

Molecular Cataloging Of Pakistani Small Wild Ruminant Species Of Family Cervidae And Bovidae Through DNA Barcoding

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ABSTRACT

In this study 7 small wild ruminant species (35 sequences) of family bovidae and cervidae along with 15 sequences (retrieved from GenBank) were phylogenetically investigated and evolutionary explored through DNA barcoding using 393 base pair region of *Cytochrome b* gene as a genetic marker. Sequences were analyzed using Bioedit, NCBI, Clustal Omega and MEGA software. Between groups evolutionary divergence was recorded as 0.070-3.542, while within group average evolutionary divergence was 0.000-0.113 and average evolutionary divergence over all sequence pairs was 0.195. Maximum likelihood phylogenetic tree was constructed. *Axis porcinus* formed monophyletic group with *Axis kuhlii*. *Axis axis* cladded with *Dama dama*. *Cervus elaphus* evolved in same clade with species of same genus. *Muntiacus muntjak* descended with *Muntiacus feae*. *Oryx leocoryx* exhibited close evolutionary relationship to *Addax nasomaculatus*. *Antelope cervicapra* displayed sister group evolutionary relationship to *Gazella bennettii* and *Gazella bennettii* formed monophyletic group with *Gazella leptoceros*. This region of *Cyt b* gene successfully identified these at species and genera level. It was concluded that owing to great resemblances in morphological characters of small wild ruminants of the family bovidae and cervidae, ought to be studied on the basis of complete mitochondrial genome and possibly its comparison to nuclear genome studies.

Key words: Molecular, Evolutionary, Pakistan, DNA Barcoding

Introduction

The small wild ruminant species of family bovidae and cervidae in Pakistan pave a diverse habitats ranging from sea level to the high altitudes of mountains Roberts (1977). The northern elevations including the higher elevations of Hindukush, Karakorum and Himalayas and the lower elevation zones of Azad Jammu and Kashmir and Potohar offer tremendous habitation for small wild ruminants of Pakistan in the form of temperate forests, sub alpine scrub and alpine grazing land. Ruminants are the main ungulate group and distributed in all continents except Antarctica and Australia “Hassanin and Douzery (2003)”. Ruminants comprise about 150 species which contain wild and domestic species. Ruminants cover antelope, few macro pods, camels, llamas, cattle, buffaloes, sheep, goats, giraffes, yaks and deer “Matsuda et al. (2011)”. The speciation of ruminants is supposed to rapid in the Mid-Eocene giving rise to 5 separate families. The genetic sorting of these ruminants is problematic owing to their rapid radiation. Out of 15, 11 phylogenies elucidate the ancestral association of family bovidae and cervidae which is the representation of factual phylogeny “Decker et al. (2009)”.

For scrutinizing biodiversity, biological properties of mitochondrial DNA declare it an appropriate unit. For addressing the systematics problem like the determination of divergence of species, the mitochondrial *Cyt b* gene is very helpful and extensively used. The supremacy of the polymorphism of the *Cyt b* gene was strengthened by “Ferreira et al. (2011)” and specified that it is an influential marker for the identification of species and might be specifically valuable for endangered/vulnerable species identification. As a genetic marker, *Cyt b* gene reconstructs phylogeny more accurately and provides relationship among species on the basis of other morphological and molecular investigations at generic, family, order and super order levels “Tobe et al. (2010)”. In this study, 7 small wild ruminant species of family bovidae and cervidae along with 15 *Cyt b* gene sequences (retrieved from GenBank) were genetically classified and evolutionary explored through DNA barcoding by using *Cyt b* as a genetic marker.

Materials and methods

Bone, meat and blood samples of small wild ruminants were collected from different localities of Pakistan with the help of Punjab wildlife and parks department, legal hunter, local taxidermist, Lahore zoo and post mortem block University of Veterinary and Animal Sciences, Lahore. In order to maintain the integrity of DNA, research samples were preserved in 95% ethanol Nagy (2010). DNA was extracted using organic DNA extraction method “Sambrook and Russel (2001)”. A set of primers for *Cyt b* gene was designed from the conserved regions of small wild ruminants using MEGA 6 software “Tamura et al. (2013)”.

