Iranian Journal of Animal Biosystematics (IJAB)
Vol.8, No.1, 47-55, 2012
ISSN: 1735-434X

Mitochondrial DNA *(CYTB)* divergences in two distinct, Old World and New World Barn Owls

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The Barn Owl, *Tyto alba* (Scopoli, 1769), occurs worldwide and shows a considerable amount of morphological and geographical variation, leading to the recognition of many subspecies around the world. Yet comprehensive study on this species needs to be done. Data from mitochondrial gene *(CYTB)* with 620bp length is analyzed for 30 individuals around the world. Maximum likelihood (ML), maximum parsimony (MP) and bayesian analysis showed considerable genetic variation between *alba* clade (Old World) and *furcata* clade (New World). The amounts of genetic variation within each of these clades are in ranges from 0.4%-1.6% but variation between clades is 7.21%. This data may suggest that Barn Owls of the Old World were a separate species from those of the New World. We found high amount of genetic variation between *T. a. stertens* from Indonesia and *alba* clade and we didn't find any support for recognition of *T. hargei* as a separate species.

Key words: *Tyto alba*- MtDNA- DNA Barcoding- Cytochrome *b*-Phylogeography

INTRODUCTION

Owls are a group at chiefly nocturnal birds which share many patterns in behaviors, morphology and anatomy (Konig and Weick, 2008). Traditionally, living owls divided into two families, Tytonidae and Strigidae. The first, separated into two genera, *Tyto* with 25 species and *Phodilus* with two species (Konig and Weick, 2008). All *Tyto* owls have well developed heart-shaped facial disks, rather small, dark eyes and relatively long legs with central and inner toe equal in length (Konig and Weick, 2008). The Common Barn Owls *Tyto alba* as one of the worldwide species of *Tyto* shows a very high geographical variations. Although, recent molecular studies shed light on systematic situation of Barn Owls, the systematic of this complex species have frequently been discussed and give clue to probable relationships.

According to Dickinson, 2003, the *Tyto* comprises of 32 subspecies in 17 species. Whereas, Konig et al. 1999, 2008 split *Tyto* into 25 species and several subspecies, a number of which have been evaluated to full species statues. Konig and Weick, 2008 distinguished in particular three groups of Barn Owls, each with several races. These three groups all given specified rank: the Common Barn Owl *Tyto alba* (Europe, Africa, Madagascar, Asia South to India and Malaysia) the American Barn Owl *Tyto furcata* (North Central and South America), and the Australian Barn Owl *Tyto delicatula* (Australia, New Zealand and Polynesia).

In a recent molecular study, Wink et al. 2004 studied sequences of mitochondrial protein coding *CYTB* (Mitochondrial cytochrome *b*) gene and nuclear marker (intron LDHb-DNA) for 80 species of Strigidae and 16 species of Tytonidae. According to this study subspecies of Barn Owls formed

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two separated clades including the New World and Old World clades. They raised the taxonomic situation of two subspecies from Australia (*T. f. delicatula* and *T. f. sumbaensis*) into species rank. Furthermore, in the next complimentary study Wink et al. 2008 using *CYTB* and nuclear *RAG-1* (Recombination Activating Gene 1) gene, found high genetic divergence between the New World and Old World samples of the Barn Owl, but could not support it with high bootstrap. In addition they introduced *T. f. bargei* as a separated species. Konig and Weick, 2008 based on Weick et al. 2008 upgraded eight subspecies of Barn Owls into species rank. They defined 25 species and 15 subspecies for Barn Owls (10 subspecies for the Old World and five subspecies for the New World). Recently Aliabadian and Nijman (submitted) based on mitochondrial protein coding *COX1* (mitochondrial cytochrome *c* oxidase subunit 1) gene for several subspecies of Barn Owl from different places of the world, showed a large genetic divergence between Barn Owl of the old and New World.

Here using mitochondrial DNA sequences (*CYTB*) for 30 samples of Barn Owl of the New World and Old World, the molecular divergence between and within in Barn Owl of the Old World and New World revisited.

MATERIAL AND METHODS

SAMPLING

We examined 30 individuals representing seven nominal subspecies of Barn Owl species (Table 1). We used blood, feathers and frozen tissue (typically pectoral muscles) from museum specimen. All DNA materials originated from either the field or from the zoological museum (Natural History Museum of Greece (NHMC), Zoological Museum of Amsterdam (ZMA) and Museum of Natural Science Louisiana State University (LSUMNSL). The taxonomy followed Konig and Weick, 2008.

