

ORIGINAL ARTICLE

Morphometric and Phylogenetic analysis of *Pipistrellus kuhlii* Captured from Fata Regions, PakistanIFTIKHAR HUSSAIN¹, ASIF ALI², SHUJAAT HUSSAIN³, AMIR HUSSAIN⁴, JAVED AHMED UJJAN⁵, FAHIM HAIDER JAMALI⁶, KHALID USMAN⁷, MUHAMMAD SHAHBAZ¹, NADIA SAEED⁸, QASIM SAEED⁹¹Department of Zoology, Women University, Azad Jammu and Kashmir, Bagh, Pakistan²Department of Biology & Zoology Pir Mehr Ali Shah University Rawalpindi Pakistan.³Department of Chemical & Life Science Qurtuba University of Science & IT Peshawar Campus.⁴Department of Zoology, Islamia College University Peshawar, KP, Pakistan.⁵Chairman Associate Professor, Department of Zoology, Shah Abdul Latif University, Khairpur Sindh Pakistan.⁶Scientific Officer PCSIR (Pakistan Council for Science & Technology) Hyderabad.⁷Department of Zoology, Qurtuba University of Science and Information Technology, Peshawar, KP, Pakistan.⁸Department of Zoology, Government Post Graduate College, Mandian, Abbottabad, Pakistan⁹Department of Environmental Sciences, National University of Sciences and Technology, Islamabad, PakistanCorresponding author's: Iftikhar Hussain, Email: iftiali33@gmail.com**ABSTRACT****Purpose:** The present study was carried out to explore the distribution of *Pipistrellus kuhlii* in selected area of FATA regions, Pakistan.**Findings & Practical implication:** Twenty six specimens of this species were collected using mist from Bara Khyber agency (33° 92' N; 71.46° E) and Tirah Aurakzai agency (33° 78' N; 70.70° E) in FATA. Mean body mass of *Pipistrellus kuhlii* was recorded 8.42g ± 2.14. Head and body length were observed 44.2 ± 3.5 respectively. Ear length was recorded 12.2 ± 0.8. For molecular analysis the sequences of CO1 gene were obtained and analyzed. The mean intraspecific divergences of *Pipistrellus kuhlii* was 0.093%. The interspecific average distance between *Pipistrellus kuhlii* and *P. tenuis* were 12.48% and distance between *Pipistrellus kuhlii* and *P. javanicus* were 18.6%. Nucleotide mean concentration was A = (24.9%), T = (32.2%), G = (18.2%) and C = (24.7%). The mean A+T contents were 57.1% and C+G were 42.9%.**Conclusion:** From the current research studied it may be concluded that the measurements of body matching with the specimens reported from Sri Lanka, Pakistan, Nepal and India. In the phylogenetic tree *Pipistrellus kuhlii* and *P. nanulus* clustered with significant bootstrap support value.**Keywords:** *Pipistrellus kuhlii*, Morphology, Kurram Agency, Bats, Pakistan**INTRODUCTION**

Pipistrellus kuhlii is a big pipistrelle distributed in the subcontinent with a normal forearm size of 34.7 mm. On account of forearm size it is difficult to distinguish *P. coromandra* smaller organisms¹. Captured this species from Asam and Calcutta (India). Normally the body weight of this species is 19 grams². Worldwide distribution of this species is Southern Europe, India, Africa and Egypt. It is also captured from Kandahar (Afghanistan). According to in Pakistan it is collected from Muzaffargarh, Lyallpur, Multan, Panjgur, Darzi, Gambat, Kashmir and Chak².

Body Measurements: According to physique examination of 15 specimens collected from Pakistan having body and head size is 49 mm, tail size 40 mm, fore arm size 35 mm (32.0 – 36.0 mm), size of 4th metacarpal 32.1 (29.7 – 33.6 mm), 3rd metacarpal 33.2 (31.7 – 34.3 mm), and ear size 11.9 mm³.

