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Molecular identification of selected parrot eggs using a non-destructive sampling method

Jung-Il Kim, Jong-Won Baek and Chang-Bae Kim*

Department of Biotechnology, Sangmyung University, Seoul 03016, Republic of Korea

Contribution to Environmental Biology

- Using a non-destructive sampling method, 43 parrot eggs were identified as seven species.
- Results of this study might help control legal and illegal trade of parrot eggs.

***Corresponding author**
Chang-Bae Kim
Tel. 02-2287-5288
E-mail. evodevo@smu.ac.kr

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Abstract: Parrots have been threatened by global trade to meet their high demand as pets. Controlling parrot trade is essential because parrots play a vital role in the ecosystem. Accurate species identification is crucial for controlling parrot trade. Parrots have been traded as eggs due to their advantages of lower mortality rates and more accessible transport than live parrots. A molecular method is required to identify parrot eggs because it is difficult to perform identification using morphological features. In this study, DNAs were obtained from 43 unidentified parrot eggs using a non-destructive sampling method. Partial cytochrome *b* (*CYTB*) gene was then successfully amplified using polymerase chain reaction (PCR) and sequenced. Sequences newly obtained in the present study were compared to those available in the GenBank by database searching. In addition, phylogenetic analysis was conducted to identify species using available sequences in GenBank along with sequences reported in previous studies. Finally, the 43 parrot eggs were successfully identified as seven species belonging to two families and seven genera. This non-destructive sampling method for obtaining DNA and molecular identification might help control the trade of parrot eggs and prevent their illegal trade.

Keywords: species identification, parrot eggs, non-destructive sampling method, DNA barcoding, mitochondrial cytochrome *b* (*CYTB*) gene

1. INTRODUCTION

Parrots (order Psittaciformes) play essential roles in the ecosystem by consuming the reproductive systems of plants and dispersing their seeds (Blanco *et al.* 2018). Despite their ecological significance, parrots are one of the most threatened species among birds because of the global trade to meet their high demand as pets (Pires 2012; Scheffers *et al.* 2019). Controlling the trade of parrots is crucial for their conservation (Scheffers *et al.* 2019). In this regard, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) controls the parrot trade. CITES has controlled wildlife trade by listing species in appendices depending on the need for controlled trade (https://cites.org). The Appendix I includes species threatened with extinction, and their trade is prohibited, while the Appendix II includes species that can be traded with export permits, and the trading of the listed species is closely monitored. A total of 413 parrot species belong-

ing to three families and 89 genera have been reported (Del Hoyo 2020). Among these, 409 parrot species are listed in the CITES Appendices I and II (https://checklist.cites.org). Despite the regulation of CITES, parrots have been illegally traded frequently in various countries (Sánchez-Mercado *et al.* 2021). Even if the trading of parrots is not regulated by CITES, these species might be captured often to sustain illegal trafficking (Olah *et al.* 2016; Formentão *et al.* 2021). In addition, most parrots are threatened due to habitat loss and fragmentation by human activity (Olah *et al.* 2016). The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (https://www.iucnredlist.org) data confirm that the wild population of parrots is rapidly decreasing. Therefore, urgent actions must be taken to prevent the extinction of all parrots regardless of the CITES Appendices designation.

Parrot eggs are widely traded due to the advantages of lower mortality rates and ease of transport than live parrots (Alacs and Georges 2008; Ortiz-von Halle 2018). These have been frequently traded illegally using purpose-built body vests to conceal the eggs (Coghlan *et al.* 2012). Hence, accurate species identification of these eggs is essential to control their legal and illegal trades (Coghlan *et al.* 2012). A molecular method is needed to identify parrot eggs because of the difficulty in identifying the species of parrot eggs based on eggshell morphology (Alacs and Georges 2008; Coghlan *et al.* 2012). Currently, the eggshell membrane has been used to extract DNA for identifying parrot eggs (Coghlan *et al.* 2012; Formentão *et al.* 2021). Previous studies have reported that high-quality DNA could be extracted from the eggshell membrane (Trimbos *et al.* 2009; Coghlan *et al.* 2012). Although this method successfully identified parrot eggs, it should destroy eggs. Typically, this destructive sampling method has been considered as a limitation to be overcome in wildlife forensics (Sahajpal and Goyal 2010; Ueland *et al.* 2020). This is because illegally traded eggs might be essential evidence in a court of law (Kumar *et al.* 2014). In addition, the use of destructive sampling method may be restricted or even forbidden for testing the eggs of endangered species (Richards *et al.* 2014). The methods that involve destroying the eggs to extract DNA should be carefully considered because those reduce the hatching success rate of the eggs (Khabisi *et al.* 2012). Therefore, a non-destructive sampling method that maintains the intactness of eggs might be helpful in identifying the eggs of both endangered and non-endangered parrots. Egloff *et al.* (2009) suggested a non-destructive method of obtaining eggshell powder by grinding the surface of eggs. This method could be used to obtain maternal DNA from the eggshell because epithelial cells are abraded from the surface of the oviduct wall and incorporated into the matrix of the eggshell (Egloff *et al.* 2009). This method may be more appropriate for controlling the trade of parrot eggs (Oskam *et al.* 2010).

The DNA barcoding technique has been globally used for species identification of wildlife (Khedkar *et al.* 2016; Dalton *et al.* 2020; Park *et al.* 2022). This technique can also be applied to assign an unknown sample that is difficult to identify morphologically to a known species (Meyer and Paulay 2005). Mitochondrial genes have been widely used as a major target in animal DNA barcoding (Hebert *et al.* 2003). Among the mitochondrial genes, cytochrome *c* oxidase subunit I (*COI*) and cytochrome *b* (*CYTB*) genes have been universally applied for the identification of avian species (Hebert *et al.* 2004; Kim *et al.* 2020). In particular, the *CYTB* has been a representative gene in identifying avian species (Branicki *et al.* 2003). There are also more available *CYTB* gene sequences of parrots than other mitochondrial genes in molecular databases, such as the GenBank (Coghlan *et al.* 2012).

Despite the importance of the identification of parrot eggs without their destruction, there have been no study to identify parrot eggs by using a non-destructive sampling method to obtain their DNA. In the present study, the DNA of 43 unidentified parrot eggs was obtained without destroying the eggs. The partial *CYTB* gene was amplified from the DNA and then sequenced. By using database searching and phylogenetic analysis, the eggs have been clearly identified into seven species.

2. MATERIALS AND METHODS

A total of 43 unfertilized parrot eggs were obtained from two pet shops located in Incheon and Seoul, South Korea. These were obtained without knowing the species that laid the eggs. Those were used without predicting species by their morphological features due to the extreme difficulty of morphological identification of the eggs. These were numbered from PE 01 to PE 43 and stored at −80°C to prevent rot. A non-destructive

sampling method suggested by Egloff *et al.* (2009) was modified and used in the present study. First, the surface of parrot eggs was cleaned using 70% ethanol and DNA AWAY (Molecular BioProducts, USA) to remove any foreign DNA on the parrot egg surface. Then, all other residues were removed using distilled water. Finally, the surface of parrot eggs was dried using sterile gauze by removing all the remaining liquids. To obtain eggshell powder, the parrot egg was placed on a weighing paper that was placed on a plastic 50-mL conical tube rack. The egg was ground using a mini grinder equipped with a round-shaped diamond grinding burr for minimal powder loss. The grinding burr was cleaned by the same method used for cleaning the parrot eggs to prevent cross-contamination among parrot eggs examined in this study. In addition, the weighing paper was replaced with a new one before collecting a new eggshell powder. 10 mg of eggshell powder was collected in a 1.5 mL Eppendorf tube. All eggshell powder samples were stored at −80°C for further experiments.

Total DNA was extracted from the eggshell powder samples using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The purity and concentration of extracted DNA were measured using the MaestroNano spectrophotometer (Maestrogen, Hsinchu, Taiwan). The purity of DNA was measured using a 260 nm/280 nm ratio (A260/A280) and 260 nm/230 nm ratio (A260/A230), which indicate the estimated levels of protein contamination and organic contamination, respectively. The partial *CYTB* gene was amplified using the primer pairs Mte (5′ GCA AAT AGG AAG TAT CAT TCT GG 3′) (Fritz *et al.* 2006) and MT-A1 (5′ CAA CAT CTC AGC ATG ATG AAA CTT CG 3′) (Wink and Sauer-Gürth 2000). PCR was performed using a 20-μL sample comprising 1.0 U of Taq polymerase with 10 μL of 2 × Dye-Mix (Enzynomics, Korea), 1 μL of each primer (10 pmol μL^{-1}), 3 to 5 μL of DNA, and distilled water up to 20 μL. The reaction conditions were as follows: initial denaturation for 2 min at 95°C, 35 cycles of 1 min at 95°C, 45 s at 48°C, 1 min at 72°C, and a final elongation step for 5 min at 72°C. The PCR products were evaluated using 1% (w/v) agarose gels in 1% tris-acetate buffer. The PCR products were directly sequenced with the primer pairs using the Sanger sequencing method.

