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Research Article
Phylogenetic Analysis of Duck Species from Tegal Indonesia Using 18S Ribosomal RNA and Mitochondrial *COI* Gene

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Abstract

Background and Objective: Tegal duck is a domestic waterfowl species known as a major producer of eggs that is traditionally used for making the famous salted egg by the local people. This study aimed to analyze the phylogenetic relationship of the Tegal duck through the gene encoding 18S ribosomal RNA (rRNA) and mitochondrial cytochrome oxidase I (COI). **Methodology:** We amplified and sequenced part of the 18S rRNA and *COI* gene of Tegal duck. These data were used to determine their similarity with other *Anas* species retrieved from GenBank and analyzing phylogenetic relationship. **Results:** The size of the PCR product of partial 18S rRNA was approximately 485 bp, whereas the size of the partial mitochondrial DNA (mtDNA) of the *COI* gene sequence was 751 bp. The 18S rRNA data showed that the Tegal duck is closely related to *A. platyrhynchos* (84.4% similarity). The *COI* gene data of the Tegal duck confirmed this relationship, with 99% homology with *A. platyrhynchos* voucher NHMO-BC399. However, the *COI* gene fragment of the Tegal duck also showed 99% homology with *A. poecilorhyncha*. **Conclusion:** Tegal duck has a close phylogenetic relationship with not only *A. platyrhynchos* but also *A. poecilorhyncha*.

Key words: Duck, cytochrome oxidase I, ribosomal, nuclear, mitochondrial, egg, *A. platyrhynchos*, *A. poecilorhyncha*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Duck is a dominant livestock with economic importance in Tegal, Central Java, Indonesia, which has the highest population after Brebes and Klaten Regencies. This region is popularly known as center for the highest quality salted duck eggs. Their egg production is unstable and showed tendency to decrease[1]. Morphological and physiological characterization has been commonly used for duck breeding programs. It resulted in instability of the progeny and the product[2,3]. The characteristics of Tegal ducks have been considered to be typologically similar with those of mallard (*Anas platyrhynchos*)[2-4]. Their variative phenotypes such as beak color, head color and tail, have been confusing in making decisions regarding in animal breeding[4,5]. Moreover, the origin of the domestic duck in Asia is still debatable[6-8]. Genetic characterization of this duck is important to improve egg production in Tegal. It is known that genetic variation is responsible for phenotypic traits. Therefore, it is very important to analyze genetic characteristics of the domestic Tegal duck for breeding program, with the possibility through their advantages. Genetic identification of the Tegal duck is conducted using two DNA barcoding methods consisting of nuclear and `mitochondrial DNA (mtDNA). The combination of mitochondrial and nuclear genes produce more credible species delineations than those based on a single gene[9]. Detection of nuclear DNA is limited due to the general low copy number of sequences and differs from the several copies of mtDNA per cell[10,11]. Mutation and recombination in the nucleus create variety in the nuclear genes, while mutation is the primary source of sequence variation in animal mitochondria. All processes can be the material for the identification of genetically related species[10,11]. Based on recent studies, *COI* gene has been effective to identify species in poultry because of their high mutation rate, which was sufficient to discriminate amongst species[11]. The advantage of mtDNA is supported at faster nucleotide substitution and lack of recombination[11,12-14]. In addition, mitochondrial genome is maternally inherited[8,15-18]. Thus, COI gene can be used in differentiating taxonomial class starting from the level of intraspecies to ordo, for example Copepoda evolution[12,14]. Another DNA barcodes suitable for animal identification is 18S rRNA gene, the central component of ribosome. This region is one of the most conserved genes in all cells[14,19,20]. Phylogenetic analysis based on molecular characterization of Tegal duck species will be important for searching a stable product for improving breeding strategies for Indonesian domestic ducks. Molecular identification of most members of the genus *Anas* has generally been performed using the *COI* mitochondrial gene. However, since 18S rRNA is also a reliable molecular barcode, it would be better if the phylogenetic analysis is performed using both of these genetic markers. In the present study, variations in mtDNA and ribosomal DNA is investigated to determine the phylogenetic relationships of the Tegal duck species at the molecular level.

MATERIALS AND METHODS

Specimen collection and DNA extraction: Tegal ducks at 3 months of age were obtained from the Center of Breeding Poultry in Banyubiru Semarang, Department of Animal Husbandry and Animal Health in Central Java Province, Indonesia. The DNA samples were extracted from the tissue samples of the Tegal ducks[21].

PCR amplification and DNA sequencing: The amplification of the 18S rRNA and *COI* gene in the mtDNA was used extracted genomic DNA. The *COI* forward primer was 5-TTCTCCAACCACAAAGACATTGGCAC-3 and the reverse primer was 5-ACGTGGGAGATAATTCCAAATCCTG-3 [22]. For the 18S rRNA gene, a universal primer was used with the forward primer 5-GTAGTCATATGCTTGTCT-3 and the reverse primer 5-GCTGGCACCACACTTGCCCT-3. The PCR for the *COI* gene was performed in a Mini Thermal Cycler (Eppendorf, Germany). The initial denaturation step was conducted at 95°C for 5 min. The next steps were 35 cycles at 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec and a final extension step at 72°C for 7 min. The PCR for the 18S rRNA gene was used an initial denaturation step at 94°C for 2 min. The PCR consist of 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. The final extension step at 72°C for 2 min. Purification and sequencing of the PCR products were performed by 1st Base Singapore.