Cranial Measurements: Largest skull size 13.4 mm (12.9-13.9 mm), Condyle canine size 12.4 mm (12 – 12.9 mm), Zygomatic size 8.4 mm (8.4 – 8.7mm). The braincase size was 6.6 mm postorbital 2.4 mm (2.2 – 4.8 mm)³. The importance of the current studied was to the phylogenetic makeup of *Pipistrellus kuhlii*.

MATERIALS AND METHODS

Study Area: Present study was conducted in Federally Administered Tribal Areas (FATA), Pakistan (Now included in Khyber Pakhtunkhwa province). The FATA is situated in the north western region of Pakistan and semi-autonomous. This area surrounds by Khyber Pakhtunkhwa and Balochistan to the east and in the south, Afghanistan provinces of Kunar, Nangrahar and Paktia to the west and north. The FATA region comprises seven tribal agencies and six frontier regions.

It is located on 33.0000° North Latitude and 70.1667° East Longitude. The elevation varies from 3600 m to 4700 m above sea level and total area covered by 27,220 km². Winters in FATA are normally moderate, with the severest weather occurring from December to March in some parts with a minimum recorded temperature of -15°C. Summers, in comparison, are fairly moderate with a maximum recorded temperature of 34°C in the

region. The average annual rainfall ranges from 250 to 500 ml in the FATA⁴ (Figure 1).

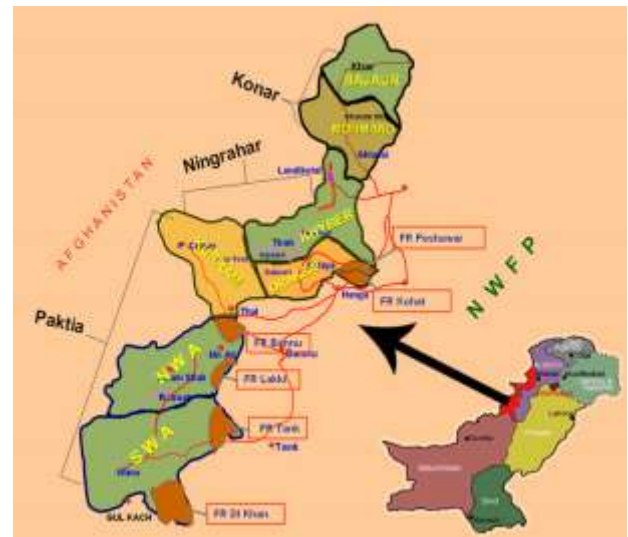


Figure 1: Map of FATA, Khyber Pakhtunkhwa, Pakistan (Hussain, 2016)

Sampling Strategy: Exploratory visits were made to locate as many bat roosts in all the sub-areas as possible. Potential bat roosts such as old and undisturbed buildings, ruins, abandoned wells, farm houses, tree groves and forest plantations were thoroughly searched. Local people were also interviewed for gaining maximum information about the exact location of various bat roosts.

A high quality, deep black, UV stable and strong mist nets were used to capture bats. Mist net was erected either in “L” or “V” shape at strategic positions on a pair of 3 m long bamboo poles in such a way that the last shelf of each net remained one foot above

the ground. The nets were ready to operate half an hour before sunset. All the nets were opened simultaneously at sunset and continued to operate, depending on the weather conditions, for two hours after sunset. Nets were checked continuously to disentangle any captured bat. The sampling effort remained the same throughout the study.

Once located, the global position of each roost was determined using Garmin etrax H Global Position System (GPS).

External Morphology: The capture bats were placed in cloth bags containing moist cotton and were brought immediately to the laboratory. The bats were weighted, their sex and age was determined⁵.

Each disentangled bat was weighed up to 0.1 g (Pesola balance 10050, Swiss made), Field number, sex, age, exact locality and district was noted. External body measurements were taken using a digital vernier caliper (0-150 mm). The age of each captured bat specimen was determined following⁶.

Cranial Measurements: For recording cranial measurements eye balls, tongue and excessive flesh was removed from each skull. Skulls thus cleaned was kept overnight in a dilute solution (0.2 % of Potassium Hydroxide (KOH)), absolute alcohol for another night before being transferred to acetone for third night.

