Short Paper

Molecular phylogeny of some avian species using *Cytochrome b* gene sequence analysis

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Summary

Veritable identification and differentiation of avian species is a vital step in conservative, taxonomic, forensic, legal and other ornithological interventions. Therefore, this study involved the application of molecular approach to identify some avian species i.e. Chicken (*Gallus gallus*), Muskovy duck (*Cairina moschata*), Japanese quail (*Coturnix japonica*), Laughing dove (*Streptopelia senegalensis*), and Rock pigeon (*Columba livia*). Genomic DNA was extracted from blood samples and partial sequence of the mitochondrial *cytochrome b* gene (358 bp) was amplified and sequenced using universal primers. Sequences alignment and phylogenetic analyses were performed by CLC main workbench program. The obtained five sequences were deposited in GenBank and compared with those previously registered in GenBank. The similarity percentage was 88.60% between *Gallus gallus* and *Coturnix japonica* and 80.46% between *Gallus gallus* and *Columba livia*. The percentage of identity between the studied species and GenBank species ranged from 77.20% (*Columba oenas* and *Anas platyrhynchos*) to 100% (*Gallus gallus* and *Gallus sonneratii*, *Coturnix coturnix* and *Coturnix japonica*, *Meleagris gallopavo* and *Columba livia*). Amplification of the partial sequence of mitochondrial *cytochrome b* gene proved to be practical for identification of an avian species unambiguously.

Key words: Avian species, *Cytochrome b* gene, Phylogenetic analysis

Introduction

Veterinary and forensic science laboratories frequently encounter samples lacking any morphological details that make it impossible to identify them as meat, leather, bones, blood stains on clothes. Therefore, there is a need to determine the origin of anonymous biological traces. In addition, species identification represents a key aspect of biodiversity studies (Ardura *et al*., 2011).

The molecular markers and DNA sequencing have been taken as good markers to classify the taxonomy and phylogenetic relationships among species. The application of PCR technique has significantly improved the efficiency of laboratorial diagnostic procedures by allowing the *in vitro* amplification of a large number of DNA copies using a specific genomic region as template, followed by complementary techniques (Fajardo *et al*., 2007a). Since it only requires a small amount of template DNA, the PCR method has been particularly useful for the identification of species in suboptimal DNA samples like forensic samples and blood stains, also in archaeological remains and museum specimens owing to the highly degraded and fragmented nature of ancient DNA (Pereira *et al*., 2008).

Analysis of mitochondrial DNA (mtDNA) sequences is useful for phylogenetic studies. The mtDNA is independent, simpler than genomic DNA, and is of maternal inheritance and has no recombination in all vertebrates, so the sequence of mtDNA is more conservative (Rokas *et al*., 2003). The rate of base substitution on mtDNA is 5-10 times relative to nuclear gene which resulted in an accumulation of base substitutions over a long period of time, and enabled discrimination of a wide variety of birds, even closely related species belonging to the same families and genera (Russell *et al*., 2000).

Among mitochondrial genes, *cytochrome b* (mt *Cytb*) gene has been proved as an efficient tool with high power of discrimination for species identification and characterization in both taxonomy and forensic science (Kuwayama and Ozawa, 2000; Saif *et al*., 2012), and is also used in studies of molecular evolution (Prusak *et al*., 2004). The gene length is 1140 bp and has some stable sequences which were used for suggestion of universal primers for typical PCR-based methods (Parson *et al*., 2000).

The aim of this study was to unambiguously identify some avian species through amplifying and sequencing of a partial sequence of mt *Cytb* gene.

Materials and Methods

Animals and blood samples

Five avian species (chicken -*Gallus gallus*, muscovy duck -*Carinia moschata*, Japanese quail -*Coturnix*

japonica, and rock pigeon -*Columba livia*) were obtained from retail markets while Laughing dove (*Streopelia senegalensis*) was captured by hunters. Whole blood samples were collected aseptically in sterilized vacutainer tubes containing EDTA as anticoagulant, stored at -20°C until DNA extraction.

DNA extraction

Genomic DNA was extracted using Gene JET whole blood genomic DNA purification mini kit (Fermentas, Thermo Fisher Scientific, USA) following the manufacturer's protocol. The quality and quantity of DNA were analyzed using 1% agarose gel electrophoresis and spectrophotometric method, respectively. The intact DNA was selected for further analysis.