Data analysis: For data analysis, published sequences of some members from the genus *Anas* were downloaded from GenBank. Sequence data of the duck *COI* gene and 18S rRNA are listed in Table 1. Sequences were aligned using the ClustalX program[23]. Identification of nucleotide variations in duck species and construction of the neighbor-joining phylogenetic tree were conducted using MEGA software version 5 (Japan)[24]. The phylogenetic tree was constructed by substitution model using the Jukes-Cantor model. It assumes equal base frequencies and equal mutation rates.

Table 1: The 18S rRNA and mitochondrial *COI* gene sequences of *Anas* species from GenBank used in this study

Species	Accesion number
A. platyrhynchos voucher NHMO-BC399	GU571240.1
A. platyrhynchos	EU123318.1
A. platyrhynchos isolate Moulard AF23	FJ416869.1
A. platyrhynchos	AF173614.1
A. platyrhynchos	D38362.1
A. platyrhynchos	EU009397.1
A. platyrhynchos breed Beijing	EU755252.1
A. platyrhynchos breed Sichuan	KX592536.1
A. platyrhynchos breed Longsheng	KJ739616.1
A. platyrhynchos	KJ778676.1
A. platyrhynchos breed mallard	EU755253.1
A. platyrhynchos breed Xilin	KJ833586.1
A. platyrhynchos voucher DCB-WD1	KF992022.1
A. platyrhynchos	KJ883269.1
A. platyrhynchos breed Rongshui	KJ833587.1
A. platyrhynchos	KJ637997.1
A. platyrhynchos	KJ689447.1
A. platyrhynchos breed Shaoxing	HM010684.1
A. platyrhynchos breed Jianchang	FJ167857.1
A. bahamensis	FJ027081.1
A. rubripes	AY666221.1
A. laysanensis	JF498830.1
A. superciliosa	JN801396.1
A. flavirostris	N01487.1
A. crecca	GU571238.1
A. laysanensis	JF498830.1
A. poecilorhyncha	JN703235.1
A. poecilorhyncha	AY164517.1
A. poecilorhyncha	KC466567.1
A. poecilorhyncha	EU009397.1
A. poecilorhyncha	KF156760.1
A. poecilorhyncha	KF751616.1
A. poecilorhyncha	JN793235.1
A. fulvigula	DQ432723.1
A. georgica	FJ027095.1
A. acuta	GU571235.1
A. carolinensis	DQ434280.1

The rate of nucleotide substitution is the same for every pair of nucleotides. Frequencies of A, T, C and G will eventually become equal to 0.25[25,26].

RESULTS AND DISCUSSION

Amplification of 18S rRNA gene by universal primer: Figure 1 shows the products of the Tegal duck 18S rRNA amplification. The annealing temperature that showed positive bands and exhibited the best sequencing results was 55°C. These data confirmed that the primer we chose in this study was suitable for amplifying the conserved region in the Tegal duck. The size of the PCR product of the 18S rRNA fragment was approximately 485 bp. These sequences showed high homology with 18S rRNA partial sequences

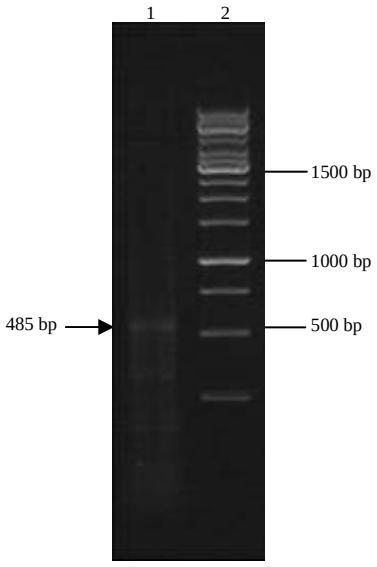


Fig. 1: The 18S rRNA fragment of the Tegal duck
lane 1 = 18S rRNA fragment, lane 2 = 1 kb DNA ladder

homology with those of *A. platyrhynchos* (Fig. 2). Sequence of the 18S rRNA fragment with gene resources from GenBank showed approximately 99% similarity between the Tegal duck and *A. platyrhynchos* (accession number EU123318.1) from Saudi Arabia, *A. platyrhynchos* (accession number AF173614.1) from the USA and *A. platyrhynchos* (accession number D38362.1) from Ibaraki, Japan. Approximately 98% similarity was also observed between the Tegal duck and the *A. platyrhynchos* isolate Moulard AF23 (accession number FJ416869.1) but only in the short region (data not shown). The 18S rRNA sequences from Tegal duck and *A. platyrhynchos* species identified 98 and 99% similarity in the region. *A. platyrhynchos* was grouped with *A. poecilorhyncha* and had 0.0047% difference[26]. This result also supported the idea that the region with high intra-species conservation, such as the 18S rRNA genes with similarities close to 100%, could be used for species-level analyses in the case of copepods[12]. Analysis and comparison of the sequences of the Tegal duck and *A. platyrhynchos* identified six single nucleotide polymorphisms and one deletion in the conserved 18S rRNA region. The transition and transversion mutation can be seen in Fig. 2 accomplished by a deletion at position 474 bp.