Molecular identification and Phylogentic analysis DNA Extraction: For the DNA extraction Thermo scientific Gene JET Genomic DNA purification Kit was used. First Wings membrane was cut with scissor from ethanol-preserved specimens and was grinded in liquid nitrogen using mortar. The grinded samples were used for DNA extraction.

Amplification of COI gene: PCR reaction was performed in a final volume of 20 μ L containing 10 μ L of Thermo scientific Dream Taq Green PCR Master Mix, 1 μ L forward primer, 1 μ L reverse primer, 0.5 μ L Taq polymerase, 1 μ L template DNA and 6.5 μ L nuclease free water. The amplification conditions were 2 cycles, 94 °C for 5 minutes, 35 cycles, 94 °C for 30 seconds, 48 °C for 30 seconds, 72 °C for 35 s, 1 cycles, 72 °C for 10 minutes, hold at 4 °C.

DNA sequence analysis: The sequences were used for the identification of species through NCBI BLAST search. Divergence at species, generic and family level were employed the K2P model of base substitution⁷. Sequences of related specimens deposited by other workers were taken from GeneBank using Blast tool for comparisons and analysis of genetic distance. The sequences were aligned in clustalW. Neighbour-Joining tree was constructed using K2P parameter and 1000 bootstrap replicates. The Maximum likelihood was constructed using best substitution model with 1000 bootstrap replicates. Maximum Parsimony approach was also employed by using Tree-Bisection-Reconnection (TBR) parameter and 1000 bootstrap replicates. All analysis was performed by using MEGA 7 software⁸.

RESULTS

Distribution: Twenty six specimens of this species were collected using mist from Bara Khyber agency (33° 92' N; 71.46° E) and Tirah Aurakzai agency (33° 78' N; 70.70° E) in FATA during the present research work (Figure 2).

Dental Formula: 2123/3133=36

Morphometry and body mass: Mean body mass of *Pipistrellus kuhlii* was recorded 8.42g \pm 2.14. Head and body length were observed 44.2 \pm 3.5 respectively. Ear length was recorded 12.2 \pm 0.8 forearm length was measured 34.8 \pm 0.9 and length of 3rd metacarpal was observed 33.5 \pm 1.1 The mean length of 4th metacarpal was recorded 31.2 \pm 0.09 length of 5th metacarpal was observed 32.2 \pm 0.9 and length of hind foot was recorded 6.5 \pm 0.7 (Table 1).

Morphometry of Cranial: The mean greatest length of condyle canine was recorded 12.5 \pm 0.3, length of braincase was observed 6.5 \pm 0.2 length of post orbital constriction was observed 3.5 \pm 0.1 and length of condyle basal was recorded 5.3 \pm 0.2. The anterior and posterior plate widths were recorded 5.5 \pm 0.1 and 5.3 \pm 0.3 respectively (Table 2).

Table 1: Morphometry of Body parameters (mm) of *Pipistrellus kuhlii* collected from FATA region of Pakistan

Body Parameters	Mean \pm SD (n=26)	Range
HB	44.2 \pm 3.5	35.0-49.0
E	12.2 \pm 0.8	10.0-13.0
FA	34.8 \pm 0.9	33.0-36.0
3mt	33.5 \pm 1.1	31.8-34.6
4mt	31.2 \pm 1.0	31.6-43.3
5mt	32.2 \pm 0.9	30.7-33.6
HF	6.5 \pm 0.7	6.0-8.0
T	41.2.2 \pm 0.6	33.0-45.0

Table 2: Morphometry of the cranial parameters (mm) of *Pipistrellus kuhlii* collected from FATA region of Pakistan.

