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Short Communication

Mitochondrial *Cyt-b* and *Cox1* Genes Based Molecular Diversity and Phylogenetic Analysis of Chukar Partridge (*Alectoris chukar*)

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ABSTRACT

The taxonomy of *Alectoris chukar (A. chukar)* has been a complex debate with different classifications and revisions suggested based on their morphology, geographical distribution and chromosome number. In Pakistan the Chukar partridge *(A.chukar)*), is an important member of Phasianidae family, however a scarce molecular data is reported that urged us to investigate its genetic diversity and phylogeny using mitochondrial DNA, *Cyt-b* and *Cox1* genes. A total of 749bp of *Cox1* and 472 bp of *Cyt-b* complete coding regions of both genes were amplified by PCR followed by sequencing. The sequences were aligned and edited using Bio-Edit software and single nucleotide polymorphisms (SNPs) were identified. The boot strapped Neighbor joining tree constructed from MEGA7 explained the genetic relationships of *A.chukar* with related members of Phasianidae family. The phylogenetic analysis also showed the genetic positioning of *A. chukar* with respect to other different reported species as well. The study gave us useful genomic information about genetic diversity in *A. chukar* and its phylogenetic relationships with related taxa, emphasizing on need of execution of conservation strategies to protect this unique genetic resource of Pakistan.

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MK and ZF collected blood samples and perfumed PCR. KA and TH helped in PCR and data analysis. MK and KA wrote the article.

Key words
Chukar, Cyt-b, COX1, Phylogenetic, PCR

Chukar Partridge (*Alectoris chukar)* is national bird of Pakistan. This 13-15 inches long medium sized bird (Nowaczewski *et al*., 2014) belongs to family Phasianidae (Kerr *et al*., 2009; Shen *et al*., 2010; Sutherland *et al*., 2004) consisting of 16 reported sub species (Song and Liu, 2013). *A. chukar* shows most similarity to *Alectoris rufa (*also known as red- legged partridge) which exists in Western areas of the world (Barbanera *et al*., 2007; Christensen, 1996). Worldwide it is present in Palestine, Turkey, Iran, Lebanon, India, Central Nepal, Middle East, Afghanistan, Pakistan, Western Himalayas, Europe, Africa, Israel, Jordan and Dead Sea area (Grewal and Bhatia, 2017; Whistler, 2007; Baker, 1928). In Pakistan it is distributed in Sindh, Salt Range, Chitral, Swat, Kohistan, Gilgit, Punjab, Baluchistan, Sindh and Azad Jammu Kashmir (Pathan *et al*., 2014). According to North American Breeding Bird Survey, Chukar populations have been stable and are slightly increasing, since being introduced into North America (Christensen, 1996) so it's at the status of Least Concern (LC) (IUCN Red-list 2010) and there are no widespread conservation measures in place for this bird (Bird Life International). But for Pakistan its importance being our unique genetic national resource cannot be denied.

mtDNA has many advantageous reasons to be chosen above other markers for phylogenetic analysis and molecular diversity studies (Hussain *et al*., 2015). In this study we explored mitochondrial *Cyt-b* and *Cox1* genes in *A. chukar* from different locations of Pakistan to have insight about its genetic architecture by measuring polymorphism and phylogenetic relationships within *A. chukar* and related Phasianidae family members.

Materials and methods

Blood samples (n=30) were collected from *A.chukar* of Bahawalpur Zoo and Gatwala Wildlife Park Faisalabad with the support and permission from Pakistan Wildlife Foundation (PWF). The DNA was extracted using standard organic method (Sambrook and Russell, 2006) DNA samples concentration was measured using gel electrophoresis.

A specific pair of primers for *Cytb*-Fw5'TACCATGAGGACAAATATCATTCTG Rev5' CCTCCTAGTTTGTTAGGGATTGATCG) was taken from Naseer *et al*. (2018) and *Cox1*-Fw5'TCTCAACCAACCACAARGAYATYGG

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Rev5'TAGACTTCTGGGTGGCCRAARAAYCA was taken from Hassanin *et al*. (2012). The amplified 472bp product was for *Cyt-b* and 749bp for *Cox1*.