EXTRACTION AND SEQUENCING

The total genomic DNA was extracted from 96% ethanol-preserved tissue samples by incubating them for overnight at 55°C in extraction buffer (2% sodium dodecylsulphate (SDS), 0.5mg/ml proteinase K). Standard salt extraction method was followed for further extraction (Bruford et al. 1992). The mitochondrial *CYTB* gene was amplified and sequenced using standard primers (H15915; 5'-AAC TGC AGT CAT CTC CGG TTT ACA AGA C-3' and L14841; 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3'), described in Johansson et al. 2002. PCR condition followed Kocher et al. 1989. According to this method each cycle of the polymerase chain reaction consisted of denaturation for 1 min at 93°C, hybridization for 1 min at 50°C, and extension for 2-5 min at 72°C. This cycle was repeated 25-40 times depending on the initial concentration of template DNA in the sample. PCR products were purified using QIA quick PCR purification Kit (Qiagen), following manufacturer instruction. The purified PCR products were sequenced using dye-labeled dideoxy terminator cycle sequencing with Big Dye V.3.1 (Applied Biosystems, Inc). Sequences of the mitochondrial protein coding *CYTB* gene was edited and aligned and finally data set with 620 bp was studied for each taxon.

ALIGNMENT AND SEQUENCE ANALYSIS

The *CYTB* sequences were aligned using BioEdit7.0.5 (Hall, 1999) and checked by eye for the presence of stop codons or insertions/deletions that would have disrupted the reading frame. We conducted Bayesian inference (Huelsenbeck and Ronquist, 2003), as implemented in MrBayes 3.1.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) to estimate phylogenetic relationships. Two independent analyses were performed for a full partitioned strategy for protein-coding *CYTB* gene using MrBayes 3.1.2 (Huelsenbeck and Ronquis, 2001). Each analysis included four

independent Metropoliscoupled Markov chains, which started from random trees and we ran them for 10 million irritations each Chains were sampled every 1000 irritations. All trees obtained before convergence as burn-in were discarded, and the log-Likelihood values (-ln L) and posterior probabilities for nodes were calculated from the remaining trees.
Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were performed for the complete *CYTB* data set, using PAUP 4.0b10 (Swofford, 2002). MP analysis was performed using heuristic searches with tree bisection reconnection (TBR) branch swapping, stepwise addition starting tree, and random addition sequence with 1000 replicates. ML models and parameters were determined using the Akaike Information Criterion (AIC) as implemented in Modeltest (Posada and Crandall, 1998). The estimated model was used in a subsequent ML heuristic tree search with 10 random addition sequence replicates, and TBR branch swapping. To assess the modal support for each branch 100 and 2000 bootstrsp replicates were run under ML and MP, respectively.
TCS software package was used for constructing a minimum spanning network for *CYTB* (Clement et al., 2000), by using the method of probability of parsimony according to Templeton et al. 1992. It evaluated the number of mutational steps based on pairwise haplotypes differ and calculated the probability of parsimony for pairwise differences until the probability exceeds 0.95 (Templeton et al., 1992). The number of mutational differences associated with the probability just before the 0.95 cut-off is then the maximum number of mutational connections between pairs of sequences justified by the parsimony criterion, and these justified connections are applied in a haplotype network (Clement et al., 2000).

RESULTS

GENE PROPERTIES

In total 620 base pairs of the mitochondrial *CYTB* gene were aligned and analyzed in 30 individuals of Barn Owl. The tree was rooted by *Accipiter gentilies* as an out group. There were 49 variable site and 27 parsimony-informative base pairs, excluding outgroup sequences, 10 haplotypes were defined for the data set. Modeltest (AIC) results showed that the GTR+I model had the best fit for *CYTB* gene. Intraspecific K_2P distances ranged from 0.4-1.6% while interspecific K_2P distances ranged from 0.9-7.20% (Table2). Maximum amount of intraspecific K_2P variation found in *T. alba* and minimum amount of interspecific K_2P variation belonged to *T. brgei* and *T. furcata.*

BAYESIAN ANALYSIS

The strict consensus tree supported three major clades (Fig. 1). The first clade contains all subspecies of North America and Curacao (*furcata*), the second one includes subspecies of Asia, Europe and Africa (*alba*). These two clades were separated with a high statistical support (100%). The third clade provided good statistical support for separating Indonesian Barn owl taxa (*Tyto alba stertens*) from the common *alba* clade. Individuals of *T. bargei* placed within the *furcata* clade along with other subspecies of *furcata* (*T. f. pratincola, T. f. hellmeyeri*) (Fig. 1).

