 2016 increased to 433 individuals; where male and female ratio was 0.63 and calf to cow ratio was 0.36 whereas male to female ratio was lowest (0.38) in 2014 and highest (1.12) in the 1988. High fecundity rate affects the calves to cow ratio; higher is the ratio more is the viability of the population. Calves: cow ratio was highest in 1976 (0.61) which was gradually declined thereafter but the lowest was 0.19 in 2009 because flood in 2008 affected population and habitat badly. There was a rise in male to female ratio in 1987 which was gradually declined till 2009 and again regained but found to be in a zigzag manner. Gradual downfall of the calves to cows ratio till 2009 was also evident which gradually increases in a similar way as like male to female ratio. There is a strong correlation (R^2 Linear = 0.099) between male to female ratio and calves to cows ratio. The overall population growth (ri) over past 40-years (1976 to 2016) is 0.048. Growth rate between 1976 and 1987 was 0.033 and between 1988 and 2000 was 0.037. The population growth rate was highest from 2011 to 2014 (0.106). Negative growth (-0.018) was recorded in 2009. Populations of wild buffalo bounced back after decimating impact of the flood in 2008. The average rate of growth (0.048) between 1976 and 2016 shows an imperative development towards the sustenance of this species in the future. This research has come up with a significant value of policy interventions and management implications as the size of the wild buffalo population at KTWR are increasing every year.

While studying behaviors of the wild buffaloes, Daily, monthly and seasonal activities and activity budget performed by the Asian wild buffaloes at KTWR were recorded to know the time budget biology of the animals. Major categories of behavioral activities that wild buffaloes undertake during foraging are mainly Grazing, Moving, Resting, Running, Chewing, Wallowing, Drinking, Grooming, Social interaction, etc. within its territory. Focal animal sampling technique was carried out to record social and ecological behaviors. Selection of focal animal was random for each session of observation over the areas of the wildlife reserve. Adult animals were preferred for sampling as a focal animal since it has influences upon others in performing their activities and ranging. Variation between different months and seasons, and years was reflected while calculating the mean and standard error (mean \pm S.E) of each of the behaviors. To elaborate the seasonal variations in behavioral time budget for a year is divided into 4 main seasons as winter (Jan-Mar); summer (Apr-June); monsoon (July-Sept); and autumn (Oct-Dec). The

Kruskal-Wallis one-way ANOVA was also applied to evaluate the activity budgets of the different focal animals. Different categories of behavioral activities were tested for significance of time budget with the percentage of time devoted to each of the activities. Significance was set at $p < 0.05$ for all statistical tests. The data on time spent on different activities across the seasons during the years 2015 and 2016 were analyzed for seasonal variations among different activities.

Monthly pattern of behavioral time budget (in a minute) in 2015, the resting time was highest in the month of February (243.3 ± 5.3 SE) followed by January (236.7 ± 6.5 SE) and December (185.0 ± 13.3 SE). While the time spent in grazing was maximum in June (218.3 ± 8.9 SE) followed by May (165.4 ± 8.4 SE) and October (158.8 ± 6.8 SE), and the wallowing was performed more during August (29.2 ± 5.9 SE) followed by July (28.8 ± 4.0 SE) and no wallows were recorded during October through February. On the other hand, animals were found moving more during March (91.3 ± 4.3 SE) followed by April (83.3 ± 2.5 SE) and least during February (33.1 ± 3.1 SE) due focusing on sun basking for thermoregulation. They were seen to moving in all the months of both the study years in an equal proportion. While in the year 2016, monthly activities budgets of wild buffaloes in Koshi Tappu shows that the major activity categories like resting time in January (244.6 ± 5.1 SE) was highest followed by February (225.6 ± 15.7 SE) and least during June (72.08 ± 8.08 SE). The total time spent in grazing was maximum in June (221.7 ± 7.6 SE) followed by May (162.9 ± 9.05 SE) and October (157.5 ± 8.6 SE), and least during January (76.67 ± 4.4 SE) and February (74.58 ± 2.9 SE), while the wallowing was performed higher during June (22.92 ± 4.9 SE) followed by July (21.25 ± 5.9 SE) and no wallows, was recorded from October through February which was same as in the year 2015.

Based on the seasonal observation of the activities budgets in the year 2015, each category of activities significantly varies across the seasons ($H = 16.59$, $HC: 16.59$, $p = 0.002326$). To understand seasonal variation, the observations were repeated in 2016, activity pattern was found almost similar as in 2015 across the seasons ($H = 16.59$, $HC: 16.59$, $p = 0.002326$). This indicates seasonal influences on time spent on each activity remain mostly the same in both 2015 and 2016 provided that the biophysical and environmental conditions remain the same. Time spent on different activities during 2015 and 2016 shows no significance over the years ($H = 8.727$, $HC = 8.727$, $p = 0.06829$). The weather and season of the year have an influence on the allocation of time budget on different activities. They were involved in grazing more time during winter and

more wallowing in summer and monsoon. Patterns of habitats used by wild buffaloes at KTWR based on the total observation (N=144) of the animal during the behavioral studies, wild buffaloes were found to use grassland and ecotone habitat the most (58.21%) followed by Sissoo, Sissoo Savannah and Sissoo mix forest (32.09%). An additional area of *Typha* dominated grassland stands for 6.72%.

