Distribution pattern of amphibians along Teesta and Rangeet valley in Sikkim and Phylogeny of select species

A Thesis Submitted

to

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Degree of Doctor of Philosophy

By

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DECLARATION

I, Prerna Dhakal, hereby declare that the Ph.D thesis entitled **"Distribution pattern of amphibians along Teesta and Rangeet valley in Sikkim and Phylogeny of select species"** submitted by me in partial fulfilment of the requirement for the award of the degree of Doctor of Philosophy in Zoology, Sikkim University under the supervision of Dr. Basundhara Chettri, Assistant Professor, Department of Zoology, Sikkim University and co-supervision of Dr. Namrata Thapa, Associate Professor, Nar Bahadur Bhandari Government College, Tadong is my original research work carried out by me in the Department of Zoology, Sikkim University .

This thesis has not been submitted for any other degree of this university or any other university. This thesis contains no materials previously published or written without due acknowledgement in the text of the thesis nor does the thesis contain any material that infringes copyright.

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This is to certify that the Ph.D thesis entitled "Distribution pattern of amphibians along Teesta and Rangeet valley in Sikkim and Phylogeny of select species" submitted to Sikkim University for the degree of Doctor of Philosophy embodies the research work carried out by Ms. Prerna Dhakal from 2015 to 2022 at the Department of Zoology, School of Life Sciences, Sikkim University under our supervision.

The work described here is original and no part of this thesis has been submitted elsewhere for the award of any Degree, Diploma, Associateship, Fellowship at this or any other University or Institution of higher learning. Ms. Dhakal is conversant with techniques and literature is cited in the thesis and has fulfilled the requirements of the degree of Doctor of Philosophy in Science (Zoology) of Sikkim University. In character and demeanor, Ms Dhakal is fit to submit thesis for Ph.D degree.

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"Distribution pattern of amphibians along Teesta and Rangeet valley in Sikkim and Phylogeny of select species"

Submitted by **Miss. Prerna Dhakal** under the supervision of **Dr.Basundhara Chettri**, Assistant Professor, Department of Zoology, Sikkim University and Co-Supervised by **Dr. Namrata Thapa**, HoD & Associate Professor, Nar Bahadur Bhandari Government College,Tadong.

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Dedication

I would like to dedicate my thesis to the mighty Himalaya, the quest is arduous but the insights are monumental.

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This thesis represents the culmination of a childhood dream which began when I started watching nature documentaries. Ever since, I always wondered how ecologists learn and survive in the wild and made a career in various areas of ecology. It was fantasy fulfilled when I was selected as a Research Scholar in Department of Zoology, Sikkim University. The passage through the course of PhD is a wonderful journey, but not without grit and perseverance. Many people and institutions helped me throughout the entire tenure of my adventurous PhD journey.

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I feel fortunate to have been given this great opportunity to explore this part of the enormous Himalaya, which humbles me and makes me realise how faith and persistence is like the mountains, each peak ascended teaches something.

Prerna Dhakal
September, 2022

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SUMMARY

The variation of biodiversity across spatial scales has been one of the fundamental questions in ecology. Understanding the factors driving geographical patterns of species remains the core element in macroecology and biogeography (Gotelli et al, 2009). Several studies across the world have reported variations in species distribution patterns and their underlying factors among taxa and regions (Rahbek, 1997; Sanders, 2003; Chettri et al, 2010; Wu et al, 2013). Although, many studies over decades have attempted to comprehend the spatial distribution patterns of species and its mechanisms, no general explanation for the same has been accepted (Brown, 2001; Lomolino, 2001). In the Himalaya, there is dearth of studies on the elevational distribution pattern of amphibians compared to other taxa despite amphibians being bio-indicator of ecological disturbance and apparent climate change. Studies on elevational patterns of distribution of species have been significantly employed in conservation planning and predicting the impact of climate change. Along with macroecological studies, community dynamics such as species-microhabitat relationships, niche breadth and overlap reflect an important aspect of community ecology. Studies on such aspects aids in formulating effective conservation strategies considering the microhabitat preferences of species. However the information on microhabitat associations of amphibians of Sikkim Himalaya is completely lacking. The species belonging to the genus Amolops show significant adult morphology and easily confused with congeneric species (Bain et al, 2006). This genus is characterized by several cryptic species which alleviates the problem of misidentification of species (Lyu et al, 2019; Wu et al, 2020). In Sikkim, a total of four species of *Amolops* have been recorded in previous studies (Chettri and Acharya, 2020). However the

phylogenetic status of species belonging to the genus *Amolops* have never been explored using molecular and morphological data.

Hence, considering the above mentioned lacuna, the present study was initiated with the following objectives: (i) to explore the distribution patterns of amphibians along the elevation gradients of Teesta and Rangeet valley of Sikkim Himalaya, (ii) to understand the factors governing the distribution patterns along the two valleys, (iii) to compare the amphibian community composition between the two valleys, (iv) to undertake phylogenetic analysis of select amphibian species, using morphological and molecular data.

Sikkim (27° 5′ - 28° 10′ N and 88° 4′ - 88° 58′ E), located in the Eastern Himalaya has an area of 7096 km^2 and is a part of Himalaya biodiversity hotspot. Two major rivers traverse the state namely Teesta and its tributary Rangeet. The Teesta valley lying in the East and North district of Sikkim extends from 300 to 5500 m. The valley is characterized by high variation in climatic conditions from warm tropical climate in lower elevations to moderate cool climate at mid-elevations and arctic cold in higher elevations. The vegetation also changes along the elevation with gradation in climate. The Rangeet valley encompasses the West and South districts of Sikkim drained by river Rangeet and its tributaries. The valley extends from 300 to 7412 m. The valley shows marked variation in climatic conditions from humid climate in lower elevations to cool temperate at mid-elevations and tundra in higher elevations. The sharp change in climatic conditions along the elevation gradient induces transition in vegetation at roughly 900 m (Acharya & Sharma, 2013).

The present study was conducted in the Teesta valley and Rangeet valley in Sikkim Himalaya. In the Teesta valley, a total of 5150 m elevation range was covered (300–

5150 m). Similarly, in the Rangeet valley, a total of 3300 m elevation range was covered (300–3300 m). Sampling in higher elevations above 3300 m in the Rangeet valley was logistically not feasible due to extreme weather conditions and reduced accessibility. A total of 141 sites were sampled in the Teesta valley whereas a total of 137 sites were sampled in the Rangeet valley. The study area in the Teesta valley was categorized into 16 elevation bands with an interval of 300 m between each band for data collection. However, in the Rangeet valley the study area could be categorized only into 10 elevation bands with 300 m interval. Field sampling were conducted during May 2016- August 2019. Visual encounter survey (VES) (Heyer et al, 1994) was followed for sampling amphibians searching all probable microhabitats conducive for amphibians. Considering the nocturnal behavior of many amphibians, night stream transect walk was conducted along streams and rivers. The elevation range in both Teesta and Rangeet valley was divided into ten elevation zones with 300 m interval: zone I (300-600 m), II (600-900 m), III (900-1200 m), IV (1200-1500 m), V (1500-1800 m), VI (1800-2100 m), VII (2100-2400 m), VIII (2400-2700 m), IX (2700-3000 m) and zone X (3000-3300 m). In order to make the two valleys comparable for amphibian richness patterns and factors driving such patterns, five elevation zones (i.e. 3300–3600 m, 3600–3900 m, 3900–4200 m, 4200-4800 m, 4800-5100 m) from the Teesta valley were not included for further analysis. Data on several predictor variables such as Mean annual temperature (MAT), Mean annual precipitation (MAP), Potential evapotranspiration (PET), Annual evapotranspiration (AET), Moisture index (MI), Isothermality, water-energy dynamics (WED) and canopy cover was accessed using secondary sources and through primary sampling.

The microhabitats of amphibian species recorded in this study were assigned to one of the 10 categories: on rocks = OR (including above and under rocks and rock crevices), inside water = IW (including mud puddles and water pools), under boulders = UB (including above boulders), under logs = UL (including fallen trees, logs), on shrub stem = OS (on top or beneath stems/ branches), open ground = OG (including grass and paddy fields), on herbs = OH, inside mud = IM, artificial water points= AP (including under metal pipes, manmade probable habitats etc) and leaf litter = LL. Niche breadth was calculated based on the relative frequency of use of different microhabitats by different amphibian species. Niche overlap analysis was carried out between amphibian species to see the overlap in microhabitat resource use patterns.

Specimens of genus *Amolops* were collected from various sampling locations in the Teesta valley and Rangeet valley with prior permissions from the authorities. The morphological evaluations were conducted and genomic DNA isolation and sequencing was carried out using standard methods. The sequences were analyzed and phylogenetic tree was constructed using appropriate tools.

Data were analyzed and visualized using various packages in R environment and using softwares such as Mid-Domain Null Model, Ecosim (Version 7.0), Sequence Scanner (Version 2), MEGA (Version 11.13), jModelTest 2 (Version 0.1.7), BEAST, Figtree, Clustal Omega, BioEdit.

In the Teesta valley Visual encounter survey of 1188 man-hours resulted in 1725 individuals of amphibians representing 25 species and 11 genera under six families. In the Rangeet valley Visual encounter survey of 1140 man-hours resulted in 3283 individuals of amphibians representing 22 species, 10 genera and five families. Observed and estimated (Chao 2 and Jackknife 2) species richness of amphibians showed a hump shaped pattern in both Teesta valley and Rangeet valley with high species richness upto 1800 m after which the number of species gradually declined.

The highest number of species was observed between 1500 m- 1800 m elevational band with a distinct peak at 1500 m in both the valleys indicating the need to conserve areas in mid-elevations.

The elevational range size distribution revealed 17 species (74%) in the Teesta valley and 14 species (64%) in the Rangeet valley with narrow elevational width indicating the sensitivity of amphibians to rapid changes in environmental variables along elevation gradients and specificity to particular microhabitats. In both valleys, significant correlation between the range size and upper limit of the distribution range of amphibians were observed (Teesta valley: $r = 0.59$; $p < 0.01$; Rangeet valley: $r = 0.53$, $p = 0.01$) and the correlation between the range size and lower limit of the distribution range was not statistically significant suggesting amphibian species occurring at higher elevations have wider ranges relative to species at lower elevations. The amphibian species richness in the Teesta valley did not fit the MDE null model and indicated deviation from the model. The low value of regression between empirical and simulated richness ($R^2 = 0.02$; $P = 0.25$) suggests that the distribution of amphibians in the Teesta valley are not constrained by geographical hard boundaries. Whereas in the Rangeet valley, amphibian species richness showed good fit to the MDE null model. The regression of empirical richness and simulated richness was significant ($R^2 = 0.66$; $p < 0.01$) indicating that amphibian species richness along the elevation gradient of Rangeet valley are constrained by hard boundaries. Actual Evapotranspiration (AET) was the most crucial factor for substantial variation in total species richness patterns of amphibians along the elevation gradients in the Teesta valley. Mean Annual Precipitation (MAP) and Canopy cover were found to be the most important factor for considerable variation in species richness of amphibians along the elevation gradients of Rangeet valley.

Amphibian species composition between Teesta and Rangeet valley showed high similarity (Sorensen Index, Cs = 0.85) with five species (*Nanorana blanfordii, Scutiger sikkimensis, Scutiger boulengeri, Rhacophorus maximus and Ichthyophis sikkimensis*) unique to Teesta valley and two species (*Nanorana* sp *and Amolops* sp) unique to Rangeet valley. The most recorded microhabitat for amphibian species (43.83%) was on rocks (OR) with 22 species and 2087 observations followed by leaf litter (LL = 28.20%) with 19 species and 1343 observations, on stem (OS = 10.94%) with 18 species and 521 observations. Minimum number of species was found in artificial water points (AP = 0.06%) with two species and three observations and inside mud (IM = 0.78%) with one species and 17 observations. Mean niche breadth values were highest for the family *Bufonidae* (n = 3 species, β = 3.17), followed by *Dicroglossidae* (n = 10 species, β = 2.47), *Rhacophoridae* (n = 4 species, β = 2.23), *Ranidae* (n = 5 species, β = 1.73) and *Megophryidae* (n = 4 species, β = 1.51). The correlation between microhabitat niche breadth and elevational range size was not statistically significant ($r = 0.15$; $p = 0.448$). The pairwise niche overlap analysis in the microhabitat dimension resulted in 276 pairs. Four pairs exhibited complete overlap (niche overlap value = 1.00) and seven pairs showed no overlap (niche overlap value = 0). Non-metric multidimensional scaling plot (NMDS) (stress = 0.097) indicated overlap in amphibian community composition in the microhabitat dimension. Permutational multivariate analysis of variance (PERMANOVA) analysis indicated that microhabitats in general have a significant effect on amphibian composition ($p < 0.001$). The microhabitat niche overlap values of pairs of amphibian species was negatively correlated to their phylogenetic distances ($r = -0.61$; $p = 0.05$). Simple linear regression showed that that there is no statistically significant effect of

body size of amphibian species on the number of microhabitats used ($R^2 = 0.00$; $p = 0.876$).

Four Amolops species were recorded in the study which were subsequently identified as *Amolops himalayanus*, *Amolops marmoratus, Amolops cf. monticola* , *Amolops cf. formosus* based on external morphology and morphometry. Phylogenetic analysis based on mt*COI* recovered four groups of Amolops as monophyletic clades: *A. ricketti* group, *A. marmoratus* group, *A. viridimaculatus group*, and *A. monticola group*. The two new sequences viz. *A. cf. formosus* and *A. himalayanus* were nested in *A. viridimaculatus group.* The two sequences were closely related to a candidate species *Amolops* sp. 5 from Illam, Nepal (Wu et al, 2020). The sequence representing *A. marmoratus* clustered within *A. marmoratus* group and exhibited evolutionary relation to this group confirming the validity of this species and its occurrence of *A.* in Sikkim. The species identified preliminarily as *A. cf. monticola* clustered in the in *A. marmoratus* group making the validity of this potential species unresolved. These two sequences were closely related to a candidate species *Amolops* sp. 7 from Illam, Nepal (Wu et al, 2020) suggesting evolutionary close relation with this potential new species.

Climatic factors such as temperature and precipitation are the principal drivers of amphibian distribution in Sikkim which indicates the vulnerability of these ectothermic species to changing climatic regimes. Amongst the vertebrates, 41% of amphibians are estimated to be threatened with extinction (Silla & Bryne, 2019). Himalaya being one of the most sensitive zones, the impact of global climate change is extremely apparent (Masson-Delmotte, 2021). Moreover, the high association of amphibians to microhabitats such as rocks and boulders indicates the significance of rocky outcrops for the survival of amphibians. The lower and mid- elevations of Sikkim are under immense pressure due to anthropological activities and mega developmental projects such as hydropower and pharmaceutical industries. Based on these findings, the present study recommends framing of conservation policies taking into account the high diversity of smaller vertebrates such as amphibians in mid-elevations. Most of protected areas lies above 1500 m whereas the diversity is higher below this elevation. Below 1500 m elevation is highly dominated by human being, and hence rear scope of establishment of protected areas. Hence advocacy regarding conservation of human modified landscape such agro-ecosystem seems to be realistic. Likewise, the preservation of rocky outcrops along streams and rivers is important to maintain the microhabitat of amphibians which is particularly disturbed due to mining or quarrying activities. Additionally, the highly cryptic diversity of amphibians of the Himalaya emphasizes the need for more extensive explorations in order to reveal the cryptic diversity. Recent discovery of not only new species of amphibians but a new family from north-east India might resolve many biogeographical paradoxes.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

The diversity of plant and animal species on earth is not uniformly distributed along latitudinal and altitudinal gradients and these geographical gradients of diversity have long fascinated biogeographers and ecologists (Lomolino & Weiser, 2001). Species richness is expected to inversely correlate with elevation, just as species richness declines from the tropics to the arctic (MacArthur, 1972). Stevens (1992) proposed that patterns of species richness along elevational gradients are considered to mirror species richness patterns along latitudinal gradients since both span a transition from warm to cold climatic conditions. Thus, indicating that similar factors could have resulted in structuring the spatial pattern of species richness along altitudes. The pattern in species richness along elevational gradients in different parts of the world varies considerably and the processes that govern them are also equally varied (Rahbek, 1995). Therefore one of the key topics of research in biogeography and conservation biology is to understand the distribution pattern of organisms across spatial gradients and the factors governing such patterns (Stevens, 1992; Acharya et al, 2011).

Species diversity along elevational gradients provide many features that make them more appropriate than latitudinal gradients for examining the governing factors of spatial variation (Sanders & Rahbek, 2012). The high mountain ranges provide gradients of habitat along the elevations thus making it suitable natural laboratory to test different hypothesis. Further, it is also convenient to collect data in elevational gradients which are comparatively smaller in area than the latitudinal gradients (Sanders & Rahbek, 2012). Besides, the mountain ranges also facilitate to test for universality of factors determining species distribution patterns because there are numerous mountain ranges that serve as replicates of elevation diversity gradients.

Initially the distribution pattern of organisms along elevation gradient was perceived to surrogate that of latitudinal gradient (Stevens, 1992), however numerous studies demonstrate distribution patterns along elevation gradients are not uniform (Rahbek, 1995; McCain, 2009; Szewczyk & McCain, 2016). This is attributed largely to the complex biophysical processes underlying the fine scale patterns along elevation gradients. Studies across the world have reported various elevation diversity trends, namely, (i) monotonic decline with increasing elevation, (ii) mid-elevation peak, (iii) increasing trend with elevation, (iv) low-elevation plateau with linear decline, and (v) low-elevation plateau with mid-elevation peak (Rahbek, 2005; McCain & Grytnes, 2010). Likewise the key drivers of distribution pattern and diversity trends are extensive and still contentious. Multitudes of factors have been proposed to explain the elevational gradients of biodiversity which can be grouped into four broad categories, viz., (i) contemporary climate, (ii) spatial aspects, (iii) habitat heterogeneity, (iv) evolutionary history (Wiens et al, 2007; McCain & Grytnes, 2010). Therefore, examination of the mechanisms that result in species diversity along elevational gradient is pivotal in identifying the effects of such processes responsible for distribution of biodiversity and in developing conservation strategies (Lomolino, 2001; Vetaas & Grytnes, 2002).

Amphibians are important components of the ecosystem; they are the bio-indicators of ecosystem degradation since they have sensitive permeable skin and dual mode of life (Beebee and Griffiths, 2005). Populations of amphibians are declining and disappearing worldwide at an increasing rate as compared to pre-1980 decades, even from protected areas (Blaustein & Wake, 1990; Phillips, 1994; Stuart et al, 2004). Though there is a global decline of amphibians (Gibbons et al, 2000), much of the amphibian diversity is unexplored and unrecognized which calls for more scientific explorations and interventions of taxonomists for correct identification of many species. The effect of climate change and anthropogenic pressures are predicted to be more pronounced in the Himalaya than any other region in the world which makes biogeographical studies in these regions increasingly important (Singh et al, 2011).

It is believed that the conservation of biodiversity requires an understanding of species-habitat relationships and fine-scale distributions including microhabitat preferences (Quirt et al, 2006; Michael et al, 2010). Among the vertebrates, amphibians exhibit high diversity with 8483 species (AmphibiaWeb, 2022). Amphibians often display fine-scale heterogeneity depending on the microhabitat they inhabit (Thorpe et al, 2018). The species richness of anurans are directly associated with availability of microhabitats (Figueirido et al, 2019). The interaction between physiological requirements of anurans and the physical characteristics of microhabitats determine their spatial distribution (Angulo et al, 2006). Ecological studies including microhabitat preference contribute to conservation of amphibians through habitat management (Eterovick et al, 2010; Thomas et al, 2019).

Research on cryptic species has flourished over the past two decades, facilitated largely by the increasing availability of DNA sequences. Molecular techniques such as DNA sequencing have revolutionized the ability of biologists to enumerate biological diversity (Burns et al, 2008). Cryptic species usually occur in organisms that communicate reproductive signals via non-visual means (e.g. sound, vibration, pheromones or electrical signals) because changes in signals conveyed in these modes need not involve morphological change (Bickford et al, 2007). One such taxa possessing all these modes and harboring wide number of unidentified cryptic species are the amphibians (Gerhardt, 2005; Stuart, 2006). Despite a worldwide decline in amphibian populations (Stuart et al, 2004), the number of species of amphibians new to science has increased dramatically in recent years (Das et al, 2019; Li et al, 2020; Patel et al, 2021; Mahoney et al, 2022). DNA barcoding has proved to be an effective molecular tool for species identification in many groups of organisms, and also shows utility for revealing cryptic diversity (Hebert et al, 2003). The most commonly used gene in molecular taxonomy and phylogenetics is the mitochondrial cytochrome oxidase subunit I (COI), cytochrome b, 12S and 16S rRNA (Glaw & Vences 2006; Che et al, 2009; Mcleoad et al, 2015). Thus, the use of molecular approach in identification of cryptic species of amphibians may help detect the occurrence of species new to science.

Sikkim is located in the Eastern Himalayan region extending over a small geographical area (7096 km^2). Despite having small area representing just 0.02% of India's landmass, it is an ecological hotspot of India hosting a diverse range of flora and fauna. The sharp transition in climate and vegetation within short geographical area makes it well suited for diversity studies. There is a paucity of studies on amphibians in the Himalayan region (Khatiwada et al, 2015, 2019; Chettri & Acharya, 2020). Therefore, distribution studies along with ecological and phylogenetic analyses will aid in understanding the diversity of the region.

1.2 Review of literature

1.2.1 Distribution pattern of species along elevation gradients and their potential factors

Studies of spatial variation in the number of species along environmental gradients across the earth predate the origins of biogeography (Lomolino, 2001). Early naturalists in the nineteenth century including Linnaeus, Willdenow, von Humboldt, Darwin and Wallace provided the first extensive accounts of decline of species richness from low to high latitudes (Lomolino, 2001). Similar trends for elevation were documented in the detailed explorations of von Humboldt along Mount Chimborazo in the Ecuadorian Andes, Darwin in the Chilean Andes and Wallace in Indonesia (Lomolino, 2001). Quantitative analyses for evaluation of patterns of species richness along elevational gradients were not assembled until the twentieth century (McCain & Grytnes, 2010). The first exhaustive quantitative study of elevational species richness pattern of numerous vertebrates such as bats, breeding birds, non-flying small mammals, amphibians and reptiles by Grinnell and Storer (1924) in Yosemite, USA showed a unimodal richness pattern. Contrastingly, subsequent studies along the elevation gradient of Mount Lassen, California, USA, revealed unimodal pattern for non-flying small mammals and birds whereas a decreasing pattern of richness was found for reptiles and bats. Likewise, Whittaker (1952, 1960) reported unimodal pattern of species richness for plants and a decreasing pattern for insects along the elevation gradients of various mountains in USA. The pioneering studies of Terborgh and Weske (1975) and Terborgh (1977, 1985) reported a decreasing richness pattern of birds along the elevation gradients of tropical Peruvian Andes. Henceforth, decreasing diversity along elevation gradients was accepted as the universal pattern across all taxonomic groups assuming diversity patterns along elevations mirrored the latitudes (McCain & Grytnes, 2007).

Rahbek (1995) examined 97 papers to analyse if the general assumption of patterns of species diversity along elevational gradients is in concordance with latitudinal gradients and concluded that the species richness decreased with increase in elevation but the pattern of monotonic decline was however not the general pattern as previously assumed. Further, Rahbek (1997) reported that a hump shaped pattern of distribution was observed more generally irrespective of region and taxa than monotonic decline. Finally, elevational studies across various taxonomic groups reported three general patterns viz., monotonic decline, mid-elevation peak and linear increase of species diversity along elevation (Rahbek, 2005). Additionally, non linear patterns of species richness such as the low plateau pattern and low plateau pattern with mid-elevation peak have also been reported (McCain & Grytnes, 2010). However, diversity pattern often depend on the spatial scale, region and taxonomic group considered for the study (Rahbek, 2005; McCain & Grytnes, 2010).

The monotonic decline or decrease in species richness along elevation gradient has been documented in a number of taxa such as plants (Zhao et al, 2004; Cirimwami et al, 2019), bees (Classen et al, 2015; Perillo et al, 2017), butterflies (Acharya & Vijayan, 2015; Dewan et al, 2021), fishes (Bhatt et al, 2012; Askeyev et al, 2017) , reptiles (Chettri et al, 2010; Jins et al, 2021) and birds (Jankowski et al, 2013; Sam et al, 2019). Similarly, the most commonly observed mid-elevation pattern of species richness has been reported for plants (Vetaas & Grytnes, 2002; Bhattarai et al, 2004), trees (Acharya et al, 2011; Rana et al, 2019), beetles (Yu et al, 2013; da Silva et al, 2018), reptiles (Fu et al, 2007), birds (Acharya et al, 2011; Wu et al, 2013; Pan et al,

2016) and mammals (Hu et al, 2017; Chen et al, 2020). The increase of species richness with increase in elevation is rare and has been reported in few taxonomic groups such as ferns (Bhattarai et al, 2004), herbs (Cirimwami et al, 2019) and amphibians (Naniwadekar & Vasudevan, 2007).

Hitherto studies by numerous researchers around the globe have reported various factors and mechanisms of differing patterns of species richness along elevation gradients. McCain and Grytnes (2010) grouped the probable factors into four main categories: climatic variables, spatial aspects, evolutionary history and biotic processes.

a. Climatic variables

The variation in different abiotic variables such as temperature, rainfall, productivity, humidity and cloud cover represent the climatic factors. Temperature decreases with increasing elevation (Barry, 2008) and it indirectly limits diversity as it restricts the physiology, behavior or ranges of individuals (Kerr & Packer, 1997; Kaspari et al, 2000). This is especially true in case of ectotherms as low temperatures restrict energy assimilation even at environments with high resource availability (Sanders et al, 2007; Buckley et al, 2012). However, variation in herpetofaunal diversity may not be explained by temperature alone rather other factors such as productivity or water availability might be more strongly related (McCain & Sanders, 2010). The More Individuals Hypothesis (MIH) posits that increase in productive areas support more individuals leading to more species hence a positive relationship between diversity and productivity (Srivastava & Lawton, 1998). Similarly, both local and regional diversity patterns have been explained by precipitation (Hawkins et al, 2003; Evans et al, 2005). Water energy dynamics (WED) has also been suggested a key driver of biodiversity patterns (O’Brien, 2006; Vetaas, 2006). WED has been reported to be a stronger predictor of species richness of various taxonomic groups at mid-elevations and not the warm lowlands of tropical and subtropical regions including amphibians (Vetaas et al, 2019, and references therein).

b. Spatial factors

The concept of species-area relationship (SAR) states that the number of species increases with increase in sampling area (Rosenweig, 1995). The variation in bird species richness along elevation gradient in the Neotropics was strongly predicted by availability of area (Rahbek (1997). However it is argued that in montane gradients, the mountain base having more area should harbor more species than higher elevations with less area (Rahbek, 1997). However, it is opined that in some mountains the greatest area is in mid-elevations (McCain & Grytnes, 2007). Nevertheless, there are mixed support for SAR along elevation gradients (Hu et al, 2011; Khatiwada et al, 2019; Chettri et al, 2020).

Another hypothesis known as the mid-domain effect (MDE) assumes that geographic boundaries such as oceans and mountain tops limit species ranges and the simple overlap of many, variously sized ranges create a peak in species richness at mid-elevations (Colwell & Hurtt, 1994; Colwell & Lees, 2000; Colwell et al, 2004). Thus, regardless of other ecological factors, MDE predicts a mid-elevation pattern of species richness with a decline at lower and higher elevations. Several authors have documented the mid-domain effect for amphibians along elevational gradients (Fu et al, 2006; VanDerWal et al, 2008).

c. Evolutionary factors

Montane regions are thought to be important centers of diversity and endemism in tropical regions, possibly even more important than lowland rainforests (e.g., Rahbek & Graves, 2001; Jetz & Rahbek, 2002). Various evolutionary hypotheses have been proposed for explaining the mid-elevation diversity patterns in the mountains such as the "montane species pump" hypothesis and the "montane museum" hypothesis (Smith et al, 2007; Weins et al, 2007). The montane species-pump hypothesis predicts that topographic complexity and zonation of climatic variables in mountains drive allopatric and parapatric speciation (faster rate of diversification) thus increasing species richness (Fjeldsa˚ and Rahbek, 2006; Kozak and Wiens, 2007). In contrast, the montane museum hypothesis states that mid-elevations have increased species richness because these habitats were occupied for extended periods of time relative to higher or lower elevations (greater time for diversification) thereby facilitating speciation and abundance of species (Stephen & Weins, 2003). The mid-elevational species richness pattern of glass frogs in Andean elevation gradient were explained by the montane museum hypothesis (Hutter et al, 2013). Similarly, the mid-elevation species richness pattern of Meso-American salamanders was attributed to the early colonization process (Weins et al, 2007).

d. Biotic processes

Species richness patterns have been explained by numerous biological processes such as competition, habitat heterogeneity and habitat complexity (McCain & Grytnes, 2010). Heterogeneous habitats provide an array of niches to be occupied by different species thus facilitating coexistence and greater species richness (Whittaker, 1998; Potts et al, 2004; Ben-hur & Kadmon, 2020). The availability of heterogenous habitats is predicted to increase species richness of amphibians (Meza-Joya & Torres, 2016; Zhu et al, 2020; Schimdt et al, 2021). Biological interactions such as competition and predation influence the distribution of species (Pianka, 1966). Competition predicts a negative linear relationship with diversity (McCain & Grytnes, 2010). The predation hypothesis posits that less competition is facilitated by the high abundance of predator populations limiting the population of prey populations resulting in less competition between the prey species. Whereas the possibility of competitive exclusion is reduced by lower competition thus enhancing coexistence and increase in species richness (Schemske & Mittelbach, 2017).

Amphibians have a rich history of use as models in ecological research to address elemental questions in population and community ecology. More recently, amphibians have been used to explore principal mechanisms in macroecology and biogeography. Amphibians are vital to an ecosystem as they are the bio-indicators of ecosystem degradation due to their physiology and dual mode of life (Zaghloul, 2020). The poikilothermic physiology of amphibians gives them an advantage to use energy poor resources which makes them important links between the lowest and highest trophic levels in a community (Hopkins, 2007). Among the vertebrates, amphibians constitute the most diverse group currently under risk of extinction globally (Wake & Vredenburg, 2008). There are 8485 species of amphibians reported in the world out of which 7492 belong to the order anura, 779 species to caudata and 214 species to gymnophiona (Frost, 2022). Many herpetologists and ecologists have analyzed data on different groups of amphibians especially anurans from several regions of the world and have found various distribution trends along elevation gradients.

Early investigations on the amphibian species richness patterns along elevation gradients have reported a monotonic decline in species richness with increasing elevation (Fauth, 1989; Owen, 1989). This pattern was attributed to the high leaf litter depth at lower elevations (Fauth, 1989) and mean annual precipitation (Owen, 1989). Buckley and Jetz (2007) examined the richness and environmental relationships for amphibians at the global scale and concluded that this relationship varies between regions, suggesting it necessary to examine the amphibian-richness-environment relationship for different regions separately as many variables change across regions. However, since then many studies across the globe have examined the distribution pattern of amphibians and have found varying trends. The monotonic decline of amphibian species richness along the elevation gradients have been reported by a number of studies (Rodriguez et al, 2005; Zancolli et al, 2013; Agyei & Wiafe, 2013; Villacampa et al, 2019). A suite of factors such as temperature, precipitation, land surface area, annual evapotranspiration (AET), litter level and reproductive habitat have been documented to be the major driving factors for such distribution pattern. However, many studies have recorded a mid-elevation peak pattern for amphibians along elevation gradients. Fu et al (2006) reported a hump shaped pattern of amphibian distribution in Hengduan Mountains, China and demonstrated that a number of interacting factors such as climate and geometric constraints (MDE) explained such elevational patterns. Similarly, Hu et al (2012) and Wang et al (2020) showed a diversity peak at mid-elevations for amphibians in China. Likewise Meza-Joya and Torress (2016) showed a hump shaped pattern of *Prismantis* frogs across the elevational gradients of Tropical Andes and attributed that spatial processes such as area, latitude and mid-domain effect (MDE) partially explain the trend. Smith et al (2007) used an evolutionary perspective to explain the high species richness of

Middle American treefrogs in the mid-elevations and reported that higher rates of diversification and early colonization of mid-elevations hence longer time for speciation to be primary cause of such pattern. A mid-elevation peak pattern of amphibian richness has also been reported in other elevation gradients in Brazil and South America (Carvalho-Rocha, 2021; Matavelli et al, 2022). Abiotic factors such as temperature and habitat is explained to be the key driver of such distribution pattern of amphibians in these areas. The increase in amphibian species richness along elevation gradients has rarely been reported. Naniwadekar and Vasudevan (2006) reported a linear increase in species richness along the elevation gradient of Western Ghats which was explained by optimal soil moisture and temperature conditions of higher elevations for occurrence of amphibian species.

The entire mountain system of the Himalaya represents one of the 34 global biodiversity hotspot with rich flora and fauna. Although several studies in the Himalaya have assessed the diversity trends of many organisms however there is paucity of empirical studies on the species richness patterns of amphibians along elevation gradients from the region. A linear decline of amphibian species richness have been observed in Eastern Nepal Himalaya (Khatiwada & Huagaasen, 2015; Khatiwada et al, 2019; Gautam et al, 2020; Zhao et al, 2022). A number of factors such as area, humidity, number of trees, water pH were reported to be the key factors for such distribution of amphibians. In contrast, many studies have also reported a mid-elevation peak pattern of amphibian species richness from the Himalaya (Fu et al, 2007; Zheng et al, 2014; Wang et al, 2020, Chettri et al, 2020). Many factors such as Mean Annual Precipitation (MAP), Mean Annual Temperature (MAT), Annual evapotranspiration (AET) were attributed for such pattern. Similar to global trends increase in amphibian species richness along elevation gradients in the Himalaya is also occasional (Zhu et al, 2020). The availability of suitable habitats was explained as the primary driver of increase of amphibian richness along elevation.

There is acute shortage of literature related to the study of amphibians of the Eastern Himalayan region. In Sikkim, part of Eastern Himalaya Biodiversity Hotspot, the number of amphibian species varies considerably. In the Fauna of British India, 17 species were reported (Boulenger, 1890), the Gazetteer of Sikkim (Risley, 1894) reported 16 species and Gammie (1928) recorded 16 species of amphibians from Sikkim. Studies on the geographical and altitudinal distribution of amphibians and reptiles from Sikkim (Waltner, 1973) have enlisted 30 species. In the documentation of amphibians occurring in India, 21 species were found to occur in Sikkim (Chanda, 2000). These studies have mostly focused on species occurrences and natural history. The study by Chettri and Acharya (2020) remains as one of the most exhaustive works on the elevational distribution pattern of amphibians in the Eastern Himalayan region. This robust study was carried out along the a large spatial gradient covering a large gradient of 4000m elevation range in which various potential factors were analyzed. The study reported a unimodal pattern with a peak at middle elevation of amphibian distribution in Sikkim Himalaya. Environmental variables such as actual evapotranspiration (AET) and mean annual precipitation (MAP) were shown as the most significant factors that explained such distribution trend.

Elevation gradients harbor tremendous opportunities to comprehend the drivers of global biodiversity as there are numerous gradients across the world with varying floral and faunal species richness and diverse climatic, spatial, historical and biotic conditions. These variabilities give a premise to test different hypotheses and determine significant factors based on similarities and differences of diversity trends across mountains (McCain & Grytnes, 2010). Sikkim Himalaya provides an ambient prospect of testing different models and generality of trends along elevation gradients considering the lack of studies from this region.

