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Application of Cytochrome *b* Gene Sequences for Identification of Parrots from Korean Zoos

Jung-il Kim[1], Thinh Dinh Do[1], Duri Lee[1], Yonggu Yeo[2], Chang-Bae Kim[1,*]

[1]Department of Biotechnology, Sangmyung University, Seoul 03016, Korea
[2]Conservation and Health Center, Seoul Zoo, Gwacheon 13829, Korea

ABSTRACT

Parrots are common targets for illegal trade because of their beauty and high price. Accurate identification is necessary for the prevention of illegal trade and conservation of parrots. In the present study, mitochondrial markers of cytochrome *b* (*CYTB*) gene were used to identify parrot species from Korean zoos. Totally, 27 samples were collected from Seoul Zoo, Cheongju Zoo, and Uchi Zoo. After collection, total DNA of samples was extracted and used for PCR amplification. *CYTB* fragments were sequenced from all samples examined. The obtained sequences were used for GenBank blast, distance estimation, and phylogenetic analysis. All species were identified using *CYTB* sequences that determined 27 samples belong to 13 species in 7 genera, and 3 families. Our finding demonstrated the usefulness of *CYTB* sequences for identifying parrot species in Korean zoos.

Keywords: zoo, illegal trade, DNA barcoding, *CYTB* gene

INTRODUCTION

Natural exploitation together with human activities has resulted in the loss of world biodiversity. Conservation measures are necessary to protect wild species from extinction. Because of threats to biodiversity in nature, conservation of species in its natural habitat may be challenging. In this circumstance, zoos have changed the roles from animal exhibitions to institutions that handle multiple missions such as conservation, scientific research, and education (Witzenberger and Hochkirch, 2011). It is well-known that zoo is important to raise people's awareness on wildlife conservation and protection. In addition, many endangered species are growed and bred in zoos for conservation targets and they could be reintroduced to their natural habitats in the future (Wirtz et al., 2018). The accumulation of specimens may result in difficult tasks for zoo staff in species recognition. A reliable and accurate method for species identification is useful for zoo staff to conduct their role in conservation and management.

High demand for wildlife products from humans has resulted in the illegal wildlife trade. The illegal wildlife trade is a serious problem that threats the survival of wild organisms and shows negative effects on wildlife conservation. Because of their colorful plumage and mimicry ability, parrots (the order Psittaciformes) are in high demand as pets and highly prized. It is reported that parrot is the most traded bird among all avian orders (Bush et al., 2014). According to the International Union for Conservation of Nature (IUCN), 37 parrot species are endangered, 18 species are critically endangered and 16 species are already extinct (https://www.iucnredlist.org). Most parrots are protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (http://checklist.cites.org/#/en). Therefore, accurate identification of parrots is necessary to support authorities to manage parrot trade. However, morphological identification of parrots is challenging due to their slight difference in morphology.

DNA barcoding has been proven to be a powerful tool for discrimination of wildlife products that are obtained from illegal trade (Lahaye et al., 2008; Presti et al., 2015). For animals, the mitochondrial protein-coding genes and ribosomal RNA genes are popular markers for DNA barcoding technique. Of mitochondrial markers, in addition to cytochrome *c* oxidase subunit I (*COI*) gene, cytochrome *b* (*CYTB*) gene is also used for molecular analysis (Arif and Khan, 2009). In avian, particularly *CYTB* gene has been used for the studies of phlogenetics, so that is more represented than other genes on

*To whom correspondence should be addressed
Tel: 82-2-2287-5288, Fax: 82-2-2287-0070
E-mail: evodevo@smu.ac.kr

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Table 1. Parrot samples collected and examined in this study