For reintroduction of Asian wild buffaloes on policy decision taken by Government of Nepal, maximum 20 numbers of Asian wild buffaloes to be captured from founder population at KTWR and to translocate to the recently evacuated Padampur area of the CNP in the first phase. The decision is implemented and the standard protocol is developed and devised to be adopted even in the future. Captured individuals were to be rehabilitated into a pre-release or soft-release holding enclosure within the north-east part of CNP near Rapti River. To avoid heat related complications, capture and translocations were timed to coincide with the cooler hours of the day (< 25⁰C) or night hours. Hence the most suitable months are during February-March.

As a preparatory work, Species Translocation Advisory Committee studied the feasibility of operations and ecological requirements such as availability of food, space, water, cover, evaluation of protection strategies against anthropogenic factors, identifying a source for founder stock. The action plan was developed and standard veterinary protocols for wild buffalo capture and translocation were prepared and activities were undertaken. A field-level coordination committee under the supervision of Chief Warden of CNP was formed to coordinate and carry out all the management and technical arrangement of the translocation. Arrangement were made for the construction of soft release enclosure at CNP, protection of the area by security provisions, technical and physical preparation for transportation, and handling all other tasks. The transport containers were designed according to the size of the target animals. For the soft release of the translocated wild buffalo, an enclosure at Padampur area inside the Chitwan National Park was designed. Then holding enclosure at CNP was constructed at the release site, called as a soft-release enclosure to keep them prior to their free movement at new open habitat. For the capture operation, a joint team including a team of veterinarians and technicians detailed out a technical plan of operation for capture and translocation from KTWR and reintroduction to soft release site at CNP. The essential veterinary supplies, dart equipment, and veterinary requirement were also reviewed before getting into the operation. Considering the field

requirement for translocation, team of monitoring and locating animal herd, capture and darting, immobilization, loading to a tractor, shifting immobilized animals to a cage and to push into the truck, emergency management and logistics in Koshi Tappu, transport team between Koshi Tappu and Chitwan; unloading and post-release monitoring team in Chitwan were identified and specific roles were assigned to each team.

To select appropriate animals for capture and translocation, experienced observers were involved to distinguish wild from domestic and feral. Darting of the selected animal was done from an approachable distance by foot or from a vehicle. Body health condition, physiological status, age and estimated weight were considered while determining the drug dosages. Correlation between drug dosage and body weight had been considered carefully. The weight of the selected animal was estimated based on published information and past experiences that ranged between 250kg and 700kg for sub-adults to adults. Etorphine Hydrochloride mixed with Azaperone at approximate doses was remotely injected with a dart gun. Other captured animals were darted using *EtorphineHydrochloride* mixed with *Xylazine*. Antidote M 50-50 was used for Etorphine Hydrochloride and Antisedan for *Xylazine*. The animals were to be transported more than 400km from Koshi Tappu to Chitwan, while *Haloperidol* was administered to each captured animal for relaxation to minimize travel stress. When the target animal was selected from the herd, they were approached from a close distance for efficiently capturing through the drug delivery. The capture of wild buffalo was done in two ways: 1) by darting, and 2) by traditional chase method. For darting the target animals, remote drug delivery was facilitated using all-plastic darts delivered through an air-pressurized Dan-Inject (Model JM) projector from close distance preferably using branched trees or elephant back or on foot from a mount. Altogether, 16 wild buffalo were captured using both of the methods as explained above. Out of them, 06 were captured using traditional method and others were captured by darting. Out of 16 animals, 13 were captured from Koshi Tappu and 3 were from Central Zoo, Nepal. Among 13 captured from Koshi Tappu, 12 (10 F & 02 M) were translocated to Chitwan and one (01 M) was translocated to Central Zoo; and, three captured from Central Zoo (2 F and 1 M) were also translocated to Chitwan. For the Sample Collection of darted wild buffaloes, two 10ml un-heparinized tubes of blood were collected from a superficial vein of each buffalo.