The sequencing result of the PCR product followed by similarity analysis for the Tegal duck showed close homology with those of the 18S rRNA gene of *A. platyrhynchos*. The 18S rRNA sequences of the Tegal duck and *A. platyrhynchos* exhibited conserved regions between the sequences. The 18S rRNA region was also highly conserved

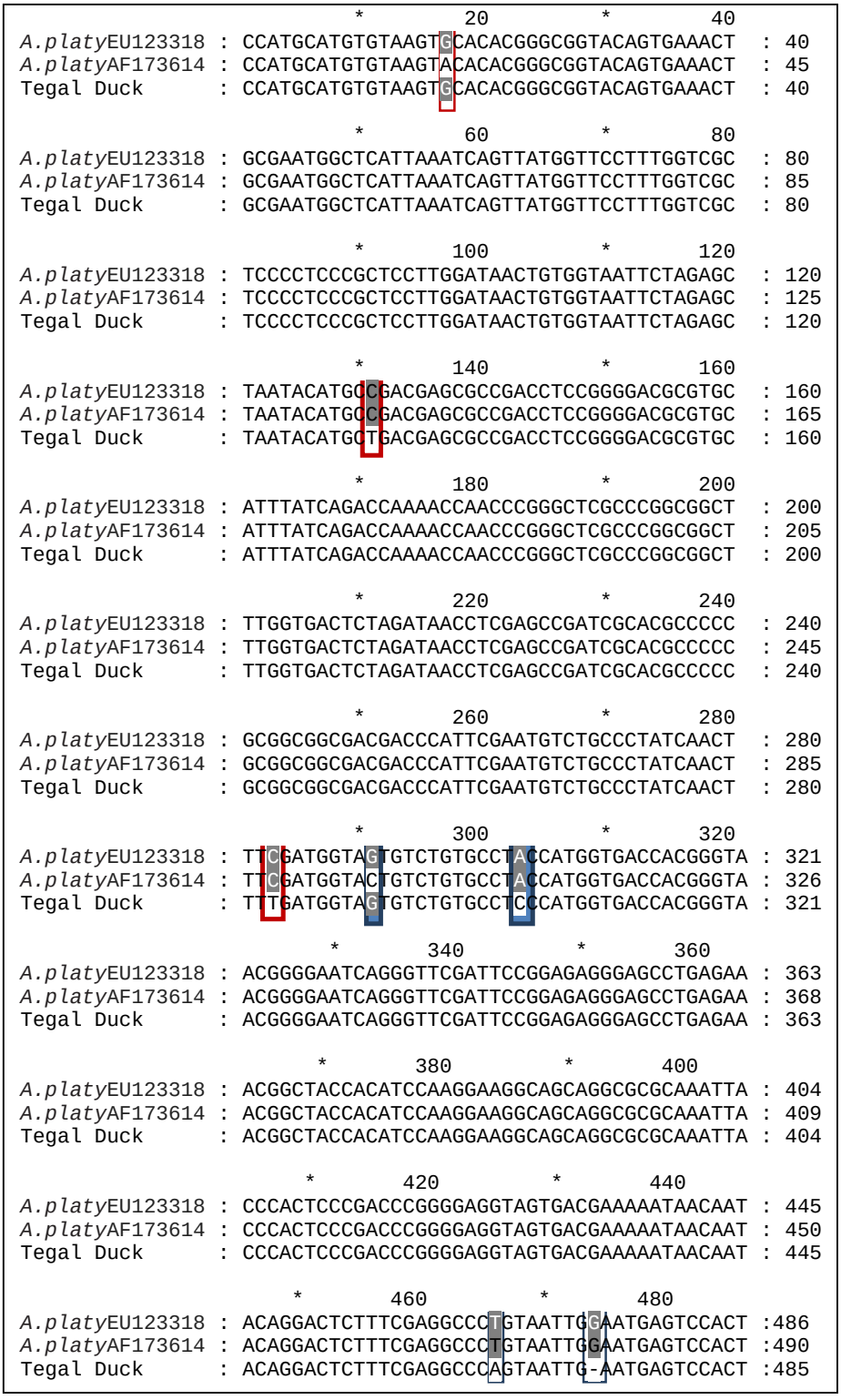


Fig. 2: Comparison of 18S rRNA sequences of the Tegal duck and the interspecies *A. platyrynchos*
Orange box indicates a transition mutation and blue box indicates a transversion mutation

of *A. platyrhynchos* (accession number EU123318.1) and *A. platyrhynchos* (accession number AF173614.1).

The sequencing results of the PCR product of the partial fragment of 18S rRNA gene of the Tegal duck showed close

among species and had been used for species-level analyses for example *A. rubripes*[8] and copepods[12].

Amplification of Tegal duck *COI* gene: The Tegal duck's partial fragment of the *COI* gene of approximately 751 bp are shown in Fig. 3. This *COI* gene fragment showed 99% similarity with sequences from *A. poecilorhyncha* and *A. platyrhynchos*.

Homology analysis revealed that the Tegal duck shared a common ancestor with *A. poecilorhyncha* and *A. platyrhynchos*, as illustrated on its cladogram (Fig. 4). Similarity analysis of the COI gene fragment of 751 bp length revealed that the Tegal duck, compared with some *A. platyrhynchos* species, had several base substitutions as detailed in Table 2. The difference between *A. platyrhynchos* and *A. poecilorhyncha* was the position of the COI gene in the complete mitochondrial genome. The *A. platyrhynchos* COI gene consistently stayed at position 6476-7225, whereas the *A. poecilorhyncha* COI gene was found at several positions, such as 5426-6175 in *A. poecilorhyncha* (accession number KF156760.1) and *A. poecilorhyncha* (accession number KC466567.1) but at other positions such as 6476-7225 in *A. poecilorhyncha* (accession number KF751616.1).