PCR (Bio-Rad Berkeley, California) conditions were optimized for maximum amplification by verifying the concentrations of PCR reagents and PCR conditions. The PCR products were purified using 80% ethanol. The reactions which generated amplified products were sequenced through Center for Applied Molecular Biology (CAMB) University of the Punjab, Lahore using Sanger sequencing method “Olsvik et al. (1993)”. Sequenced reactions were analyzed through Big Dye v 3.1. Sequences were analyzed using Bioedit, NCBI, Clustal Omega and MEGA software.

Results

All Cyt *b* gene sequences were aligned, 145 (36.9%) positions were conserved, 248 (63.1%) were variable, 113 (28.8%) substitutions were informative for parsimony analysis and 135 (34.4%) positions were singleton data not shown. Evolutionary divergence between the groups of small wild ruminant species of family bovidae and cervidae was estimated (Table 1) with highest divergence (3.542) found between *Oryx leucoryx* and *Muntiacus muntjak*. This may be due to habitat change of these species because *O. leucoryx* is found in Arabian and African regions while *M. muntjak* is mainly inhabited in India, China, Indonesia, Vietnam and Pakistan. The diet of both the species is also changed. The *O. leucoryx* feeds mostly on grasses but it also eats huge diverse vegetation like roots, tubers, fruit, herbs and buds. The *M. muntjak* eats small animals, eggs of birds, seeds, shoot, fruits and grass. This finding is supported by previous study because with the help of low bootstrap provision, *O. leucoryx* cladded with *O. gazelle* “Khan et al. (2008)”. In other study “Xiang-Rong et al. (2002)” reported mitochondrial DNA phylogenetic relationship on the basis of 7 species of cervidae by constructing maximum parsimony and neighbor joining methods (Fig. 1). Their results termed that *M. crinifrons*, *M. reeves* and *M. muntjak* have been evolved from a common ancestor. The shortest genetic distance or evolutionary divergence (0.070) was recorded between *A. axis* and *A. porcinus*. This finding is consistent with the preceding study in which the mitochondrial genome of *A. porcinus* was sequenced and this species exhibited 99.8 % resemblance to the already published genome of *A. axis* “Hill et al. (2017)”.

The within group evolutionary divergence was assessed with lowest evolutionary divergence (0.000) was found in *Axis porcinus* and *Cervus elaphus* (Table 2). The finding in this study that *A. porcinus* has no genetic diversity is confirmed from the previous study from Pakistan by stating that Pakistani Hog deer (*A. porcinus*) possesses lower genetic diversity “Abbas et al. (2017)”. Likewise the finding of the present research regarding *C. elaphus* was supported by previous finding that no noteworthy genetic dissimilarities were found in the genetic diversity between fenced and wild populations of *C. elaphus* and comparatively lower genetic diversity was recorded in captive population of *C. elaphus* “Galarza et al. (2017)”. Highest genetic divergence (0.113) was evaluated in the individuals of *Oryx leucoryx*. This finding was confirmed from the preceding discovery by “Eljarah et al. (2017)” and they investigated so high genetic diversity within *O. leucoryx* population that it can live on its own.

Table 1: Evolutionary divergence over sequence pairs between groups

	<i>Oryx leucoryx</i>	<i>Antilope cervicapra</i>	<i>Muntiacus muntjak</i>	<i>Gazella bennettii</i>	<i>Axis porcinus</i>	<i>Axis axis</i>	<i>Cervus elaphus</i>
<i>Oryx leucoryx</i>							
<i>Antilope cervicapra</i>	2.533						
<i>Muntiacus muntjak</i>	3.542	3.317					
<i>Gazella bennettii</i>	2.614	1.399	3.154				
<i>Axis porcinus</i>	1.970	0.748	3.360	1.407			

<i>Axis axis</i>	1.893	0.767	3.241	1.383	0.070		
<i>Cervus elaphus</i>	1.888	0.721	3.228	1.307	0.145	0.128	

The number of per site base substitutions between groups is exhibited. The analysis of 35 nucleotide sequences was performed through Maximum Composite Likelihood model (Tamura and Kumar, 2004). The analysis of evolution was done in MEGA 6 (Tamura et al., 2013).

