Pakistan J. Zool., vol. 53(3), pp 1157-1160, 2021 DOI: https://dx.doi.org/10.17582/journal.pjz/20190410130412

Short Communication

Morphometric and Phylogenetic Analysis of Short-Nosed Fruit Bat *Cynopterus sphinx* (Vahl, 1797) Captured from FATA Regions, Pakistan

Iftikhar Hussain[1], Sardar Azhar Mehmood[1], Muzafar Shah[2]*, Mohammad Salim[3], Shabir Ahmed[1], Sana Ullah[1], Sidra Batool[4], Nadia Saeed[1] and Khalid Usman[1]

[1]*Department of Zoology, Hazara University, Mansehra, Pakistan*
[2]*Centre for Animal Sciences & Fisheries, University of Swat, Swat, Pakistan*
[3]*Department of Forestry and Wildlife Management, University of Haripur, Haripur, Pakistan*
[4]*Department of Zoology, Islamia College University, Peshawar, Pakistan*

Article Information
Received 10 April 2019
Revised 15 June 2019
Accepted 28 January 2020
Available online 30 March 2021

Authors' Contribution
IH, SAM, M Shah and M Salim carried out the research work. SA and SU analysed of data. SB and NS wrote the manuscript. KU proofread the manuscript.

Key words
Bats, FATA, CO1, Bootstrap, Nucleotide, Barcodes

ABSTRACT

During the present survey, extending from the May 2015 to November 2016 the short-nosed fruit bat *Cynopterus sphinx* (n = 4) was captured from Frontier Region Kohat and Shublan, Kurram Agency in Khyber Pakhtunkhwa. The morphometry and cranial characteristics of the captured specimens were compared with literature. *C. sphinx* is being reported form the study area for first time. Morphometry of all captured *C. sphinx* showed that head and body length was 77.13 mm, ear length 19.51 mm, forearm length 65.49 mm, length of tibia 26.61 mm, length of hind foot 17.95 mm and length of tail 7.35 mm. Phylogenetic analysis was performed on the bases of *CO1* gene sequences. The mean intraspecific divergences of *C. sphinx* were 0.011%. The nucleotide composition of the sequences showed higher concentration of A+T as compared to G+C. The A+T contents were 53.1% and C+G were 46.8%. In phylogenetic tree the sequences of *C. sphinx* clustered together with 100% bootstrap support. The relationship between *C. sphinx* and *C. horsefield* was supported by 83% bootstrap value. The relationship between *C. titthaecheilus* and *C. horsefield* was supported by 98% bootstrap value. These analyses confirm the utility of the COI gene sequence of C. *sphinx,* obtained in the present study for species level identification and phylogenetic relationships.

The genus Cynopterus (Cuvier, 1824), represented by seven species (Simmons, 2005) of which two are *C. brachyotis* and *C. sphinx* reported from Indian subcontinent in which *Cynopterus sphinx* is most common in Sri Lanka and India (Bates and Harrison, 1997). In Pakistan the same species has been reported from Malir area of Karachi, Sindh (Roberts, 1977). This species has also been reported from Jhajjar Kotli, Jammu and Kashmir, (Chakraborty, 1983), whereas the same species reported from Rajasthan, Bundi, Banswara and Jhalawar (Advani,1982) from Surat and Silvassa (Sinha,1981). *Cynopterus* are mainly seasonally migratory bats of Himalayan mountain in the summer where fruit trees present up to 1200m (Roberts, 1997; Beg and Khan, 1984), *C. sphinx* is present in Appendix II of IUCN SSC Action Plan (1992) Not Threatened, at Low Risk-IUCN 2003 and in the Least Concerned-CAMP 2003, CAMP 2002; IUCN 2008 (Mahmood-ul-Hassan *et al*., 2009). The current study was conducted in different area of FATA, Khyber Pakhtunkhwa to mention the distribution of *C. sphinx.*

* Corresponding author: muzafar@uswat.edu.pk
0030-9923/2021/0003-1157 $ 9.00/0

For identification instead of typical taxonomic characters the uses of mitochondrial and nuclear genes have been more beneficial. In mt-DNA various parts change with various ratios playing a significance role in solving discrepancies at various taxonomic levels. For identification of large number of mammalian fauna molecular technique is used but studies on Chiroptera are rare so far (Michael *et al*., 2014). The exploration of Chiroptera from Pakistan is inadequate. The current investigation is the first effort to study the distribution and phylogeny of *C. sphinx* in FATA region of Pakistan.