Cranial Parameters	Mean \pm SD (n=26)	Range
CCL	12.5 \pm 0.3	12.0-12.9
C-M ⁿ	4.9 \pm 0.1	4.6-5.0
C-M _n	5.3 \pm 0.2	5.0-5.5
GTL	13.5 \pm 0.2	12.9-13.9
M	9.8 \pm 0.3	9.3-10.4
M ⁿ -M _n	5.5 \pm 0.1	5.5-5.9
BB	6.5 \pm 0.2	6.4-6.8
PC	3.5 \pm 0.1	3.2-3.7



Figure 2: Localities and distribution of *Pipistrellus kuhlii* in Pakistan

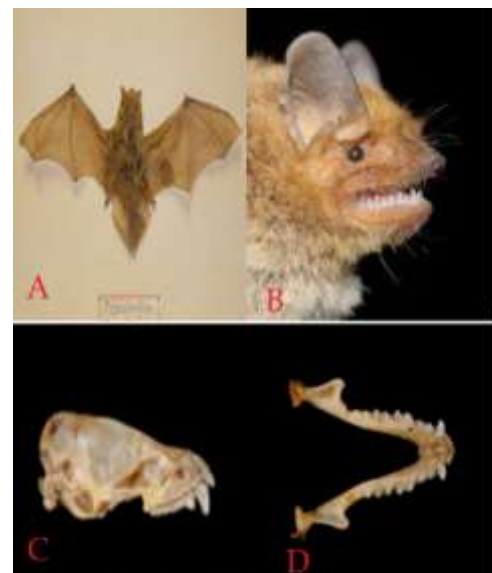


Figure 3: A= *Pipistrellus kuhlii* B= Head C= Skull lateral view D= Dorsal view of lower jaw

DISCUSSIONS

Global Distribution: Its specimens were reported from Southern Europe, India, Africa and Egypt. This species was also recorded from Kandar (Afghanistan)³. Kurup (1968) reported this species from Asam and Calcutta (India)¹.

Previous record from Pakistan

Pipistrellus kuhlii was reported from Muzaffargarh, Lyallpur, Multan, Panjgur, Darzi, Gambat, Kashmor and Chak².

Habitat description: Pipistrellus kuhlii was found in different habitat from 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

IUCN status: Pipistrellus kuhlii is not yet has been assessed for the IUCN Red List.

Remarks: Twenty six specimens of Pipistrellus kuhlii were captured from various sites. This shows that population of subject species is widespread and their complete description about habitat is preferencely the distribution. In Pakistan Pipistrellus kuhlii is reported from Muzaffargarh, Lyallpur, Multan, Panjgur, Darzi, Gambat, Kashmor and Chak².

New range reported: All the localities were recorded new from FATA

The sequence of COI gene of species belonging to genus Pipistrellus, obtained in the present study were compared to other gene sequences using NCBI, BLAST. The similarity indexes with other sequences of the same species were from 99-100%. These sequences were also studied with nucleotide deviation and their composition. The sequences DNA of seven species related to single genus was procured with Gene Bank contributed by other coworker for matching. The final aligned data had seven sequences of more than 657 bp length show seven species. All the barcode gaps were distinct among species. In sequence no overlap deviation was observed. The collected data aligned was shown by 657 characters to which conserved sites were 438, variable sites were 219, parsimony informative sites were 192 and singleton sites were 27. Increase in the mean K2P deviations was observed. The mean intraspecific divergence of P. kuhlii was 0.06%. The average intraspecific divergence of P. javanicus 0.093% was lower than mean intraspecific divergence of P. tennius 0.126%. The mean divergence between P. coramandra was 0.40%. The interspecific average distance between P. javanicus and P. tennius were 10.48% and distance between P. coromandra and P. tennius were 12.8%. The interspecific average between P. javanicus and P. abramus were 13.3% and P. kuhlii and P. javanicus were 18.65%. The average interspecific distance were 19.1% between P. kuhlii and P. coromandra, while 19.3% between P. kuhlii and P. javanicus (Table 4.24).

Table 4: Composition of base percentage wise in gene COI of genus Pipistrellus Kaup, 1829

Base	A	T	G	C
Mean percentage%	24.9	32.2	18.2	24.7

The descending order of contents in A+T were Pipistrellus paterculus 59.4%, P. nathusii 59.2%, P. abramus 58.1%, P. annulus 57.7%, P. javanicus 57.2%, P. kuhlii 56.6%, P. coromandra 56.5 and P. tennius 55.7%. Similarly descending order of content in C+G were P. tennius 44.4%, P. kuhlii 43.7%, P. javanicus 42.7%, P. abramus 58.1% and P. nathusii 40.8%.

