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1. INTRODUCTION

1.1. *Pipistrellus* Kaup 1829

Taxonomic Classification:

Kingdom: Animalia

Phylum: Chordata

Group: Vertebrata

Subphylum: Gnathostomata

Class: Mammalia

Subclass: Theria

Infraclass: Eutheria

Order: Chiroptera

Suborder: Yangochiroptera

Superfamily: Vespertilionoidea

Family: Vespertilionidae

Subfamily: Vespertilioninae

Tribe: Pipistrellini

Genus: *Pipistrellus*

Pipistrellus is a genus of family Vespertilionidae (Evening bats). To the date, the genus comprises of 31 species globally (Simmons, 2005); eight species in South Asia (Srinivasulu *et al.*, 2010) whereas only three species in Nepal (Thapa, 2010); (Table 5). Pipistrelles are common term or English name for the bats falling under genus *Pipistrellus* normally. However, nowadays some bats out of this genus are also collectively called pipistrelles for eg. Some species of genera *Arielulus*, *Hypsugo*, *Falsistrellus*, *Neoromicia*, *Parastrellus*, *Perimyotis*, *Scotozous*. Pipistrelles are averagely smaller within Vespertilionid bats. The nostrils are directed antero-laterally with a

distinct inter-narial groove. Well marked and nearly naked pararhinal glandular swellings on the muzzle can be unveiled. Ears are short, but broad. The antitragus is in the form of a minute lobular projection at the base of the external border of the pinna. The tragus stands halfway of the pinna height. Anterior border of pinna is poorly concave. Just extreme tip of tail exceeds the interfemoral membrane.

The dental formula for the genus is a key for identification of the genus, which is given below:

$$\mathbf{i}\ \frac{-\,2\,3}{1\,2\,3}\quad \mathbf{C}\ \frac{1}{1}\quad \mathbf{pm}\quad \frac{-\,2\,-\,4}{-\,2\,-\,4}\ \mathbf{m}\ \frac{1\,2\,3}{1\,2\,3} = 34$$

The genus is widely distributed throughout the range from central Southern Africa, Eurasia, South East Asia, Solomon Islands and northern Australia.
Among the three species of *Pipistrellus* in Nepal; *P. javanicus* has been recorded only from hilly regions whereas *P. coromandra* and *P. tenuis* has been documented from hills and Tarai (Plain) both (Bates and Harrison, 1997).

Pipistrellus coromandra (Gray, 1838)

Myotis parvipes Blyth, 1853
Pipistrellus coromandra Gaisler, 1870 ssp. *afghanus*
Scotophilus coromandelianus Blyth, 1863
Sctophilus coromandra Gray, 1838
Vespertilio coromandelicus Blyth, 1851
Vesperugo blythii Wagner, 1855
Vesperugo micropus Peters, 1872
Vesperugo nicobaricus Fitzinger, 1861

Common Name: Coromandel Pipistrelle, Indian Pipistrelle
Nepali Name: Buchhe chamero (Baral and Shah, 2008): Coromandel ko pipistrelle chamero

Conservation Status: World-wide: LC (IUCN, 2010)

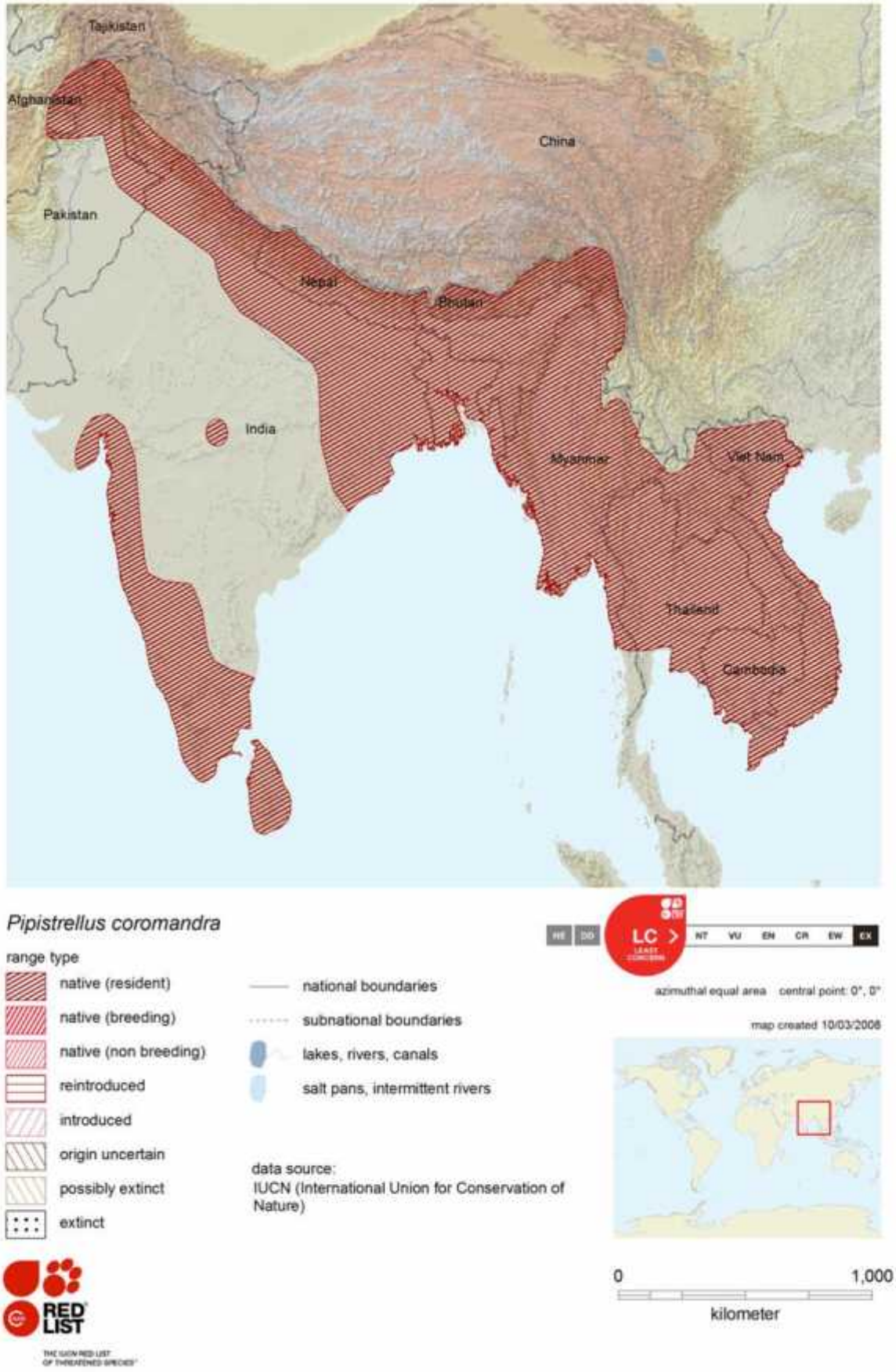
In South Asia: LC (Molur *et al.*, 2002)

In Nepal: LC (National Red List of Nepal Mammals, 2010)

Range Description: This widely distributed species is found throughout most of South Asia, parts of southern China and much of mainland Southeast Asia. In South Asia this species is presently known from Afghanistan (Nangarhar Province), Bangladesh (no exact location), Bhutan (no exact location), India (Andaman and Nicobar Islands, Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Goa, Gujarat, Jammu and Kashmir, Jharkhand, Karnataka, Madhya Pradesh, Maharashtra, Manipur, Meghalaya, Nagaland, Orissa, Sikkim, Tamil Nadu, Tripura, Uttarakhand, Uttar Pradesh and West Bengal), Nepal (Central), Pakistan (North West Frontier Province and Punjab) and Sri Lanka (Central, North Central, North Western Northern, Southern and Uva provinces) (Das, 2003; Khan, 2001; Korad *et al.*, 2007; Molur *et al.*, 2002; Simmons 2005; Srinivasulu and Srinivasulu, 2005; Vanitharani, 2006). In South Asia, it has been recorded from 100 to 2,769 m a.s.l. (Molur *et al.*, 2002). In China it has been recorded from Xizang (Smith and Xie, 2008). In Southeast Asia, it is present in Myanmar, Thailand, Lao PDR, Vietnam, Cambodia and Peninsular Malaysia (IUCN, 2010).

Distribution in Nepal:

Annapurna CA, Makalu-Barun NP, Bardia NP, Chitwan NP [Suwal *et al.*, 1995 (BPP)]: Hazaria (85° 20' E , 26° 51' N); Bairia (85° 23' E, 27° 00' N); Bairaglia (85° 23' E, 26° 45' N): probably in Sarlahi districts along Bagmati River (Hinton and Fry, 1923); Barabisse (85° 35' E, 27° 35' N), Sindhupalchowk district (FMNH); (Bates and Harrison, 1997): Central Zoo, Jawalakhel, Lalitpur, Kathmandu Valley, Forestry Campus, IOF, Pokhara (Daniel, 2007): Dudora Nala/Park road (84° 27.4' E, 27°33.6' N), 4.3 km SW of Sauraha; Tamar Tal (84° 20.3' E, 27°31.9' N), approx. 13 km E of Sauraha; Dhangari Khola (84° 11.5' E, 27°32.2' N), Tiger Tops, 33 km W of Sauraha; Simal Ghol Tal (84° 28.6' E, 27°33.9' N) 2.5 km SW of Sauraha; Bardhaha Khola (84° 28.2' E, 27°30.8' N), in the Churia range of Siwalik hills at an elevation of 500m a.s.l., 3 km SW of Bwanipur Chowki: CNP (Myers *et al.*, 2000).



Map 1. Range distribution map of *P. coromandra* (Source: IUCN, 2010).

Population: In South Asia, it is a widely distributed and common species and the population seems to be doing well in its range (Molur *et al.*, 2002). It is fairly common in Viet Nam, even in cities and similar urban habitats (IUCN, 2010).

Habitat and Ecology: This species is found in varied habitat types from forested regions, agricultural landscapes to urban areas. It roosts in trees, crevices and cracks in walls and ceilings of houses, tiles of huts, old buildings, temples, under bark and in holes of large trees, signboards, tree hollows in small groups of few individuals. It is an early flyer with a slow fluttering flight and hunts on flies, ants and other small insects. There are three breeding seasons and two young ones are born (Bates and Harrison, 1997).

Pipistrellus tenuis (Temminck, 1840)

Pipistrellus mimus Wroughton, 1899
Pipistrellus mimus Wroughton, 1912 *ssp. glaucillus*
Pipistrellus mimus Wroughton, 1899 *ssp. mimus*
Pipistrellus mimus Thomas, 1915 *ssp. principulus*
Pipistrellus principulus Thomas, 1915
Vespertilio tenuis Temminck, 1840

Common Name: Least Pipistrelle, Indian Pygmy Bat
Nepali Name: Sano chamero (Baral and Shah 2008): Sano pipistrelle chamero

Conservation Status: World-wide: LC (IUCN, 2010)
In South Asia: LC (Molur *et al.*, 2002)
In Nepal: LC (National Red List of Nepal Mammals, 2010)

Range Description: This widespread species is found throughout much of South Asia, southeastern China and Southeast Asia. In South Asia, this species is presently known from

Afghanistan (Nangarhar Province), Bangladesh (Chittagong and Sylhet divisions), India (Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Chhattisgarh, Gujarat, Haryana, Himachal Pradesh, Jharkhand, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Manipur, Meghalaya, Nagaland, Orissa, Rajasthan, Tamil Nadu, Tripura, Uttar Pradesh, Uttarakhand and West Bengal), Nepal (Central and mid western), Pakistan (North West Frontier Province, Punjab and Sind) and Sri Lanka (Central, North Central, North Western, Sabaragamuwa, Southern, Uva and Western provinces) (Das, 2003, Korad *et al*., 2007; Molur *et al*., 2002; Vanitharani, 2006). It has been recorded from sea level to 769 m a.s.l. (Molur *et al*., 2002). It is present over much of southeastern China, including the island of Hainan (Smith and Xie, 2008). In Southeast Asia, it is found throughout the mainland, ranging into Indonesia (Sumatra, Java, Bali, Lombok, Sulawesi, Seram and Serasan), the island of Timor (East Timor and Indonesia), Borneo (Indonesia, Malaysia and possibly Brunei; the species is possibly more widespread than currently known) and the Philippines, where it has been recorded on Cebu (Paguntalan pers. comm., 2006), Luzon (Rizal Province), Mindanao (Taylor, 1934), Negros, and Sibuyan (Heaney *et al*., 1998) where it occupies an altitudinal range from sea level to 800-1700 m (P. Bates and L. Heaney pers. comm. 2006) (IUCN, 2010).

Distribution in Nepal: Hazaria (85° 20' E , 26° 51' N); Bairia (85° 23' E, 27° 00' N): probably in Sarlahi district along Bagmati River (Hinton and Fry, 1923); Banke (81° 47' E , 27° 57'N) at an altitude of 160 m a.s.l. (Mitchell, 1980); (Bates and Harrison, 1997): In the vicinity of The Nepal Conservation Research and Training Center (84° 29.5' E, 27°34.2' N), Sauraha, CNP at an elevation of 200m ; Dudora Nala/Park road (84° 27.4' E, 27°33.6' N), 4.3 km SW of Sauraha; Tamar Tal (84° 20.3' E, 27°31.9' N), approx. 13 km E of Sauraha ;Dhangari Khola (84° 11.5' E, 27°32.2' N), Tiger Tops, 33 km W of Sauraha; Simal Ghol Tal (84° 28.6' E, 27°33.9' N) 2.5 km SW of Sauraha: CNP(Myers *et al*., 2000): Central Zoo, Jawalakhel, Lalitpur (Daniel, 2007).

Population: In South Asia, this species is widely distributed and common, and the population is stable and seems to be doing well (Molur *et al*., 2002). It is widespread and moderately common in the Philippines (Heaney *et al*., 1998). It is locally very common in Myanmar and Lao PDR (Bates *et al*., 2005) (IUCN, 2010).

Pipistrellus tenuis

range type

- native (resident)
- native (breeding)
- native (non breeding)
- reintroduced
- introduced
- origin uncertain
- possibly extinct
- extinct

- national boundaries
- subnational boundaries
- lakes, rivers, canals
- salt pans, intermittent rivers

data source:
IUCN (International Union for Conservation of Nature)

NE DD LC NT VU EN CR EW EX

azimuthal equal area central point: 0°, 0°

map created 10/03/2008

0 1,600

kilometer

Map 2. Range distribution map of *P. tenuis* (Source: IUCN, 2010).

Habitat and Ecology: In South Asia, this species is found from arid zones to wet and humid areas. It is equally abundant in forested areas, in rural and urban landscapes (C. Srinivasulu pers. comm., 2007). It roosts in hollows of trees, holes, crevices and cracks in walls and ceilings of old buildings, dead leaves of trees. In Southeast Asia this is a largely forest species inhabits primary and secondary hill, montane and montane mossy forest (Heaney *et al*., 1998). It is adapted to highly disturbed habitats, gardens, and mangrove forests (P. Bates pers. comm., 2006). It is an early flyer, with a varied flight pattern from a jerky flight with many twists and turns to a slow fluttering and floating flight to an erratic flight as the evening progresses. Its diet is varied and seasonal. It feeds on beetles, cockroaches and wingless ants in winter, on a wide variety of insects in summer and on winter termites, moths, hymenopterans, dipterans and beetles during monsoon. There are two breeding seasons one in February-March and the other in July-August and between one to three young are born (Bates and Harrison, 1997).

1.2. Complication in taxonomy of genus *Pipistrellus*

The genus *Pipistrellus* cannot be diagnosed globally by any universally applicable morphological characters, however, distinguished from most of the Vespertilionine genera by the bacular morphology (Hill and Harrison, 1987). Miller, 1907 distinguished them primarily on the basis of dental characters. The first upper incisor (i^2) is simple and usually has a well developed secondary cusp; the second upper incisor (i^3) is reduced and smaller than i^2 while absent in case of *Scotozous dormeri* (Previously *Pipistrellus dormeri*) but usually extends above the cingulum of i^2 if present. The upper canine is relatively short, which usually but not always has an incipient secondary cusp on its posterior edge. Upper First Premolar (pm^2) is small and present in all except *P. savii* (now *Hypsugo savii*) and also absent in *Eptesicus*. So Upper First Premolar can be an important character in distinguishing *Pipistrellus* from others as it is situated internally to the toothrow. pm^3 and pm_3 are absent (Bates and Harrison, 1997).