Materials and methods

Bats were collected from FATA region during 2015-2016. The caves in mountain were searched properly. The location of bat roosts was recorded by using a global positioning system device (Garmin etrax H GPS). The mist was used for capturing bats and each specimen was weighed by using Pesola weight balance 10050, made by Swiss weighting up to 0.1 g. The collected bats were euthanized and assigned identification number to every specimen and then were transferred to the laboratory. The external measurements of the different parameters of body specimens were also recorded following Bates

and Harrison (1997). The different cranial parameters measurements were taken following Bates *et al*. (2005) while the baculam were also prepared following Bates *et al*. (2005) and measured taken according to Lidicker and Yang (1986).

DNA was extracted from wings membrane using Thermo Scientific Gene JET Genomic DNA purification kit. This work was carried out at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar. Polymerase chain reaction for amplification of *COI* gene was done in a volume of 22 µL containing 10 µL of Thermoscientific Dream Taq Green PCR Master Mix, 2 µL forward primer, 2 µL reverse primer, 0.5 µL polymerase, 1 µL template DNA and 6.5 µL nuclease free. The thermal cycles comprised incubation of 94ºC for 6 min, 35 cycles, each of 96ºC for 40 seconds, 48 ºC for 40 seconds, 70ºC for 35 s and one cycles of 70ºC for 12 min. The PCR products were gotten sequenced from BGI Bio Solutions (Hong Kong) Co., Limited.

The *CO1* gene sequences were used for the identification of species through NCBI BLAST search and BOLD. Sequences of related specimens deposited by other workers were taken from GeneBank using Blast tool for comparisons. The sequences were aligned in clustalW using MEGA7. Neighbour-Joining tree was constructed using K2P parameter and 1000 bootstrap replicates. The Maximum likelihood was constructed using best substitution model. Maximum Parsimony approaches were also employed by using Tree-Bisection-Reconnection (TBR) parameter and 1000 bootstrap replicates (Kumar *et al*., 2016).

Results and discussion

Four specimens in all belonging to *C. sphinx* were collected from FR Kohat and Shublan and their morphological characteristics were compared with those given by Bates and Harrison (1997) and Aziz (2007). Head and the body length of all captured bats was 77.13 mm, the ear length 19.51 mm, the forearm length 65.49 mm, the length of tibia 26.61 mm, length of hind foot 17.95 mm and length of tail 7.35 mm. Aziz (2007) observed range of mass of the body between 330.0-501.0 g, length of head and body 85.0-115.0 mm, length of ear 13.0-19.0 mm, length of forearm 59.0-75.0 mm, length of tibia 21.0-30.0 mm, length of hind foot 12.0-21.0 mm and length of tail between 2.0-13.0 mm (Supplementary Table I). The range of length of head and body of specimen observed by Aziz (2007) was somewhat larger than the present finding. The length of head and body of the present *C. sphinx* is within ranges observed by Bates and Harrison (1997). The length of ear, length of forearm, length of third metacarpal, length of forth metacarpal, length of fifth metacarpal, length of hind foot and length of tail are comparable with parameters recorded by Bates and Harrison (1997) and Aziz (2007). Srinivasulu *et al*. (2010) gathered specimen from the South Asia having length of forearm ranging 64.0-79.0 mm, length of head and body 76.0-113.0 mm, length of hind foot 12.6-18.0 mm, length of tail 4.5- 19.0 mm and length of ear 17.5-24.0 mm. The length of head and body, length of tail, length of ear, length of forearm, tibia length and length of hind foot was 99.2 mm (89-109), 15.1 mm (13- 17.5), 20.7 mm (19-22), 71.2 mm (67-74.5), 27.2 mm (25-29) and 17.6 mm (16-20.5), respectively, from Indian state of Bengal (Das and Sinha, 1971). All the parameters except length of tail of the collected specimen were in the line with ranges mention by Das and Sinha (1971) (Supplementary Table II). The rostrum was slightly broad and short. The zygomatic arch was anteriorly rounded and comparatively shorter as compared with Rousettus. Braincase was weak sagittal crest. The postorbital process was developed. The mean length condylo-canine was found 28.85 mm, the length of maxillary tooth row 10.86 mm, length of mandibular tooth row 12.64 mm, length of greatest of skull 32.20 mm, length of mandible was 24.75 mm, width of posterior palatal 9.63 mm. The breadth of zygomatic was 18.81 mm, the braincase 14.50 mm; constriction of interorbital 6.61 mm and width of anterior palatal was 6.35 mm. All of the above parameters and measurements were found in range with comparative results.

