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Genetic Relationships Analysis of Some various Species Using Cytochrome b Genes as a Phylogenetic Marker

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Abstract: This study examined the genetic relationships among six species—chicken (*Gallus gallus*), quail *(Coturnix coturnix*), duck (*Anas platyrhynchos*), goose (*Anseranser domestica*), and turkey (*Meleagris gallopavo*) and rabbit (*Lepus capensis*), using partial mitochondrial cytochrome b (*Cyt-b*) gene sequences. PCR amplification of the mitochondrial *Cyt-b* gene resulted in an approximately 358 *bp* amplicon in size for all species, and sequences alignment and phylogenetic analyses were performed with Bio Edit and MEGA X software's to determine genetic similarities and distances. Results revealed high nucleotide similarity between chicken and quail (89.1%) and chicken and turkey (83.6%). The closest genetic relationship was between duck and domestic goose, with 87.4% identity and the smallest genetic distance of 0.126. Chicken (*Gallus gallus*) demonstrated a relatively close relationship with both duck and domestic goose, with identities (83.6%). While, Turkey (*Meleagris gallopavo*) exhibited a slightly more distant relationship with avian species, with the lowest similarity at 81.6% with domestic goose. The rabbit, positioned at the root of the phylogenetic tree, exhibited the greatest genetic distance from avian species, with a 28.3% distance and 71.7% identity with chicken. This study provides solid evidence of the effectiveness of using *Cyt-b* gene sequences as a reliable tool for species identification across various applications, underscoring the effectiveness of partial *Cyt-b* gene sequences for avian species identification and genetic analysis, which is valuable for breeding and diversity studies.

Keywords: *Cyt-b* gene, genetic distance,phylogenetic tree, sequence analysis, various species

Introduction

Avian species, such as chickens, quails, turkeys, ducks, and geese, are valuable domesticated birds raised by humans for their eggs and meat (Farrell, 2014). They provide high-quality animal proteins and are often included in various meat products. However, substitution of these meats with lower-cost alternatives has led to fraudulent practices to increase financial gains. Such adulteration undermines fair competition and disregards consumer interests (Abbas *et al.,* 2019; Bohme *et al.,* 2019).To enforce legislation aimed at protecting endangered bird species, it is crucial to accurately identify avian species. DNA testing is often employed for this purpose, particularly when morphological information is limited, such as in cases involving only feather fragments

or powdered remains. Sequence-based identification of cytochrome b genes in mitochondria is the most popular exploited among DNA-based techniques (Hebert et al., 2003; Lee *et al.,* 2008). Accurately identifying a species is also an important element of the studies of biodiversity (Ardura *et al.,* 2011). Other approaches that have been applied for species identification include but are not limited to morphological features, immune systems, and their techniques, gel electrophoresis, and chromatography (Taylor *et al.,* 1993; Andrasko & Rosen, 1994; Espinoza et al., 1999; Czesny *et al.,* 2000). Many recent identification approaches have particularly failed to separate some closely related bird species and therefore, approaches with better resolution and sensitivity are needed (Bellis *et al.,* 2003). Molecular genetic analysis, particularly using mitochondrial DNA (mt-DNA) sequences, offers a promising solution. Mitochondrial DNA, due to its simpler structure compared to genomic DNA, maternal inheritance, and lack of recombination in vertebrates, makes its sequence more conserved (Rokas *et al.,* 2003). The rate of base substitution in mt-DNA is approximately 5 - 10 times higher than in the nuclear genes which enables the differentiation of a wide range of bird species even those that are closely related in the same families and genera. This is so as a result of over some time the number of base substitutions increases (Russell *et al.,* 2000). These scenarios point out that lineage with differential mt-DNA is a useful source for species identification and characterization in both taxonomy and phylogenetic research (Noro *et al.,* 1998; Olschewsky & Hinrichs, 2021). Cytochrome b (*Cyt-b*) proteins are favored by biologists for their active involvement in the taxonomic and phylogenetic analysis of organisms at various levels, including species and family, etc. The mt-cyb gene has been functional in testing distances among taxa, short of the order among several groups of animals. The *mt-Cyb* gene offers information specific to the species concerned within the purported specimens whose forensic applications range from the development of the phylogenetic tree to forensic caseworks investigations including source determination of the biological matter within the caseworks (Yacoub *et al.,* 2015; Hartatik *et al.,* 2019). The goal of this study was to use cytochrome b (*Cyt-b*) sequenceing as a reliable tool for species identification.