The consensus sequence was extracted from forward and reverse direction sequences by alignment using Geneious 9.1 software (Kearse *et al.* 2012). These final sequences were deposited in GenBank under accession numbers OQ413731−OQ413773. The sequences were compared with those from the GenBank through the BLAST search, and the top one sequence showing the highest sequence similarity selected (Altschul *et al.* 1997). The additional *CYTB* gene sequences of congeneric species were retrieved from the GenBank to analyze the phylogeny of species examined in this study. The sequences of closely related species of each species were selected as an outgroup of the phylogeny based on the phylogenies of the parrots analyzed in previous studies (Ribas and Miyaki 2004; Ribas *et al.* 2006; Manegold and Podsiadlowski 2014; Kim *et al.* 2021; Kim *et al.* 2022). The sequences were aligned using MAFFT (Katoh and Standley 2013) in Geneious 9.1 software with the default setting. A suitable region for phylogenetic analysis was selected using GBlocks (Talavera and Castresana 2007) in the Phylogeny.fr pipeline with the default setting (Dereeper *et al.* 2008). The best-fit substitution model was determined using jModelTest (Darriba *et al.* 2012). Length and best-fit substitution model for each *CYTB* gene sequence dataset to construct phylogenies of the species examined in this study are presented in Table S1. Phylogenetic analysis was conducted by Maximum Likelihood (ML) using IQ-TREE v1.6.12 (Nguyen *et al.* 2015). Node supports were calculated using 5,000 bootstrap replicates. ML method has been commonly used to construct phylogeny because this typically presents the compromise between accuracy and computational requirements (Kang *et al.* 2022; Maio *et al.* 2023). The resulting trees were visualized and edited using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Genetic distances were evaluated using Kimura-2-parameter (K2P) distance model (Kimura 1980) in MEGA X software (Kumar *et al.* 2018) using the same dataset of analyzing to construct each phylogeny.

3. RESULTS AND DISCUSSION

The values of A260/A280 and A230/A260 of the egg samples in this study varied from 1.092 to 2.703 and from 0.515 to 2.903, respectively (data not shown). The values of A260/A280 and A260/A230 for pure DNA are 1.8 and 2.0, respectively (Qamar *et al.* 2017; Sloan *et al.* 2021). The purity of the extracted DNA was lower than that of the pure DNA. The concentration of DNA

varied from 5.09 to 101.61 ng μL^{-1} (data not shown). The eggshell consists of calcium carbonate and an organic matrix such as a cuticle layer (Oskam *et al.* 2010). Typically, calcium ions and cuticles have been known as inhibitors of DNA extraction (Mohammadi *et al.* 2017; Sloan *et al.* 2021). The low purity and concentration of DNA extracted from 43 parrot eggs might be due to these components of eggs. The DNA samples extracted from parrot eggs were successfully amplified by PCR for the marker on the mitochondrial *CYTB* gene despite the low purity and concentration of DNA. Among the examined samples, seven representative PCR products identified as different species; *Nymphicus hollandicus* (Kerr 1792), *Pyrrhura molinae* (Massena and Souancé 1854), *Agapornis roseicollis* (Vieillot 1818), *Aratinga solstitialis* (Linnaeus 1758), *Myiopsitta monachus* (Boddaert 1783), *Eclectus roratus* (Müller 1776), and *Melopsittacus undulatus* (Shaw 1805), are presented in Fig. 1. The partial *CYTB* gene sequences were obtained from the PCR products of all parrot egg samples.

The sequence having the highest sequence similarity with the 43 sequences that were newly sequenced in the study are presented in Table 1. The *CYTB* gene sequences of the samples demonstrated >99.71% sequence similarity with the available sequences of seven species in the database (Table 1). Among those, 14 sequences (accession number: OQ413753–OQ413766) showed the highest similarity with the sequences of *Eclectus roratus*. Further, 13 sequences (accession number: OQ413731–OQ413743) presented the highest similarity with sequences of *Nymphicus hollandicus*. In addition, eight (accession number: OQ413744–OQ413751), four (accession number: OQ413769–OQ413772), two (accession number: OQ413767 and OQ413768) sequences showed the highest similarity with sequences of *Agapornis roseicollis*, *Myiopsitta monachus*, and *Melopsittacus undulatus*, respectively. The sequences designated as accession numbers: OQ413752 and OQ413773 showed the highest similarity with the sequences of *Aratinga solstitialis* and *Pyrrhura molinae*, respectively.

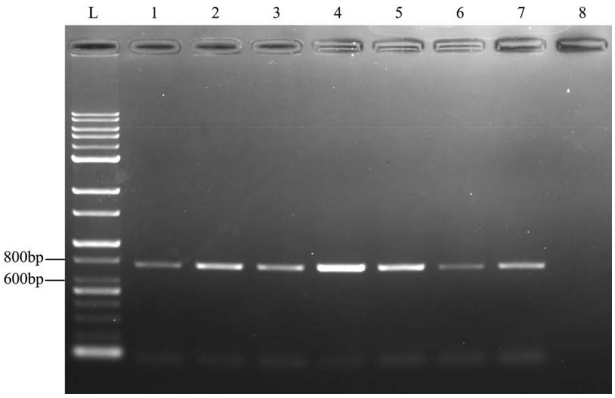

Fig. 1. Seven representative PCR products for partial mitochondrial cytochrome *b* (*CYTB*) gene of egg samples identified as different species. L, 1 kb ladder; 1. PE 01 identified as *Nymphicus hollandicus*; 2, PE 05 as *Pyrrhura molinae*; 3, PE 10 as *Agapornis roseicollis*; 4, PE 14 as *Myiopsitta monachus*; 5, PE 18 as *Eclectus roratus*; 6, PE 27 as *Melopsittacus undulatus*; 7, PE 29 as *Aratinga solstitialis*; 8, Negative control. Sample numbers are presented in Table 1.

For species identification, the phylogenies of these seven species were analyzed using the new sequences obtained in this study and the *CYTB* gene sequences were retrieved from the database. The phylogenies of *Agapornis roseicollis*, *Aratinga solstitialis*, and *P. molinae* were analyzed using the available sequences of each congeneric species. The best-fit substitution model of each dataset used to analyze the phylogenies of these species is presented in Table S1. The phylogeny of *Agapornis roseicollis* is presented in Fig. 2. The sequence *Psittaculirostris desmarestii* (Desmarest 1826) and *Psittaculirostris edwardsii* (Oustalet 1885) closely related to *Agapornis*, were used as an outgroup (Manegold and Podsiadlowski 2014). Eight sequences (accession number: OQ413744–OQ413751) that were newly sequenced in the present study were clustered along with the sequences of *Agapornis roseicollis*, and this branch was supported with high bootstrap values (Fig. 2). *Agapornis roseicollis* is a sister taxon of the *Agapornis personatus* group comprising *Agapornis fischeri* (Reichenow 1887), *Agapornis lilianae* (Shelley 1894), and *Agapornis personatus* (Reichenow 1887) in the phylogeny analyzed in this study. The relationship among species in the *Agapornis* was congruent with the finding of a previous study (Manegold and Podsiadlowski 2014). The phylogenetic relationship of *Aratinga* is presented in Fig. 3. For constructing this relationship, the outgroup consisted of the sequence of *Primolius couloni* (Sclater 1876) and *Primolius maracana* (Vieillot 1816), closely related to *Aratinga* (Ribas and Miyaki 2004). A sequence that was newly obtained in this study (accession number: OQ413752) was included in the branch of *Aratinga solstitialis* (Fig. 3). However, the bootstrap support value of this branch was relatively low, probably due to the close relationship between *Aratinga solstitialis*, *Aratinga auricapillus* (Kuhl 1820), and *Aratinga jandaya* (Gmelin

Table 1. BLAST searching results comparing partial mitochondrial cytochrome *b* (*CYTB*) gene sequences obtained from 43 parrot eggs to sequences of GenBank database

