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Original Article

Molecular identification of genus Pipistrellus (Mammalia: Chiroptera) from Fata region, Pakistan

Identificação molecular do gênero Pipistrellus (Mammalia: Chiroptera) da região de Fáta, Paquistão

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Abstract

A total of 10 specimens were captured from selected sites of Bajaur Agency FATA, Pakistan using mist nets. The captured specimens were morphologically identified and various morphometric measurements were taken. The head and Body length (HB) of *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* (n=10) was 43±0.11 mm and 45±1.1 respectively. Morphologically identified *Pipistrellus kuhlii* confirmed as *Pipistrellus kuhlii lepidus* based on 16S rRNA sequences. The DNA sequences were submitted to GenBank and accession numbers were obtained (MN 719478 and MT430902). The available 16S rRNA gene sequences of *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* were retrieved from NCBI and incorporated in N-J tree analysis. Overall, the interspecific genetic variations among *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* were 8% and 1% respectively. In our recommendation, a comprehensive molecular identification of bats is need of hour to report more cryptic and new species from Pakistan.

Keywords: Pipistrellus, *Pipistrellus kuhlii lepidus*, *Pipistrellus kuhlii*, *Pipistrellus coromondra*.

Resumo

Um total de 10 espécimes foi capturado em locais selecionados da Bajaur Agency FATA, Paquistão, usando redes de neblina. Os espécimes capturados foram identificados morfologicamente e várias medidas morfométricas foram realizadas. O comprimento da cabeça e do corpo (HB) de *Pipistrellus coromondra* e *Pipistrellus kuhlii lepidus* (n = 10) foi de 43 ± 0,11 mm e 45 ± 1,1, respectivamente. *Pipistrellus kuhlii* identificado morfologicamente e confirmado como *Pipistrellus kuhlii lepidus* com base em sequências de rRNA 16S. As sequências de DNA foram submetidas ao GenBank e os números de acesso foram obtidos (MN 719478 e MT430902). As sequências do gene 16S rRNA disponíveis de *Pipistrellus coromondra* e *Pipistrellus kuhlii lepidus* foram recuperadas do NCBI e incorporadas na análise da árvore N-J. No geral, as variações genéticas interespecíficas entre *Pipistrellus coromondra* e *Pipistrellus kuhlii lepidus* foram de 8% e 1%, respectivamente. Em nossa recomendação, uma identificação molecular abrangente de morcegos precisa de uma hora para relatar mais espécies crípticas e novas do Paquistão.

Palavras-chave: Pipistrellus, *Pipistrellus kuhlii lepidus*, *Pipistrellus kuhlii*, *Pipistrellus coromondra*.

1. Introduction

The work of systematics has started from the last 250 years, despite majority of the species is still unidentified. Currently, the task of species identification has been resolved by molecular identification through short segment of DNA. Generally, the technology of DNA sequencing has resolved the taxonomic disputes of many taxa, but some higher taxa have not yet been resolved precisely as a species. Molecular identification helped scientist to resolve many taxonomic problems such as cryptic species, synonymous species or matching the juvenile with adults (Clare et al., 2011; Wilson et al., 2014).

In most of the areas of the world, the bat fauna is either rare or least known, consequently they have a low abundance along with their lifestyle and hence

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these are the least explored taxa of mammals. The genus *Pipistrellus* is comprising of 51 species throughout the world (Koopman, 1994), 12 from subcontinent (Bates and Harrison, 1998) and 8 species from Pakistan (Roberts, 1997). The distributional range extends from Eurasia to Japan, central southern Africa, Solomon Islands, Indonesia, northern Australia, Canada, New Guinea, USA and Mexico (Roberts, 1997). Bats are also presenting minimal morphological variations and overlapping measurements, highly cryptic species which have been explored by molecular analyses (Clare, 2011; Dool et al., 2016; Gager et al., 2016; Miranda et al., 2011). Bat fauna of Pakistan is poorly explored, so an extensive chiropteran survey is recommended to study these environment friendly creatures (Javid et al., 2014, 2015). Therefore, this pioneer study was design to identify bats of genus *Pipistrellus* from Bajaur Agency, Federally Administered Tribal Areas (FATA), Pakistan.