1.2.2 Studies on microhabitat associations of amphibians

A key focus in addition to these macroecological patterns in species richness have been the community patterns including niche relationships, coexistence and inter-specific competitions (Pianka, 1973). Joseph Grinnell first coined the term ecological niche to define the basic habitat required by a species to survive and reproduce (Grinnell, 1917). However, the distinction between fundamental niche and realized niche was made by Hutchinson (1917). The three important dimensions of an ecological niche of an organism are space, time and food (Pianka, 1973; Peterson, 2011). The ecological niche of an organism reflects the effect of various environmental factors combined with habitat features (Whittaker et al, 1973). Studies on niche breadth are helpful in distinguishing specialist from generalist species (Clavel et al, 2010). However, species may be specialized along one axis and a generalist in the other, thus different types of specialists exist such as habitat, climate, diet or behavior (Williams et al, 2006; Devictor et al, 2010). Hence such studies provide information on the extinction risk of species as well as their vulnerability to anthropogenic activities (Botts et al, 2013). Additionally, studies on resource partitioning aids in understanding effect of habitat modifications on species through niche conservatism (Wiens & Graham, 2005). The tendency of organisms to retain their ancestral ecological traits is termed as niche conservatism (Wiens et al, 2010). Since closely related species have similar ecological preferences due to the slow evolution of their niches (Wiens et al, 2010), species that are genetically close would have similar affects of habitat alteration relative to genetically distant species. The species microhabitat association, niche breadth and niche overlap have been reported to be an important aspect of conservation in various taxa such as reptiles (Michael et al, 2015; Jins et al, 2022), birds (Stratford & Stouffer, 2013), fishes (Goudinho & Lotufo, 2010) and mammals (Melo et al, 2013). The large diversity within the taxa, differences in morphology, life history and physiology make amphibians suitable model organisms for studying community dynamics including niche segregation and coexistence (Toft, 1985). Besides, studies on microhabitat partitioning of amphibians are scarce. Microhabitat associations and niche partitioning in amphibian communities have been addressed by many studies (Afonso & Eterovick, 2007; Dure et al, 2009; Nath et al, 2012: Zainudin et al, 2017; Thorpe et al, 2018). Hitherto, studies on microhabitat associations and resource partitioning of amphibians are lacking from the Himalayan region. Microhabitat serves as a significant resource dimension that is partitioned between ecologically and genetically similar species (Whitfield & Pierce, 2005). Studies focusing on microhabitat associations and availability can ascertain resource limitations (Abrams, 1980). Moreover, species-habitat association is an important aspect that determines the diversity and distribution of organisms. Investigating species habitat relationship is fundamental to develop long term conservation and management of species (Sutton et al, 2010). Amphibians are one of the most threatened taxa with habitat loss and degradation being the contributing factors for the decline in amphibian species (Ficetola et al, 2015). Hence, studies on species habitat associations will aid in recognizing vulnerable species and conservation planning.

1.2.3 Studies on phylogeny of *Amolops* species

Research on cryptic species has increased markedly in the past two decades with the availability of DNA sequences. The taxonomic challenge caused by cryptic species has been recognized even before the Linnaean classification system was adopted (Winker, 2005). Molecular techniques (primarily DNA sequencing) have equipped biologists to distinguish morphologically similar species (Bickford et al, 2006). One such group likely to hide cryptic species diversity are the amphibians essentially due to their non-morphological characteristics of species recognition and reproductive behavior (Bickford et al, 2006). Moreover amphibians presumably have more cryptic diversity as they are generally considered less lucrative due to their small size, inaccessible habitats and no commercial value and hence ignored (Bickford et al, 2006). Additionally the worldwide decline of amphibians at an alarming rate makes it even urgent to uncover cryptic species. Amongst the amphibians, the *genus Amolops* contains several species that have conservative morphology and hence difficult to identify by morphology alone (Mahony et al, 2022) hence subjected to misidentification of species (Bain et al, 2006). Nevertheless there has been an exponential increase in new species descriptions from the *genus* Amolops (Zhao et al, 2005; Khatiwada et al, 2020; Wu et al, 2020; Mahony et al, 2022). In Sikkim, previous studies have thoroughly explored the amphibian diversity along the elevation gradient (Chettri & Acharya, 2021) however very few studies have examined the cryptic diversity (Subba et al, 2015; Patel et al, 2021). Given the high number of cryptic lineages within the genus *Amolops*, it is imperative to explore species diversity using contemporary molecular approach. Additionally, given the global degradation of natural ecosystems and impending decline of amphibian species (Alroy, 2015) there is an urgency to identify species using molecular tools and catalogue their threats for efficient species specific conservation planning.

1.3 Research Gaps

The diversity and patterns of organisms have captivated researchers since time immemorial. Understanding geographical distribution of species and the factors underlying such distributions have been a central focus in community ecology (Pianka, 1966; McArthur, 1972). Many exhaustive studies have been carried out along the elevation gradients in different taxa (Rahbek, 1997; Vetaas & Grytnes, 2002; Marathe et al, 2020; Smith et al, 2007). However, the generality of the pattern remains disputed as there are significant variations in the mechanisms and patterns across regions and taxa. In Himalaya many studies have explored the diversity trends of amphibians (Khatiwada et al, 2019; Gautam et al, 2020; Zhao et al; 2022). However studies on the distribution pattern of amphibians from Sikkim has been sporadic (Chettri et al, 2020) despite their importance in proposing conservation strategies. Moreover, in Sikkim, previous study has explored the distribution pattern of amphibians only along the elevation gradient of Teesta valley. Whereas the amphibian diversity and distribution along elevation gradients of the Rangeet valley remains hitherto unexplored. Moreover, studies comparing two valleys in the same landscape are entirely lacking.

Microhabitat reflects an important dimension of resources that are partitioned between ecologically similar species (Zainudin et al, 2017). Microhabitat availability considerably influences the species richness and distribution of amphibians (Figueiredo et al, 2019). In frog assemblages spatial niche is an essential aspect that governs relationships among individuals (Toft, 1985). Studies on resource utilization and microhabitat association of amphibians in the Eastern Himalayan region including Sikkim is entirely lacking.

Lastly, the taxonomic position of the *Amolops* species occurring in Sikkim is poorly understood and has never been explored using molecular data.

Hence, considering the above gap in studies the present study was carried out to address the following specific objectives:

1.4 Objectives

1. Explore the distribution patterns of amphibians along the elevation gradients of Teesta and Rangeet valley of Sikkim Himalaya.
2. Understand the factors governing the distribution patterns along the two valleys
3. Compare the amphibian community composition between the two valleys.
4. Undertake phylogenetic analysis of select amphibian species, using morphological and molecular data.

1.5 Organization of thesis

The thesis has a total of five chapters of which three are technical chapters addressing the four major objectives and are organized as follows:

1. Chapter I provide a brief background of the study and review the literature on distribution pattern of organisms along elevation gradients across the globe. Here I present the major gaps in research and the objectives of the study.
2. Chapter II describes the study area with a general outline of the Sikkim Himalaya, vegetation and climate in the Teesta valley and Rangeet valley.
3. Chapter III explains the distribution pattern of amphibians along the elevation gradients of Teesta valley and Rangeet valley in Sikkim Himalaya. The patterns of amphibian distribution are compared between the two valleys and their underlying factors are discussed in this chapter.
4. Chapter IV compares the amphibian community composition between the Teesta valley and the Rangeet valley. The niche breadth, niche overlap and microhabitat associations of amphibians occurring in Sikkim are provided.
5. Chapter V provides the taxonomic status of species belonging to *Amolops* genus of Sikkim. The morphological assessment and molecular phylogeny of four Amolops species occurring in Sikkim has been discussed.

CHAPTER 2

STUDY AREA

2.1 Sikkim Himalaya: a brief outline

The Himalaya is the youngest and geographically complex mountain system in the world that surpasses Nanga Parbat (Pakistan) in the east to Namcha Barwa (China) in the west, measuring about 2500 km and a width ranging from 240-350 km (Roy & Purohit, 2018). The extensive Himalayan mountain range separates the fertile alluvial plains of Indian subcontinent from the Tibetan plateau. The Himalayan range passes through the nations of Afghanistan, Pakistan, India, Nepal, Bhutan, China, Bangladesh and Myanmar. The Himalayan range possesses the world's highest peaks including Mt. Everest (8848.86 m), Mt K2 (8611 m) and Mt Khangchendzonga (8686 m). The mountain range represents elements from four zoogeographic realms viz. the Palearctic, Saharo-Arabian, Sino-Japanese and Oriental (Holt et al, 2013; White et al, 2019). The Himalaya harbors rich repositories of biodiversity owing to the substantial variation in eco-biogeography that gives rise to diverse eco-climatic zones. Further, the variation in climate resulting in wide ranging habitats and colonization by flora and fauna from different realms has given rise to a biodiversity hotspot of global importance (Myers et al, 2000). The Himalayan region encompassing a total of 5.95000 km^2 is home to 10,000 species of plants, 270 fishes, 175 reptiles, 105 amphibians, 980 birds and 300 mammals, (CEPF, 2020). The Himalayan range consists of four parallel mountain belts from south to north i.e., the outer Himalaya (Siwalik range), the lesser Himalaya, and the greater Himalaya and the Tibetan Himalaya (Valdiya, 2001). Similarly from west to east, the Himalayan mountain ranges are divided broadly into three regions, Western, Central and Eastern. The Eastern Himalaya consists of portions of three of the 34 biodiversity hotspots which accounts for a total of 39% of the Himalayan hotspot (Chettri et al, 2010). It is the confluence of three biogeographical realms namely the Indo-Malayan, Palearctic and Sino- Japanese realm (Chettri et al, 2010). In India, the Eastern Himalayan region encompasses Indian states of West Bengal, Sikkim, Assam and Arunachal Pradesh. The moisture regime decreases toward the Western region due to the Himalayan range in the North blocking the southwest monsoon from the Bay of Bengal, while the Eastern Himalaya receives relatively more rain (Chettri et al, 2010). Thus, the moisture laden Eastern Himalayan region harbors increased biodiversity relative to drier western Himalaya. The Eastern Himalayan region includes 25 ecoregions (Olson & Dinerstein, 2002) and has been identified as a Priority I Endemic Bird Area (Birdlife International, 2001). However, different habitats and ecoregions in the Eastern Himalayan region are recognized to be threatened to a considerable degree by human induced activities (Myers et al, 2000; CEPF, 2020.

Sikkim (27^{O} 5′ - 28^{O} 10′ N and 88^{O} 4′ - 88^{O} 58′ E) is amongst the smallest states located in the northeast India and is a part of Himalayan biodiversity hotspot. Sikkim is bordered by the Tibet Autonomous Region of China to the north and northeast, Bhutan to the southeast, Indian state of West Bengal to the south, and Nepal to the west. The physical landscape of the state is characterized by striking variations in elevation as low as 300 m to extremes as high as 8586 m (Mt. Khadchendzonga). Sikkim is bounded by high ridges and ranges such as the Trans Himalaya in the northern side which separates the state from Tibetan highlands, the Chola range in the Eastern side demarcates the state from Tibet and the Singalila range in the western side separates the state from Nepal. These ranges also regulate the climate and large scale wind movements of the region and adjoining regions (Rawat & Tambe, 2011). The two major rivers Teesta and Rangeet originates from the glaciers and ice fed lakes which sculpts the mountains into deep ravines and valleys before its confluence with river Brahmaputra in the Bay of Bengal. The abrupt rise in elevation in Sikkim Himalaya facilitates rapid transition of eco-climatic zones within relatively short distance. An altitudinal gradation in vegetation zones is seen within a distance of 100 km from tropical moist forests in the south to dry alpine steppe in the north (Rawat & Tambe, 2011). There are 10 biogeographic zones and 25 biotic provinces across the world consisting of 16 key forest types of which nine types are found in Sikkim and is grouped under Himalayan biogeographic zone and central Himalaya biotic province (Champion & Seth, 1968). Owing to the great variation in vegetation and climatic conditions, Sikkim with just 0.2% (7096 km^2) geographical area of India has been recognized as a hotspot of biodiversity and one among the three ecoregions in India (O'Neil, 2019).

Sikkim Himalaya harbors rich floral and faunal diversity with almost 40% of the total diversity of India (Acharya & Sharma, 2013). The floral diversity includes over 4458 flowering plant species .Similarly, the faunal diversity consists of more than 7,000 moths, 5,892 insects, 994 beetles, 40 bees, 689 butterflies, 48 fish , 574 birds and 169 mammals (Acharya & Sharma, 2013; Banerjee et al, 2019). The herpetofaunal diversity consists of 42 species of reptiles (snakes and lizards) and 25 species of amphibians (Chettri et al, 2011: Chettri & Acharya, 2020). The enormous biodiversity of Sikkim is sustained by the maintenance of pristine habitats. Sikkim has a total of 47.08% (3341.04 km^2) of geographical area under forest cover (FSI, 2021). The state has seven wildlife sanctuaries and one national park, the Khangchendzonga National

Park which is declared as the UNESCO World Heritage Site and 30.77% of forest is under the Protected Area Network.

2.2 Vegetation type

The vegetation changes rapidly as one traverse from lower to higher elevations. Many naturalists and biologists have documented the rich floral and faunal diversity of Sikkim Himalaya and categorized the vegetation into different classifications however depending on the elevation, physical and ecological factors; it can be classified into six major types (Haribal, 1992; Acharya & Sharma, 2013).

i). Tropical Semi deciduous forests (300 – 900 m)

It includes the lower elevation zones which consist of areas bordering the state of Sikkim and West Bengal. This zone is dominated by deciduous plants such as *Shorea robusta*, *Ailanthus grandis*, *Terminalia myriocarpa*, *Pandanus furcatus*, *Castanopsis indica* etc. Species such as *Strobilathes* sp, *Barleria* sp, *Polygonum* sp, *Tridax* sp. etc occur as secondary growths. It also consists of Kitam Bird sanctuary (the only protected area) in this zone.

ii). Tropical moist or broad-leaved forests (900 -1800 m)

This zone is represented by broad-leaved species such as *Schima wallichi*, *Alnus nepalensis*, *Castanopsis* sp, *Acer* sp, *Litsea* sp, *Engelhardia* sp etc. Secondary growths such as *Girardinia* sp, *Boehmeria* sp, *Maesa* sp, *Melastoma* sp, *Edgeworthia* sp etc dominate this zone. This zone also consists of several bamboo species.

iii). Temperate broad-leaved forest (1800 – 2800 m)

This zone has a mix of broad leaved species and coniferous species such as *Acer campbelli*, *Taxus baccata, Abies densa, Betual utilis, Rhododendron arboretum, Tsuga dumosa* etc. Plant species such as *Hypercium* sp, *Viburnum* sp, *Urtica* sp, *Vaccinium* sp, etc represent the undergrowth. A large percentage of protected area comes under this zone and hence consists of pristine natural forests.

iv). Temperate coniferous forests (2800 – 3800 m)

This zone is predominated by coniferous species such as *Tsuga dumosa*, *Abies densa* etc and various *Rhododendron* species such as *Rhododendron arboreum, Rhododendron campanulatum, Rhododendron grande*. Species of *Berberis* sp, *Rubus* sp, *Spirae* sp etc represent the secondary growth. Moss covered floor is the characteristic of coniferous forest.

v). Sub- Alpine vegetation (3800 – 4500 m)

This zone is characterized by coniferous trees and smaller shrubs. The steep terrain and deforestation how come deforestation cease the treeline at lower level causes the treeline in east Sikkim to cease at relatively lower elevations at 3800 m compared to west Sikkim which is upto 4000 m. The harshness of the climate causes underdevelopment of several rhododendron species in this zone. However, several flowering species of *Azalea* sp, *Primula* sp, *Aste*r sp, *Anemone* sp etc become very common.

vi). Alpine zone (>4500 m)

This zone represents the high altitude cold desert and consists of plants that appear during spring time as at other times of the year it is covered under perpetual snow. Plant species such as *Potentilla* sp, *Saxifraga* sp, *Lagotis* sp, *Pedicularis* sp, *Iris* sp, *Corydalis* sp etc are found in this zone. The strong winds cause these plant species to grow flat across the land and their flowers are directed towards the ground.

2.3. Teesta Valley and Rangeet Valley

The river Teesta originates from the snowmelt and spill waters of Teesta Khangse glacier and Tso Lhamo lake in the northern part of Sikkim. The catchment area in the upper portions of Teesta river is mostly snow whereas the lower portion is covered with forest (Goyal & Goswami, 2018). River flows southwards and is joined by several tributaries and sub-tributaries forming deep canyons and narrow valleys. The river Teesta descends steeply for around 3.03 miles before covering a distance of about 50 miles before merging with the plains of West Bengal. The Teesta valley is characterized by high variation in climatic conditions. The present climatic data is retrieved from m climatologist at high resolution for the Earth's land surface areas (CHELSA; Karger et al, 2017 a,b). Temperature shows a linear decline with elevation whereas precipitation shows an almost hump shaped pattern with peaks at 1200–1800m. Lower elevations of the Teesta valley experience warm tropical hot weather in the summers whereas the winters are cold and dry. The annual temperatures range from 19 ºC to 23 ºC in lower elevations (<1000 m) and precipitation ranges from 1663-1811 mm. Similarly, climate in middle elevation between 1000-2000m experiences a moderate weather where the annual temperatures range from 14 ºC -19 ºC and annual precipitation is 2012-2209 mm. This is the most humid region in the elevation gradient of Teesta valley. Maximum precipitation is received during the monsoons from June through September. The high elevations between 2000-3000 m is cold with an annual temperature ranges from 10 to 14 ºC and an annual precipitation from 1018-2128 mm. Similarly, the zones between 3000-4000 m is characterized by an annual temperature ranging from 4–7 ºC and an annual precipitation of 446- 645 mm. This elevation zone exhibits chilling winters and cool summers. As the elevation increases the regions above 4000m shows sub-alpine conditions with an annual temperature ranging from sub-zero temperatures to 1.96 ºC and the annual precipitation ranges from 264- 419 mm in the form of snow.

The river Rangit is a major right bank tributary of river Teesta and drains the west Sikkim district (Vellozi et al, 2017). The catchment of Rangit River is about 80 km in length with almost 2200km^2 of area originating from a glacier of Mount Kabru (7412 m) at the southern side of Kangchendzonga massif (Vellozi et al, 2017). The Rangeet valley watershed area lies in the west and south districts of Sikkim which is drained by River Rangeet and its tributaries. The Rangit river flows east till it meets the river Teesta in Triveni at Melli, West Bengal at around 300 m elevation. The Teesta and the Rangeet valley are separated by the north- south ridge that runs through the centre of the Sikkim before ending at the confluence of the two rivers. The temperature and precipitation regime in the Rangeet valley varies considerably with elevation. The lower elevations experience temperatures ranging from 20 – 24 ºC and precipitation from 1180 mm-1195 mm. The elevations between 1000- 2000 m exhibit annual temperatures ranging from 11-21 ºC and an annual precipitation between 2142-2172 mm. The areas along elevation between 2000 - 3000 m is humid with an annual temperature range between 12 - 9.5 ºC while the precipitation ranges between 2321 - 2194.4 mm. This zone represents the most humid areas along the Rangeet valley. The elevation zone above 3000m exhibits cold and chilling annual temperatures ranging between 3.5 – 8 ºC while the precipitation is high between 2184 - 2234 mm. However the upper edges of this zone can show harsh weather conditions with temperatures decreasing to 0 ºC.

2.4 Sampling sites

The present study was conducted along the elevation gradient ranging from 300 m to 5150 m in the Teesta valley and from 300 to 3300 m in the Rangeet valley (Photo Plate 2.1). Sampling above 3300 m in the Rangeet valley was logistically not feasible due to extreme climatic conditions and reduced accessibility. In the Teesta valley, entire elevational zone (5150 m, considered for the study) was divided into 16 elevational zones. Likewise, in the Rangeet valley, entire elevational zone (5150 m, considered for the study) was divided into 10 elevational zones. The elevational distance between two consecutive zones was 300 m in both valleys. In each zone, prior to sampling suitable sites conducive for amphibians were identified and the accessibility to the sites were assessed. Each zone consisted of multiple sampling sites. A total of 140 sites were sampled in the Teesta valley and 137 sites in Rangeet valley.

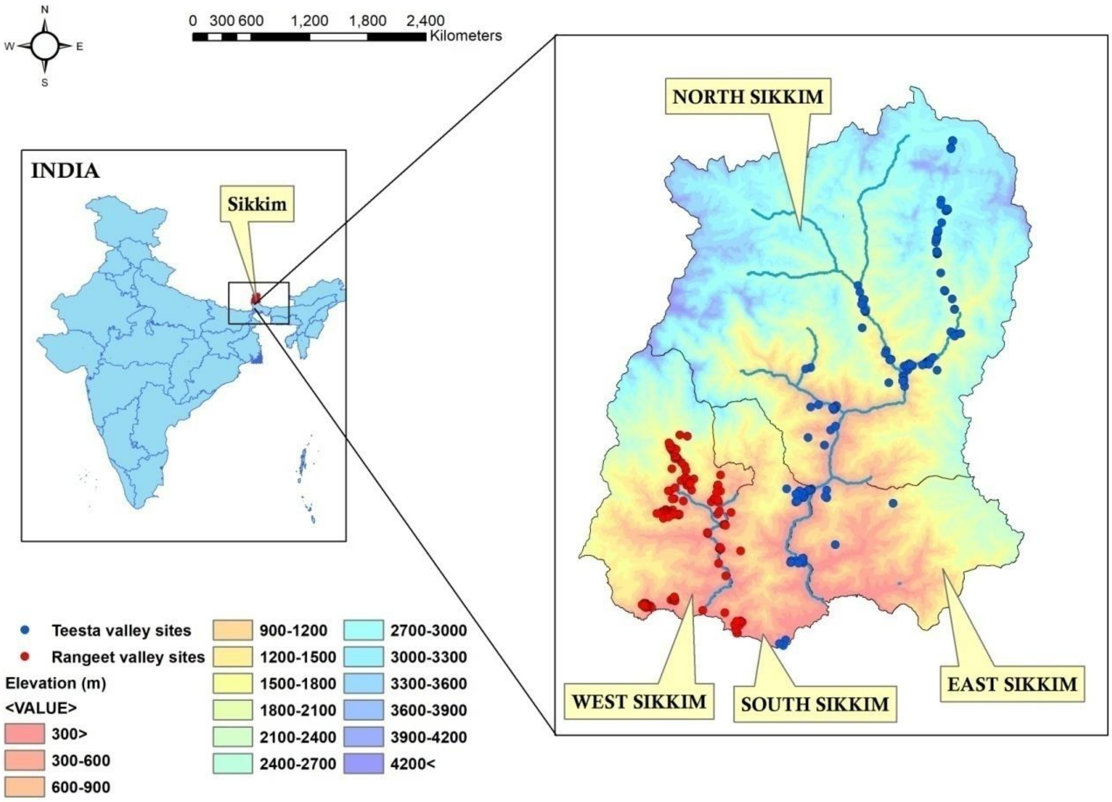


Photo plate 1.1: Map showing sampling locations in the Teesta and Rangeet valley, Sikkim Himalaya

CHAPTER 3

SPECIES RICHNESS PATTERNS AND THE FACTORS GOVERNING DISTRIBUTION OF AMPHIBIANS ALONG ELEVATION GRADIENTS

3.1 Introduction

Species diversity, composition and abundance remarkably vary across environmental and spatial gradients. A general decline in species richness with increasing geographical distance from the equator is well recognized latitudinal diversity gradient (Rosenweig, 1995; Willig et al, 2003). Elevation is considered to be one of the notable gradients that determine species distribution patterns at smaller spatial scales (Sanders & Rahbek, 2012). As outlined in Chapter I of this thesis, species richness and abundance follow three general patterns along elevational gradients: monotonic decline, mid elevation peak and linear increase (Rahbek, 2005). Several factors have been proposed as drivers of elevational gradients of biodiversity and can be broadly classified into four main categories: climate (past and contemporary), spatial aspects (area and geometric constraints), evolutionary history and habitat characteristics (heterogeneity, structural complexity) (McCain, 2009; McCain & Grytnes, 2010; Rahbek et al, 2019).

Among the vertebrate groups, amphibians serve as suitable ecological indicators and are particularly sensitive to environmental change due to their biphasic life cycle, permeable skin and ectothermic physiology (Halstead et al, 2022; Hopkins, 2007). Additionally, amphibians can be easily observed and monitored in the field (Halstead et al, 2022). Such features make amphibians favorable taxa for assessing various biogeographic hypotheses. Several studies on amphibians across the world have reported all three patterns of species richness as mentioned above, however, the mid-elevation peak pattern is the most frequently observed (Smith et al, 2007; Weins et al, 2007; Hutter et al, 2013; Meza-Joya & Torres, 2016). Similarly, the most frequently observed and discussed drivers are the climatic factors (Qian et al, 2007; McCain and Sanders, 2010; Malonza, 2015), area and geometric constraints (Fu et al, 2006; Khatiwada et al, 2019) and evolutionary history (Wiens et al, 2007; Hutter et al, 2017; Rahbek et al, 2019). Nonetheless, species distribution patterns vary due to the interplay of numerous biophysical processes that influence the smaller scale spatial patterns along elevation gradients. Furthermore, patterns of species richness and its potential drivers may vary due to climate, topography and spatial scale of the study (Rahbek, 2005). Therefore, region specific studies are necessary to ascertain species distribution model along elevation gradients. Many studies have investigated the factors of species distribution along elevation gradients (McCain & Grytnes, 2010; Sanders and Rahbek, 2012), however the mechanisms and factors governing such patterns are still contentious (Lomolino, 2001; Sanders & Rahbek, 2012; Stevens et al, 2013). Predicting the species richness patterns and the factors responsible for such patterns is a fundamental approach for planning conservation strategies (Nogués-Bravo & Martínez-Rica, 2004).

The Eastern Himalaya forms the most diverse mountain regions in the world (CEPF, 2007) and part of the globally significant ecological biodiversity hotspots (Mittermeir et al, 2005, 2011; CEPF, 2020). It supports a vast diversity of life forms owing to its unparalleled elevation range and its location at the junction of the Oriental and Palearctic geographical realms (Mani, 1974). A wide variation in climatic conditions and vegetation types within a small geographic range makes it a unique region to test patterns of species richness along elevation gradients. In Himalaya, the elevational pattern of species distribution has been documented in various taxonomic groups such as mammals (Hu et al, 2017), birds (Hunter & Yonzon, 1993; Acharya et al, 2011a; Wu et al, 2013a), reptiles (Chettri et al, 2010; Fu et al, 2007), fishes (Fu et al, 2003; Bhatt et al, 2012), butterflies (Acharya et al, 2015; Dewan et al, 2021), trees (Acharya et al, 2011b) and different plant groups (Yang et al, 2018; Sharma et al, 2019). Although limited studies on the distribution patterns of amphibians in the Himalaya have shown mixed patterns (Fu et al, 2006; Khatiwada et al, 2019; Chettri & Acharya, 2020), patterns comparing two valleys in the same landscape has never been considered. Moreover to achieve a greater understanding of the general patterns of diversity, studies for montane biogeography should include comparisons among mountain ranges which can provide valuable insights into the patterns and processes underlying elevational diversity (Lomolino, 2001).

Amphibians are facing immediate extinction crisis with nearly a third of species categorized as threatened (Stuart et al, 2004) due to multiple factors with habitat destruction, alteration and fragmentation being the most prominent ones (Beebee and Griffiths, 2005; GAA, 2006; Hussain, 2012). Among the vertebrates, amphibians are considered as the most endangered group (Ripple et al, 2019).While many new species of amphibians are regularly described, amphibians are also the most data deficient group of vertebrates with a lack of knowledge on geographical distribution and population trends of many species (Howard & Bickford, 2014). Therefore, there is an urgent need to understand richness trends of amphibians and their drivers which consecutively will help identify their potential threats as well. Additionally, in the face of climate change, mountainous regions such as the Himalaya have been most vulnerable with predictions of more prominent consequences relative to other parts of the world (IPCC, 2014). In particular, amphibians are presumed to be hugely impacted in future climate change scenarios (Zellmer, 2020). Given the global declines in amphibians, knowledge on the diversity patterns will offer vital information on priority areas for conservation planning of such under represented group. Furthermore, knowledge on species distribution patterns and their potential mechanisms along elevation gradients is pivotal to formulate conservation policies and to identify areas of conservation importance (Hunter and Yonzon, 1993; Shrestha et al, 2010).

Therefore, this chapter addresses the following questions: what is the distribution pattern of amphibians along the elevational gradients of Teesta valley and Rangeet valley, Eastern Himalaya? Are the patterns consistent between the two valleys i.e. Teesta valley and Rangeet valley? Are the underlying mechanisms similar in both valleys?

3.2 Methodology

3.2.1 Study Area

Sikkim (27^{O} 5′ - 28^{O} 10′ N and 88^{O} 4′ - 88^{O} 58′ E) is a small state of India located in Eastern Himalaya. Despite the small geographical area (7096 km^{2}), Sikkim is one of biodiversity rich regions of the world. The present study was conducted covering two valleys of Sikkim Himalaya, Teesta valley and the Rangeet valley (Fig 1). Teesta valley encompasses North, East and South district of Sikkim, and it is formed by expansive watershed area drained by river Teesta. The Teesta river originates from the Teesta Khangse (or Pauhunri) glaciers at 7068 m a.s.l and flows southward (Meetei et al, 2007) and adjoins many snow fed lakes and small tributaries in its course before entering the state of West Bengal, India. The elevation of the Teesta valley ranges from 300 m to 7068 m. The river Rangeet is a major tributary of Teesta and originates as Rathong chu river at 4674 m a.s.l from the Rathong glacier in West Sikkim and spans major parts of West and South Sikkim. The elevation of the Rangeet valley ranges from 300 to 7412 m. The river Teesta and Rangeet confluence at a place called Melli at around 300 m. The numerous watersheds and sub-watersheds formed by several streams and tributaries of these rivers and the moist conditions along the riverbeds facilitate formation of diverse habitats conducive for the amphibians. Both Teesta and Rangeet valley experience a wide range of climatic conditions from hot tropical at lower elevations to freezing arctic conditions at higher elevations. The temperature displays a monotonic decline with increase in elevation in both the valleys. The sharp changes in climatic conditions along the elevation gradients induces transition in vegetation from tropical semi-deciduous in the low elevations, temperate broad-leaved forest at mid elevations and sub-alpine to alpine at higher elevations. Simultaneously, other abiotic factors such as precipitation, humidity and temperature change along these elevation gradients The considerable elevational range and marked distribution of bioclimatic zones makes these valleys ideal regions for elevational studies.

In the Teesta valley the sampling site started from Melli at 350 m in the south and ended at 5150 m in Gurudongmar lake in the north. A total of 141 sites were sampled in the Teesta valley covering areas adjoining to Melli, Dalep, Lingee, Dzongu, Toong, Theeng, Chungthang, Lachung, Yumthang, Yumesamdong, Lachen, Thangu and Gurudongmar (Fig 3.1). Similarly, in the Rangeet valley, the sampling site started from Kitam at 310 m in the south and ended at 3250 m in Tshoka in the west. Sampling in higher elevations above 3300 m in the Rangeet valley was logistically not feasible due to extreme weather conditions and reduced accessibility. A total of 137 sites were sampled in the Rangeet valley covering areas adjoining to Kitam, Jorethang, Reshi, Legshep, Tashiding, Darap, Rimbi, Khecheopalri, Yuksom, Sachen, Bakhim, Tshoka, Dzongri, Gangyap, Kongri, Labdang, Malbasey and Daramdin (Fig. 3.1). The study area in the Teesta valley was categorized into 16 elevation bands based on elevation with an interval of 300 m between each band for data collection. However, in the Rangeet valley the study area could be categorized only into 10 elevation bands with 300m interval. Previous studies on amphibian distribution pattern have used elevation bands with either 1000 m or 500 m interval (Khatiwada et al, 2019; Chettri & Acharya, 2020) but studies on finer scale has been lacking.

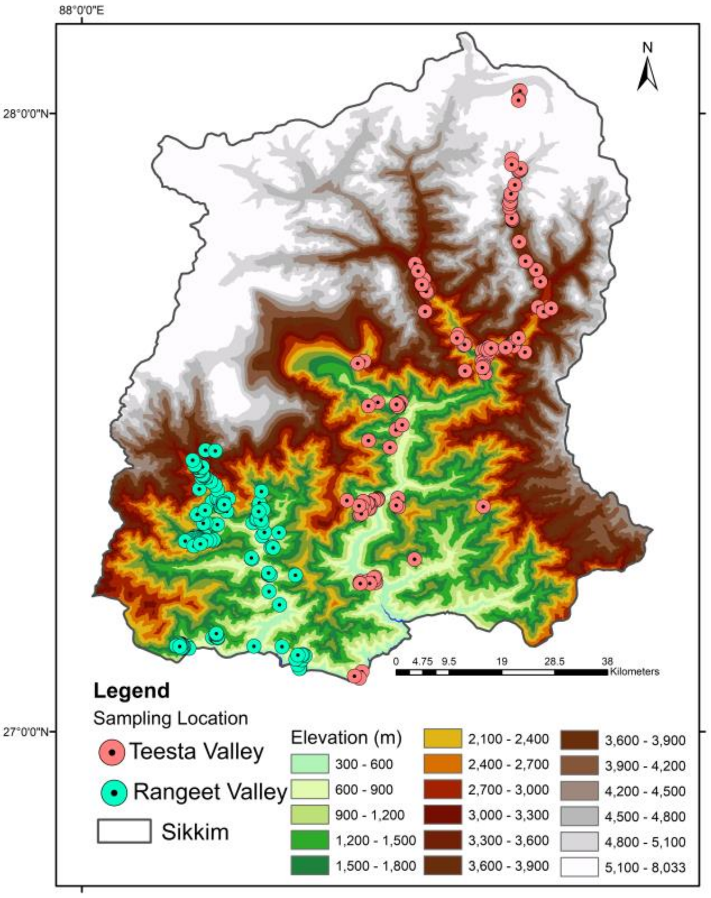


Photo Plate 3.1: Map of Sikkim showing the study sites in Teesta valley and Rangeet valley, Sikkim, Eastern Himalaya

3.2.2 Data collection

Field sampling were conducted during May 2016- August 2019 with more rigorous effort during May to August each year coinciding with the breeding season for most of the species of amphibians. Sampling sites in each valley were determined on the basis of elevation and likelihood of viable microhabitats conducive for amphibians. Visual encounter survey (VES) (Heyer et al, 1994) was followed for sampling amphibians searching all probable microhabitats such as under boulders, logs, leaf litters, grasses, herbs, shrubs, tree holes, rocks, and mosses. The search was time constrained and was carried out by two personnels (researcher and a field assistant) for 3-4 hours per day from 9:30 to 14:00 hrs. The same sampling site was not repeated in successive sampling to refrain from repetitive accounts of the same individuals. Additionally, considering the nocturnal behavior of many amphibians, night stream transect walk was conducted along streams and rivers. Streams were identified, measured and marked prior to sampling during the day. Amphibians were searched along the marked transect with the help of search lights during dusk to early night hours (18:00-21:00) and only amphibians observed above ground were recorded without disturbing their microhabitats. The collected specimens were identified, photographed and amphibian species that were difficult to identify in the field were taken to the laboratory for identification. Identification was done with help of literatures such as Boulenger (1890 & 1920); Schleich and Kastle (2002); Chanda (2002); Zoological survey of India (2010).

3.2.3. Creation of elevational bands using Digital Elevation Model (DEM) imagery

Cartosat-1 Digital Elevation Model (DEM) raster imagery (covering the Sikkim Himalayan region) was generated using Bhuvan, an online geospatial platform (https://bhuvan.nrsc.gov.in, built and operated by the ISRO, India). Each of the pixel (resolution of 2.5 m) of the imagery contains the value of elevation of the area it overlays. The DEM raster was classified into series of 300 m elevational zones (e.g. 300-600 m, 600-900 m and so on) using the spatial analyst toolbox in ArcGIS 10.4. The classified bands were then separated into unique elevational bands using the reclassification method using zonal geometry tool in ArcGIS 10.4. The reclassified data was used as an overlay data for extracting various environmental datasets (outlined in the section below) and to create a map of the study area.