Present samples and sequences		Top one sequence showing the highest sequence similarity with present sequences		Identity (%)	E-value
Sample number	Accession number	Accession number	Species		
PE 01	OQ413731	MH133968	*Nymphicus hollandicus*	100	0
PE 02	OQ413732	MH133968	*Nymphicus hollandicus*	100	0
PE 03	OQ413733	MH133968	*Nymphicus hollandicus*	99.86	0
PE 04	OQ413734	MH133968	*Nymphicus hollandicus*	100	0
PE 05	OQ413773	AY751641	*Pyrrhura molinae*	100	0
PE 06	OQ413735	MH133968	*Nymphicus hollandicus*	100	0
PE 07	OQ413736	MH133968	*Nymphicus hollandicus*	100	0
PE 08	OQ413737	MH133968	*Nymphicus hollandicus*	100	0
PE 09	OQ413738	MH133968	*Nymphicus hollandicus*	100	0
PE 10	OQ413744	KM372554	*Agapornis roseicollis*	100	0
PE 11	OQ413745	KM372554	*Agapornis roseicollis*	100	0
PE 12	OQ413739	MH133968	*Nymphicus hollandicus*	100	0
PE 13	OQ413740	MH133968	*Nymphicus hollandicus*	99.86	0
PE 14	OQ413769	KM611471	*Myiopsitta monachus*	99.86	0
PE 15	OQ413741	MH133968	*Nymphicus hollandicus*	100	0
PE 16	OQ413742	MH133968	*Nymphicus hollandicus*	100	0
PE 17	OQ413743	MH133968	*Nymphicus hollandicus*	100	0
PE 18	OQ413753	MT275996	*Eclectus roratus*	99.86	0
PE 19	OQ413754	MT275996	*Eclectus roratus*	99.86	0
PE 20	OQ413755	MT275996	*Eclectus roratus*	99.86	0
PE 21	OQ413746	KM372554	*Agapornis roseicollis*	100	0
PE 22	OQ413747	KM372554	*Agapornis roseicollis*	100	0
PE 23	OQ413748	EU410486	*Agapornis roseicollis*	99.86	0
PE 24	OQ413749	KM372554	*Agapornis roseicollis*	100	0
PE 25	OQ413750	KM372554	*Agapornis roseicollis*	100	0
PE 26	OQ413751	EU410486	*Agapornis roseicollis*	99.86	0
PE 27	OQ413767	MT276003	*Melopsittacus undulatus*	100	0
PE 28	OQ413768	MN356136	*Melopsittacus undulatus*	100	0
PE 29	OQ413752	JX441869	*Aratinga solstitialis*	99.86	0
PE 30	OQ413770	KM611471	*Myiopsitta monachus*	99.86	0
PE 31	OQ413756	MT275996	*Eclectus roratus*	99.72	0
PE 32	OQ413757	MT275996	*Eclectus roratus*	99.86	0
PE 33	OQ413758	MT275996	*Eclectus roratus*	99.86	0
PE 34	OQ413759	MT275996	*Eclectus roratus*	99.86	0
PE 35	OQ413760	MT275996	*Eclectus roratus*	99.86	0
PE 36	OQ413761	MT275996	*Eclectus roratus*	99.86	0
PE 37	OQ413762	MT275996	*Eclectus roratus*	99.86	0
PE 38	OQ413763	MT275996	*Eclectus roratus*	99.86	0
PE 39	OQ413764	MT275996	*Eclectus roratus*	99.86	0
PE 40	OQ413765	MT275996	*Eclectus roratus*	99.72	0
PE 41	OQ413766	MT275996	*Eclectus roratus*	99.86	0
PE 42	OQ413771	KM611471	*Myiopsitta monachus*	99.71	0
PE 43	OQ413772	KM611471	*Myiopsitta monachus*	100	0

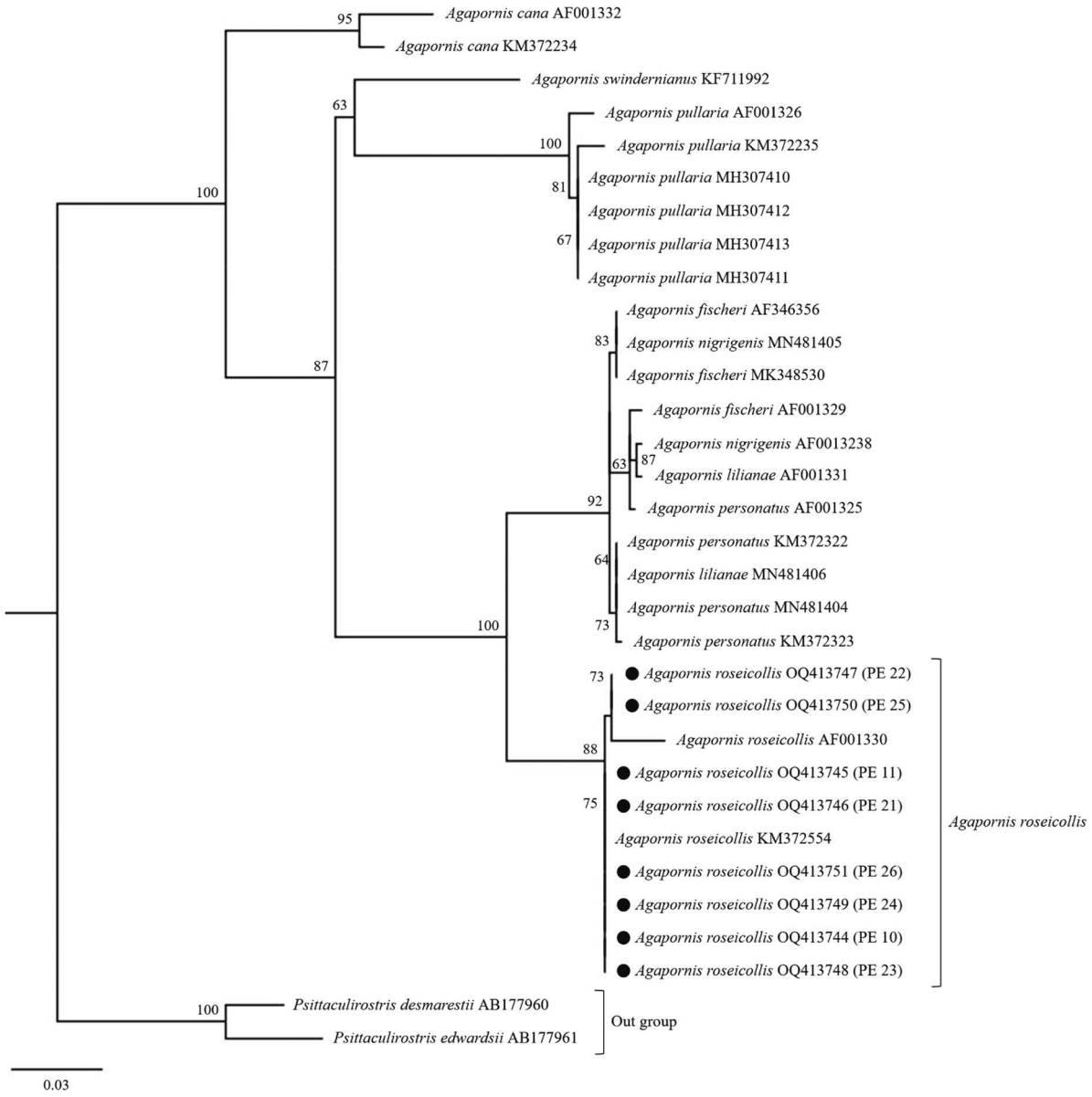

Fig. 2. Phylogeny of genus *Agapornis* based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circles indicate eight individuals investigated in this study. Their sample numbers are presented in parentheses. Accession numbers of *CYTB* sequences retrieved from GenBank are presented with species. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at nodes. Scale bar indicates nucleotide substitutions per site.

1788) (Ribas and Miyaki 2004). This close relationship among these species might be caused by the recent divergence of these three species during the Pleistocene (Ribas and Miyaki 2004).