The Neighbor Joining tree was constructed into two clades i.e. Clade I and Clade II. Clade I was divided into four subclades i.e. subclade IA, subclade IB, subclade IC and subclade ID. Subclade IA comprised upon three sequences, the sequences of P. javanicus of the present study was among them. Subclade IB comprised upon three sequences, the sequences of P. tenuis of the present study was among them. Subclade ID comprised upon three sequences, the sequences of P. coromandra of the present study was among them. Clade II comprised upon four sequences, the sequences of P. kuhlii of the present study was among them (Figure 4.33).

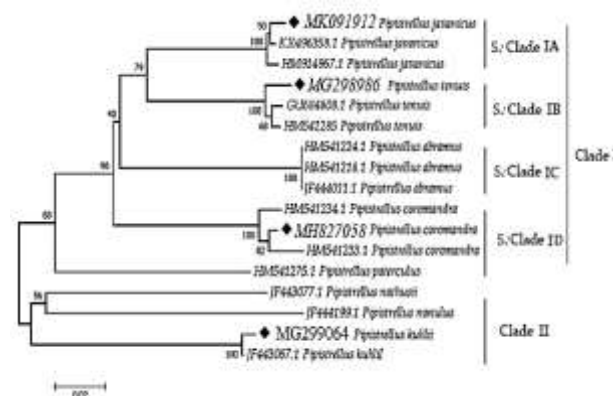


Figure 4: Genus Pipistrellus peculiar to COI by Neighbour Joining method. Numbers show percent of 1000 bootstraps replication above 50. Shows the sequences of species of the present research. The Maximum Likelihood tree (-2812.83) represented the same topography with neighbor joining tree. Conspecific taxa clumped with 50-100% supported bootstrap values (Figure 5). The length of Maximum Parsimony tree was (469), consistency index (0.5419199) retention index (0.714078) and composite index (0.414135) was contracted with same topography to Neighbor Joining tree and Maximum Likelihood tree (Figure 6). These observations justify utility of COI gene sequence of Pipistrellus, obtained in the current analysis for identification to species.

Table 3: pairwise genetic distance of genus Pipistrellus Kaup, 1829

Species	MF443077	Pipistrellus nathusii	JF444199	Pipistrellus nanulus	Pipistrellus kuhlii	JF443067	Pipistrellus kuhlii	HM541275	Pipistrellus paterculus	Pipistrellus coromandra	HM541234	Pipistrellus coromandra	HM541233	Pipistrellus coromandra	HM1914967	Pipistrellus javanicus	Pipistrellus javanicus	KX496358	Pipistrellus javanicus	Pipistrellus tenuis	HM541285	Pipistrellus tenuis	GU864808	Pipistrellus tenuis	HM541285	Pipistrellus tenuis	HM541224	Pipistrellus abramus	HM541218	Pipistrellus abramus	JF444011	Pipistrellus abramus				
JF443077 Pipistrellus nathusii																																				
JF444199 Pipistrellus nanulus	0.20																																			
Pipistrellus kuhlii	0.18	0.20																																		
JF443067 Pipistrellus kuhlii	0.19	0.20	0.01																																	
HM541275 Pipistrellus paterculus	0.20	0.24	0.18	0.18																																
Pipistrellus coromandra	0.22	0.24	0.20	0.19	0.17																															
HM541234 Pipistrellus coromandra	0.22	0.23	0.19	0.19	0.17	0.01																														
HM541233 Pipistrellus coromandra	0.22	0.24	0.20	0.20	0.17	0.02	0.03																													
HM1914967 Pipistrellus javanicus	0.21	0.23	0.19	0.19	0.16	0.13	0.14	0.15																												
Pipistrellus javanicus	0.21	0.24	0.20	0.19	0.17	0.13	0.13	0.14	0.01																											
KX496358 Pipistrellus javanicus	0.21	0.23	0.20	0.19	0.17	0.12	0.13	0.14	0.01	0.01																										
Pipistrellus tenuis	0.21	0.23	0.21	0.21	0.18	0.12	0.13	0.14	0.11	0.11	0.10																									
HM541285 Pipistrellus tenuis	0.21	0.23	0.21	0.21	0.17	0.12	0.13	0.14	0.10	0.10	0.10	0.02																								
GU864808 Pipistrellus tenuis	0.21	0.22	0.22	0.21	0.17	0.12	0.13	0.14	0.10	0.11	0.10	0.02	0.01																							
HM541285 Pipistrellus tenuis	0.21	0.23	0.21	0.21	0.17	0.12	0.13	0.14	0.10	0.10	0.10	0.02	0.00	0.01																						
HM541224 Pipistrellus abramus	0.20	0.23	0.22	0.21	0.17	0.14	0.15	0.15	0.13	0.14	0.13	0.14	0.13	0.13	0.13																					
HM541218 Pipistrellus abramus	0.20	0.23	0.22	0.21	0.17	0.14	0.15	0.15	0.13	0.14	0.13	0.14	0.13	0.13	0.13	0.00																				
JF444011 Pipistrellus abramus	0.20	0.23	0.22	0.21	0.17	0.14	0.15	0.15	0.13	0.14	0.13	0.14	0.13	0.13	0.13	0.00	0.00																			