2. Materials and Methods

2.1. Sample collection and preservation

A total of 10 specimens were captured using mist nets from various sites of Bajaur Agency, FATA, Pakistan (N 34° 43' 48.7812", E 71° 28' 45.9012"). The samples were identified in the field on the basis of morphology and preserved in 70% ethanol.. The preserved samples brought the Lab at Institute of Biochemistry and Biotechnology (IBBt), University of Veterinary and Animal Sciences, Lahore.

2.2. DNA extraction and amplification

DNA was extracted from ethanol (70%) preserved specimens (wing tissue) by standard phenol-chloroform method (Hoelzel and Green, 1992). The Purity of DNA was checked through agarose gel electrophoresis. Total genomic DNA was amplified using 16S rRNA universal primers set (Forward: 5´-AAAGACGAGAAGACCC-3´ and Reverse: 5´-GATTGCGCTGTTATTCC-3´).

Amplification was performed in a 100 µl of a solution containing 67 mM Tris (pH 8.8), 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, each dNTP at 1 mM, each primer at 1 µl, genomic DNA (10-1000 ng), and 2-5 units of Thermus aquaticus polymerase (Perkin-Elmer/Cetus).

The PCR amplification comprised of 93 °C for 1 min, 40 cycles at93 °C for 1 min, 50 °C for 30 seconds, 72 °C for 2 min and a final 10 min at 72 °C. The PCR products were checked through 1% agarose gel. PCR products were purified by the Qiagen purification kit and all the samples were Sanger sequenced on AB3730xl sequencer Applied Bio-system, Korea.

2.3. Data analysis

The obtained DNA sequences checked on BioEdit version 7.2 and aligned using ClustalX (Ali et al., 2020; Hussain et al., 2020). After trimming ambiguous bases, DNA sequences were submitted to GenBank and accession numbers were obtained. All the sequences of *Pipistrellus kuhlii lepidus* and *Pipistrellus coromondra* were subject to BLAST analysis to retrieved closely matched sequences. Genetic variation between and within the species were calculated using MEGA 10 based on p-distance. Neighbor-joining tree was constructed using 100 bootstraps in MEGA 10 (Kumar et al., 2018).

3. Results

3.1. Distribution

A total of 10 specimens were collected from selected sites of Bajaur Agency, FATA, Pakistan (34°24′17.76″N 72°33′32.16″E) (Figure 1).

3.2. Taxonomic position

Least Pipistrelle (Temminck, 1840): *Pipistrellus tenuis*
Indian Pipistrelle (Gray, 1838): *Pipistrellus coromondra*
Common Pipistrelle (Schreber, 1774): *Pipistrellus pipistrellus*
Pipistrellus kuhlii lepidus (Blyth, 1845)

3.3. Morphology

Various morphological parameters viz., head and body length (HB), tail length (TL), hind foot length (HL), forearm length (FL), wing span (WS), 5th metacarpal length (ML 5th), 4th metacarpal Length (ML 4th) and ear length (EL) were taken. During the study, head and Body length (HB) of *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* (n=10) was 43±0.11 mm and 45±1.1 respectively.

3.4. Phylogenetic relationship

The obtained DNA sequences have shown reliable and clear species identification. Morphologically identified *Pipistrellus kuhlii* confirmed as *Pipistrellus kuhlii lepidus* based on 16S rRNA sequences. The DNA sequences were submitted to GenBank and accession numbers were obtained (MN 719478 and MT430902). In Pakistan, the genus *pipistrellus* is represented by 8 species based on their morphological parameters. Till date no molecular based identification of bats has been conducted and mtDNA sequences are not available for chiropteran species belonging to Pakistan. The available 16S rRNA gene sequences of *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* and were retrieved from NCBI and incorporated in N-J tree analysis. Overall, the interspecific genetic variations among *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* were 8% and 1% respectively. Neighbor-joining tree based on p-distance is shown in Figure 2. Genetic divergence of *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* is mentioned in Table 1.