For the amplification of mitochondrial *Cyt-b* and *Cox1* genes the PCR was carried out using BioRad (USA) thermocycler in a reaction volume of 25 µl containing genomic DNA, PCR buffer, dNTPs, MgCl2, forward and reverse primers, *Taq* DNA polymerase (BioRon , Germany) and nuclease-free water. The conditions used were for *Cyt-b:* initial denaturation 95˚C for 5 min, followed by 5 cycles of 95˚C for 45 sec; 45˚C for 1 min 72˚C for 1 min, then 30 cycles of 95˚C for 45 sec, 48ºC for 1min and 72˚C for 1 min and final extension at 72˚C for 10 min. For *Cox1:* initial denaturation 94˚C for 3 min, followed by 10 cycles (with 1 ºC decrease in each cycle) of 94˚C for 1 min; 65˚C for 1 min and 72˚C for 1 min, then 25 cycles of 94˚C for 1 min, 55ºC for 1min and 72˚C for 1 min and final extension at 72˚C for 7 min. The PCR products (3 µL of PCR product and 2µL of loading dye mixed) were run on 1.2 % Agarose gel at 90 Voltages for 35 min in 1X TAE buffer and seen by gel documentation system (Bio Rad, USA) under UV light. The positive samples were sent for sequencing to 1st Base Laboratories Singapore. The obtained sequences were aligned with the help of online NCBI BLAST (http/www.ncbi.nlm.nih.com) to see relevant reported sequences. The sequences were edited and assembled through Bio-Edit software (Hall, 1999) for the identification of single nucleotide polymorphisms. DnaSP v. 5software (Librado and Rozas, 2009) was used to reconfirm SNPs and to observe haplotypes. MEGA7 program package was used (Tamura *et al*., 2013) to construct Neighbor-Joining (Saitou and Nei, 1987) evolutionary trees ((1000 bootstrap value) for Alectoris chukar and other the related taxa assembled together are shown next to the branch as a percentage of replicate trees (Felsenstein, 1985). The Maximum Composite Likelihood method was used to calculate evolutionary distances (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site.

Results

SNPs were identified by using DnaSP (Librado and Rozas, 2009) in Alectoris chukar in 423bp and 599 bp of *Cyt-b* and *Cox1* respectively. In *Cyt-b* there were 412 invariable (monomorphic) sites while variable (polymorphic) sites found are only 11 (53, 56, 86, 94, 124, 136, 161, 190, 198, 258, 271) which were all parsimony informative sites with two variants and there was no Singleton variable site. In *Cox1* there were 517 invariable (monomorphic) sites while 73 variable (polymorphic) were observed out of which 61 were Singleton variable sites, 12 Parsimony informative sites. (Table I).

Table I. Single nucleotide polymorphisms (SNPs) identified in 599 bp fragment of *Cox1* in 20 samples of *Alectoris chukar.*

Nucleotide Position	Variation
2, 3, 4, 8, 11, 21, 22, 30, 53, 56, 57, 63, 64, 66, 69, 73, 78, 81, 86, 93, 99, 103, 112, 114, 117, 142, 144, 149, 152, 157, 161, 166, 168, 169, 178, 194, 195, 197, 199, 208, 224, 228, 256, 258, 261, 271, 274, 276, 303, 330, 445, 484, 554, 574, 575, 577, 587, 589, 590, 593.	Singleton variable sites of (two variants)
7, 14, 15, 27, 34, 39, 128, 198, 200, 211, 223	Parsimony informative sites (two variants)
44	Singleton variable sites (three variants)
192	Parsimony informative sites (three variants)

In *Alectoris chukar* for *Cyt-b* gene 11 haplotypes (h) with 1.0000 haplotype (gene) diversity (hd) , variance of Haplotype diversity was found 0.00150, with standard deviation of 0.039, and per site nucleotide diversity (Pi) 0.49587, The sampling variance of Pi was calculated as 0.0016083 and standard deviation of Pi was 0.04010 and in *Cox1* gene 10 haplotypes (h) with 0.711 haplotype (gene) diversity (hd), variance of haplotype diversity was found 0.01288, with standard deviation of 0.113 and per site nucleotide diversity (Pi) 0.01509, The sampling variance of Pi was calculated as 0.0000476 and standard deviation of Pi was 0.00690.