Table 1: Average Evolutionary Divergence over Sequence Pairs within Groups

1	<i>Oryx leucoryx</i>	0.113
2	<i>Antilope cervicapra</i>	0.019
3	<i>Muntiacus muntjak</i>	0.069
4	<i>Gazella bennettii</i>	0.002
5	<i>Axis porcinus</i>	0.000
6	<i>Axis axis</i>	0.002
7	<i>Cervus elaphus</i>	0.000

The number of per site base substitutions within groups is exhibited. The analysis of 35 nucleotide sequences was achieved with the help of Maximum Composite Likelihood model (Tamura and Kumar, 2004). The analysis of evolution was done in MEGA 6 (Tamura et al., 2013).

Discussion

All the amplicons of Cyt *b* gene were retrieved in which some intraspecific variations in *O. leucoryx*, *A. axis*, *M. muntjak*, were observed which have been reported in previous studies from other mammal species “Borisenko et al. (2008), Clare et al. (2007)”. This intraspecific variation was may be distinct sampling localities or sub species population of these species. *Axis porcinus* formed monophyletic group with *A. kuhlii* (Accession# HQ893538.1), species reported from Indonesia by Grubb (2005), “Lachenmeier and Melisch (1996)”. However our finding did not inveterate the finding of Hill because they discovered that *A. porcinus* formed clad with *A. axis*. But our study confirmed the classical theory regarding this species. On morphological basis *A. porcinus* was comprised of two dispersed but alike species; *Hyelaphus calamianensis* (Calamian deer) which was found in the Calamian Islands of Philippines and *Hyelaphus kuhlii* (Bawean deer) which was habitant of Bawean Island of Indonesia “Timmins et al. (2008)”. The other economically important species *A. axis* shared ancestor within back in time with *Dama dama* (JN622629.1) which is a Eurasian species. According to “Pitra et al. (2004)”, Stefano and Petronio (2000)”, *A. axis* made a cluster with *Rucervus schomburgki* (a species from Central Thailand) and *R. duvaucelii* which was found in India, Nepal but near to extinction in Pakistan and Bangladesh “Duckworth et al. (2015)”. The current study is not in line with the theory of Pitra pitraand co-workers and Stefano and Petronio regarding the phylogenetic position of *A. axis*. The *C. elaphus* evolved from common ancestor with *C. e. hispanicus* (AF489281.1) which is found in Iberia, *C. e. hippelaphus* (AY044860) a species found in France, Switzerland, Austria, Germany, and Denmark, *C. e.*