The genus *Pyrrhura* is phylogenetically divided into three major clades, and *P. molinae* was included in the clade along with *P. frontalis* (Vieillot 1818), *P. lepida* (Wagler 1832), and *P. perlata* (Spix 1824) (Ribas *et al.* 2006). The sequences of the ten *Pyrrhura* species belonging to the clade, including *P. molinae* were retrieved to analyze the phylogenetic relationships of this clade. The sequences of *Anodorhynchus hyacinthinus* (Latham 1790) and *Anodorhynchus leari* (Bonaparte 1856) were used as an outgroup (Ribas *et al.* 2006). The new sequence reported in this study (accession number: OQ 413773) was clustered with the sequences of *P. molinae*,

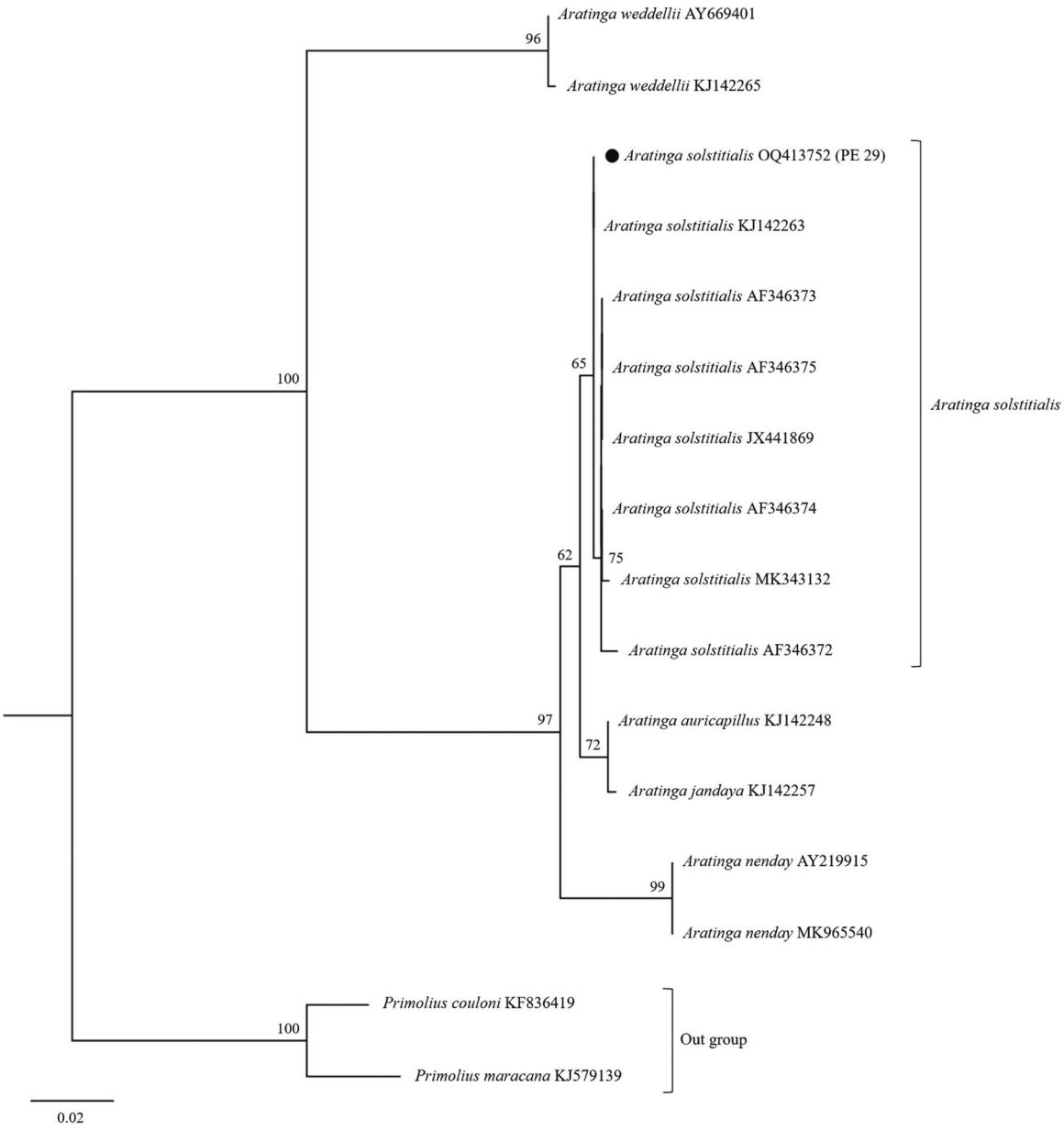

Fig. 3. Phylogeny of genus *Aratinga* based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circle indicates the one individual examined in this study. The sample number of that is presented in parentheses. Accession numbers of the *CYTB* sequences retrieved from GenBank are presented with the species. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at nodes. Scale bar indicates nucleotide substitutions per site.

and it was supported with high bootstrap values (Fig. 4). The sister taxon of *P. molinae* was *P. frontalis* in the phylogeny constructed in this study. However, *P. molinae* was a sister taxon of the branch that included *P. lepida* and *P. perlata* in the phylogeny based on the mitochondrial *CYTB* gene and control region in a previous study (Ribas *et al.* 2006). The difference in the phylogenetic relationships among these species may be attributed to the use of only *CYTB* gene in constructing the phylogeny in the present study. In future studies, more mitochondrial genes should be used to analyze the phylogenetic relationship among *P. frontalis*, *P. lepida*, *P. molinae*, and *P. perlata*.

The four monotypic species whose phylogenies were

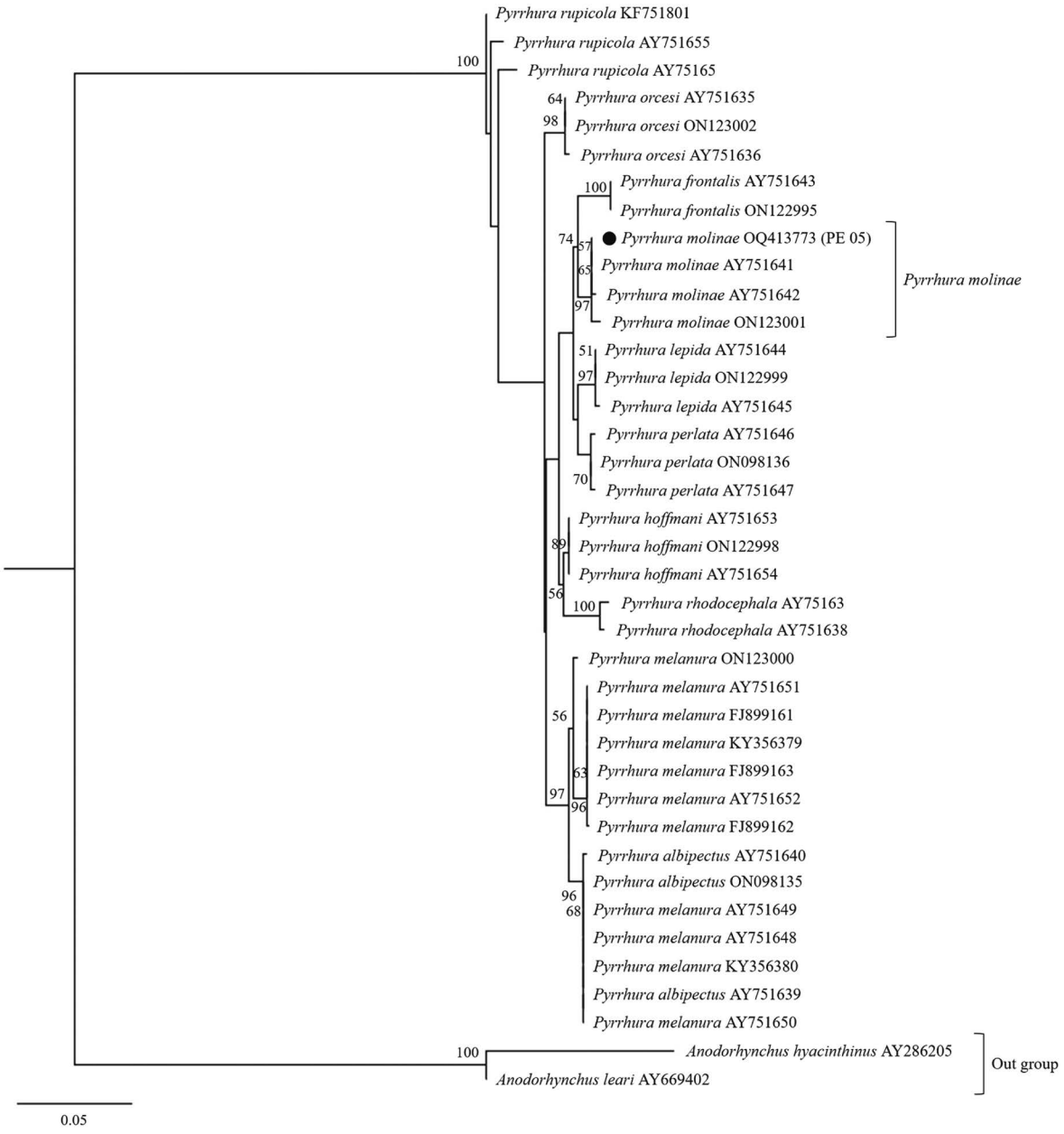

Fig. 4. Phylogeny of one major clade of genus *Pyrrhura*, including *Pyrrhura molinae*, based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circle indicates the one individual examined in this study. Sample number is presented in parentheses. Accession numbers of *CYTB* sequences retrieved from GenBank are presented with the species. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at nodes. Scale bar indicates nucleotide substitutions per site.