The cladogram on Fig. 4 indicates that some *A. platyrhynchos* and *A. poecilorhyncha* species had been bred and had a descendant possessing both of their characters, whereas others still retained the dominant original character of *A. poecilorhyncha*. The COI gene of Tegal duck showed a unique character combination resemble with 13 subspecies *A. platyrhynchos* and it also gained the character of 3 subspecies of *A. poecilorhyncha.* This result strongly implied that the domestic Tegal ducks from Indonesia may have ancestral A. platyrhynchos predominantly and also little character of *A. poecilorhyncha* species. Similarity analysis of the COI gene fragment with a length of 751 bp showed that the Tegal duck had several base substitutions almost in the same position as that in some *A. platyrhynchos* and *A. poecilorhyncha* species. We have resumed some differences in bases substitution, transition and transversion among *A. platyrhynchos* and *A. poecilorhyncha* as depicted in Table 2.

Further analysis also found 99-100% similarity in the *COI* gene of the Tegal duck with other members of the *Anas* genus as shown in Fig. 5. They exhibited some variations in this region, all with respect to species in the genus *Anas*. In all the *COI* gene fragments, the rate of transition was higher than the rate of transversion.

Similarity analysis of the *COI* gene sequences of the Tegal duck in comparison with the mitochondrial *COI* gene of other members of the *Anas* genus identified 99-100% similarity in their *COI* regions. However, the differences among members in the genus *Anas* is much greater than the difference between Tegal duck with *A. platyrhynchos* and *A. poecilorhyncha*.

Similarity analysis also revealed the number of base substitutions between the Tegal duck and *A. platyrhynchos* with transition mutation in three base positions and a transversion mutation in three base positions, which can be used to distinguish between species. This result creates a bias in the ratio of the number of transitions to the number of transversions. As shown in Table 3, the maximum likelihood estimation of transition/transversion bias (R) for all the sequences is 15.30, which was still within the range for mtDNA[27]. The probability of transition was greater than that of

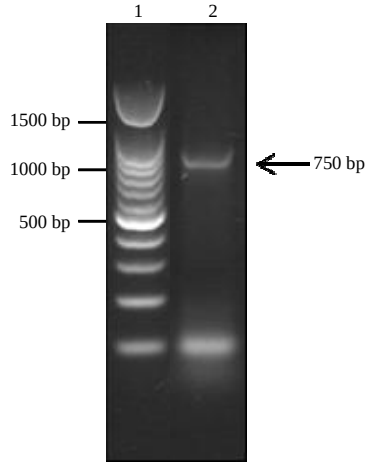


Fig. 3: The *COI* gene fragment of the Tegal duck
lane 1 = 100 bp DNA Ladder, 2 = *COI* gene fragment

Table 2: Base substitutions in the Tegal duck COI gene fragment compared with those of several *A. platyrhynchos* and *A. poecilorhyncha* species

	Tegal Duck and *A. platyrhynchos*	Tegal Duck and *A. poecilorhyncha*
Position of base substitution	7 (T→A),12(C→T),24 (T→C), 27(C→T), 732(T→C), 739(T→A), 740(T→C, 744 (C→A)	7 (T→A), 12(C→T),24 (T→C), 732(T→C), 739(T→A), 740(T→C), 744(C→A) KF156760, KF751616, 27(C→T) KC466567
Type of base substitution	7(transversion), 12(transition), 24 (transition), 27(transition), 732(transition), 739(transversion), 740(transition), 744(transversion)	7(transversion), 12(transition), 24 (transition), 27(transition) 732(transition), 739(transversion), 740(transition), 744(transversion)
Position of base insertion	746 (C→-)	746(C→-)

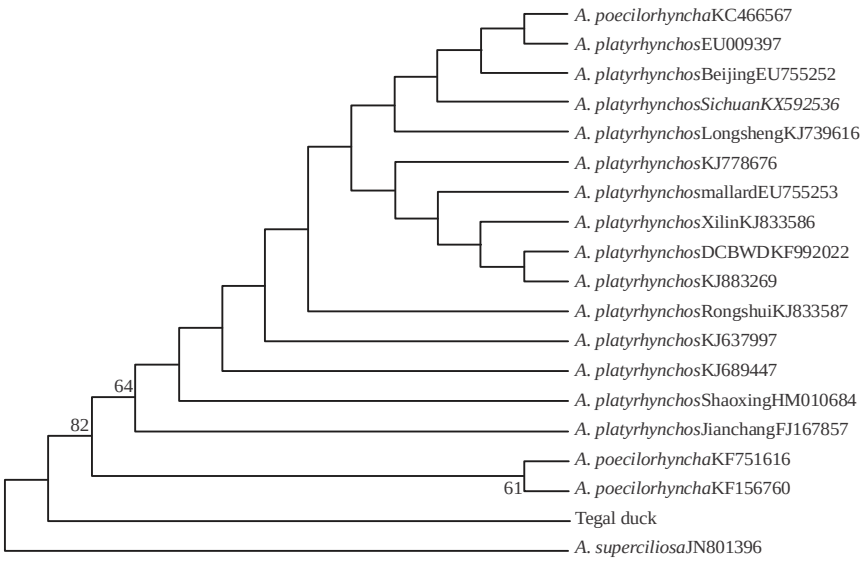


Fig. 4: The phylogenetic relationship of the *COI* gene fragment of Tegal duck with *A. platyrhynchos* and *A. poecilorhyncha*

Table 3: Maximum likelihood estimation of substitution matrix

	A	T/U	C	G
A	-	0.65*	1.05*	24.06**
T/U	0.70*	-	22.55**	0.50*
C	0.70*	13.87**	-	0.50*
G	33.72**	0.65*	1.05*	-

The probability of substitution (r) from one base (row) to another base (column), *Rates of different transversion substitutions are shown, **Those of transition substitutions are shown

transversion, indicating that the changes in the COI gene of the Tegal duck could be silent mutations and therefore do not significantly alter the genes to ensure its taxon in the *Anas* group.