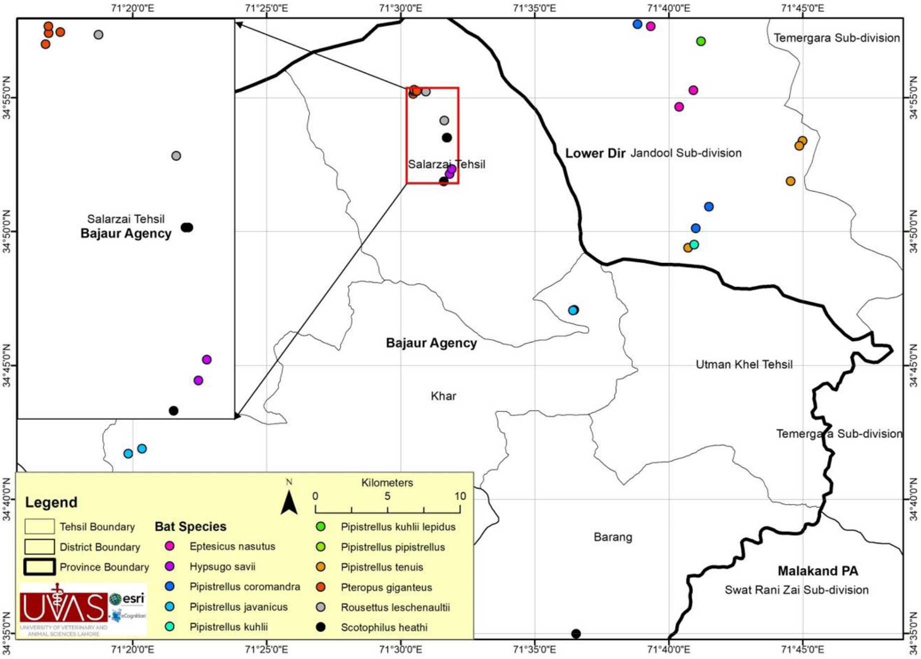

Figure 1. Collection sites of bats species from FATA, Pakistan.

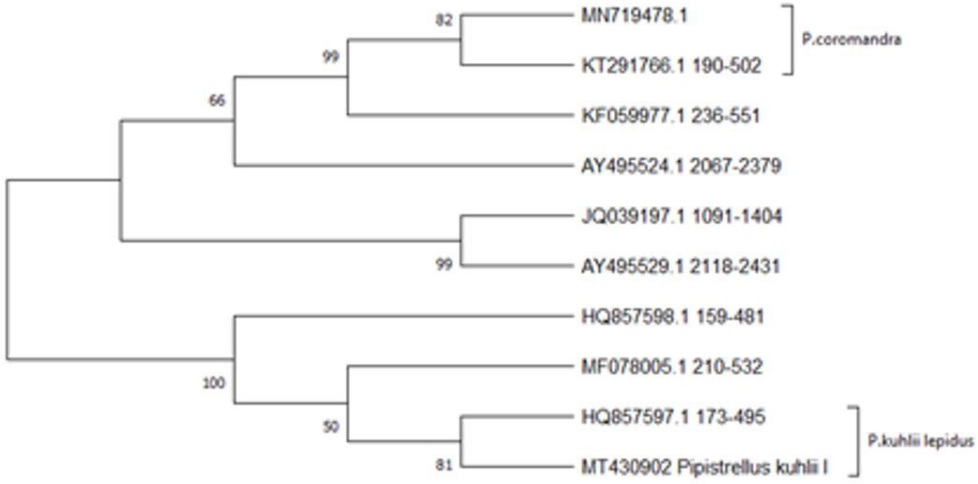

Figure 2. Neighbour-joining tree of bats species captured from study area.

Table 1. Genetic variation between *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* from FATA, Pakistan.

Ac. No	MF078005	HQ857598	HQ857597	MT430902	AY495524	MN719478	KT291766	JQ039197	AY495529	KF059977
MF078005										
HQ857598	0.004									
HQ857597	0.000	0.004								
MT430902	0.000	0.004	0.000							
AY495524	0.216	0.210	0.216	0.216						

Table 1. Continued....

Ac. No	MF078005	HQ857598	HQ857597	MT430902	AY495524	MN719478	KT291766	JQ039197	AY495529	KF059977
MN719478	0.204	0.197	0.204	0.204	0.095					
KT291766	0.196	0.190	0.196	0.196	0.100	0.012				
JQ039197	0.180	0.174	0.180	0.180	0.090	0.102	0.107			
AY495529	0.180	0.174	0.180	0.180	0.090	0.102	0.107	0.000		
KF059977	0.207	0.201	0.207	0.207	0.086	0.020	0.024	0.104	0.104	

4. Discussion

The partial sequence of 16S rRNA confirms the species identity and this information could be used for conservational and other ecological related studies. Another important implication of mtDNA study is to assess the genetic diversity at inter-specific and intra-specific level. Similar results were reported by Ali et al. (2020). Genetic diversity is an important component of biodiversity and it could be used to formulate conservation and management planes to preserve the evolutionary history of a species. It is estimated that the bats are constituting about 28% of mammalian fauna in Pakistan but it is debatable for exact number of bats' fauna within the territorial boundary of the country (Roberts, 1977; Walker and Molur, 2003; Wilson and Reeder, 2005).

