VETERINARSKI ARHIV 80 (5), 653-661, 2010

Identification of avian species using polymerase chain reaction and sequence analysis of mitochondrial 12S rRNA gene

Nagappa S. Karabasanavar[1]*, Suresh P. Singh[1], Sunil N. Shebannavar[2], Angadi M. Kotresh[3], Patil S. Girish[4], and Pillai V. Umapathi[5]

[1]Department of Veterinary Public Health, College of Veterinary and Animal Sciences, G. B. Pant University of Agriculture and Technology, Patnagar, Uttarkhand, India

[2]Gennova Biopharmaceutical Pvt. LTD. Pune, Maharashtra, India

[3]Department of Veterinary Biochemistry, Veterinary College, Karnataka Veterinary, Animal and Fishery Sciences University (KVAFSU) Shivamogga, Karnataka, India

[4]NRC on Meat, Indian Council of Agricultural Research, Hyderabad, Andra Pradesh, India

[5]Indian Council of Agricultural Research, Palampur, Himachal Pradesh, India

---

KARABASANAVAR, N. S., S. P. SINGH, S. N. SHEBANNAVAR, A. M. KOTRESH, P. S. GIRISH, P. V. UMAPATHI: Identification of avian species using polymerase chain reaction and sequence analysis of mitochondrial 12S rRNA gene. Vet. arhiv 80, 653-661, 2010.

ABSTRACT

Authentic identification and differentiation of avian species is a vital step in conservative, taxonomic, forensic, legal and other ornithological interventions. The present investigation involved the application of molecular biological approach to identify and differentiate avian species i.e. two species of birds, namely black kite (*Milvus migrans*) and parakeet (*Psittacula krameri*). The DNA was isolated from blood samples of each species and a part of the mitochondrial 12S rRNA gene was amplified through polymerase chain reaction (PCR). The PCR products were sequenced and aligned using Basic Local Alignment Search Tool (BLAST) of the GenBank (NCBI). Based on the alignment and similarity/divergence, these avian species were accurately identified and differentiated.

Key words: 12S rRNA gene, polymerase chain reaction, sequence analysis, avian species

---

Introduction

The task of correct morphological identification of avian species at times poses a challenge to investigators since there are numerous avian species, ultimately demanding

*Corresponding author:

Dr. Nagappa S. Karabasanavar M.V.Sc, Ph.D, Assistant Professor, Department of Veterinary Public Health, Mumbai Veterinary College, Parel, Mumbai-400 012, Maharashtra, India, E-mail: docnagappa@rediffmail.com

ISSN 0372-5480
Printed in Croatia

intervention of systematic, morphological and taxonomic ornithologists. However, consequent to the developments in the field of molecular biology, genome analysis has paved alternative ways. Now it has become an easy task even to less skilled persons to identify avian species correctly, since an enormous amount of data pertaining to the DNA sequence of a species is available for comparison in a database in the public domain (NCBI, EMBL, etc). Further, ever-evolving, consistent and comprehensive research in avian mitochondrial (mt) genomics is paving the way for their application in avian species identification (KOCHER et al., 1989; AVISE et al., 1994; TREWICK 1997; EDWARDS et al., 2005). Keeping this in view, the present investigation was undertaken with the objective of establishing the value of PCR amplification of the mitochondrial 12S rRNA gene with universal primers, amplicon sequencing and its sequence analysis technique, for the authentic identification of avian species, so that problems in the field that require identification of avian species may be easily solved and that this in turn would enhance the conservation of birds in the wild.