The amino acid analysis of the *COI* gene from the members of the *Anas* genus showed the characteristics of the mitochondrial genetic code, in which codon AUA is used to encode methionine. Furthermore, codon UGA, usually a termination codon, codes for tryptophan. The nucleotide frequencies are A = 24.06%, T/U = 22.38%, C = 36.39% and G = 17.17%. These *COI* regions generally have the highest transition/transversion ratios and are more significant in closely related species. In general, the percentage of amino acids in the *COI* gene fragment in Tegal duck is dominated by alanine (14.5%), followed by isoleucine (13.9%), leucine and glycine (13.2%). It is also observed that codons ending in C are always the most frequently observed in the *COI* gene of *Anas*, followed by codons ending in T and then A or G. Codons ending in C appear to be somewhat more frequently used than those ending in T, among the twofold degenerate codons. The least common third position nucleotide in all categories, except for arginine and glycine codons, is codon G (G having the same frequency with C and T but less than A). The number of base substitutions in the Tegal duck and *A. platyrhynchos* with both transition and transversion mutations in three base positions can be used to distinguish between species. The probability of transition substitution was higher than that of transversion substitution, wherein the substitution of adenine with guanine and vice versa was the greatest. This is affected by the similarity of their molecular structure. Therefore, the probability is smaller for transversion substitution, which is caused by the purine and pyrimidine nucleotides that have a different larger molecular structure[27]. The substitution at the nitrogen bases causes a mismatch in the sequence and there is turnover among the same base pairs (transitions) and the base pairs with its opponent (transversion).

The principal problem of evolution in the nuclear and mitochondrial genomes is a mutation that changes the genes. A gene or DNA sequence with a mutation will cause nucleotide substitution that might be spread into the population by natural selection and genetic drift and