examined in this study are listed as follows: *E. roratus*, *Myoipsitta monachus*, *Melopsittacus undulatus*, and *N. hollandicus*. The phylogenies were constructed using the available *CYTB* gene sequences of the most closely related genera of each species. The most closely related genera of the four species were selected based on the phylogenies of the parrots analyzed using complete mitochondrial genomes reported in previous studies (Kim *et al.* 2021, 2022). The available sequences of the genera *Psittacula*, *Brotogeris*, and *Probosciger* were used to construct phylogenies of *E. roratus*, *Myiopsitta monachus*, and *N. hollandicus*, respectively. Since *Lorius* and *Tri-*

Fig. 5. Representative images of seven species examined in this study. A, *Nymphicus hollandicus*; B, *Agapornis roseicollis*; C, *Aratinga solstitialis*; D, *Eclectus roratus* (male); E, *Eclectus roratus* (female); F, *Melopsittacus undulatus*; G, *Myiopsitta monachus*; H, *Pyrrhura molinae*. Photo credit: A, Zefry; B, Tim; C, H. Zell; D, Sheba; E, Dany Sloan; F, Benjamint444; G, Bernard DUPONT; H, Brandon Lim.

choglossus genera have been reported as sister taxa of *Melopsittacus undulatus*, the available sequences of both genera were used to construct the phylogenetic tree of this species (Kim *et al.* 2022). The species list and accession numbers of the sequences of the most closely related genera of each of four monotypic species retrieved from the database are presented in Table S2. The best-fit substitution model of each dataset used to construct the phylogenies of these four species is presented in Table S1. 14 sequences (accession number: OQ413 753–OQ413766) were clustered with the sequences of *E. roratus*, that showed high bootstrap support values (Fig. S1). 13 sequences (accession number: OQ413 731–OQ413743) were included in the branch of *N. hollandicus*, supported with high bootstrap values (Fig. S2). In addition, four sequences (accession number: OQ 413769–OQ413772) were clustered with the sequences of *Myiopsitta monachus*, and it showed high bootstrap support values (Fig. S3). Two sequences (accession number: OQ413767 and OQ413768) were included in the branch of *Melopsittacus undulatus*, and were supported with high bootstrap values (Fig. S4).

The genetics distance of seven species examined in this study was calculated using the same dataset analyzed to construct the phylogeny (Table S3). The maximum intra-specific distance varied from 0.003 for *Myiopsitta monachus* to 0.027 for *E. roratus*. The lowest minimum inter-specific distance was 0.010 for *Aratinga solstitialis*, and the highest was 0.135 for *N. hollandicus*. In all seven species examined in the present study, the minimum inter-specific distance was higher than the maximum intra-specific distance.

Representative images of seven species are presented in Fig. 5. The native distribution of those is presented in Table S4. Three species, *N. hollandicus*, *E. roratus*, and *Melopsittacus undulatus*, are native to Australia and Southeast Asia countries. The other three, *Aratinga solstitialis*, *Myiopsitta monachus*, and *P. molinae*, are natively distributed in South America countries, and another species, *Agapornis roseicollis*, are native to Angola, Namibia, and South Africa. *Aratinga solstitialis* is categorized as Endangered, and others are Least Concern on the IUCN Red List of Threatened Species (Table S4). In addition, four of seven species are listed in CITES Appendix II for controlling their trade (Table S4). According to the report from the National Institution of Biological Resources (NIBR) in 2016, 50 parrots were imported into Korea from 2009 to 2014 (NIBR 2016). Among seven species examined in this study, four species, *Aratinga solstitialis*, *E. roratus*, *Myiopsitta monachus*, and *P. molinae*, were listed in the list of imported parrots (NIBR 2016).

In conclusion, DNA was successfully obtained from 43 unidentified parrot eggs by using a non-destructive sampling method, and then PCR and DNA sequencing were executed from the extracted DNA. As a result, these eggs were identified as seven parrot species belonging to two families and seven genera by sequence comparison with sequences of the GenBank using database search and phylogenetic analysis using available sequences retrieved from the GenBank. The non-destructive sampling method to obtain DNA from parrot eggs and molecular identification might help to control the trade of parrot eggs and prevent illegal trade of those. However, only 43 samples from seven parrot species were analyzed in this study. To ensure the usefulness of molecular identification and the non-destructive sampling method to identify parrot eggs, more comprehensively sampled parrot eggs, particularly heavily traded legally or illegally, should be investigated in future studies.

CRediT authorship contribution statement

JI Kim: Conceptualization, Methodology, Investigation, Writing-Original Draft. JW Baek: Methodology, Investigation, Data Curation. CB Kim: Conceptualization, Methodology, Writing-Original Draft, Writing-Review & Editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Table S1. Length and best-fit substitution model for each mitochondrial cytochrome *b* (*CYTB*) gene sequence dataset to construct phylogenies of seven species examined in this study

CYTB gene sequence dataset	Length (bp)	Best-fit substitution model
Agapornis roseicollis	494	TPM3uf + G
Aratinga solstitialis	526	HKY + G
Eclectus roratus	455	HKY + G
Melopsittacus undulatus	536	HKY + G
Myiopsitta monahcus	619	TPM2uf + G
Nymphicus hollandicus	684	TIM2 + I
Pyrrhura molinae	579	TrN + G

Table S2. Species list and accession numbers of mitochondrial cytochrome *b* (*CYTB*) gene sequences of five genera retrieved from GenBank to construct phylogenies of *Eclectus roratus*, *Melopsittacus undulats*, *Myiopsitta monachus*, and *Nymphicus hollandicus* in this study

Family	Genus	Species	Accession number
Cacatuidae	*Probosciger*	*Probosciger aterrimus*	AB177953
		Probosciger aterrimus	AB177981
		Probosciger aterrimus	AF346392
		Probosciger aterrimus	MH133970
		Probosciger aterrimus	MN356334
Psittacidae	*Brotogeris*	*Brotogeris chiriri*	DQ143281
		Brotogeris chiriri	FJ652855
		Brotogeris chiriri	FJ652857
		Brotogeris chiriri	FJ652858
		Brotogeris chiriri	FJ652859
		Brotogeris chrysoptera	FJ652876
		Brotogeris chrysoptera	FJ652877
		Brotogeris chrysoptera	FJ652878
		Brotogeris chrysoptera	FJ652879
		Brotogeris chrysoptera	FJ652880
		Brotogeris chrysoptera	FJ652881
		Brotogeris chrysoptera	FJ652882
		Brotogeris chrysoptera	FJ652883
		Brotogeris chrysoptera	FJ652884
		Brotogeris chrysoptera	FJ652885
		Brotogeris chrysoptera	FJ652886
		Brotogeris chrysoptera	FJ652887
		Brotogeris chrysoptera	FJ652888
		Brotogeris chrysoptera	FJ652889
		Brotogeris chrysoptera	FJ652890
		Brotogeris chrysoptera	FJ652891
		Brotogeris chrysoptera	FJ652892
		Brotogeris chrysoptera	FJ652894
		Brotogeris cyanoptera	FJ652867
		Brotogeris cyanoptera	FJ652868
		Brotogeris cyanoptera	FJ652869
		Brotogeris cyanoptera	FJ652870