Materials and methods

DNA extraction. Blood samples (~2 mL) from black kite (*M. migrans*) and parakeet (*P. krameri*) were collected in EDTA (0.2 mL, 0.5 M) from wing veins and erythrocytes were lysed. The white blood cell pellet was used as a source of DNA. Total DNA was extracted from the white blood cells using the Wizard® Genome DNA purification kit (Promega, USA) as per the manufacturers' instructions Briefly, the WBC pellet was placed in a 1.5 mL micro centrifuge tube; a digestion solution (275 µL) was added to each tube consisting of 200 µL of nuclei lysis solution, 50 µL of 0.5 M EDTA (pH 8), 20 µL of proteinase K (20 mg/mL) and 5 µL of RNase A (4 mg/mL). After incubation (16-18 hrs, at 55 °C on a dry bath), 250 µL of Wizard® SV lysis buffer was added; vertexed and the contents were loaded onto Wizard® SV mini column assembly to be centrifuged at 10,000 rpm for 3 minutes. The mini columns were washed 4-6 times with 650 µL of Wizard® SV wash solution and dried by centrifugation for 2 min. The mini columns were then transferred to fresh 1.5 mL tubes; 250 µL of nuclease-free water (NFW) was added; incubated for 2 min at room temperature and centrifuged for 2 minutes, and this was repeated with an additional 250 µL of NFW. The pooled elutes were collected for use or stored at -20 °C until further use.

Agarose gel (0.8%, w/v) electrophoresis was performed to check the quality of total DNA. Ethidium bromide stained DNA bands were visualized under UV transilluminator and the gel was documented to evaluate the quality. The purity of the isolated DNA was checked by taking the OD_{260}/OD_{280} ratio and the concentration was calculated as per the standard protocols (SAMBROOK and RUSSEL, 2001).

Amplification of mt 12S rRNA gene by PCR. The PCR was performed using universal primers flanking the mt 12S rRNA gene. In a 25 µL reaction volume, based on the initial trials, the PCR reaction mixture was optimized as follows: 2.5 µL of 10X assay buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM $MgCl_2$ from Bioron, GmbH, Germany), 0.5 µL (200 µM each) of dNTP mix (sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5 from Promega, Madison, WI USA), 0.5 µL or 20 Pico moles of forward (5′-CAA CTG GGA TTA GAT ACC CCA CTA T-3′) and reverse (5′-GAG GGT GAC GGG CGG TGT GT-3′) primers (KOCHER et al., 1989) procured from Integrated DNA Technologies (IDT), 1 U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH, Germany), 50 ng of purified DNA and autoclaved NFW to make up the volume. The PCR tube containing the reaction mixture was flash spun and amplification was performed in a Thermal cycler (Applied Biosystems).

The cycling conditions were optimized for the initial denaturation (94 °C for 5 min) followed by 30 cycles of denaturation (94 °C for 45 s), primer annealing (60 °C for 45 s) and elongation (72 °C for 1 min). After the final extension (72 °C for 5 min), the PCR products were electrophoresed (agarose gel 2%) with a 100 bp marker and the amplicons were sequenced.

Sequencing of the PCR products. The PCR products were custom sequenced using ABI DNA sequencer- inst model/name/3100/ABI3100-1699-013 (Bangalore Genei, India). Both black kite and parakeet sequences were submitted to EMBL nucleotide sequence database and related sequences were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov) for comparison.

Alignment of amplified mt 12S rRNA gene sequences using BLAST. The sequences were aligned using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and the nearest species were identified. Closely related species` mitochondrial 12S rRNA gene sequences were downloaded from the GenBank database; and aligned by the '*Clustal'* method of '*Megalign'* software (DNAStar Inc., 1996). Afterwards the related species (Table 1) were considered for similarity/divergence and phylogenetic analysis.

Results

The PCR amplification of the mitochondrial (mt) 12S rRNA gene using universal primers yielded a product of 456 bp in both black kite and parakeet (Fig. 1). The amplified product was sequenced and a partial sequence of the mt 12S rRNA gene of black kite (*Milvus migrans*) with accession number AM778107 was aligned and compared through the *Clustal* method (DNAstar) with the related species (Table 1).

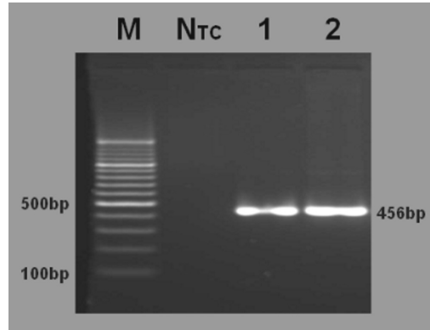

Fig. 1. Amplification of a mt 12S rRNA gene fragment from 1. Parakeet (*Psittacula krameri*), and 2. Black Kite (*Milvus migrans*). M- 100 bp molecular weight marker, NTC- No template control.