3.2.4 Environmental variables

Climatic variables

Seven climatic variables that may influence species richness patterns of amphibians were investigated in this study: Mean annual temperature (MAT), Mean annual precipitation (MAP), Potential evapotranspiration (PET), Annual evapotranspiration (AET), Moisture index (MI), Isothermality and water-energy dynamics (WED). The high resolution datasets developed by CHELSA (Climatologies at high resolution for Earth’s land surface areas) (Karger et al. 2017a, b) having a resolution of 30 arc seconds (1 km^2 grid) were used for extraction of the climatic variables data for the study sites. This dataset consists of a total of 19 bio-climatic variables amongst which only MAT, MAP and Isothermality were used. The values of MAP and MAT for consecutive elevation bands (300m) in the study sites were obtained by averaging the grid values falling into each band by using the reclassified DEM dataset as an overlay boundary in ArcGIS 10.4. The other variables were derived using standard equations from the values of MAT and MAP as follows:

PET is considered a measure of ambient energy (Hawkins & Porter, 2003). Moisture related variables are considered as potential factor for amphibian distribution. PET and MI was calculated following Holdridge et al (1971) and Bhattarai et al (2004)-

$PET = T \text{ x } 58.93$

where T is the absolute temperature >0°C

$MI = PET/MAP$

Likewise, AET is assumed to be a measure of ambient energy (Hawkins & Porter, 2003) and it was calculated using Turc's formula (1954)-

$AET = P/ [0.9+ (P/ L)\ ^{2}]^{1/2}$ with $L = 300 + 25T + 0.05T3$

where

P = mean annual precipitation

T = mean annual temperature

Water –energy dynamics is regarded as an important variable considering the physiology of amphibians (Vetaas et al, 2019). The bivariate WED was calculated as-

$WED = MAP + PET + PET^{2}$

Canopy cover

Canopy cover (%) of the vegetation was visually estimated at 2 m radius in each sampling site following Khatiwada et al (2019) due to its potential importance in shaping amphibian composition .

Normalized Difference Vegetation Index (NDVI)

The Normalized Difference Vegetation Index (NDVI) is often regarded as an important predictor of amphibian species richness (Radeloff et al, 2019; Vasconselos et al, 2019). The Normalized Difference Vegetation Index (NDVI) for three years (2016-2018) of Landsat8 imagery data (30 m resolution) available from USGS (https://earthexplorer.usgs.gov) for the study sites was used as a surrogate for primary productivity (Levanoni, 2011). The red and near-infrared imagery data was averaged for three years and NDVI was calculated using the formula –

NDVI = (Near Infrared- Red) / (Near infrared +Red) in ArcGis 10.4.

The final output of the NDVI which consisted of values in each pixel was averaged for each elevational band.

3.2.5 Data analysis

In the Rangeet valley, sampling sites in the elevation zone above 3300 m could not be surveyed due to logistical issues whereas in the Teesta valley the elevation zone till 4800 m - 5100 m was sampled. In order to make the two valleys comparable for amphibian richness patterns and factors driving such patterns, five elevation zones (i.e. 3300-3600 m, 3600-3900 m, 3900-4200 m, 4200-4800 m, 4800-5100 m) from the Teesta valley were not included for further analysis. The total species richness recorded within an elevational band during the study was considered as the observed species richness. As there may be discrepancies in the actual number of species present during sampling, estimators provide reliable estimates of species richness (Hortal, 2006). Non parametric species richness estimators were estimated using software EstimateS version 9.1.0 (Colwell, 2005). The Chao 2 estimator is assumed to be precise and less sensitive to rare species and patchiness of species distributions (Hortal, 2006). Also, non parametric estimator such as Jackknife 2 takes into consideration the unique species (Gotelli & Colwell, 2001). The completion of sampling was assessed by generating species accumulation curves for each elevational band in respective valleys using observed and estimated (Chao 2 and Jackknife 2) species.

Since the relationship between estimates of richness and elevation is not necessarily linear, quadratic function was used in the model to evaluate response variables (observed richness, Chao 2 and Jackknife 2) with elevation and selected the best fit-curve based on r^2 and p values. Additionally, multivariate analysis was performed to explore the potential factors for the elevational species richness patterns in amphibians of Teesta valley and Rangeet valley. Since all measures of species richness showed similar pattern (details in the result section) I only used observed species richness in multivariate analysis as a dependent variable. Prior to performing any analysis, I calculated the variance inflation factors (VIF) of nine predictor (independent) variables (MAT, MAP, PET, AET, MI, Isothermality, WED, NDVI and Canopy cover) in these models to account for multicollinearity of datasets. In order to reduce the multicollinearity, only those predictor variables with VIF<10 were selected (Hair et al, 1995). Generalized linear modeling (GLM) with a log link function assuming Poisson distribution error was used to investigate the relationship of selected variables with amphibian species richness in each valley. In the Teesta valley, four variables AET, NDVI, Isothermality and Canopy cover showed less multicollinearity (VIF<10) amongst the nine predictor variables mentioned above . Therefore, AET, NDVI, Isothermality and Canopy cover were considered for further analysis in GLM. Correspondingly in the Rangeet valley, three variables namely MAP, NDVI and Canopy cover exhibited low multicollinearity out of the nine predictor variables. Consequently, MAP, NDVI and Canopy cover were regarded as significant factors responsible for amphibian species richness in the Rangeet valley. Subsequently, I selected the best fit model based on the lowest AICc value (corrected Akaike Information Criterion). Models showing a difference of AICc<2 from the best model are regarded as equivalently possible (Burnham & Anderson, 2002). All the statistical analysis were conducted in R environment (R core team 2019) using the packages *ggplot2* (Wickham, 2016*), vegan, glmulti* (Calcagno & de Mazancourt, 2010) and *MuMin* (Barton, 2013).

3.2.6 Mid-domain Null Model

In order to test the influence of physiographical constraints or the mid-domain effect on total species distributions of the Teesta valley and Rangeet valley, we used Monte Carlo simulation (Mid-Domain Null program; McCain, 2004). This program employs empirical range sizes or range midpoints within two hard boundaries and simulates species richness curves based on analytical stochastic models (Colwell & Lees, 2000; McCain, 2004). To evaluate the effect of spatial constraints, 95% prediction curves were generated based on 50,000 simulations (without replacement) using empirical range sizes at 100 m interval. The difference between the lowest and highest elevational observation of each species was used to determine the range size of species. Some species had an elevational range of zero due to single- site observation. Therefore, to avoid the loss of species during randomization of the mid points, species recorded in a single site were assigned a 100m range (±50m of the recorded elevation), presuming the occurrence of species within this range (Stevens, 1992). To examine the potential contribution of geometric constraints on amphibian species richness, the observed empirical values was regressed with the values of simulated species.

To assess whether the distribution of amphibians along the elevation gradients of Teesta and Rangeet valley follow Rapoport's elevation rule (Stevens, 1992), I performed correlation analysis between elevational range size with lower and upper distribution limit of each species. This analysis is also helpful in understanding the increase or decrease of range size along elevation gradients.

3.3 Results

3.3.1 Species richness and abundance of amphibians

In the Teesta valley, a total of 1188 man-hours of visual encounter survey were conducted (Table 3.1). I recorded a total of 1725 individuals of amphibians representing 25 species and 11 genera under six families. *Dicroglossidae* is the most dominant family with nine species followed by *Ranidae, Megophryidae* and *Rhacophoridae* each with four species, *Bufonidae* with three species and *Ichthyophiidae* with only one species (Fig. 3.1). The elevation zone above 3300 m consisted of only two species (i.e. *Scutiger sikkimensis* and *Scutiger boulengeri*).

Table 3.1. Elevational zones sampled in Teesta and Rangeet valley, Sikkim Himalaya with respective sampling effort, species richness and abundance

Elevation zone	**Elevation (m)**	**Teesta valley**			**Rangeet valley**		
		Sampling effort (Visual Encounter Survey in hours)	**Species richness**	**Abundance**	**Sampling effort (Visual Encounter Survey in hours)**	**Species richness**	**Abundance**
Zone I	300-600	104	9	167	121	8	436
Zone II	600-900	96	12	165	118	9	265
Zone III	900-1200	98	12	231	116	11	294
Zone IV	1200-1500	103	11	263	112	10	539
Zone V	1500-1800	102	13	221	107	14	750
Zone VI	1800-2100	106	9	128	102	13	532
Zone VII	2100-2400	102	9	126	98	10	282
Zone VIII	2400-2700	107	6	74	96	7	116
Zone IX	2700-3000	94	7	82	84	3	66
Zone X	3000-	88	3	54	66	1	3

	3300						
Zone XI	3300-3600	71	2	30	54	8	436
Zone XII	3600-3900	47	2	30	48	9	265
Zone XIII	3900-4200	30	2	28	18	11	294
Zone XIV	4200-4500	12	1	25	-	-	-
Zone XV	4500-4800	16	1	60	-	-	-
Zone XVI	4800-5100	12	1	41	-	-	-

Likewise, I conducted a total of 1140 man-hours of visual encounter survey in the Rangeet valley (Table 3. 1) and a total of 3283 individuals of amphibians representing 22 species, 10 genera and five families were recorded. *Dicroglossidae* is the most dominant family with nine species, followed by *Ranidae* with five species, *Bufonidae* and *Rhacophoridae* with three species each and *Megophryidae* with two species (Fig 3.1).

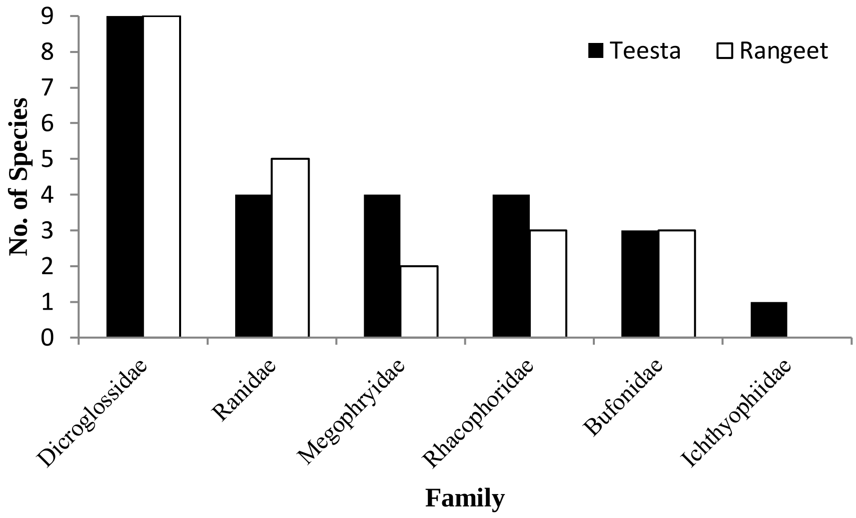
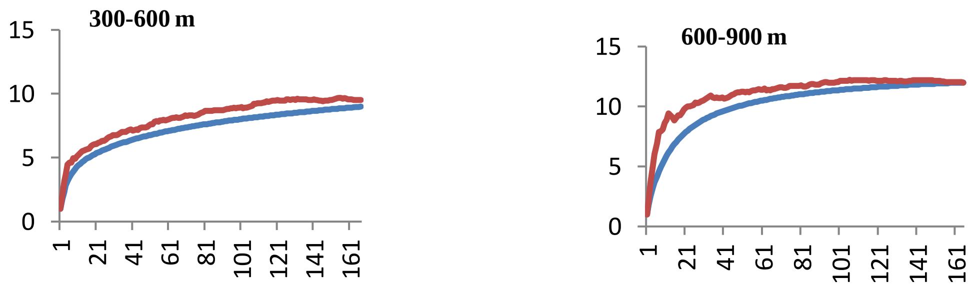


Fig. 3.1. Familywise amphibian species observed in the Teesta and Rangeet valley, Sikkim Himalaya

3.3.2. Elevational patterns of species richness

The species accumulation curves for the Teesta valley approached an asymptote for almost all the elevational bands indicating adequate sampling and only very few species would be added with increased sampling (Fig 3.2).



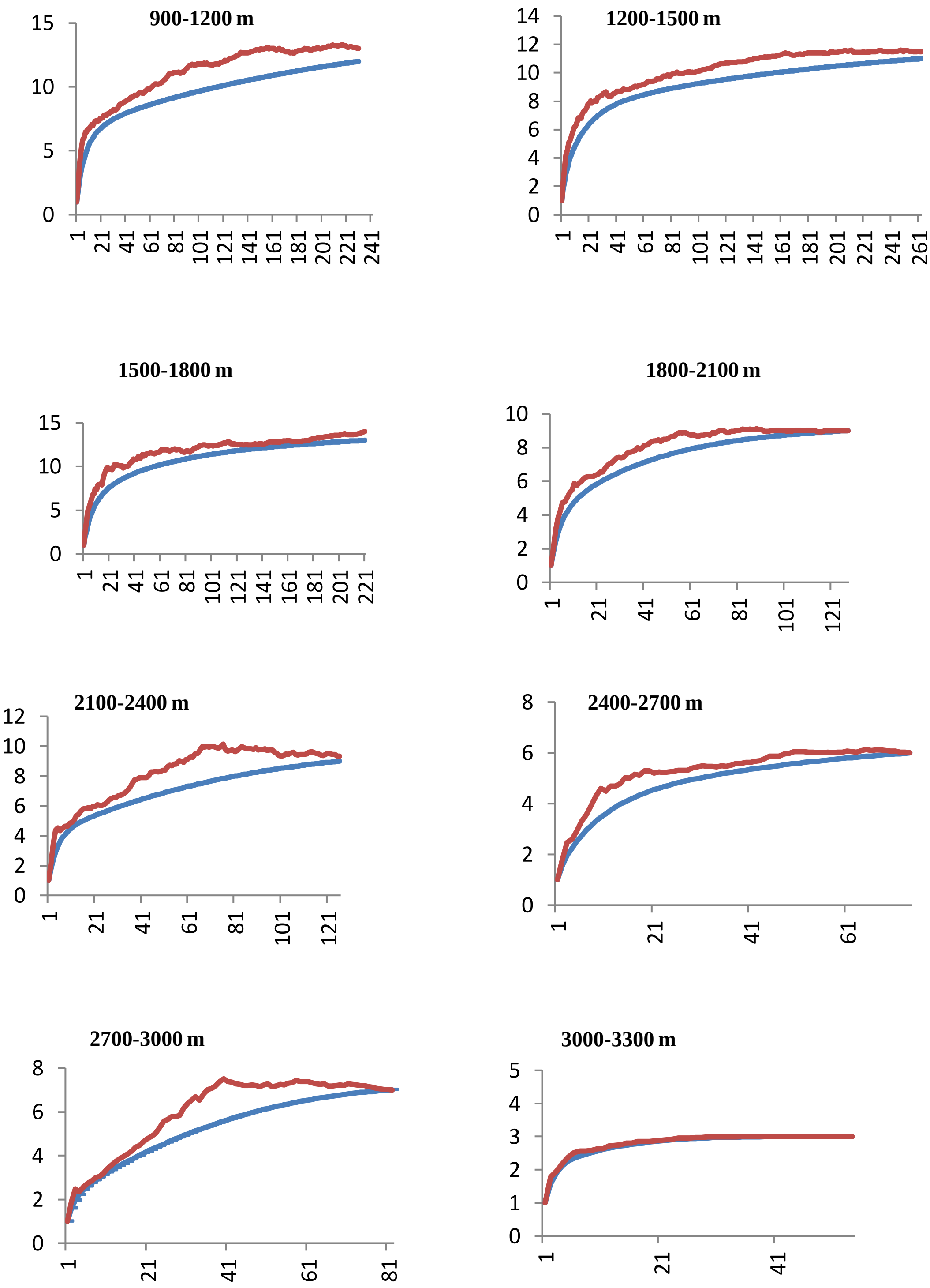
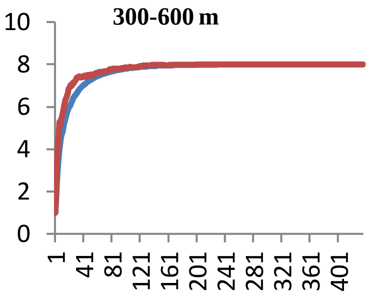
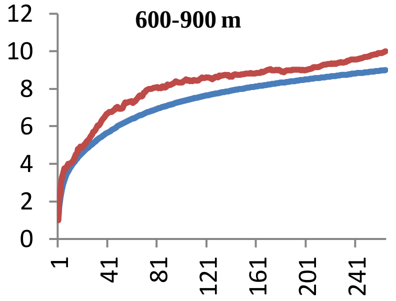
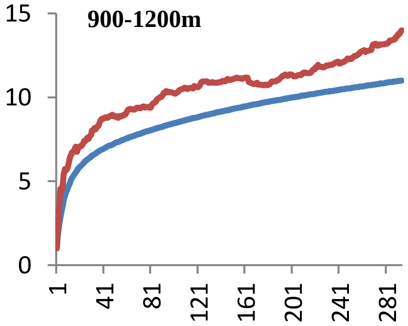


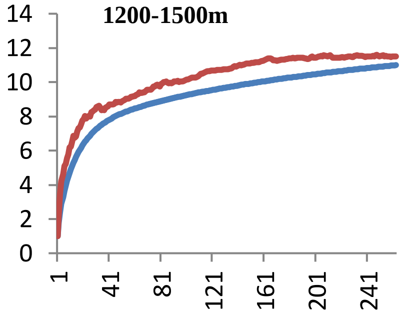
Fig 3.2. Species accumulation curves of amphibians observed at different elevations (in m above sea level) in Teesta Valley, Sikkim, Eastern Himalaya. The blue line represents observed richness, the red represents Chao2 estimates. x axis= Total number of point counts, y axis= Species richness

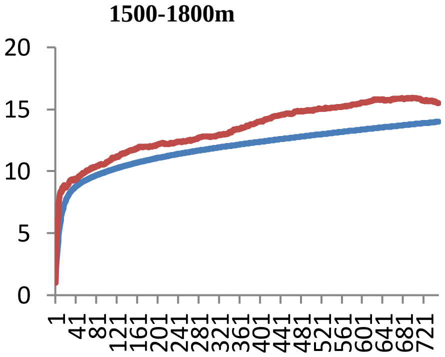
However, for the Rangeet valley the species accumulation curve approached an asymptote for all the elevational bands except 2100-2400 m suggesting more species could be observed with further sampling, however, the number of species added would be considerably less (Fig 3.3).

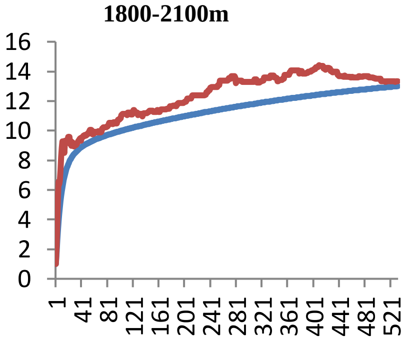












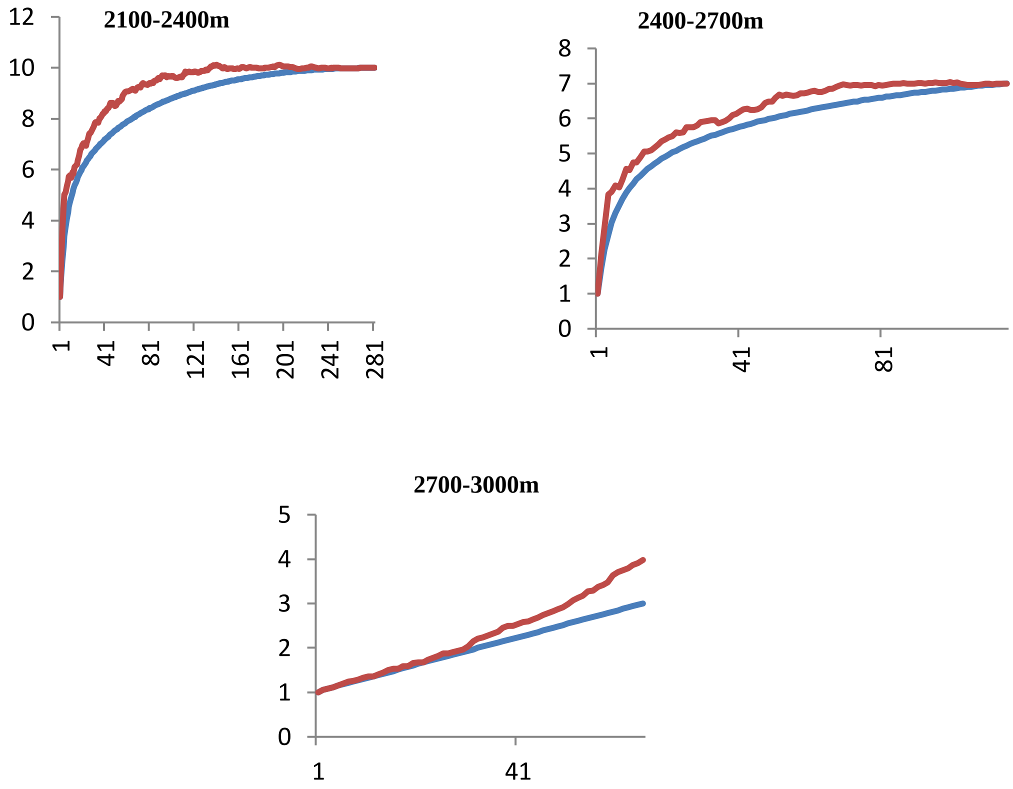
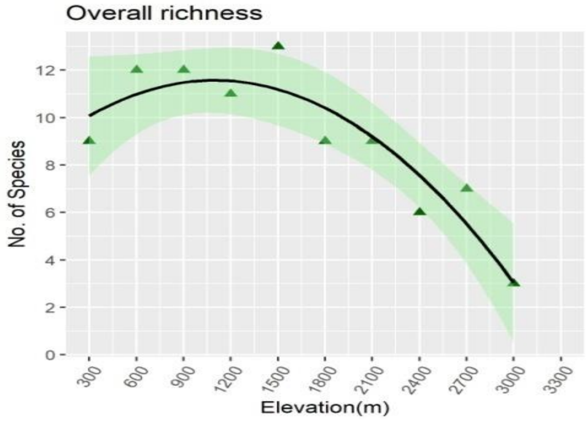
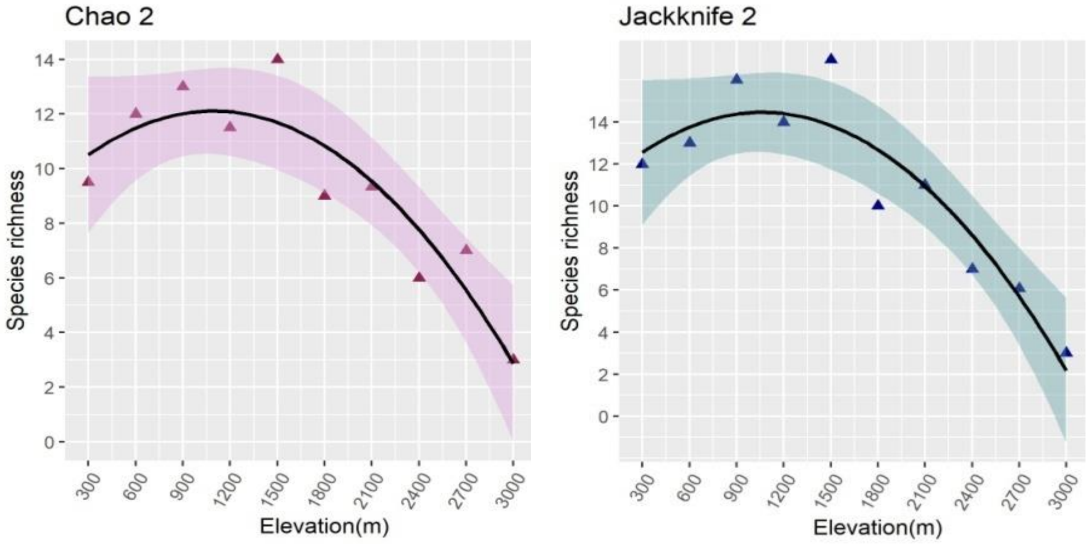


Fig 3.3. Species accumulation curves of amphibians observed at different elevations (in m above sea level) in Rangeet Valley, Sikkim, Eastern Himalaya. The blue line represents observed richness, the red represents Chao2 estimates. x axis= Total number of point counts, y axis= Species richness.

The observed species richness of amphibians showed a hump shaped pattern in both Teesta and Rangeet valley with high species richness upto 1800m after which the number of species gradually declined (Fig 3.4 & Fig 3.5). Concordantly, the highest number of species was observed between 1500-1800 m elevational band with a distinct peak at 1500 m in both the valleys. In the Teesta valley the estimated species richness (Chao 2) also exhibited a mid-elevation peak pattern with a declining trend at higher elevations with highest species richness between 1500 m -1800 m ($R^{2\,=}\,0.79$; $p \leq 0.01$) (Fig 3.4b). Likewise, the Jackknife 2 confirmed the hump shaped pattern of amphibian distribution with decreasing richness at higher elevations ($R^2 = 0.82$; $p \leq 0.01$) with a distinct peak at 1500-1800 m (Fig 3.4c).



a. Observed Species richness

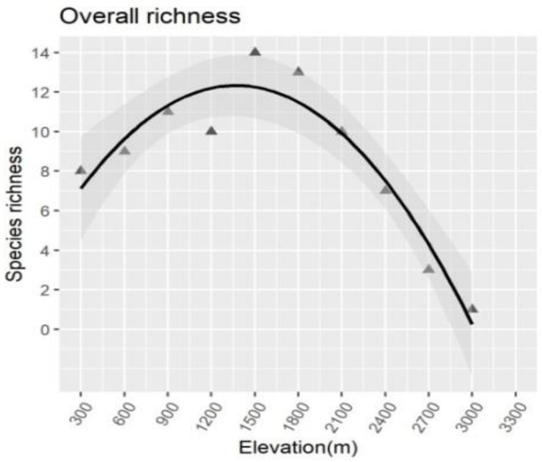


b. Chao 2 estimated richness

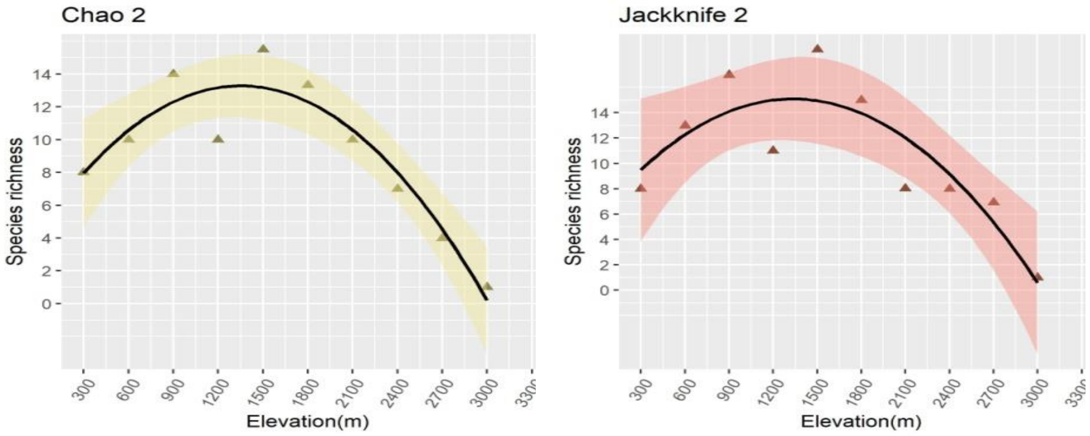
c. Jackknife 2 estimated richness

Fig 3.4. Species richness pattern of amphibians along the elevation gradient of Teesta valley, Eastern Himalaya
*Shaded areas along the line represent 95% confidence interval

Similarly in the Rangeet valley the non-parametic Chao 2 ($R^2 = 0.85$, $p\leq 0.01$) and Jackknife 2 ($R^2 =0.84$, $p\leq 0.01$) also showed a mid- elevation peak at 1500-1800 m elevation band (Fig 3.5b and 3.5c).



a. Observed species richness



b. Chao 2 estimated richness

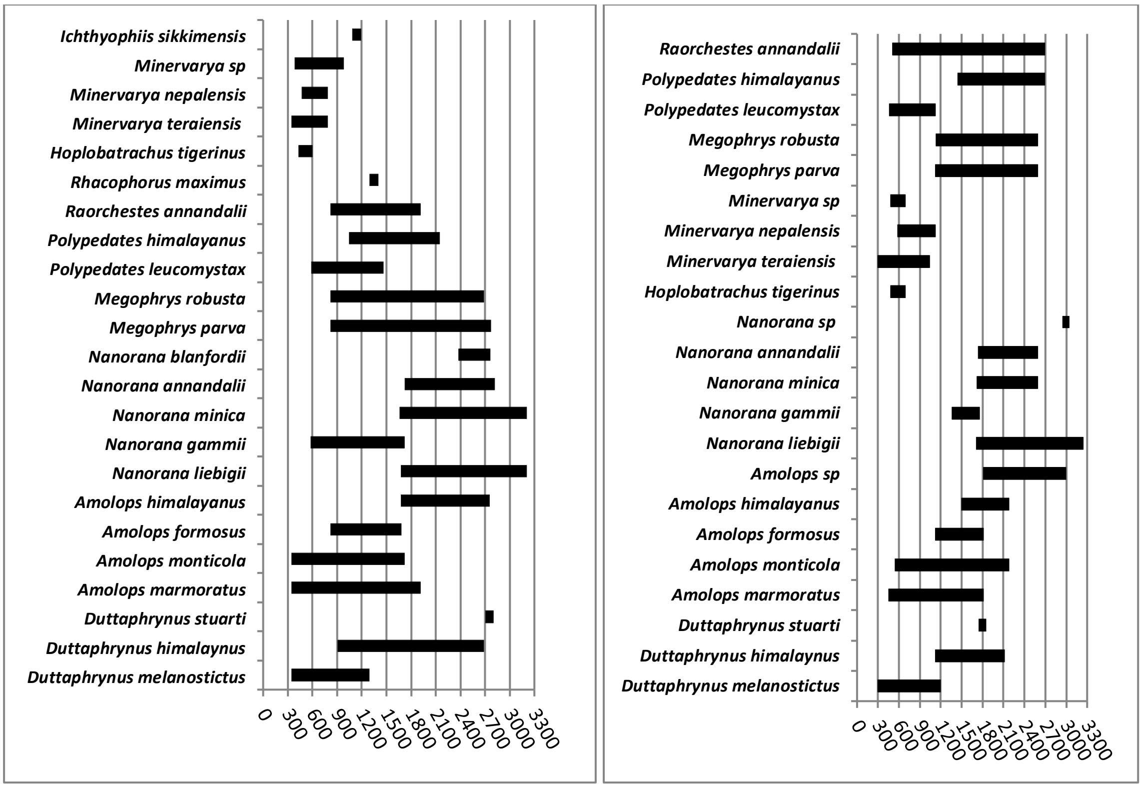
c. Jackknife 2 estimated richness

Fig 3.5. Species richness pattern of amphibians along the elevation gradient of Rangeet valley, Eastern Himalaya

*Shaded areas along the line represent 95% confidence interval

3.3.3 Elevational range size distribution

In the Teesta valley, majority of the amphibians exhibited a narrow distribution range along the elevation gradient (Fig 3.6 a), with 17 species (74%) occupying a narrow elevational width (elevational width less than 1500 m) and seven species showing an elevational width more than 1500 m. Eight species were restricted below 1500m while only two species extended their ranges beyond 3000m (Fig 3.6a). A significant correlation between the range size and upper limit of the distribution range of amphibians of the Teesta valley was observed ($r = 0.59$; $p < 0.01$), whereas no statistically significant correlation was detected between the range size and lower limit of the distribution range. Similarly, in the Rangeet valley the majority of amphibian species showed a narrow elevational distribution range with 14 species (64%) having a narrow elevational width (<1100 m), seven species (32%) occupying mid-elevational width (1100-2100 m) and only one species exhibited wider elevational width (>2100 m) (Fig. 3.6b). Similar to Teesta valley, only six species were restricted below 1500 m and only two species extended their range beyond 3000 m. A statistically significant correlation between range size and upper limit of the distribution range of amphibians was found ($r = 0.53$, $p = 0.01$), however, no significant correlation was found between the range size and the lower limit of the distribution range of amphibians of Rangeet valley.



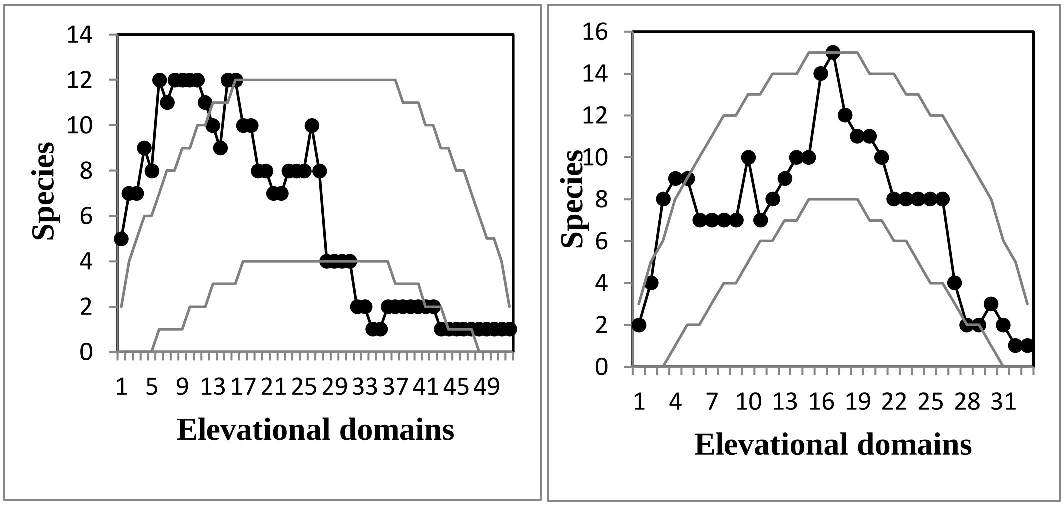
a. b.

Fig 3.6. Elevational range distribution profile of amphibians in Teesta valley (a) and Rangeet valley (b), Sikkim Himalaya.

3.3.4 Factors governing elevational distribution pattern of amphibians

Mid domain effect

As the emperical richness showed low elevation plateau, mid-domain effect was not supported for amphibians in the Teesta Valley. More than half of the emperical points (31 out of 51 i.e. 60%) fell outside the 95% prediction curve (Fig 3.7a) obtained from 50,000 simulations. The mid domain effect was not significant in shaping the species richness in the Teesta valley and indicated a poor fit to the null model when empirical richness and mean of simulated richness were regressed ($R^2 = 0.02$; $P = 0.25$). However, the geometric contraints played significant role in determining the species richness patterns of amphibians in the Rangeet valley. More than 80% (27/33) of the empirical points occurred within 95% prediction curve indicating good fit of emprirical richness with simulated data (Fig. 3.7b). Regression of empirical richness with mean of the simulated richness was significant ($R^2 = 0.66$; $p < 0.01$) indicating a good fit to the null model suggesting crucial role of mid domain effect in defining species richness patterns in the Rangeet valley.



a. b.

Fig 3.7. Mid domain effect null model using Monte Carlo simulation in the Mid domain Null Program. Species richness patterns of amphibians along the elevation gradients of Teesta valley (a) and Rangeet valley (b), Sikkim Himalaya. The empirical species richness (black solid lines with data points) and the 95% confidence intervals of prediction curves (grey lines) are shown.

3.3.5 Ecological determinants of patterns of species richness

In the Teesta valley 16 candidate models were generated in the GLM, however, two sets of models having the lowest AICc best predicted the overall amphibian distribution (Table 3.2). AET appears to be the best explanatory variable for substantial variation in total species richness patterns of amphibians along the elevation gradients in the Teesta valley as suggested by model averaged inference of the two most likely models (Table 3.3).

Table 3.2 Best fit generalized linear models depicting relationship between overall species richness of amphibians with the selected variables along the elevation gradients in the Teesta and Rangeet valley, Sikkim Himalaya. Models with the lowest AICc and a difference ofΔAICc< 2 from the top models are shown

		Models	**AICc**	**ΔAICc**	***wi***
Teesta valley	Species richness	~1+ AET	59.47	0	0.337
		~1+ AET+ Canopy cover	68.11	2.79	0.008
Rangeet valley	Species richness	~1+ MAP+ NDVI+ Canopy cover	58.61	0	0.064
		~1+ MAP + NDVI	62.56	3.95	0.022

Correspondingly, in the Rangeet valley, a total of 16 candidate models were generated out of which two sets of best models with the lowest AICc explained the overall amphibian distribution (Table 3.2). Model averaged inference of the two potential models indicated MAP and Canopy cover as the best explanatory variables for considerable variation in species richness of amphibians along the elevation gradients in Rangeet valley (Table 3.3).