Materials and Methods

Avian Species Blood Samples

Ten blood samples from each of the following species: chicken (*Gallus gallus*), quail (*Coturnix coturnix*), duck (*Anas platyrhynchos*), goose (*Anseranserdo mestica*), turkey (*Meleagris gallopavo*), and rabbit (*Lepus capensis*), were collected for molecular analysis. Blood was drawn from the jugular vein using a 10 ml syringe. Prior to collection, the area was cleaned, hair was trimmed, and the site was sterilized with 70% ethanol. The samples were positioned in test tubes that included EDTA and were held at -20°C until the DNA extraction procedure was performed.

DNA Isolation

The DNA was extracted from blood samples of eight bird species using a GeNet Bio, Korea blood DNA extraction kit. Following the manufacturer's instructions, the extracted DNA's quality and quantity were determined using a Nanodrop 1000 spectrophotometer (UK) and a 1% agarose gel electrophoresis method, respectively.

PCR and gel electrophoresis

The methodology of the experiment involved two primers that were specifically chosen to target and amplify a segment of the mitochondrial cytochrome b (*Cyt-b*) gene:(5'CCATCCAACATCTCAGCATGATGAAA3')and(5'CCCCTCAGAATGATATTTGTCCTCA-3'), as indicated by (Awad *et al.,* 2015). The PCR reaction was carried out in a total volume of 20 µL, which included a mixture of 10 µL of Green Master Mix (featuring 25 units/mL of Taq DNA polymerase, 200 µM of each dNTP, and 1.5 mM of $MgCl_2$), 2 µL of genomic DNA, 1 µL of each primer (at a concentration of 10 µM), and 6 µL of DNase-free water.The PCR amplification was conducted utilizing a Tprofessional thermo cycler (Biometra, Germany), under the specified thermal conditions: an initial denaturation step at 95°C for 10 minutes, followed by 33 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 50°C for 40 seconds, and extension at 72°C for 50 seconds. The amplification concluded with a final extension at 72°C for 6 minutes. Afterward, 10 µL of the PCR products were applied to a 2% agarose gel, which was stained with Safe Dye (Catalog No. B-2010, GeNet Bio, Korea) and viewed under ultraviolet (UV) light. The gel images were captured utilizing a Gel Documentation System (Bio Doc. Analyse, Biometra, Germany).

DNA sequencing

The PCR products were sequenced using the set of amplifying primers, both forward and reverse. This particular sequencing was performed by Macrogeninc, Inc. a South Korean commercial company offering both sequencing and purification services.

Aligning Sequences and Evolutionary Tree Construction

The amplified mitochondrial cytochrome b (*Cyt-b*) gene sequences were aligned with BLAST available at http://www.ncbi.nlm.nih.gov/BLAST, and then retrieved mitochondrial *Cyt-b* genes of closely related species from GenBank. The genetic diversity among accessions was estimated by using the MEGA X software (version 10.2.6) (Kumar *et al.,* 2018) with multiple sequence alignment file. Counting the number of base pair substitutions between sequences and using Kimura's 2-parameter model to correct for evolutionary rates, we calculated genetic distances (d) for each locus among the accessions. Phylogenetic tree construction was based on the maximum likelihood (ML) method with the neighbor-joining algorithm. Figure 3 shows that 1,000 bootstrap replicates were conducted to test the reliability of the resulting phylogenetic trees (Tripathi *et al.,* 2013).

Table (1): Phylogenetic tree, based on the mitochondrial cytochrome b gene of avian species understudy and also retrieved from Gen Bank.