As with most *Pipistrellus*, the problem of identification coupled with taxonomic uncertainties means that the abundance and the distribution of the species cannot be easily defined. Previous

records referable *Pipistrellus* species have often been confused (G. Csorba pers. comm., in IUCN, 2010). Quandary still exists in taxonomy at intra-genus or species level. One of good example is between *P. coromandra* and *P. tenuis* as well as other species where the external measurement overlaps and field identification is perplexed.

In the skull and baculum morphology there can be a way for distinction. The cranio-dental measurement of *P. coromandra* is larger in comparison to *P. tenuis*. Rostrum and palate are more elongated while braincase exceeds in size in comparison to *P. tenuis*. The first upper incisor (i^2) is bicuspidate; the secondary cusp is occasionally small or absent, but if present is usually half the height of the anterior cusp. The second upper incisor (i^3) is well developed, with a larger principal cusp and a smaller lateral accessory cusp. i^3 usually exceeds in height to the secondary cusp of i^2 and is separated from upper canine (C^1). C^1 has a secondary cusp and a distinct cingular cusp posteriorly in *P. coromandra* but only distinct secondary cusp in *P. tenuis*. pm^2 is intruded from the upper tooth row and is equal to crown area of i^2. The canine and second upper premolar (pm^4) is not in contact rather closely adjacent. The first lower premolar (pm_2) is slightly extruding from the lower tooth row and is half to three-quarters the crown area of second lower premolar (pm_4) in *P. coromandra* while three -quarters the crown area and two-thirds the height of second lower premolar (pm_4) in *P. tenuis* (Bates and Harrison, 1997).

The baculum has a straight or slightly sinuous shaft (in *P. coromandra*) as well as long thin shaft (in *P. tenuis*), with a distinctly bifid tip and with the basal lobes (well developed in *P. tenuis*) deflected ventrally (Hill and Harrison, 1987).

Review and reconstruction of systematic of this genus has been ongoing because of quagmire. *P. dormeri* (Ellerman and Morrison-Scott, 1951; Corbet, 1978; Koopman, 1993, 1994; Bates and Harrison, 1997) was misinterpreted under *Scotoecus* Thomas, 1901 (Menu, 1987), and now is treated under a distinct genus *Scotozous* (Tate, 1942; Corbet and Hill 1980, 1992; Hill and Harrison, 1987; Roberts, 1977). Earlier forms of *Falsistrellus affinis* from India and Sri Lanka were included under *Pipistrellus mordax* Peters, 1866 (Wroughton, 1916, 1918; Ellerman and Morrison-Scott, 1951; Phillips, 1980), but, are presently considered distinct by Simmons, 2005. *Arielulus* circumdatus has been raised from *P. circumdatus*. *P. murrayi* was considered distinct (Kitchener *et al*., 1986) and listed as separate species (Hill and Harrison, 1987) which was suggested con-specific with *P. tenuis* (Koopman, 1973, 1993). The genetically and

morphologically distinct population of pipistrelle bats belonging to the *pygmaeus* genetic clade and occurring in the Cyrenaica/Libya was recently described (Benda *et al.*, 2004) as species: *P. hanaki*. Earlier *P. paterculus* was treated as subspecies of *P. abramus* (Temminck, 1840) (Ellerman and Morrison-Scott, 1951; Hill, 1962; Soota and Chaturvedi, 1980), it has been accorded specific status by (Hill and Harrison, 1987). *P. malagasyensis* Peterson, Eger and Mitchell, 1995, was formerly considered to be a subspecies of *P. somalicus* (Goodman and Ranivo, 2004). *P. vordermanni* may be conspecific with *P. macrotis* (Corbet and Hill, 1992). Egyptian pipistrelle *P. deserti* Thomas, 1902 was formerly included within *P. kuhlii*, but is now recognized to be a separate species (Simmons, 2005). Also *P. hesperidus* separated from *P. kuhlii* by Kock, 2001. Simmons, 2005 considers sub-Saharan African and Canary Island populations of *P. kuhlii* belong to a different species, *P. hesperidus*. Bacular morphology and sound analysis as well as genetic approaches recently have produced above results.

The taxonomy of this group is challenging because many species of this genus has been splitted off and raised to new genera: *Arielulus*, *Falsistrellus*, *Hypsugo*, *Neoromicia*, *Parastrellus*, *Perimyotis*, *Scotozous*. Numerous cryptic species complex exist among this genus and species for example; *P. pipistrellus* and *P. pygmaeus* have been documented. The genus *Hypsugo* is again retained in *Pipistrellus* following the continental-level classification adopted by Happold and Happold (in press). The taxonomy is inconsistent of this genus at greater extent.

1.3. Bats from Koshi Tappu Wildlife Reserve

Thapa, 2009 carried out the bat survey for the first time in Eastern Tarai including Koshi Tappu Wildlife Reserve (KTWR). He focused his study at KTWR headquarters, Kusaha V.D.C. (Buffer Zone of KTWR) where he searched house and tree roosts, museum collection and could report three species: *Megaderma lyra*; *Pipistrellus sp.*; *Scotophilus heathii* respectively.

PHOTO PLATE 1

Photo 1A, B. Unidentified pipistrelles at Kusaha, KTWR (above and below)

1.4. Issue of the Research

As already stated inter-specific identification is hectic in this genus. One of such condition come in-between *P. coromandra* and *P. tenuis*. Externally, it is often difficult to distinguish these two species. In external measurements generally *P. coromandra* are larger on an average while *P. tenuis* are smaller averagely. Still, significant overlaps occur. The slight variation in pelage colors is also not significant in field identification of these bats. In the occasional cases, where the localities of these both species overlap, the identification becomes further complex. Keen observation of their skull and baculum morphology can help in distinguishing.

Pipistrelles from KTWR could not be identified during the survey as they show overlapping morpho-metrics of *P. coromandra and P. tenuis*. Present study is concerned in identifying those unidentified Pipistrelles through skull-baculum morphology and PCR based method. This study will aid in further upcoming taxonomical researches on bats.

1.5. Objectives

The main objective of this study is to identify the *Pipistrellus* complex in the study area.

Specific objectives of the research:

- carry on comparative study of skulls and bacula morphology of the bat specimens
- conduct comparative study of PCR products of the bat specimens.

1.6. Limitations of the study

This study does not represent whole pipistrelles from KTWR as:

- the survey was carried out at a single Village Development Committee (Paschim Kusaha V.D.C.) of KTWR

- Specimens were captured from roosts only, not a single specimen was caught during mist-nettings.
- Very less quantity of specimens were collected, taking in consideration of conservation issues.

The attempt of PCR approach failed:

- Because of lack of literatures and sophisticated molecular laboratory
- Caused by inexperience for molecular lab work
- Due to insufficient funding and
- In need of long time running process and continuity of PCR optimization.

2. STUDY AREA

2.1. Brief Description

Koshi Tappu Wildlife Reserve, a well known Ramsar site (wetland of international importance) since December 1987, also famous destination for migratory birds and the last remnant population of the Wild Water Buffalo (*Bubalus bubalis*) is the only Wildlife Reserve in Eastern Nepal. The reserve was established and gazetted in 1976. It occupies an area of 175 sq.km (149.6 sq.km from GIS survey in the year 2000) excluding its buffer zone within 86° 55' 15" E 26° 33' 57" N to 87° 05' 02" E 26° 43' 40" N (DNPWC/PPP, 2001), extending in three districts namely; Sunsari, Saptari and Udaypur. The protected area is rectangular in shape bounded by dykes, each in East and West along the Sapta Koshi river.

The field site lies within a periphery of 1 km south and east from headquarters office of KTWR (87° 34' 52.4" E 26° 17' 35.2" N) at Kusaha in Pashim Kusaha V.D.C. The study site lies within the buffer zone area (For Geographic gazetteer, Appendix I).

2.2. Climate

The climate of the reserve falls under tropical monsoon type. The average daily maximum temperature ranges from 23.5℃ to 33.4℃ and average daily minimum temperature ranges from 7.8℃ to 25.3℃ (Sah, 1997). The mean monthly temperature ranges from 15.7℃ to 29.2℃ (Singh, 2001). The mean annual precipitation recorded at Fathepur, Saptari district is about 1300mm. This rainfall occurs from mid June to mid September (Sah, 1997). The mean annual precipitation at Kusaha ranges 1601-2000mm (DNPWC/PPP, 2001).

2.3. Floral diversity

According to factsheet (Ramsar information sheet) of Koshi Tappu Wildlife Reserve, The existing vegetation in the area consists of diverse physiognomic types as submerged and floating aquatic plants, tall reed stands, seasonally flooded grassland/ savannah and structurally complex

forest communities in various conditions of spatial arrangements. Among 514 species of plants, *Dalbergia sissoo*, *Bombax ceiba*, *Saccharum sp*, *Phragmites sp.*, *Typha sp.*, *Imperata sp.*, *Valisneria sp.*, *Eichornia sp.*, *Hydrilla sp.*, *Azolla sp.*, *Lotus sp.* are common species found in the wetlands. Six species of plants found in this area, *Rauwolfia serpentina*, *Alstonia scholoris*, *Oroxylum indicum*, *Acacia catechu*, *Butea monosperma* and *Dalbergia latifolia*, are listed in the different threat categories and appendices of IUCN and CITES respectively (W.W.F., 2009).

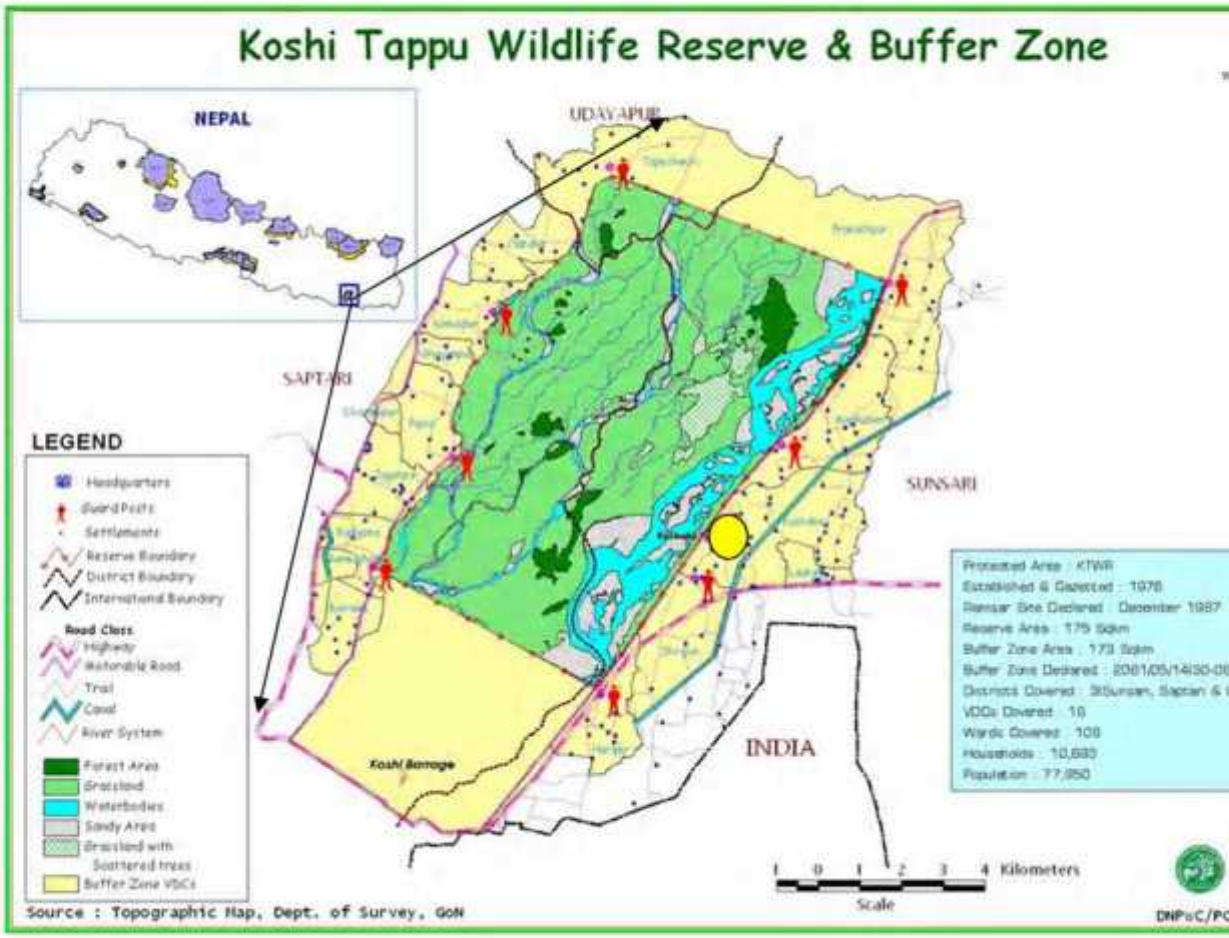
Around four percent of KTWR is occupied by forest. Cutch Tree *Acacia catechu* (Khair) and Indian Rosewood *Dalbergia sisso* (Sisso) forest grows in narrow stripes on alluvium along the Sapta Koshi river channels. *Dalbergia sisso* majors in the new plantation at eastern dyke and wetlands (near Kusaha) where few old Silk Cotton Tree *Bombax ceiba* (Simal) gets intermixed. About 67 percent of reserve is covered by grassland (Heinen, 1993), which reflects as pseudo steppes dominated with tall elephant grasses (*Typha sp.* and *Saccharum spontaneum*), patches of Imeperata and Shrub (*Phragmites kharka*) (Dodman, 1990). The study site is dominated by agricultural land and scattered trees (mainly Sisso).

2.4. Faunal diversity

According to Factsheet (Ramsar information sheet) of Koshi Tappu Wildlife Reserve, the reserve harbors: 485 species of Birds, 31 species of Mammals, 200 species of Fishes, 11 Amphibian, 24 Reptiles, 77 species of Butterfly. Notable birds recorded in the site include *Gallicrex cinerea*, *Caprimulgus asiaticus*, *Bubo coromandus*, *Coracina melanoptera*, *Saxicola leucura* and *Megalurus palustris*. At least 114 species are water birds, 176 species breed in the reserve and 180 species are passage migrant or winter visitors. It is the only area in Nepal where water cock, *(Gallicrex cinerea)* and Abbott's babbler are found. Out of these 485 species of birds, 12 species are globally threatened and 101 species are nationally threatened. Of the 31 species of mammals recorded, Nepal's last remaining population of Wild Water Buffalo *Bubalus bubalis* (Arna) inhabits the area and the Gangetic dolphin *Platanista gangetica* (Sons) has been recorded in the Koshi river. Large mammals like Gaur *Bos gaurus* (Gauri Gai) and Blue bull *Boselaphus tragocamelus* (Nil Gai) are almost disappearing from the area. Other mammals found are Wild Elephant *Elephus maximus* (Jangali Hatti), Wildboar *Sus scrofa* (Bandel), Hog deer *Axis*

porcinus, spotted deer *Axis axis* (Chittal), Smooth coated Otter *Lutra perspicillata* (Oont) and Jackal *Canis aureus* (Syal). Of the 200 species of fishes, 91 species are resident, 21 species are local migratory and 5 species are migratory. Of these, 9 species are listed in the different threatened categories, 8 species as vulnerable and 1 species as endangered. 11 Amphibian (2 toads and 9 frogs) and 24 reptiles (2 crocodiles, 11 turtles, 6 lizards and 5 snakes) are recorded till now. 17 species of herpetofauna are nationally threatened out of which 6 species are globally threatened. 77 species of butterfly are recorded in the area (W.W.F., 2009).

Thapa, 2009 recently reported three species of bats from Kusaha area namely Greater False Vampire *Megaderma lyra* (Nakkali Boksi chamero), Pipistrelle *Pipistrellus sp.* (pipistrelle chamero) and Asiatic Greater Yellow House bat *Scotophilus heathii* (Thulo Asiali Pitta chamero).



Study site (Kusaha, Paschim Kusaha V.D.C.)

Map 3. Koshi Tappu Wildlife Reserve (Nepal in inset) (Source: DNPWC/PCP, 2006).

3. LITERATURE REVIEW

This is the first species specific study of bats in Nepal except Flying Fox study in context of limited bat researches and literatures in the country.