Table 1. Mitochondrial 12S rRNA gene sequences of black kite and parakeet and their comparison with similar avian species

Common name	Latin Name	Bases aligned	Accession No.
Black Kite	*Milvus migrans*	21-392	AM778107
Black Kite	*Milvus migrans lineatus*	480-851	AB219546
Black Kite	*Milvus migrans*	480-851	U83721
White-tailed Eagle	*Haliaeetus albicilla albicilla*	482-852	AB219552
Steller's Sea Eagle	*Haliaeetus pelagicus pelagicus*	481-852	AB219553
The White-bellied Sea-eagle	*Haliaeetus leucogaster*	22-392	U88011
Bald Eagle	*Haliaeetus leucocephalus*	480-850	U83722
Blue-bellied Parrot	*Triclaria malachitacea*	493-881	DQ143222
Derbyan parakeet	*Psittacula derbiana*	6-392	U88021
Rose-ringed or Ring-necked Parakeet	*Psittacula krameri*	8-391	AM778108
White-crowned Parrot or Plum-crowned Pionus	*Pionus senilis*	459-846	U89233
Yellow bellied parrot	*Amazona collaria*	2-389	AY301342
Kawall's Parrot	*Amazona kawalli*	2-389	AY301352
Cuban Parrot	*Amazona leucocephala*	2-389	AY301353
Monk or Quaker Parakeet	*Myiopsitta monachus*	499-888	AF362932
Scaly-headed Parrot	*Pionus maximiliani*	492-879	DQ143220
Pesquet's Parrot / Vulturine Parrot	*Psittrichas fulgidus*	459-848	U89231

The comparison revealed that *M. migrans* with accession number AM778108 was closely related to *Haliaeetus pelagicus pelagicus* (92.5%), *Haliaeetus albicilla albicilla* (91.6%), *Haliaeetus leucogaster* (91.1%) and *Haliaeetus leucocephalus* (90.8%). The phylogenetic relationship and similarity/divergence among these species are shown in Table 2 and Fig. 2.

Table 2. Percent similarity and divergence between black kite and related avian species

		Percent Similarity								
		1	2	3	4	5	6	7		
Percent Divergence	1		98.9	99.2	94.9	91.6	91.6	91.6	1	*Haliaeetus albicilla albicilla* AB219552
	2	1.1		99.7	93.8	90.8	90.8	90.8	2	*Haliaeetus leucocephalus* U83722
	3	0.8	0.3		94.1	91.1	91.1	91.1	3	*Haliaeetus leucogaster* U88011
	4	3.3	4.5	4.2		92.5	92.5	92.5	4	*Haliaeetus pelagicus pelagicus* AB219553
	5	7.2	8.1	7.8	7.2		100.0	100.0	5	*Milvus migrans lineatus* AB219546
	6	7.2	8.1	7.8	7.2	0.0		100.0	6	*Milvus migrans* U83721
	7	7.2	8.1	7.8	7.2	0.0	0.0		7	*Milvus migrans* AM778107
		1	2	3	4	5	6	7		

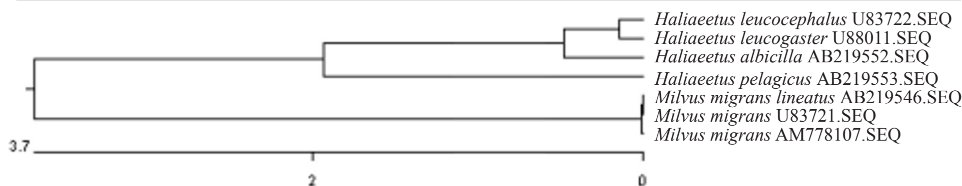

Fig. 2. Phylogenetic tree of black kite and its comparison with other related species

Table 3. Similarity and divergence of *Psittacula krameri* based on mt 12S rRNA gene sequence with closely related species