	Taxonomic name	Popular name	Accession No
1-	*Coturnix coturnix*	quail	PQ356919
2-	*Gallus gallus*	chicken	PQ356920
3-	*Anseranser domestica*	duck	PQ356917
4-	*Anas Platyrhynchos*	goose	PQ356918
5-	*Meleagris gallopavo*	turkey	PQ356922
6-	*Lepus capensis*	rabbit	PQ356921

Results and Discussion

The investigation revealed that using universal primers for PCR amplification targeting a segment of the mitochondrial cytochrome b (*Cytb*) gene resulted in a single PCR product. This product was analyzed using a 2% agarose gel, revealing a fragment size of approximately 358 base pairs (*bp*) across the six various species examined, as shown in (Fig. 1).

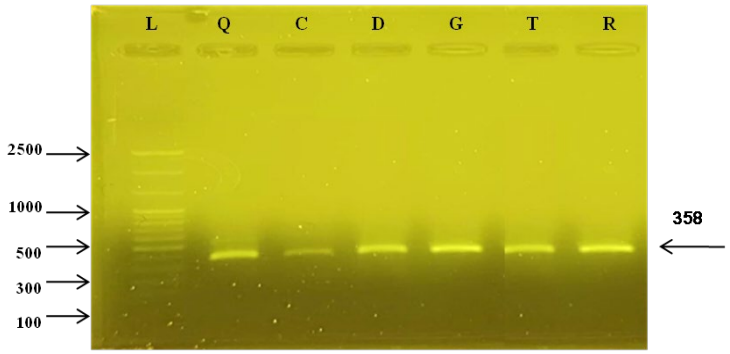

Fig.(1): The *mt-Cytb* universal primer was employed to amplify PCR products from various species.Lane L: 100 bp DNA marker, Lane Q is quail, Lane C is chicken, Lane D is duck, Lane G is goose, Lane T is turkey, and Lane R is rabbit.

The study involved submitting mitochondrial cytochrome b (*Cyt-b*) gene sequences from various species chickens (*Gallus- gallus*), quails (*Coturnix- coturnix*), ducks (*Anas-platyrhynchos*), geese (*Anseranser-domestica*), turkeys (*Meleagris- gallopavo*), and rabbits (*Lepus-capensis*)to Gen Bank under accession numbersPQ356920, PQ356919, PQ356917, PQ356918, PQ356922 and PQ356921. The Bio Edit software (version7.2.5) was utilized to align and compare these sequences with those of other species. The partial sequencing analysis of the *mt-Cytb* gene, excluding primer regions, was conducted over a length of 280 base pairs (*bp*), as detailed in (Table 1 and Fig. 2). These results are consistent with the observations reported by Abdul-Hassan & Tauma, (2014) as well as (Awad *et al.,* 2014).

In a comparative analysis of the *mt-Cytb* gene sequences from five bird species

alongside *Lepus capensis,* it was determined that the nucleotide similarity was 89.1% between chicken (*Gallus gallus*) and quail (*Coturnix coturnix*), and 83.6% between chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*). In contrast, the nucleotide identity of rabbit (*Lepus capensis)* was found to be lower when compared to the avian species under investigation. Furthermore, Sequencing generally involves examining part or all of the mitochondrial genome and then comparing it to existing sequences in Gen Bank (NCBI). The nucleotide similarity between the sequenced mitochondrial genomes and the reference sequences for the same species varied between 98.22% and 99.66%.