ML AND MP

Maximum likelihood analysis base on the selected model resulted in a single tree with $-\ln L = 1688.5381$. The strict consensus tree was similar to Bayesian Inference analysis in Figure 1. Maximum parsimony analyses for the 620 bp datasets recovered a single tree which was well similar to that of Bayesian inference or Maximum likelihood analyses.

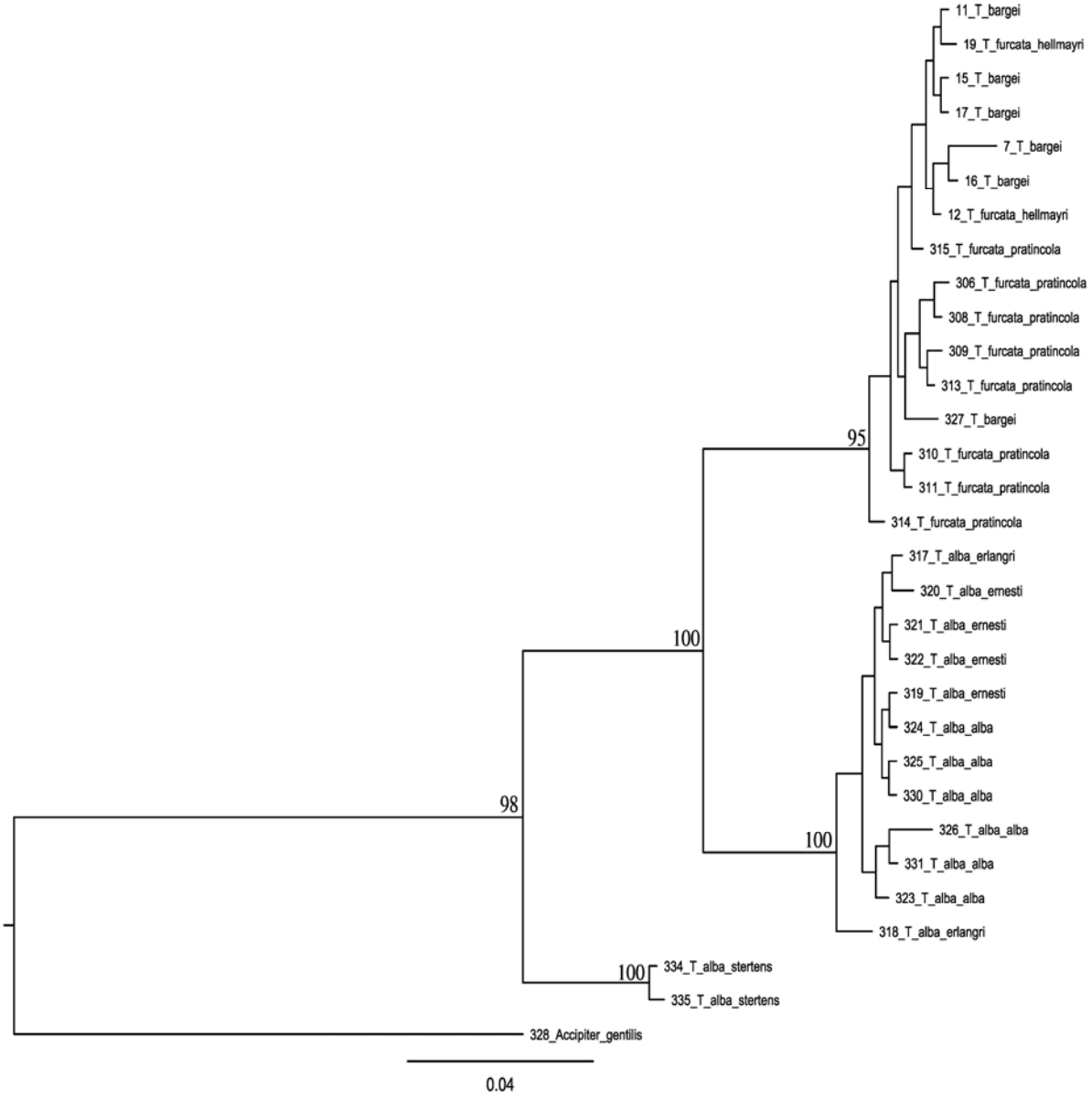

FIGURE 1. Ninety-percent majority-rule consensus tree sampled from the posterior distribution of the most-partitioned analysis. Posterior probability values from the Bayesian analysis are indicated at the >99% (**) >95% (*) significance levels. Numbers represent ML and MP bootstrap values (500/2000 replicates; given only if >70%).