Family	Species name	Zoos	Form	No. of samples
Cacatuidae	*Cacatua alba*	Seoul zoo	Blood	1
	Cacatua ducorpsii	Uchi zoo	Feather	1
	Cacatua galerita	Seoul zoo	Blood	1
	Cacatua goffiniana	Seoul zoo	Blood	1
	Cacaua moluccensis	Seoul zoo	Feather	1
		Cheongju Zoo	Feather	1
	Nymphicus hollandicus	Seoul zoo	Feathers	2
		Cheongju Zoo	Feathers	2
Psittacidae	*Ara ararauna*	Seoul zoo	Feather	1
		Cheongju Zoo	Feather	1
		Uchi zoo	Feather	1
	Ara chloropterus	Seoul zoo	Feather	1
		Uchi zoo	Feather	1
	Ara macao	Seoul zoo	Feather	1
	Myiopsitta monachus	Seoul zoo	Feathers	2
Psittaculidae	*Eclectus roratus*	Seoul zoo	Feather	1
		Uchi zoo	Feather	1
	Lorius garrulus	Seoul zoo	Feather	1
		Cheongju Zoo	Feather	1
	Melopsittacus undulatus	Seoul zoo	Feathers	2
		Cheongju Zoo	Feathers	2
		Uchi zoo	Feather	1

Table 2. Cytochrome *b* (*CYTB*) gene primers used in this study

Primer name	Direction	Sequence (5′–3′)	Reference
MT-A1	F	CAACATCTCAGCATGATGAAACTTCG	Wink and Sauer-Gürth (2000)
Mte	R	GCAAATAGGAAGTATCATTCTGG	Fritz et al. (2006)
ND5L 14754	F	GGACCAGAAGGACTTGCCGACCTA	Ribas (2004)
H15400	R	AAGAATCGGGTTAGGGTGGGG	Braun (2014)
L15311	F	GTCCTACCATGAGGTCAAATATC	Braun (2014)
HThr 16082	R	TCTTTTGGTTTACAAGACCAATG	Kornegay et al. (1993)

reference databases such as GenBank (Branicki et al., 2003; Coghlan et al., 2013). Also, *CYTB* gene have been widely applied for avian species identification (Lee et al., 2008; Aliabadian et al., 2009; Boonseub et al., 2009) and estimating interspecific genetic relationship (Moore and DeFilippis, 1997). In this study, partial mitochondrial protein-coding genes (*CYTB* gene) were used to identify parrot species from Korean zoos. For this purpose, the partial *CYTB* gene of all samples was sequenced and analyzed.

MATERIALS AND METHODS

There were 15 feather and blood samples collected from Seoul Zoo (Seoul). Another set of samples including 12 feathers were collected from Cheongju Zoo (Cheongju), and Uchi Zoo (Gwangju). The sample information used in this study is presented in Table 1. The ethical procedure for using blood and muscle tissue was approved by the Seoul Zoo IACUC (no. 2019-001).

Upon sample collection, total DNA was extracted and measured purity and concentration. The partial mitochondrial *CYTB* gene was amplified for all collected samples. *CYTB* primers used in this study are listed in Table 2. The 20 μL of PCR reaction mixture contained 10 μL of 2× DyeMix (Enzynomics, Korea) with 1.0 U of Taq polymerase, 1 μL of each primer (5 pmole/μL), 100 ng DNA and distilled water up to 20 μL. The amplification protocol was as follows: ini-

Table 3. Cytochrome *b* (*CYTB*) gene sequences of parrot species in the present study in comparison to GenBank database

No.	Family	Species name	No. of samples	Accession no.	Identity (%)	E-value
1	Cacatuidae	*Cacatua alba*	1	MT275977	99.0	0
2		*Cacatua ducorpsii*	1	MT275978	100	3E-163
3		*Cacatua galerita*	1	MT275979	100	0
4		*Cacatua goffiniana*	1	MT275980	99.9	0
5		*Cacatua moluccensis*	2	MT275981–MT275982	100	0
6		*Nymphicus hollandicus*	4	MT275983–MT275986	100	0
7	Psittacidae	*Ara ararauna*	3	MT275987–MT275989	100	0
8		*Ara chloropterus*	2	MT275990–MT275991	99.7	0
9		*Ara macao*	1	MT275992	100	0
10		*Myiopsitta monachus*	2	MT275993–MT275994	99.9–100	0
11	Psittaculidae	*Eclectus roratus*	2	MT275995–MT275996	100	0
12		*Lorius garrulus*	2	MT275997–MT275998	99.7	0
13		*Melopsittacus undulatus*	5	MT275999–MT276003	99.7–100	0

Table 4. Intraspecific distance and interspecific distance (%) of congenetic species based cytochrome *b* (*CYTB*) sequences