PCR amplification and gel electrophoresis

A pair of universal primers was used to amplify partial sequence of *mt Cytb* gene. Primers' sequences were as follow: L14816 (5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′), H15173 (5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′) (Kocher *et al*., 1989). PCR amplification reactions were performed in a total volume of 25 µL. Each reaction mixture contained: 12.5 µL HotStar Taq® Master mix (Qiagen GmbH, Germany), 1 µL of each primer (10 µM), 8.5 µL RNAse/DNase free water and 2 µL of DNA. PCR was carried out in a Tprofessional thermal cycler (Biometra, Germany). The cycling conditions included a single initial denaturation at 95°C for 11 min followed by 35 cycles of 94°C for 30 s (denaturation), 50°C for 45 s (annealing), 72°C for 45 s (extension) and a final extension step at 72°C for 7 min. PCR products (10 µL) were separated by 2% agarose gel electrophoresis at 120 V for 20 min. A 100 bp plus DNA ladder (Fermentas, Thermo Fisher Scientific, USA) was used to estimate sizes of the products. The resulting DNA fragments were visualized by UV transillumination and analyzed using Gel Documentation System (Bio Doc Analyse, Biometra, Germany).

DNA purification and DNA sequencing

DNA fragment was excised from the gel using sterile, sharp cutter and purified using Gene JET PCR purification kit (Fermentas, Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Purified products were directly sequenced using both the forward and reverse primers of PCR amplification. The sequencing process was performed by European Custom Sequencing Centre (GATC Biotech AG, Germany).

Sequence alignment and phylogenetic analysis

Sequences were edited manually using Chromas Lite Ver. 2.1.1, (http://technelysium.com.au) and then imported into nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve similar sequences from NCBI GenBank (Table 1). Comparative alignment and phylogenetic analyses were performed by CLC main workbench program version. 6.9.1 (http://www.clcbio.com). The phylogenetic tree was generated by distance-based neighbor-joining method (N-J) and the liability of internal branches was assessed by 100 bootstrap replicates.

Table 1: Mitochondrial *cytochrome b* gene sequences of five studied avian species and sequences retrieved from GenBank for phylogenetic tree construction

Latin name	Common name	Accession No.
Gallus gallus	Chicken	KF964328
Coturnix japonica	Japanese quail	KF964327
Cairina moschata	Muscovy duck	KF964329
Columba livia	Rock pigeon	KF964326
Streptopelia senegalensis	Laughing dove	KF964325
Gallus varius	Green jungle fowl	AP003324.1
Gallus sonneratii	Grey jungle fowl	AB044989.1
Francolinus erckelii	Sudanin frankoliini	FR691589.1
Coturnix coturnix	Common quail	L08377.1
Sarkidiornis melanotos	Knob-billed duck	AF059111.1
Chloephaga melanoptera	Andean goose	AF173763.1
Neochen jubatus	Orinoco goose	AF173762.1
Aix galericulata	Mandarin duck	U46484.1
Anas platyrhynchos	Mallard or wild duck	EU755252.1
Streptopelia mayeri	Pink pigeon	AF483322.1
Ectopistes migratorius	Passenger pigeon	AF483351.1
Meleagris gallopavo	Wild turkey	FM205719.1
Columba rupestris	Turkestan hill dove	AF353410.1
Columba oenas	Stock dove	AF375961.1
Columba palumbus	Common wood pigeon	AF483335.1

Results

The PCR amplification of partial mt *Cytb* gene using universal primers yielded a single amplification product, when separated on a 2% agarose gel, with the size being approximately 358 bp in the five avian species (Fig. 1).

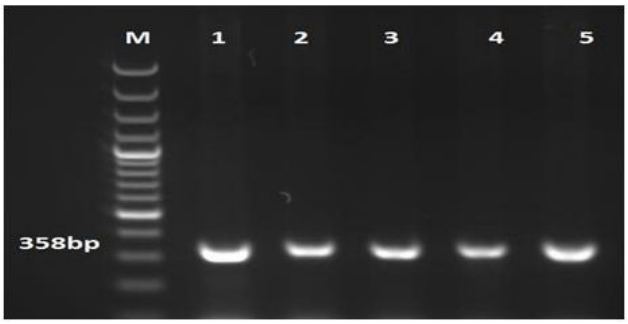

Fig. 1: Electrophoretic analysis of PCR product amplified with mt *Cytb* universal primer. Lane M: 100 bp plus DNA marker, Lane 1: Chicken (*Gallus gallus*), Lane 2: Japanese quail (*Coturnix japonica*), Lane 3: Muskovy duck (*Cairina moschata*), Lane 4: Laughing dove (*Streptopelia senegalensis*), and Lane 5: Rock pigeon (*Columba livia*)