In Pakistan there are about 8 families of bats, 26 genera and 54 species has so far been discovered based on their morphological basis (Mahmood-ul-Hassan, 2009), this is equivalent to any region of the world with same climatic and topographic conditions and no data is yet available on barcoding of bats up till now in the country. Species identification and characterization has a crucial role in taxonomy and classification of organisms. Modern taxonomy, originated in mid18th century has described up to 1.7 million species of organisms (Stoeckle, 2003). Besides this, to study the relationship of living beings with each other various behavioral and morphological parameters are taken into consideration. It is very unsurprising that the larger animals are given a priority for description and the smaller ones mostly remain unknown in sciences (Blaxter, 2003).

The available 16S rRNA gene sequences of *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* were retrieved from NCBI and incorporated in N-J tree analysis. Overall, the interspecific genetic variations among *Pipistrellus coromondra* and *Pipistrellus kuhlii lepidus* were 8% and 1% respectively. In our recommendation, a comprehensive molecular identification of bats is need of hour to report more cryptic and new species from Pakistan.

Genetic analysis of species provides a useful information about the scales at which the wild species are impacted by anthropogenic activities but also provides the information about a successful demographic management of wild species (Sovic et al., 2016). Advancement in molecular techniques has revolutionized the field of systematics and improved the taxonomy of some more complex chiropteran species. Molecular genetics highlighted many new discoveries in taxonomy of understudied and species rich tropical areas (Clare et al., 2007; Francis et al., 2010), besides this, in temperate fauna where the relative species diversity is low, the molecular genetics has also resolved taxonomic uncertainties (Mayer et al., 2007; Mayer and von Helversen, 2001).

Pipistrellus kuhlii lepidus from Pakistan making a clade with a species having accession number HQ857597 which is also a *Pipistrellus kuhlii lepidus* as a sister species from Sardinia, this subspecies of *Kuhl's pipistrelle* was not previously reported from the study area, which may be due to its morphological non-differentiation due to cryptic species. Such a report of a new record also highlights the importance of genetic identification as compared to the conventional methods for taxonomy. An extensive survey should be carried out in the country to explore and compare the conventional taxonomic methods with barcoding.

Pipistrellus coromandra Indian Pipistrelle (Gray, 1838) is distributed in Afghanistan, Bangla Desh, India (including Nicobar Isls), Sri Lanka, Pakistan, Nepal, Bhutan, Burma, Cambodia, Thailand, S China. In Pakistan it is has been collected from Dir, Chitral and Swat districts of North Western Frontier Province. This species is often difficult to distinguish from *P. tenuis*. In general *P. coromandra* averages larger than *P. tenuis* but there is a significant overlap in all external measurements. In BLASTn results our query sequence (MN719478) has shown a 97.45% percentage identity and 99% query coverage with *Pipistrellus coromandra* (KT291766) which has been reported from India.

Since last two decades, genetics has played a major role in ecology and conservation biology (Frankham, 2005; Frankham et al., 2002; Hedrick, 2001). Genetics has significant contributions to understand the effects of habitat fragmentation, genetic erosion on extinction and endangerment of the species, the dynamics of adaptation of species to the new environmental circumstances are added, results in the formation of a modern scientific filed of biology called "Conservation Genetics" (Ouborg et al., 2006). Whereas several conservation efforts measured at native scale or regional levels, they could affect the biotic consequences of universal phenomenon, specifically the recent climatic changes and their consequences on populations' extinction rate that is now believed to be on the top of the background levels (McLaughlin et al., 2002).

5. Conclusions and Recommendation

The bats considered as fearsome and pest animals in Pakistan. Bats always take less concern to scientific community in Pakistan regardless of their many environment services. In Pakistan, the genus *pipistrellus* is represented by 8 species based on their morphological parameters. During present study, morphologically identified *Pipistrellus kuhlii* confirmed as *Pipistrellus kuhlii lepidus* based on 16S rRNA sequences. In our recommendation, a comprehensive molecular identification of bats is need of hour to report more cryptic and new species from Pakistan.