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atlanticus (AY070221.1) which is originated in Norway, *Elaphurus davidianus* (JN399997.1) which is a species from China and *Rucervus eldii eldii* (Accession# KU133959.1) which is an endangered and endemic sub species of brow-antlered deer commonly inhabited in the Manipur of India. However difference was recorded in the branching pattern between *C. elaphus* and *E. davidianus* and *R. e. eldii*. According to the finding of (Pitra et al., 2004), it was reported from 33 taxa of cervinae and on the basis of complete mitochondrial *Cyt b* gene, the cluster includes *sika*, *cervus rusa*, *panolia* and perhaps *przewalskium*. The present study inveterate the findings of Pitra et al. up to some extent because according to Pitra and co-workers, in addition to genus *sika* (*nippon*), the genus *cervus* also formed a cluster with *cervus rusa*, *panolia* and *przewalskium*. Rendering the traditional theories regarding *C. elaphus* until recently biologists assumed that wapiti or elk and Red deer are identical species creating a constant spreading through North America and moderate Eurasia. Such certainty was on the basis of completely productive crosses that might be shaped in the captive environment “Perez-Espona et al. (2013)”, “Shackell et al. (2003)”, “Moore and Littlejohn (1989)”. The present study did not approve the traditional theory regarding the taxonomy of *C. elaphus* because if wapiti or elk (*C. e. xanthopygus*, *C. e. songaricus*, *C. e. sibiricus* and *Megaloceros giganteus* and red deer were same species then these would be in the same clade or would descend simultaneously. The *M. muntjak* cladded with the species of the same genus, the *M. feae* (Accession# AF042721.1) and *Cervus schomburgki* (AY607036.1) within back in time. The analysis of “Wang and Lan, (2000a)” which was based on the phylogeny of mitochondrial DNA, revealed that *M. muntjak* descended from a common ancestor along with *M. feae*. This finding supported our investigation regarding the phylogeny of *M. muntjak*. Some intraspecific splits were observed in *M. muntjak*. This sequence divergence may be owing to very rapid rate of chromosome number alteration in this species “Wang and Lan, 2000b). According to a classical theory, England has a huge feral population of *M. muntjak*. *Muntiacus reeves* was introduced in England. The wild population of the deer had been raised from the fugitives of the state of Woburn Abbey in 1925 Kenneth (1964). Muntjacs exist in most of the regions of England. These are lower in number in north-west. The British Deer Society conducted a survey in UK on the wild deer in 2005-2007 described that *muntjak* has been expanded in 2000 ‘Deer Distribution Survey (2007). The present findings are not in line with the classical theory regarding the molecular classification of *M. muntjak* because *M. muntjak* made clad with *M. feae* which is a native species of Thailand and Western Myanmar. The findings of the present study contradicted the observations of (Kenneth, 1964) because we discovered the Asiatic origin of *M. muntjak* rather than European. The *Oryx leucoryx* formed monophyletic group with *Oryx beisa* (DQ13195.1) which is inhabitant of Africa. Previous research inveterate our finding by declaring that *O. beisa* is a sub species of *Oryx* “Masembe et al. (2006)”. It also exhibited close evolutionary relationship to *Addax nasomaculatus* (JN632591.1) because the ancestor of *O. leucoryx* has been evolved from the ancestor of *A. nasomaculatus*. This finding is supported by the investigation of “Khan et al. (2008) that genus *Oryx* is closely related to genus *Addax*. It should be noted that highest intraspecific variation was recorded in *O. leucoryx* because all its individuals evolved unparalleled. This analysis has been confirmed from the preceding investigation in

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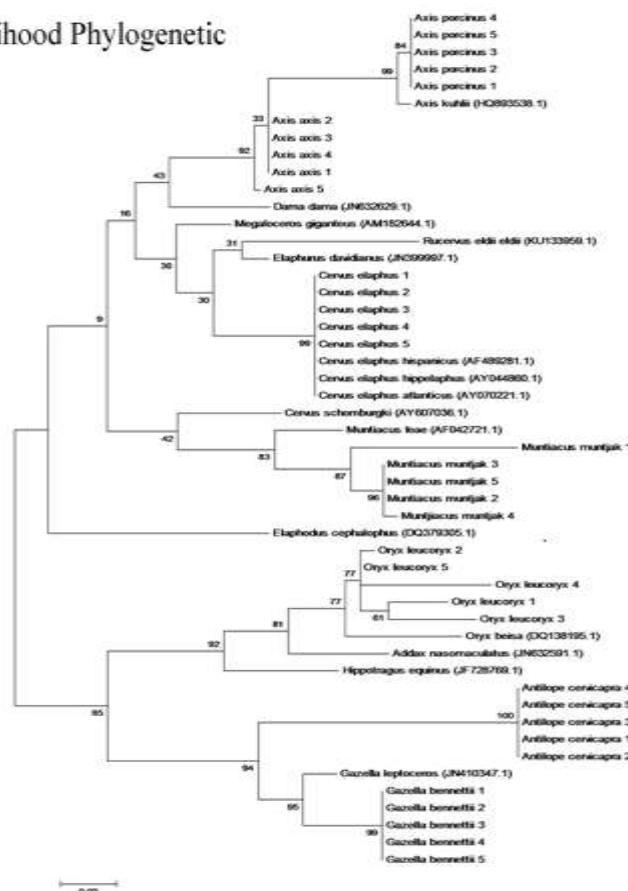
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which high level genetic diversification was concluded within the population of *O. leucoryx* “Eljarah et al. (2017)”. The *A. cervicapra* exhibited sister group evolutionary relationship with the species of genus *Gazella*, the *G. leptoceros* (Acc# JN410347.1) and *G. bennettii* because the ancestor of these species has been evolved from the ancestor of *A. cervicapra*. According to the phylogenetic analysis of gazelles, it was found that Blackbuck (*A. cervicapra*) was the sister group of the *Gazella* genus “Lerp et al. (2016), which is mostly found in Africa southwest, central Asian & Indian subcontinent. *Gazella bennettii* formed a monophyletic clade with the species of the same genus; *G. leptoceros*. (JN410347). However the lateral species evolved earlier within forward in time. According to the findings of Lerp et al. *G. bennettii* cladded with *G. subgutturosa*, *G. marica*, *G. leptoceros* and *G. cuvieri*. Previously this species was considered a subspecies of *Gazella dorcas* Groves (1969) and Corbet (1979). Our findings confirmed the genetic theory of Lerp but negated the classical theory regarding the taxonomy of *G. bennettii*.