Altogether, blood samples of wild (n=20) including domestic (n=11) and hybrids (n=11) were acquired for genetic analysis. Samples of 7 wild, 15 hybrid, and 20 domesticated buffalo from Flamand *et al.* (2003) and used by Zhang *et al.* (2016) were also included. PCR was carried out and samples were amplified following standard protocols. The PCR products were electrophoresed; the amplified DNA fragments were purified. Out of 45 selected samples, PCR optimization was successful only in 36 samples. High quality purified PCR amplicons were subjected to Dideoxy sequencing was done in an automated DNA sequencer. Raw sequence fragments of 422 bp length were assembled, checked and edited with Sequencer 5.0 and the contigs of both reverse and forward primers were created. Sequences were aligned with ClustalX in BioEdit. In our 36 sequences, we have added 42 reported sequences of Nepalese buffaloes of all the three groups from Zhang *et al.*, 2016 (KR009964NPH01-42). To understand the possible haplotypic variation among the river buffaloes of the neighboring region, we further added four *Cytochrome b* haplotypes identified by Kumar *et al.*, 2007 from Indian river buffaloes (EF409942H1-4) and 15 *cytochrome b* haplotypes defined by Kikkawa *et al.*, 1997 representing both river buffaloes from Bangladesh, Srilanka, Italy and Pakistan, and swamp buffaloes from Japan, Taiwan, Thailand, Philippines, Indonesia and Bangladesh (D34637, D88629-38). This yielded a total of 97 sequences (36 from the present study, 42 from Zhang *et al.* (2016), four from Kumar *et al.* (2008) and 15 sequences from Kikkawa *et al.* (1997) of buffaloes representing river and swamp types.

The lengths of the 36 partial *cytochrome b* sequences of all the three types of buffaloes of this study have 422 bp. The aligned matrix was 422 characters and contained 2 variable sites. However, the complete *cytochrome b* sequences of the 42 samples of Nepal used in Zhang *et al.* (2016) have 1140 characters and revealed 4 variable sites among the three groups of river buffaloes. The partial *cytochrome b* sequence used in our study is shorter than that described by Zhang *et al.* (2016) and thus has fewer variable sites. However, when our data set was compared with the accession of Kumar *et al.* (2007) and Kikkawa *et al.* (1997), base substitutions at 13 nucleotide positions (variable sites) were obtained and among them three nucleotide positions were specific for river buffaloes and ten positions were specific for swamp buffaloes. The sequence divergence within buffaloes of Nepal and India was 0.24-0.49 % however when compared with river buffaloes sequence of Kikkawa *et al.* (1997), the divergence was slightly

higher 0.24-0.74 %. Sequence divergence within swamp buffaloes was 0.24-0.98%. The sequence divergence between swamp and river buffaloes was calculated to be 1.49-2.49%.

Analysis of a total of 97 *cytochrome b* partial sequences of 422 bp length of the river buffaloes and swamp buffaloes showed 13 variable sites defining 9 haplotypes (H1-H9). Among the nine observed haplotypes, Nepalese buffaloes both in this study and that of Zhang *et al.* (2016) were represented by just the three common haplotypes (H1, H2, and H3). These three haplotypes are identified by Kumar *et al.* (2007) as well. The most common haplotype (H2) is widely distributed among groups and represented by 66 percent of sequences (Nepal: D=23, W=18, and H=17; one of Kumar *et al.*, 2007 and five of Kikkawa *et al.*, 1997). The second common haplotype (H1) was represented by 20 percent of the samples (D=7, W=2 and H=8 and one each of Kumar *et al.*, 2007 and Kikkawa *et al.*, 1997). Three sequences of Nepalese samples (two from present study and one from Zhang *et al.*, 2016) and one from Kumar *et al.*, 2007 were restricted to the third haplotype (H3). Of the remaining six haplotypes, one was reported (H4) by Kumar *et al.*, 2007 while the remaining five (H5-H9) were defined by Kikkawa *et al.*, 1997. Within groups, haplotype diversity was highest in hybrid groups followed by domesticated groups and slightly lower in wild breeds. Overall haplotypes and nucleotide diversities were 0.403 and 0.00105 respectively. Interestingly, haplotypes were divided into two branches corresponding to river and swamp buffaloes with six nucleotide mutations. We could not find specific haplotypes for wild, hybrid and domesticated groups of buffaloes. In terms of genetic diversity, river buffaloes were found to be less diverse than swamp buffaloes.

Maximum parsimonious analysis of 97 partial sequences of *cytochrome b* performed under heuristic search and 1000 bootstraps revealed four distinct clusters or clades (Clade A-D) with moderate to high bootstrap values. Among four sub-clusters, one cluster with high bootstrap values (99%) showed strong differences between swamp and river buffaloes. Surprisingly relationship among haplotypes revealed by median-joining network corroborates with the four lineages observed in MP tree. In MP analysis, a total of 3 most parsimonious trees (CI = 1.00, RI = 1.00, Length = 15 steps) were recovered. Phylogenetic analysis and evolutionary history of 97 partial sequences of *cytochrome b* inferred using the Neighbor-Joining (NJ) with 10000 bootstrapping replicates also depicted identical topology of the phylogenetic tree.

On the basis of the partial *cytochrome b* sequences of 422 bp length, we were able to define very few (only three) haplotypes in the Nepalese buffaloes. None of the haplotypes were wild, domestic and hybrid specific. NJ and MP analysis performed (result not shown) with complete length (1120 bp) *cytochrome b* sequences of Zhang *et al.*, 2016 (42 Nepalese sequences), Kumar *et al.* (2007) (four river buffalo haplotypes) Kikkawa *et al.* (1997) (seven river and eight swamp haplotypes) provided essentially the same topography of the *tree butan* addition of one more haplotype represented by *Genbank accession* KR009945NP_D07 alone.