References

ALI, W., JAVID, A., HUSSAIN, A., HAFEEZ-UR-REHMAN, M., CHABBER, A. and HEMMATZADEH, F., 2020. First record of Euphlyctis kalasgramensis (Anura: Dicroglossidae) from Punjab, Pakistan. *Mitochondrial DNA. Part B, Resources*, vol. 5, no. 2, pp. 1227-1231. http://dx.doi.org/10.1080/23802359.2020.1731337. PMid:33366922.

BATES, P. and HARRISON, D., 1998. Bats of the Indian subcontinent. *Biodiversity and Conservation*, vol. 7, no. 10, pp. 1383-1386. http://dx.doi.org/10.1023/A:1017113501563.

BLAXTER, M., 2003. Molecular systematics: counting angels with DNA. *Nature*, vol. 421, no. 6919, pp. 122-124. http://dx.doi.org/10.1038/421122a. PMid:12520286.

CLARE, E.L., 2011. Cryptic species? Patterns of maternal and paternal gene flow in eight Neotropical bats. *PLoS One*, vol. 6, no. 7, pp. e21460. http://dx.doi.org/10.1371/journal.pone.0021460. PMid:21814545.

CLARE, E.L., LIM, B.K., FENTON, M.B. and HEBERT, P.D., 2011. Neotropical bats: estimating species diversity with DNA barcodes. *PLoS One*, vol. 6, no. 7, pp. e22648. http://dx.doi.org/10.1371/journal.pone.0022648. PMid:21818359.

CLARE, E.L., LIM, B.K., ENGSTROM, M.D., EGER, J.L. and HEBERT, P.D., 2007. DNA barcoding of Neotropical bats: species identification and discovery within Guyana. *Molecular Ecology Resources*, vol. 7, no. 2, pp. 184-190.

DOOL, S.E., PUECHMAILLE, S.J., FOLEY, N.M., ALLEGRINI, B., BASTIAN, A., MUTUMI, G.L., MALULEKE, T.G., ODENDAAL, L.J., TEELING, E.C. and JACOBS, D.S., 2016. Nuclear introns outperform mitochondrial DNA in inter-specific phylogenetic reconstruction: lessons from horseshoe bats (Rhinolophidae: Chiroptera). *Molecular Phylogenetics and Evolution*, vol. 97, pp. 196-212. http://dx.doi.org/10.1016/j.ympev.2016.01.003. PMid:26826601.

FRANCIS, C.M., BORISENKO, A.V., IVANOVA, N.V., EGER, J.L., LIM, B.K., GUILLÉN-SERVENT, A., KRUSKOP, S.V., MACKIE, I. and HEBERT, P.D., 2010. The role of DNA barcodes in understanding and conservation of mammal diversity in Southeast Asia. *PLoS One*, vol. 5, no. 9, e12575. http://dx.doi.org/10.1371/journal.pone.0012575. PMid:20838635.

FRANKHAM, R., 2005. Genetics and extinction. *Biological Conservation*, vol. 126, no. 2, pp. 131-140. http://dx.doi.org/10.1016/j.biocon.2005.05.002.

FRANKHAM, R., BRISCOE, D.A. and BALLOU, J.D., 2002. *Introduction to conservation genetics*. Cambridge: Cambridge University Press. http://dx.doi.org/10.1017/CBO9780511808999.

GAGER, Y., TARLAND, E., LIECKFELDT, D., MÉNAGE, M., BOTERO-CASTRO, F., ROSSITER, S.J., KRAUS, R.H., LUDWIG, A. and DECHMANN, D.K., 2016. The value of molecular vs. morphometric and acoustic information for species identification using sympatric molossid bats. *PLoS One*, vol. 11, no. 3, e0150780. http://dx.doi.org/10.1371/journal.pone.0150780. PMid:26943355.

HEDRICK, P.W., 2001. Conservation genetics: where are we now? *Trends in Ecology & Evolution*, vol. 16, no. 11, pp. 629-636. http://dx.doi.org/10.1016/S0169-5347(01)02282-0.

HOELZEL, A., and GREEN, A., 1992. Analysis of population-level variation by sequencing PCR-amplified DNA. In: A. HOELZEL, ed. *Molecular genetic analysis of populations: a practical approach*. Oxford: Oxford University Press, pp. 159-187.

