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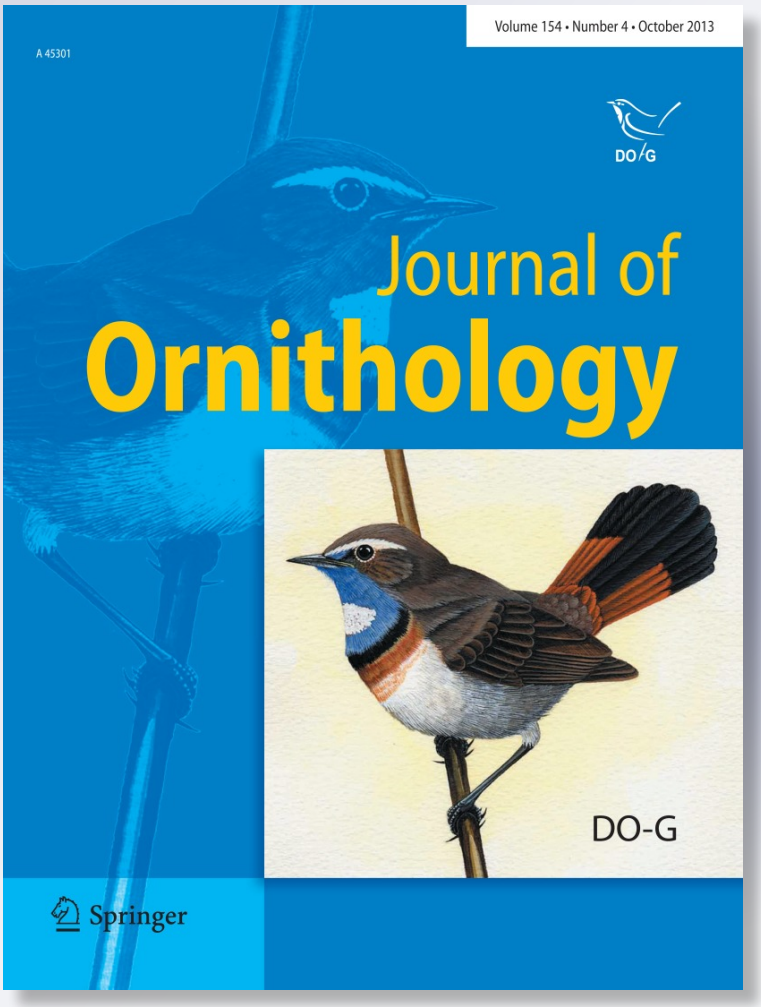
Journal of Ornithology

ISSN 2193-7192
Volume 154
Number 4

J Ornithol (2013) 154:987-994
DOI 10.1007/s10336-013-0966-z



Springer

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ORIGINAL ARTICLE

Genetic differentiation between migratory and sedentary populations of the Northern Boobook (*Ninox japonica*), with the discovery of a novel cryptic sedentary lineage

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Received: 11 September 2012 / Revised: 6 April 2013 / Accepted: 6 May 2013 / Published online: 21 May 2013

Abstract Species that exhibit intraspecific variation in migratory behavior provide a valuable opportunity to study the evolution of avian migration. The Northern Boobook (*Ninox japonica*) has two subspecies in East Asia, one sedentary (*N. j. totogo*) and one migratory (*N. j. japonica*). The validity and residential status of the two subspecies has never been examined through genetic analysis. Their coexistence in Taiwan provides an excellent opportunity to explore their genetic differentiation and migratory behavior. Analyzing the mitochondrial cytochrome *b* gene of 77 samples including ascertained breeders, ascertained migrants, and topotypes of the sedentary *N. j. totogo* from Lanyu, we found a coexistence of two clades with a 1.72 % sequence divergence, and both clades were highly supported in phylogenetic analyses. The clade containing ascertained breeders occurs year round in Taiwan and is the only resident population during the breeding season. The other clade containing ascertained migrants appears only in non-breeding seasons and coexists with the former during these months. Topotypes of *N. j. totogo* from Lanyu were clustered with *N. j. japonica*, which undermines its classification as a subspecies. We suggest treating *N. j. totogo* as an invalid taxon and treating the sedentary population in Taiwan as a unique cryptic lineage until further information is available. The discovery of this lineage will improve our understanding of the owls in terms of animal conservation, genetic biodiversity, and the evolution of their migratory behavior.

Keywords Cryptic species · Genetic diversity · Migration route · Orchid Island · Rescue center · Sympatric distribution

Communicated by M. Wink.