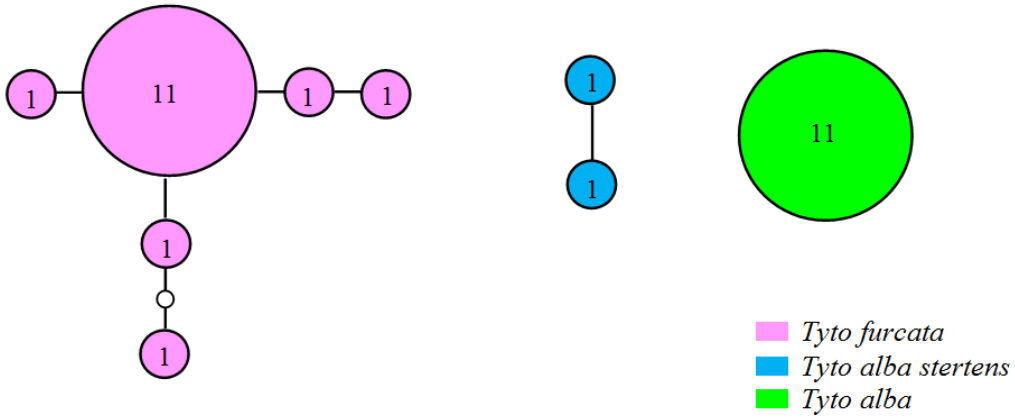

FIGURE 2. Haplotype networks of species of *Tyto* (30 individuals), based on 620bp of the Mitochondrial cytochrome*c*oxidase subunit I gene (*CYTB*). The number that was written inside the haplotypes indicated the samples of them.

TABLE 1- Specimen, collection number and GenBank accession numbers for the samples used in the study that were collected all 5 geographical regions.

Species	Sample ID Number ZMFUM	Location
Tyto alba alba	ZMA 58962	Netherlands
Tyto alba alba	ZMA 58963	Netherlands
Tyto alba alba	ZMA 58964	Netherlands
Tyto alba alba	ZMA 58965	Netherlands
Tyto alba alba	ZMA 58843	Netherlands
Tyto alba alba	ZMA 58844	Netherlands
Tyto alba stertens	ZMA334	Indonesia
Tyto alba stertens	ZMA335	Indonesia
Tyto alba erlangeri	MFUM800002	Iran
Tyto alba erlangeri	MFUM800003	Iran
Tyto alba ernesti	NHMC80.4.108.9	Greece
Tyto alba ernesti	NHMC80.4.108.8	Greece
Tyto alba ernesti	NHMC80.4.108.7	Greece
Tyto alba ernesti	NHMC80.4.108.6	Greece
Tytobargie	ZMA55930	Curacao
Tytobargie	ZMA55939	Curacao
Tytobargie	ZMA55941	Curacao
Tytobargie	ZMA55942	Curacao
Tytobargie	ZMA55943	Curacao
Tytobargie	ZMA 58966	Curacao
Tytofurcatahellmayri	ZMA55945	Burner
Tytofurcatahellmayri	ZMA58259	Burner
Tytofurcatapratincola	LSUMZ.16306	Louisiana
Tytofurcatapratincola	LSUMZ.20610	Louisiana
Tytofurcatapratincola	LSUMZ.20485	Louisiana
Tytofurcatapratincola	LSUMZ.49512	Florida
Tytofurcatapratincola	LSUMZ.49511	Florida
Tytofurcatapratincola	LSUMZ.49509	Florida
Tytofurcatapratincola	LSUMZ.21734	Texas
Tytofurcatapratincola	LSUMZ.29566	Colorado

HAPLOTYPE NETWORK

Haplotype network demonstrated three distinct networks, one of them including all subspecies of *T. alba* (The Old World), the other one enclosed subspecies of *T. furcata* and *T. bargei* (The New World) and the last one included samples of subspecies of Indonesia (*T. a. stertens*) (Fig. 2).