The significance of the present study to the scientific community is that they can use the results of the present study as a guide to conduct their studies regarding the small wild ruminant species of other countries. Similarly the scientific community can assess the power and importance of this region of mitochondrial Cyt *b* gene marker regarding molecular classification and evolutionary studies through DNA barcoding.

Fig 1: Maximum Likelihood Phylogenetic Tree



Conclusions

It was concluded that DNA barcoding successfully identified the small wild ruminant species of families' bovidae and cervidae at genus and species level. The small wild ruminant species are greatly similar in morphological characteristics. In most cases it was challenging to thoroughly discriminate these species. It is essential to discriminate these species on the basis of complete mitochondrial genome and possibly its comparison to nuclear genome analysis.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

References

- Abbas, G., Nadeem, A., Babar, M. E., Hussain, T., Tahir, M. S., Shehzad, W., Iqbal, R. Z., Tayyab, M., and Javed., M. (2017). Molecular phylogeny and diversity analysis of Hog deer (*Axis porcinus*) in Pakistan Pak. J. Zoology. 49: 1701-1712.
- Borisenko, A. V., Lim, B. K., Ivanova, N. V., Hanner, R. H., and Hebert., P. D. N. (2008). DNA barcoding in surveys of small mammal communities: a field study in Suriname. Mol. Ecol. Resour. 8: 471-479.
- Clare, E. L., Lim, B. K., Engstrom, M. D., Eger, J. L., and Hebert., P. D. N. (2007). DNA barcoding of Neotropical bats: species identification and discovery within Guyana. Mol. Ecol. Notes. 7: 184-190.
- Corbet., G. B. (1979). The mammals of the Palaearctic Region: a taxonomic review. British Museum (natural history) and Cornell University press, London and Ithaca (NY). J. Mammal. 60: 656-657.
- Decker, J. E., Pires, J. C., Conant, G. C., McKay, S. D., Heaton, M. P., Chen, K., Cooper, A., Vilkki, J., Seaburi, C. M., Caetano, A. R., Johnson, G. S., Brenneman, R. A., Hanotte, O., Eggert, L. S., Wiener, P., Kim, J. J., Kim, K. S., Sonstegard, T. S., Van Tassell, C. P., Neibergs, H. L., McEwan, J. C., Brauning, R., Coutinho, L. L., Babar, M. E., Wilson, G. A., McClure, M. C., Rolf, M. M., Kim, J. W., Schnabel, R. D., and Taylor., J. F. 2009. Resolving the evolution of extant and extinct ruminants with high-throughput phylogenomics. Proc. Natl. Acad. Sci. 106: 18644-18649.
- Deer Distribution Survey (2007). The British Deer Society. Retrieved 6 September 2011.
- Duckworth, J. W., Kumar, N. S., Anwarul, I. M., Sagar, B. H., and Timmins., R. (2015). *Axis axis*: The IUCN Red List of Threatened Species 2015: e.T41783A22158006. doi:10.2305/IUCN.UK.2015-4.RLTS.T41783A22158006.en.