The compositions of nucleotide sequences of all species were also observed. Nucleotide mean concentration was A = (24.9%), T = (32.2%), G = (18.2%) and C = (24.7%). The mean A+T contents were 57.1% and C+G were 42.9% (Table 4).

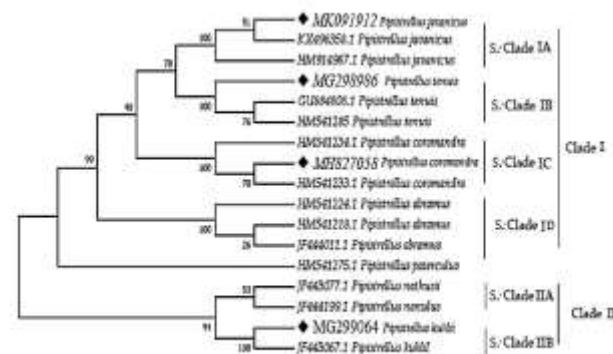


Figure 5: Genus Pipistrellus peculiar to COI by Maximum likelihood method. Numbers show percent of 1000 bootstraps replication above 50. Shows the sequences of species of the present research.

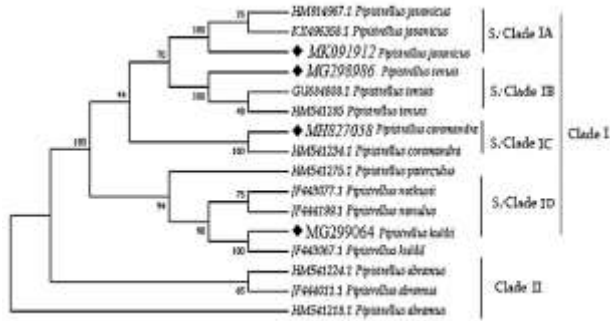


Figure 6: Genus *Pipistrellus* peculiar to COI by Maximum parsimony method. Numbers show percent of 1000 bootstraps replication above 50. During present study the important taxonomic characters like length of skull, condylocanine, mandibular tooth row, mandible and breadth of zygomatic, braincase, posterior palatal, postorbital constriction of the specimens captured and matched exactly with had the similar findings in same study. In the present study of *Pipistrellus kuhlii* the length of hind leg, forearm, ear and body weight were similar with those specimens reported. In the present study the measurements of body matching with the specimens reported from Sri Lanka, Pakistan, Nepal and India³⁻¹².

CONCLUSION

In the phylogenetic tree *Pipistrellus kuhlii* and *P. nanulus* clustered with significant bootstrap support value. From the current study it may be concluded that the measurements of body matching with the specimens reported from Sri Lanka, Pakistan, Nepal and India.

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