3.1. Taxonomic studies on *Pipistrellus*

Pipistrellus species has been confirmed to species level by baculum preparation and its comparative studies during surveys and reviews. On the basis of bacular morphology Heller and Volleth, 1984 and Hill and Harrison, 1987 suggested that *Eptesicus* and *Pipistrellus* could be distinguished from each other by small, triangular baculum in *Eptesicus* and *Pipistrellus* having a medium to large, 'stick-like' elongated baculum. Applying these characters, Hill and Harrison, 1987 transferred Southern African species of *Eptesicus* namely; *capensis*, *melckorum*, *somalicus*, *zuluensis*, *rendalli* to a new sub genus *Neoromicia* in the genus *Pipistrellus* (Kearney *et al.*, 2002). Koopman, 1973, 1993 suggested *P. murrayi* was con-specific with *P. temuis* without presentation of any data. Kitchener *et al.*, 1986 considered *P. murrayi* distinct, though with little information given, but more information was given in a letter to Chris Tidemann. Hill and Harrison, 1987 listed *P. murrayi* as a separate species in a revision of all Vespertilioninae based on baculum (IUCN, 2010).

Bates *et al.*, 2005 applied bacular characters and measurements during review of the five genera of Vespertilionidae from Myanmar (Burma) including *Pipistrellus*. They differentiated *P. coromandra* and *P. temuis* as well as revealed an incorrectly identified *Pipistrellus pipistrellus* to *P. coromandra* on the basis of study of bacula of specimens. *P. raceyi* Racey's pipistrelle bat a new species and new records of other five pipistrelles including *P. hesperidus* has been described comparing cranio-dental, tragus and baculum characters and measurements (Bates *et al.*, 2006).
The cranial, mandibular, and dental differences observed between *P. pipistrellus* and *P. pygmaeus* through the geometric morpho-metrics approach to the morphological differentiation of the cryptic species showed the cryptic species differed significantly in terms of size and shape of the mandible. The shape of the mandible proved to be a good factor differentiating the species

if the leave-one-out cross-validation of discriminant function of only three specimens out of 26 (11%) that were misclassified, be considered. Results (differences in elevation of coronoid process, mandible body length, canine length and molar breadth) strongly point to the diet as a differentiating factor between the species and supports the probability that the dietary differences might have underlain the speciation process (e.g. Nogueira *et al.*, 2005), which led to the evolution of these cryptic species (Sztencel-Jabłonka *et al.*, 2009).

3.2. Molecular studies on pipistrelles

This is the first attempt of molecular approach in bat studies and rare practice of other wildlife in Nepal. Also this is the initiation of genetic study of Pipistrelle in Nepal as well as in South Asia. However, numerous molecular applications on different *Pipistrellus* species have been undertaken in other parts of the world.

Differences in resulting fragment number and fragment size between the two species allowed for accurate and consistent distinction between the species (Zinck *et al.*, 2004; Weller *et al.*, 2007). Theoretically, based on a low number of mutations in sequences where the primers are designed, the PCR can also be successfully used that could help in the investigation of the species-specific ecological requirements, and in general, it can be widely used in studies concerning the ecology and evolution of cryptic vertebrate species (Barlow and Jones, 1999; Davidson-Watts and Jones, 2006; Nicholls and Racey, 2006; Racey *et al.*, 2007; Kaňuch *et al.*, 2007).

Barratt *et al.*, 1995, 1997 showed the common Pipistrelle comprised of two distinct species, *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus* through subsequent genetic differences in the nucleotide sequences of 308-bp segment of cytochrome *b* gene (*cyt b*) (Kaňuch *et al.*, 2007). Barratt *et al.*, 1997 subsequently identified two highly divergent mitochondrial DNA (mtDNA) groupings (termed clades I and II), which show high levels of sequence divergence both at the 630-bp of aligned cytochrome *b* sequence (Barratt *et al.*, 1997) and the *ND1*gene (Mayer and von Helversen, 2001). The two mtDNA clades correspond completely to the two phonic types (46-kHz bats have the clade II haplotype, whereas 55-kHz bats are clade I) and all individuals

within a single colony are of the same phonic type and mtDNA clade (Jones & van Parijs, 1993; Barratt *et al.*, 1997) (Hulva *et al.*, 2004; Racey *et al.*, 2007).

Barragán *et al.*, 2003 described a Hind III family of repetitive DNA sequences that is located in the centromeric heterochromatin of the autosomes and the X chromosome in *Pipistrellus pipistrellus*, indicating that could be a species/genus specific repetitive DNA family.
Wicht *et al.*, 2003 confirmed the first record of *P. pygmaeus* in southern Switzerland by echolocation analysis and mitochondrial DNA sequencing. Hulva *et al.*, 2004 provided the genetic architecture of *P. Pipistrellus*/*P. pygmaeus* complex throughout western palaearctic range and supported geographic (allopatric) speciation model for the complex species performing phylogenetic analysis of 402-bp portion of the cytochrome *b* gene.

Jarzembowski, 2004 supported the hypothesis: migration routes and migration strategies of *Pipistrellus nathusii* in Europe may suggest that there are several different populations of this species through Control region variability of the fragment of around 450-bp from the D-loop of mitochondrial DNA. Hoofer and Van Den Bussche, 2003 justified recognition of *subflavus* within *Perimyotis* and *hesperus* within *Parastrellus* which wereincluded under genus *Pipistrellus* before, by providing the 1st cladistic assessment of molecular data for *hesperus*, *subflavus*, and several other Pipistrellus-like bats. Hoofer *et al.*, 2006 supplemented additional morphological (penial structure) and genetic data demonstrating the marked divergence among *hesperus*, *subflavus*, *pipistrellus*, and other *Pipistrellus*-like genera, and also provided a formal description of a new generic name for the nominal species *Parastrellus hesperus*.

Ibáñez *et al.*, 2006 investigated the contribution of the Iberian bat fauna to the cryptic diversity in Europe using mitochondrial (558 to 803-bp *cyt b* and *ND1*) and nuclear (*RAG2*) DNA sequences. In their study almost 20% of the Iberian species showed important mitochondrial discontinuities (K2P distance values > 5%) including *Pipistrellus kuhlii*, suggesting the existence of further cryptic diversity.
Mayer *et al.*, 2007 showed substantial sequence divergence suggesting an unexpected high number of undiscovered species of bats including *Pipistrellus* species applying sequencing of the

mitochondrial protein-coding gene NADH dehydrogenase, subunit 1 (nd1) from 534 bats of the Western Palaearctic region.

Racey *et al.*, 2007 identified differences in genetic population structure between *P. Pipistrellus* and *P. Pygmaeus*, with *P. pipistrellus* showing a wider range of levels of genetic differentiation among colonies and a stronger relationship between genetic and geographical distance than *P. pygmaeus*. Their findings supported the hypothesis that the species are reproductively isolated.

Kaňuch *et al.*, 2007 presented multiplex panels of polymorphic microsatellites for two closely related cryptic species *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*. In another research they described a non-destructive and quick polymerase chain reaction (PCR)-based technique amplifying 320-bp fragment of *cyt b*, providing easy and reliable identification of two cryptic species of the genus *Pipistrellus* namely *pipistrellus and pygmaeus*; and tested the efficacy of the method for species identification of ethanol-fixed and dry museum material as well as dropping samples.

4. MATERIALS AND METHODS

4.1. Field study

4.1.1. Bat Capture

Bats were captured by following methods:

4.1.1.1. Mistnetting

A single mist net was deployed every evening to 22:00 hr from March 29 to April 2, 2009. Mistnetting was randomly applied in the study area near wetlands (near Aqua Tourist Hotel), pond (Milanchowk).

4.1.1.2. Capturing from the Roost

During the day time, thatched houses and sheds were searched for the bat species with the help of focusing torch-light. Cloth bags were tied to the bamboo roosted by bats and left hanged in the late afternoon, in which those bats were captured during evening.

4.1.2. Morpho-metric study

The following external measurements was taken six times to minimize the biasness with the help of millimeter graded steel scale to the nearest 1 mm: HB-Head body, T- Tail length (from the anus to last vertebra), TIB - Tibia length, HF- hind foot length (excluding claws), FA – Forearm Length, Thumb-Thumb length, 3mt-Third metacarpal, 1ph3mt- First Phalange Third metacarpal, 2ph3mt- Second Phalange Third metacarpal, 4mt-Fourth metacarpal, 1ph4mt- First Phalange Fourth metacarpal, 2ph4mt- Second Phalange Fourth metacarpal, 5mt-Fifth metacarpal, 1ph5mt- First Phalange Fifth metacarpal, 2ph5mt- Second Phalange Fifth metacarpal, E – Ear length,

PHOTO PLATE 2

Photo 2A. Mist netting at a pond in the study area

Photo 2B. *Pipistrellus sp.* inside the hollow bamboo

Photo 2C. Searching bats in attics of a house at Hattisar

Photo 2D. Cotton bag tied on the mouth of a bat roosting bamboo of hut

Tragus-Tragus length from the lower border of the external auditory meatus (Posterior to tragus to the tip of pinna). The body weight was measured with the help of simple spring balance graded with gram. On the basis of these morpho-metric data and referring the reference book (for e.g. Bates and Harrison, 1997) taxonomic identification was done in the field up to genus level. Additionally, the reproductive stage of them was noted by observing their genitalia and nipples (in case of female).

4.1.3. Collection of bat specimens

Four male (KT1, KT6, KT3 and KT4) and two female (KT5 and KT2) Pipistrelles were collected in the field from three houses at Samsul tole and one house and one shed from Goithi tole. The voucher specimens were preserved at 70% alcohol in air-tight plastic bottles, transferred to, labeled and stored at Museum of Central Department of Zoology (CDZ), Tribhuavan University, Kathmandu after taking wing tissue sample at Research Laboratory for Agricultural Biotechnology and Biochemistry (RLABB), Sanepa, Lalitpur.

4.2. Lab study

Skull and baculum study was carried out in Laboratory of CDZ while PCR was carried out at RLABB.

4.2.1. Baculum preparation and storage

Bacula of three specimens (KT1, KT6, KT3 and KT4) were prepared and stored as follows:

1. Penis of the specimen was cut off (very closer from the body surface) and placed in a 1.5 ml micro centrifuge tube
2. The tube was labeled the sample number
3. The cut off penis was transferred to a test tube half filled with cold water and brought to boil and simmered for two minutes

4. The micro centrifuge tube half filled with 5% Potassium hydroxide (KOH) and a pinch of alizarin red powder which was used to stain the baculum
5. The penis was transferred to the tube (step 4) and left for 24 hours
6. The baculum was dissected out from the penis tissue under a dissecting microscope with very fine forceps and entomological pin
7. The baculum was photographed by using Canon digital power shot camera at normal from the eyepiece of the electronic compound microscope
8. Length of the baculum was measured with the help of simple vernier caliper from below of the glass slide
9. Their sketches were drawn by observing them under the electronic compound microscope as well as looking photographs
10. The baculum was carefully stored in a cleaned micro centrifuge tube half filled with glycerine
11. The tube was marked with accession number, species name, date and locality of collection
12. The tube with baculum was stored in upright position.

4.2.2. Skull preparation and storage

Skulls of six specimens were prepared and stored as follows:

4.2.2.1. Skull extraction

1. A cut was made from the corner of the mouth on one cheek to enlarge the hole
2. The facial skin was peeled off from the mandible and rostrum using forceps taking care not to damage the zygomatic arches
3. The skull was cut at the neck taking care not to damage the skull.

4.2.2.2. Skull cleaning

1. Each skull was dropped into a glass beaker partially filled with cold water temporarily labeled with sample number
2. The skull was heated and gently brought to boil and left for 10 minutes in simmering water

3. The boiled skull was left in the same beaker for 24 hours
4. Then muscles in and around the skull were peeled off gently taking care not to damage zygomatic arches
5. The tongue was removed by forcep
6. Brain was removed through the foramen magnum using forceps
7. The skull was cleaned maximum as much as possible.

The skull was dried in air and kept in plastic pot with secure lid. The skull was supported in between cotton. Few naphthalene balls were kept inside along with the skull for preservation. A pencil written tracing paper was tagged to the skull. Photographs of skull and dentitions was taken with the aid of AROMA 52mm close-up lenses (+1, +2, +4 and Macro) at 55mm zoom using AF-S NIKKOR 18-55 mm lens fitted with Nikon D40X camera. Cranio-dental sketches were drawn directly observing skulls with lenses as well as looking photographs.

4.2.3. Cranio-dental measurements

Following cranio-dental measurements were taken from the skull six times to minimize the biasness was taken with the help of vernier caliper to the nearest 1 millimeter: GTL-greatest length of skull; CCL-condylo-canine length; BB-breadth of braincase; PC-postorbital constriction; C-M^3-maxillary toothrow length; C-M_3-mandibular toothrow length; M-mandible length; C^1- C^1-anterior palatal width; M^3- M^3-posterior palatal width; RW-rostral width. The measurements were tallied with the available reference (for e.g. Bates and Harrison, 1997).

4.2.4. PCR Approach

4.2.4.1. DNA extraction and Purification

QIAGEN DNeasy® Blood and Tissue Kit was used to extract and purify the DNA

Protocol for tissue extraction and purification as followed is given below:

1. 3 mm sample of wing tissue was punched out using biopsy punch from each voucher specimen and kept in 1.5ml micro centrifuge tube. Each tube was marked with sample number

PHOTO PLATE 3

Photo 3A. Large penis of *Scotozous dormeri* specimen

Photo 3B. Boiling cut off penis

Photo 3C. Dissecting baculum under dissecting microscope

Photo 3D. Photographing baculum

Photo 3E. Unclear skull left for 24 hrs. after boiling

2. 20 µl Proteinase K was added, immediately mixed thoroughly by vortexing and incubated at 56℃ and occasionally vortexed during incubation to disperse the sample, until the tissue was completely lysed
3. The sample in micro centrifuge tube was again vortexed for 15 seconds. 200 µl Buffer AL was added to the sample and mixed thoroughly by vortexing. Then 200 µl ethanol (96%-100%) was added and again mixed thoroughly by vortexing
4. The mixture (including precipitate) from the step 3 was transferred through pipette into the Dneasy® Mini Spin Column placed in a 2ml collection tube. The Dneasy® Mini Spin Column placed in a 2ml collection tube was centrifuged at 8000 rpm for 1 min. The collection tube with the flow was discarded
5. The same Dneasy® Mini Spin Column was placed in a new 2ml collection tube, 500 µl Buffer AW1 was added through pipette and then centrifuged at 8000 rpm for 1 min. The collection tube with the flow was discarded
6. The same Dneasy® Mini Spin Column was placed in a new 2ml collection tube, 500 µl Buffer AW2 was added through pipette and then centrifuged at 14000 rpm for 3 min to dry the Dneasy membrane. The collection tube with the flow was discarded
7. The same Dneasy® Mini Spin Column was placed in a new 2ml collection tube, 200 µl Buffer AE was added through pipette directly onto Dneasy membrane, incubated at room temperature for 1 min. and then centrifuged at 8000 rpm for 1 min to dry the Dneasy membrane. Elute was collected in the collection tube
8. The step 7 was repeated (without discarding the collection tube and only adding 200 µl Buffer AE) to get maximum DNA yield.

4.2.4.2. Gel-electrophoresis of DNA

Following chemicals were prepared as follows:

Buffers

TE Buffer (300ml)

10mM (Milli Molar) Tris-HCl 0.363 gm

1mM Ethylene Diamine Tetra Acetic Acid (disodium salt) (EDTA) 0.116gm

pH adjusted to 8.0 and autoclaved

TAE Buffer (10 X stock in 100ml)

Tris-base 4.84 gm

0.5M EDTA 0.372gm

Glacial acetic acid added to maintain pH 8.0 and autoclaved.

NB: 10 ml 10X TAE buffer added to 90ml distilled water to make 1X for electrophoresis.

DNA dye (Blue juice) (25ml: 10X)

Glycerol 350µl

0.25M EDTA 40µl

20% SDS 5µl

10% bromophenol blue 30 µl

NB: 1 ml 10X DNA dye (blue juice) added to 9 ml distilled water to make 1X for electrophoresis.

Ethidium Bromide solution (10mg/ml stock)

Ethidium bromide 0.05gm

Added to 5ml distilled water in a vile, shaked and wrapped with aluminum foil and stored at 4°C.

NB: Thick gloves were used during the preparation as Ethidium Bromide is a potential carcinogen.

37.5 µl Ethidium Bromide solution was added to mild agarose solution.

Agarose gel (1.5 %)

TAE buffer 1X 35ml

Agarose 0.28gm

Heated to boiling for well mixing of Agarose solution and filled in the well and left it to cool and convert into gel.

The loading sample was prepared of 5µl DNA template with 10 µl DNA dye (blue juice) was mixed with pulse vortexing. The sample (10µl) was loaded in the well of the gel dipped into the TAE buffer and gel was run at 80 volt until the dye travelled the third-fourth (¾) length of the gel. The run gel was visualized using Fotodyne UV trans-illuminator.