Table 3.3. Summary of model averaged estimates of predictor variables using multimodal inference in explaining variation in species richness of amphibians along the elevation gradients in Teesta and Rangeet valley.

		Variables	**Estimate**	**Std Error**	**Z value**	**P-value**
Teesta valley	Species richness	**AET**	**0.007**	**0.003**	**2.243**	**<0.001****
		Canopy cover	0.011	0.010	0.911	0.024
Rangeet valley	Species richness	**MAP**	**0.004**	**0.001**	**7.110**	**<0.001****
		NDVI	0.315	0.248	1.051	0.293
		Canopy cover	**0.002**	**0.001**	**3.721**	**<0.001****

3.4 Discussion

3.4.1 Species richness patterns

The present study examined the species richness pattern of amphibians along the elevation gradients in two major river valleys (Teesta and Rangeet valley) in Sikkim, Eastern Himalaya. A total of 27 species were recorded in the study including two unidentified species. While previous studies have documented the amphibian species richness pattern along the elevation gradient of Teesta valley (Chettri & Acharya, 2020), the same has not been explored in the Rangeet valley elevation gradient. Moreover, distribution patterns of amphibians comparing two elevation gradients in the same landscape have hitherto not been conducted in the eastern Himalayan elevation gradient. This study showed a hump shaped pattern of amphibian species richness in both Teesta and Rangeet valley which is concordant with previous studies in the region (Chettri & Acharya, 2020). However, the elevational band size considered for analysis in this study (300 m) is at a finer resolution than the previous study which considered 500m band . In the Himalaya, mid elevation peak pattern has been reported in various other taxa such as trees (Oommen & Shanker, 2005; Acharya et al, 2011; Rana et al, 2019; Sharma et al, 2019), ants (Bharti et al, 2013), birds (Acharya et al, 2011a; Wu et al, 2013a; Acharya & Vijayan, 2017) mammals (Wu et al, 2013b; Hu et al, 2017; Hu et al, 2018). Some studies suggest that amphibian species richness decreases monotonically along elevation because of larger areas at lower elevations providing suitable habitats for amphibians to occur (Khatiwada et al, 2019). However, a higher number of studies indicate a mid-elevation peak pattern (Fu et al, 2006; Hu et al, 2011; Wang et al, 2020). Hence a hump shaped pattern with highest species richness at mid-elevations might be the general pattern of distribution of amphibians along mountain elevation gradient.

3.4.2 Elevational Range size distribution and Rapoport's rule

Majority of the amphibian species in both Teesta and Rangeet valley have narrow elevational range distributions. The narrow elevational distribution range indicates that amphibians are sensitive to rapid changes in environmental variables along elevation gradients and specilaise only in certain microhabitat types. As the temperature in a region increases, these specialist species may not be able to survive unless they disperse to higher elevations. Thus, such specilaist species display less climatic tolerance and may be vulnerable to declines or extinction considering the current global climate change. The climate induced elevational range shift has been reported in many taxa from the Himalya (Telwala et al, 2013; He et al, 2019; Li et al, 2016; Dahal et al, 2021; Girish & Srinivasan, 2022). Previous study have also reported narrow elevation range width of 76% of amphibian species in Sikkim (Chettri & Acharya, 2020). Species with narrow ranges are reported to be susceptible to extinction (Isik, 2011).

The Rapoport's rule hypothesizes that there is a positive correlation between elevation and the elevational range of species (Stevens, 1992). In both Teesta valley and Rangeet valley a significant correlation of elevation range with the upper limits of the elevation range was found which suggests that amphibian species occurring at higher elevations have wider ranges relative to species at lower elevations. Species at higher elevations are adapted to frequent variability in climatic conditions, hence have wider range size relative to species at lower elevations which experience approximately constant climatic regimes (Mamantov et al, 2021).

3.4.3 Mid-domain null model

Several studies have reported that the mid-domain effect substantially explains the species richness patterns along elevation gradients (McCain 2004; Bachman et al, 2004; Oomen & Shankar 2005; Kluge et al, 2006). In this study, roles of the geometric constraints in shaping richness patterns vary between gradients in the two valleys. The amphibian species richness in the Teesta valley did not fit the MDE null model and indicated deviation from the model which is in accordance with the previous study (Chettri & Acharya, 2020). Several other taxa along the elevation gradient in the same valley and other areas of the Himalaya did not show support to the MDE null model (Chettri et al, 2010; Acharya et al, 2011; Khatiwada & Haugaasen, 2015). However, in the Rangeet valley the amphibian species richness showed a good fit to the null model indicating that geometric constraints indeed contribute to richness patterns of amphibians. Some studies in the Himalaya have shown support to the mid domain null models (Fu et al, 2006; Shuai et al, 2017). With respect to the Teesta valley, the Trans Himalayan cold desert (Tibetan plateau) extends beyond upper side of the Teesta valley possessing no hard boundaries to the movement of animals. However, amphibians being ectothermic and having limited mobility are likely to be constrained by physiological tolerances rather than geographic boundaries. Likewise, in the lower side, the landmass of the Teesta valley continues towards the plains of West Bengal, India possessing no hard boundaries which is apparent from the high species richness in the lower elevations of the elevation gradient in Teesta valley. Hence, MDE does not account for species richness patterns of amphibians in the Teesta valley instead other environmental variables are likely the possible causal factors. In the Rangeet valley, the Khangchendzonga range starts from west Sikkim and hence the mountain ranges exist as a hard boundary in the upper side. However, similar to Teesta valley there are no hard boundaries in the lower side of Rangeet valley as it also continues with the plains of West Bengal in the lower elevation. The strong mid- domain effects in the Rangeet valley could be due to its narrower valley compared to Teesta. It may perhaps also be spurious and warrants more explorations for the mid-domain effect along the elevation gradient of Rangeet valley.

3.4.4 Environmental factors governing amphibian richness patterns

In this study, the findings indicate that amphibian species richness patterns along the Teesta and Rangeet valley are determined by different sets of factors. In the Teesta valley, among the seven variables MAT, MAP, PET, AET, MI, Isothermality and WED, actual evapo-transpiration (AET) was the most important in shaping amphibian distribution along the elevation gradient, whereas in the Rangeet valley, MAP and

Canopy cover were found to be the primary variables determining amphibian distribution along the elevation gradient. Both MAP and AET are identified as a key determinant of amphibian distribution along the elevation gradient of Eastern Himalaya (Chettri & Acharya, 2020). AET is a fundamental part of the hydrological cycle and amongst the significant physical processes in natural ecosystems. The exchange of water and energy between soil, land surface and the atmosphere is explained by AET (Ochoa-Sánchez et al, 2019). AET is considered to be the measure of water- energy balance (Rodriguez et al, 2005) and has been identified as one of the predominant predictor of amphibian species richness (Buckley &Jetz, 2007). MAP and AET are derived from precipitation and temperature which are known as principal factors for distribution of ectothermic species (Aragon et al, 2010; Pyron, 2014). AET is also considered a proxy of productive energy but is dependent on both solar (ambient) energy and water-energy balance. This combination is markedly relevant for amphibians that are sensitive to both ambient energy and moisture (Rodriguez et al, 2005; Buckley & Jetz, 2007). Amphibians are peculiar in habit because they are constrained both by water availability and temperature, and the former is frequently recognized as a key driver of distribution of amphibians (Rodríguez et al. 2005; Buckley & Jetz 2007; Whittaker et al, 2007). Amphibians, being ectothermic, are sensitive to temperature, however, rely increasingly on water availability for their general physiological functions and reproduction (Rodríguez et al, 2005).

In the Himalaya, species richness patterns of various taxa have been explained by the direct and indirect effects of AET (Acharya & Vijayan, 2015; Vetaas et al, 2019; Dewan et al, 2021, 2022). It is asserted that warm and wet climates are more favorable for species diversity in mountainous regions (Wiens & Graham, 2005). The mid-elevations of the Teesta valley and Rangeet valley possess suitable climatic conditions (warm and wet) thus harbor higher amphibian richness. Humidity is crucial for species such as amphibians which are sensitive to desiccation. Likewise, the physiological, behavioral and ecological functioning of amphibian species is largely affected by temperature (Navas et al, 2008). Environmental temperatures limit mobility and energy acquisition in amphibians (Bennet, 1990). In ectotherms, interspecific interactions, resource utilization and rates of energy use is influenced by environmental temperatures (Bennet, 1990).

In the Rangeet valley, canopy cover was a strong predictor of amphibian species richness along the elevation gradient. This valley encompasses the southern part of the Khangchendzonga National Park (KNP) which is characterized by pristine dense forests and vegetation. The protected areas in the mid-elevations of Rangeet valley has helped retain the natural forests and canopies thereby maintaining conducive habitats for amphibians. Moreover, lower elevation of the Rangeet valley are increasingly disturbed (Dewan et al, 2019) which may explain high amphibian richness at mid-elevations. Many studies have reported that amphibian species are affected by the degree of canopy cover (Halverson et al, 2003; Skelly & Golon, 2003). Previous studies have also found significant effects of canopy cover on species composition of amphibians along elevation gradients (Zhu et al, 2020). Canopy cover is associated with forest structure and affects the transpiration rates, microclimates and the amount of rain reaching the soil (Wanger et al, 2010). Canopy cover is also related with the oviposition rates of anurans (Pizzini, 2007) and protects eggs against desiccation and predation (Hazell et al, 2001). The decrease in canopy cover has also been reported to reduce the vagility of amphibians which is crucial at their stage of life when they need to move to newer water bodies for reproduction (Cushman, 2006). Canopy cover is assumed to regulate the micro-climate and structural heterogeneity of vegetation by providing a greater diversity of microhabitats and resources which reduces competition for resources thereby facilitating coexistence (Urbina-Cardona & Perez-Torres 2002). Canopy cover shields the near-ground areas from solar radiation and decreases the mixing of air which in turn modulates the day and night temperature and humidity levels (Von Arx et al, 2013).

The difference in the factors determining amphibian richness patterns along the elevation gradients of Teesta valley and Rangeet valley may possibly be due to the presence of hard boundary (Mt. Khanchendzonga) in the latter causing a strong mid-domain effect (MDE). However, it is suggested that at smaller spatial scales MDE alone cannot predict variations in species diversity and other (biological and environmental) explanations are necessary (Dunn et al, 2007). Therefore, MDE alone is not likely to drive richness patterns but can act in conjunction with environmental variables such as MAP and canopy cover which is high at mid elevations. This suggests that trends in species richness may vary among gradients in the same region and could be regulated by landscape features combined with local environmental variables rather than factors that are considered to be important for large scale patterns of diversity. Therefore, gradient specific studies are necessary to understand species distribution patterns and their potential determinants.

3.5. Conclusion

This study examined the amphibian species richness pattern and potential determinants in two river valleys in Sikkim, Eastern Himalaya. Amphibian species richness in the Teesta valley and Rangeet valley displayed a hump shaped pattern along the elevation gradient. However, the abiotic and biotic factors shaping this distribution pattern varied between the valleys. The combined effect of temperature and precipitation is the key driver of amphibian distribution in Sikkim regardless of the valley which indicates the vulnerability of these ectothermic species to changing climatic regimes. It is estimated that 41% of amphibians are threatened with extinction (Hoffman et al, 2010) which is the highest percentage amongst all other vertebrate taxa (Stuart et al. 2004; Wake & Vredenburg 2008). In recent years, many studies have reported potential range shifts in amphibians in response to climate change (Sillero 2010; Acharya & Chettri, 2012; Iosif et al, 2014). Himalaya being one of the most sensitive zones, the impact of global climate change is extremely apparent (IPCC, 2014). Pristine forests in Sikkim are under tremendous anthropological pressure due to rapid population growth and developmental projects such as hydro power plants and pharmaceutical industries which adversely impact amphibian habitats and, hence, their population. In Sikkim, most of the protected areas are above 2000 m despite 34% of the total geographical area being under protection (Forest, environment and Wildlife Management Department, 2020). However, only the occurrences of larger vertebrates were acknowledged for notification of protected areas neglecting the diversity of smaller vertebrates which are mostly species rich in low to mid elevations. Therefore, the present study highly advocates the extension of protected area network in the lower and mid - elevations taking into consideration the diversity of smaller vertebrates such as amphibians.

CHAPTER 4

ECOLOGICAL STUDY OF MICROHABITAT ASSOCIATIONS OF AMPHIBIANS OF SIKKIM HIMALAYAS

4.1. Introduction

Ecologists have long been fascinated to understand the distribution, diversity and abundance of species based on the differential resource use by species (Toft, 1985). The differential use of resources by species was termed as “resource partitioning” (Schoener, 1974). According to Pianka (1975), there are three conventional categories of resource dimensions: habitat, food and time. However, considering the ecological differences between organisms, these categories can be further sub-divided into six categories: macrohabitat, microhabitat, food type, food size, diel time and seasonal time (Schoener, 1977). Based on the available habitat heterogeneity, species partition resources in a variety of way. Study of resource utilization pattern helps to understand community structure such as species diversity, abundance and coexistence (Tokeshi, 2009).

Though, the niche concept has been very popular and important in community ecology, there is much debate on the concept of niche (McInerny & Etienne, 2012; Soberon, 2014). Many authors defined niche in various context in the past (see Grinnell, 1917; Hutchinson, 1957), it was Pianka (2000), who defined ecological niche as “the sum total of all adaptations of a species” to occur in their environment and is used in many different ways. Niche breadth of a species refers to the set of environmental conditions and resources in the broadest sense, that a species can inhabit or use (Gaston & Blackburn, 1996). Species with wider niche breadth have better tolerance to range of environmental conditions (Evans et al, 2011) than species with narrow niche breadth as the conditions become harsher and less predictable (Korner, 2003). Slatyer et al (2013) suggested that niche breadth-range size relationship is a general ecological pattern among different taxa. Moreover, measuring niche breadth (food and habitat) is important to assess the ability of a species to subsist in cases of habitat alteration or climate change (Swihart et al, 2003). Generally, many species share the same resources, hence there may be overlap in resource use patterns at various levels. The concept of ecologically similar species likely being phylogenetically related has been widely accepted (Losos, 2008). The phenomenon of closely related species to be more similar than expected under randomness is termed as phylogenetic niche conservatism (Blomberg & Garland, 2002). It posits that ecological niches evolve slowly through time resulting in closely-related species having similar environmental preferences (Wiens, 2010). "Niche overlap" is the extent to which two organisms use similar resources in one or more dimensions (Noon, 1981). Communities with higher resource sharing or greater niche overlap will support more species than that with less niche overlap (Pianka, 1974). The closely related species compete strongly under natural conditions which often results in efficient resource partitioning (Toft, 1985). Therefore, partitioning of available resources among species in an ecological community determines the diversity of coexisting species (Griffin & Silliman, 2011). The interaction of many biotic and abiotic factors determines species coexistence in a region (Chase, 2003). According to the coexistence theory, competing species coexist at similar sites achieved by some form of resource partitioning and interspecific competition can promote niche differentiation as a mechanism enabling species coexistence (Chesson, 2000). For a given resource, competition may occur between individuals of the same species (intraspecific competition) or between different species (interspecific competition). Assemblage structure can either be explained by historical or evolutionary changes that affected interactions of species over time or by current interactions (Thorpe et al, 1994; Vitt & Zani, 1996). The current competitive abilities of species which also retain ancestral differences may affect partitioning of resources (Vitt & Pianka, 2000). Consequently, among phylogenetically distant species, the historical effects would be highest and interactions would be insignificant whereas historical effects would be lowest and interactions predominant among phylogenetically related species (Vitt & Pianka, 2005; Bellini et al, 2015).

Microhabitats generally play an important part as resources that are partitioned between ecologically similar species (Luiselli, 2006; Zainudin et al, 2017). Among the vertebrates, amphibians exhibit high species richness with 8381 species distributed worldwide (Frost, 2021). Amphibians display fine-scale heterogeneity depending on the microhabitats they inhabit thus their high diversity and endemism can be attributed to their microenvironment (Thorpe et al, 2018). Species richness and distribution of amphibians is influenced considerably by microhabitat availability which indicates that microhabitats positively impact species assemblages (Santos-Barrera, 2011; Balaji et al, 2014; Figueiredo et al, 2019). The spatial niche seems to be one of the most important primary factors governing the relationships among individuals in frog assemblages (Eterovick et al, 2010; Hodl, 1977; Toft, 1985). The utilization of varied microhabitats thus permits the dynamic utilization of the habitat and access to different resources (Abrunhosa et al, 2014; Cardoso et al, 1989; Duré & Kehr, 2004). Thus, specific microhabitat preferences, species interactions and possible niche-partitioning in the microhabitat dimension can explain the amphibian assemblage structure and diversity. The ecological role of body size plays a vital role in structuring a community (Khatiwada et al, 2019) as it provides information about animals life history (Gouveia & Correia, 2016) and extinction risks (Cooper et al, 2008). Besides, the microhabitat use can also vary with the body size of the individuals (Lynch & Duellman, 1997). Given the wide range of variation in body size of anurans, there is an opportunity of exploring the relationship between microhabitats and body size which affects many other ecological traits (LaBarbara, 1986). Moreover, amphibians are highly sensitive to habitat alterations which are one among many factors responsible for the global declines in amphibian populations (Wake & Vredenburg, 2008). Therefore, information on microhabitat resource partitioning of amphibians may be useful for conservation management and also to see response of amphibians towards changing or altered habitat.

Hitherto, studies on microhabitat of amphibians have mainly focused on the Salamanders (Crawford et al, 2008), or on pond breeding amphibians (Brodman et al, 2002; Burne et al, 2005; Earl et al, 2015). In India, studies on amphibian community structure and microhabitat associations have been sporadic and mostly limited to southern part of the country (Ganesh & Arumugam, 2015; Thorpe et al, 2018; Vasudevan et al, 2001). However, studies on niche partitioning and species coexistence of amphibian communities in India as a whole and Eastern Himalaya in particular are sporadic.

Further, Sikkim is recognized as the hotspot of biodiversity located in the Eastern Himalayan region and a key priority for conservation (Banerjee et al, 2019). Considering amphibians as globally vulnerable data deficient vertebrate group, it is of paramount importance that this gap in ecological data is prioritized and explored. Many species of amphibians of Sikkim Himalaya are listed as data deficient or not evaluated category in IUCN redlist criteria. (IUCN, 2021). Moreover, with rapid developmental and anthropogenic activities such as construction of dams, quarrying and establishment of series of pharmaceutical industries on the river bank in Sikkim, amphibian species associated with streams and rivers are under threat (Chettri & Acharya, 2020). The stream overbank areas which are often flooded by water during peak monsoon season are most vulnerable to mining at different scales to meet the demands of the construction industry (Chettri et al, 2011). Such extraction of sand, gravel and boulders which serves as potential microhabitat of amphibians of Sikkim poses substantial threat to the population. Species that rely on rocky outcrops are at a risk of local extinction due to gradual degradation of such critical microhabitats. Thus, management schemes should include restoration and retention of rocky outcrops on the riverbanks for conservation of species associated with such microhabitats (Michael et al, 2015). Hence, given the various threats to the ecologically fragile mountains of Sikkim including habitat fragmentation, global warming and numerous micro and mega hydrothermal projects, that urgency is acute. The present study attempts to examine the resource partitioning in the microhabitat dimension structuring amphibian communities in Sikkim Himalaya and effect of body size on microhabitat use. This study on the microhabitat association of amphibians of Sikkim will be useful as a baseline to formulate conservation programs to safeguard survival of these highly vulnerable and threatened taxa.

4.2 Methods

4.2.1 Study Area

Field studies were conducted from June 2016 to September 2019 with intensive sampling during monsoon season from May to August when the species are actively breeding. Amphibian species were sampled across elevation gradient covering different habitat types in the Teesta and Rangeet valley of Sikkim Himalaya. Sikkim (ca. 7096 sq. km, 27° 5' - 28° 9' N and 87° 59' - 88° 56' E) is a small Himalayan state of North East India, bordered by three international borders - Nepal in the West, Tibet in the North, Bhutan in the East and one national border with the state of West Bengal in the south. The eastern Himalaya, including Sikkim and North Eastern parts of India is one of the biodiversity hotspots of the world (Mittermeier et al, 2005). Sikkim experiences hot and humid weather during summers, heavy rainfall during the monsoon season and cold and dry weather in the winters. The interplay of tropical, temperate and alpine climatic factors; the admixture of Palaearctic and Oriental elements, young geological rock formation and several streams of different order have created varieties of niches to harbor astounding degree of biodiversity in such a small area of land.

Teesta and Rangeet Valley

The largest river of Sikkim, Teesta, is a major physical feature of the state which originates from the glaciers of Tso Lhamu lake (4800 m above sea level) in North Sikkim and descends steeply traversing wide variations in altitude (300-4800 m), climate (tropical-arctic) and vegetation types (Alpine- Tropical deciduous) (Chettri & Acharya, 2020). The river Rangeet is a major tributary of Teesta which originates from Rathong glacier in West Sikkim (Ali, 1962) and confluences with the river Teesta at the border between Sikkim and West Bengal. Both Teesta and Rangeet are fed by snow and rain that accumulate in the catchments during monsoon. The rivers of Sikkim are perennial and do not dry out even in winters thus possessing ideal habitats for developing tadpoles. Teesta and Rangeet valley of Sikkim Himalaya harbor rich biodiversity but recent rampant developmental activities and expanding human settlement has lead to loss of natural habitats of flora and fauna, and amphibian species are perhaps the most affected due to their sensitive physiological requirements and dual mode of life.

4.2.2 Field Investigation

Field sampling was conducted from June 2016 - September 2019 with rigorous sampling during May-August of all the years considering the breeding activity of amphibian species and highest probability of sightings. A total of 140 sampling points in the Teesta valley and 137 sampling points in the Rangeet valley were sampled. Considering the rough terrain and huge gradient of elevation to be covered along the valleys of Teesta and Rangeet river, time constrained visual encounter survey (VES) method (Heyer et al, 1994) was employed for sampling amphibians. Sampling was carried for 3-4 hours by two personnels wherein all probable microhabitats such as inside leaf litters, under boulders, inside tree hollow, tree trunks, etc were carefully inspected in particular area. Since most amphibians are either nocturnal or crepuscular, VES was supplemented with nocturnal stream surveys. The stream survey was carried out from dusk (6:00 pm onwards) to early night hours (upto 9:00 pm) with the help of search lights without disturbing the microhabitats. For each observations, data on the type of microhabitat used by species was recorded and were assigned to one of the 10 categories, namely: on rocks = OR (including above and under rocks and rock crevices), inside water = IW (including mud puddles and water pools), under boulders = UB (including above boulders), under logs = UL (including fallen trees, logs), on shrub stem = OS (on top or beneath stems/ branches), open ground = OG (including grass and paddy fields), on herbs = OH, inside mud = IM, artificial water points= AP (including under metal pipes, man-made probable habitats etc) and leaf litter = LL. The rocks and boulders were classified on the basis of size (mm) with size >300mm considered as rocks and size <300mm as boulders. These categories were used to calculate the niche breadth, niche overlap based on their occurrence records in each microhabitat type. Standard morphometric measurements for body size was taken as SVL (Snout- Vent Length: tip of the snout to posterior margin of the vent) using a digital vernier caliper (Mitutoyo, accuracy: ±0.02mm).

4.2.3. Data analysis

Niche breadth was calculated based on the relative frequency of use of different microhabitats by different amphibian species. The status of amphibian species of Sikkim was ascertained based on IUCN red list (IUCN, 2021).Niche breadth (β) for microhabitat was calculated using Simpson's diversity index.

$$\beta = 1/\sum pi^2$$

Where, pi is the proportion of individuals of species in i^{th} microhabitat category.

The elevational range size of the species was calculated as the difference between the highest and lowest elevation of amphibian occurrence in the sampling points. The relationship between microhabitat niche breadth and elevational range size was explored to see if the species with wider niche breadth also has wide elevation range.

Niche overlap (Pianka's overlap index) between amphibian species was calculated in the microhabitat dimension. EcoSim (Version 7.0) was used for niche overlap analysis (Gotelli & Entsminger, 2000). Niche overlap analysis was carried out between amphibian species to see the overlap in resource use patterns. The amphibian species with single observations i.e. singletons were excluded from niche overlap as well as niche breadth analysis.

Permutational multivariate analysis of variance (PERMANOVA) and non-metric multidimensional scaling (NMDS) was performed on Bray–Curtis dissimilarity to visualize and understand the variation between the different microhabitat community assemblages. Point of occurrence in each microhabitat type was used for NMDS ordination. In the NMDS ordinate system, microhabitats that are closer to each other are more likely to represent ecologically similar species than more distant ones. Permutational multivariate analysis of variance (PERMANOVA) was performed to test the differences in species composition at the microhabitat level. All statistical analysis was performed in R software version 3.4.3 using *vegan* (Oksanen et al, 2016) and *betapart* (Baselga et al, 2018) packages. Furthermore, to see the phylogenetic signal of niche overlap, the phylogenetic distance of 11 pairs of species were calculated from 12S and 16S sequence data of 11 species retrieved from GenBank database (The remaining 13 species were excluded as the 12S and 16S sequence data for those species were unavailable and also two species were unidentified). The sequences were aligned in multiple sequence alignment program Clustal Omega (Sievers et al, 2011). The alignment file was converted to FASTA format using BioEdit software (Hall, 1999). The pairwise phylogenetic distance between the species was computed using MEGA 11.0.8 (Kumar et al, 2018). The niche overlap values of the 11 species pairs were correlated with their pairwise genetic distance. Additionally, the effect of bodysize on the number of microhabitats used by each species was tested by simple linear regression.

4.3. **Results**

4.3.1 **Species composition**

A total of 4762 individuals belonging to 27 species, 11 genera, six families under two orders of amphibians were recorded in the study. In the Teesta valley, 1532 individuals belonging to 25 species were recorded. Maximum species observed belonged to the family *Dicroglossidae* (*n* = 9 species) and only one species was observed from the family *Ichthyophidae* in the Teesta valley. In the Rangeet valley, 3230 individuals belonging to 22 species were recorded. Similarly, the family *Dicroglossidae* represented the highest number of species (*n* = 9) and the minimum number of species was observed from the family *Megophryidae* in the Rangeet valley.

Amphibian species composition between Teesta and Rangeet valley showed high similarity (Sorensen Index, Cs = 0.85) with five species (*Nanorana blanfordii, Scutiger sikkimensis, Scutiger boulengeri, Rhacophorus maximus and Ichthyophis sikkimensis*) unique to Teesta valley and two species (*Nanorana* sp *and Amolops* sp) unique to Rangeet valley. These two species were encountered only once during the entire sampling period whereby the species could not be ascertained and was identified only upto genus level (Table 4.1).

In the present study, out of the 4762 sightings of amphibians in both Teesta and Rangeet valley, nearly, 35.26% sightings comprised of only two amphibian species namely *Amolops marmoratus* (*n* = 978 individuals, 20.54%) and *Amolops monticola* (*n* = 701 individuals, 14.72%). Three species, *Ichthyophis sikkimensis*, *Nanorana* sp and *Amolops sp* and *Rhacophorus maximus* were singletons (i.e. observed only once during the sampling).

Table 4.1. Amphibian species observed in Teesta and Rangeet valley of Sikkim Himalaya with their niche breadth values (β), no. of microhabitat used by species, BODYSIZE (SVL in mm), IUCN status and range size (m)

Sl. No.	Species	Teesta Valley	Rangeet Valley	No. of observations	Niche Breadth (β)	No. of recorded microhabitats (N=10)	IUCN Red list category	SVL (mm)	Elevation range size (m)
	Order: *Anura*								
	Family: *Bufonidae*								
1.	*Duttaphrynus melanostictus*	✓	✓	223	3.79	8	LC	107.2	988
2.	*Duttaphrynus himalayanus*	✓	✓	439	3.91	8	LC	119.0	1771
3.	*Duttaphrynus stuarti**	✓	✓	3	1.80	2	DD	68.8	1054

	Family: ***Ranidae***								
4.	*Amolops marmoratus*	✓	✓	978	1.80	8	LC	88.6	1562
5.	*Amolops monticola*	✓	✓	701	1.40	7	LC	64.5	1830
6.	*Amolops formosus*	✓	✓	11	1.81	4	LC	61.3	982
7.	*Amolops himalayanus**	✓	✓	222	1.62	7	NE	84.7	1254
8.	*Amolops* sp	✗	✓	2	2.00	2	-	68.2	1188
	Family: ***Dicroglossidae***								
9.	*Nanorana liebigii*	✓	✓	374	3.04	8	LC	123.8	1564

10.	*Nanorana gammii**	✓	✓	194	2.59	7	NE	67.7	1176
11.	*Nanorana minica**	✓	✓	15	2.53	3	VU	41.4	1537
12.	*Nanorana annandalii**	✓	✓	9	2.45	3	NT	48.3	1093
13.	*Nanorana blanfordii*	✓	✗	26	1.55	2	LC	43.5	1012
14.	*Nanorana* sp	✗	✓	1	-	1	-	42.2	100
15.	*Hoplobatrachus tigerinus*	✓	✓	35	2.31	4	LC	79.6	261
16.	*Minervarya teraiensis*	✓	✓	109	2.80	6	LC	38.3	745
17.	*Minervarya nepalensis*	✓	✓	60	2.53	6	LC	33.8	656

18	*Minervarya* sp	✓	✓	35	2.46	3	-	35.6	598
	Family: *Megophryidae*								
19.	*Megophrys parva*	✓	✓	388	1.98	7	LC	53.8	1941
20.	*Megophrys robusta**	✓	✓	420	1.33	8	DD	88.9	1856
21.	*Scutiger sikkimensis**	✓	✗	15	1.74	3	NE	63.1	1489
22.	*Scutiger boulengeri*	✓	✗	55	1.00	1	LC	61.3	1544
	Family: *Rhacophoridae*								
23.	*Polypedates leucomystax*	✓	✓	23	2.39	5	LC	79.8	997
24.	*Polypedates*			36	2.99	6			

	*himalayanus**	✓	✓				NE	81.4	1648
25.	*Raorchestes annandalii*	✓	✓	386	1.31	8	LC	17.7	2188
26.	*Rhacophorus maximus*	✓	×	1	-	1	LC	93.9	100
	Family: *Ichthyophiidae*								
27.	*Ichthyophis sikkimensis*	✓	×	1	-	1	DD	112.9	100

4.3.2 Microhabitat Use

The most recorded microhabitat for amphibian species (43.83%) was on rocks (OR) with 22 species and 2087 observations followed by leaf litter (LL = 28.20%) with 19 species and 1343 observations, on stem (OS = 10.94%) with 18 species and 521 observations. While minimum number of species was found in artificial water points (AP = 0.06%) with two species and three observations and inside mud (IM = 0.78%) with one species and 17 observations (Figure 4.1).

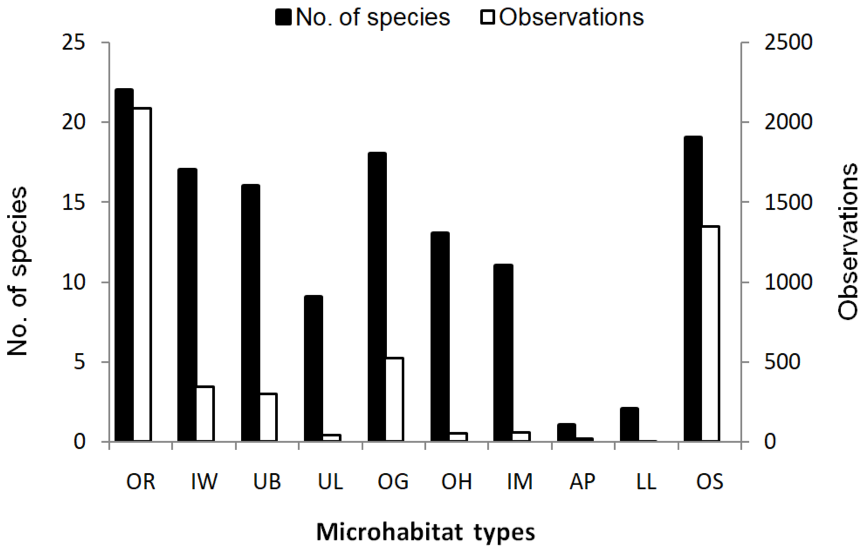


Fig 4.1. Number of species and observations of amphibians found in different microhabitat types: OR- On rocks; IW- Inside water; UB- Under boulders; UL- Under logs; OS- On shrubs/stem/branches; OG- Open ground; OH- On Herbs; IM- Inside mud; AP- Artificial water points; LL- Leaf Litter.

4.3.3 **Niche Breadth**

Microhabitat niche breadth (β) values of amphibians in Sikkim Himalaya ranged from 1.00 to 3.91 (Table 1). We removed species with less than two observations (*Rhacophorus maximus*, *Ichthyophis sikkimensis* and unidentified *Nanorana* sp and *Amolops* sp) for niche breadth analysis. Mean niche breadth values were highest for the family *Bufonidae* (*n* = 3 species, β = 3.17), followed by *Dicroglossidae* (*n* = 10 species, β = 2.47), *Rhacophoridae* (*n* = 4 species, β = 2.23), *Ranidae* (*n* = 5 species, β = 1.73) and *Megophryidae* (*n* = 4 species, β = 1.51). The Himalayan toad *Duttaphrynus himalayanus* occupied 8 out of 10 microhabitats and had the highest niche breadth (3.91) whereas *Scutiger boulengeri* occupied only one microhabitat and had the lowest niche breadth (1.00). Additionally, the threat status of each species was ascertained using IUCN redlist. A total of 14 (58.3%) amphibian species were categorized as least concern (LC), 4 (16.7%) species were not evaluated (NE), 2 (8.3%) species were data deficient (DD), 2 (8.3%) species required further taxonomic identification hence we could not ascertain their threat status, 1 (4.2%) species each was listed as near threatened and vulnerable. A total of 8 amphibian species (33%) listed as either data deficient, not evaluated, near threatened or vulnerable were classified as specialists (Table 4. 1).

4.3.4 Niche breadth- range size relationship

Species with wider niche breadth is expected to correlate positively with elevational range size as they are able to utilize an array of resources. However, the correlation between microhabitat niche breadth and elevational range size was not statistically significant (r = 0.15; p = 0.448).

4.3.5 **Niche Overlap**

Niche overlap values in the microhabitat dimension ranged from 0 to 1 (Figure 4.2, Table 4.2). After removing three species with less than two observations, 24 species was considered for niche overlap analysis from a total of 27 species. The pairwise niche overlap analysis in the microhabitat dimension resulted in 276 pairs (Table 4.2).

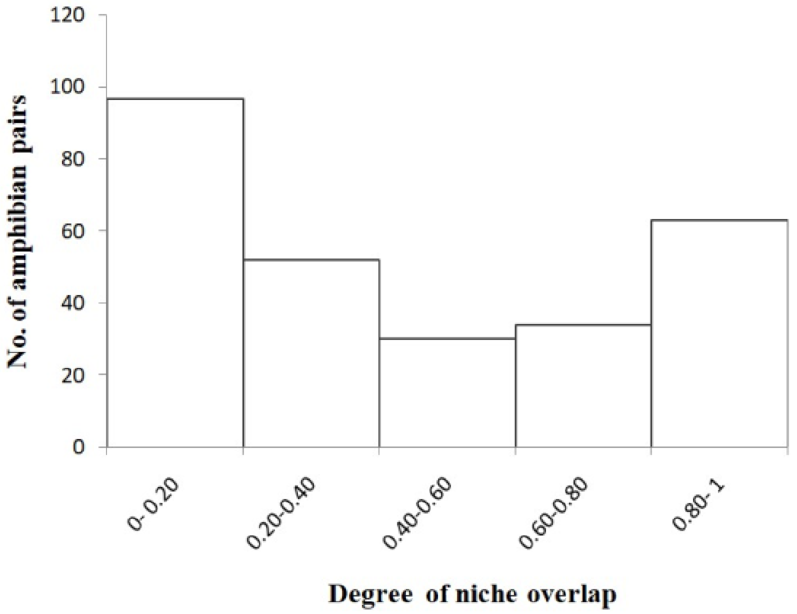


Figure 4.2. Degree of niche overlap in the microhabitat dimension of amphibian pairs of Sikkim.