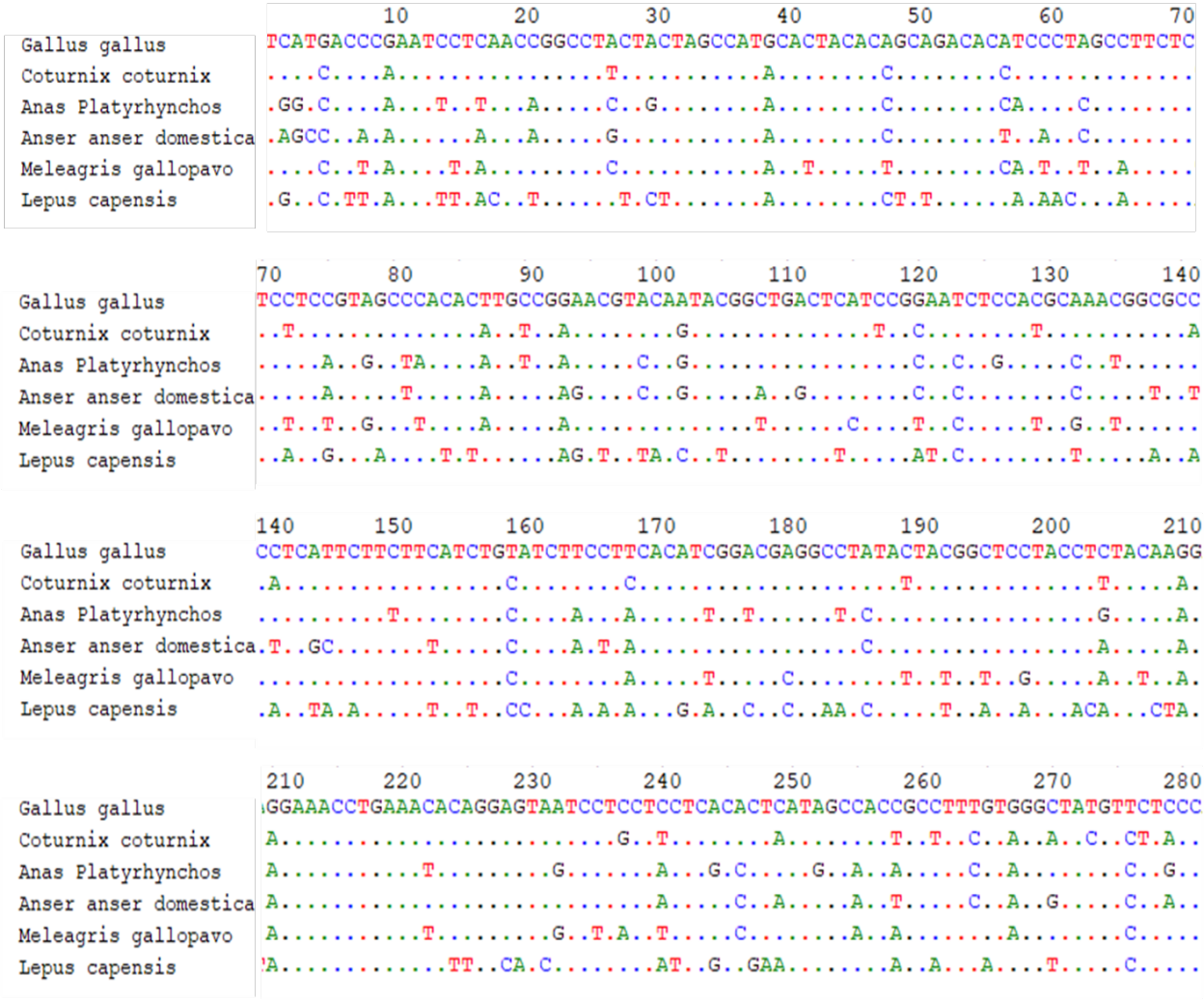

Fig. (2): The Bio Edit software was used to align the partial mitochondrial cytochrome b (*mt-Cytb*) gene sequences from chicken (*G. gallus*), quail (*C. coturnix*), duck (*A. platyrhynchos*), goose (*A. anserdomestica*), turkey (*M. gallopavo*), and rabbit (*L. capensis*), with sequence identities represented by dots.

The results indicate that *Anas platyrhynchos* (Duck) and *Anseranser domestica* (Domestic Goose) share the closest genetic relationship, with a high percentage of identity (87.4%)

and the smallest genetic distance (0.126). *Gallus gallus* (Chicken) sequence also shows a relatively close genetic relationship with both *Anas platyrhynchos* (Duck) and *Anseranser domestica* (Domestic Goose), with a percentage of identity around 83.6%. The derived sequence *Meleagris gallopavo* (Turkey) has a slightly more distant relationship with the other avian species, with percentage identities ranging from 84.6% with those of *Anas platyrhynchos* (Duck) and *Coturnix coturnix*, but showed less similarity 81.6% with *Anseranser domestica* (Domestic Goose). The genetic distances among the avian species are generally low, reflecting their closer evolutionary relationships. In contrast, *Lepus capensis* shows significantly greater genetic distances and lower percentage identities with the avian species, emphasizing its distinct evolutionary lineage.