4.2.4.3. Quantification

The template DNA was quantized using the SSI UV 2101 Spectrophotometer. DNA template (20 µl) was added to TE buffer to make 2 ml total volume in quartz cuvette. The absorbance reading was noted at 260 and 280 nm. Concentration was calculated according to the formula given below:

$$\text{Concentration of DNA (µg/µl)} = \frac{\text{A260* dilution factor* 50 µg/µl}}{1000}$$

NB: The purity of the DNA at 260/280 absorbance should be 1.8 and Absorbance of 50µg/ml solution at 260nm is 1.

4.2.4.4. Polymerase Chain Reaction (PCR)

Stock Template DNA was diluted as follows:

Sample	Original concentration of DNA (µg/µl) (S1)	Required concentration of DNA for PCR (ng/µl) (S2)	Manipulated Volume (µl) (V2)	Required Volume (µl) (V1)	Diluent (SdDw) (µl)
KT1	0.585	25	20	0.85	19.15
KT2	0.585			0.85	19.15
KT3	0.5			1	19
KT4	0.66			0.75	19.25
KT5	0.1			5	15
KT6	0.1			5	15

NB: V1 was calculated using formula: S1*V1=S2*V2 and for the ease calculation was done in 100 µl and 50 µl final manipulated volumes for KT1-KT4 and KT5-KT6 respectively.

Primers designed for amplification of whole Cytochrome *b* gene (Irwin *et al.*, 1991) were used.

Primer name	Sequence 5'-3'
L14724 ()	CGA AGC TTG ATA TGA AAA ACC ATC GTT G
H15915	AAC TGC AGT CAT CTC CGG TTT ACA AGA C

Reaction Mixture was prepared:

Final Vol. = 25 µl

Template concentration taken = 25 ng/ µl
Template volume taken = 1 µl
Primer L 14724 vol. taken = 2.5 µl at conc^{n}. 10 µM
H 15915 vol. = 2.5 µl at conc^{n}. 10 µM

SdDw (ddH_2O) = 19 µl

Six reaction mixtures were poured into illustra™ PuRe Taq Ready-To-Go PCR Beads which constituted:

PuRe Taq™ DNA polymerase = ~2.5 units
Tris-HCl (pH 9.0 at room temp.) = 10 mM
KCL = 50 mM
$MgCl_2$ = 1.5 mM
dATP,dCTP, dGTP, dTTP and stabilizers = 200µM
including BSA

The beads were spinned (2000 rpm/5 sec) in REMNI C24 cooling centrifuge. All reaction mixtures were covered with gently adding Paraffin. Paraffin was also poured into wells of Thermocycler (Programmable Thermal Controller, MJ Research Inc.). The beads and negative control were placed into the former. Thermal Cycler Program was run as below:

Steps	Temp.	Time
Predenaturation	95˚C	3:00
Denaturation	95˚C	1:00
Annealing	56˚C	1:00
Extension	72˚C	3:00
Cycles 35		
Final Extension	72˚C	10:00
Storage	4˚C	24:00:00

4.2.4.5. Gel-electrophoresis of PCR Product

Gel electrophoresis of PCR product was immediately carried out after the Thermal Cycler Program ends. The PCR Product and negative control was carefully pipetted out avoiding Paraffin. The negative control contained reaction mixture without template DNA and it was placed to test the contamination during handling. The process was carried out accordingly to 4.2.4.2. In addition, DNA ladder (marker, PCR Marker 50bp-2000bp, USB Corporation, USA) was run along with the loading samples. The visualized gel was photographed.

PHOTO PLATE 4

Photo 4A. Using REMNI® C24 cooling centrifuge

Photo 4B. Template DNA in micro-centrifuge tubes

Photo 4C, D. Placing PCR Beads (left) and running program (right) in Programmable Thermal Controller, MJ Research Inc.

Photo 4E, F. Pipetting in loading sample (left) and pipetting out loading samples into agarose gel well

5. RESULTS

This study was carried within September 2008 to September 2010.

5.1. Bats Captured and Collected

Mist netting was empty hand. Six specimens (two female remaining male) of pipistrelles were captured from three houses at Samsul tole and one house and one shed from Goithi tole.

Specimens (KT2, KT3, KT4, KT5 and KT6) exhibited overlaps in all external character measurements range of *P. pipistrellus*, *P. paterculus*, *P. coromandra*, and *P. tenuis* while one specimen (KT1) exhibited overlaps in all external character measurements range of *P. javanicus*, *P. ceylonicus*, *P .savii*, *Scotozous dormeri*.

5.2. Skull and Bacular Morphology

The bacular morphology (Photo plates 5-7) confirmed two species namely; *P. tenuis* (KT3) and *P. coromandra* (KT4 and KT6). Dental characters (Photo plates 8-13) revealed the specimens of three different species namely *Scotozous dormeri* (KT1); *P. tenuis* (KT2 and KT3) and *P. coromandra* (KT4, KT5 and KT6); respectively.

Specimen KT1 exhibited all external and cranio-dental measurements tallying with *S. dormeri*. Following distinguishing characters were also exhibited: i^2 without a secondary cusp but with a distinct cingular cusp posteriorly. i^3 absent. The upper canine without a secondary cusp rather cingular cusps were present anterior and posterior. pm^2 was intruded from the upper tooth row and its crown area was two-thirds to crown area of i^2. Unfortunately, the baculum of KT1 was totally damaged. However, these distinguishing characters confirmed the specimen of *S. dormeri*.

In Specimens KT2 and KT3, the baculum had a long thin shaft with a distinctly bifid tip and with the well developed basal lobes deflected ventrally (in specimen KT3 while specimen KT2 was

female). The specimens exhibited all external and cranio-dental measurements tallying with *P. tenuis*. Following distinguishing characters were also exhibited: C^1 had a distinct secondary cusp. The first lower premolar (pm_2) was slightly extruding from the lower tooth row and was three – quarters the crown area and two-thirds the height of second lower premolar (pm_4). These distinguishing characters confirmed the specimen of *P. tenuis*.

In Specimens KT4, KT5 and KT6, The baculum had a straight or slightly sinuous shaft with a distinctly bifid tip and with the basal lobes deflected ventrally (in specimen KT4 and KT6) while specimen KT5 was female (T). Unfortunately baculum of KT6 was partially damaged (However, species confirmed on the basis of shape of basal lobes). The specimens exhibited all external and cranio-dental measurements tallying with *P. coromandra* larger in comparison to *P. tenuis*. Following distinguishing characters were also exhibited: rostrum and palate are more elongated while braincase exceeds in size in comparison to *P. tenuis*. The upper canine (C^1 had a secondary cusp and a distinct cingular cusp posteriorly. The first lower premolar (pm_2) was slightly extruding from the lower tooth row and was three-quarters the crown area of second lower premolar (pm_4). These distinguishing characters confirm the specimen of *P. coromandra*.

Table 1. Pipistrelles collected from Kusaha, KTWR

Sample No.	Accession No.	Specimens collected	No. of Indiviuals collected	Sex	Age	Repro-status
KT1	CDZ TU_BAT 019	*Scotozous dormeri*	1	U	A	R
KT2	CDZ TU_BAT 020	*Pipistrellus tenuis*	1	T	A	NR
KT3	CDZ TU_BAT 021		1	U		R
KT4	CDZ TU_BAT 022	*P. coromandra*	1	U	A	R
KT5	CDZ TU_BAT 023		1	T		NR
KT6	CDZ TU_BAT 024		1	U		NR

Note: U=Male; T=Female; A=adult; NR=Non-Reproducing; R= Reproducing.

Table 2. Selected external, cranial, and dental measurements (in mm.) of Pipistrelles from Kusah

	Kusaha, KTWR CDZ TU_BAT 019 (KT 1)		Kusaha, KTWR CDZ TU_BAT 020 (KT2), 021 (KT3)			Kus CDZ
		n	mean	range	n	me
HB	53.0	1	37.5	36.0-39.0	2	41
T	30.0	1	27.8	25.5-30.0	2	31
TIB	10.0	1	11.0	11.0	2	11
HF	8.0	1	5.0	-	1	5
FA	33.5	1	27.0	26.0-28.0	2	30
Thumb	6.5	1	4.5	4.0-5.0	2	5
3mt	32.5	1	26.5	26.0-27.0	2	29
1ph3mt	12.0	1	10.5	10.0-11.0	2	11
2ph3mt	9.0	1	15.0	15.0	2	15
4mt	32.0	1	25.5	24.0-27.0	2	29
1ph4mt	12.0	1	10.5	10.0-11.0	2	10
2ph4mt	8.0	1	6.8	6.0-7.5	2	7
5mt	32.0	1	26.0	25.0-27.0	2	28
1ph5mt	9.0	1	7.0	7.0	2	7
2ph5mt	6.0	1	5.0	5.0	2	5
E	10.0	1	11.3	10.0-12.5	2	10
Tragus	5.0	1	3.5	3.0-4.0	2	4
GTL	14.1	1	11.4	11.3-11.4	2	12
CCL	13.2	1	10.3	10.3	2	11
BB	7.0	1	6.4	6.3-6.4	2	6
PC	3.5	1	3.3	3.2-3.4	2	3
C-M3	5.3	1	3.9	3.8-4.0	2	4
c-m3	5.7	1	4.3	4.1-4.4	2	4
M	10.7	1	8.2	8.2	2	9
C1-C1	4.5	1	3.8	3.7-3.8	2	4
M3-M3	6.5	1	5.2	5.0-5.3	2	5
RW	5.6	1	4.2	4.0-4.3	2	4
Baculum length	-	-	3.5	-	1	2

PHOTO PLATE 5

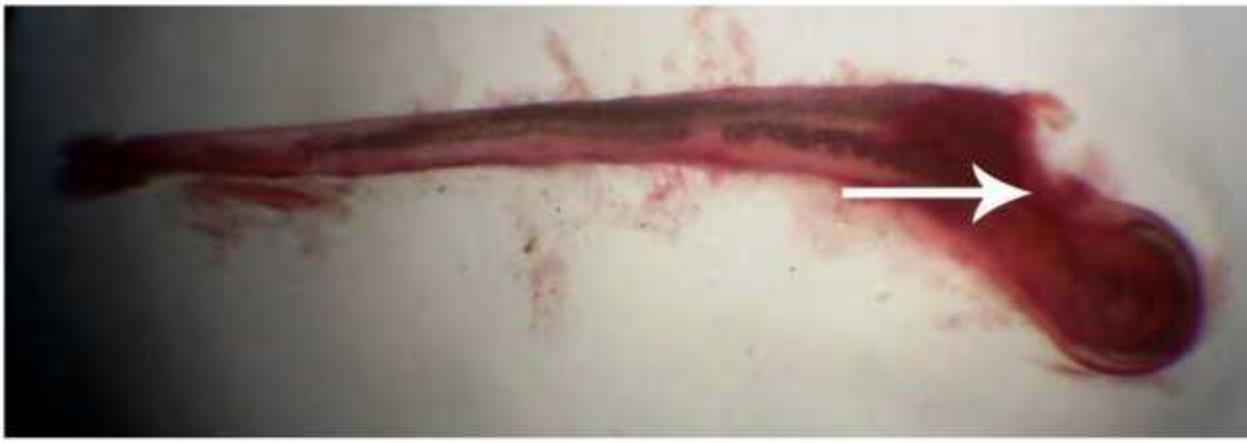


Photo 5A. Lateral view of baculum of *P. tenuis* (demarcation at basal lobes pointed) (KT3)

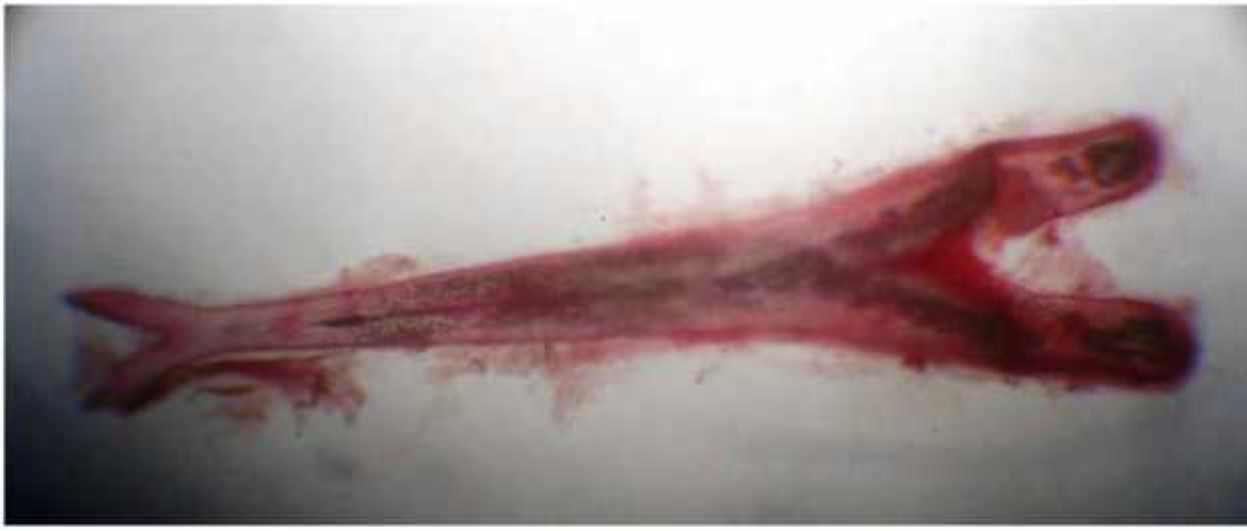


Photo 5B. Dorsal view of baculum of *P. tenuis* (KT3)

PHOTO PLATE 6

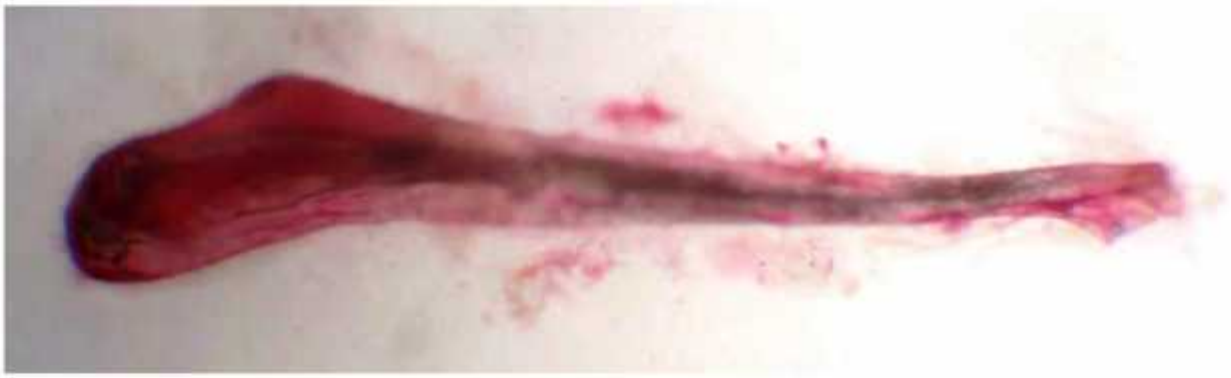


Photo 6A. Lateral view of baculum of *P. coromandra* (KT4)

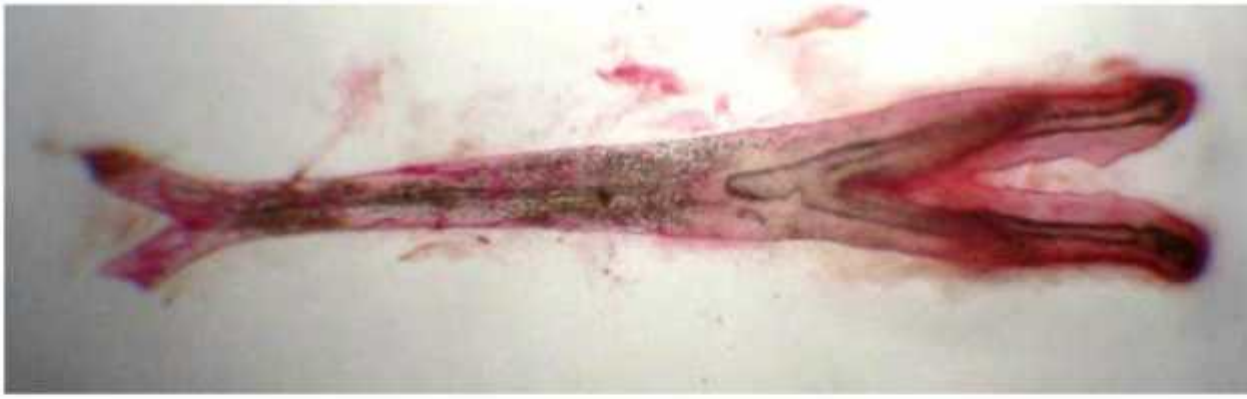


Photo 6B. Dorsal view of baculum of *P. coromandra* (KT4)