For *Cyt-b the o*verall genetic distance among all *Alectoris chukar* sequences 0.0142. The evolutionary history was inferred using the neighbor-Joining method. The optimal tree with the sum of branch length = 0.04688547 (Supplementary Fig. 1). For *Cox1* the overall genetic distance among all *Alectoris chukar* sequences 0.0147. Optimal branch length of the tree is 0.41892384 (Supplementary Fig. 2). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The genetic distance comparison between sample Chukars and other sequences in Phasianidae family obtained from NCBI was performed for both *Cox1* and *Cytb* genes. The analysis showed that for *Cytb* there is 90% to 99% (Table II) similarity amongst sample sequences and for *Cox 1* there is 93% to 100 % similarity observed (Table II).

Table II. Comparison of *Alectoris chukar* samples sequences with sequences of Phasianidae family available in NCBI for *Cyt-b and Cox1*.

Samples	Similarity (%)	Accession #
Cyt-b		
A. chukar _ 1_Cytochrome b	95%	GU214294.1
A. chukar _ 2_Cytochrome b	99%	KY829450.1
A. chukar _ 3_Cytochrome b	94%	AM850828.1
A. chukar _ 4_Cytochrome b	93%	FJ752426.1
A. chukar _ 5_Cytochrome b	99%	GU214293.1
A. chukar _ 6_Cytochrome b	98%	GU214292.1
A. chukar _ 7_Cytochrome b	94%	AM850784.1
A. chukar _ 8_Cytochrome b	93%	AM850752.1
A. chukar _ 9_Cytochrome b	97%	EU893475.1
A. chukar _ 10_Cytochrome b	96%	AM850788.1
A. chukar _ 11_Cytochrome b	90%	AM850752.1
Cox1		
Chukar_1_Cox1	99%	KY829450.1
Chukar_2_Cox1	97%	KT806484.1
Chukar_3_Cox1	93%	JF498827.1
Chukar_4_Cox1	99%	JF498826.1
Chukar_5_Cox1	99%	GQ481315.1
Chukar_6_Cox1	99%	DQ432706.1
Chukar_7_Cox1	99%	AY666409.1
Chukar_8_Cox1	98%	GU951807.1
Chukar_9_Cox1	99%	FJ808621.1
Chukar_10_Cox1	99%	KT803621.1
Chukar_11_Cox1	99%	GQ481314.1
Chukar_12_Cox1	99%	AY666409.1
Chukar_13_Cox1	99%	JF498828.1
Chukar_14_Cox1	100%	DQ432706.1
Chukar_15_Cox1	98%	FJ465298.1
Chukar_16_Cox1	99%	KT806484.1
Chukar_17_Cox1	100%	DQ432706.1
Chukar_18_Cox1	99%	JF498827.1
Chukar_19_Cox1	99%	KT803621.1
Chukar_20_Cox1	100%	DQ432706.1

Discussion

According to North American Breeding Bird Survey, Chukar populations have been stable and are slightly increasing, since being introduced into North America (Christensen, 1996). Although in some areas it has been affected by habitat destruction, for example in Azerbaijan. In Turkey it has been affected due to pesticide usage, while in USA and Canada hunters and poachers can be a source of discomfort for this bird (McCarthy, 2006). Overall in world it has a large range and stable population, so is not currently considered to be at risk of extinction. Chukar has a status of Least Concern (LC) according to International Union of Conservation of Nature (IUCN Red-list 2016) and there are no widespread conservation measures in place for this bird some proposed conservation actions include assessment of the impacts of pesticides on this species and identification of key areas of habitat for this species to be introduced, protected and expanded. Chukar is our National Bird so it has a significant importance and its conservation for future is need of our country.

In the past, this important bird has not been considered for its characterization and evaluation level at which this study has been conducted. Previously mtDNA has been widely used to investigate closely related animals (Achilli *et al*., 2012; Achilli *et al*., 2009). Here, DNA samples of several species were isolated and the molecular diversity and phylogenetic were explored using *Cyt-b* and *Cox1* mitochondrial genes in *Alectoris chukar* from different areas of Pakistan. The phylogenetic analysis showed that Chukar have adapted to the environment independently according to their unique capacity which represents that they have achieved lineage specific variations in that particular genetic region.

Conclusion

As being national bird of Pakistan, Chukar must be considered as a rare animal to get attention for suitable conservation actions. This study demonstrates the genetic diversification and phylogenetic differentiations of this unique bird. Our study provided useful material for supporting conservation strategies and breeding plans for this important bird, however further genomic investigations should be carried out at larger scale.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20181011191004

Statement of conflict of Interest

The authors have declared no conflict of interest.