		Percent Similarity											
		1	2	3	4	5	6	7	8	9	10		
Percent Divergence	1		80.1	80.5	87.9	87.6	88.4	87.6	100.0	88.4	84.1	1	*Triclaria malachitacea* DQ143222
	2	12.0		87.5	84.4	84.4	84.6	84.4	80.1	84.6	83.3	2	*Psittacula derbiana* U88021
	3	13.1	6.5		84.1	83.3	84.4	83.3	80.5	84.4	82.3	3	*Psittacula krameri* AM778108
	4	8.9	10.1	9.6		96.9	98.2	96.9	87.9	98.2	87.3	4	*Pionus senilis* U89233
	5	9.2	10.4	9.9	3.2		97.2	100.0	87.6	97.2	88.7	5	*Amazona collaria* AY301342
	6	8.3	9.5	9.6	1.6	2.6		97.2	88.4	100.0	88.1	6	*Amazona kawalli* AY301352
	7	9.2	10.4	9.9	3.2	0.0	2.6		87.6	97.2	88.7	7	*Amazona leucocephala* AY301353
	8	0.0	12.0	13.1	8.9	9.2	8.3	9.2		88.4	84.1	8	*Myiopsitta monachus* AF362932
	9	8.3	9.5	9.6	1.6	2.6	0.0	2.6	8.3		88.1	9	*Pionus maximiliani* DQ143220
	10	10.3	10.2	9.3	8.9	7.4	7.7	7.4	10.3	7.7		10	*Psittrichas fulgidus* U89231
		1	2	3	4	5	6	7	8	9	10		

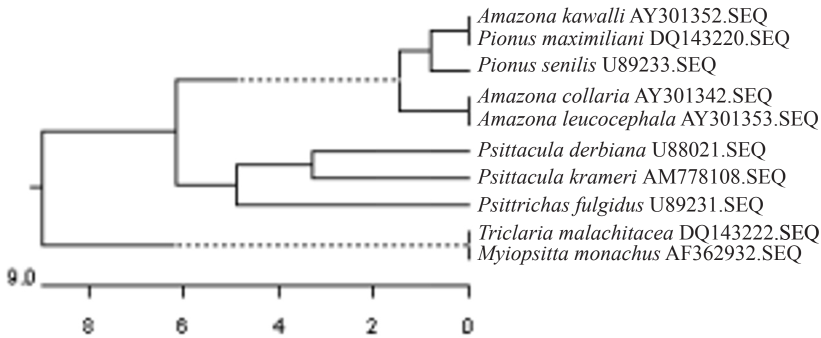

Fig. 3. Phylogenetic relationship of parakeet (*Psittacula krameri*) with closely related parakeets / parrots based on the mt 12S rRNA gene sequence

Similarly, in the case of the parakeet (*Psittacula krameri*), based on the partial mt 12S rRNA gene sequence alignment, similarity/divergence and phylogenetic relationships (Table 3 and Fig. 3), the most closely related species were *Psittacula derbiana* (87.5%), *Amazona kawalli* and *Pionus maximiliani* (84.4%), *Pionus senilis* (84.1%), *Amazona collaria* and *Amazona leucocephala* (83.3), *Psittrichas fulgidus* (82.3), *Myiopsitta monachus* and *Triclaria malachitacea* (80.5%).

Discussion

Mitochondria possess extra-chromosomal DNA and have many desirable features such as a high copy number (2-10 copies per cell and as many as 1,000 mitochondria per somatic cell) compared to just a single (or a few) copy of nuclear gene(s). Hence, mitochondrial DNA sequences are preferred for forensic, zoological and molecular analysis of avian species (HARLID et al., 1997; LEE et al., 1997; MINDELL et al., 1999; BUDOWLE et al., 2003). The recent developments in the field of molecular biology have revolutionized wildlife forensics. For instance, a wildlife crime was detected by PCR amplification of the mt gene and its sequence analysis for the identification of an endangered and protected national bird of India, i.e. the peafowl (GUPTA et al., 2005). Similarly, on the basis of phylogenetic relationships, various species have been identified to solve the taxonomic puzzle (HEDGES et al., 1995).