PHOTO PLATE 7

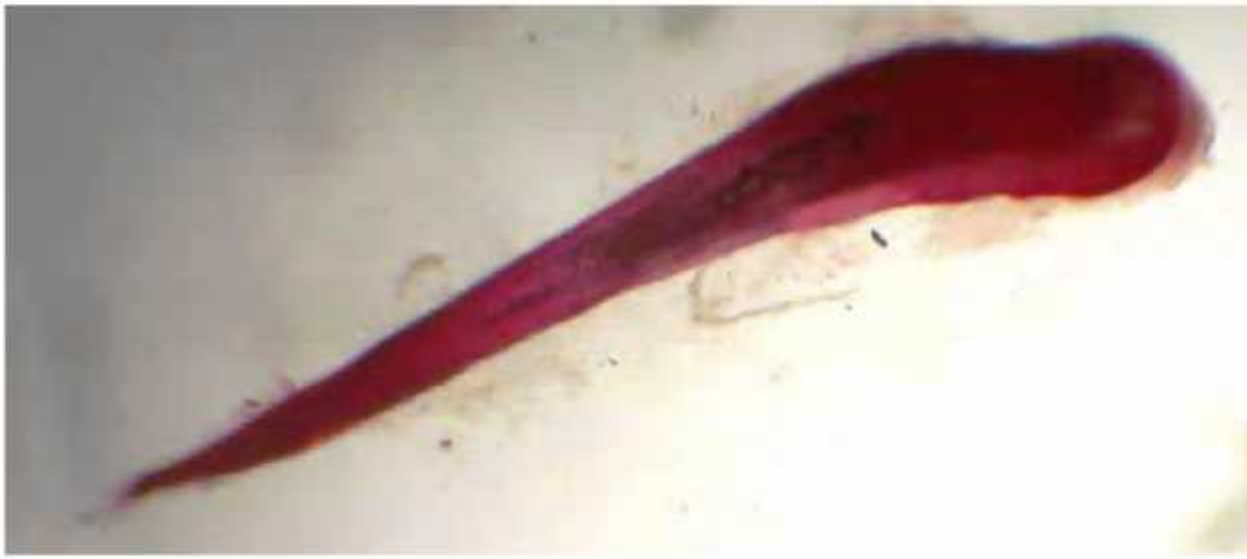


Photo 7A. Lateral view of baculum of *P. coromandra* (KT6)

Photo 7B. Dorsal view of baculum of *P. coromandra* (KT6)

PHOTO PLATE 8

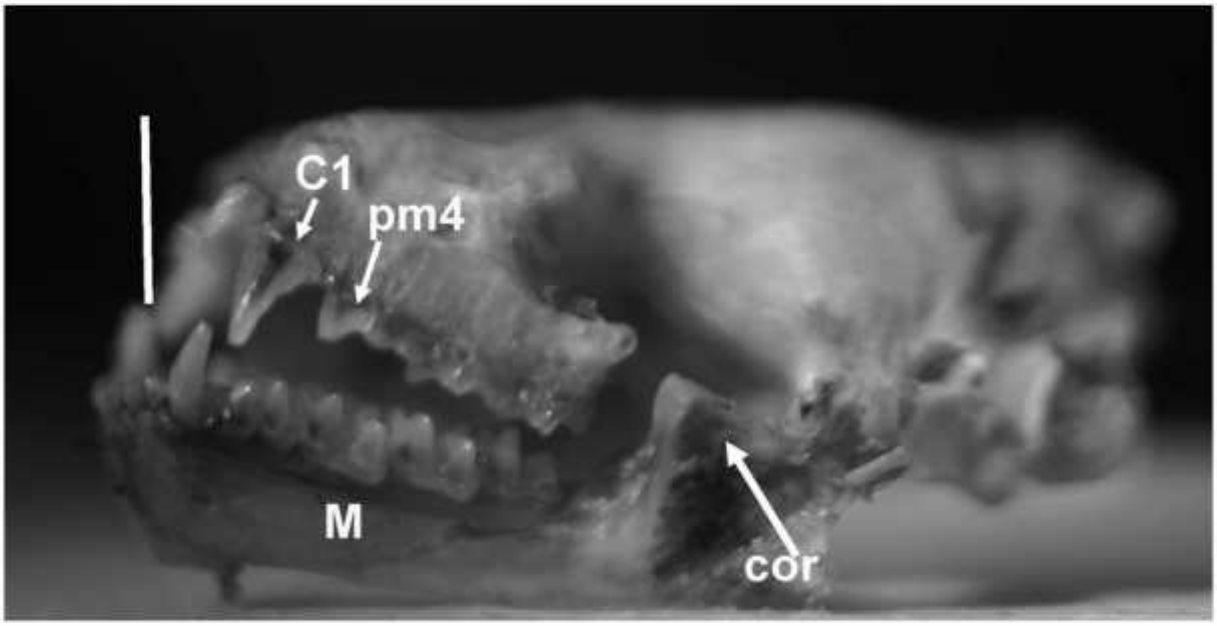


Photo 8A. Lateral View of skull of *Scotozous dormeri.* CDZ TU_BAT 019, Kusaha, KTWR, Nepal. (Scale=3mm) (KT1).

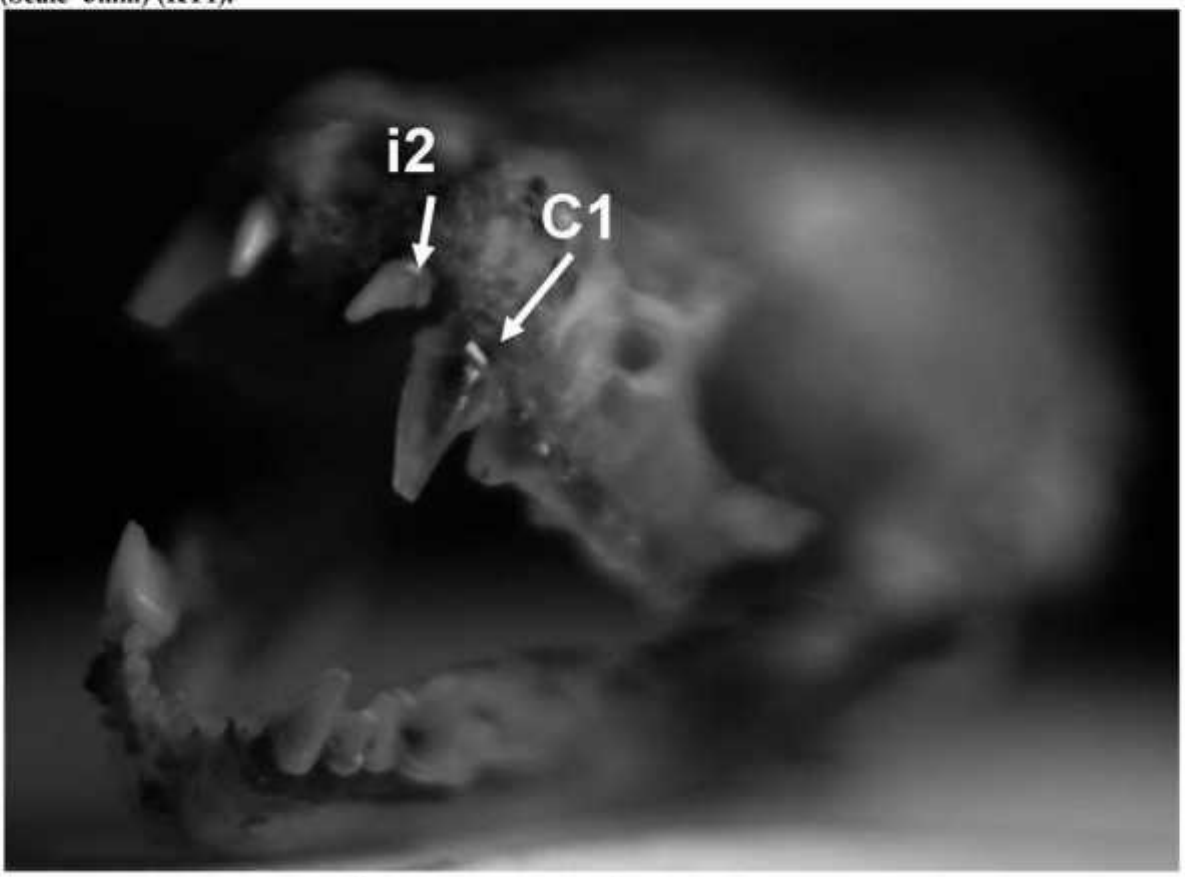


Photo 8B. First upper incisor (i^2) and upper canine (C^1) of skull of *Scotozous dormeri.* CDZ TU_BAT 019, Kusaha, KTWR, Nepal (KT1).

PHOTO PLATE 9

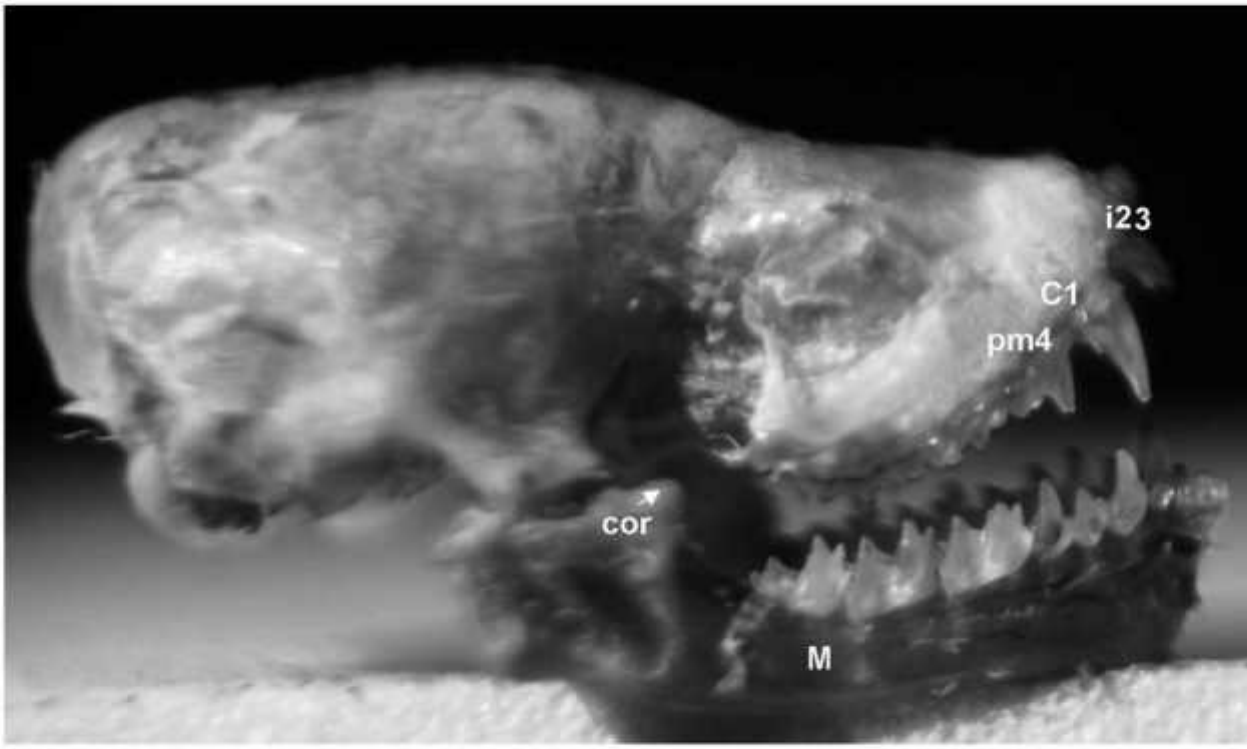


Photo 9A. Lateral View of skull of *Pipistrellus tenuis.* CDZ TU_BAT 020, Kusaha, KTWR, Nepal (KT2).

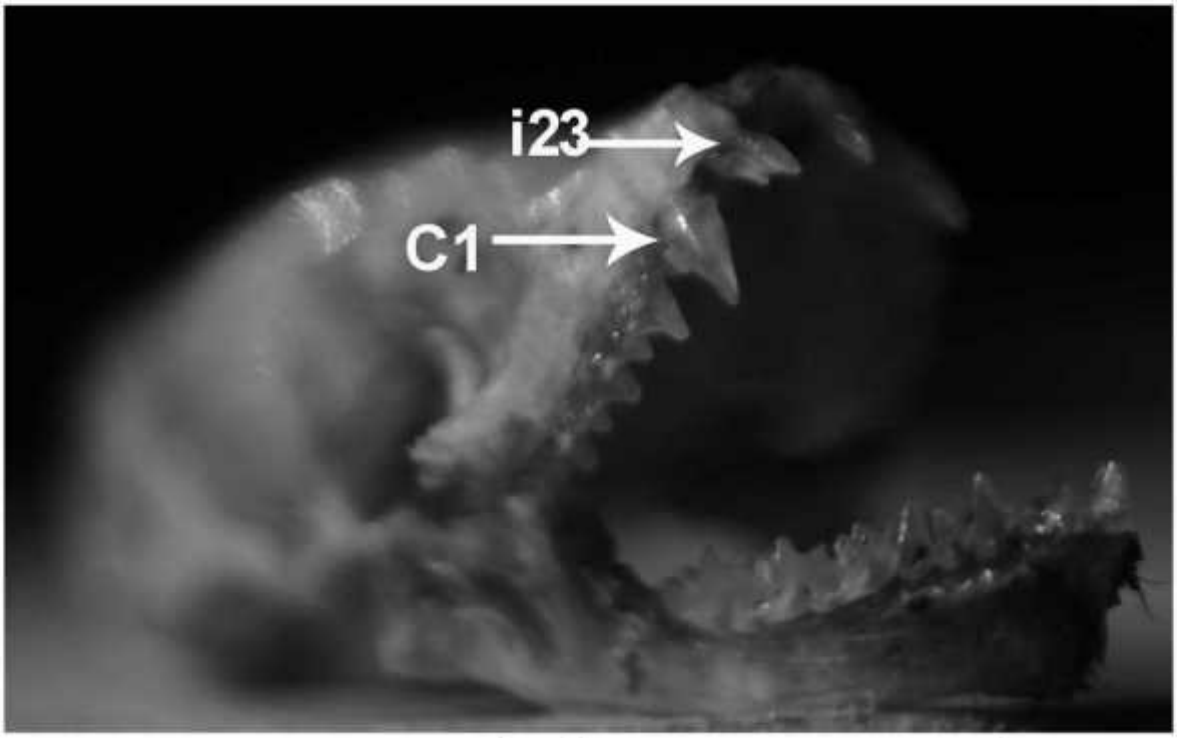


Photo 9B. First and second upper incisors (i^2 and i^3 denoted as i23) and upper canine (C^1) of skull of *P. tenuis.* CDZ TU_BAT 020, Kusaha, KTWR, Nepal (KT2).