The mt *Cytb* gene sequences of chicken (*G. gallus*), Japanese quail (*C. japonica*), muskovy duck (*C. moschata*), laughing dove (*S. senegalensis*) and rock pigeon (*C. livia*) were deposited in GenBank with accession numbers KF964328, KF964327, KF964329, KF964325, KF964326, respectively and were aligned and compared with other species using CLC main workbench program Ver. 6.9.1 (Table 1). Excluding the primer region, the partial sequence analysis of the mt *Cytb* gene is based on a total of 307 bp Fig. 2.

Pair wise comparison of the mt *Cytb* gene sequences of five avian species revealed that, the nucleotide

Fig. 2: Alignment of the partial sequence of mitochondrial *cytochrome b* (mt *Cytb*) gene from chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*), muskovy duck (*Cairina moschata*), laughing dove (*Streptopelia senegalensis*) and rock pigeon (*Columba livia*). Sequences were aligned using CLC Main Workbench program. Dots indicate sequence identity

Table 2: Genetic distance (below diagonal) and percentage of identity (above diagonal) between five studied avian species and its comparison with other related species

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*Gallus gallus*		88.60	81.76	80.46	81.76	100.00	99.35	88.60	88.93	84.04	84.36	85.99	83.39	81.43	80.46	80.46	80.13	80.46	81.11	80.46
2	*Coturnix japonica*	0.12		83.71	82.41	83.71	88.60	88.93	100.00	90.55	82.74	83.06	85.99	83.71	82.41	82.41	82.41	82.41	80.78	81.11	82.08
3	*Cairina moschata*	0.21	0.18		80.78	82.08	81.76	81.76	83.71	83.06	90.23	89.90	91.86	88.27	89.90	80.78	81.43	79.80	80.13	80.78	79.48
4	*Columba livia*	0.23	0.20	0.22		86.64	80.46	80.46	82.41	80.78	79.48	79.48	80.78	78.83	80.78	100.00	98.05	92.18	87.95	89.25	87.30
5	*Streptopelia senegalensis*	0.21	0.18	0.20	0.15		81.76	82.08	83.71	81.43	83.06	83.06	82.74	81.76	80.46	86.64	86.64	87.95	88.93	88.93	88.60
6	*Gallus sonneratii*	0.00	0.12	0.21	0.23	0.21		99.35	88.60	88.93	84.04	84.36	85.99	83.39	81.43	80.46	80.46	80.13	80.46	81.11	80.46
7	*Gallus varius*	0.01	0.12	0.21	0.23	0.20	0.01		88.93	88.93	83.39	83.71	85.34	83.71	81.43	80.46	80.46	80.13	80.46	81.11	81.11
8	*Coturnix coturnix*	0.12	0.00	0.18	0.20	0.18	0.12	0.12		90.55	82.74	83.06	85.99	83.71	82.41	82.41	82.41	82.41	80.78	81.11	82.08
9	*Francolinus erckelii*	0.10	0.08	0.17	0.20	0.19	0.10	0.10	0.08		81.76	82.08	84.36	83.39	82.74	80.78	80.78	79.80	82.84	79.15	80.53
10	*Neochen jubatus*	0.18	0.19	0.10	0.24	0.19	0.18	0.18	0.19	0.18		99.02	92.51	89.25	89.87	79.48	79.15	78.18	80.13	81.11	80.46
11	*Chloephaga melanoptera*	0.17	0.19	0.11	0.24	0.19	0.17	0.18	0.19	0.18	0.01		92.18	82.87	90.85	79.48	79.15	78.18	80.13	80.46	79.80
12	*Sarkidiornis melanotos*	0.16	0.16	0.09	0.22	0.20	0.16	0.16	0.16	0.15	0.08	0.08		90.23	90.23	80.78	80.78	79.80	80.78	81.11	80.78
13	*Anas platyrhynchos*	0.19	0.18	0.13	0.25	0.21	0.19	0.18	0.18	0.17	0.11	0.12	0.10		88.60	80.78	78.50	77.20	79.15	79.15	79.80
14	*Aix galericulata*	0.21	0.20	0.11	0.22	0.22	0.21	0.21	0.20	0.17	0.11	0.10	0.10	0.12		78.83	79.48	77.85	79.48	77.85	77.52
15	*Meleagris gallopavo*	0.23	0.20	0.22	0.00	0.15	0.23	0.23	0.20	0.20	0.24	0.24	0.22	0.25	0.22		98.05	92.18	87.95	89.25	87.30
16	*Columba rupestris*	0.23	0.20	0.21	0.02	0.15	0.23	0.23	0.20	0.20	0.24	0.24	0.22	0.25	0.24	0.02		92.83	87.95	90.55	87.30
17	*Columba oenas*	0.23	0.20	0.24	0.08	0.13	0.23	0.23	0.20	0.21	0.25	0.25	0.24	0.27	0.26	0.08	0.08		86.97	88.60	85.67
18	*Streptopelia mayeri*	0.21	0.21	0.22	0.12	0.11	0.21	0.21	0.21	0.19	0.21	0.21	0.21	0.23	0.22	0.12	0.12	0.13		89.90	90.43
19	*Columba palumbus*	0.22	0.22	0.22	0.12	0.12	0.22	0.22	0.22	0.22	0.21	0.22	0.22	0.24	0.26	0.12	0.10	0.12	0.09		87.30
20	*Ectopistes migratorius*	0.21	0.19	0.23	0.13	0.11	0.21	0.20	0.19	0.22	0.21	0.22	0.21	0.22	0.25	0.13	0.13	0.15	0.10	0.13	