Fortunately, mitochondrial DNA sequences of most avian species are presently available (http://www.ncbi.nlm.nih.gov). Further, PCR amplification and sequencing of mitochondrial DNA using universal primers has been a reliable tool for the forensic analysis of samples in many laboratories (KOCHER et al., 1989; BARTLETT and DAVIDSON, 1992; ZEHNER et al., 1998; PARSON et al., 2000; VERMA et al., 2003; VERMA and SINGH 2003; GUPTA et al., 2005). For instance, the increasing international market for a few

costly forms of avian meat and products often raises the problem of adulteration. Hence, COLOMBO et al. (2000) differentiated ostrich and emu by using PCR, targeting the mt genes. The 12S rRNA has been proven to be a good candidate for the identification of species for such forensic analysis (PRAKASH et al., 2000; SHUKLA et al., 2001; GIRISH et al., 2004).

The limitation of this approach could be the non-availability of the sequence of a rare avian species in the database. Hence, prior authentic availability of the 12S rRNA gene sequence in the database is a mandatory criterion. Also, for confirmation, a second alternative tool is necessary to increase the validity of the results in a court of law. However, recent developments in varied fields of molecular biology have left no species behind, and the mitochondrial DNA sequences of almost all known species are available in the databases, making the mt DNA a forerunner in forensic/ taxonomic analysis.

The population of many species has declined and hence their status has reached critical limits. In order to strengthen legislation and assist in conservation of wild avi-fauna we need to have reliable taxonomic/ forensic analysis tools. In this direction the approach given in this article will provide a comprehensive insight for the identification and differentiation of an unknown avian species. To conclude, PCR amplification of the mt 12S rRNA gene, amplicon sequencing and sequence analysis would help to solve the problem of identification of an avian species unambiguously.

Acknowledgements

The authors thank the Dean, College of Veterinary and Animal Sciences, and the Director, Experiment Station, G. B. Pant University of Agriculture and Technology, Pantnagar for providing the necessary laboratory facilities for the conduct of this study. The authors also thank the authorities and staff of Pt. G. B. Pant High Altitude Zoo, Nainital, Uttarakhand, for providing the samples.

References

AVISE, J. C., W. S. NELSON, C. G. SIBLEYT (1994): DNA sequence support for a close phylogenetic relationship between some storks and New World vultures. Proc. Natl. Acad. Sci. USA 91, 5173-5177.

BARTLETT, S. E., W. S. DAVIDSON (1992): FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. Biotechniques 12, 408-411.

BUDOWLE, B., M. W. ALLARD, M. R. WILSON, R. CHAKRABORTY (2003): Forensics and mitochondrial DNA: applications, debates, and foundations. Annu. Rev. Genomics Hum. Genet. 4, 119-141.

COLOMBO, F., R. VIACAVA, M. GRIARETTI (2000): Differentiation of the ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*) species by polymerase chain reaction using an ostrich-specific primer pair. Meat Sci. 56, 15-17.

EDWARDS, S. V., S. B. KINGAN, J. D. CALKINS, C. N. BALAKRISHNAN, W. B. JENNINGS, W. J. SWANSON, M. D. SORENSON (2005): Speciation in birds: genes, geography, and sexual selection. Proc. Natl. Acad. Sci. USA 102, 6550-6557.

GIRISH, P. S., A. S. R. ANJANEYULU, K. N. VISWAS, M. ANAND, N. RAJKUMAR, B. M. SHIVAKUMAR, B. SHARMA (2004): Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat Sci. 66, 551-556.

GUPTA, S. K., S. K. VERMA, L. SINGH (2005): Molecular insight into a wildlife crime: the case of a peafowl slaughter. Forensic Sci. Int. 154, 214-217.

HARLID, A., A. JANKE, U. ARNASON (1997): The mt DNA sequence of the ostrich and the divergence between paleognathous and neognathous birds. Mol. Biol. and Evol. 14, 754-761.