Table S2. Continued

Family	Genus	Species	Accession number
Psittacidae	*Brotogeris*	*Brotogeris cyanoptera*	FJ652871
		Brotogeris cyanoptera	FJ652872
		Brotogeris cyanoptera	FJ652873
		Brotogeris cyanoptera	FJ652874
		Brotogeris cyanoptera	FJ652875
		Brotogeris cyanoptera	HM627323
		Brotogeris jugularis	FJ652903
		Brotogeris jugularis	FJ652904
		Brotogeris jugularis	FJ652905
		Brotogeris jugularis	FJ652906
		Brotogeris jugularis	FJ652907
		Brotogeris jugularis	FJ652908
		Brotogeris jugularis	FJ652909
		Brotogeris jugularis	FJ652910
		Brotogeris jugularis	JX877360
		Brotogeris jugularis	KM372271
		Brotogeris jugularis	KM372272
		Brotogeris pyrrhoptera	FJ652860
		Brotogeris pyrrhoptera	FJ652864
		Brotogeris pyrrhoptera	FJ652865
		Brotogeris sanctithomae	FJ652895
		Brotogeris sanctithomae	FJ652896
		Brotogeris sanctithomae	FJ652897
		Brotogeris sanctithomae	FJ652898
		Brotogeris sanctithomae	FJ652899
		Brotogeris sanctithomae	FJ652900
		Brotogeris sanctithomae	FJ652901
		Brotogeris sanctithomae	FJ652902
		Brotogeris tirica	FJ652848
		Brotogeris tirica	FJ652849
		Brotogeris versicolurus	FJ652850
		Brotogeris versicolurus	FJ652851
		Brotogeris versicolurus	FJ652852
		Brotogeris versicolurus	FJ652853
		Brotogeris versicolurus	FJ652854
	Lorius	*Lorius chlorocercus*	KM372225
		Lorius chlorocercus	KM372226
		Lorius chlorocercus	MN515396
		Lorius domicella	KM372227
		Lorius domicella	KM372228
		Lorius garrulus	AB177951
		Lorius garrulus	AF346335
		Lorius garrulus	AF346336
		Lorius garrulus	MT275997
		Lorius garrulus	MT275998
		Lorius lory	AB177952
		Lorius lory	KM372229
		Lorius lory	KM372315

Table S2. Continued

Family	Genus	Species	Accession number
Psittacidae	*Psittacula*	*Psittacula alexandri*	AB177958
		Psittacula alexandri	AB177970
		Psittacula alexandri	GQ996507
		Psittacula alexandri	KJ456433
		Psittacula alexandri	KM372495
		Psittacula alexandri	KM372558
		Psittacula alexandri	KM372559
		Psittacula alexandri	KM372560
		Psittacula alexandri	MK986660
		Psittacula calthorpae	GQ996512
		Psittacula columboides	KF803278
		Psittacula columboides	MH645639
		Psittacula cyanocephala	GQ996508
		Psittacula cyanocephala	KJ456434
		Psittacula cyanocephala	MT433093
		Psittacula derbiana	KM372562
		Psittacula derbiana	KM372563
		Psittacula derbiana	MK343133
		Psittacula eques	LN614517
		Psittacula eupatria	KF803277
		Psittacula eupatria	KM372564
		Psittacula eupatria	KM372565
		Psittacula eupatria	KM372566
		Psittacula eupatria	KM372567
		Psittacula eupatria	MH645640
		Psittacula eupatria	MK343134
		Psittacula finschii	GQ996510
		Psittacula finschii	KM372568
		Psittacula finschii	KM372569
		Psittacula himalayana	KJ456436
		Psittacula himalayana	KM372570
		Psittacula krameri	GQ996517
		Psittacula krameri	KC876642
		Psittacula krameri	KC876643
		Psittacula krameri	KC876644
		Psittacula krameri	KC876645
		Psittacula krameri	KC876646
		Psittacula krameri	KC876647
		Psittacula krameri	KC876648
		Psittacula krameri	KC876649
		Psittacula krameri	KC876650
		Psittacula krameri	KC876651
		Psittacula krameri	KC876652
		Psittacula krameri	KC876653
		Psittacula krameri	KC876654
		Psittacula krameri	KC876655
		Psittacula krameri	KC876656
		Psittacula krameri	KC876657

Table S2. Continued

Family	Genus	Species	Accession number
Psittacidae	*Psittacula*	*Psittacula krameri*	KC876658
		Psittacula krameri	KC876659
		Psittacula krameri	KC876660
		Psittacula krameri	KC876661
		Psittacula krameri	KC876662
		Psittacula krameri	KC876663
		Psittacula krameri	KC876664
		Psittacula krameri	KC876665
		Psittacula krameri	KF803279
		Psittacula krameri	KJ456437
		Psittacula krameri	KM372571
		Psittacula krameri	KM372572
		Psittacula krameri	KM372573
		Psittacula krameri	KM372574
		Psittacula krameri	KM372575
		Psittacula krameri	KU609544
		Psittacula krameri	KU609545
		Psittacula krameri	KU609546
		Psittacula krameri	KU609547
		Psittacula krameri	KU609548
		Psittacula krameri	KU609549
		Psittacula krameri	KU609550
		Psittacula krameri	KU609551
		Psittacula krameri	KU609552
		Psittacula krameri	KU609553
		Psittacula krameri	KU609554
		Psittacula krameri	KU609555
		Psittacula krameri	KU609556
		Psittacula krameri	KU609557
		Psittacula krameri	KU609558
		Psittacula krameri	KU609559
		Psittacula krameri	KU609560
		Psittacula krameri	KU609561
		Psittacula krameri	KU609562
		Psittacula krameri	KU609563
		Psittacula krameri	KU609564
		Psittacula krameri	KU609565
		Psittacula krameri	KU609566
		Psittacula krameri	KU609567
		Psittacula krameri	LN614519
		Psittacula krameri	LN614520
		Psittacula krameri	MH645641
		Psittacula krameri	MN065674
		Psittacula longicauda	GQ996509
		Psittacula longicauda	KM372576
		Psittacula roseata	KF851356
		Psittacula roseata	KJ456438
		Psittacula roseata	KM372577

Table S2. Continued

Family	Genus	Species	Accession number
Psittacidae	*Psittacula*	*Psittacula roseata*	MH645642
		Psittacula roseata	MK986661
	Trichoglossus	*Trichoglossus capistratus*	KM372516
		Trichoglossus capistratus	KM372517
		Trichoglossus capistratus	KM372522
		Trichoglossus capistratus	MG429726
		Trichoglossus euteles	AB177943
		Trichoglossus euteles	AB177963
		Trichoglossus flavoviridis	KM372231
		Trichoglossus forsteni	KM372520
		Trichoglossus forsteni	KM372525
		Trichoglossus forsteni	KM372526
		Trichoglossus forsteni	MW755300
		Trichoglossus haematodus	AB177942
		Trichoglossus haematodus	KM372514
		Trichoglossus haematodus	KM372515
		Trichoglossus haematodus	KM372523
		Trichoglossus haematodus	KM372524
		Trichoglossus haematodus	KM372529
		Trichoglossus haematodus	KM372530
		Trichoglossus haematodus	MN652920
		Trichoglossus moluccanus	KM372527
		Trichoglossus moluccanus	KM372528
		Trichoglossus moluccanus	MW755301
		Trichoglossus ornatus	KM372320
		Trichoglossus rubritorquis	KM372531
		Trichoglossus rubritorquis	KM372532
		Trichoglossus rubritorquis	MN182499
		Trichoglossus weberi	KM372533
		Trichoglossus weberi	KM372534

Table S3. Intra-specific and inter-specific distances (%) analyzed using partial *CYTB* gene sequences of 43 parrot eggs newly sequenced in the present study and those of congeneric species retrieved from GenBank

Family	Species	Accession number*	Intra-specific distance (%)	Inter-specific distance (%)
Cacatuidae	*Nymphicus hollandicus*	OQ413731–OQ413743	0.000–0.007	0.135–0.205
Psittacidae	*Agapornis roseicollis*	OQ413744–OQ413751	0.000–0.018	0.055–0.146
	Aratinga solstitialis	OQ413752	0.000–0.006	0.010–0.095
	Eclectus roratus	OQ413753–OQ413766	0.000–0.027	0.068–0.118
	Melopsittacus undulatus	OQ413767–OQ413768	0.000–0.006	0.104–0.122
	Myiopsitta monachus	OQ413769–OQ413772	0.000–0.003	0.120–0.146
	Pyrrhura molinae	OQ413773	0.000–0.005	0.014–0.047

*Accession numbers of the newly sequenced in the present study.