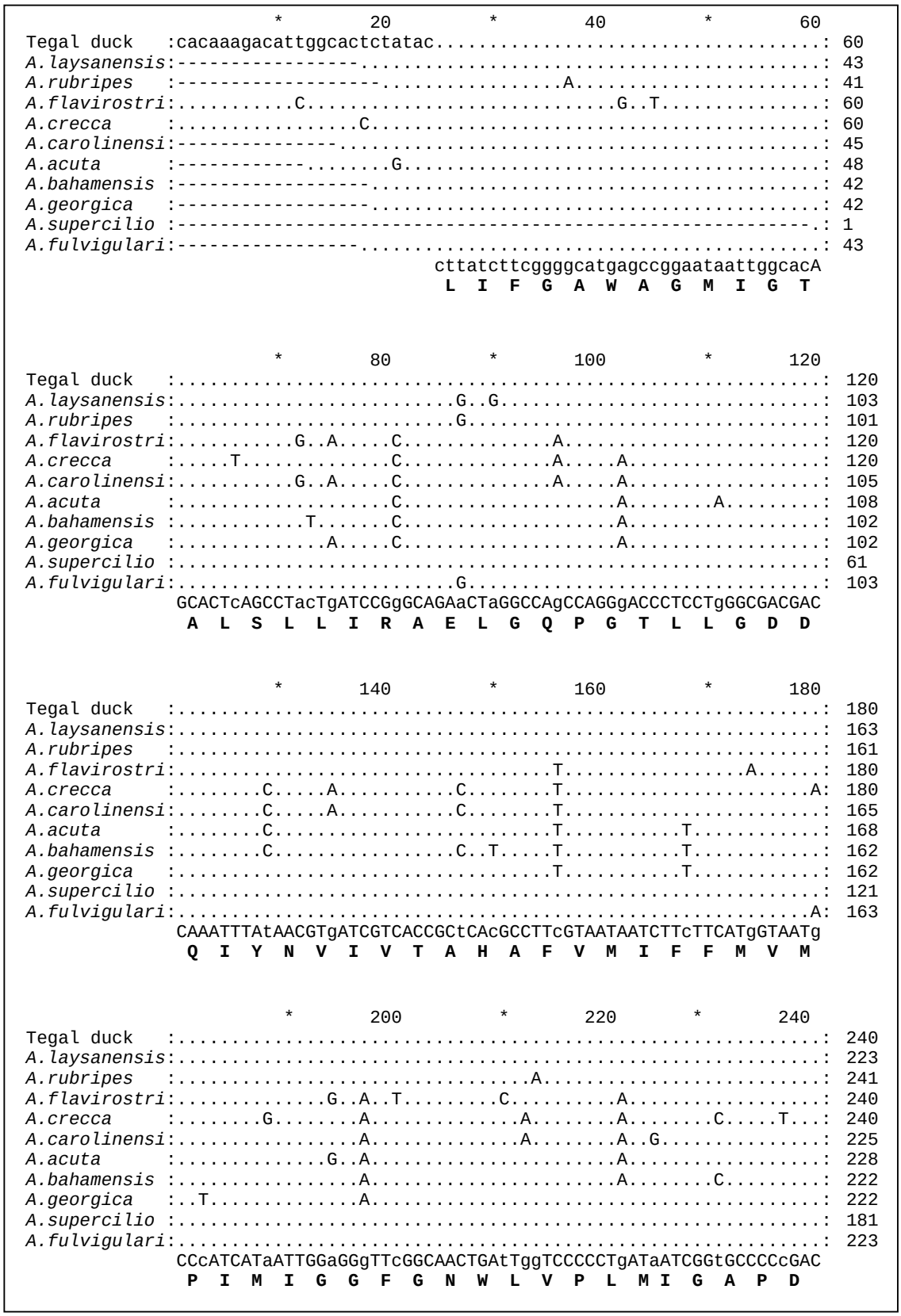
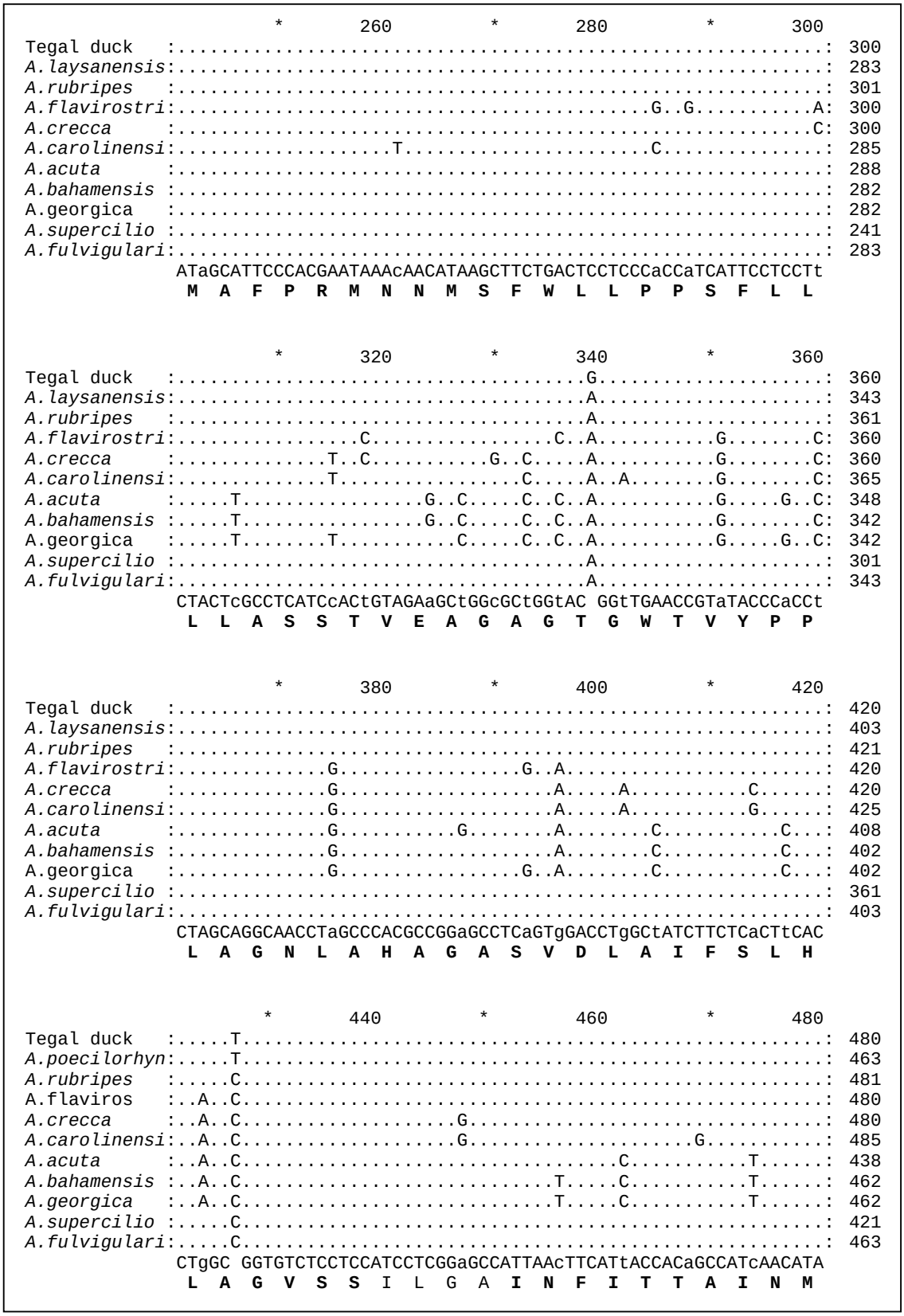