The cytochrome b (*Cyt-b*) gene has been fully or partially sequenced in a wide range of animals, including birds, mammals, fish, amphibians, reptiles (Chow *et al.,* 1993; Ram *et al.,* 1996; Quinterio *et al.,* 1998; Lindstrom, 1999; Parson *et al.,* 2000), as well as some invertebrates (Lee *et al.,* 2009). Additionally, *Cyt-b* gene sequences have been utilized to identify meats and meat products from birds like song thrush, quail, and sparrow, and other species such as red deer, roe deer, Pyrenean ibex, and chamois (Chikuni *et al.,* 1994; Matsunaga *et al.,* 1998; La Neve *et al.,* 2008).

Table (2): The genetic distance is represented below the diagonal, while the percentage of identity among the various species analyzed is illustrated above the diagonal.

No.	Species	1	2	3	4	5	6
1	*Gallus gallus*		89.1	82.9	83.6	83.6	71.7
2	*Coturnix coturnix*	0.109		84.3	86.0	84.6	70.6
3	*Anas Platyrhynchos*	0.171	0.157		87.4	84.6	71.0
4	*Anseranser domestica*	0.164	0.140	0.126		81.6	73.4
5	*Meleagris gallopavo*	0.164	0.154	0.154	0.184		71.0
6	*Lepus capensis*	0.283	0.294	0.290	0.266	0.290	

The greatest genetic distance observed is between *Lepus capensis* and *Gallus gallus* (Chicken) at 28.3%, with the percentage of identity being the lowest at 71.7%. The table 2 highlights the closer genetic relationships among the avian species compared to the more distantly related *Lepus capensis*. The genetic distance ranged from 0.109 to 0.294 among various species as shown in Table 2 below the diagonal. Awad *et al.,* (2014) conducted a comparative analysis of *mt-Cytb* gene sequences from five bird species, revealing nucleotide similarity percentages of 88.60% between the chicken (*Gallus-gallus*) and the Japanese quail (*Coturnix-japonica*), as well as 86.64% between the laughing dove (*Spilopelia-senegalensis*) and the rock pigeon (*Columba livia*). Conversely, the sequences of the Muscovy duck (*Cairina-moschata*) demonstrated a lower degree of sequence identity than the

other avian species included in the study. Dave *et al.,* (2021) conducted a comparison between two indigenous chicken populations, Aravali and Ankleshwar, with various domestic chicken breeds. They concluded that the Red Jungle Fowl and Gray Jungle Fowl are the wild species that all domestic chicken breeds descend from.

The phylogenetic tree evaluates the relationships among several bird species (*Gallus gallus, Coturnix coturnix, Meleagris gallopavo, Anas platyrhynchos,* and *Anseranser domestica*) and one mammal (*Lepus capensis)* using genetic data. The tree reveals that *Gallus gallus* (domestic chicken) and *Coturnix coturnix* (common quail) share the most recent common ancestor, with *Meleagris gallopavo* (wild turkey). These three species form a closely related group. *Anas platyrhynchos* (mallard duck) and *Anseranser domestica* (domestic goose) also form a close relationship but they are less closely related to the first three species. Although *Lepus capensis* is a mammal, it appears at the base of the phylogenetic tree due to its substantial genetic distance from the avian species.