Table S4. Detailed information of examined species

Family	Species	Common name	Native distribution	IUCN red list	CITES appendix
Cacatuidae	*Nymphicus hollandicus*	Cockatiel	Australia	Least concern	–
Psittacidae	*Agapornis roseicollis*	Rosy-faced Lovebird	Angola, Namibia, South Africa	Least concern	–
	Aratinga solstitialis	Sun Parakeet	Brazil, Guyana	Endangered	II
	Eclectus roratus	Moluccan Eclectus	Australia, Indonesia, Palau, Papua New Guinea, Solomon Islands	Least concern	II
	Melopsittacus undulatus	Budgerigar	Australia	Least concern	–
	Myiopsitta monachus	Monk Parakeet	Argentina, Bolivia, Brazil, Paraguay, Uruguay	Least concern	II
	Pyrrhura molinae	Green-cheeked Parakeet	Argentina, Bolivia, Brazil, Paraguay	Least concern	II

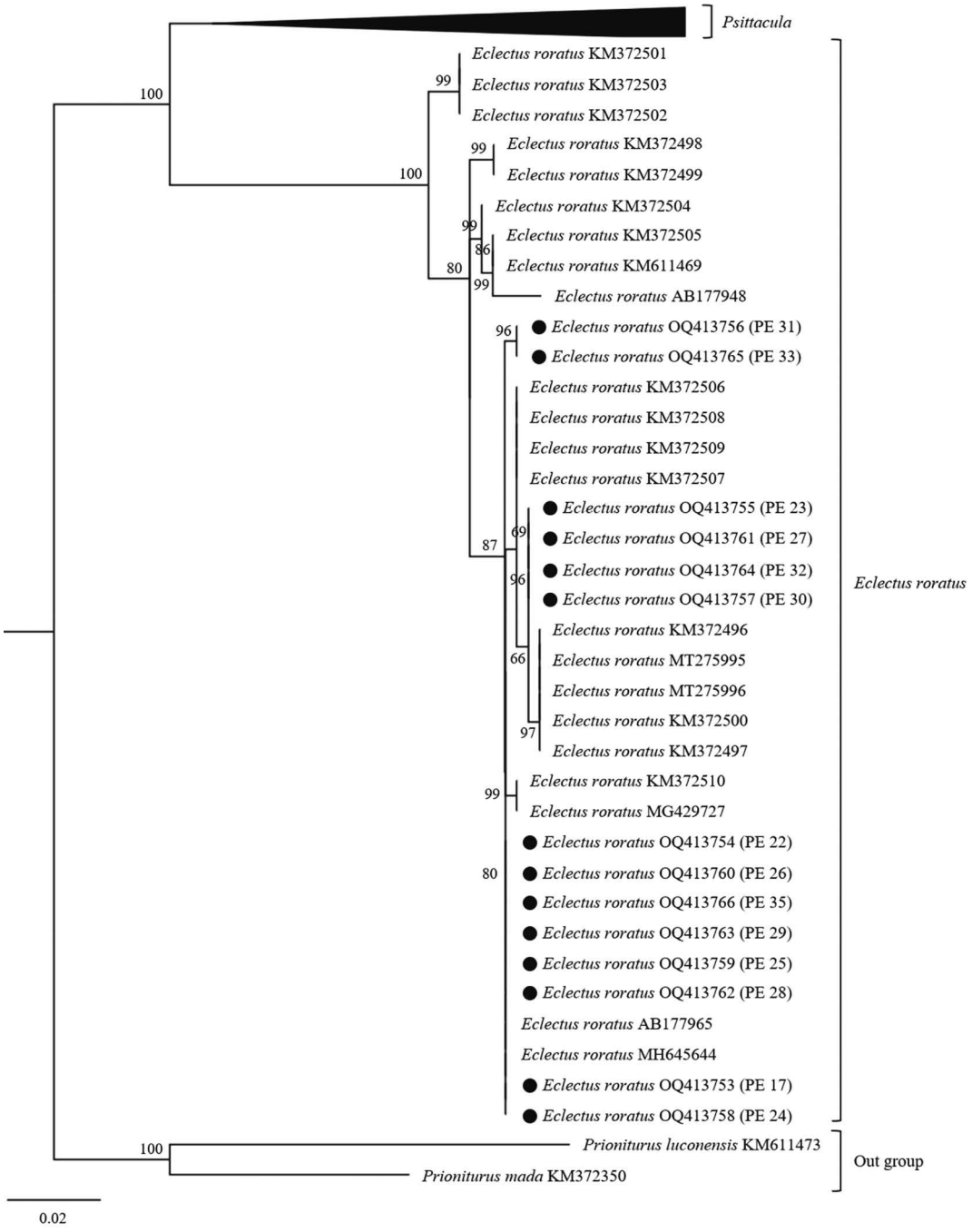

Fig. S1. Phylogeny of *Eclectus roratus* based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circles indicate fourteen sequences investigated in this study. Sample numbers are presented in parentheses. Sequences of *Prioniturus luconensis* and *Prioniturus mada*, a closely related genus to *Eclectus* and *Psittacula*, were used as outgroup. Accession numbers of *CYTB* sequences retrieved from GenBank are presented with the species. Branch of genus *Psittacula* is presented as collapsing. Accession numbers of *CYTB* sequences of the genus *Psittacula* retrieved from GenBank are presented in Table S1. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at nodes. Scale bar indicates nucleotide substitutions per site.

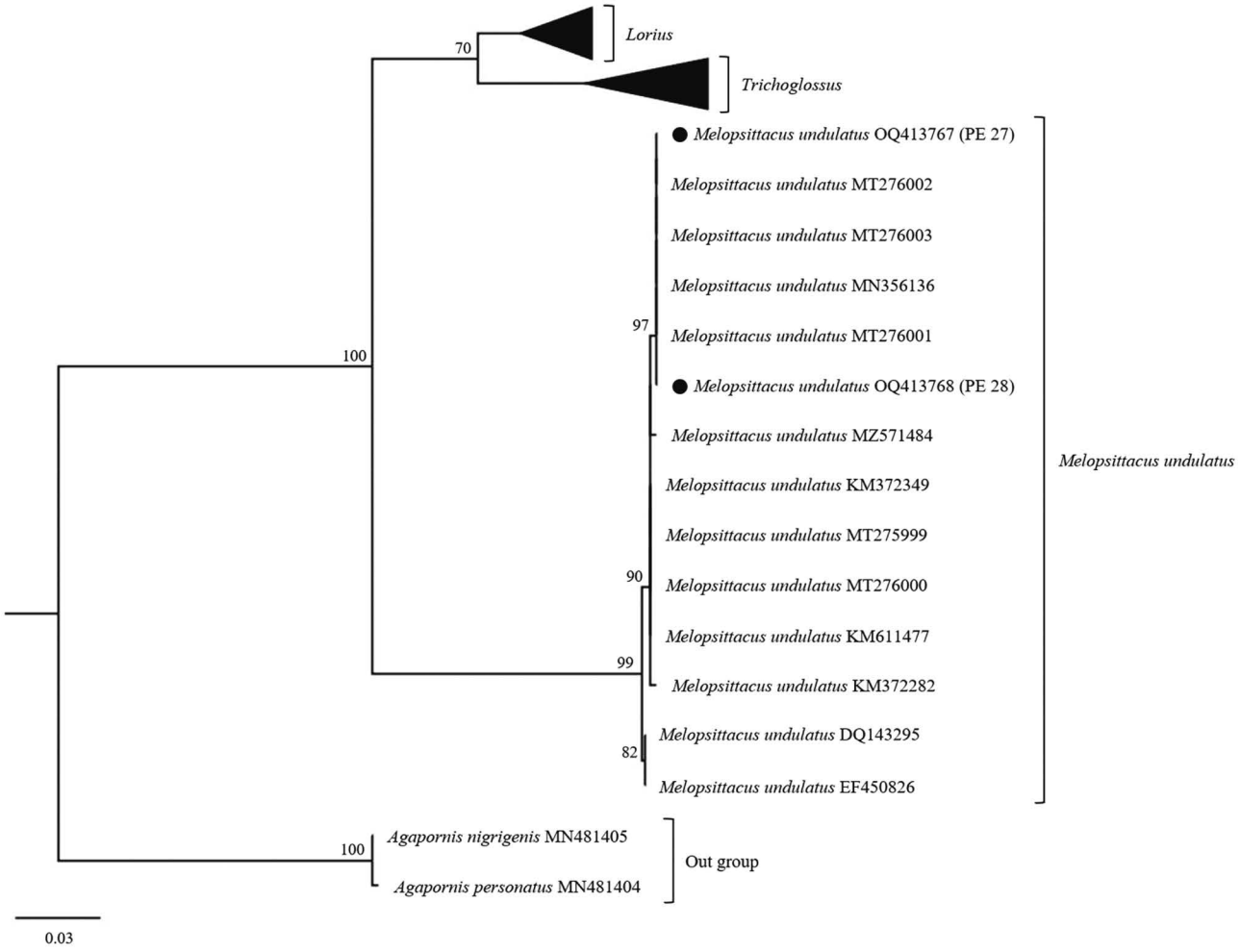

Fig. S2. Phylogeny of *Melopsittacus undulatus* based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circles indicate two sequences investigated in this study. Sample numbers of those are presented in parentheses. Sequences of *Agapornis nigrigenis* and *Agapornis personatus*, a closely related genus to those three genera, were used as outgroup. Accession numbers of *CYTB* sequences retrieved from GenBank are presented with the species. Branch of genera *Lorius* and *Trichoglossus* is presented as collapsing. Accession numbers of *CYTB* sequences of genera *Lorius* and *Trichoglossus* retrieved from GenBank are presented in Table S1. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at nodes. Scale bar indicates nucleotide substitutions per site.