Among them, a total of four pairs exhibited complete overlap, congeneric species such as *Amolops monticola* showed complete overlap (niche overlap value = 1.00) with *Amolops marmoratus* and *Amolops himalayanus*. Similarly, *Raorchestes annandali* showed complete overlap with *Megophrys robusta* while *Minervarya teraiensis* with *Minervarya* sp (unidentified sp). A total of seven pairs showed no overlap (niche overlap value = 0), *Duttaphrynus stuarti* showed no overlap with *Nanorana* spp (*Nanorana annandalii* and *Nanorana blanfordii*) and both species of Scutiger (*Scutiger sikkimensis* and *Scutiger boulengeri*). Similarly, *Hoplobatrachus tigerinus* showed no overlap with *Nanorana minica*, *Scutiger boulengeri* and *Amolops* sp (Table 4.2).

Table 4. 2. Microhabitat niche overlap of amphibian species in Sikkim Himalaya

Species	*Dm*	*Dh*	*Ds*	*Am*	*Amnt*	*Af*	*Ah*	*Nl*	*Ng*	*Nm*	*Na*	*Nb*	*Mp*	*Mr*	*Ss*	*Sb*	*Pl*	*Ph*	*Ra*	*Ht*	*Mt*	*Mn*	*M sp*	*A sp*
Duttaphrynus melanostictus (Dm)		0.11	0.66	0.26	0.19	0.26	0.22	0.47	0.21	0.20	0.40	0.65	0.11	0.06	0.29	0.16	0.39	0.57	0.07	0.95	0.95	0.98	0.94	0.13
Duttaphrynus himalayanus (Dh)			0.42	0.19	0.15	0.18	0.14	0.10	0.50	0.24	0.09	0.10	0.87	0.90	0.09	0.07	0.80	0.65	0.91	0.07	0.08	0.12	0.07	0.68
Duttaphrynus stuarti (Ds)				0.15	0.06	0.16	0.05	0.04	0.23	0.09	**0.00**	**0.00**	0.44	0.45	**0.00**	**0.00**	0.81	0.94	0.48	0.78	0.76	0.69	0.76	0.32
Amolops marmoratus (Am)					**1.00**	0.99	0.99	0.86	0.93	0.70	0.89	0.31	0.45	0.23	0.96	0.98	0.23	0.26	0.18	0.10	0.39	0.18	0.37	0.78
Amolops monticola (Amnt)						0.99	**1.00**	0.86	0.92	0.70	0.89	0.29	0.42	0.19	0.96	0.99	0.16	0.18	0.14	0.03	0.32	0.11	0.29	0.76
Amolops formosus (Af)							0.98	0.82	0.91	0.65	0.85	0.28	0.44	0.22	0.95	0.97	0.23	0.27	0.18	0.11	0.39	0.17	0.37	0.78
Amolops himalayanus (Ah)								0.88	0.90	0.69	0.91	0.33	0.38	0.15	0.97	0.99	0.13	0.16	0.10	0.05	0.34	0.14	0.31	0.73
Nanorana liebigii (Nl)									0.80	0.79	0.99	0.64	0.32	0.11	0.88	0.83	0.11	0.15	0.08	0.24	0.48	0.38	0.45	0.60
Nanorana gammii (Ng)										0.76	0.82	0.29	0.74	0.55	0.85	0.87	0.49	0.43	0.51	0.04	0.30	0.14	0.27	0.94
Nanorana minica (Nm)											0.81	0.18	0.48	0.29	0.62	0.64	0.28	0.27	0.25	0.00	0.22	0.08	0.17	0.60
Nanorana annandalii (Na)												0.58	0.33	0.10	0.90	0.87	0.08	0.12	0.05	0.17	0.43	0.31	0.39	0.62
Nanorana blanfordii (Nb)													0.11	0.04	0.45	0.29	0.02	0.03	0.02	0.46	0.51	0.66	0.51	0.20
Megophrys parva (Mp)														0.97	0.33	0.33	0.86	0.69	0.96	0.03	0.13	0.10	0.11	0.90
Megophrys robusta (Mr)															0.10	0.10	0.88	0.07	**1.00**	0.01	0.04	0.08	0.04	0.77
Scutiger sikkimensis (Ss)																0.97	0.08	0.93	0.05	0.11	0.37	0.20	0.34	0.68
Scutiger boulengeri (Sb)																	0.07	0.10	0.05	0.00	0.29	0.08	0.27	0.71

Species	***Dm***	***Dh***	***Ds***	***Am***	***Amnt***	***Af***	***Ah***	***Nl***	***Ng***	***Nm***	***Na***	***Nb***	***Mp***	***Mr***	***Ss***	***Sb***	***Pl***	***Ph***	***Ra***	***Ht***	***Mt***	***Mn***	***M sp***	***A sp***
Polypedates leucomystax (Pl)																		0.94	0.89	0.42	0.42	0.40	0.42	0.67
Polypedates himalayanus (Ph)																			0.71	0.63	0.64	0.58	0.64	0.54
Raorchestes annandalii (Ra)																				0.04	0.05	0.09	0.05	0.74
Hoplobatrachus tigerinus (Ht)																					0.95	0.96	0.95	**0.00**
Minervarya teraiensis (Mt)																						0.95	**1.00**	0.21
Minervarya nepalensis (Mn)																							0.95	0.09
Minervarya* sp *(M* sp*)																								0.19
Amolops* sp *(A* sp*)																								

4.3.6 Community Assemblage

Non-metric multidimensional scaling plot (NMDS) (stress = 0.097) indicated overlap in amphibian community composition in the microhabitat dimension (Fig 4.3). Though majority of the amphibian species assemblages overlapped, however, this might be due to the wider use of different types of microhabitat by generalist species which can utilize wider range of microhabitat types. Amphibian species with similar requirements at the microhabitat scale tend to cluster together. Congeneric species from the *Amolops* group (*Amolops marmoratus*, *Amolops monticola and Amolops formosus*), *Nanorana* group (*Nanorana annandalii*, *Nanorana liebigii* and *Nanorana minica*) and *Scutiger* group (*Scutiger sikkimensis* and *Scutiger boulengeri*) clustered together. Species from the genus *Megophrys* (*Megophrys parva* and *Megophrys robusta*) clustered with *Rhacophorid* species (*Polypedates himalayanus*, *Polypedates leucomystax*, *Rhacophorus maximus*, and *Raorchestes annandalii*). However, *Nanorana* sp did not cluster with the other amphibian species and exhibited unique microhabitat requirement (Figure 4.3). *Nanorana* sp was observed at an altitude of 3000m on vegetation surrounding ephemeral water body during the monsoon season.

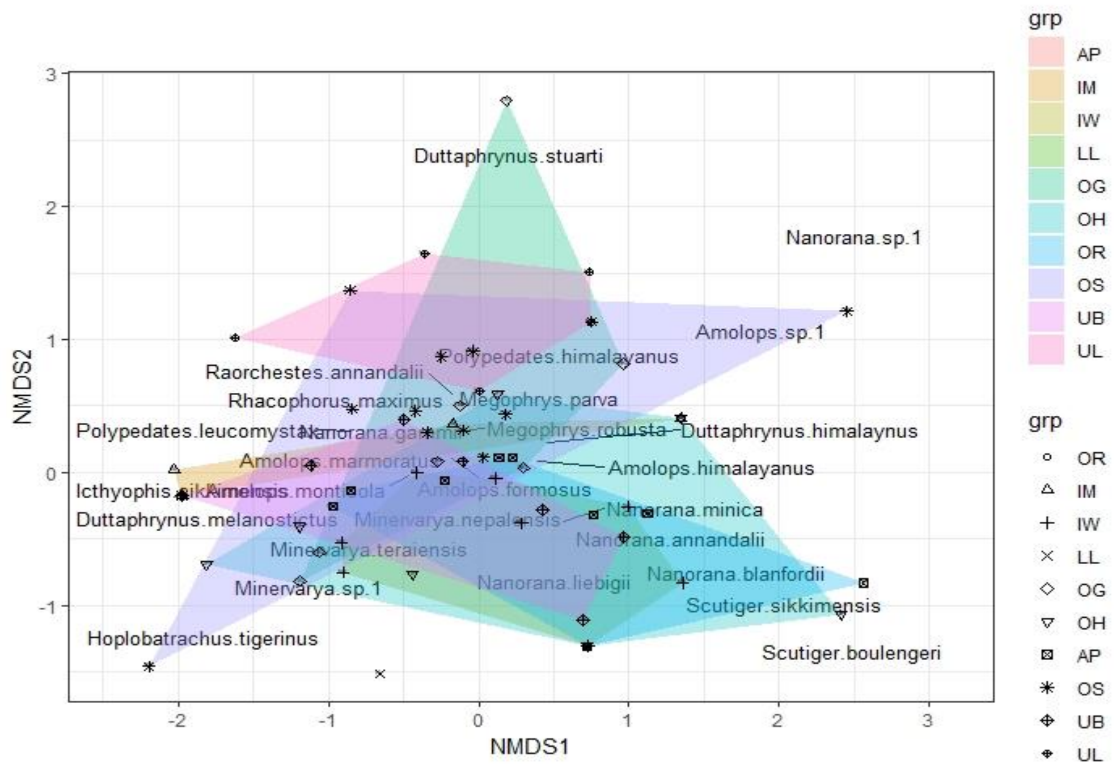


Fig 4.3. Non-metric multidimensional scaling (NMDS) showing differences in species composition between microhabitats (based on Bray–Curtis dissimilarity).

However, PERMANOVA analysis indicated that microhabitats in general indeed have a significant effect on amphibian composition ($p < 0.001$) (Table 4.3) but it does not show the effect of specific microhabitat types which suggests that amphibian communities of Sikkim Himalaya uses diverse microhabitats.

Table 4.3. Results of PERMANOVA based on Bray–Curtis dissimilarity analyzing changes in community structure at the microhabitat level.

	d.f.	**SS**	**MS**	**Pseudo F**	**$R^{2a,b}$**
Microhabitat	9	6.1428	0.68253	1.7	0.221***
Residuals	54	21.6523	0.40097		0.779
Total	63	27.7951			1

Abbreviations: SS, sum of squares; MS, mean sum of squares.

[a] Significance value based on 999 permutations.

[b] Significance level*** $p<0.00$

4.3.7 Phylogenetic distance and niche overlap

The pairwise phylogenetic distance of 11 species showed that the least genetic distance was shown by congeneric species whereas the largest distance was shown by species belonging to different genus (Table 4.4). The microhabitat niche overlap values of these 11 pairs of amphibian species is negatively correlated to their phylogenetic distances ($r = -0.61$; $p = 0.05$).

Table 4.4. Pairwise genetic distance of amphibians of Sikkim from available 12S rRNA sequences retrieved from GenBank database, NCBI

GenBank Accn no. Use same font		***Megophrys robusta***	***Megophrys parva***	***Scutiger sikkimensis***	***Scutiger boulengeri***	***Duttaphrynus stuarti***	***Duttaphrynus melanostictus***	***Duttaphrynus himalayanus***	***Polypedates leucomystax***	***Amolops marmoratus***	***Nanorana liebigii***	***Hoplobatrachus tigerinus***
MH647513.1	*Megophrys robusta*											
MH406568.1	*Megophrys parva*	51.31%										
KR781486.1	*Scutiger sikkimensis*	24.17%	28.63%									
EF397273.1	*Scutiger boulengeri*	62.35%	26.08%	**7.03%***								
MW111526.1	*Duttaphrynus stuarti*	43.57%	61.72%	35.41%	61.35%							
KJ697684.1	*Duttaphrynus melanostict*	41.12%	36.34%	31.75%	40.08%	10.35%						

	us											
MW111525.1	*Duttaphrynus himalayanus*	36.19%	31.88%	33.98%	39.79%	9.94%	**5.79%***					
AB728168.1	*Polypedates leucomystax*	47.41%	67.45%	39.20%	59.93%	42.79%	37.47%	33.98%				
MG909645.1	*Amolops marmoratus*	54.83%	71.79%	44.79%	62.57%	71.62%	46.53%	40.31%	61.37%			
MN012005.1	*Nanorana liebigii*	67.31%	66.36%	43.35%	49.63%	57.78%	53.07%	51.36%	58.98%	27.31%		
AB290422.1	*Hoplobatrachus tigerinus*	63.73%	63.92%	34.93%	57.85%	68.51%	48.49%	43.20%	65.32%	29.63%	29.19%	

4.3.8 Effect of body size on microhabitat

Species with larger body size is expected to explore diverse microhabitats than smaller sized species. Simple linear regression of the body size on the number of microhabitats showed that that there is no statistically significant effect of body size of amphibian species on the number of microhabitats used ($R^2 = 0.00$; $p = 0.876$).

4.4 **Discussion**

The present study recorded 4762 individuals belonging to 27 species, 11 genera under six families which include two unidentified species, with 25 species in the Teesta valley and 23 species in the Rangeet valley, respectively. Additionally, two of three unidentified species are from the Rangeet valley. Chettri et al (2020) reported 25 species from the Teesta valley out of which the present study could record 20 species and Subba et al (2017) reported 23 species out of which 21 species was recorded in the present study. Three species (*Minervarya* sp, *Nanorana* sp and *Amolops* sp) were identified only upto genus level and needs further taxonomic assessment. Though the species richness was higher in the Teesta valley relative to the Rangeet valley, there was high similarity in community composition of amphibian species (Sorensen Index, Cs = 0.85) in both valleys which is probably due to the homogeneity in the vegetation and climatic conditions of the two valleys as a consequence of close proximity.

In the present study, rocks (OR) were the most used microhabitat by amphibian species of Sikkim Himalaya (n = 22), as the majority of the species are stream dwellers and inhabit torrential and perennial streams. These rocks serve as calling sites or as desirable oviposition sites for stream dwelling species during breeding season (Khan, 2014; Zhao, 2017) to prevent washing away by turbulent water (Fei et al, 2012). However, microhabitat use in amphibians depend on a number of factors such as temperature of shelter microhabitats (Seebacher, 2002), seasonal variation of moisture levels in environment (Seebacher, 1999), sexes and age classes (juvenile, metamorph, or adult) due to individual behavioral and resource requirements (Bartelt et al, 2004; Bull, 2006; Valdez et al, 2016). Refuge shelters such as under/above rocks usually tend to have higher temperature and humidity than the surrounding water or vegetation temperature which may also provide optimum temperature for ectotherms such as amphibians or reptiles. Leaf litter (LL) was found to be another microhabitat preferred by majority of the amphibian (28.20%, n = 19), presumably because leaf litters provide ambient humidity and substrate temperature. Chinchilla-Lemus (2020) reported that Prismantis frogs favor leaf litter microhabitat because leaf litter may provide optimum humidity required by the amphibians (Miyamoto, 1982). Leaf litter may also provide refuge from predators (Fauth et al, 1989).

Niche breadth analysis along microhabitat dimension revealed almost half (45%) of the amphibian species recorded in this study have low niche breadths. Low niche breadth value indicates that these species are specialist in their microhabitat requirement. In the IUCN Redlist, a total of eight species present in the study area are not evaluated, data deficient, near threatened or vulnerable category. Amphibian species such as *Polypedates himalayanus*, *Nanorana gammi*, *Scutiger sikkimensis* and *Amolops himalayanus* are distributed sporadically in the eastern Himalayan region and this study has also found that the latter two species also have narrow niche breadth which makes them highly specialized to the habitat types they occur. The IUCN red list is heavily biased towards large charismatic species and excludes species with small body sizes, limited distribution range and low dispersal abilities especially the local endemics (Cardoso et al, 2012), hence the threat status of many amphibian species are not evaluated. Similarly, *Duttaphrynus stuarti* and *Megophrys robusta* have low niche breadth and are listed as data deficient. However, data deficient species are found to be potentially endangered due to their low abundance and rare sightings or even be cryptic which makes it difficult to estimate their population trends (Parsons, 2016). The present study recorded only one sighting of *Ichthyophis sikkimensis* which is data deficient in the IUCN red list database. *Nanorana minica* and *Nanorana annandalii* are fairly common species and are locally abundant however they are geographically restricted which explains the categories (VU and NT) in the IUCN red list. These species are endemic to the Eastern Himalaya and have narrow distribution range and are habitat specialists. The ability of an organism to use a suite of microhabitat makes it more tolerant to a wide range of biotic and abiotic conditions, hence higher chance of tolerance to disturbances. While, specialists have higher susceptibility to habitat alteration and anthropogenic activities relative to generalists (Michael et al, 2015) since they are able to use only limited resources for their reproduction and survival. Habitat specialists are vulnerable to threats leading to population decline and extinction (Devictor et al, 2010; Murray et al, 2011; Sodhi et al, 2008). In the present study, three species were singletons while other three species were recorded less than 10 times, which reflects the rareness of these species in this region. Thus, understanding the causes of the rarity of these amphibian species in this region including data on their resource use patterns will aid in understanding the ecological requirement of the amphibian species of Sikkim Himalaya. The microhabitat niche breadth did not show significant relationship with range size of amphibian species as the values of niche breadth in the present study was taken from a single variable of number of microhabitats used. Perhaps, range size is rather influenced by dispersal abilities of amphibian species (Trakimas et al, 2016;

Penner & Rodel, 2019). In spatially heterogeneous environment, dispersion can facilitate niche separation as each species disperses to suitable habitats. In conditions where similar species have the same requirements, spatial heterogeneity in fitness permits coexistence with differing proportions of dispersers due to fluctuating conditions beneficial to dispersal (McPeek & Holt 1992; Holt & McPeek, 1996).

The co-utilization of resources in one or more dimensions by two species is known as niche overlap and such indices have been often used as estimators of competitions in ecology (Pianka, 1973). Species coexistence is often ensured by niche separation. Congeneric species (*Amolops monticola*, *Amolops marmoratus* with *Amolops himalayanus*) and (*Minervarya teraiensis* with *Minervarya* sp1) exhibited complete overlap presumably because these species belonging to their respective genus are of common descent and are thus more likely to be similar in ecological traits and may utilize similar resources (microhabitat in the present study). Similarly, closely related species such as *Scutiger sikkimensis* and *Scutiger boulengeri*, *Duttaphrynus melanostictus* and *Duttaphrynus himalaynus* showed a higher niche overlap in the microhabitat dimension with less genetic distance. This is also known as phylogenetic niche conservatism which is the tendency of lineages to retain ancestral ecological characteristics over time (Wiens & Graham, 2005). The high degree of niche overlap between congeneric species indicate that selective pressures may not be influential for complete species differentiation in microhabitat use while they may be significantly affected by phylogenetic constraints of species colonizing specific microhabitats. Previous studies on microhabitat use by amphibians have shown that phylogenetic relationships influence microhabitat utilization (Eterovick & Barros, 2003; Leite-Filho, 2017). Niche conservatism is applicable to a number of traits including those determining abiotic niches of a species such as thermal tolerance to that determining resource utilization such as diet microhabitat and other aspects of species interactions (Weins et al, 2010). However, to substantially progress the understanding of phylogenetic niche conservatism there is a need to use advanced tools such as Ecological Niche Modeling (ENMs), newly available phylogenies, databases of occurrence records and fine-scale environmental variables (McCormack et al, 2010; Sober´on, 2007).

According to Pianka (1973), high overlap in one dimension is often compensated by low overlap in other dimensions (food, time) thereby reducing competition. Hence, it is possible for similar species to co-exist in similar habitats. Coexistence of species has also been explained by competitive abilities of species and exclusion rate which posits that niche overlap (the extent of species sharing the same resources) reflects simple possibilities for exclusion and induces the process of competitive exclusion however, the differences in competitive abilities of species determines actual exclusion rate (Carmel et al, 2017). Under this premise, increased competition is likely between similar species with greater niche overlap whereas there is less competition between species with smaller niche overlap and hence no potential for competitive exclusion which leads to coexistence of species.

Amphibian species which are largely terrestrial (*Duttaphrynus* spp) or occur in paddy fields (*Minervarya* spp) exhibited either low or medium niche overlap with species occurring in torrential streams (*Amolops* spp) or in vegetation that are in close proximity of rapid streams (*Megophrys* spp) because these species have different types of adaptations to specific microhabitat. Studies incorporating additional data on resource use patterns in other dimensions will assist in understanding ecological prerequisites of amphibian species (Toft, 1985). Species occurring at higher elevations such as *Scutiger sikkimensis* and *Scutiger boulengeri* and the ones occurring at lower elevations have adapted to extreme climatic conditions whereas species such as *Nanorana minica*, *Amolops* sp and *Hoplobatrachus tigerinus* occur either at mid elevations or lower elevations (Chettri & Acharya, 2020). Thus, these species show no overlap in microhabitat niche as they are segregated along elevation gradients. Both species of *Scutiger* being resident of higher elevation have different physiological requirement than other amphibians of the study area (Subba et al, 2015). They were found along the edges of lakes or along the streams within thick moss covered boulders to protect them from freezing conditions prevailing in the area.

The high overlap of amphibian communities in NMDS plot (Figure 4) is perhaps due to shared resources by common generalist species such as *Duttaphrynus melanostictus*, *Duttaphrynus himalayanus* which occupy a wide range of microhabitats. Such extensive overlapping has also been supported by other studies (Zainudin, 2017). Additional factors such as availability of oviposition sites (horizontal positions) or exposure to direct sunlight (vertical positions) might affect the microhabitat use of amphibians and may help in partitioning microhabitat resources in order to co-exist. The species belonging to the genus *Amolops* (*Amolops marmoratus*, *Amolops monticola*, *Amolops formosus*) *Nanorana* (*Nanorana annandalii*, *Nanorana liebigii*, *Nanorana minica*) and *Scutiger* (*Scutiger sikkimensis* and *Scutiger boulengeri*) are clumped together probably because these species usually occur in water bodies such as streams and pools and use similar kinds of microhabitat. However, these species are segregated widely along the elevation gradient thus avoiding competition. Species such as *Scutiger boulengeri* and *Scutiger sikkimensis* occur only at elevations > 2500m inside water or under moss covered boulders in streams or glacier fed lakes. The *Megophryid* species (*Megophrys parva* and

Megophrys robusta) clustered with the *Rhacophorid* species (*Polypedates himalayanus*, *Polypedates leucomystax*, *Rhacophorus maximus*, and *Raorchestes annandalii*). Species from the family *Rhacophoridae* are known to be arboreal (AmphibiaWeb, 2021), however, in the present study, the method for canopy sampling was not included and hence these species were often observed either on forest floors or on stem of shrubs along with *Megophryid* species. However, frogs such as *Nanorana* sp probably exhibit unique microhabitat requirements with higher canopy coverage as recorded in the present study. The distributions of some amphibian species is correlated with overhead canopy cover and they prefer habitats with higher degree of shading (Collins, 1993; Werner & Glennemeier, 1999). Yang et al (2019) suggested water temperature, air temperature, pH, humidity, riparian canopy, river width, river width in flood season, and vegetation coverage to be the vital factors that influenced microhabitat utilization in species of *Feirana*. As amphibians are mostly limited by factors such as physiological tolerances and climate (temperature, precipitation, humidity), species with similar microhabitat requirements tend to group together. Although, amphibians of most body sizes can be found in varied type of microhabitats, amphibian body size failed to explain the variation in microhabitat use in the present study. The relationship between body size and microhabitat may be potentially effective when biotic and abiotic factors such as prey availability and microclimate are taken into consideration (Womack and Bell, 2020). Therefore, additional data on such factors are required to ascertain the effect of body size on microhabitat use. This study highlighted that amphibian species in Sikkim coexist by microhabitat partitioning which is mostly driven by phylogenetic relatedness. However, newer phylogenetic tools and fine-resolution ecological data will be helpful to ascertain niche conservatism.

4.5 **Conclusion**

Almost 33% of the species were microhabitat specialists which indicate that maintenance of the complex structure of such microhabitats is critical for the conservation of these species in Sikkim Himalaya. The present study highlighted rocks or boulders as the most used microhabitat by majority of amphibians (n = 22) which is likely because it provides shelter from climatic extremes, refuge from predators and suitable oviposition sites to avoid the eggs being washed away from the rapid water currents of the torrential streams. Thus, the preservation of rocky outcrops along the stream and river is essential to conserve the diversity of amphibians in Sikkim. Additionally, the microhabitats such as boulders and water bodies associated with the specialist amphibian species are key priority for conservation. However, in recent years rapid developmental activities such as construction of pharmaceutical industries and hydropower projects has been seen to be a major threat to amphibian communities (Pandit & Grumbine, 2012). Moreover, the rapid demand of construction materials such as sand and gravel has promoted alteration of stream beds and water bodies for mining/quarrying activities which adversely affects the microhabitat availability of amphibian species associated with rocky outcrops. Therefore, amphibian species associated with occurrence of rocks and boulders as an important microhabitat resource are at risk due to mining activities (Thorpe et al, 2018). Hence, future conservation policies should focus on retention and restoration of rocky outcrops along the streams or river bank for maintenance of amphibian diversity in Sikkim. The Amphibian Conservation Action Plan (ACAP) recognized the importance of understanding the causes of declines in amphibian population and diversity (Gascon, 2007) which can be achieved only after obtaining baseline data on ecological requirements of amphibian species (Williams and Hero, 2001). Therefore, the shortfall in data relating to habitat specificity can only be compensated with increased study on habitat features and microhabitat requirements of amphibians which is critical for assessment of conservation status in the face of climate change scenarios and land use changes.

CHAPTER 5

UNDERESTIMATED CRYPTIC DIVERSITY WITHIN THE GENUS *AMOLOPS* IN SIKKIM HIMALAYA

5.1 Introduction

The ranid frogs of the genus *Amolops* Cope 1865 are the most species rich with 69 species distributed widespread across Nepal, India, China, Bhutan and Southeast Asia (Frost, 2021) and one of the oldest genera in the family Ranidae (Pyron and Wiens, 2011; Wu et al, 2020). *Amolops* are montane species and generally known as cascade frogs due to their preference for swift rocky stream habitats, including torrents and small fast flowing waterfalls or cascades in hilly streams. The frogs belonging to this genus are habitat specialists (Khatiwada & Haugaasen, 2015) and this habitat specialization is characterized by the presence of enlarged digital pads in adults and abdominal suckers in tadpoles. *Amolops* species are characterized by adult morphological similarity and easily confused with other species occurring in similar habitats which has contributed to misidentification of these species (Bain et al, 2006). Also many cryptic species have been reported from this genus which further aggravates the problems in taxonomy and species delimitation (Lyu et al, 2019; Onn et al, 2018). Currently, based on morphological similarities six species groups of *Amolops* has been classified from the Himalaya and Southeast Asia consisting of *Amolops marmoratus*, *Amolops monticola*, *Amolops mantzorum, Amolops daiyunensis, Amolops ricketti* and *Amolops hainanensis* (Fei et al, 2009; Lyu et al, 2019). Previous studies have supported the monophyly of *Amolops* however the phylogenetic relationships within the genus remains confusing (Stuart et al, 2008; Lyu et al, 2019). The species belonging to the genus *Amolops* exhibit a high degree of local endemism. Endemic species are characterized by narrow geographic range and habitat specificity which makes them vulnerable to extinction (Isik, 2011). Thus, many species from the *Amolops* genus is susceptible to extinction risks. Moreover, factors such as climate change, pollution and anthropogenic activities coupled with their extent of local endemism have contributed to declines in species belonging to *Amolops* (IUCN, 2019). Hence there is an urgent need to assess the phylogenetic relationships within this group.

Many species of the genus *Amolops* are widely distributed across China and southeast Asia however from the northeast India only eight species have been recorded viz. *Amolops assamensis*, *Amolops formosus*, *Amolops himalayanus*, *Amolops kaulbacki*, *Amolops marmoratus*, *Amolops monticola* and *Amolops viridimaculatus* (Ao et al, 2003; Das & Dutta, 2007; Sailo et al, 2007; Sengupta et al, 2008; Biju et al, 2010). In the Western Himalaya however, two additional species of the genus *Amolops* is recorded, *Amolops chakratensis* and *Amolops jaunsari* (Ray, 1992) which is absent from the remaining geographical regions of the country. In Sikkim, four species of *Amolops* have been recorded (Chettri et al, 2020) viz. *Amolops formosus* (Günther, 1875), *Amolops marmoratus* (Blyth, 1853), *Amolops monticola* (Anderson, 1871) and *Amolops himalayanus* (Boulenger, 1888). Sikkim has a complex topography with some of the highest mountains in the world. The deep gorges and valleys created by fast torrential streams and large rivers can promote speciation and geographical isolation leading to reduced gene flow (Qu et al, 2014), hence these species might have small or possibly allopatric distributions.

In India, the identification and phylogenetic status of species belonging to the genus *Amolops* were mostly done on the basis of morphology (e.g Sengupta et al, 2008; Biju et al, 2010) and very few have used molecular data for species delimitation (e.g Patel et al, 2021). However since species of the genus *Amolops* are known to have morphological similarities (Wu et al, 2020), the application of molecular data for determining the phylogenetic status of these cascade frogs is urgent. Although, much of the distribution and diversity of amphibians in Sikkim has been documented (Chettri & Acharya, 2020), the phylogenetic status of these species remain a matter of contention. Moreover, only one study has delineated two amphibian species occurring in high altitudes in Sikkim using molecular data which were previously thought to be same species due to similar morphology (Subba et al, 2015). Thus the application of molecular phylogeny for inferring phylogenetic status is important to ascertain the unknown cryptic diversity of amphibian species in this region. The use of genetic markers such as *Cytochrome c Oxidase subunit I* (*COI*) barcode gene has been often used in inferring phylogenetic relationships and species delimitation among closely related species (Herbert et al, 2004). Molecular phylogeny using DNA barcode gene has witnessed wider attention in recent times for ascertaining phylogenetic relationships and status of several amphibian species (Xia et al, 2012; Chet et al, 2012; Murphy et al, 2013). The present chapter attempts to infer the phylogenetic relationship and status of the *Amolops* species occurring in Sikkim through morphological and molecular analysis.

5.2 Methodology

5.2.1 Field Surveys and Sampling

With prior permission from the Department of Forest, Environment & Wildlife Management, Government of Sikkim (Permit No: 78/GOS/FEWMD/BDR/CCF (HQ)/189) specimens of genus *Amolops* were collected from various sampling locations in the Teesta valley and Rangeet valley. All the four reported species from Sikkim were encountered and could be collected in this study. A total of three to four individuals of each species were collected. The collected individuals were carefully placed in a plastic container with small holes for aeration and brought back to the laboratory. In the laboratory, the live characteristics of the specimen were noted for taxonomic identification using standard literatures (Schleich & Kastle, 2002; Yang, 1991). Thereafter the individual specimens were then euthanized following standard ethical protocol using a local anesthesia (Heyer et al, 2014). The samples were then fixed in 10% formalin for a couple of days, and were washed and preserved in 70% ethanol. Prior to fixing, abdominal cavity of the specimens was opened using sterilized forceps and scissors and liver tissue samples were collected. Additionally muscle tissue samples from the thighs were also collected. The tissue samples were quickly placed in screw cap tubes containing 70% ethanol for preservation. The tubes were labeled and stored in -20°C refrigerator for further analysis. The whole specimens and tissue samples were kept in the Department of Zoology, Sikkim University.

5.2.2 Morphological study

Sex of collected individuals was determined by the presence of secondary sexual characters such as loose skin on the throat and presence of a nuptial pad on the finger. The following measurements were taken to the nearest 0.1 mm using digital callipers: SVL (snout-vent length), HW (head width, at the angle of the jaws), HL (head length, from rear of mandible to tip of snout), SL (snout length, from tip of snout to anterior orbital border), EL (eye length, horizontal distance between bony orbital borders), EN (distance from the front of the eye to the nostril), IN (internarial distance), NS (distance from the nostril to the tip of the snout), TYD (greatest tympanum diameter), TYE (distance from the tympanum to the back of the eye), FAL (forearm length, from flexed elbow to base of outer palmar tubercle), HAL (hand length, from base of outer palmar tubercle to tip of third finger), FD (maximum disc width of finger), FW (width of finger, measured at the base of the disc), TL (thigh length), SHL (shank length), FOL (foot length, from base of inner metatarsal tubercle to tip of fourth toe), TFOL (total foot length, from heel to tip of fourth toe), TD (maximum disc width of toe), IMTL (length of inner metatarsal tubercle). The measurements are in millimeters (mm).

Principal component analysis (PCA) was performed using the FactorMineR and factoextra packages from Kassambara (2017), to evaluate the possible separation of the four species based on morphometric characters.

5.2.3 Isolation of genomic DNA, amplification and sequencing

The total genomic DNA from the collected specimens was extracted from the preserved liver tissue and muscle tissue samples. The liver tissue and muscle tissue are considered to be amongst the best tissue samples for extracting total genomic DNA (Wong et al, 2012). About 25mg of tissue samples were carefully weighed and thawed in a sterile petridish to prevent discrepancies in measurements. The tissue samples were then rinsed in phosphate buffered saline (PBS) and ground using the same saline for the extraction of DNA. Commercially available DNA isolation kit (QIAGEN DNeasy Blood & Tissue kit) was used for the isolation of genomic DNA following the protocol provided in the kit by the manufacturer (Qiagen, Germany). PCR amplification of the isolated genomic DNA targeting four gene regions was performed in triplicates. The gene regions considered for PCR were three mitochondrial gene regions: *Cytochrome c oxidase Subunit I* (*COI*), *16S ribosomal RNA* and *12S ribosomal RNA*, and one nuclear gene: *Recombination activating gene subunit 1* (*RAG 1*) (Table 5.1). The mitochondrial *COI* gene is considered to be efficacious in resolving phylogenetic relationship among closely related species (Lu et al, 2011). The mitochondrial *16rRNA* and *12SrRNA* gene is commonly used in amphibian systematics to infer phylogenetic relationships (Matsui et al, 2010; Chan et al, 2022). *Recombination activating genes* have proved to be efficient targets for phylogenetic reconstructions and have been extensively used in studies in wide range of organisms including amphibians (Groth et al, 2002; San Mauro et al, 2004). PCR was performed with a total reaction volume of 25 µl (dH20 = 18µl; taq buffer = 2.5 µl; taq polymerase = 0.5µl; dNTPs = 1µl; forward primer =1µl; reverse primer = 1µl; DNA sample = 1µl) in ProFlex PCR system (Thermo Fisher Scientific, USA). The following PCR conditions were employed for amplification: 72°C for 5 min (initial denaturation); 94°C for 1 min (denaturation) for 35 cycles, 46-48°C for 1 min (annealing), 72°C for 2 min (extension); 72°C for 10 min (elongation). Gene Elute PCR clean up kit (Sigma-Aldrich) was used for purification of amplicons. Sequencing of the purified PCR products were outsourced to Macrogen, Korea and Barcode Biosciences, Bangalore.