Online ISSN

3007-3197

Print ISSN

3007-3189

<http://amresearchreview.com/index.php/Journal/about>

- Eljarah, A., Ababneh, M., Jwasreh, K., and Ismail, Z. B. (2017). Estimation of genetic diversity of Arabian *Oryx* (*Oryx leucoryx*) in Wadi Rum area of Jordan by microsatellite markers. *The J. Anim. Plant Sci.* 27: 1861-1866.
- Ferreira, P. B., Torres, R. A., and Garcia, J. E. (2011). Single nucleotide polymorphisms from *Cyt b* gene as a useful protocol in forensic genetics against the illegal hunting of manatees: *Trichechus manatus*, *Trichechus inunguis*, *Trichechus senegalensis* and Dugong dugon (*Eutheria: Sirenia*). *Zoologia*, 28: 133-138.
- Galarza, J. A., Sánchez-Fernández, B., Fandos, P., and Soriguer, R. (2017). Intensive management and natural genetic variation in Red deer (*Cervus elaphus*). *J. Hered.* 108: 496-504.
- Groves, C. P. (1969). On the smaller gazelles on the genus *gazella de Blaindveli*. 1816 *Zeitschrift. Fur. Saugetierkunde*. 34: 38-60.
- Grubb, P. (2005). Artiodactyla. In: D.E. Wilson and D.M. Reeder (eds), *Mammal species of the world. A taxonomic and geographic reference*, (3rd ed), pp. 637-722. Johns Hopkins university press, Baltimore, USA.
- Grubb, P. (2005). "*Muntiacus muntjak*". In Wilson, D. E. Reeder, D. M. *Mammal Species of the World: A Taxonomic and Geographic Reference*, (3rd Ed.) Johns Hopkins University Press p. 667 ISBN 978-0-8018-8221-0. OCLC 62265494.
- Hassanin, A., and Douzery, E. J. P. (2003). Molecular and morphological phylogenies of ruminantia and the alternative position of the Moschidae. *Syst. Biol.* 52: 206-228.
- Hill, E., Linacre, A. M. T., Toop, S., Murphy, N. P., and Strugnell, J. M. (2017). The complete mitochondrial genome of *Axis porcinus* (Mammalia: cervidae) from Victoria, Australia, using MiSeq sequencing. *Mitochondrial DNA*. 2: 453-554.
- Kenneth, W. G. (1964). *The deer of Great Britain and Ireland: an account of their history, status and distribution*. London: Routledge & K. Paul. 597 pages and retrieved 6 September 2011.
- Khan, H. A., Arif, L. A., Al-Farhan, A. H., and AL-Homaidan, A. A. (2008). Phylogenetic analysis of *Oryx* species using partial sequences of mitochondrial *rRNA* genes. *Genet. Mol. Res.* 7: 1150-1155.
- Lachenmeier, K., and Melisch, R. (1996). The Bawean deer (*Axis kuhli*): status and protection of a little known deer species of Indonesia. *Zoological society for the conservation of species and populations*. 12: 23-25.
- Lerp, H., Klaus, S., Allgöwer, S., Wronski, T., Pfenninger, M., and Plathf., M. (2016). Phylogenetic analyses of gazelles reveal repeated transitions of key ecological traits and provide novel insights into the origin of the genus *Gazella*. *Mol. Phylogenet. Evol.* 98: 1-10.
- Masembe, C., Muwanika, V. B., Nyakaana, S., Arctander, P., and Siegismund, H. R. (2006). Three genetically divergent lineages of the *Oryx* in Eastern Africa: Evidence for an ancient introgressive hybridization. *Conserv Genet.* 7: 551-562.
- Matsuda, I., Murai, T., Clauss, M., Yamada, T., Tuuga, A., Bernard, H., and Higashi, S. (2011). "Regurgitation and re-mastication in the foregut-fermenting

