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), Muscovy 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 sonneratti, 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 -Carinina moschata, Japanese quail -Coturnix
japonica, and rock pigeon -Columbia livia) were obtained from retail markets while Laughing dove (Streptopelia 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’), H1573 (5’-CCC CTC AGA ATGATA 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.

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<th>Latin name</th>
<th>Common name</th>
<th>Accession No</th>
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**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: Muscovy duck (Cairina moschata), Lane 4: Laughing dove (Streptopelia senegalensis), and Lane 5: Rock pigeon (Columbia livia) ![](https://example.com/image.png)](https://example.com/image.png)

The mt Cytb gene sequences of chicken (G. gallus), Japanese quail (C. japonica), muscovy 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
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 muskow 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% (C. oenas and A. platyrhynchos) to 100% (G. gallus and G. sonneratii, Coturnix coturnix and C. japonica, C. albicollis and C. livia). The derived sequence of chicken (G. gallus) of this study was highly matched (100%) with G. sonneratii, while less similarity was seen with C. oenas (80.13%). Moreover, the sequence of Japanese quail (C. japonica) was closely related to that of C. coturnix (100%), which showed less identity (80.78%) compared with that of C. palumbus.

Also, muskow 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 (C. livia) was closely related to those of Meleagris gallopavo (99.48%) but less identity (78.83%) with A. galericulata. The sequence of laughing dove (S. senegalensis) showed slightly high similarity (88.93%) with those of Streptopelia mayeri and C. palumbus, but showed less similarity (80.46%) with A. platyrhynchos.

The genetic distance among different species ranged from 0.00 (G. gallus with G. sonneratii, C. japonica with C. coturnix and C. livia with M. gallopavo) to 0.27 (A. galericulata and C. 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 erckelli*. 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.

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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