Table 5.1. Primers used for molecular analysis of species of genus *Amolops* recorded in Sikkim, Eastern Himalaya

Gene region	Primers	Sequence	Annealing Temperat- -ure	Reference
Recombination Activating Gene	L-RAG1Ran	5'-CTG GTC GTC AGA TCT TTC AGC-3'	55°C	Pyron et al, 2011
	H-RAG1Ran	5'-GCA AAA CGT TGA GAG TGA TAA C-3'		
16SrRNA	L2188	5'- AAA GTG GGC CTA AAA GCA GCC A-3'	52°C	Matsui et al, 2005
	16H1	5'-CTC CGG TCT GAA CTC AGA TCA CGT AGG-3'		
COI	Chmf4	5'-TYT CWA CWA AYC AYA AAG AYA TCG G-3'	46°C	Che et al, 2012
	Chmr4	5'-ACY TCR GGR TGR CCR AAR AAT CA-3'		
12SrRNA	12SH	5'-AAA GGT TTG GTC CTA GCC TT-3'	60°C	Matsui et al, 2005
	H1458	5'- TAC CAT GTT ACG ACT TTC CTC TTC T-3'		

5.2.4 Sequence alignment and sequence retrieval

The quality of the sequences was assessed using Sequence Scanner Software (version 2). Amongst the four gene regions only the barcode gene *COI* provided better results whereas the *RAG1*, *12SrRNA* and *16SrRNA* could not provide good length of sequences for analysis. The trace score of the *16SrRNA*, *12SrRNA* and *RAG1* were relatively low (<30) hence were unsuitable for phylogenetic analysis. In total, four sequences of *COI* region of four potential species were included in the study. Morphometric characterization of the collected samples suggests that they belong to genus *Amolops*. Hence BLAST analysis for the *COI* gene region of amphibians of the genus *Amolops* was performed using the nucleotide BLAST suite of NCBI (www.ncbi.nlm.nih.gov). Consequently, 165 *COI* sequences belonging to 49 genetically known species and some potential candidate species including four outgroups were retrieved from GenBank (NCBI) (Table 5.2). As outgroup, four *COI* sequences of Asian specimens belonging to the genus *Huia* and *Staurios* were used, under the hypothesis that latter are evolutionary close relative of mostly Asian native *Amolops* genus. The sequences were aligned using the ClustalW Multiple Alignment algorithm (Thompson *et al*, 1994) as implemented in Bioedit v7.0.5.3 (Hall, 1999). The alignment was manually edited and the final alignment consisted of 527 nucleotide positions.

Table 5.2. Details of the DNA sequences of *Amolops* used for molecular phylogenetic analysis. NCBI accession numbers of gene (*COI*) sequences along with location of sample collection is provided

S.No.	Taxa	Collection Locality	Accession Number	References
	Genus *Amolops*		***COI***	
1	*A. chunganensis*	China: Anxian, Sichuan	n.a.	Wu et al. 2020
2	*A. chunganensis*	China: Anxian, Sichuan	MN961363	Stuart et al. 2010; Wu et al. 2020
3	*A. chunganensis*	China: Anxian, Sichuan	MN961467	Wu et al. 2020
4	*A. chunganensis*	China: Gansu	KX645666	Yuan et al. 2016
5	*A. chunganensis*	China: Bijie, Guizhou	MN961468	Wu et al. 2020
6	*A. chunganensis*	China: Mt. Jinggang, Jiangxi	MG991915	Lyu et al. 2018
7	*A. chunganensis*	China: Mt. Wugong, Anfu, Jiangxi	KX507330	Sung et al. 2016
8	*A. chunganensis*	China: Shenglongjia, Hubei	KF771328	Xia et al. 2014
9	*A. chunganensis*	China: Chengkou, Chongqing	MN961364	Wu et al. 2020
10	*A. chunganensis*	China: Chengkou, Chongqing	MN961463	Wu et al. 2020
11	*A. chunganensis*	China: Fangxian, Hubei	MN961365	Wu et al. 2020
12	*A. compotrix*	Laos: Nakai	MN961366	Stuart et al. 2010; Wu et al. 2020
13	*A. compotrix*	Vietnam: Dak Glei, Kon Tum	MN961367	Stuart et al. 2010; Wu et al. 2020
14	*A. cucae*	Vietnam: Van Ban, Lao Cai	MN961372	Stuart et al. 2010; Wu et al. 2020
15	*A. cucae*	Vietnam: Van Ban, Lao Cai	MN961373	Stuart et al. 2010;

				Wu et al. 2020
16	*A. wenshanensis*	China: Xichou, Yunnan	MN961426	Yuan et al. 2018; Wu et al. 2020
17	*A. wenshanensis*	China: Xichou, Yunnan	MN961427	Yuan et al. 2018; Wu et al. 2020
18	*A. wenshanensis*	China: Jinxiu, Guangxi	MN961425	Stuart et al. 2010; Wu et al. 2020
19	*A. vitreus*	Laos: Phongsaly, Phongsaly	MN961439	Stuart et al. 2010; Wu et al. 2020
20	*A. vitreus*	Laos: Phongsaly, Phou Dendin National Biodiversity Conservation Area	n.a.	Wu et al. 2020
21	*A. iriodes*	Vietnam: Vi Xuyen, Ha Giang	MN961400	Stuart et al. 2010; Wu et al. 2020
22	*A. iriodes*	Vietnam: Vi Xuyen, Ha Giang	MN961401	Stuart et al. 2010; Wu et al. 2020
23	*A. mengyangensis*	Vietnam: Sa Pa, Lao Cai	KR087618	Grosjean et al. 2015
24	*A. mengyangensis*	Vietnam: Sa Pa, Lao Cai	KR087619	Grosjean et al. 2015
25	*A. daorum* 1	China: Jingdong, Yunnan	MN961377	Wu et al. 2020
26	*A. daorum* 1	China: Jingdong, Yunnan	MN961378	Wu et al. 2020
27	*A. mengyangensis*	Vietnam: Sa Pa, Lao Cai	n.a.	Stuart et al. 2010
28	*A. daorum* 2	Laos: Vieng Tong, Huaphahn	MN961379	Stuart et al. 2010; Wu et al. 2020
29	*A. daorum* 2	Laos: Vieng Tong, Huaphahn	MN961380	Stuart et al. 2010; Wu et al. 2020

30	*A. archotaphus*	Thailand: Amphoe Chom Thong, Chiang Mai	MN961356	Wu et al. 2020
31	*A. archotaphus*	Thailand: Doi Inthanon, Chiang Mai	MN961357	Wu et al. 2020
32	*A. archotaphus*	Thailand: Doi Inthanon, Chiang Mai	MN961358	Wu et al. 2020
33	*A. mengdingensis*	China: Mengding, Yunnan	MK501812	Yu et al. 2019
34	*A. mengdingensis*	China: Mengding, Yunnan	MK501813	Yu et al. 2019
35	*A. mengdingensis*	China: Mengding, Yunnan	MK501811	Yu et al. 2019
36	*A. akhaorum*	Laos: Vieng Phou Kha, Luang Namtha	MN961350	Stuart et al. 2010; Wu et al. 2020
37	*A. akhaorum*	Laos: Vieng Phou Kha, Luang Namtha	MN961351	Stuart et al. 2010; Wu et al. 2020
38	*A. deng*	China: Zayü, Tibet	MN961395	Wu et al. 2020
39	*A. deng*	China: Zayü, Tibet	MN961396	Wu et al. 2020
40	*A. deng*	China: Zayü, Tibet	MN961397	Wu et al. 2020
41	*A. deng*	China: Zayü, Tibet	MN961398	Wu et al. 2020
42	*A. deng*	China: Medog, Tibet	MN961399	Wu et al. 2020
43	*A. nyingchiensis*	China: Mainling, Tibet	MN961418	Wu et al. 2020
44	*A. nyingchiensis*	China: Mainling, Tibet	MN961420	Wu et al. 2020
45	*A. nyingchiensis*	China: Mainling, Tibet	MN961417	Wu et al. 2020
46	*A. nyingchiensis*	China: Mainling, Tibet	MN961419	Wu et al. 2020
47	*A. nyingchiensis*	China: Medog	KU243068	Jiang et al. 2016; Wu et al. 2020
48	*A. bellulus* 1	China: Teng Chong Co., Yunnan	KU243078	Jiang et al. 2016; Wu et al. 2020

49	*A. bellulus* 1	China: Teng Chong Co., Yunnan	KU243079	Jiang et al. 2016; Wu et al. 2020
50	*A. bellulus* 1	China: Teng Chong Co., Yunnan	MN961361	Stuart et al. 2010; Wu et al. 2020
51	*A. aniqiaoensis* 1	China: Medog, Tibet	MN961352	Wu et al. 2020
52	*A. aniqiaoensis* 1	China: Medog, Tibet	MN961353	Wu et al. 2020
53	*A. aniqiaoensis*1	China: Tibet	MN961355	Wu et al. 2020
54	*A. aniqiaoensis* 2	China: Medog, Tibet	MN961354	Wu et al. 2020
55	*A. bellulus* 2	China: Teng Chong Co., Yunnan	MN961362	Stuart et al. 2010; Wu et al. 2020
56	*A. chayuensis*	China: Baxoi, Tibet	KU243074	Jiang et al. 2016; Wu et al. 2020
57	*A. chayuensis*	China: Baxoi, Tibet	KU243075	Jiang et al. 2016; Wu et al. 2020
58	*A. loloensis*	China: Xichang, Sichuan	MN961407	Wu et al. 2020
59	*A. loloensis*	China: Xichang, Sichuan	MN961458	Wu et al. 2020
60	*A. loloensis*	China: Xichang, Sichuan	MN961456	Cai et al. 2007; Wu et al. 2020
61	*A. loloensis*	China: Xichang, Sichuan	MN961457	Wu et al. 2020
62	*A. loloensis*	China: Yuexi, Sichuan	JN700802	Che et al. 2012; Wu et al. 2020
63	*A. jinjiangensis*	China: Deqing, Yunnan	MN961402	Wu et al. 2020
64	*A. jinjiangensis*	China: Deqing, Yunnan	MN961403	Cai et al. 2007; Wu et al. 2020
65	*A. jinjiangensis*	China: Chuxiong, Yunnan	MN961404	Wu et al. 2020
66	*A. mantzorum*	China: Wolong, Sichuan	MN961408	Wu et al. 2020

67	*A. mantzorum*	China: Dayi, Sichuan	MN961409	Wu et al. 2020
68	*A. xinduqiao*	China: Kangding, Sichuan	MN961465	Wu et al. 2020
69	*A. xinduqiao*	China: Kangding, Sichuan	MN961466	Wu et al. 2020
70	*A. tuberodepressus*	China: Jingdong, Yunnan	MN961432	Wu et al. 2020
71	*A. tuberodepressus*	China: Jingdong, Yunnan	MN961433	Wu et al. 2020
72	*A. shuichengicus*	China: Shuicheng County, Guizhou	MK605603	Lyu et al. 2019
73	*A. shuichengicus*	China: Shuicheng County, Guizhou	MK605604	Lyu et al. 2019
74	*A. granulosus*	China: Dayi, Sichuan	JN700804	Che et al. 2012; Wu et al. 2020
75	*A. granulosus*	China: Anxian, Sichuan	MN961381	Wu et al. 2020
76	*A. lifanensis*	China: Maoxian, Sichuan	MN961405	Wu et al. 2020
77	*A. lifanensis*	China: Maoxian, Sichuan	MN961406	Wu et al. 2020
78	*A. viridimaculatus* 1	China: Tengchong, Yunnan	MN961434	Wu et al. 2020
79	*A. viridimaculatus* 1	China: Tengchong, Yunnan	MN961435	Wu et al. 2020
80	*A. viridimaculatus* 1	China: Pianma, Yunnan	MN961436	Wu et al. 2020
81	*A. viridimaculatus* 1	China: Pingbian, Yunnan	n.a.	Wu et al. 2020
82	*A. viridimaculatus* 1	China: Pingbian, Yunnan	n.a.	Wu et al. 2020
83	*A.*	China: Gongshan, Yunnan	n.a.	Wu et al. 2020

	viridimaculatus 1			
84	*A. viridimaculatus* 2	China: Pianma, Yunnan	MN961437	Wu et al. 2020
85	*A. viridimaculatus* 2	China: Pianma, Yunnan	MN961438	Wu et al. 2020
86	*A. beibengensis*	China: Medog, Tibet	MN961359	Wu et al. 2020
87	*A. beibengensis*	China: Medog, Tibet	MN961360	Wu et al. 2020
88	*A. medogensis*	China: Medog, Tibet	MN961412	Wu et al. 2020
89	*A. medogensis*	China: Medog, Tibet	MN961413	Wu et al. 2020
90	*A. wangyufani*	China: Zayü, Tibet	MN961440	Wu et al. 2020
91	*A. wangyufani*	China: Zayü, Tibet	MN961441	Wu et al. 2020
92	*A. gyirongensis*	China: Gyirong, Tibet	MN961382	Wu et al. 2020
93	*A. gyirongensis*	China: Gyirong, Tibet	MN961386	Wu et al. 2020
94	*A. gyirongensis*	China: Gyirong, Tibet	MN961384	Wu et al. 2020
95	*A. gyirongensis*	China: Gyirong, Tibet	MN961383	Wu et al. 2020
96	*A. gyirongensis*	China: Gyirong, Tibet	MN961385	Wu et al. 2020
97	*A.* “sp. 5”	Nepal: Rakshe Village, Mechi	MN961414	Wu et al. 2020
98	*A.* “sp. 5”	Nepal: Mabu, Ilam	MN961415	Wu et al. 2020
99	*A.* “sp. 5”	Nepal: Maimajhuwa, Ilam	MN961416	Wu et al. 2020
100	*A. afghanus*	China: Husa, Yunnan	MN961349	Wu et al. 2020
101	*A. afghanus*	Myanmar: Indawgyi Lake Wildlife Sanctuary, Kachin	MN961474	Wu et al. 2020
102	*A. afghanus*	Myanmar: Myitkyina	MN961475	Wu et al. 2020
103	*A. indoburmanensis*	Myanmar: Baw village, Chin	MN961392	Wu et al. 2020

104	*A. indoburmanensis*	Myanmar: Upper Bee Hoe village, Mindat, Chin	MN961394	Wu et al. 2020
105	*A. indoburmanensis*	Myanmar: Haka Township, Chin	MN961393	Wu et al. 2020
106	*A. marmoratus*	Thailand: Huai Hea, Chiang Mai	MN961410	Wu et al. 2020
107	*A. marmoratus*	Thailand: Wachiratarn Falls, Amphoe Chom Thong, Chiangmai	MN961411	Wu et al. 2020
108	*A. yarlungzangbo*	China: Medog, Tibet	MN961444	Wu et al. 2020
109	*A. yarlungzangbo*	China: Medog, Tibet	MN961445	Wu et al. 2020
110	*A. yarlungzangbo*	China: Medog, Tibet	MN961446	Wu et al. 2020
111	*A. yarlungzangbo*	China: Medog, Tibet	MN961447	Wu et al. 2020
112	*A.* “sp. 7”	Nepal: Mabu, Ilam	MN961450	Wu et al. 2020
113	*A. panhai*	Thailand: Huay Yang National Park, Prachuap Khiri Khan	MN961421	Wu et al. 2020
114	*A. panhai*	Myanmar: Pakchan Reserve Forest, Kawthoung, Tanintharyi	MN961423	Wu et al. 2020
115	*A. panhai*	Thailand: Ngao Falls National Park, Ranong	MN961422	Wu et al. 2020
116	*A. yatseni*	Vietnam: Sa Pa, Lao Cai	MN961424	Wu et al. 2020
117	*A. yatseni*	China: Jingxi, Guangxi	MN961459	Wu et al. 2020
118	*A. yatseni*	China: Zhongshan City, Guangdong	MK263324	Lyu et al. 2019
119	*A. yatseni*	China: Shangchuan Island, Guangdong	MK263304	Lyu et al. 2019
120	*A. sinensis*	China: Mao’er Shan, Guangxi	MN961448	Wu et al. 2020

121	*A. sinensis*	China: Mao'er Shan, Guangxi	MN961449	Wu et al. 2020
122	*A. sinensis*	China: Guidong, Hunan	MN961476	Wu et al. 2020
123	*A. sinensis*	China: Gongcheng, Guangxi	MN961464	Wu et al. 2020
124	*A. albispinus*	China: Mt. Wutong, Guangdong	KX507333	Sung et al. 2016; Lyu et al. 2019
125	*A. albispinus*	China: Mt. Wutong, Guangdong	KX507334	Sung et al. 2016; Lyu et al. 2019
126	*A. yunkaiensis*	China: Yunkaishan Nature Reserve, Guangdong	MG991910	Lyu et al. 2018, 2019
127	*A. yunkaiensis*	China: Ehuangzhang Nature Reserve, Guangdong	MG991909	Lyu et al. 2018, 2019
128	*A. wuyiensis*	China: Wuyishan, Fujian	MN961442	Wu et al. 2020
129	*A. wuyiensis*	China: Wencheng, Zhejiang	MN961461	Wu et al. 2020
130	*A. wuyiensis*	China: Yiwu, Zhejiang	MN961462	Wu et al. 2020
131	*A. rickettii*	China: Wuyishan, Fujian	MN961443	Wu et al. 2020
132	*A. rickettii*	China: Shicheng, Jiangxi	MN961460	Wu et al. 2020
133	*A. daiyunensis*	China: Daiyunshan, Fujian	MN961374	Wu et al. 2020
134	*A. daiyunensis*	China: Daiyunshan, Fujian	MN961375	Wu et al. 2020
135	*A. daiyunensis*	China: Daiyunshan, Fujian	MN961376	Wu et al. 2020
136	*A. hongkongensis*	China: Hong Kong	MN961389	Wu et al. 2020
137	*A. hongkongensis*	China: Hong Kong	MN961391	Wu et al. 2020
138	*A. hongkongensis*	China: Hong Kong	MN961390	Wu et al. 2020
139	*A. hainanensis*	China: Wuzhishan, Hainan	MN961387	Wu et al. 2020
140	*A. hainanensis*	China: Wuzhishan, Hainan	MN961388	Wu et al. 2020
141	*A. torrentis*	China: Hainan	MN961430	Cai et al. 2007; Wu

				et al. 2020
142	*A. torrentis*	China: Diao Luo Shan forest Park, Lingshui, Hainan	MN961431	Wu et al. 2020
143	*A. spinapectoralis*	Vietnam: Ngoc Linh vicinity, Kon Tum	MN961428	Wu et al. 2020
144	*A. spinapectoralis*	Vietnam:Central Highland, Ngok Linh, Kon Tum	MN961472	Wu et al. 2020
145	*A. spinapectoralis*	Vietnam:Kon Ka Kinh, Gia Lai	MN961429	Wu et al. 2020
146	*A. spinapectoralis*	Vietnam:Tram Lap, Gia Lai	MN961471	Wu et al. 2020
147	*A. spinapectoralis*	Vietnam:Phong Dien Nature Reserve, Phong Dien, Thua Thien Hue	MN961470	Wu et al. 2020
148	*A. spinapectoralis*	Vietnam:Phong Dien Nature Reserve, Phong Dien, Thua Thien Hue	MN961473	Wu et al. 2020
149	*A. spinapectoralis*	Vietnam: Bana resort, Da Nang	MN961469	Wu et al. 2020
150	*A. cremnobatus*	Vietnam: Puhu National Reserve, Thanh Hoa	MN961368	Wu et al. 2020
151	*A. cremnobatus*	Vietnam: Puhu National Reserve, Thanh Hoa	MN961369	Wu et al. 2020
152	*A. cremnobatus*	Laos: Kasi, Vientiane	MN961370	Stuart et al. 2010; Wu et al. 2020
153	*A. cremnobatus*	Thailand: Doi Phuka, Chom Poo Phuka nature trail, Nan	MN961371	Wu et al. 2020

154	*A. gerutu*	Malaysia: Gunung Tebu, Terengganu	n.a.	Chan (unpublished)
155	*A. australis*	Malaysia: Endau-Rompin, Johor (Peta)	n.a.	Chan (unpublished)
156	*A. larutensis*	Malaysia: Bukit Larut, Perak	n.a.	Chan (unpublished)
157	***A. marmoratus***	India: Teesta Valley, South Sikkim	SUZ181021	Present study
158	***A. cf. monticola***	India: Teesta Valley, Sikkim	SUZ181022	Present study
159	***A. himlayanus***	India: Rangeet Valley, Sikkim	SUZ181023	Present study
160	***A.cf. formosus***	India: Rangeet Valley Sikkim	SUZ181024	Present study
	Outgroups			
162	*Huia cavitympanum*	Malaysia: Marak Parak, S. Tahobang, Kota Marudu, Sabah	MN961452	Wu et al. 2020
163	*Huia cavitympanum*	China: Jingdong, Yunnan	MW322850	Wu et al. 2020
164	*Huia cavitympanum*	China: Jiemuxi, Hunan	MW322850	Wu et al. 2020
165	*Staurois natator*	Philippines: Sitio San, Gingoog City, Barangay Lumotan, Misamis Oriental	MN961454	Wu et al. 2020

5.2.5 Molecular Phylogenetic analysis

The evolutionary relationships of the *Amolops* species were inferred using three different methods: the Neighbor-Joining method (Saitou and Nei, 1987), the Maximum Likelihood method, and Bayesian Inference. The Neighbor-Joining phylogeny was inferred using the Kimura 2-parameter (Kimura, 1980) and 1000 bootstrap replicates to compute the evolutionary distances between taxa in MEGA v.11.13 (Kumar et al, 2018).

The model of evolution for my dataset was determined using the software jModelTest 2 v. 0.1.7 (Darriba et al, 2012). The software returned The GTR (Generalized Time Reversal) + γ (Gamma) + I (Invariant sites) as the best-fit model according to BIC as well as AICc criteria (Table 5.4). The Maximum Likelihood-based evolutionary history of the species was thus inferred applying the General Time Reversible (GTR + γ + I) substitution model (Nei & Kumar, 2000) with 1000 bootstrap replicates in MEGA v.11.13. In the Maximum Likelihood phylogeny, the previously inferred Neighbor-Joining was used as the initial tree in the heuristic search. Bayesian Inference was performed using the BEAST platform, using the GTR + γ + I DNA substitution model, a strict clock model and setting the priors for the speciation model to the Yule model. The length of the chain was set to 10000000. The trees were visualized in software Figtree.

Table 5.3 Best Fitting Models based on BIC and AICc criteria

Model	#Parameter	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G
GTR+G+I	875	38880.80003	29785.23527	-14014.45616	0.517028983	1.082237664	7.075928375	0.246899222	0.294559562	0.291522484	0.167018732
TN93+G+I	872	38895.00138	29830.59975	-14040.16007	0.519135522	1.064391373	6.90250984	0.246899222	0.294559562	0.291522484	0.167018732
HKY+G+I	871	38903.01194	29848.99806	-14050.36643	0.516968173	1.072703124	7.044162082	0.246899222	0.294559562	0.291522484	0.167018732
T92+G+I	869	39062.72921	30029.49087	-14142.62722	0.518414717	1.100156256	6.40168403	0.270729392	0.270729392	0.229270608	0.229270608
GTR+G	874	39071.11534	29985.93827	-14115.81489	n/a	0.260922642	7.740150854	0.246899222	0.294559562	0.291522484	0.167018732
HKY+G	870	3909	30048.	-	n/a	0.262453127	5.889457682	0.246899	0.294559	0.2915224	0.167018732

		2.11755	49144	14151.12031				222	562	84	
TN93+G	871	39116.01391	30062.00003	-14156.86741	n/a	0.261906039	5.773515435	0.246899222	0.294559562	0.291522484	0.167018732
K2+G+I	868	39128.44186	30105.59131	-14181.68462	0.51664953	1.128933185	6.227592895	0.25	0.25	0.25	0.25
T92+G	868	39298.89241	30276.04187	-14266.9099	n/a	0.280470625	4.931512287	0.270729392	0.270729392	0.229270608	0.229270608
K2+G	867	39344.00127	30331.53854	-14295.66541	n/a	0.276068534	6.505865352	0.25	0.25	0.25	0.25
GTR+I	874	39972.12675	30886.94968	-14566.3206	0.516965744	n/a	5.234848283	0.246899222	0.294559562	0.291522484	0.167018732
TN93+I	871	40016.282	30962.26871	-14607.001	0.519106629	n/a	5.015746474	0.246899222	0.294559562	0.291522484	0.167018732

		59		75							
HKY+I	870	40056.63501	31013.00889	-14633.37904	0.516997776	n/a	4.974631913	0.246899222	0.294559562	0.291522484	0.167018732
T92+I	868	40178.90372	31156.05318	-14706.91556	0.517799054	n/a	4.833662521	0.270729392	0.270729392	0.229270608	0.229270608
K2+I	867	40188.84264	31176.37991	-14718.0861	0.517785942	n/a	4.832335111	0.25	0.25	0.25	0.25
JC+G+I	867	42580.94516	33568.48243	-15914.13735	0.521737871	1.606997985	0.5	0.25	0.25	0.25	0.25
JC+G	866	42722.41435	33720.33945	-15991.07303	n/a	0.173086252	0.5	0.25	0.25	0.25	0.25
JC+I	866	43399.81152	34397.73662	-16329.77161	0.516960993	n/a	0.5	0.25	0.25	0.25	0.25

GTR	873	44887.15588	35812.36652	-17030.03624	n/a	n/a	3.654348628	0.246899222	0.294559562	0.291522484	0.167018732
T92	867	44975.81845	35963.35572	-17111.574	n/a	n/a	4.130789246	0.270729392	0.270729392	0.229270608	0.229270608
TN93	870	44981.33939	35937.71327	-17095.73123	n/a	n/a	4.123389266	0.246899222	0.294559562	0.291522484	0.167018732
HKY	869	44982.53549	35949.29715	-17102.53036	n/a	n/a	4.128954418	0.246899222	0.294559562	0.291522484	0.167018732
K2	866	44988.83524	35986.76034	-17124.28347	n/a	n/a	4.123401977	0.25	0.25	0.25	0.25
JC	865	47991.56512	38999.87806	-18631.84949	n/a	n/a	0.5	0.25	0.25	0.25	0.25

5.3 Results

5.3.1 Morphological identification of species of genus *Amolops*

The morphological characters of the collected amphibian specimens were assessed following relevant literature (Cope, 1865; Boulenger 1890; Schleich & Kastle, 2002; Yang, 1991). A total of four species of *Amolops* were recorded which were placed under the species viz. *Amolops himalayanus*, *Amolops marmoratus, Amolops cf. monticola* , *Amolops cf. formosus* based on the following set of observed morphological characters. ***Amolops himalayanus***

Amolops himalayanus is assignable to *Amolops viridimaculatus* group based on having the following combination of characters: Smooth skin on the back with clusters of horny spinules on the side of head; dorsolateral fold absent; temporal fold thick and distinct; presence of small tubercles under anus and rear of thigh; rounded snout projecting beyond lower jaw; nostril is nearer to eye than to tip of snout; tympanum distinct; slender fingers with first finger shorter than second; fingers expanded into large disks with circummarginal grooves except on first finger; Large, long supernumerary tubercles present at bases of three outer fingers; outer palmar tubercle absent; males possess a velvety nuptial pad on inner and upper surfaces of first finger; coloration is greenish yellow with distinct brown spots on the back and yellowish on the belly; belly interspersed with numerous tiny tubercles.

Amolops marmoratus

Amolops marmoratus is assignable to *Amolops marmoratus* group based on having the following set of characters: skin granular with dark mottling; dorsolateral fold absent; narrow temporal fold; hind parts of thighs granulated; snout round, projecting slightly beyond the mouth; tympanum distinct; nostril is midway between tip of snout and eye; fingers very long with first finger shorter than second; tips of the fingers are expanded into large disks; disks of the toes smaller than those of fingers; toes completely webbed; subarticular tubercles bulging, oval and well developed; inner metatarsal tubercle oval and flat; outer tubercle present; tarsal fold and tarsal gland absent; coloration bluish green with tiny black spots and marblings; limbs present numerous black crossbars; belly grayish and granulated ; hind parts of thighs yellowish.

Amolops cf. monticola

Amolops cf. monticola is assignable to *Amolops monticola* group based on having the following set of characters: smooth skin on the back with or without granular projections; snout rounded or obtuse; weakly developed glandular dorsolateral folds; tympanum distinct; ventrum smooth; subarticular tubercles are rounded and bulging; supernumerary tubercles indistinct with outer palmar tubercle absent; disks of toes smaller than those of fingers; webbing between fingers absent; no tarsal fold; light cream colored stripe extending from the snout tip along the upper lip present; lateral surface of head dark-brown; dorsum dark to light brown with numerous black speckles.

Amolops cf. *formosus*

Amolops cf. *formosus* is assignable to *Amolops viridimaculatus* group based on having the following set of characters: Smooth Skin with distinct dorsolateral fold; snout round projecting beyond lower jaw; tympanum partially distinct; fingers long; first finger is shorter than second; circummarginal grooves absent in first finger; subarticular tubercle round and bulging with two palmar tubercles; hind limbs long almost equal to the length of foot; toes fully webbed; inner metatarsal tubercle present; outer metatarsal tubercle absent; bright green colored dorsum with sharply defined blackish / brownish spots often with green dots on head and body; crossbars on limbs and hind sides of thighs marbled with black.

The genus inhabits similar microhabitats in small torrential streams, brooks in well forested areas and rapid waterfalls within the elevation of 300-3000 m in the Sikkim Himalaya. While *Amolops marmoratus a*nd *Amolops monticola* occur mostly in lowland tropical areas, *Amolops formosus* and *Amolops himalayanus* occur at mid-elevations.

a.

b.

c.

d.

Photo Plate 5.1: Amolops species recorded in the study a. *Amolops cf. formosus*, b. *Amolops himalayanus*, c. *Amolops marmoratus*, d. *Amolops cf. monticola*

The morphometric measurements of the species collected in this study is provided in Table 5.4.

Table 5.4. Morphometric measurements of *Amolops himalayanus*, *Amolops cf. formosus*, *Amolops marmoratus* and *Amolops cf. monticola* included in the study.

Species	**SVL**	**HL**	**HW**	**SL**	**EL**	**TYD**	**TYE**	**EN**	**NS**	**IN**	**FAL**	**HAL**	**FDI**	**FWI**	**TL**	**SHL**	**FOL**	**TFOL**	**TDIV**	**IMTL**
A.cf.monticola	62.3	20.8	21.3	6.9	6.7	3.2	2.6	4.2	4.4	6.4	16.5	17.6	1.6	1.6	38.9	42.6	32.1	52.7	2.3	3.2
A.cf.monticola	66.7	21.2	20.6	7.1	6.9	3.1	2.4	4.3	4	6.3	15.8	17.8	1.8	1.7	39.2	42.8	32.8	53.1	2.5	3.3
A.cf.monticola	41.6	16.2	15.5	5.2	5.1	2.5	1.4	2.5	2.4	4.1	10.1	11.6	1	1.1	22.8	25.2	21.7	34.4	1.6	1.5
A.cf.monticola	42.1	15.8	14.8	4.8	4.6	2.6	1.3	2.3	2.5	4.2	10.2	11.8	1.1	1	23.3	25.8	21.9	34.7	1.7	1.6
A.cf.monticola	68.4	22.3	21.4	7.2	7.1	3.4	2.8	4.4	4.5	6.1	16.6	17.9	1.9	1.8	39.4	43.3	33.4	53.6	2.8	3.5
A.marmoratus	75.8	27.9	28.6	8.9	6.9	3.1	2.2	4.5	4.6	9.5	23.2	26.7	1.9	1.8	51.7	47.6	37.7	53.2	2.6	5.6
A.marmoratus	76.2	28.2	29.8	8.6	7	3.2	2.4	4.6	4.1	9.3	24.4	26.9	1.8	1.7	52.1	48.1	38.8	53.6	2.5	5.2

A.marmoratus	78.6	28.9	30.1	9.2	7.1	3.3	2.7	4.5	4.7	9.1	24.8	27	1.6	1.5	52.9	48.6	39.6	54	2.6	5.8
A.marmoratus	70.2	26.4	26.5	7.7	6.5	2.5	1.7	2.4	2.6	8.2	22.9	25.7	1.4	1.5	50.3	46.7	37.2	51.2	2.1	5
A.marmoratus	69.9	26.2	26.4	7.4	6.3	2.4	1.3	2.7	2.9	8.1	22.4	25.2	1.3	1.4	49.9	46.1	36.9	52.5	2	4.9
A.cf.formosus	53.7	17.8	17.7	5.1	5.7	2.3	1.6	2.8	2.7	4.3	13.6	14	1.2	1.2	39.1	39.8	29.2	39.5	1.8	2.7
A.cf.formosus	54.1	18.1	18	5	5.9	2.4	1.5	2.6	2.5	4.2	13.9	14.2	1.2	1.1	40	41.3	29.8	39.3	1.6	2.4
A.cf.formosus	63.8	20.8	21.7	6.3	6.1	3.2	2.3	4.4	4.5	6.5	16.7	17.2	1.5	1.3	42	41.9	32.9	53	2.2	3.3
A.cf.formosus	63	21.1	21	6	6	3	2	4.2	4.2	6.7	16.5	17.8	1.4	1.2	41.2	41.5	32.6	52.6	2.1	3.2
A.cf.formosus	62.7	19.5	19.3	6.2	6.2	3.1	2.2	4.3	4.5	6.8	15.9	16.8	1.6	1.5	40.9	40.9	31.8	51.8	2	3.1
A.himalayanus	71	22.2	21.6	9	8.7	3.2	1.2	4	4.2	8.1	21.3	24.7	1.8	1.7	50.7	44.5	35.6	53.8	2.5	5.4
A.himalayanus	70.3	22	21	8.8	8.6	3	1.1	4.3	4	8	20.8	24	1.6	1.6	49.8	44.2	35.4	53.5	2.3	5.1
A.himalayanus	70.1	22.1	20.9	8.7	8.4	2.9	1.8	3.9	4.1	7.9	20.5	23.9	1.5	1.6	49.5	44	34.9	53.1	2.1	5

A_himalayanus	68.7	19.9	19	6.4	6.1	2.5	0.7	3.6	4	6.9	19.6	21.7	1.1	1.2	48.6	43.5	33.8	51.9	2	4.3
A_himalayanus	69	21.3	20.9	6.8	6.6	2.7	0.4	3.5	4	7	19.4	22.2	1	0.9	49.2	43.8	33.7	51.7	2	4.1

5.3.2 Principal Component Analysis

In the PCA, *A. marmoratus, A. cf. monticola, A. himalayanus* and *A. cf. formosus* clustered separately from each other along the ordination of principal components (PC)1 and 2 (Fig. 5.1), which together explained 61% of the total variation. PC1 accounted for 40.7% of the variation in the morphometric data, with the following variables contributing the most: TYD, FAL, TDIV, TL, HAL, IMTL, FDI and TFCL in that order. PC2 accounted for additional 20.4% variation in the data, with the following variables contributing the most: SL, IN, SVL and HAL. PC3 accounted for another 15.3% of the variation with the following variables contributing the most: FCL, HL, HW and NS. PC4 accounted for 8.8 % variation with the following variables contributing the most: EL and SL. Altogether, the *Amolops* species in this study was found to be distinct from each other based on morphometric characters.