similarities percentage was 88.60% between chicken (*G. gallus*) and Japanese quail (*C. japonica*) and 86.64% between laughing dove (*S. senegalensis*) and rock pigeon (*C. livia*), while muskovy duck (*C. moschata*) sequences showed less identity with those of other studied avian species (Table 2).

The comparison of the obtained mt *Cytb* gene sequences with those available in GenBank revealed nucleotide similarities percentage ranged from 77.20% (*Columba oenas* and *Anas platyrhynchos*) to 100% (*Gallus gallus* and *Gallus sonneratii*, *Coturnix coturnix* and *Coturnix japonica*, *Meleagris gallopavo* and *Columba livia*). The derived sequence of chicken (*G. gallus*) of this study was highly matched (100%) with *G. sonneratii*, while less similarity was seen with *Columba oenas* (80.13%). Moreover, the sequence of Japanese quail (*C. japonica*) was closely related to that of *C. coturnix* (100%), whichshowed less identity (80.78%) compared with that of *Columba palumbus*.

Also, muskovy duck (*C. moschata*) sequence displayed high similarity (91.86%) with that of *Sarkidiornis melanotos* but showed less similarity (79.48%) with *Ectopistes migratorius*. The sequence of rock pigeon (*Columba livia*) was closely related to those of *Meleagris gallopavo* (99.48%) but less identity (78.83%) with *Aix galericulata*. The sequence of laughing dove (*Streptopelia senegalensis*) showed slightly high similarity (88.93%) with those of *Streptopelia mayeri* and *Columba palumbus*, but showed less similarity (80.46%) with *Anas platyrhynchos*.

The genetic distance among different species ranged from 0.00 (*Gallus gallus* with *Gallus sonneratii*, *Coturnix japonica* with *Coturnix coturnix* and *Columba livia* with *Meleagris gallopavo*) to 0.27 (*Aix galericulata* and *Columba oenas*) as shown in Table 2 below the diagonal.

The phylogenetic tree revealed three major clusters referred to as A, B, and C. Each cluster was divided into

branches with highly supported relationships. Cluster A includes *Cairina moschata*, *Aix galericulata*, *Chloephaga melanoptera*, *Neochen jubatus*, *Sarkidiornis melanotos* and *Anas platyrhynchos*. Cluster B contains *Gallus gallus*, *Gallus varius*, *Gallus sonneratii*, *Coturnix japonica*, *Coturnix coturnix* and *Francolinus erckelii*. Cluster C contains *Columba livia*, *Meleagris gallopavo*, *Columba rupestris*, *Columba oenas*, *Ectopistes migratorius*, *Streptopelia senegalensis*, *Columba palumbus* and *Streptopelia mayeri* as shown in Table 2 and illustrated in Fig. 3.