HEDGES, S. B., M. D. SIMMONS, M. A. M. VAN DUK, G. J. CASPERS, W. W. DE JONG, C. G. SIBLEY (1995): Phylogenetic relationships of the hoatzin: an enigmatic South American bird. Proc. Natl. Acad. Sci. USA 92, 11662-11665.

KOCHER, T. D., W. K. THOMAS, A. MEYER, S. V. EDWARDS, S. PAABO, F. X. VILLABLANCA, A. C. WILSON (1989): Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196-6200.

LEE, K., J. FEINSTEIN, J. CRACRAFT (1997): Phylogenetic relationships of the ratite birds: resolving conflicts between molecular and morphological data sets. In: Avian Molecular Evolution and Systmatics. (Mindell, D. P., Ed.). Academic Press. New York.

MINDELL, D. P., M. D. SORENSON, D. E. DIMCHEFF, M. HASEGAWA, J. C. AST, T. TURI (1999): Interordinal relationships of birds and other reptiles based on whole mitochondrial genomes. Syst. Biol. 48, 138-152.

PARSON, W., K. PEGORARO, H. NEIDERSTATTER, M. FOGER, M. STEINLECHNER (2000): Species identification by means of cytochrome b gene. Int. J. Legal Med. 114, 23-28.

PRAKASH, P. S., M. S. GHUMATKAR, S. V. NANDODE, S. K. YOGESH, Y. S. SHOUCHE (2000): Mitochondrial 12S rRNA sequence analysis in wild life forensics. Current Sci. 78, 1239-1241.

SAMBROOK, J., D. W. RUSSELL (2001): Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

SHUKLA, M. S., V. PIDIYAR, N. BHAVE, M. S. PATOLE, Y. S. SHOUCHE (2001): PCR amplification and sequencing of mitochondrial 12S rRNA gene fragment from *Muntiacus muntjak* (Indian muntjak). Current Sci. 80, 617-618.

TREWICK, S. A. (1997): Flightlessness and phylogeny amongst endemic rails (Aves: Rallidae) of the New Zealand region. Phil. Trans. R. Soc. Lond. B. 352, 429-446.

VERMA, S. K., K. PRASAD, N. NAGESH, M. SULTANA, L. SINGH (2003): Was elusive carnivore a panther? DNA typing of faeces reveals the mystery. Forensic Sci. Int. 137, 16-20.

VERMA, S. K., L. SINGH (2003): Novel universal primers establish identity of enormous number of animal species for forensic application. Mol. Ecol. Notes 3, 28-31.

ZEHNER, R., S. ZIMMERMANN, D. MEBS (1998): RFLP and sequence analysis of the cytochrome b gene of selected animals and man: methodology and forensic application. Int. J. Legal Med. 111, 323-327.

Received: 27 September 2009
Accepted: 9 July 2010

KARABASANAVAR, N. S., S. P. SINGH, S. N. SHEBANNAVAR, A. M. KOTRESH, P. S. GIRISH, P. V. UMAPATHI: Identifikacija ptičjih vrsta lančanom reakcijom polimerazom i analizom slijeda mitohondrijskoga gena 12S rRNA. Vet. arhiv 80, 653-661, 2010.

SAŽETAK

Autentična identifikacija i razlikovanje ptičjih vrsta od presudnoga su značenja u različitim konzervirajućim, taksonomskim, sudbenim, zakonskim i drugim ornitološkim aktivnostima. Ovo istraživanje bavi se molekularnobiološkim pristupom identifikaciji i razlikovanja dviju ptičjih vrsta: crvenkaste lunje (sokola) (*Milvus migrans*) i papige (*Psittacula krameri*). DNA je bila izdvojena iz uzoraka njihove krvi te je dio mitohondrijskoga 12S rRNA bio umnožen lančanom reakcijom polimerazom. Proizvodi PCR-a bili su sekvencirani i analizirani upotrebom Basic Local Alignment Search Tool (BLAST) genske banke GenBank (NCBI). Na osnovi sličnosti odnosno različitosti nalaza identificirane su te dvije pretraživane vrste.

Ključne riječi: gen 12S rRNA, lančana reakcija polimerazom, analiza slijeda, *Milvus migrans*, *Psittacula krameri*