Fig. 5: Continue



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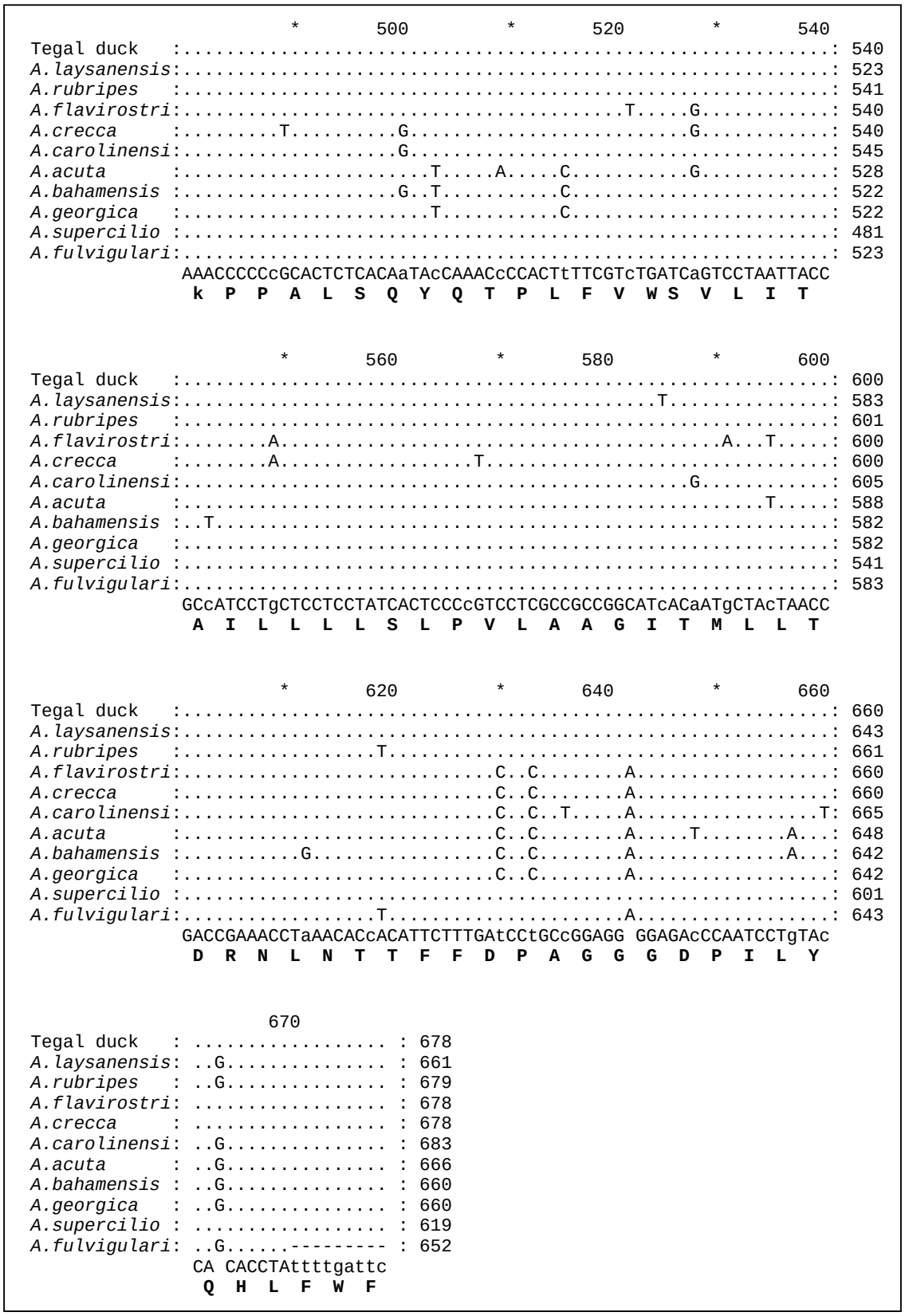

Fig. 5: Similarity analysis of the *COI* gene fragment of the Tegal duck compared with other members of the genus *Anas*

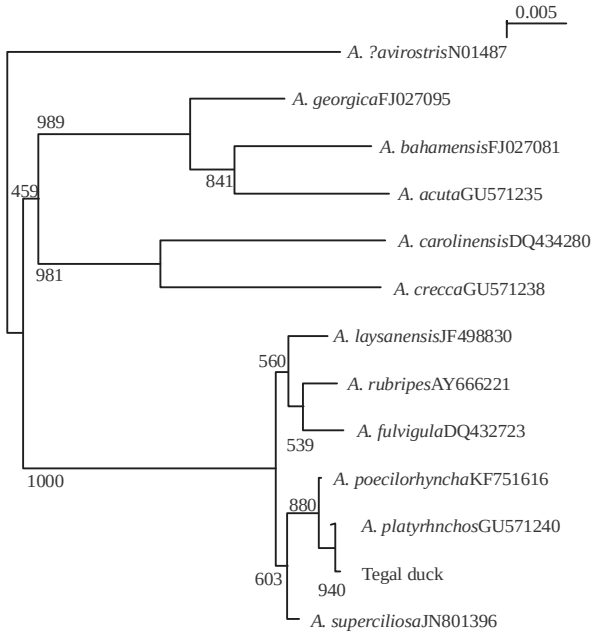

Fig. 6: Phylogenetic evolutionary tree of the *COI* gene displaying the evolutionary relationship of the Tegal duck within a lineage shared by other members of *Anas* species
The scale bar in the upper right represents 0.05 substitutions per nucleotide site

sometimes will be fixed in a species. This character will be inherited into their descendant species if the mutated gene produces new morphological or physiological characters. Single nucleotides can also cause polymorphisms in RNA that will exhibit genetic differences between individuals and have an important function when the mutation occurs in a specific region. A mutation in the 18S rRNA is a heritable change in the DNA sequence that may or may not potentially affect the phenotype of the organism, since the mutations are very important in producing genetic variations. These genes can influence the characteristics of an organism. Changes in any part of the environment could make the organism extinct. The theory of evolution assumes that mutations are random because of their adaptive value. For example, some nucleotides are more mutable than others. The *COI* regions generally have high transition/transversion ratios of mutation and are more significant in closely related species. Nucleotide substitution with a transition bias is a common phenomenon in animal mtDNA. Purified selection can aid in tolerating synonymous mutations and eliminating nonsynonymous mutations[27]. Transition mutations occur at a higher frequency than that of transversion mutations. Transitions are less likely to result in amino acid substitutions and are therefore more likely to be considered silent substitutions in populations[28].