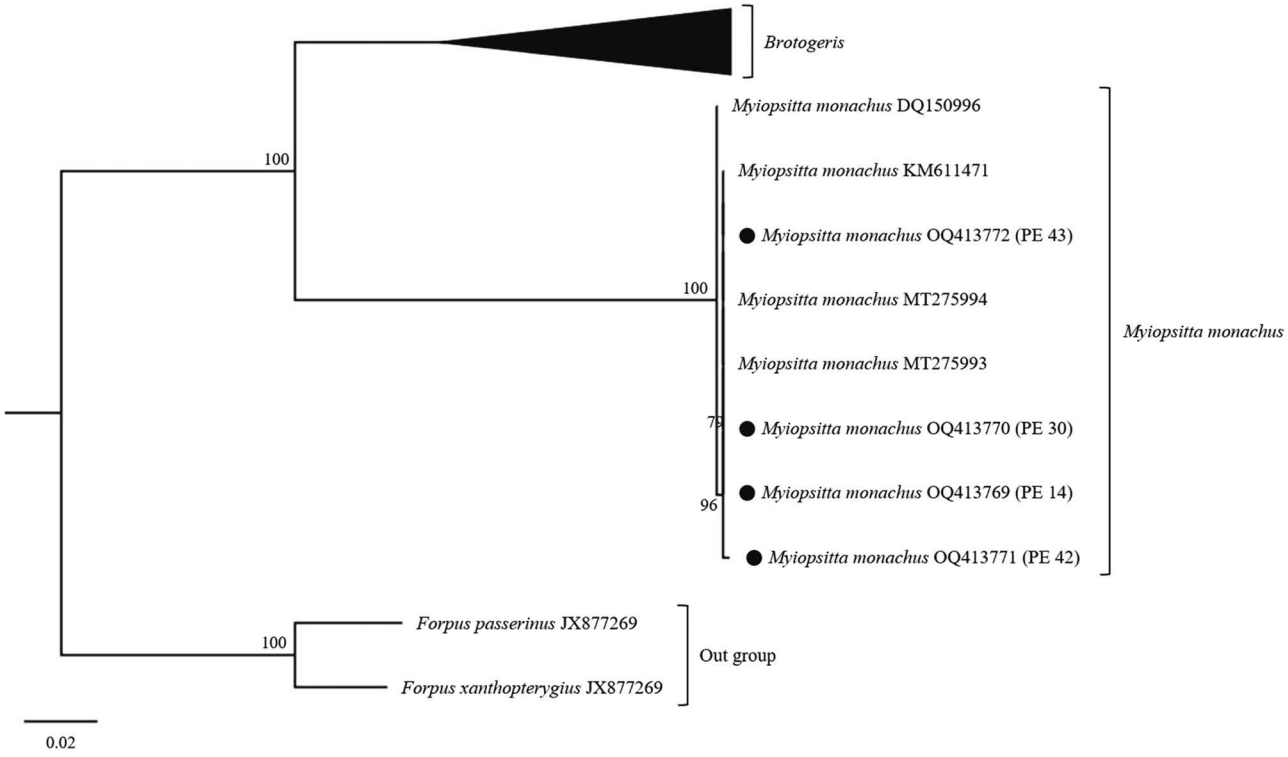

Fig. S3. Phylogeny of *Myiopsitta monachus* based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circles indicate four sequences investigated in this study. Sample numbers of those are presented in parentheses. Sequences of *Forpus passerinus* and *Forpus xanthopterygius*, a closely related genus to *Myiopsitta* and *Brotogeris*, were used as outgroup. Accession numbers of *CYTB* sequences retrieved from GenBank are presented with the species. Branch of genus *Brotogeris* is presented as collapsing. Accession numbers of *CYTB* sequences of the genus *Brotogeris* retrieved from GenBank are presented in Table S1. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at the nodes. Scale bar indicates nucleotide substitutions per site.

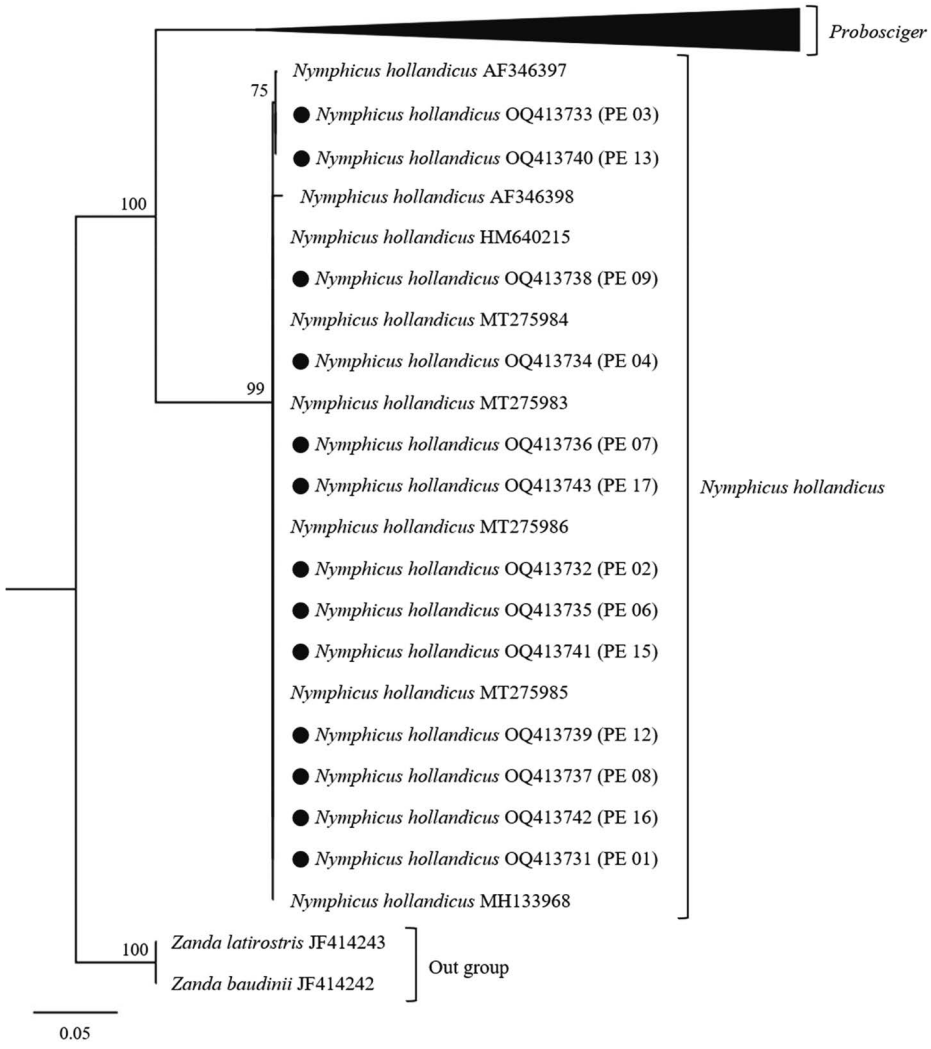

Fig. S4. Phylogeny of *Nymphicus hollandicus* based on partial mitochondrial cytochrome *b* (*CYTB*) gene. Black circles indicate thirteen sequences investigated in this study. Sample numbers of those are presented in parentheses. Sequences of *Zanda baudinii* and *Zanda latirostris*, a closely related genus to *Nymphicus* and *Prosobsciger*, were used as outgroup. Accession numbers of *CYTB* sequences retrieved from GenBank are presented with the species. Branch of genus *Prosobsciger* is presented as collapsing. Accession numbers of *CYTB* sequences of genus *Prosobsciger* retrieved from GenBank are presented in Table S1. Maximum Likelihood (ML) bootstrap values ≥ 50 are shown at nodes. Scale bar indicates nucleotide substitutions per site.