No.	Family	Species name	Intraspecific distance (%)	Interspecific distance (%)
1	Cacatuidae	*Cacatua alba*	0.00–0.33	5.97–11.30
2		*Cacatua ducorpsii*	0.00	2.03–11.82
3		*Cacatua galerita*	0.00–0.67	3.07–8.60
4		*Cacatua goffiniana*	0.00–0.33	1.34–11.00
5		*Cacatua moluccensis*	0.00–1.68	5.23–13.52
6	Psittacidae	*Ara ararauna*	0.00–0.52	6.00–13.39
7		*Ara chloropterus*	0.00–0.52	4.32–10.24
8		*Ara macao*	0.00–0.78	3.76–11.16
9	Psittaculidae	*Lorius garrulus*	0.16	2.30–3.48

Distances were estimated based sequences in this study and GenBank sequences.

tial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 1 min, extension at 72°C for 1 min and final elongation at 72°C for 5 minutes. The PCR products were analyzed by electrophoresis in 1% (w/v) agarose gels in 1% tris-acetate buffer. The PCR products were sequenced directly by Sanger sequencing.

DNA sequence data sets were verified quality, and the consensus sequence was extracted from forward and reverse sequences using Geneious 9.1 software (Kearse et al., 2012). The consensus sequence was blasted on GenBank database and identified species with the highest similarity. For the genus with multiple species and sequences available on Genbank, additional sequences were retrieved from GenBank for further analyses. Sequence distances were estimated using Kimura-2-parameter (K2P) distance model (Kimura, 1980) in MEGA X software (Kumar et al., 2018). For reconstruction of the phylogenetic tree, the Maximum likelihood method with 1,000 bootstrap replicates in MEGA X software was used (Kumar et al., 2018).

RESULTS AND DISCUSSION

Totally, 27 sequences of *CYTB* gene were generated. The blast result of *CYTB* sequences showed identity >99.0% with sequences available on Genbank for all samples (Table 3). Intraspecific distance and interspecific distance for examined species are presented in Table 4. Among five *Cacatua* species collected in this study, maximum intraspecific distance varied from 0% (*C. ducorpsii*) to 1.68% (*C. moluccensis*). These values are lower than minimum interspecific distance of the same species. The similar pattern was also found in *Ara* spp. and *Lorius garrulus* (Table 4). Combination of blast result and comparision of sequence distances, there were 13 parrot species identified from three Korean zoos. The examined parrots belonged to 7 genera and 3 families of the order Psittaciformes. The finding based on *CYTB* sequences is consistent