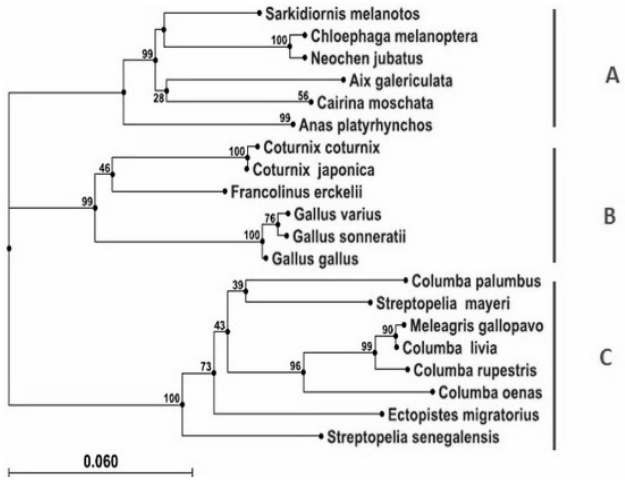

Fig. 3: Phylogenetic analysis based on the *cytochrome b* gene sequences of five avian species and other related species. The tree was analyzed by neighbor-joining (N-J) analysis

Discussion

Identification of avian species is a vital step in conservative, taxonomic, forensic, legal and other ornithological interferences. The obtained result is not only useful for taxonomic purposes and evolutionary analysis, but also has important inclusions for species conservation (Baling and Brunton, 2005).

DNA-based molecular techniques developed over the last two decades have increased the possibility of developing authentic and reliable methods for species identification, due to the stability of DNA at high temperatures and the fact that its structure is conserved within all tissues of an individual (Saini *et al*., 2007). Many authors applied various DNA based methods to identify different animals and avian species, (Calvo *et al*., 2001; Sasazaki *et al*., 2004; Tanabe *et al*., 2007; Asensio *et al*., 2008; Gruszczyńska and Michalska, 2013).

Identification of some avian species based on direct sequencing of mt *Cytb* gene was applied. Although, sequencing is more expensive than the other PCR-based methods, it provides information for all positions of a target sequence. For this reason direct sequencing can be successfully used for differentiation between taxonomically close species or determining the origin of anonymous samples that is of particular interest in veterinary control and animal (Budowle *et al*., 2003).

The short length fragment of the mt *Cytb* gene was used because of its widest taxonomic representation in nucleotide databases. Despite the relatively short size of the fragment, it provides enough sequence divergence, which fulfills the objectives of the study. Furthermore, only one pair of primers was used in this study to amplify a 358 bp of the mt *Cytb* gene for all samples. This is in agreement with studies that reported the use of universal primers as complements to conserved region of mt *Cytb* gene in vertebrates (Kocher *et al*., 1989).

In current study the obtained DNA sequence had been deposited in GenBank. Therefore, there is a high chance that the unknown sample will match a DNA sequence from a reference sample deposited on the database. Hence, these results will contribute to developing the GenBank database and can be applied for further phylogenetic and forensic studies on other avian and animal species.

Phylogenetic tree was constructed by N-J method which is based on distances between sequences revealing a variation between different avian species, even closely related ones. Such tree could identify the ancestors and closest relatives of the group and also have great practical value, where trees have helped solve criminal cases, and epidemiology. The phylogenetic analysis reflects the historical agreement on the point (Prager and Wilson, 1976) that the studied birds belong to different orders and families [chicken and Japanese quail; *Galliformes* (*Phasianidae*), rock pigeon and laughing dove; *Columbiformes* (*Columbidae*) and muskovy duck; *Anseriformes* (*Anatidae*)].

Chickens (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) are species of considerable economic importance in a number of countries including Egypt. Moreover they are frequently used as a model laboratory animal (Kayang *et al*., 2004). Our findings are consistent with the earlier results reported by Pang *et al*. (1999), Inoue-Murayama *et al*. (2001) and Kayang *et al*. (2006).

In cluster C, it is shown that pigeons and doves belong to a typical group within this group, *Streptopelia* (turtledoves including laughing dove) and *Columba* (old world pigeons including rock pigeon) are supported in a well-supported sister *Streptopelia-Columba* clade (Fulton *et al*., 2012). To conclude, PCR amplification of the mt *Cytb* gene, amplicon sequencing and sequence analysis would help to solve the problem of identification of an avian species unambiguously.

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