PHOTO PLATE 10

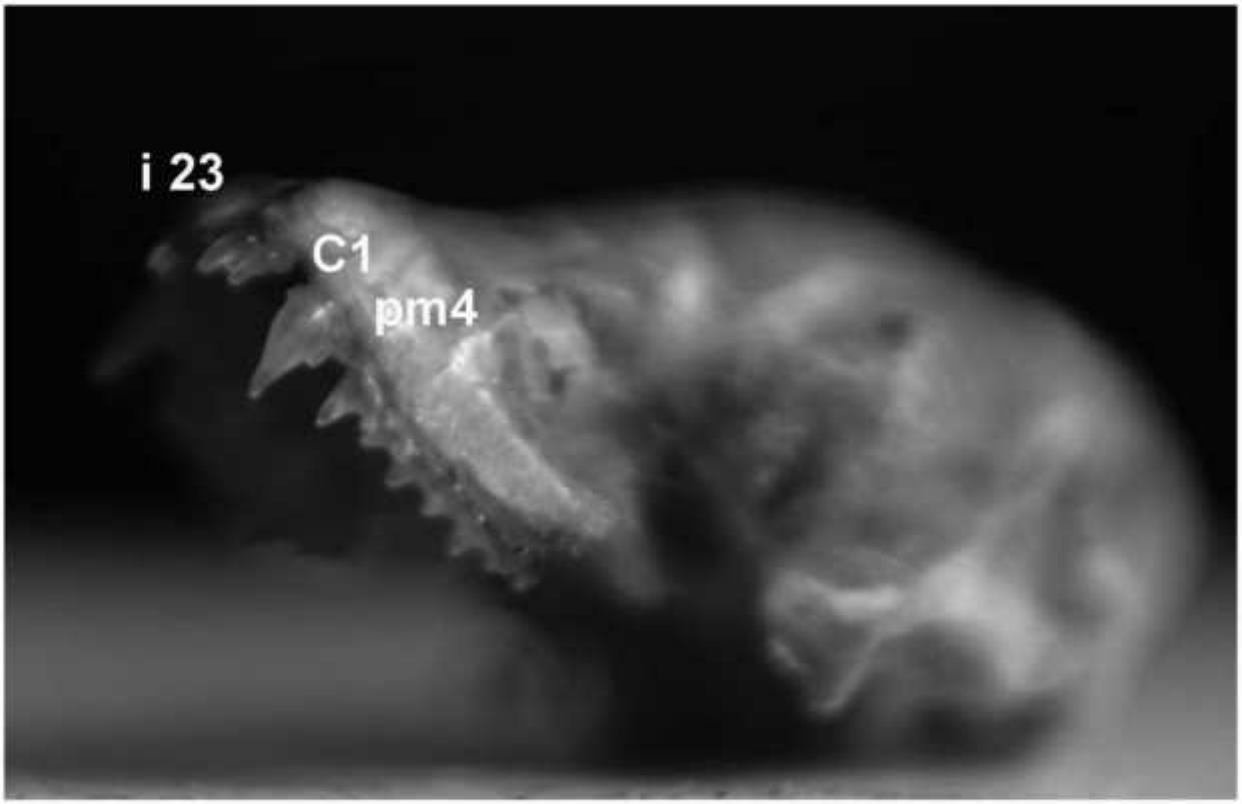


Photo 10A. Lateral View of skull of *P. tenuis* (Excluding mandible). CDZ TU_BAT 021, Kusaha, KTWR, Nepal (KT3).

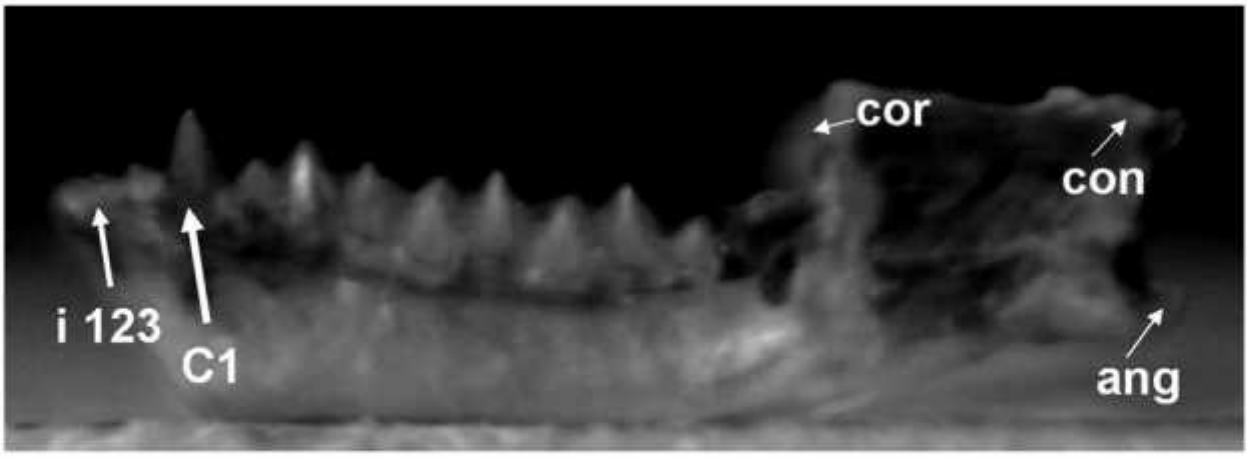


Photo 10B. Lateral View of mandible of *P. tenuis.* CDZ TU_BAT 021, Kusaha, KTWR, Nepal (KT3).

PHOTO PLATE 11

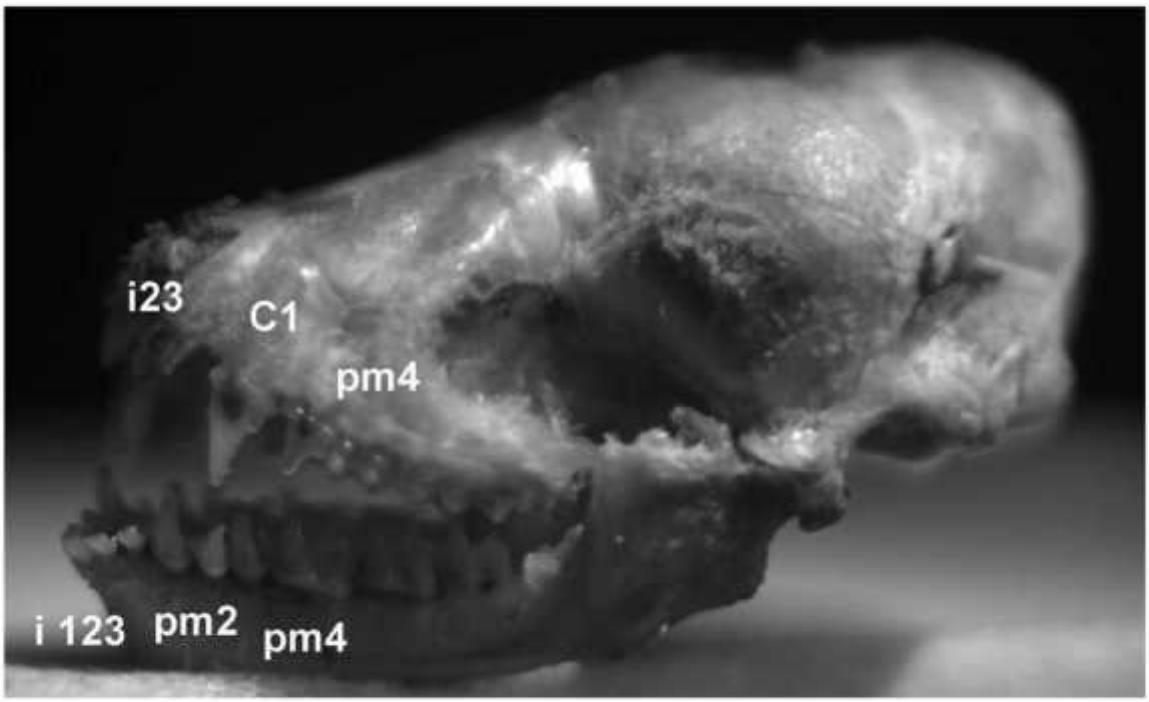


Photo 11A. Lateral View of skull of *P. coromandra.* CDZ TU_BAT 022, Kusaha, KTWR, Nepal (KT4).

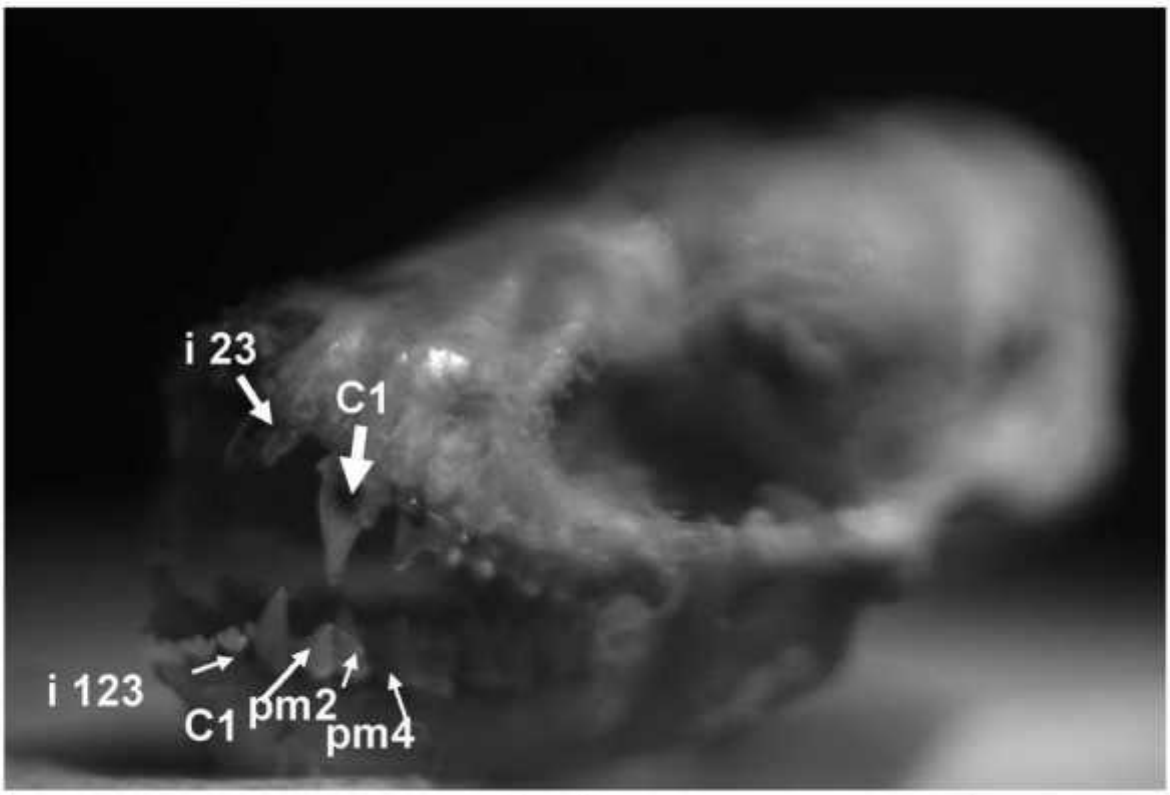


Photo 11B. First and second upper incisors (i^2 and i^3 denoted as i23), upper canine (C^1), first, second and third lower incisor (i123), lower canine (C_1), first lower premolar (pm_2)and second lower premolar (pm_4) of skull of *P. coromandra.* CDZ TU_BAT 022, Kusaha, KTWR, Nepal (KT4).

PHOTO PLATE 12

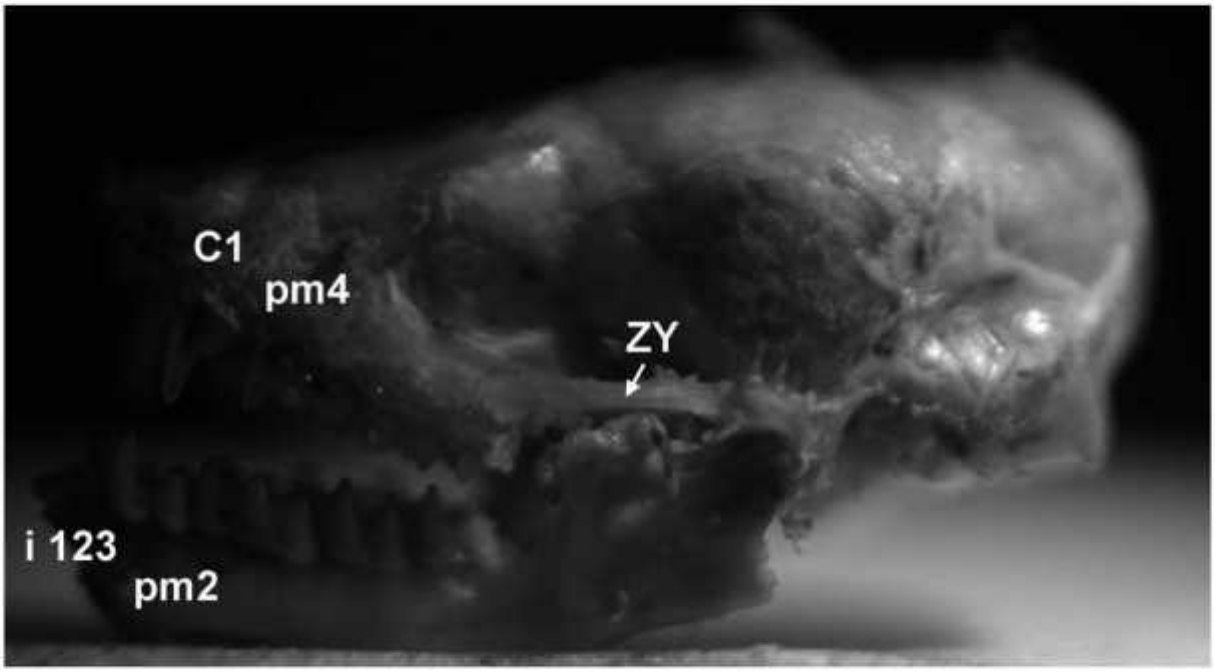


Photo 12A. Lateral View of skull of *P. coromandra.* CDZ TU_BAT 023, Kusaha, KTWR, Nepal (KT5).

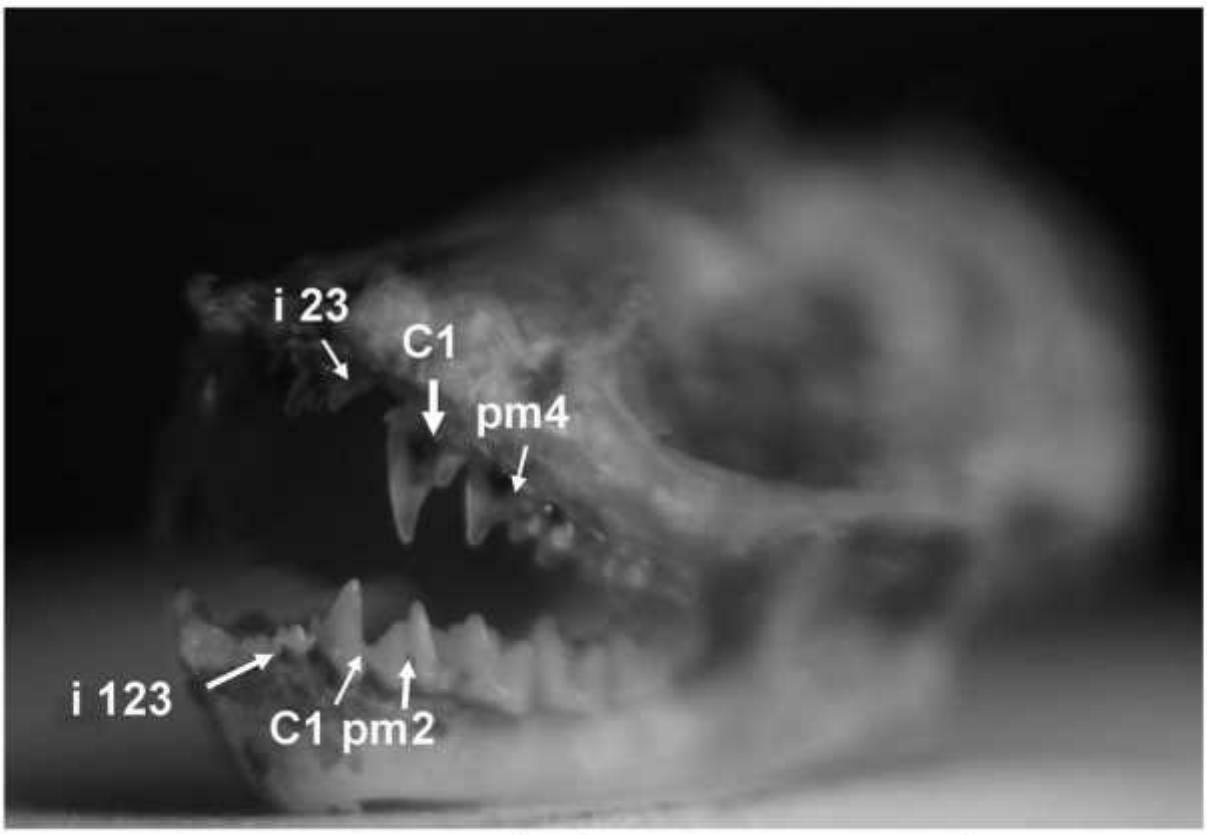


Photo 12B. First and second upper incisors (i^2 and i^3 denoted as i23), upper canine (C^1), first, second and third lower incisor (i123), lower canine (C_1), first lower premolar (pm_2)and second lower premolar (pm_4) of skull of *P. coromandra.* CDZ TU_BAT 023, Kusaha, KTWR, Nepal (KT5).