HUSSAIN, S., BUKHARI, S.M., JAVID, A., HUSSAIN, A., RASHID, M. and ALI, W., 2020. Molecular identification of genus Duttaphrynus from Punjab, Pakistan. *Mitochondrial DNA. Part B, Resources*, vol. 5, no. 3, pp. 3218-3238. http://dx.doi.org/10.1080/23802359.2020.1810143. PMid:33458117.

JAVID, A., MAHMOOD-UL-HASSAN, M., HUSSAIN, S.M. and IQBAL, K.J., 2014. Recent record of the Asiatic lesser yellow house bat (*Scotophilus kuhlii*) from Punjab, Pakistan. *Mammalia*, vol. 78, no. 1, pp. 133-137. http://dx.doi.org/10.1515/mammalia-2013-0012.

JAVID, A., SHAHBAZ, M., MAHMOOD-UL-HASSAN, M. and HUSSAIN, S.M., 2015. The Blasius' horseshoe bat Rhinolophus blasii (Chiroptera, Rhinolophidae) still extends to Pakistan. *Mammalia*, vol. 79, no. 2, pp. 249-251.

KOOPMAN, K., 1994. Chiroptera: systematics. In: J. NIETHAMMER, H. SCHLIEMANN and D. STARCK, eds. *Handbook of Zoology*. Berlin: Walter de Gruyter, vol. VIII. Mammalia. Part 60.

KUMAR, S., STECHER, G., LI, M., KNYAZ, C. and TAMURA, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, vol. 35, no. 6, pp. 1547-1549. http://dx.doi.org/10.1093/molbev/msy096. PMid:29722887.

MAHMOOD-UL-HASSAN, M., 2009. *The bats of Pakistan: the least known creatures*. Saarbrücken: VDM Publishing.

MAYER, F. and VON HELVERSEN, O., 2001. Cryptic diversity in European bats. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, vol. 268, no. 1478, pp. 1825-1832. http://dx.doi.org/10.1098/rspb.2001.1744. PMid:11522202.

MAYER, F., DIETZ, C. and KIEFER, A., 2007. Molecular species identification boosts bat diversity. *Frontiers in Zoology*, vol. 4, no. 1, pp. 4. http://dx.doi.org/10.1186/1742-9994-4-4. PMid:17295921.

MCLAUGHLIN, J.F., HELLMANN, J.J., BOGGS, C.L. and EHRLICH, P.R., 2002. Climate change hastens population extinctions. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 9, pp. 6070-6074. http://dx.doi.org/10.1073/pnas.052131199. PMid:11972020.

MIRANDA, J., BERNARDI, I. and PASSOS, F., 2011. *Chave ilustrada para a determinação dos morcegos da Região Sul do Brasil*. Curitiba: João MD Miranda.

OUBORG, N., VERGEER, P. and MIX, C., 2006. The rough edges of the conservation genetics paradigm for plants. *Journal of Ecology*, vol. 94, no. 6, pp. 1233-1248. http://dx.doi.org/10.1111/j.1365-2745.2006.01167.x.

ROBERTS, T., 1977. *The mammals of Pakistan*. London: E. Benn.

ROBERTS, T., 1997. *The mammals of Pakistan*. Revised ed. Karachi, Pakistan: Oxford University Press, 525 p.

SOVIC, M.G., CARSTENS, B.C. and GIBBS, H.L., 2016. Genetic diversity in migratory bats: results from RADseq data for three tree bat species at an Ohio windfarm. *PeerJ*, vol. 4, pp. e1647. http://dx.doi.org/10.7717/peerj.1647. PMid:26824001.

STOECKLE, M., 2003. Taxonomy, DNA, and the bar code of life. *A.I.B.S. Bulletin*, vol. 53, no. 9, pp. 796-797.

WALKER, S. and MOLUR, S., 2003. *Summary of the Status of South Asian Chiroptera. Extracted from the CAMP 2002 Report.* Coimbatore, India: Zoo Outreach Organization, CBSG. South Asia and WILD.

WILSON, D.E. and REEDER, D.M., 2005. *Mammal species of the world: a taxonomic and geographic reference*. Baltimore: Johns Hopkins University Press.

WILSON, J., SING, K., HALIM, M., RAMLI, R., HASHIM, R. and SOFIAN-AZIRUN, M., 2014. Utility of DNA barcoding for rapid and accurate assessment of bat diversity in Malaysia in the absence of formally described species. *Genetics and Molecular Research*, vol. 13, no. 1, pp. 920-925. http://dx.doi.org/10.4238/2014.February.19.2. PMid:24634112.