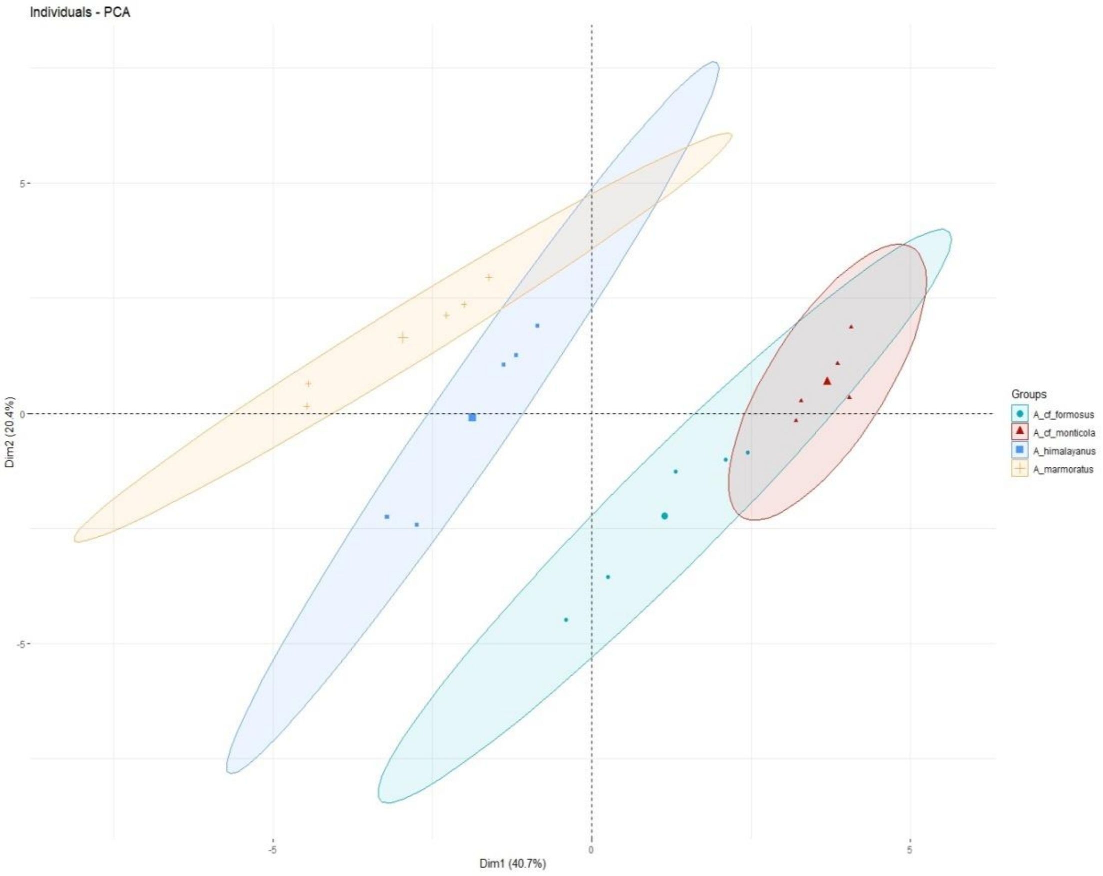


Fig. 5.1. Plot of the first two principal components (PC1 and PC2) from the from principal component analysis based on morphometric data for *Amolops marmoratus*, *Amolops cf. monticola*, *Amolops himalayanus* and *Amolops cf. formosus.*

5.3.3 Phylogenetic Inference

A total of 586 bp of mitochondrial *COI* of the four species (*Amolops marmoratus*, *Amolops cf. monticola*, *Amolops himalayanus* and *Amolops cf. formosus)* was generated and apparently all the four sequences were novel (Fig. 5.2). For the phylogenetic analysis a target fragment of 564 bp of COI for each species was used for the phylogenetic analysis. While all the three methods i.e. BI, ML and NJ gave similar results, BI method gave higher node support. The phylogenetic analysis recovered four of the eight groups of Amolops species recognized by Wu et al. (2020) as monophyletic clades: *A. ricketti* group, *A. marmoratus* group, *A. viridimaculatus* *group*, and *A. monticola group*. Most of these groups were well supported in the BI (BPP ≥ 95%) and ML framework (BS ≥ 70%). The lineage representing *Amolops spinapectoralis*, showed a distinct and well-supported phylogenetic relationship with the clade containing *A. ricketti* group. This lineage was not assigned to any of the species groups by Wu et al. (2020). The two new sequences in the present study, namely *A. cf. formosus* and *A. himalayanus* were nested in *A. viridimaculatus group* and clearly belong to this group. In particular, both sequences are evolutionary close relative to one another. These two sequences were closely related to a candidate species *Amolops sp. 5* from Illam, Nepal (Wu et al, 2020). Similarly, *A. marmoratus* was nested within *A. marmoratus* group and exhibit evolutionary relation to this group. However, interestingly the species identified potentially as *A.cf. monticola* also clustered in the in *A. marmoratus* group making the validity of this potential species unresolved. These two sequences were closely related to a candidate species *Amolops sp. 7* from Illam, Nepal (Wu et al, 2020).

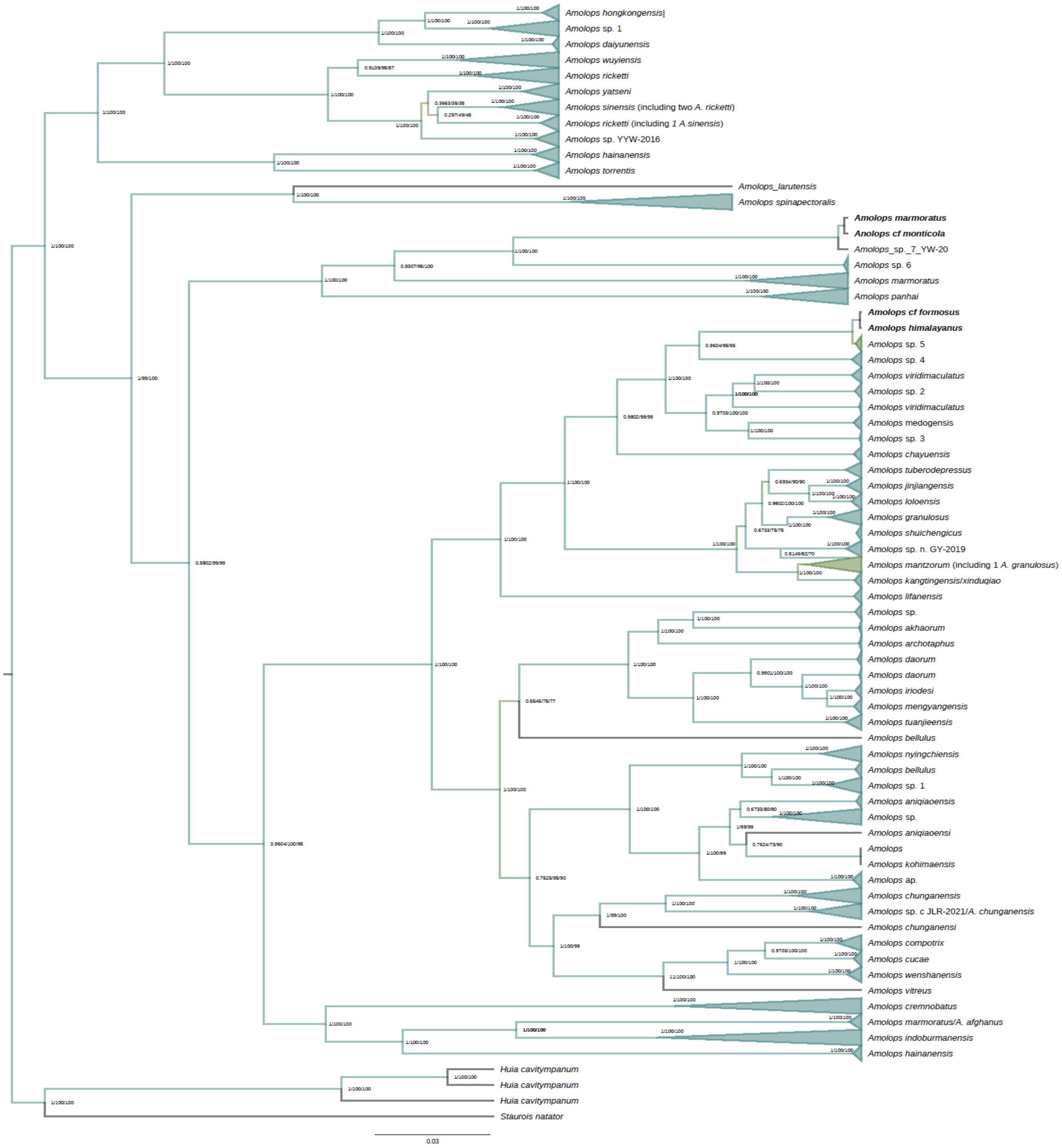


Fig 5.2. Phylogenetic tree of genus *Amolops* based on COI gene using Bayesian Inference (BI) , Maximum likelihood(ML) and Neighbor Joining(NJ) approach. The values on the node represent BI posterior probability, NJ bootstrap and ML bootstrap values.

5.4 Discussion

The classification of species within *Amolops* has undergone several systematic rearrangements (Fei et al, 1999, 2005, 2009) however the taxonomy has remained a matter of dispute. Since *Amolops* species have similar adult morphology, they are easily confused with other species occurring in similar habitats which has created confusion and misidentification of *Amolops* species (Bain et al, 2006). In this study we recorded a total of four *Amolops* species from Sikkim. The morphological evaluation of species demonstrated potential affinities to *Amolops marmoratus*, *Amolops monticola*, *Amolops himalayanus* and *Amolops formosus*. Hence, based on morphological characters recorded in this study, these four species were subsequently identified as *Amolops marmoratus*, *Amolops cf. monticola*, *Amolops himalayanus* and *Amolops cf. formosus* with the help of relevant literatures (Boulenger, 1920; Yang, 1991; Schleich & Kastle, 2004). The distribution of these *Amolops* species has been reported by previous studies in Sikkim (Subba et al, 2017; Chettri & Acharya, 2020). Principal component analysis based on morphometric characters exhibited significant variation among the species in the study thus confirming that the species in this study are likely to be distinct species. Although, this study demonstrated taxonomic identities based on morphological variations (Boulenger, 1920; Yang, 1991; Schleich & Kastle, 2004), however, detailed comparisons with type specimens deposited in different natural history museums in India and around the world would further aid in validating the taxonomic identities of these species. Morphological study of the Amolops group can be particularly challenging considering the substantial amount of morphological similarity and cryptic diversity (Patel et al, 2020). Nonetheless, our current findings can facilitate preliminary identification of *Amolops* species from Sikkim and possible misidentifications from the region. Morphological characters typically employed for the identification of species such as dorsal and ventral coloration and texture, presence or absence of nuptial pads, dorsal spots etc vary depending on the sampling season (Patel et al, 2020). Hence, the use of molecular techniques have greatly aided in evaluating species diversity within the Amolops group (Matsui et al, 2006; Wu et al, 2020; Mahony et al, 2022).

For the molecular anlaysis, I attempted phylogenetic reconstruction of three mitochondrial genes (*COI*, *16srRNA* and *12SrRNA*) and one nuclear (*RAG1*) gene however, only *COI* yielded quality sequences for further analysis. Molecular phylogenetics and genetic markers such as the *Cytochorme Oxidase Subunit I* gene (*COI*) or the barcode gene has significantly aided in the assignation of species of the genus *Amolops*. The use of approximately 650bp of mitochondrial (COI) gene has been considered efficient for animals (Herbert et al, 2003). The result of the phylogenetic anlaysis of this study is comparable with the phylogeny of *Amolops* based on combined mitochondrial and nuclear genes (Wu et al, 2020; Patel et al, 2021). The sequences for species identified as *Amolops himalayanus* and *Amolops cf. formosus* in the present study were found nested with the *A. viridimaculatus group* (five known species and four candidate new species). Interestingly the sequences of these two species are closely related to a potential new candidate species *Amolops* sp *5* from Illam, Nepal (Wu et al, 2020). The *Amolops viridimaculatus* group is a newly erected group that contains many new candidate species (Wu et al, 2020). Hence, the validity of this group needs to be further assessed considering the high amount of candidate species. Similarly, the sequence representing *Amolops marmoratus* in this study nested in the *Amolops marmoratus* group (three known species and two unknown species) and is evolutionarily closely related to *Amolops* sp. *7* which is collected from Illam, India (Wu et al, 2020). Thus, it validates the taxonomic identity of *Amolops marmoratus* based both on morphological and molecular data of this study. However, the sequence representing *A. cf. monticola* in this study clustered within *A. marmoratus* group and is closely related to *Amolops* sp 7 which brings into question the validity of this species or its occurrence in Sikkim. Although only recently discovered by means of molecular analyses (Wu et al, 2020), *Amolops* cf. *monticola* and *Amolops* sp 7 are from the same environmental range (Himalaya) which reinforces the possibility of new candidate species from Sikkim. In the past two decades, many new species of amphibians have been reported from the Himalaya (Khatiwada et al, 2015, 2020; Jiang et al, 2016; Qi et al, 2019). Hence, this result reflects the trend of undescribed hidden diversity in the Himalaya. It is sometimes criticized that single-locus barcoding analyses do not provide enough information for new species description (DeSalle, 2006). Therefore, the present study recommends further analysis with multi-gene phylogenies of species belonging to *Amolops* group from Sikkim to strengthen the present findings.

5.5 Conclusion

The phylogeny of genus *Amolops* was inferred using mitochondrial (COI) genetic marker for *Amolops* species occurring in Sikkim Himalaya. The morphological identification of *Amolops* species occurring in Sikkim indicated the presence of distinct species namely *Amolops marmoratus*, *Amolops monticola*, *Amolops formosus* and *Amolops himalayanus*. Molecular phylogeny revealed that *A. himalayanus* and *A. cf. formosus* identified on the basis of morphological characters clustered in *A. viridimaculatus* group . Both species exhibited close evolutionary relationship with *Amolops* sp 5 currently unidentified from Nepal (Wu et al, 2020). Similarly *Amolops marmoratus* from this study nested within *A. marmoratus* group which validates concordance with morphological observations and its occurrence in Sikkim. However, sequence representing *Amolops cf. monticola* also nested within *A. marmoratus* group and was closely related to *Amolops* sp 7 which is unidentified and potential candidate species (Wu et al, 2020). This result in particular reveals the presence of cryptic diversity within the *A. marmoratus*. The unidentified *Amolops* sp 5 and *Amolops* sp 7 were collected from Nepal which is from the same environmental range and just 57.9 km away from present location. Hence this study is in concordance with the present trend of cryptic diversity of amphibians reported from the Himalayas and reflects the possibility of new species in this region. In the present study only one genetic marker was used (*mt COI*) which is generally considered insufficient for the description of new species. Hence, future studies should incorporate different sets of molecular markers and more sequences of known species for phylogenetic analyses which could aid in revealing the cryptic diversity within the genus *Amolops* and possibly the identification of new species from the region.

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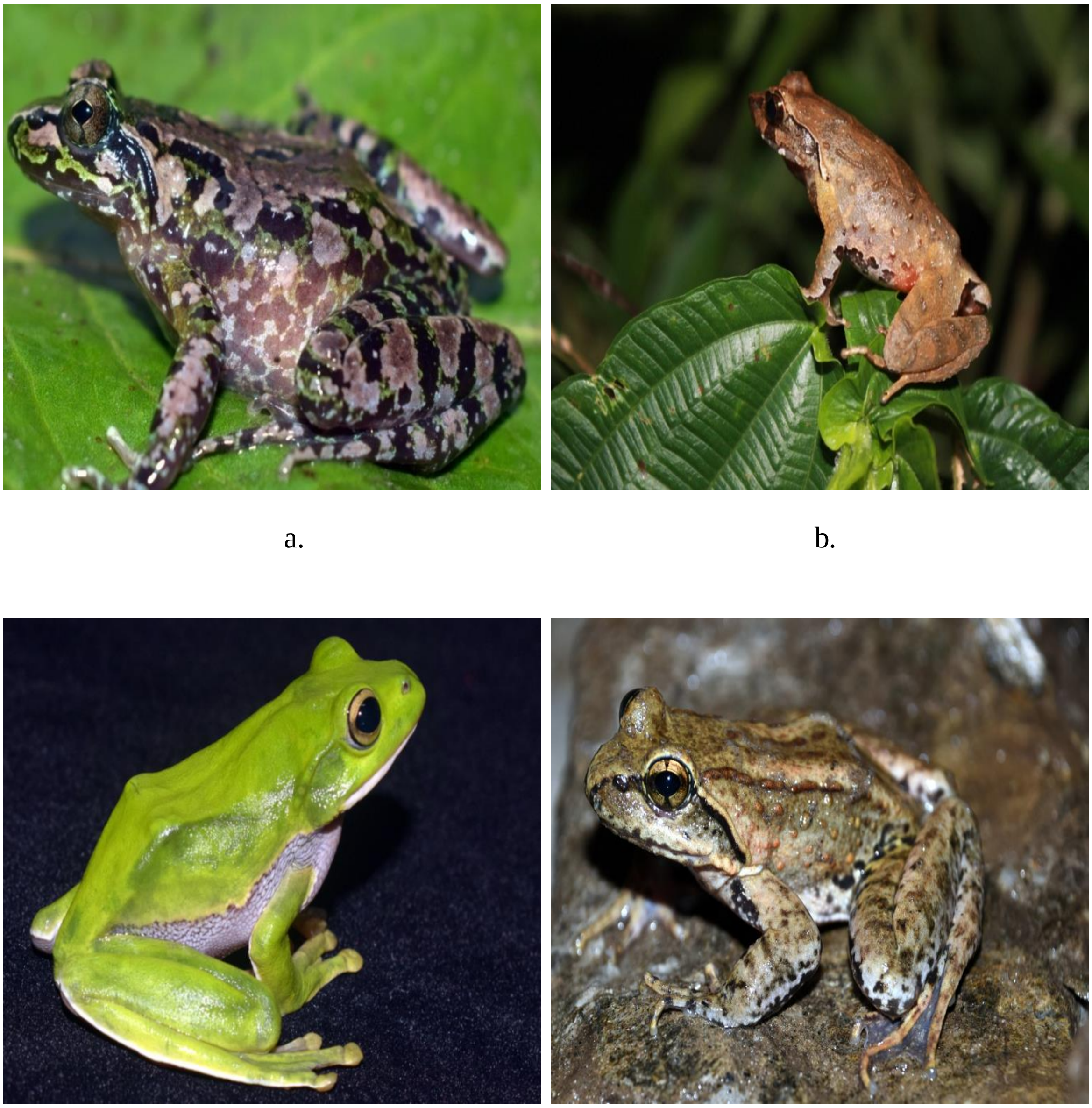
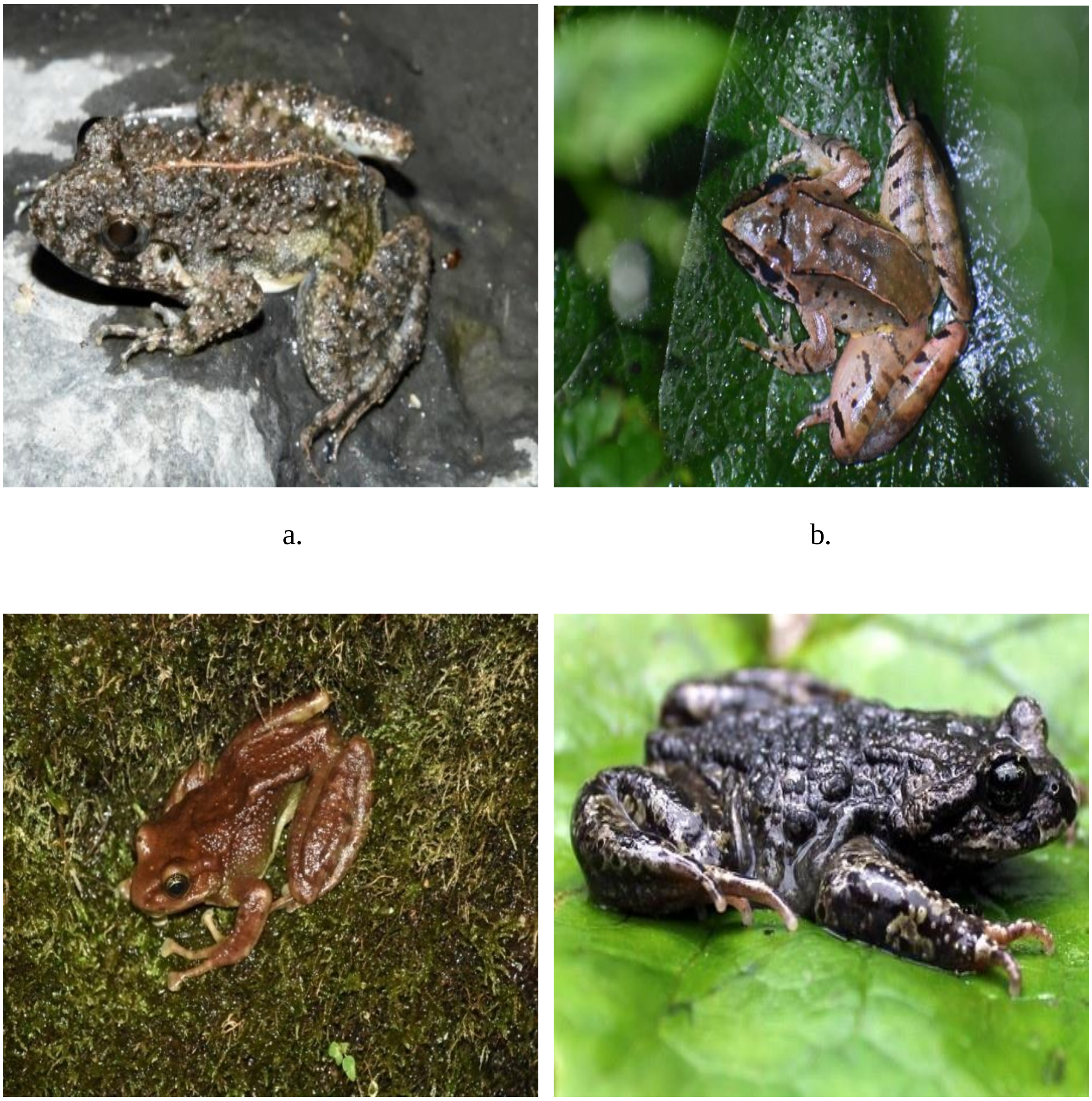


a. b.

c. d.

Photo plate 6. Amphibian species recorded in the study a. *Nanorana blanfordii* , b. *Megophrys parva*, c. *Rhacophorus maximus* d. *Nanorana liebigii*



a. b.

c. d.

Photo plate 7. a. *Minervarya teraiensis* b. *Nanorana gammii*, c. *Amolops* sp. d. *Scutiger boulengeri*.

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Appendix

Table A1. Species records among recent studies on amphibians of Sikkim Himalaya

Species	**Present study**	**Chettri et al., 2020**	**Subba et al., 2017**
Duttaphrynus melanostictus	✓	✓	✓
Duttaphrynus himalayanus	✓	✓	✓
Duttaphrynus stuarti	✓	✗	✓
Amolops marmoratus	✓	✓	✓
Amolops monticola	✓	✓	✓
Amolops formosus	✓	✓	✓
Amolops himalayanus	✓	✓	✓
Amolops sp1	✓	✗	✗
Nanorana liebigii	✓	✓	✓
Nanorana gammii	✓	✓	✓
Nanorana minica	✓	✗	✗
Nanorana annandalii	✓	✓	✓
Nanorana blanfordii	✓	✓	✓
Nanorana sp1	✓	✗	✗
Hoplobatrachus tigerinus	✓	✓	✓
Minervarya teraiensis	✓	✓	✓
Minervarya nepalensis	✓	✓	✓
Minervarya sp1	✓	✗	✗
Megophrys parva	✓	✓	✓
Megophrys robusta	✓	✓	✓
Scutiger sikkimensis	✓	✓	✓
Scutiger boulengeri	✓	✓	✓
Polypedates leucomystax	✓	✓	✓
Polypedates himalayanus	✓	✗	✗
Raorchestes annandalii	✓	✓	✓
Rhacophorus maximus	✓	✗	✓
Ichthyophis sikkimensis	✓	✓	✓

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Rich yet undocumented ethnozoological practices of socio-culturally diverse indigenous communities of Sikkim Himalaya, India

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ABSTRACT

Aim of the study: The indigenous people of Sikkim Himalaya possess indispensable traditional knowledge including the use of flora and fauna due to their close association with nature. The present study aims to explore the rich and undocumented ethnozoological practices of different indigenous communities of the Sikkim Himalaya

Materials and methods: Data was collected using semi-structured questionnaire interviews among the traditional healers and indigenous communities of Sikkim. A total of 91 respondents covering all four districts of Sikkim were interviewed from September 2017–April 2018.

Results: This study recorded a total of 59 species belonging to nine taxonomic groups used for traditional ethnozoological practices by the indigenous communities of Sikkim. Mammals represented 58% of the total animals followed by birds (22%). Among the total, 71% of animal species were used for zootherapy whereas 29% were used for religious customs or shamanistic practices. Almost 64% species were used for treatment of more than one disease and chief mode of preparation was by boiling the body parts. Among different parts used in traditional medicine, meat was most preferred while horns and hairs were mostly used for religious purposes.

Conclusion: Present findings suggest that traditional medicine including zootherapy still serves as a source of primary healthcare in rural areas of Sikkim and provides an identity to the culture of a region. We recommend documentation of more such traditional medicinal systems along with scientific validation of traditional practices with modern tools. Biodiversity Management Committees (BMCs), the legally formed bodies at local level, may be entrusted in documentation of such practices which might contribute significantly in the conservation of traditional practices and also preserves the associated traditional knowledge as per the provision of Biological Diversity Act of India.

1. Introduction

Human beings, more specifically the ethnic communities, depend on wild flora and fauna for a range of services because of their cultural association with natural resources since prehistoric times. Such associations still prevail to the present day as socio-ethnic practices in the form of traditional medicine and folk culture (Lev, 2003; Alves, 2009). "Traditional medicine is the sum total of knowledge, skills and practices based on theories, beliefs and experiences used in healthcare for prevention, diagnosis, improvement or treatment of physical and mental illness"-World Health Organization (2013). According to WHO (1993), the primary health care requirement in about 80% of the world population is fulfilled by traditional folk medicine especially in low-income countries making it only access to health care. In India, people in rural areas are dependent mostly on traditional medicine system relative to modern health care facilities which are either unavailable or expensive (Borah and Prasad, 2017).

Around the world, many studies have focused on ethno-medicinal practices using plants or plant parts (Polat et al., 2015; Silambarasan and Ayyanar, 2015; Faruque et al., 2018). However studies pertaining to usage of animals for medicine and cultural reasons are scanty (Alves and Rosa, 2005). Some studies conducted on animals have highlighted its significance in numerous religious rituals and mystical practices among various indigenous communities (Walter and Fridman, 2004). Ancient civilizations have historical records involving the use of animal parts or products for curing various diseases (Lev, 2003). Traditional Chinese Medicine uses more than 1500 animal species comprising different taxonomic groups (Alonso-Castro, 2014). Similarly, Brazil and Latin America use 326 (Costa-Neto and Alves, 2010) and 584 (Alves and Alves, 2011) animal species respectively for curing different ailments. Kim and Song (2013) reported 40 animal species that are potentially used for medicinal purposes in Jeju Island, Korea, while 163 animal species were used in ethno-zoological practices in Mexico (Alonso-Castro, 2014).

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In India, research on medicinal uses of animals or their parts or products are scanty and most of our knowledge is based on ancient texts such as Ayurveda and Charaka Samhita. These texts highlight the importance of ethnobiology mentioning the use of almost 380 types of animal substances (Unnikrishnan, 1998). A review of traditional zootherapeutic studies of India (Mahawar and Jaroli, 2007) reported 109 animal species with its 270 medicinal uses. In Chhattisgarh state, over 500 species of insects, mites, spiders are used as medicines to cure various illnesses (Oudhia, 1995). Similarly, in Tamil Nadu, 16 species of animals including mammals, birds, reptiles, annelids, arthropods are used for the treatment of over 17 kinds of diseases (Solavan et al., 2004). In Meghalaya, the Khasi tribe uses 13 animal species for the treatment of cough, fever, diarrhoea and anemia (Solavan et al., 2004). Similarly, in Arunachal Pradesh, the Galo tribe collects 12 species of edible insects for zootherapeutic use (Kato and Gopi, 2009) and in Mizoram, a total of 35 animal species are used for therapeutic purposes (Chinlampianga et al., 2013). Vijayakumar et al. (2015) reported 69 species of animals used by traditional healers of Silent Valley, Kerala to treat over 85 medical conditions.

Sikkim, located in the lap of Himalayas in India, has diverse ethnic communities and cultures representing three major groups- Lepcha, Bhutia and Nepali. The Nepali community consists of 23 sub-communities, each having their own unique traditions, rituals, food habits and ethnomedicinal practices (Anon, 2017). Despite rich ethnic demography, systematic study on traditional medicines in Sikkim is very scanty and focus is given mainly on medicinal uses of plants (Pradhan and Badola, 2008; Sherpa et al., 2015). The study of ethnozoology not only aids in preserving the socio-cultural identity of a community or society but also helps in validating the scientific observation which ultimately contributes to the well being of the people. In this paper, we have studied the ethnozoological practices of the indigenous people living in Sikkim Himalaya which remains undocumented till date.

2. Methodology

2.1. Study area

The hilly state of Sikkim spread between 27° 03′ to 28° 07′ N and 88° 03′ to 88° 57′ E is located in the Eastern Himalaya, India. Eastern Himalaya including Sikkim contributes to three global biodiversity hotspots of the world (Chettri et al., 2010). Its location at the junction of Indo-Malayan, Palaearctic and Sino-Japanese biogeographical realms and interplay of various climatic factors has created varieties of niches to harbor an astounding degree of biodiversity within a small geographical area. Sikkim comprises of only 0.2% of the total geographical area of India but makes one of the biodiversity-rich states of India harboring 144 species of mammals, 690 butterfly species, 574 bird species and more than 4500 flowering plant species (Acharya and Sharma, 2013). Thus, the plentitude of natural resources has been significant in shaping the cultural heritage of Sikkim.

2.2. Socio-cultural diversity of Sikkim

The state of Sikkim is bounded by Tibetan Autonomous Republic of China on the North and East, Kingdom of Bhutan on the East, the Indian state of West Bengal in the South and Nepal on the West. Thus, this transboundary creates an amalgamation of rich ethnic and cultural diversity of people. Ethnographically Sikkimese people show affinity with Aryan, Mongoloid and Tibeto-Burman stocks (Vandenhelsken, 2016). The majority of Sikkimese population resides in rural areas and agriculture is the major source of livelihood for the indigenous communities. The collection of wild edibles for food and medicine has been a major practice of these communities in the past and the same continues even today. Their association and dependence on natural resources have endowed them with vast information on the use and conservation of these resources using their traditional knowledge practices.

The low to mid-elevation areas in all four districts of Sikkim are mostly dominated by different sub-communities belonging to Nepali ethnic groups such as Bahun, Chettri, Pradhan, Rai, Limbu, Bhujel, Gurung, Damai, Kami, etc. In addition to their involvement in government and private sector jobs, these communities practice terrace cultivation in the indigenous farming systems. The ethnic Lepcha tribe is concentrated in isolated valleys and forest-clad mountains of North Sikkim, especially in Dzongu which is known as 'Lepcha Reserve'. Agriculture and animal husbandry being their main occupation, animals are not only significant for sustenance but also have important roles in their religion and shamanistic sacrificial rituals (Gorer, 1938). The Bhutia community is spread all across the state but Lachenpa and Lachungpa tribe within this community predominate the higher elevations, with their main concentration in the North district with agro-pastoralism and semi-settled agriculture (Gulia, 2005; Roy Burman, 2008). The flourishing tourism in higher elevations is adding new dimensions to their socio-economic profile in recent years thus bringing new sources of income generation.

2.3. Data collection

The data on the zootherapeutic uses of animals were obtained through field surveys for a period of seven months from September 2017–April 2018 in all four districts (North, South, East and West) of Sikkim. A total of 30 respondents from Chungthang, Lachung, Dzongu in North district, 20 respondents from Kaw, Lingee in South, 20 respondents from Kongri and Labdang in West and 21 respondents from Ranka, Samdong in East district (Fig. 1). In total 91 people were interviewed with a semi-structured questionnaire regarding the use of zootherapy (Questionnaire is given Annexure I). Sample size from North district was more as this covers almost 50% of the total geographical area of the state and comprises of mainly tribal communities where ethno-zoological practices is relatively more. Using this approach we have fairly covered all the three major ethnic communities from all four districts of Sikkim. The respondents were selected based on their knowledge on traditional medicine who practice/have been practicing zootherapy at local level. They comprised of local healers, farmers, shamans (Dhami, Jhakri, Bijuwa, Phedangma), and elderly people. Data including the local name of the animal, parts or products used, mode of preparation, application, ailments treated and mode of administration was collected during the questionnaire survey following (Huntington, 2000). The total population of different ethnic groups of study area was obtained from the Department of Economics, Statistics, Monitoring and Evaluation (DESME), Government of Sikkim, India.

Permission from the respondents was obtained for the questionnaire and digital records during the survey prior to the interviews. Digital records are available in the Ecology, Biodiversity and Conservation Biology Laboratory of the Department of Zoology, Sikkim University, India for further identification by experts and future references. The animals were identified on the basis of their occurrence in the study area, available digital records and identifying characters narrated by the respondents following standard literature (Schleich and Kastle, 2002; Grimmett et al., 2013; Menon, 2014). While majority of species were identified up to species level, certain species were identified only up to genus level and scientific taxonomy could not be ascertained for few species because many species within the animal group are known by same local names.

2.4. Data analysis

We converted the number of respondents into relative percentage with regard to gender, age, educational qualification and monthly in-

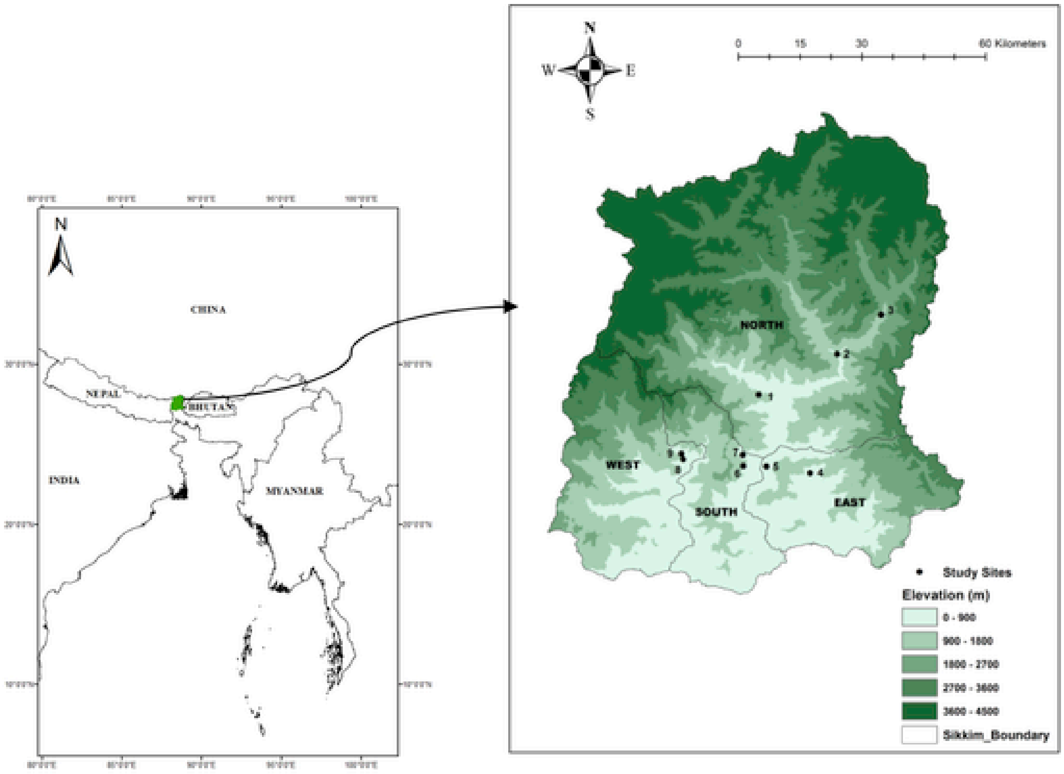

Fig. 1. Map showing study sites located in four districts of Sikkim, Eastern Himalaya: 1. Chungthang, 2. Lachung, 3. Dzongu, 4. Ranka, 5. Samdong, 6. Kaw, 7. Lingee, 8. Kongri, 9. Labdang.

come, and grouped them into different categories (see Table 1). The variation in number of practitioners in different categories were then assessed using χ^2 test. The number of species and different body parts used by the ethnic communities were represented graphically.

The fidelity level (FL) was used to ascertain the local importance of animal species used for treating the disease by the respondents of the study area (Friedman et al., 1986; del Rosario Jacobo-Salcedo et al., 2011; Kim and Song, 2013). The fidelity level signifies the most preferred species for treatment of ailments by respondents. FL was calculated using the formula: FL (%) = Np x 100/N, where Np is the number of respondents mentioning the particular animal species used to treat certain ailments and N is the total number of respondents who used those animals as medicine for treating any given ailment.

Table 1
Demographic characteristics of the ethno-zoological practitioners (selected as respondents) of Sikkim Himalaya.