Phylogenetic analysis: Tree construction for a closely related gene using DNA sequences would be better than using protein because it avoids any silent substitution that is phylogenetically informative. The results of the phylogenetic analysis of the members of the *Anas* genus in Fig. 6 showed that the domestic Tegal duck from Indonesia could more likely to be a hybrid of *A. platyrhynchos* and *A. poecilorhyncha* as they are found together in a monophyletic cluster in their phylogenetic tree. Other duck species are well separated using the *COI* gene in mitochondrial DNA. This result was also supported by an adequate bootstrap value of approximately 94% (the number of bootstrap replications was 1000). The result shown in Fig. 6 also reveals that in addition to *A. platyrhynchos* and *A. poecilorhyncha*, the Tegal duck was also a member of the monophyletic cluster with *A. laysanensis, A. rubripes, A. fulvigula* and *A. superciliosa*. This result supports the finding that *A. platyrhynchos* species have hybridized extensively with other closely related species worldwide such as *A. superciliosa* in Australia and New Zealand, *A. rubripes* and *A. platyrhynchos* from North America[29] and *A. laysanensis*[30].

The genus *Anas* is primarily derived from mallard ducks. Moreover, the evolution of *Anas* species is a dominant factor that affects the production of hybrid ducks[29-32]. Based on the *COI* gene fragment of the Tegal duck analysis results which showed 100% homology with *A. platyrhynchos* voucher

NHMO-BC399 (GenBank accession number GU571240), Scandinavian birds from the trans-Atlantic species[33] which indicated that the Tegal duck originated from migrating wild ducks.

The results for the 18S rRNA gene of the Tegal duck confirmed by the results of the *COI* gene showed that it was a hybrid of *A. platyrhynchos* and *A. poecilorhyncha* with dominant character of *A. platyrhynchos*. Combination of both genetic marker had resulted more accurate molecular identification of species.

The results of the phylogenetic evolutionary tree analysis of the *COI* gene also support the phylogenetic analysis result using 18S rRNA, which showed that the Tegal duck have a common ancestor *A. platyrhynchos* and *A. poecilorhyncha*. This result is in agreement with another study which grouping *A. platyrhynchos* with *A. poecilorhyncha*. A phylogenetic relationship was shown among the Korean duck derived from the mallard (*A. platyrhynchos*) and the spot-billed duck (*A. poecilorhyncha*)[34]. However, another study reported that based on their mtDNA, the domestic Korean ducks originated only from *A. platyrhynchos*[6]. Some South and East Asian ducks may live in areas with suitable food and habitat because they are migratory birds and have characteristics by which they can be easily adapted[26]. Hybrids from mallards and spot-billed ducks often appear in natural habitats, which makes it difficult to identify. In current study, phylogenetic analysis also supported the result that the mallard and spot-billed duck breeds are not separated.

The *COI* regions generally have the highest transition/transversion ratios and are more significant in closely related species. These patterns are usually similar for almost all vertebrate groups, except Xenopus[31]. The Tegal ducks are genetically close to *A. platyrhynchos* as they show a closer relationship with other *A. platyrhynchos* species compared with *A. poecilorhyncha*. Moreover, the pairwise distance among the Tegal duck and other members of *Anas* demonstrated a relatively low-level interspecific distance. This smaller difference is possibly due to the crossbreeding of spot-billed ducks and mallards influenced by the same habitats[32]. Phylogenetic analysis also revealed that mallards and spot-billed ducks are genetically very close. The phylogenetic tree analysis also showed that in the monophyletic cluster, the Tegal duck has a close relationship with *A. laysanensis* from Hawaii, *A. rubripes* and *A. fulvigula* from Canada and *A. superciliosa* from Australia. These vast areas have been proven as the migratory habitats of most mallards, covering an area of several thousand kilometers. Mallard populations are highly genetically connected, which accounts for the decrease in the domestic mallard numbers and the genetic diversity exacerbated by alterations in the local environment[6]. Morphologically, mallard and spot-billed duck breeds are highly similar in shape, behavior and habitat and are even genetically compatible for crossbreeding[32,33,35]. Although animal mitochondrial sequences are known to evolve rapidly, their gene arrangements often remain unchanged over long periods of evolutionary time[32,36-37].

The *COI* gene provided a better coverage and wider spectrum of species characterization than the 18S rRNA gene. The use of 18S rRNA in combination with the *COI* gene had overcome the limited availability of the data set in GenBank to conduct a more accurate analysis, which made it possible to find more genetic diversity within *A. platyrhynchos*. This study provides important information for improving egg production of Tegal duck. Since the egg production of *A. platyrhynchos* (average 140-150 each year) was higher than *A. poecilorhyncha* (84-108 each year)[38-40], this study will help in developing the breeding program strategy and selection for the Tegal duck. Thus, a new approach on selection for identification and selection on superior domestic ducks based on the integration of morphological and physiological characterization with molecular characterization may be achieved.

CONCLUSION

In conclusion, phylogenetic analysis based on the nuclear 18S rRNA gene and mitochondrial *COI* gene characterization was gain representative identification of the Tegal duck. The Tegal duck had showed close genetic relationship with *A. platyrhynchos* and *A. poecilorhyncha.*

SIGNIFICANCE STATEMENTS

This study discover that integration of both genetic markers 18S rRNA and COI gene had provided a comprehensive genetic information for Tegal duck and showed their close similarities with not only *A. platyrhynchos* but also *A. poecilorhyncha*. The identification of domestic duck species and genetic variation would be very useful to gain consistency of the progeny and the egg production. Thus, this study provide a new approach on identification and selection on superior domestic ducks based on the integration of morphological and physiological characterization with molecular characterization to guide the development of breeding program for Tegal duck and possibly for another local duck in Indonesia.

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