References

Achilli, A., Bonfiglio, S., Olivieri, A., Malusa, A., Pala,

M., Kashani, B.H. and Semino, O., 2009. *PLoS One,* **4**: e5753. https://doi.org/10.1371/journal.pone.0005753

Achilli, A., Olivieri, A., Soares, P., Lancioni, H., Kashani, B.H., Perego, U.A. and Capomaccio, S., 2012. *Proc. natl. Acad. Sci.,* **109**: 2449-2454. https://doi.org/10.1073/pnas.1111637109

Baker, E.S., 1928. Fauna of British India-birds, Vol, IV. *Indian Forest,* **54**: 183-184.

Barbanera, F., Guerrini, M., Hadjigerou, P., Panayides, P., Sokos, C., Wilkinson, P. and Dini, F., 2007. *Genetica,* **131**: 287-298. https://doi.org/10.1007/s10709-006-9138-x

Christensen, G.C., 1996. *Chukar:* Alectoris chukar. American Ornithologists' Union. https://doi.org/10.2173/bna.258

Felsenstein, J., 1985. *Evolution,* **39**: 783-791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Grewal, B. and Bhatia, G., 2017. *A photographic field guide to the birds of India, Pakistan, Nepal, Bhutan, Sri Lanka, and Bangladesh*. Princeton University Press.

Hall, T.A., 1999. *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* Paper presented at the Nucleic acids symposium series.

Hassanin, A., Delsuc, F., Ropiquet, A., Hammer, C., van Vuuren, B.J., Matthee, C. and Nguyen, T.T., 2012. *C. R. Biol.,* **335**: 32-50. https://doi.org/10.1016/j.crvi.2011.11.002

Hussain, T., Babar, M., Musthafa, M., Saif, R., Hussain, F., Aqeel, M. and Ziaullah, S., 2015. *J. Anim. Pl. Sci.,* **25**: 311-318.

Kerr, K.C., Birks, S.M., Kalyakin, M.V., Red'kin, Y.A., Koblik, E.A. and Hebert, P.D., 2009. *Front. Zool.,* **6**: 29. https://doi.org/10.1186/1742-9994-6-29

Librado, P. and Rozas, J., 2009. *Bioinformatics,* **25**: 1451-1452. https://doi.org/10.1093/bioinformatics/btp187

McCarthy, E.M., 2006. *Handbook of avian hybrids of the world*. Oxford University Press, USA.

Naseer, J., Anjum, K.M., Khan, W.A., Imran, M., Ishaque, M., Hafeez, S. and Nazir, M.A., 2018. *Indian J. Anim. Res.,* **52**: 343-346. https://doi.org/10.18805/ijar.v0iOF.9150

Nowaczewski, S., Kolanos, B., Krystianiak, S., Kontecka, H. and Gorecki, M., 2014. *Bulgarian J. agric. Sci.,* **20**: 962-966.

Pathan, A.J., Khan, S., Akhtar, N. and Saeed, K., 2014. *Adv. Zool*., **2014**: 1-7. https://doi.org/10.1155/2014/430297

Saitou, N. and Nei, M., 1987. *Mol. Biol. Evolut.,* **4**: 406-425.

Sambrook, J. and Russell, D.W., 2006. *Cold Spring Harbor Protoc.,* pii: pdb.prot4455 https://doi.org/10.1101/pdb.prot4455

Shen, Y.Y., Liang, L., Sun, Y.B., Yue, B.S., Yang, X.J., Murphy, R.W. and Zhang, Y.P., 2010. *BMC Evolut. Biol.,* **10**: 132. https://doi.org/10.1186/1471-2148-10-132

Song, S. and Liu, N., 2013. *Acta Ecol. Sin.,* **33**: 4215-4225. https://doi.org/10.5846/stxb201205010624

Sutherland, W.J., Newton, I. and Green, R., 2004. *Bird ecology and conservation: a handbook of techniques*. OUP Oxford. https://doi.org/10.1093/acprof:oso/9780198520863.001.0001

Tamura, K., Nei, M. and Kumar, S., 2004. *Proc. natl. Acad. Sci. USA.,* **101**: 11030-11035. https://doi.org/10.1073/pnas.0404206101

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. *Mol. Biol. Evolut.,* **30**: 2725-2729. https://doi.org/10.1093/molbev/mst197

Whistler, H., 2007. *Popular handbook of Indian birds.* Read Books, London.