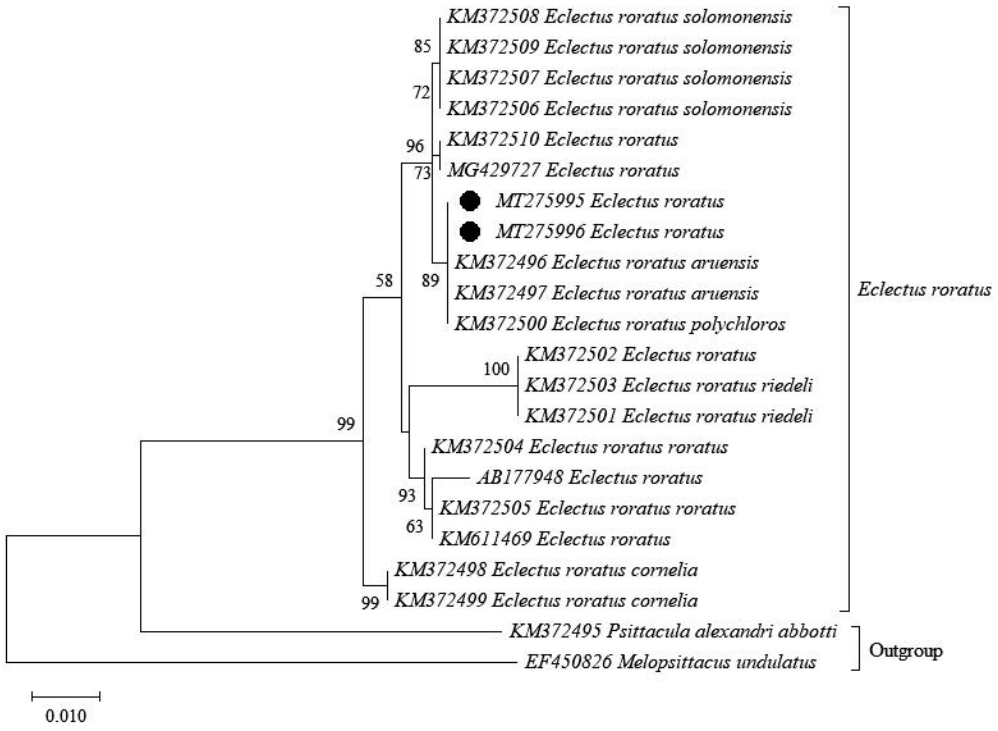

Fig. 1. Phylogenetic analysis of *Eclectus roratus* complex based on cytochrome *b* (*CYTB*) sequences. GeneBank accession numbers of sequences are next to species. The sequences in this study were lablled with black dots. Bootstrap values >50 are shown at nodes.

to species labelled by zoos (Table 1).

Further analyses of *Eclectus roratus* sequences indicated that *E. roratus* is divided into 4 clusters and 2 samples in this study belonged to a cluster that includes 3 subspecies: *E. r. aruensis*, *E. r. polychloros* and *E. r. solomonensis* (Fig. 1). Our finding is congruent with the results reported by Braun et al. (2016). According to NCBI Taxonomy (http://www.ncbi.nlm.nih.gov/taxonomy), *E. roratus* is a single species of the genus *Eclectus* and includes six subspecies: *E. r. aruensis*, *E. r. cornelia*, *E. r. polychloros*, *E. r. riedeli*, *E. r. roratus*, and *E. r. solomonensis*. However, based on analyses of *CYTB* sequences together with morphological and geographical data, Braun et al. (2016) revealed that *Eclectus roratus* is species complex and its 6 subspecies could be devided into 4 independent species: *E. roratus*, *E. cornelia*, *E. riedeli*, and *E. polychloros*. Accordingly, *E. polychloros* consisted of 3 subspecies: *E. r. aruensis*, *E. r. polychloros*, and *E. r. solomonensis* while each remaining species consisted of 1 subspecies. This demonstrated that *CYTB* marker is a useful tool for discrimination of species complex in parrot.

Due to their beauty and ability, parrots are one of the most common captive birds (Frynta et al., 2010). The illegal trade that meets human's high demand is a serious threat to parrots. Under biodiversity threats zoo has emerged its role in species conservation. The accurate identification of parrots particularly which taxonomy is under discussion or recently identified in zoo is necessary for management and protection of parrots. In this study, DNA barcoding was applied to identify parrots from different Korean zoos. With *CYTB* marker, all collected species were identified based on blast results and the comparison between intraspecific and interspecific variations. Especially, *E. roratus* in this study might be identified to *E. polychloros* following Braun et al. (2016). It could be helpful to for management and protection of parrots in Korean zoos by correct identification. Since the first study of Hebert et al. (2004), numerous studies have used DNA barcoding to identify bird species, including parrots. Together with other mitochondrial markers, more *CYTB* sequences have been generated and accumulated on databases. With the increasing accumulation of *CYTB* sequences from different localities in the world, *CYTB* gene becomes a powerful approach for avian. Therefore, the gene has been applied to study avian identification and phylogeny (Astuti et al., 2006; Lee et al., 2008). This step is necessary to protect endanger bird species like many parrots from extinction.

In this study, *CYTB* gene sequences were applied to identify parrot species from three Korea zoos. The result showed that the use of *CYTB* sequences was effective for parrot identification. In addition, the marker is able to separate independent species from species complex group as the case of *E.*

eclectus. More species should be included in the future study to show the effectiveness of *CYTB* gene sequences and resolve better phylogeny of parrots.

ORCID

Jung-il Kim: https://orcid.org/0000-0001-7851-2493
Thinh Dinh Do: https://orcid.org/0000-0001-8945-1518
Duri Lee: https://orcid.org/0000-0002-5784-3653
Yonggu Yeo: https://orcid.org/0000-0002-5578-2877
Chang-Bae Kim: https://orcid.org/0000-0002-1040-7600

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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