Online ISSN

3007-3197

Print ISSN

3007-3189

<http://amresearchreview.com/index.php/Journal/about>

- proboscis monkey (*Nasalis larvatus*)". Biol Lett. 7: 786–789.
- Moore, G. H., and Littlejohn, R. P. (1989). "Hybridization of farmed wapiti (*Cervus elaphus manitobensis*) and red deer (*Cervus elaphus*)". New. Zeal. J. Zool. 16: 191–198.
- Nagy, Z. T. (2010). A hands-on overview of tissue preservation methods for molecular genetic analyses. Org. Divers. Evol. 10, 91–105
- Olsvik, O., Wahlberg, J., Petterson, B., Uhlen, M., Popovic, T., Wachsmuth, I. K., and Fields, P. I. (1993). Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit b in *Vibrio cholerae* 01 strains. J. Clin. Microbiol. 31. 22–25.
- Perez-Espona, S., Hall, R. J., Perez-Barberia, F. J., Glass, B. C., Ward, J. F., and Pemberton, J. M. (2013). The impact of past introductions on an iconic and economically important species, the Red deer of Scotland. J. Hered 104: 14–22.
- Pitra, C., Fickel, J., Meijaard, E., Groves, P. C. (2004). Evolution and phylogeny of Old world deer. Mol. Phylogenet. Evol. 33: 880–895.
- Purty, R. S., and Catterjee, S. (2016). DNA barcoding: An effective technique in molecular taxonomy. Austin. J. Biotechnol. Bioeng. 3:1059.
- Roberts, T. J. (1977). The Mammals of Pakistan. London and Tonbridge: Ernest Benn Limited (First Ed).
- Sambrook, J., and Russell, D. W. (2001). Molecular Cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
- Shackell, G. H., Drew, K. R., Pearse, A. J. T., and Amer, P. R. (2003). "A cost-benefit analysis of the value of investment in a wapiti hybridization research program and the returns to New Zealand venison producers". New. Zeal. J. Agr. Res. 46: 133–140.
- Stefano, G. D., and Petronio, C. (2000). "Systematics and evolution of the Eurasian Plio-Pleistocene tribe Cervini (Artiodactyla, Mammalia). Geol. Rom. 36: 311–334.
- Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512–526.
- Tamura, K., Nei, M., and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the Neighbor-joining method. Proc. Acad. Nat. Sci. 110: 11030–11035.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.
- Timmins, R. J., Duckworth, J. W., Samba, K. N., Islam, A., Baral, S., Long, B., and Maxwell, A. (2008). " *Axis porcinus*". IUCN red list of threatened species. Version 2008. International Union for Conservation of Nature. Retrieved 8 April 2009.
- Tobe, S. S., Kitchener, A. C., and Linacre, A. M. T. (2010). Reconstructing mammalian phylogenies: A detailed comparison of the *Cytochrome b* and *Cytochrome Oxidase* Subunit I Mitochondrial genes. Plos one. 30: 11.

Online ISSN

3007-3197

Print ISSN

3007-3189

<http://amresearchreview.com/index.php/Journal/about>

- Wang, W., and Lan., H. (2000). Rapid and parallel chromosomal reductions in *Muntjak* deer inferred from mitochondrial DNA phylogeny. *Mol. Biol. Evol.* 17: 1326-1333.
- Xiang-Rong, C. A. O., Feng-Jue, S. H. U., Xi-Ran, Z., Chun-Ming, B. I., Chao-Jun, L. I., Jun, H. U., and Jianyan., F. (2002). Phylogenetic relationships of *Elaphodus cephalophus* and three *Muntiacus* species revealed by mitochondrial *Cyt b* nucleotide sequence. *Curr. Zool.* 48: 44-49.