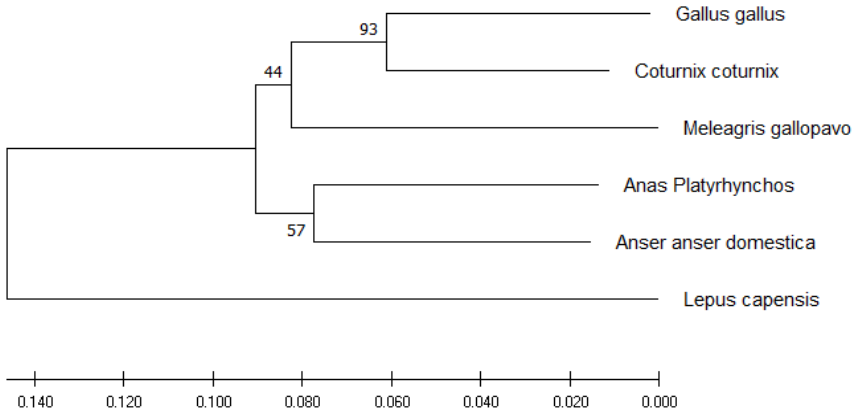

Fig. (3): Analysis of cytochrome b gene sequences was performed on species collected for the construction of a phylogenetic tree using maximum likelihood (ML) analysis.

Genetic distances among various poultry breeds or genotypes can provide an accurate picture of genetic diversity (Ahmed, 2020; Ahmed & Al-Barzinji, 2019). This type of information is important for breeders, particularly in designing mating systems for crossbreeding

Conclusion

The study investigated species differentiation by amplifying and sequencing a specific fragment of the mitochondrial cytochrome b (*mt-Cytb*) gene. The mitochondrial *Cyt-b* gene, known for its high genetic variability across species, was chosen as it provides a reliable molecular for species identification in phylogenetic studies.

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Conflict of interest

The authors affirm that this work has been submitted without any conflicts of interest.

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تحليل العلاقات الوراثية لبعض أنواع المختلفة باستخدام جين Cytochrome b كمؤشر النشوء والتطور

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المستخلص: تناولت هذه الدراسة العلاقات الوراثية بين خمسة انواع مختلفة من الطيور: الدجاج (*Gallus-gallus*)، والسمان (*Coturnix coturnix*)، والبط (*Anas-platyrhynchos*)، والإوز (*Anser-anserdomestica*)، والديك الرومي (*Meleagris gallopavo*) فضلا عن الأرنب كمجموعة خارجية (*Lepus-capensis*)، وذلك باستخدام تسلسلات جزئية من جين الميتوكوندريا السيتوكروم ب (*Cyt-b*) نتج عن تضخيم جين الميتوكوندريا Cyt-b باستخدام تقنية PCR حزمة بحجم 358 زوج قاعدي أساس لجميع الانواع. وتم إجراء محاذاة التسلسلات وتحليلات النشوء والتطور بواسطة Bio Edit لتحديد أوجه التشابه والمسافات الجينية. أظهرت النتائج وجود تشابه كبير في النيوكليوتيدات بين الدجاج والسمان (89.1%) والدجاج والديك الرومي (83.6%). أقرب علاقة وراثية كانت بين البط والأوز المستأنس حيث بلغت نسبة التشابه 87.4% وأقل مسافة جينية كانت 0.126. أظهر الدجاج (*Gallus-gallus*) علاقة جينية قريبة نسبيًا مع كل من البط والأوز المستأنس، حيث بلغت نسب التشابه 83.6%. في حين أظهر الديك الرومي (*Meleagris-gallopavo*) علاقة جينية أبعد قليلاً مع الأنواع الطيور الأخرى، حيث كانت أقل نسبة تشابه هي 81.6% مع الأوز المستأنس. الأرنب، الذي تم وضعه في جذر الشجرة التطورية، أظهر أكبر مسافة جينية عن الأنواع الطيور، حيث كانت المسافة 28.3% ونسبة التشابه 71.7% مع الدجاج. تسلط هذه الدراسة الضوء على العلاقات الجينية الأقرب بين الأنواع الطيور مقارنة بالأرنب، مما يبرز فعالية استخدام تسلسلات جين *Cyt-b* الجزئية في تحديد الأنواع الطيور وتحليلها جينيًا، وهو دراسة ذو قيمة في دراسات التنوع وبرامج التربية.

كلمات المفتاحية: *Cyt-b* جين, شجرة النشوء, تسلسلات الجينية, البعد الوراثي ,الانواع المختلفة