Gender		
Male	90	(99%)
Female	1	(1%)
Age group		
20-30	8	(9%)
31-40	10	(11%)
41-50	17	(19%)
51-60	24	(26%)
61-70	32	(35%)
Education		
Never attended school	30	(33%)
Primary level	35	(38%)
Junior level	11	(12%)
Secondary level	6	(7%)
Higher Secondary level	9	(10%)
Monthly Income		
Rs 1000- Rs 5000	73	(80%)
Rs 5000- Rs10000	11	(12%)
Rs 10000- Rs15000	5	(6%)
Rs 15000- Rs20000	2	(2%)

The ethnopharmacological importance of the collected animal species and the concordance of informant knowledge were identified using Informant Consensus Factor (ICF) (Heinrich et al., 1998; Kim and Song, 2013). The ICF was calculated using the formula: $ICF = (n_{ur}-n_t)/(n_{ur}-1)$, where n_{ur} is the number of times an ailment was mentioned in each category and n_t is the number of animal species used.

3. Results and discussion

3.1. Demographic characteristics of respondents

All the 91 respondents (1 female, 90 male) were interviewed based on their history and experience of using zootherapy for healing practices. The majority of the respondents were males indicating male dominance in traditional healing practices, and the trend is similar as reported in other studies in India (Mahawar and Jaroli, 2007; Verma et al., 2014). The age of the informants ranged from 25 to 70 years with a maximum (35%) between 60 to 70 years (Table 1). The percentage of healers in the age group of 60–70 years was significantly higher as compared to other age groups ($\chi^2 = 21.80$, df = 4, p = 0.01). The data revealed that the elderly people (61–70 years) are more into the profession of ethnozoological practices indicating low acceptance of such system among younger generations.

About 33% of the respondents had not received any formal education while only 10% had a higher secondary level of education which is similar to the observation of Borah and Prasad (2017). The number of practitioners having primary level of education was significantly higher ($\chi^2 = 38.83$, df = 4, p = 0.01) among the ethnic communities reflecting that basic education is necessary to understand and implement the ethno-zoological practices in a better and effective manner. However, the profession either does not require much formal education or better educated person get government or public sector jobs and do not learn or practice zootherapy. Communities have adopted traditional knowledge from their forefathers and continued up to their generation so that they could get some economic benefits and live respectable life as healers.

Ethnozoological practices in Sikkim is mostly used by people having low-income (Rs 1000 to Rs 5000 per month) which is significantly higher compared to other income groups ($\chi^2 = 149.83$, df = 3, p = 0.01). Most of the respondents are farmers and practice zootherapy as a part-time occupation. The income so generated is unsteady because it usually depends only on the patients seeking such treatments in the locality. Based on ailments, types of faunal ingredients used and the duration required for the treatment, the costs varies from Rs 200 to Rs 1000 which is quite meager for sustenance. Therefore, zootherapy is not regarded as a regular source of livelihood but an additional supplement for living by the local communities. It is often seen that the use of faunal resources for ethnomedicinal practices are prevalent in communities that are underprivileged and have poor socio-economic conditions (De Souza-Mazurek et al., 2000; Pattiselanno, 2004; Alves et al., 2009). Traditional ethnomedicinal systems can contribute to family income as in the case of Garasiya tribe of Rajasthan thus leading to the sustenance of such practices even today (Jaroli et al., 2010). However, due to less popularity of traditional practices, easy availability of modern health care facilities and other livelihood opportunities (such as tourism industry in Sikkim), most youngsters have not given due consideration to zootherapeutic systems. This might result in loss of rich traditional knowledge which is orally passed from one generation to another.

3.2. Ethnic communities in study sites

Among the 91 respondents, 79% belonged to the Lepcha community; the most dominant community using animals for zootherapeutic practices, 11% Limbu community followed by 6% Rai community. Only 2% of the practitioners were Gurung community and 1% Chettri community (Fig. 2). Lepcha comprises of only 7.78% of the total population of Sikkim (Rai, 2013) and represented 40% of the total population in the study area (Fig. 2). Similarly, Limbu community comprised around 7% of the total population but 11% were ethnozoological practitioners. The highest representation of Lepcha and Limbu respondents indicates their higher association with traditional practices.

The ethnic Lepcha communities mainly inhabit forest fringes and worship mother nature who have retained their rich cultural heritage in the current era of globalization (Pradhan and Badola, 2008). They are considered as the primitive tribe of Sikkim and possess a vast repository of biodiversity knowledge (Gorer, 1938). Their knowledge on birds (identification skills as parataxonomists) was highlighted by previous researchers (Acharya et al., 2009; Jha and Jha, 2012). Majority in the community, especially aged people, depend completely on traditional healing practices for various ailments.

3.3. Ethnozoological analysis

In this study, a total of 59 species belonging to nine taxonomic groups were recorded to be in use for traditional ethnozoological practices by the indigenous communities of Sikkim. Among these, 71% of animal species were used for zootherapy, whereas 29% were used for religious customs or shamanistic practices (Table 2 & Table 3).

Of the total 59 species, 34 are mammals representing the highest percentage (58%), followed by 13 bird species (22%). Insects represented three species (5%), whereas amphibians, reptiles and molluscs contributed 3% representing two species each. Similarly, arachnids, crustaceans and annelids represented one species each (2%) (Fig. 3). Among the animals, mammals have higher demand in ethnozoological practices, for example, maximum number of mammalian species were used in Theni district of Tamil Nadu (Chellappandian et al., 2014) and in Mount Abu, Rajasthan (Jaroli et al., 2010) by ethnic communities. Similarly, in Mizoram and Arunachal Pradesh 48 and 39 mammal species were used for zoo therapy (Chinlampianga et al., 2013). The increased use of mammals might be due to the large number of domesticated mammals and also due to more knowledge and fascination of ethnic communities towards mammals (being large and charismatic animals).

3.4. Animals of medicinal use

A total of 42 species of animals were recorded for medicinal use in traditional zootherapy by the different indigenous communities of Sikkim. These animals were used to treat over 26 different medical conditions using various methods (Table 2). Common ailments treated using animal parts are common cold and cough (11%) followed by fever (8%), diabetes and food poisoning (6%). About 64% of animal species were used for the treatment of more than one ailment. The results show eight modes of preparation of medicines derived from different parts or by products of animals. The preparation was mostly in the form of soup (25.5%) followed by raw consumption (23.4%), plain boiled (21.3%), powdered (14.9%), decoction (6.4%), dried

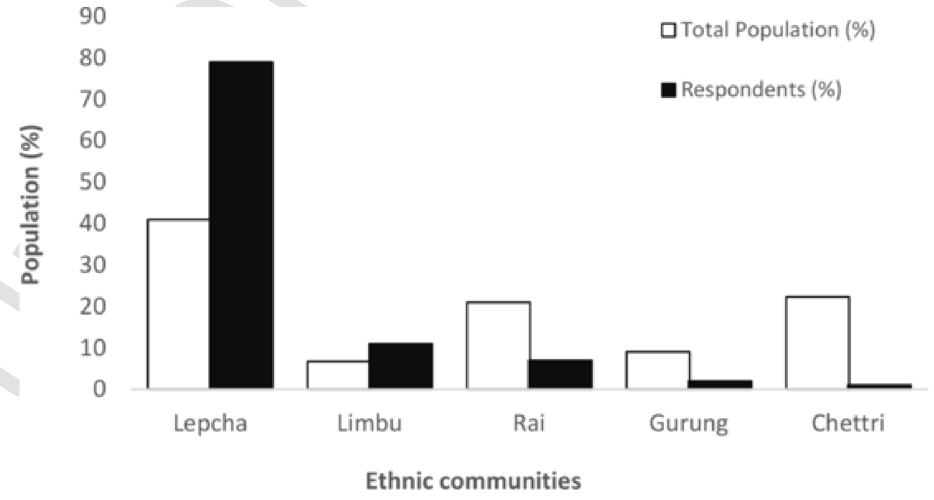

Fig. 2. Proportion of total population and ethno-zoological practitioners representing different indigenous communities of the area of Sikkim Himalaya.

Table 2
Animal species used by ethnic communities for medicinal purposes in Sikkim Himalaya.

Animal Group	Common Name	Local Name	Scientific Name	IUCN Conservation Status/CITES (Appendices)	Parts/Products Used	Ailments Treated	Preparation	Application	FL (%)
Mammal	Himalayan Black Bear	*Bhalu* (N) *Sanna* (L)	*Ursus thibetanus*	VU Appendix I	Gall Bladder	Fever Liver disorders Heart disease Diabetes Wounds Body ache Dysentery Asthma	Powder The Gall bladder is dried and made into a powder and given for consumption with water Raw The gall bladder is minced into small pieces and given for consumption everyday till relief of symptoms is observed	Oral	16.9% 16.9% 16.9% 16.9% 16.9% 5.6% 5.6% 4.0%
Mammal	Tiger	*Bagh* (N) *Sothong* (L)	*Panthera tigris*	EN Appendix I	Bones	Rheumatoid Arthritis	Powder The bones are crushed into small pieces and given to patients with water in small doses for consumption for atleast a period of three months	Oral	100%
Mammal	Jackal	*Syal* (N)	*Canis aureus*	LC Appendix III	Meat	Piles Gout	Smoke dried The skin of the animal is removed, it is cut into pieces and smoke dried and given for consumption Fermented The meat of the animal is mixed with paddy grains with addition of some yeast, fermented and distilled, and given for consumption to patients with chronic gout	Oral	37.5% 62.5%
Mammal	Red Fox	*Fyauro* (N)	*Vulpes vulpes*	LC Appendix III	Meat	Joint pain	Boil The meat of the animal is boiled and then given for consumption	Oral	100%
Mammal	Indian Wild Dog	*Ban Kukur* (N)	*Cuon alpinus*	EN Appendix II	Meat	Asthma High fever Tuberculosis	Boil The meat of the animal is cut into small pieces and boiled and given for consumption. In case of Tuberculosis, 5–6 doses within a span of six months are given to the patients	Oral	33.3% 33.3% 33.3%
Mammal	Wild Boar	*Badel* (N)	*Sus scrofa*	LC NL	Teeth Horn	Rheumatism Epilepsy Parturition	Powder The tooth/horn is ground to a fine powder and 5-10 gm is given to the patients	Oral	33.3% 33.3% 33.3%
Mammal	Barking Deer	*Mrigha* (N)	*Muntiacus muntjac*	LC NL	Skin Horn Bones	Food poisoning Constipation Ear pain	Raw The fur is removed from the animal body and de-skinned, a small piece of the meat is given raw for consumption The horn and bones are made into a paste by rubbing against a traditional stone grinder and given to the patients for consumption	Oral	83.3% 8.3% 8.3%

Table 2 (*Continued*)

Animal Group	Common Name	Local Name	Scientific Name	IUCN Conservation Status/CITES (Appendices)	Parts/Products Used	Ailments Treated	Preparation	Application	FL (%)
Mammal	Himalayan Musk Deer	*Kasturi Mrigha* (N)	*Moschus leucogaster*	EN Appendix II	Musk gland	Cough Cold Urinary tract infection Ear pain	Raw The musk gland of the animal is isolated and washed, kept in airy conditions for a day or two. It is then minced into small pieces and given to the patients till relief of symptoms In cases of cold and cough, the musk gland is inhaled to subside the symptoms	Inhalation	25% 25% 25% 25%
Mammal	Spotted Deer	*Chital* (N)	*Axis axis*	LC NL	Meat	Immunity booster Constipation	Boil (Soup) The meat from any part of the animal is minced into small pieces and boiled till broth consistency and then given to patients	Oral	50% 50%
Mammal	Himalayan Blue Sheep	*Bharal/Ban Bheda* (N)	*Pseudois nayaur*	LC Appendix III	Horn	Dysentery Food poisoning Pneumonia	Powder The horn of the animal is crushed into fine powder using mortar and pestle, then a dose of around 30 gm is given for consumption with water	Oral	33.3% 33.3% 33.3%
Mammal	Himalayan Tahr	*Thar* (N)	*Hemitragus jemlahicus*	NT NL	Meat Bones	Dysentery Bone fractures	Boil (Soup) The meat is cut into small pieces and boiled till soft and given to the patients Powder The bone of the animal is powdered and applied on fractures using bamboo splint	Oral Topical	50% 50%
Mammal	Rhesus Monkey	*Badar* (N)	*Macaca mulatta*	LC NL	Meat	Tuberculosis	Boil The animal is de-skinned and cut into small pieces, the meat is boiled till soft without adding salt and consumed	Oral	100%
Mammal	Chinese Pangolin	*Shalak* (N)	*Manis pentadactyla*	CR Appendix I	Scales Meat	Food poisoning Fever	Decoction The scales are crushed and boiled in water for hours until a concentrate is formed which is given to the patients Boil (Soup) The meat is boiled until soft and given to the patients continuously for 2–3 months	Oral	50% 50%
Mammal	Hoary - bellied Himalayan Squirrel	*Lotharke* (N) *Kelee* (L)	*Callosciurus pygerythrus*	LC NL	Meat	Common Cold Cough	Boil (Soup) The meat is cut into small pieces, boiled and given every day for consumption till relief of symptoms	Oral	50% 50%
Mammal	Red giant Flying Squirrel	*Rajpankhi* (N)	*Petaurista petaurista*	LC NL	Meat	Diabetes	Boil (Soup) The meat is minced into small pieces, boiled till soft and given for consumption	Oral	100%

Table 2 (*Continued*)

Animal Group	Common Name	Local Name	Scientific Name	IUCN Conservation Status/CITES (Appendices)	Parts/ Products Used	Ailments Treated	Preparation	Application	FL (%)
Mammal	Procupine	*Damsee* (N)	*Hystrix brachyura*	LC NL	Stomach meat	Diarrhoea Cough Cold Food poisoning Snake bite Malaria Fever Diabetes	Dried The skin of the animal is removed and the stomach is obtained which is either sun dried or smoke dried and given for consumption	Oral	32.6% 3.8% 3.8% 15.3% 27% 7.6% 5.7% 3.8%
Mammal	Indian Hare	*Kharayo* (N)	*Lepus nigricollis*	LC NL	Meat Fur	Body pain Cold Cough Cuts and wounds	Boil The meat is chopped into medium pieces and boiled in water without addition of salt and once it is soft is given to the patients for consumption Powder The fur is burnt in burning coals and then it is squashed into fine powder and applied on cuts/ wounds to stop bleeding	Oral Topical	62.5% 6.2% 6.2% 25%
Mammal	Mole	*Uttaney musa* (N)	*Bandicota* sp	–	Meat	Tuberculosis Fever Diabetes	Boil The animal is de-skinned and then cut into small pieces, it is boiled till soft and given to patients in regular doses for almost two weeks	Oral	33.3% 33.3% 33.3%
Mammal	Mongoose	*Naurey musa* (N)	*Herpestes* sp.	LC Appendix III	Meat	Tuberculosis Fever	Boil The meat is cut into small pieces, it is then boiled till soft and given for given for consumption In case of tuberculosis at least 5–6 doses within a span of six months are given to the patients	Oral	50% 50%
Mammal	Bat	*Chamera* (N) *Pilim* (L)	*Pteropus giganteus*	LC Appendix III	Meat	Bed wetting Piles Cold Cough	Boil (Soup) The meat is cut into small pieces, boiled without adding salt and then given to the patients till relief of symptoms	Oral	50% 25% 12.5% 12.5%
Mammal	Sheep	*Bheda* (N)	*Ovis aries*	NA	Butter	Burns Scars	Raw The butter is made out of the milk of the animal and is stored, applied on burns and scars for quick healing and to fade the scars	Topical	50% 50%
Mammal	Cattle	*Gai/ Goru(N)*	*Bos taurus*	NA	Urine Gall bladder	Diabetes Cold Cough	Raw Urine is collected, some water is added and then immediately given to the patient for consumption Gall bladder is extracted from the animal, cut into pieces and given raw for diabetics	Oral	37.5% 31.2% 31.2%
Mammal	Dog	*Kukur* (N)	*Canis familiaris*	NA	Blood	Asthma Fever	Raw The blood of this animal is collected immediately after sacrificing and then a dose of around 100 ml is given to the patients	Oral	50% 50%

Table 2 (*Continued*)

Animal Group	Common Name	Local Name	Scientific Name	IUCN Conservation Status/CITES (Appendices)	Parts/Products Used	Ailments Treated	Preparation	Application	FL (%)
Mammal	Yak	*Chauri* (N)	*Bos grunniens*	NA	Meat	Body pain	Boil (Soup) The animal after sacrificing is cut into small pieces and boiled adding salt till the meat is soft and given for consumption	Oral	100%
Mammal	Pig	*Sungur* (N)	*Sus domesticus*	NA	Gall bladder	Cold Cough	Boil (Soup) The gall bladder of the animal is cut into small pieces and boiled in water till soft and given to patients for consumption	Oral	50% 50%
Mammal	Human	*Manchey* (N)	*Homo sapiens*	NA	Milk	Eye pain	Raw Milk of lactating mothers is extracted and immediately poured drop by drop inside the eye and repeated until relief	Topical	100%
Bird	Peacock	*Majur (N)*	*Pavo cristatus*	LC Appendix III	Feather	Nose bleed	Powder The feathers are burnt and then crushed to fine powder and applied inside the nostrils during nose bleeds	Topical	100%
Bird	Large-billed Crow	*Kaag* (N)	*Corvus macrorhynchos*	LC NL	Meat	Piles Fever	Roasted The feathers of the bird is removed and the bird is roasted in fire and the meat is then given to patients	Oral	50% 50%
Bird	Common Myna	*Ruppi (N)*	*Acridotheres tristis*	LC NL	Meat	Piles	Boil The feathers are removed and the meat is cut into medium pieces and boiled till soft and given for consumption and continued for atleast three months	Oral	100%
Bird	Eurasian Tree Sparrow	*Bhangera* (N)	*Passer montanus*	LC NL	Meat	Immunity booster	Boil The feathers of the bird is removed and the meat is cut into small pieces and boiled in water adding salt till soft and given for consumption	Oral	100%
Bird	Pigeon	*Parewa* (N)	*Columba livia*	NA	Meat	Immunity booster	Boil (Soup) The feather of the animal is removed and the meat is minced to small pieces and boiled till broth consistency and is given for consumption	Oral	100%
Bird	Domestic fowl	*Kukhura (N)*	*Gallus domesticus*	NA	Meat	Immunity booster	Boil (Soup) The feathers of the bird is removed and the meat is minced into small pieces and boiled till broth consistency and given to newly lactating mothers	Oral	100%
Reptile	King Cobra	*Naag* (N)	*Ophiophagus hannah*	VU Appendix II	Molted skin	Cuts or wounds	Raw The molted skin is collected and then directly applied on cuts/wounds to stop bleeding and faster healing	Topical	100%

Table 2 (*Continued*)

Animal Group	Common Name	Local Name	Scientific Name	IUCN Conservation Status/CITES (Appendices)	Parts/Products Used	Ailments Treated	Preparation	Application	FL (%)
Amphibian	Frog	*Mann paa (N)*	*Nanorana liebigii*	LC NL	Meat	Cough & cold Dysentery/Diarrhoea Piles	Boil (Soup) The frog is de-skinned and cut into small pieces and boiled in water adding salt till broth consistency and given for consumption	Oral	46.2% 46.2% 7.4%
Amphibian	Frog	*Pirey paa (N)*	*Amolops himalayanus*	LC NL	Meat Egg	Cold Cough Bodyache Cuts or wounds	Boil The frog is de-skinned and the meat is minced to small pieces and boiled till soft and given for consumption Raw The egg of the frog is collected and applied directly on cuts/wounds for quick healing	Oral Topical	36.3% 36.3% 9.0% 18.1%
Insect	Red Ants	*Kamila (N)*	*Solenopsis sp.*	-	Whole insect	Improvement of vision	Decoction Ants are crushed using a pestle and then water is added and boiled till concentrated red solution, it is then cooled and given in doses of 20-30ml to the patients	Oral	100%
Insect	Honey Bee	*Mauri (N)*	*Apis sp.*	-	Honey	Cold Cough Ulcers	Decoction Raw honey is boiled with the addition of other ingredients such as ginger, fenugreek powder till it is slightly caramelized and given for consumption	Oral	49.4% 49.4% 1.0%
Insect	Stingless Bee	*Putka* (N)	*Tetragonula iridipennis*	NL NL	Honey	Food poisoning, Diarrhea & vomiting Cold Cough Wounds	Raw Raw honey is given for consumption Applied directly on wounds	Oral Topical	25% 25% 25% 25%
Arachnid	Spider	*Makura* (N)	Multiple sp., taxonomy not sorted	-	Web	Wound or cut bleeding	Heated The spider web is heated in burning coal and then applied directly on wounds or cuts to stop bleeding and faster healing	Topical	100%
Crustacean	Mud Crab	*Ganggata* (N)	Multiple sp., taxonomy not sorted	-	Meat	Jaundice Tuberculosis Diabetes	Boil (Soup) The shells of the crab is removed and it is cut into small pieces and boiled in water till broth consistency and given to patients for consumption. At least six doses are given within a span of 6-7 months	Oral	33.3% 33.3% 33.3%
Annelida	Earthworm	*Gadeula* (N)	Multiple sp., taxonomy not sorted	-	Whole worm	Asthma Snakebite	Raw The worm is crushed and then given raw for consumption	Oral	50% 50%
Mollusca	Snail	*Chipley kira (N)*	Multiple sp., taxonomy not sorted	-	Meat	Asthma	Raw The shell is removed and the meat is cut into small pieces and given raw for consumption	Oral	100%

*LC- Least Concern, VU-Vulnerable, EN- Endangered, CR-Critically Endangered, NE- Not Evaluated, NL-Not Listed, NA- Not Applicable; Local Name (L = Lepcha, N = Nepali)

(4.3%), heated (2.1%) and roasted (2.1%). Oral administration accounted for 78.7% of total applications while topical and nasal applications represented 19.1% and 2.1% respectively.

Overall 12 different body parts and products of animals were used for zootherapy in Sikkim (Fig. 4). The meat of the animal species was largely used for zootherapy and religious practices followed by urine, honey and bones. The horns of animals, beak and feathers of birds were generally used in shamanism and religious practices rather than in medicinal practices.

Table 3
Animal species used for religious/cultural purposes by ethnic communities in Sikkim Himalaya.

Animal group	Common Name	Local Name	Scientific Name	IUCN Conservation Status/CITES (Appendices)	Parts/product used	Belief/Use	Way of use
Mammal	Domestic Goat	*Bakhra* (N)	*Copra aegagrus hicrus*	NA	Horn	Protects from evil spirits	Horn is hung outside the main door
Mammal	Donkey	*Gadha* (N)	*Equus asinus*	NA	Bone	Protects from evil spirits	Used in religious rituals
Mammal	Wild Water Buffalo	*Bhainse* (N)	*Bubalus* sp.	–	Tooth	Protects from evil spirits	Hung around the neck as a talisman
Mammal	Himalayan Serow	*Thar* (N)	*Capricornis thar*	NT Appendix I	Horn	To please the gods	Used in religious rituals
Mammal	Goral	*Ghoral* (N)	*Naemorhedus goral*	NT Appendix I	Skin	To please the gods	The skin is sun dried and used during shamanistic practices
Mammal	Jungle cat	*Ban biralo* (N)	*Felis chaus*	LC Appendix II	Meat	Traditional belief to ward away spirits	The meat is dried and hung around the neck as a talisman
Mammal	Tibetan Antelope	*Chiru* (N)	*Pantholops hodgsonii*	NT Appendix I	Horn	Used in traditional rituals	Used during religious rituals
Mammal	Indian Elephant	*Hatthi* (N)	*Elephas maximus*	EN Appendix I	Hair	Prevents food poisoning	Trunk hair is made into a ring and worn on a finger
Bird	Oriental Bay Owl	*Ullu* (N)	Multiple sp., taxonomy not sorted	–	Bone	Protects from evil spirit	The bone is hung around the neck as a talisman
Bird	Crested Serpent Eagle	*Cheel* (N)	*Spilornis cheela*	LC Appendix II	Feathers	Worn by shamans	The beak is worn as a talisman and feathers are used in headbands by shamans during their traditional rituals
Bird	Himalayan Griffon Vulture	*Giddha* (N)	*Gyps himalayensis*	NT Appendix II	Bone	Protects from evil spirits	The bone is hung around the neck as a talisman
Bird	Cattle Egret	*Bakulla* (N)	*Bubulcus ibis*	LC NL	Beak	To ward off evil spirits	The beak is hung around the neck
Bird	Green-billed Malkoha	*Koilee* (N)	*Phaenicophaeus tristis*	LC NL	Tail feather	To ward off evil spirits	The feathers are worn as a headband by shamans during rituals
Bird	Kalij Pheasant	*Kalij* (N)	*Lophura leucomelanos*	LC NL	Feather	To ward off evil spirits	The feathers are worn as a headband by shamans during rituals
Bird	Red Junglefowl	*Luichey, Ban kukhura* (N)	*Gallus gallus*	LC NL	Tail feather	To ward off evil spirits	The feathers are worn as a headband by shamans during rituals
Reptiles	Snake	*Sirisey saap* (N)	*Dendrelaphis tristis*	LC NL	Skeleton	Protects from evil sprit	The skeleton is made into a necklace and worn around the neck
Mollusc	Money cowry	*Kawree* (N)	Taxonomy not sorted	–	Shell	Protects from evil spirit	The shells are made into a waistband and worn around the body

*LC- Least Concern, VU-Vulnerable, EN- Endangered, CR-Critically Endangered, NE- Not Evaluated, NL-Not Listed, NA- Not Applicable; Local Name (L = Lepcha, N = Nepali).

The meat of different animals was used in treatment of ailments such as tuberculosis, fever, common cold, cough and in food poisoning. Animal byproducts such as milk and honey are used for different ailments such as cold, cough, high fever, mouth ulcers and even bone fracture. In other parts of Northeast India, almost all animal parts such as skin, spines, hair, horn and metabolic products such as honey and urine are used in ethnomedicine (Chinlampianga et al., 2013). The indigenous communities also have faith in shamanism and magico-religious practices and it forms an important aspect of their traditional customs and rituals. Bones, horns and feathers of birds are either worn on human bodies or hung on entrances of their houses with a belief of warding off evil spirits. Similar beliefs and practices are reported from neighboring areas, such as ethnic communities in Nepal and Assam (Lohani, 2011a; Borah and Prasad, 2017).

3.5. Fidelity level

Fidelity level is calculated to identify the most frequently used species for treatment of certain diseases. A higher FL value indicates that different respondents used the same animal species for treating certain ailments (Kim and Song, 2013; Vijayakumar et al., 2015). The present study found 16 animal species with a FL of 100% (Table 2) for example flesh of monkey (*Macaca mulatta*) used for the treatment of tuberculosis, feathers of peacock (*Pavo cristatus*) used for treating nose bleed, web of spider for wounds and cuts, etc. This indicates that the communities rely on one specific animal species for a given ailments reflecting strong association of particular animal with particular disease.

3.6. Informant Consensus Factor (ICF)

The category with the highest degree of consensus from the informants was nervous system disorders and skin diseases (ICF value, 1.00). The ranking of health problems ranged from liver complaints (0.96) and circulatory system disorders (0.96) followed by cuts and wounds (0.93), respiratory system disorders (0.93) and poisonings (0.91), birth related disorders (0.89), other health problems (0.87), inflammation (0.82), gastrointestinal disorders (0.81), genitourinary system disorders (0.80) and physical pain (0.71). Among 14 ailments cate-

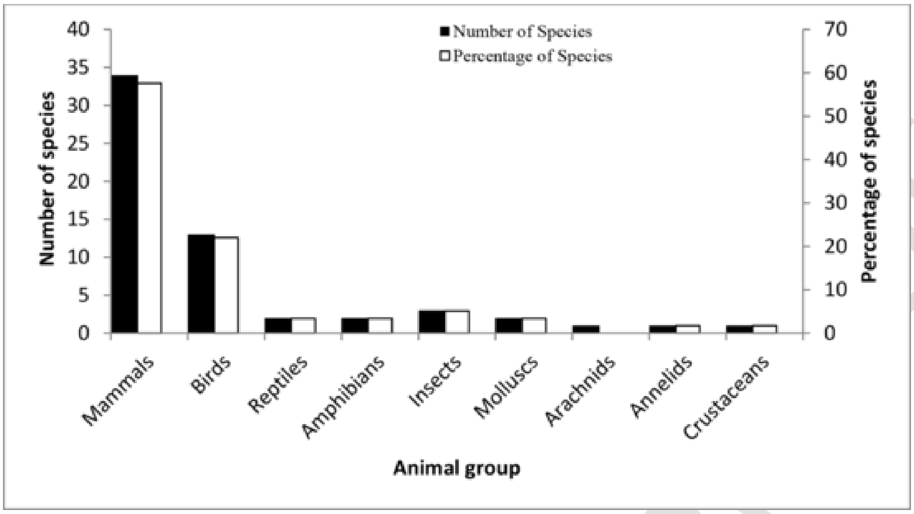

Fig. 3. Proportion of animal species used as per taxonomic category by different indigenous communities of Sikkim Himalaya.

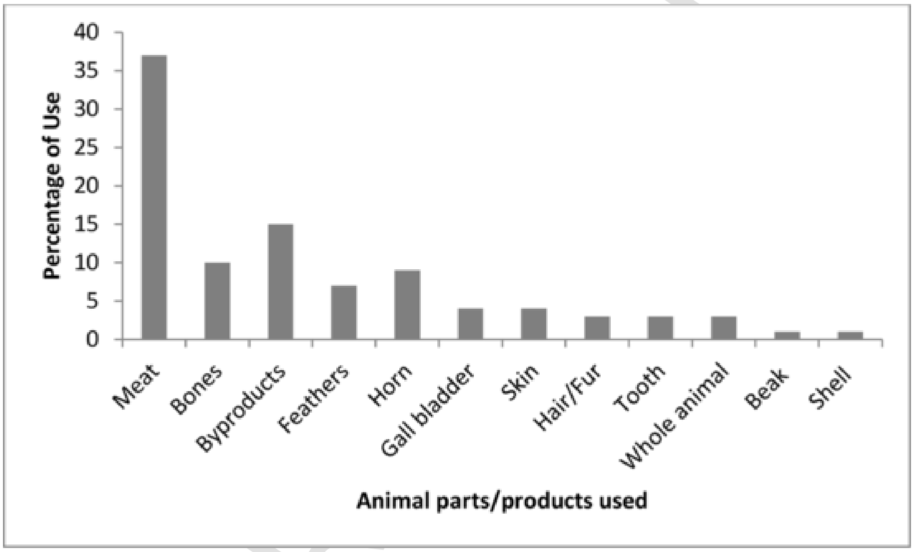

Fig. 4. Percentage of specific parts or byproducts of animals used in zootherapy in Sikkim Himalaya.

gory, the lowest degree of consensus was for muscular-skeletal disorders (0.70) (Table 4). Our results differ markedly from that of Korea (Kim and Song, 2013), where genitourinary system disorders and poisonings ranked the highest, whereas in Silent valley, India hair care, hemorrhoids, cancer and mental disorder was maximum (Vijayakumar et al., 2015). These differences have often been attributed to the geographical locations and disease association of the respective ethnic communities. Additionally, such differences may be due to the food habits and healthcare accessibility of each region.

3.7. Wildlife trade

It was observed that some parts or products of animal species used in zootherapy such as gallbladder of Himalayan Black Bear, horns of goral, scales of pangolin, flesh of monkey are rarely available. The implementation of stringent wildlife laws has been effective in curbing open market of such animal derived parts or products in Sikkim. This could be another reason for declining trend of traditional ethno-medicinal practices among the communities. Dried parts such as the bones of *Gyps himalayensis*, feathers of *Lophura leucomelanos*, horns and shells of animals which are used in shamanism or religious practices belong

Table 4
Category of ailments and their Informant Consensus Factor (ICF) in Sikkim Himalaya (according to Heinrich et al., 1998).

Symptom and ailment Category	Taxons	Use citations	ICF
Genitourinary system disorders	2	6	0.80
Poisonings	6	59	0.91
Liver complaints	2	24	0.96
Muscular-skeletal disorders	4	11	0.70
Nervous system disorders	1	2	1.00
Physical pain	9	29	0.71
Respiratory system disorders	20	264	0.93
Other health problems	7	48	0.87
Cuts and wounds	5	58	0.93
Inflammation	12	62	0.82
Skin diseases and disorders	1	12	1.00
Circulatory system disorders	2	25	0.96
Birth-related disorders	6	46	0.89
Gastrointestinal disorders	8	38	0.81

to their forefathers and are still in use. The local folk healers have smoke-dried such parts and stored for future use in traditional zootherapy.

It was also observed that few smaller vertebrates, though not exploited commercially, are in use for healing different ailments by the ethnic communities and also as a delicacy. The animal group mostly affected by such collection is the amphibian. Amphibian species such as *Nanorana liebegii* and *Amolops himalayanus* are highly valued for their medicinal properties in zootherapy and also as delicacy particularly due to their large size and taste. Streams are diverted for ease of collection of amphibians which poses a grave threat to the breeding habitat of amphibians and fishes as many tadpoles and fingerlings dehydrate and die. *Nanorana liebegii* becomes the worst victim being extensively used for medicine (diarrhoea, dysentery, vomiting, loss of appetite, etc) and as delicacy. Similar uses of amphibians in ethnomedicine have also been reported in the Magar community of Central Nepal (Lohani, 2011b).

3.8. Conservation status of animals used in zootherapy in Sikkim

It was observed that 11 animal species used by local communities are listed in the IUCN Red list as well as in any one of the three appendices of CITES (Table 2 & Table 3). Among the mammalian group, the Chinese pangolin (*Manis pentadactyla*) is critically endangered species and listed in Appendix I of CITES. Similarly, the Indian Bengal Tiger (*Panthera tigris*), Indian Wild Dog (*Cuon alpinus*) and Himalayan Musk Deer (*Moschus leucogaster*) are under endangered category with the first species under Appendix I and latter two under Appendix II of CITES. The Himalayan Black Bear (*Ursus thibethanus*) and King Cobra (*Ophiophagus hannah*) are under vulnerable category and Appendix I and Appendix II CITES species respectively. Many smaller vertebrates are not evaluated properly by IUCN, hence their threat category is still unknown. All the above mentioned red list species are locally rare and are in threat of being locally extinct if population falls below minimum viable population. It has been observed that the global trade in animal-derived medicines generates billions of dollars annually (Marques, 1997). The escalating demand for traditional medicine has led to exploitation and loss of many species (Call, 2006; Still, 2003). In Sikkim, the local market of medicinal products derived from larger vertebrates has been apparently in decline due to the rigorous surveillance of protected areas and severe consequences for a breach in Wildlife rules and regulations. However, smaller vertebrates such as amphibians and invertebrates are extracted to cater to the demands for zootherapy or as delicacy or protein source by ethnic communities. However, commercialization of the same may pose great threat to the rich and unique biodiversity of the region.

4. Conclusion

This is the pioneer study on the ethnozoological practices of indigenous communities of Sikkim. The animal species used in zootherapy by the different indigenous communities of Sikkim reflects the relationship between rich biodiversity and society. The religious practices involving the use of parts or products of animals also reflect the cultural heritage of local communities. Further, these ethno biological practices, if validated scientifically, might lead into the potential discovery of new drugs. However, traditional knowledge is on the brink of disappearance due to lack of interest of the present generation, low income and increased accessibility to modern day health care facilities. Therefore, before they disappear from the knowledge domain, documentation of the traditional knowledge should be encouraged. The knowledge base of traditional zootherapy should be expanded by its integration into education system and encourage clinical studies which will ensure safe use and recognize the potential of traditional medicines in improving health care. Biodiversity Management Committees (BMCs), the legally formed bodies at local level, may be entrusted in documentation of such practices which might contribute significantly in the conservation of traditional practices and also preserves the associated traditional knowledge as per the provision of Biological Diversity Act of India.

The traditional ethnomedicinal practices should be recognized by the authorities and incentivized training and education programmes should be developed for practitioners of such medicinal systems to ensure the quality of products and services. A database on the local indigenous knowledge system should be developed so that appropriate information including policies and regulations pertaining to use of such medicinal systems can be shared for safe use and also for the conservation of natural resources. Such documentation not only assists in preserving the diverse socio-cultural identity of the community but also helps in framing effective policies for sustainable use, conservation and management of rich and unique biodiversity of the region.

Author's contribution

BC and BKA conceived the idea and designed the study, SL collected the field data, PD, BC and BKA provided additional information on frog extraction, analysed the data and led the MS preparation. All authors significantly contributed to the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2019.112386.

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