COI gene sequence of species belonging to genus, obtained in the present study was analyzed for nucleotide divergence and composition. DNA sequences of nine species belonging single genus were procured for Gene Bank deposited by other workers for comparison. The final aligned data had nine sequences of more than 680 bp length representing nine species. Overall the barcode gap was distinct between the species. No overlap in sequence divergence was found. The aligned data was represented by 680 genetic characters of which 561 were conserved sites, 96 variable sites, 86 parsimony informative sites and 10 singleton sites. Increase in the mean K2P divergences was observed across different taxonomic levels. The mean intraspecific divergences of *C. sphinx* were 0.011% (Table I). The average intraspecific divergence of *C. horsefield* was 0.005% and *C. titthaecheilus* 0.113%. The nucleotide composition of the total sequences was also analyzed. The mean concentration of each nucleotide was A = (24.8%), T = (28.3%), G = (17.7%) and C = (29.1%). The A+T contents were 53.1%and C+G were 46.8%. The descending order of contents in A+T was *C. sphinx* 26.9%, *C. lylei* 26.5%, *C. horsefield* 26.3%, *C. titthaecheilus* 26.2% and *C. giganteus* 26.1%. Similarly descending order of contenst in C+G were *C. giganteus* 23.8%, *C. horsefield* 23.7%, *C. titthaecheilus* 23.7%, *C. lylei* 23.5% and *C. sphinx* 23.3%.

In neighbor joining tree the sequences of *C. sphinx* clustered together with 100% bootstrap support and that

Table I. pairwise genetic distance of genus *Cynopterus sphinx*.

		1	2	3	4	5	6	7	8	9
1	*KT291772.1_Pteropus_giganteus*									
2	*HM541304.1_Pteropus_lylei*	0.015								
3	*MG299065Cynopterus_sphinx*	0.198	0.194							
4	*KT291769.1_Cynopterus_sphinx*	0.221	0.216	0.023						
5	*HM540207.1_Cynopterus_horsfieldii*	0.223	0.218	0.026	0.011					
6	*HM540207.1_Cynopterus_horsfieldii*	0.239	0.230	0.086	0.073	0.082				
7	*HM540207.1_Cynopterus_horsfieldii(2)*	0.239	0.230	0.086	0.073	0.082	0.000			
8	*HM540206.1_Cynopterus_horsfieldii*	0.239	0.230	0.084	0.068	0.077	0.005	0.005		
9	*JQ599876.1_Cynopterus_titthaecheilus*	0.225	0.218	0.126	0.118	0.116	0.105	0.105	0.103	
10	*HM540241.1_Cynopterus_titthaecheilus*	0.225	0.218	0.126	0.118	0.116	0.105	0.105	0.103	0.000

of *C. horsefield* clustered together with 100% bootstrap support. The relationship between *C. sphinx* and *C. horsefield* was supported by 83% bootstrap value. The sequences of *C. titthaecheilus* clustered together with 100% bootstrap support and that of *C. horsefield* clustered together with 100% bootstrap support. The relationship between *C. titthaecheilus* and *C. horsefield* was supported by 98% bootstrap value. The Maximum Likelihood tree with log likelihood (-1420.74) showed similar topology to Neighbor Joining tree. Conspecific taxa clustered together with 50-100% bootstrap support (Fig. 2). The maximum Parsimony tree with length (108), consistency index (0.927083), retention index (0.948148) and composite index (0.886694) was also contracted having similar topology to neighbor joining tree and maximum likelihood tree (Fig. 3). These analyses confirm the utility of the *COI* gene sequence of *C. sphinx,* obtained in the present study for species level identification and phylogenetic relationships.

The intraspecific divergence of the present study is in agreement with the intraspecific divergence reported in other animal taxa, such as in marine fish 0.27%. Birds 0.39% (Ward *et al*., 2005) and 0.06% in bats (Clare *et al*., 2007). Seven species are currently recognized (Simmons 2005), but the recent identification of six genetically distinct lineages within *C. brachyotis* indicates that several additional species remain to be described (Campbell *et al*., 2004). Here, we focus on the Malay Peninsula and southern Thailand where four nominal species are broadly sympatric: *C. horsfieldi*, *C. sphinx* and *C. brachyotis*, with the latter split into two ecologically, morphologically and genetically divergent lineages, referred to herein as *C. brachyotis* Sunda and *C. brachyotis* Forest (Campbell *et al*., 2004). Morphological differentiation among the species is not concordant with genetic distance. Based on 1266 bp of mitochondrial DNA (mtDNA) (partial control region and cytochrome b combined), the smallest species, *C. brachyotis* Forest is paraphyletic with respect to the largest, *C. horsfieldi* (4.2% divergence). In contrast, despite considerable overlap in size between *C. brachyotis* Forest and *C. brachyotis* and between *C. brachyotis* Sunda and *C. sphinx*, genetic divergence between these species is 8.3% and 8.9%, respectively (Campbell *et al*., 2004).