DISCUSSION

In recent years, distance threshold (Hebert et al., 2004) has been used as an effective tool for both the identification of known species and the discovery of new ones. An attribute which characterizes a threshold of variation for each taxonomic group, above which a group of individuals does not belong to the same species but instead forms an infra-specific taxon. A concept for which the term DNA barcoding has been coined (Hebert et al., 2003). However, the initial idea proposed by Hebert et al. 2003, based on just a small portion of a single gene, comprising a 650-bp fragment from the first half of the mitochondrial cytochrome *c* oxidase subunit I gene (*COX1*), the idea has quickly been tested for other part of mitochondrial genes (e.g. Aliabadian et al., 2009; Bradley and Baker, 2001; Lemer et al., 2007). In our data, intraspecific variation of *CYTB* sequences (0.43%) was an average of 18 times smaller than interspecific variation (7.93%), and there was a clear gap between intra- and interspecific variation. Hebert et al. 2003, 2004 utilizing this barcoding gap for *COX1* gene proposed a standard sequence threshold to define species boundaries of around 10 times the mean intraspecific variation for the group under study. In a similar study Wink et al. 2008 showed 1.5-2% of *CYTB* sequences variation in Owls group as a threshold for species threshold.

In all molecular trees obtained from ML, MP, and Bayesian analyses, two clades of the Old World (*alba*) and New World (*furcata*) were split with high amount of bootstrap and statistical support. The amount of variation between these two clades is 7.21% which is above the proposed threshold of species recognition by Wink et al. 2008 (1.5-2%) or Hebert et al. 2003 (10x average intraspecific). Furthermore, in haplotype network analysis, these two clades also constructed two distinct networks. In two independent study using *CYTB* and *RAG-1* genes (Wink et al., 2008) and *COX1* gene (Aliabadian and Nijman, submitted) the split of these two clades has been confirmed, however, Wink et al. 2008 did not show a high amount of support for the split of this clade.

The constructed NJ tree based on published mitochondrial *CYTB* gene sequences (Wink et al., 2008) and our *CYTB* sequences, two main clades between subspecies of the new and Old World were supported with high bootstrap (Fig. 3). One of these clades contains subspecies of the North and South America (the *furcata*-clade) and the Caribbean (*T. bargei*) and the other one contains subspecies of Europe, Asia and Africa (the *alba*-clade). These two clades are highly in congruent with our *CYTB* data set.

Wink et al. 2008 upgraded *T. bargei* as a good species. However, the constructed NJ tree of combined data set placed this taxon within *furcata* clade along with other subspecies of *Tyto furcata* (*T. f. furcata, T. f. hellmayri, T. f. pratincola*). In our data set *T. bargei* showed low genetic divergence (0.6%) with *T. furcuta* and this amount of genetic divergence is lower than the amount that introduced by Wink et al. 2008 for recognizing a distinct species. This result also proved by haplotype network result in which no separate network has been recognized. Therefore, our results cannot support the taxonomic species level of *T. bargei*. This fact is in agreement with Aliabadian and Nijman (submitted) results.