PHOTO PLATE 13

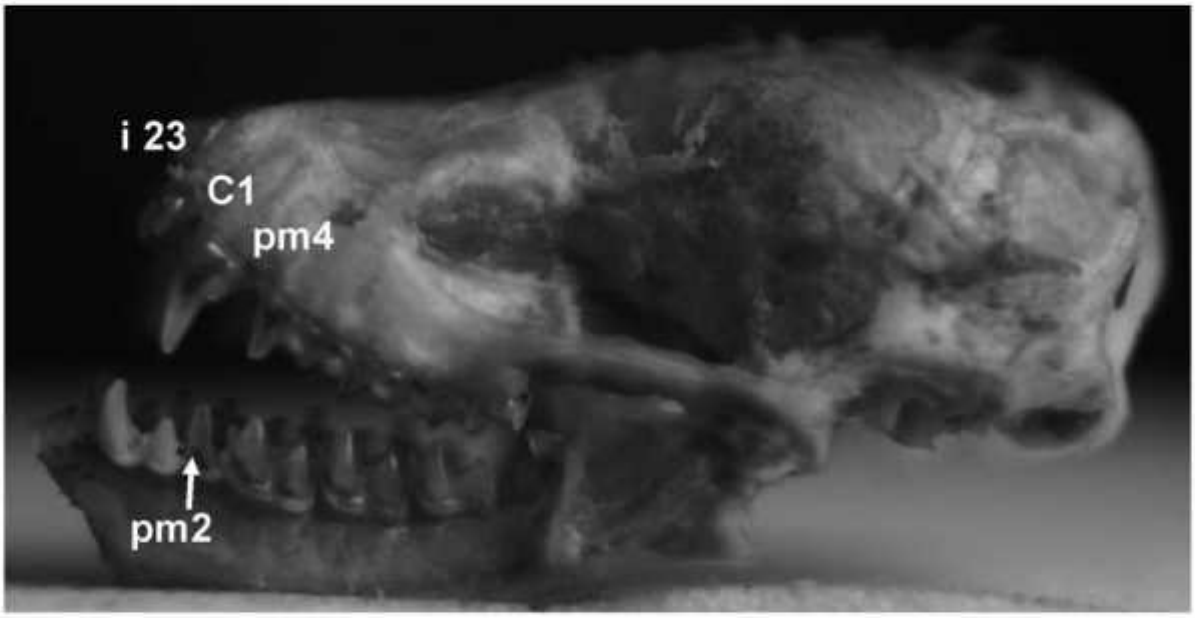


Photo 13A. Lateral View of skull of *P. coromandra.* CDZ TU_BAT 024, Kusaha, KTWR, Nepal (KT6).

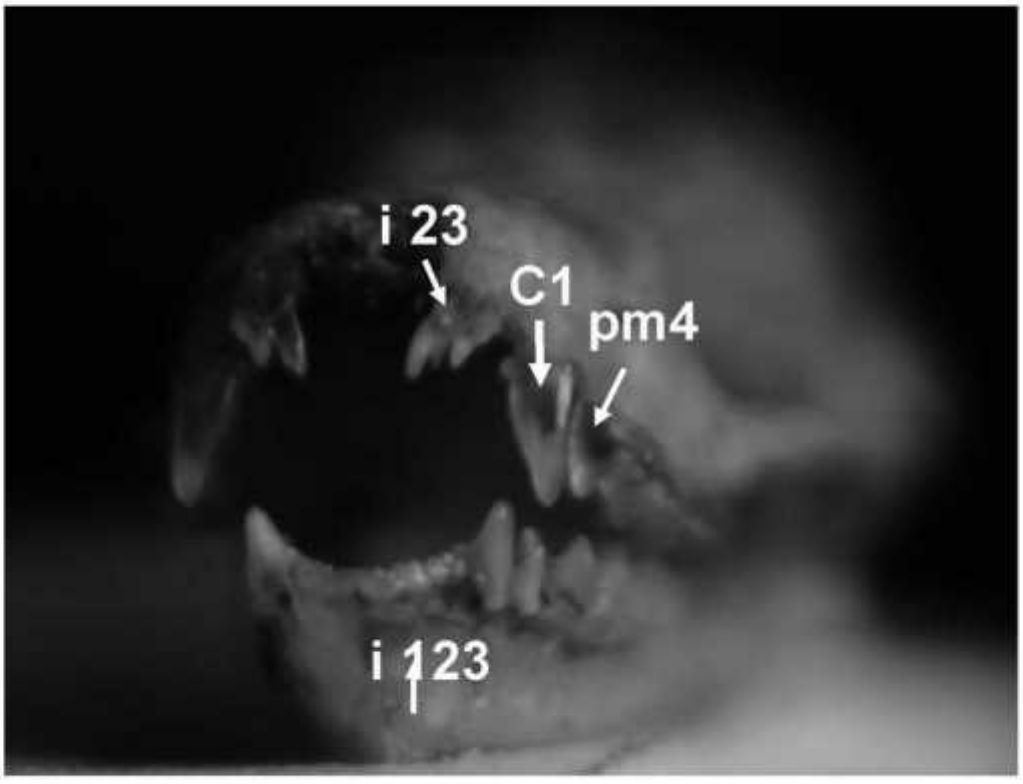


Photo 13B. Anterior view of skull of *P. coromandra*.CDZ TU_BAT 023, Kusaha, KTWR, Nepal showing First and second upper incisors (i^2 and i^3 denoted as i23), upper canine (C^1), first, second and third lower incisor (i123), lower canine (C_1), first lower premolar (pm_2) and second lower premolar (pm_4) (KT6).

5.3. Recorded species profile

Pipistrellus coromandra (Gray, 1838)

Status: Two were males (KT4 and KT6) and a female (KT5) adults.

Reproductive Status: KT4 was reproducing with swollen testes, KT5 and KT6 was non-reproducing.

Population: hundreds of these bats could be found in the study area.

External Characters: The color of the dorsal pelage was ranging from uniform mid-brown to deep clove brown. Ventral pelage were paler buffy brown and with cinnamon brown tips. The external measurements are given in table 2. Ectoparasites were absent in the body of all specimens.

Habitat: These were found roosting at the bamboo hollows and holes of the thatched roofs of the houses at Samsul tole; Goithi tole; Near Hattisar Kusaha village (N 26° 25' 54", E 87° 19' 31.4" and at an elevation of 70m from the sea level), Koshi Tappu Wildlife Reserve (KTWR); They were even found in the roosts of smoky areas like kitchen and Shed.

Threats: Children play with these bats and kill for fun. According to locals at KTWR, Kusaha after the flooding of Koshi the population of the species has decreased. Their habitat is disturbed when the house is re-thatched or maintained.

Status from the present study: Common

Pipistrellus tenuis (Temminck, 1840)

Status: One was male (KT3) and a female (KT2) adults.

Reproductive Status: KT3 was reproducing with swollen testes, KT2 was non-reproducing.

Population: hundreds of these bats could be found in the study area.

External Characters: The color of the dorsal pelage was ranging from uniform mid-brown to deep clove brown. Ventral pelage were paler buffy brown and with cinnamon brown tips. The external measurements are given in table 2. Ectoparasites were absent in the body of all specimens.

Habitat: These were found roosting at the bamboo hollows and holes of the thatched roofs of the houses at Samsul tole; Goithi tole; Near Hattisar Kusaha village (N 26° 25' 54", E 87° 19' 31.4" and at an elevation of 70m from the sea level), Koshi Tappu Wildlife Reserve (KTWR); They were even found in the roosts of smoky areas like kitchen and Shed.

Threats: Children play with these bats and kill for fun. According to locals at KTWR, Kusaha after the flooding of Koshi the population of the species has decreased. Their habitat is disturbed when the house is re-thatched or maintained.

Status from the present study: Common

Scotozous dormeri Dobson, 1875

Status: A single male (KT1)

Reproductive Status: KT1 was reproducing with large swollen testes.

Population: Unknown

External Characters: The specimen had greatest HB of 53mm. Penis was large and swollen.

Habitat: This was found roosting at the bamboo hollows of the thatched roofs of the houses at Kusaha village (N 26° 25' 54", E 87° 19' 31.4" and at an elevation of 70m from the sea level).

Threats: Children play with these bats and kill for fun. According to locals at KTWR, Kusaha after the flooding of Koshi the population of the species has decreased. Their habitat is disturbed when the house is re-thatched or maintained.

Status from the present study: Unknown.

5.4. PCR Approach

The extracted and purified DNA templates showed reading at A260 for different samples as:

KT1= 0.117; KT2= 0.117; KT3= 0.1; KT4= 0.132; KT5= 0.02; KT6= 0.02

The quantified concentration was calculated to be:

KT1= 0.585µg/µl; KT2= 0.585µg/µl; KT3= 0.5µg/µl; KT4= 0.66µg/µl; KT5= 0.1µg/µl; KT6= 0.1µg/µl.

Unfortunately negative result was encountered (DNA band could not be retrieved in PCR products separated on 1.5% agarose gel and visualized by ethidium bromide.

PHOTO PLATE 14

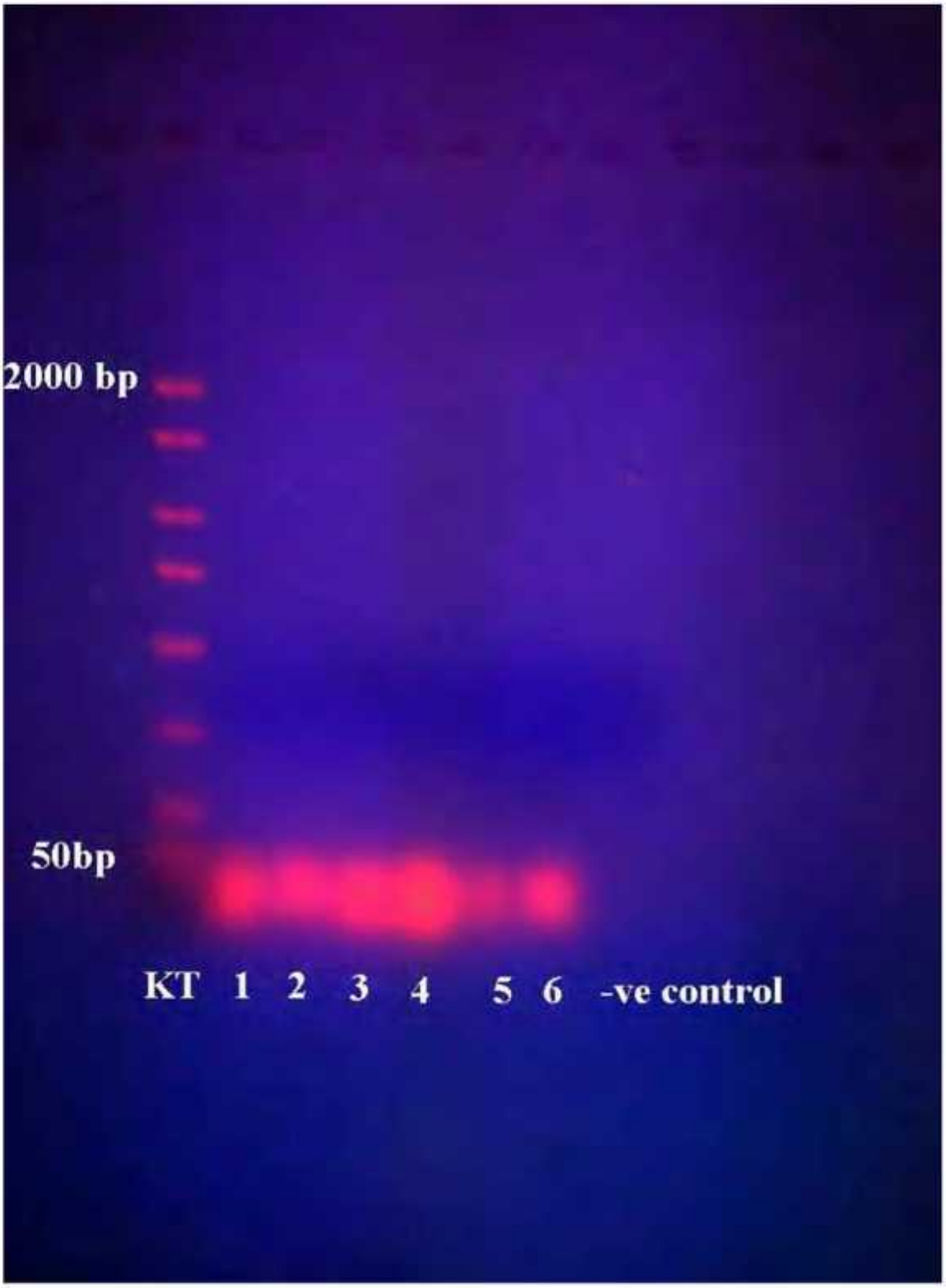
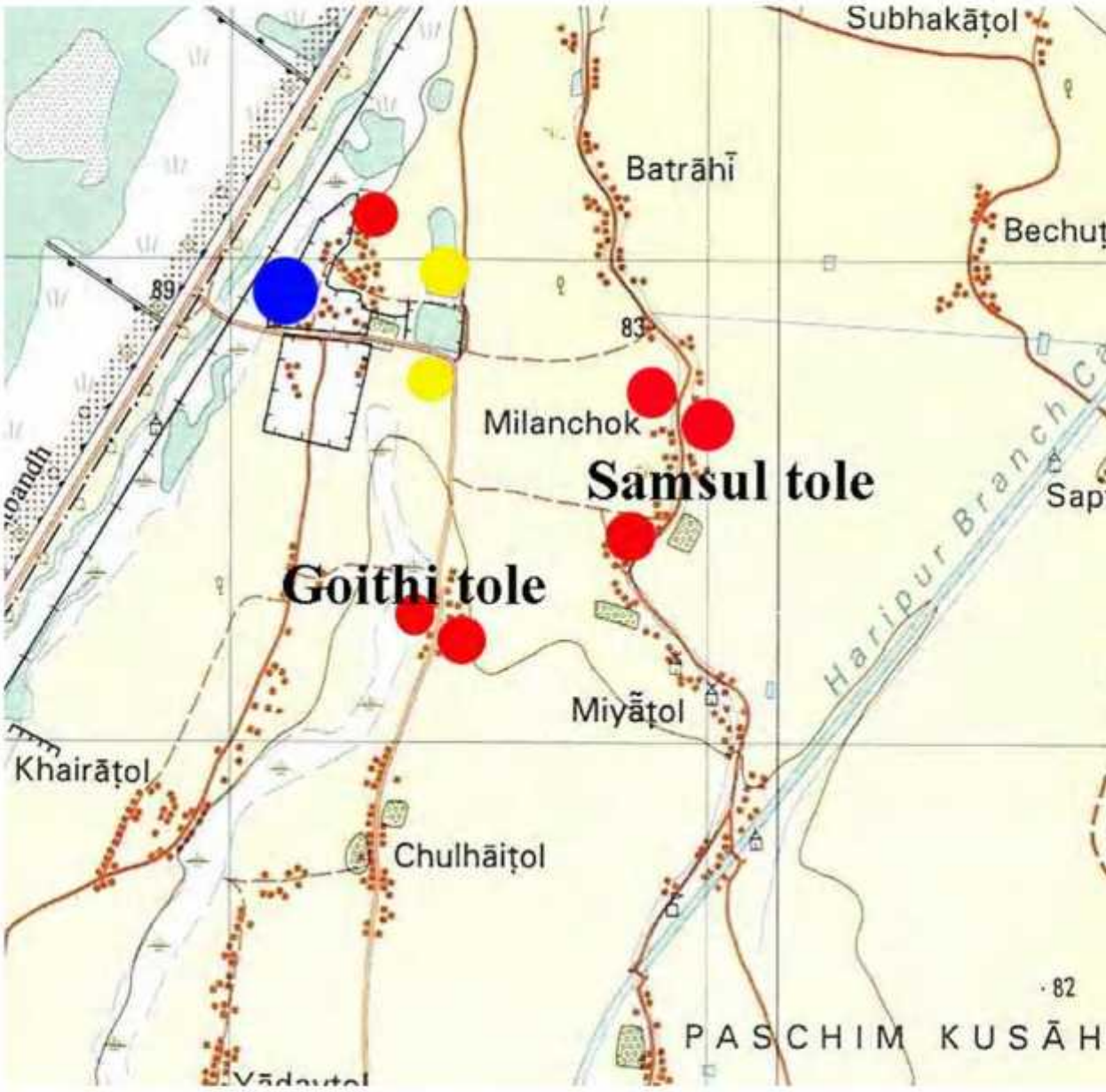


Photo13. PCR products separated on 1.5% agarose gel and visualized by ethidium bromide.



Map 4. Study area with study sites (Kusaha, KTWR).