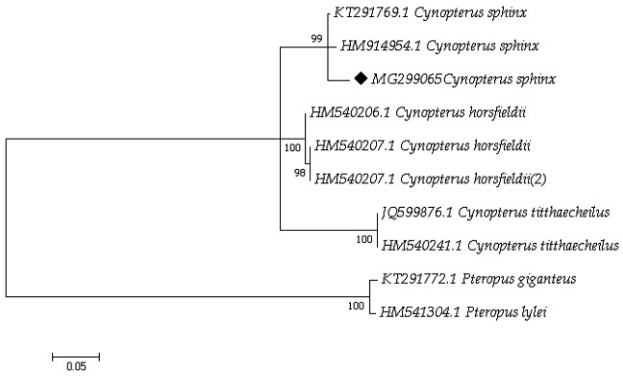

Fig. 1. Phylogenetic tree of genus *Cynopterus* based on COI using maximum likelihood method. Number indicates the percentage of 1000 bootstrap replicates greater than 50.

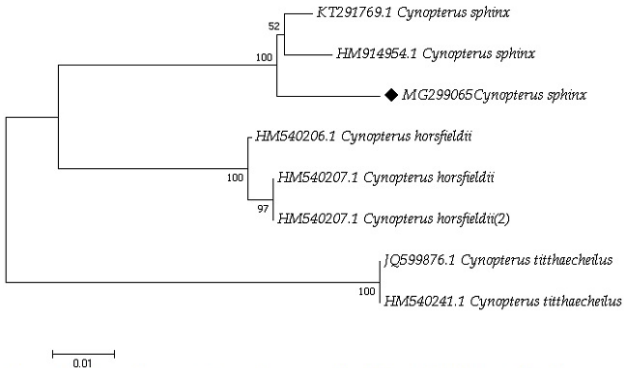

Fig. 2. Phylogenetic tree of genus *Cynopterus* based on COI (K2P model) using neighbour joining method. Number indicates the percentage of 1000 bootstrap replicates greater than 50.

The results of the present analysis are congruent with previous studies and morphology based identification.

These analyses confirm the utility of *COI* gene sequence for identification of *Cynopterus* and to elaborate their phylogenetic relationships. Further studies are needed to add more data to the BOLD database for identification of *Cynopterus* species (Figs. 1, 2 and 3).

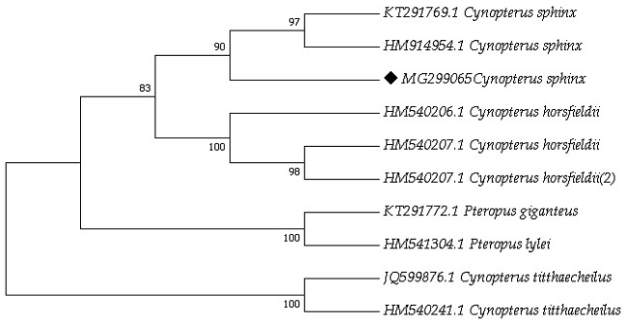

Fig. 3. Phylogenetic tree of genus *Cynopterus* based on COI using maximum parsimony method. Number indicates the percentage of 1000 bootstrap replicates greater than 50.

Conclusion

During the current study it was concluded that morphology of *Cynopterus sphinx* showed that head and body length was 77.13 mm, ear length 19.51 mm, forearm length 65.49 mm, length of tibia 26.61 mm, length of hind foot 17.95 mm and length of tail 7.35 mm whereas the phylogenetic analysis with using COI gene. The mean intraspecific divergences of *C. sphinx* were 0.011%. The nucleotide composition of the sequences showed higher concentration of A+T as compared to G+C. The A+T contents were 53.1%and C+G were 46.8%. In the phylogenetic tree *C. sphinx* clustered together with 100% bootstrap support. The relationship between C.sphinx and C. horsefield was supported by 83% bootstrap value. The relationship between C. titthaecheilus and C. horsefield was supported by 98% bootstrap value. These analyses confirm the utility of the COI gene sequence of *C. sphinx*, obtained in the present study for species level identification and phylogenetic relationships.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190410130412

Statement of conflict of interest

The authors have declared no conflict of interest.

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