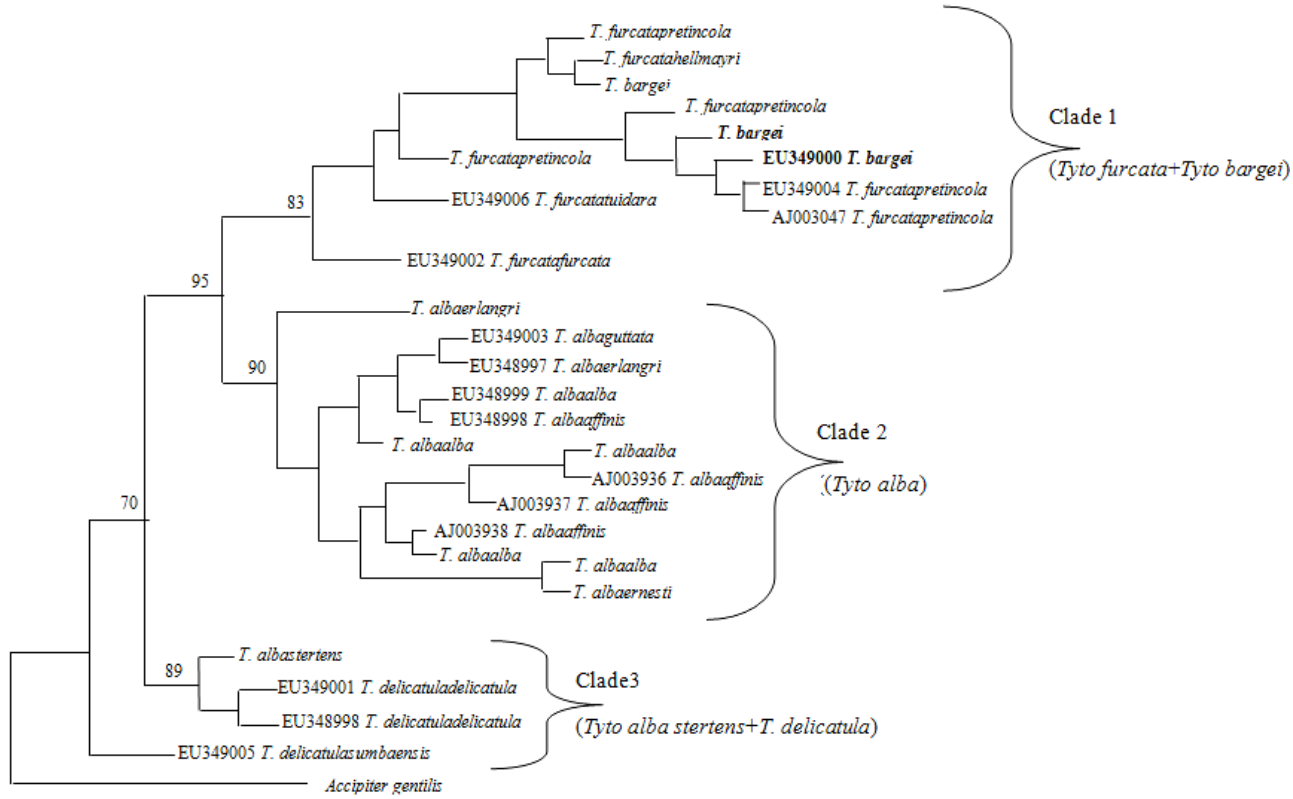

FIGURE 3. NJ tree obtained from combination of all sequences of Wink et al. 2008 and our *CYTB* data set.

TABLE 2- Kimura-2-parameter genetic distance interspecies and intraspecies (Bold numbers) in mitochondrial *CYTB* gene for species of *Tyto.*

Species	*Tytobargei*	*Tytofurcata*	*Tyto alba*
Tytobargei	**0.0066**		
Tytofurcata	0.0091	**0.0701**	
Tyto alba	0.0701	0.0721	**0.0162**

TABLE 3- Kimura-2-parameter genetic distance interspecies and intraspecies (Bold numbers) in mitochondrial *CYTB* gene for *T. alba* and *T. a. stetens.*

Species	*Tyto alba*	*Tytofalbastertens*
Tyto alba	**0.0025**	
Tyto alba stertens	0.0954	**0.0017**

Indonesian Barn owl, *Tyto alba stertens,* formed a well supported clade in all phylogenetic trees and also constructed NJ tree (Figs. 2 and 3). We found also a high level of genetic divergence (9.54%) between *T. alba* and *T. a. stertens* (Table 3). Haplotype network results also confirmed the divergence of Indonesian Barn Owl from other barn owl group. Based on these results, it's possible to introduce *T. a. stertens* as good species. In constructed NJ tree, the Australian subspecies (*T. delicatula delicatula*, *T. delicatula lulu*, *T. delicatula sumbaensis*) clustered with *T. alba stertens* from Indonesia in a distinct clade. This result may show that subspecies of *T. alba stertens* closer to Australian taxa than *alba* one. Overall our study suggests a comprehensive study using further markers for this complex species as one of the most widespread of all birds.

ACKNOWLEDGMENTS

We thank several museums that provided some of the samples for this study; Christos Barboutis (Natural History Museum of Greece, Greece University), Donna L. Dittmann (Museum of Natural Science, Louisiana State University) and Tineke Prines (Zoological Museum of Amsterdam). This study was supported by a grant from Ferdowsi University of Mashhad to N. A. K (P.1021).

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