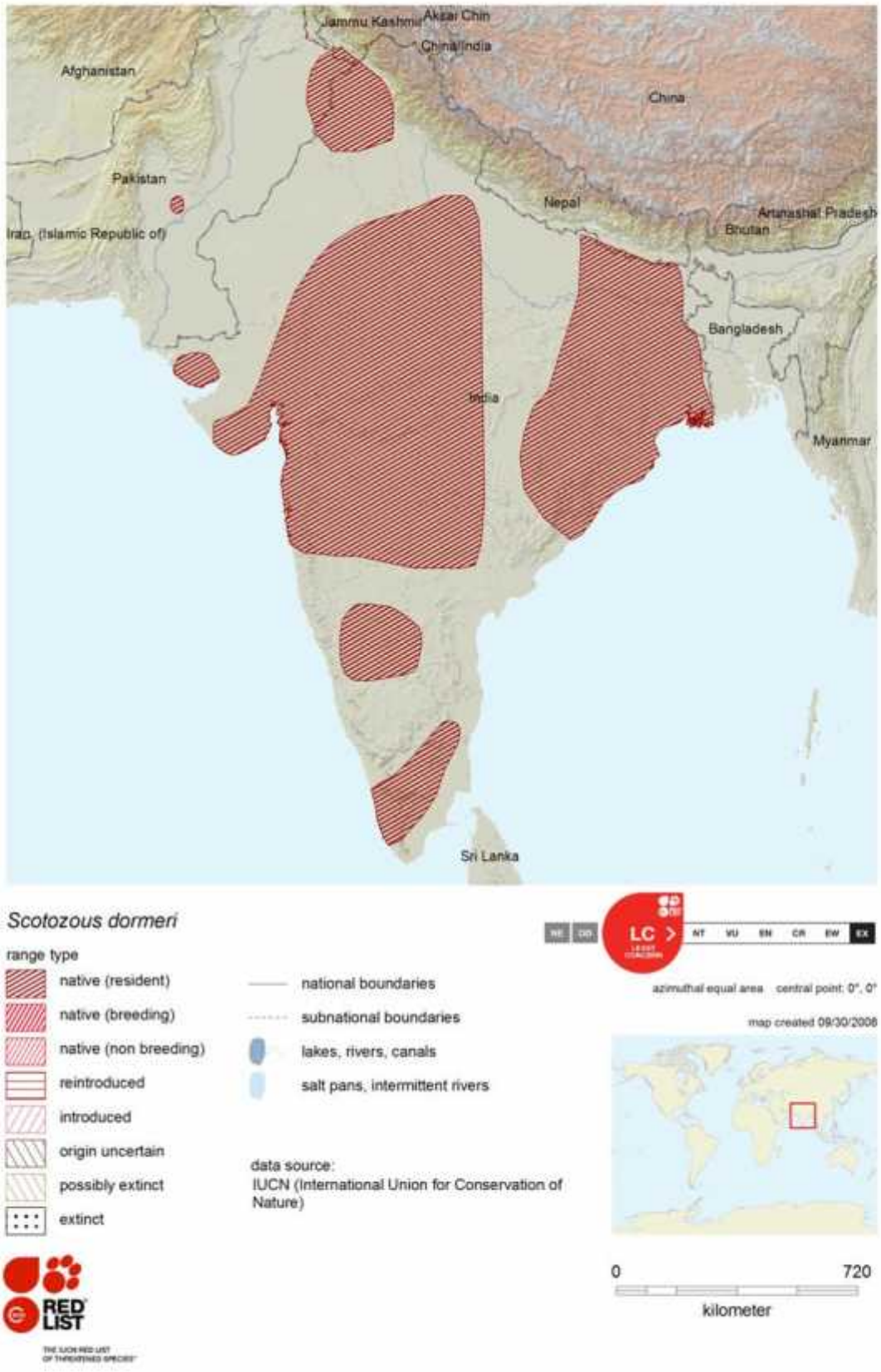
- Species recorded site
- KTWR head office, Kusaha
- Mistnetted sites

6. DISCUSSION

Chiropteran surveys have been initiated recently in Nepal and it is obvious, research on bat fauna is still missing from most of the unknown potential bat assemblage area specifically southern parts of the country. Only four species of bats were recorded from the first short duration survey in plains (Tarai) of Eastern Nepal including Kusaha area of Koshi Tappu Wildlife Reserve, when unidentified pipistrelles were found to be widely distributed throughout (Thapa, 2009). Mist netting is not so successful here rather roost survey proved to be promising methodology.

P. coromandra has been recorded from 11 localities throughout Nepal, the nearest; Hazaria, Bairia and Bairaglia (Probably from Sarlahi district along the Bagmati river) (Hinton and Fry, 1923). *P. tenuis* has been recorded from nine localities throughout Nepal, the nearest; Bairia and Hazaria (Hinton and Fry, 1923) from the study area. The collection of *P. coromandra* and *P. tenuis* from Kusaha are new materials to Nepal from the study area. However, distribution of these species has been extensively recorded in India. *P. coromandra* has been reported from 22 localities only from Bihar, likewise *P. tenuis* has been reported from 18 localities (Bates and Harrison, 1997).

This is the first record of *S. dormeri* in Nepal as well as first locality record. This species is endemic to South Asia and is presently known from Bangladesh (Dhaka and Rajshahi divisions) (Khan, 2001; Srinivasulu and Srinivasulu, 2005), India (Andhra Pradesh, Assam, Bihar, Goa, Gujarat, Haryana, Jammu and Kashmir, Jharkhand, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Manipur, Meghalaya, Mizoram, Nagaland, Orissa, Punjab, Rajasthan, Tamil Nadu, Tripura, Uttarakhand, Uttar Pradesh and West Bengal) and Pakistan (Punjab and Sind) (Das, 2003; Vanitharani, 2006; Korad *et al.*, 2007; Molur *et al.*, 2002). The single species of the genus is extensively distributed throughout India and has been reported occurring from 13 localities from Bihar (Bates and Harrison, 1997). It was restricted to Bangladesh, India and Pakistan (IUCN, 2010). It has been recorded from sea level to an elevation of 2,000 m a.s.l. The extent of occurrence is greater than 20,000 km^2 and the area of occupancy is greater than 2,000 km^2. This is a widespread and common species and the population seems to be stable and doing well. This species is found in drier climes and near human habitations in both rural and urban landscapes. It roosts in cracks, crevices, holes in old temples, old disused buildings and tombs and in holes in



Map 5. Range distribution map of *S. dormeri* (Source: IUCN, 2010).

large trees in colonies of 2-24 individuals. It is a late flyer, hunts close to its roosting site and flies steadily. Its diet varies seasonally feeding on beetles, moths, grasshoppers, crickets in winter, winged termites, beetles, moths, orthopterans, hymenopterans in summer and termites, beetles, moths, orthopterans and hymenopterans in monsoon and feeds on agriculturally important insect pests (Molur *et al.*, 2002). This species seems to breed almost throughout the year (Bates and Harrison, 1997). Overall there appear to be no major threats to this species. As this species feeds on agricultural pests its population might be declining in parts of its range because of the use of chemical pesticides (Molur *et al.*, 2002).

Cranio-dental characters have proved to be helping in identification of Pipistrelles and also bacular morphology has shown distinct divergence among *P. coromandra* and *P. tenuis*.

Unfortunately DNA band couldn't be retrieved in PCR product separated on 1.5% agarose gel and visualized by ethidium bromide. The PCR Product band is seen to be less than or nearly about 50bp comparing DNA ladder (PCR Marker 50bp-2000bp, USB Corporation, USA). The size of each Primer used was 28bp. This interprets that the unspecific band seen is primer dimer. This condition may have risen due to unsuccessful required optimization. Further practice is necessary for the success of PCR. Negative PCR results (without any bands) in tested individuals of *P. kuhlii* and *P. nathusii* museum specimens was encountered (Kaňuch *et al.*, 2007). In the rare case that a sample could not be identified to species using RFLP, DNA sequencing (Zinck *et al.*, 2004; Weller *et al.*, 2007) is recommended.

7. CONCLUSION AND RECOMMENDATIONS

Conclusion

Roost capture proved to be a better technique to capture bats in the study area than mist netting. Skull and baculum morphology revealed three species among the *Pipistrellus* complex from the same locality (Paschim Kusaha V.D.C.) with a new record of *Scotozous dormeri* Dobson, 1875 while new material of *Pipistrellus coromandra* and *P. tenuis* to Nepal. All species were found roosting in similar microhabitat. This signifies the area harbors good diversity as well as well distribution of bats. Although the PCR approach was unsuccessful in this study, application of molecular approaches can reveal further new bat records to Nepal and new to science from other parts of Koshi Tappu Wildlife Reserve (KTWR).

Recommendations

- Detailed Survey

Detailed survey of wetland visiting bats in KTWR should be carried out to access the chiropteran diversity of the area as there are chances of further new records to Nepal and even to science. Extensive application of sub-canopy and canopy levels mist nettings along with acoustic survey should be launched that would definitely be successful.

- Genetic Analysis

Further practices on molecular approaches should be focused which can reveal existence of new records to Nepal as well as to science from the area.

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APPENDIX

Table 3. Selected external, cranial and dental measurements (in mm) of *Scotozous dormeri* Dobson, 1875 from India (Bates and Harrison, 1997).

	mean	range	n	s
HB	48.9	39.0-55.0	25	3.6
T	35.3	27.0-41.0	25	3.8
TIB	-	-	-	-
HF	6.2	5.0-8.0	25	1.1
FA	34.4	32.7-36.3	25	0.9
Thumb	-	-	-	-
3mt	33.7	31.7-36.5	25	1.3
1ph3mt	-	-	-	-
2ph3mt	-	-	-	-
4mt	33.3	31.6-36.4	25	1.2
1ph4mt	-	-	-	-
2ph4mt	-	-	-	-
5mt	32.6	31.2-35	25	1.1
1ph5mt	-	-	-	-
2ph5mt	-	-	-	-
E	11.9	10.0 - 18.0	25	1.6
Tragus	-	-	-	-
GTL	14.3	13.7-15	29	0.3
CCL	13.3	12.8-13.6	29	0.2
BB	7.1	6.8-7.5	29	0.2
PC	3.9	3.6-4.2	29	0.2
C-M3	5.4	5.2-5.6	29	0.1
c-m3	5.8	5.5-6.1	29	0.1
M	10.8	10.4-11.2	29	0.3
C1-C1	-	-	-	-
M3-M3	6.7	6.3-7.0	29	0.2
RW	6.0	5.6-6.4	29	0.2

Table 4. Selected external, cranial, and dental measurements (in mm) of *Pipistrellus tenuis* (Temminck, 1840) and *P. coromandra* (Gray, 1838) from India, Pakistan, Nepal and Sri Lanka (Bates and Harrison, 1997).

	P. tenuis				*P. coromandra*			
	mean	range	n	s	mean	range	n	s
HB	39.1	33.0-45.0	37	3.0	42.3	34.0-49.0	47	3
T	28.9	20.0 - 35.0	37	3.7	32.0	22.0-39.0	48	3
TIB	-	-	-	-	-	-	-	-
HF	5.3	3.0-7.0	32	1.4	5.6	3.4-8.0	38	1
FA	27.7	25.0-30.2	39	1.2	30.0	25.5-34.3	47	1
Thumb	-	-	-	-	-	-	-	-
3mt	26.7	23.9-29.7	39	1.3	29.0	25.8-33.1	46	1
1ph3mt	-	-	-	-	-	-	-	-
2ph3mt	-	-	-	-	-	-	-	-
4mt	26.4	23.7-29.2	39	1.2	28.7	25.7-32.7	46	1
1ph4mt	-	-	-	-	-	-	-	-
2ph4mt	-	-	-	-	-	-	-	-
5mt	25.9	23.5-28.5	39	1.2	28.1	25.2-31.1	46	1
1ph5mt	-	-	-	-	-	-	-	-
2ph5mt	-	-	-	-	-	-	-	-
E	9.7	5.0 - 11.0	37	1.5	10.3	7.1-14.0	48	1
Tragus	-	-	-	-	-	-	-	-
GTL	11.5	10.7-12.1	47	0.3	12.5	11.8-13.1	51	(
CCL	10.2	9.3-10.7	47	0.3	11.2	10.6-11.9	52	(
BB	6.0	5.6-6.3	47	0.2	6.2	5.7-6.7	51	(
PC	3.3	2.9-3.7	47	0.2	3.4	3.0-3.8	51	(
C-M3	3.8	3.5-4.1	48	0.1	4.4	3.9-4.6	53	(
c-m3	4.1	3.8-4.4	44	0.1	4.7	4.1-5.1	51	(
M	7.9	7.2-8.3	42	0.2	8.9	8.2-9.5	51	(
C1-C1	-	-	-	-	-	-	-	-
M3-M3	4.9	4.5-5.2	46	0.1	5.5	5.0-6.0	51	(
RW	4.4	3.9-4.8	47	0.2	4.9	4.3-5.3	51	(

Table 5. Checklist of *Pipistrellus* spp. world-wide, in South Asia and in Nepal

S.N.	Name of the species	World-wide*	South Asia+
1.	Japanese pipistrelle, *Pipistrellus abramus* (Temminck, 1838)		
2.	Adams's Pipistrelle, *Pipistrellus adamsi* Kitchener, Caputi & Jones, 1986		
3.	Mt. Gargues Pipistrelle, *Pipistrellus aero* Heller, 1912		
4.	Angulate Pipistrelle, *Pipistrellus angulatus* Peters, 1880		
5.	Kelaart's Pipistrelle, *Pipistrellus ceylonicus* (Kelaart, 1852)		
6.	Greater Papuan Pipistrelle, *Pipistrellus collinus* Thomas, 1920		
7.	Indian Pipistrelle, *Pipistrellus coromandra* (Gray, 1838)		
8.	Egyptian Pipistrelle, *Pipistrellus deserti* Thomas, 1902		
9.	Endo's Pipistrelle, *Pipistrellus endoi* Imaizumi, 1959		
10.	Hanaki's Dwarf Bat, *Pipistrellus hanaki* Hulva & Benda, 2004		
11.	Dusky Pipistrelle, *Pipistrellus hesperidus* (Temminck, 1840)		
12.	Aellen's Pipistrelle, *Pipistrellus inexspectatus* Aellen, 1959		
13.	Java Pipistrelle, *Pipistrellus javanicus* (Gray, 1838)		
14.	Kuhl's Pipistrelle, *Pipistrellus kuhlii* (Kuhl, 1817)		
15.	Minahassa Pipistrelle, *Pipistrellus minahassae* (Meyer, 1899)		
16.	Christmas Island Pipistrelle, *Pipistrellus murrayi* Andrews, 1900		
17.	Tiny Pipistrelle, *Pipistrellus nanulus* Thomas, 1904		
18.	Nathusius's pipistrelle, *Pipistrellus nathusii* (Keyserling & Blasius, 1839)		
19.	Lesser Papuan Pipistrelle, *Pipistrellus papuanus* Peters & Doria, 1881		
20.	Mount Popa Pipistrelle, *Pipistrellus paterculus* Thomas, 1915		
21.	Dar-Es-Salaam Pipistrelle, *Pipistrellus permixtus* Aellen, 1957		
22.	Common pipistrelle, *Pipistrellus pipistrellus* (Schreber, 1774)		
23.	Soprano Pipistrelle, *Pipistrellus pygmaeus* (Leach, 1825)		
24.	Racey's Pipistrelle, *Pipistrellus raceyi* Bates, Ratrimomanarivo, Harrison & Goodman, 2006		
25.	Rüppell's Pipistrelle, *Pipistrellus rueppellii* (Fischer, 1829)		

26.	Rusty Pipistrelle, *Pipistrellus rusticus* (Tomes, 1861)			
27.	Narrow-winged Pipistrelle, *Pipistrellus stenopterus* (Dobson, 1875)			
28.	Sturdee's Pipistrelle, *Pipistrellus sturdeei* Thomas, 1915			
29.	Least Pipistrelle, *Pipistrellus tenuis* (Temminck, 1840)			
30.	Watts's Pipistrelle, *Pipistrellus wattsi* Kitchener, Caputi & Jones, 1986			
31.	Koopman's Pipistrelle, *Pipistrellus westralis* Koopman, 1984			

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Hattisar	87° 01' 49.24" E 26° 37' 15.01" N
Milanchowk	87° 01' 55.94" E 26° 37' 6.44" N
Near Aqua Tourist Hotel	87° 01' 56.02" E 26° 37' 15.49" N
Goithi tole 1	87° 01' 54.94" E 26° 36' 49.48" N
Goithi tole 2	87° 01' 55.83" E 26° 36' 48.50" N
Samsul tole 1	87° 02' 15.06" E 26° 37' 3.86" N
Samsul tole 2	87° 02' 13.83" E 26° 37' 5.82" N
Samsul tole 3	87° 02' 11.63" E 26° 36' 55.36" N