Electronic supplementary material The online version of this article (doi:10.1007/s10336-013-0966-z) contains supplementary material, which is available to authorized users.

W.-L. Lin · S.-M. Lin (✉)
Department of Life Science, National Taiwan Normal University, Taipei City, Taiwan
e-mail: fish@ntnu.edu.tw

W.-L. Lin
Taichung Wildlife Conservation Group, Taichung, Taiwan

L. L. Severinghaus
Biodiversity Research Center, Academia Sinica, Taipei City, Taiwan

L. L. Severinghaus · S.-M. Lin
Raptor Research Group of Taiwan, Taipei City, Taiwan

H.-Y. Tseng (✉)
Zoology Department, National Museum of Natural Science, Taichung, Taiwan
e-mail: hytseng1216@gmail.com

H.-Y. Tseng
Department of Life Science, Tunghai University, Taichung, Taiwan

Zusammenfassung

Genetische Unterscheidung von Standvögel- und Zugvögel-Populationen des Nördlichen Boobookkauz (*Ninox japonica; Strigidae*) im Zusammenhang mit dem Fund einer neuen, noch ungeklärten ortsabhängigen Abstammungslinie

Arten mit innerartlich unterschiedlichem Zugverhalten bieten eine wichtige Möglichkeit, die Entstehung des Vogelzugs zu untersuchen. Der Nördliche Boobookkauz (*Ninox japonica*) kommt in Ostasien in zwei Unterarten

vor; die Tiere der einen (*N. j. totogo*) sind reine Standvögel, die der anderen (*N. j. japonica*) sind Zugvögel. Bislang ist die Validität dieser Feststellung zu Stand- versus Zugvogel aber nie anhand einer genetischen Analyse überprüft worden. Das gleichzeitige Vorkommen beider Unterarten in Taiwan bietet eine ausgezeichnete Möglichkeit, ihr Zugverhalten und ihre genetischen Unterschiede zu untersuchen. Bei der Analyse des mitochondrialen Cytochrome b-Gens aus 77 Proben u.a. von eindeutig nachgewiesenen Standvögeln, Zugvögeln und Topotypen der nicht ziehenden Unterart *N. j. totogo* von der Insel Lanyu (vor der südöstlichen Küste Taiwans) fanden wir ein gemeinsames Auftreten von zwei Gruppen mit einer Sequenz-Abweichung von 1.72 %, wobei das Vorhandensein beider Gruppen auch phylogenetisch hoch absicherbar war. Die Gruppe mit den nachgewiesenen Standvögeln kann das ganze Jahr über in Taiwan angetroffen werden und ist während der Brutzeit die einzige dort vorhandene Population. Die andere Gruppe mit den Ziehern kommt dort nur außerhalb der Brutzeiten und dann gemeinsam mit der ersten Gruppe vor. Die Topotypen von *N. j. totogo* von Lanyu wurden mit *N. j. japonica* zusammengefasst, was ihre Klassifizierung als eigene Unterart untergräbt. Wir regen an, bis zum Vorliegen weiterer Informationen *N. j. totogo* als ungültiges Taxon anzusehen und die Standvögel-Population in Taiwan als eigene, noch nicht ganz geklärte Abstammungslinie zu betrachten. Die Entdeckung dieser Linie wird unser Verständnis der Eulen im Zusammenhang mit Artenschutz, genetischer Vielfalt und der Entstehung ihres Zugverhaltens verbessern.

Fig. 1 A Northern Boobook (*Ninox japonica*) flying across the ocean between Taiwan and Lanyu. This picture provides evidence for the oversea dispersal of this species, and might be one of the very few photographic records of a subtropical forest owl on migration. Photographed by Mei-Fong Liao from a boat at 14:53 hours, April 19, 2012

Introduction

The evolution of genetic and phenotypic traits in migratory birds has attracted much attention of ornithologists in recent years (Webster et al. 2002; Cotton 2003; Liedvogel et al. 2011). Active areas of inquiry include investigations of the time needed for migratory behavior to arise, or the genetic differentiation between closely related lineages representing different migratory phenomena (Pérez-Tris et al. 2004; Bearhop et al. 2005; Rolshausen et al. 2009; Bensch et al. 2009). Separate lineages can evolve in allopatric breeding sites and meet at wintering sites, or be sympatrically distributed but with different migratory routes (Bearhop et al. 2005; Bensch et al. 2009; Rolshausen et al. 2009). The genetic differentiation between such lineages provides a valuable opportunity to examine the development of migratory behaviors.

Ninox, also known as Hawk Owls or Boobooks, is an owl genus with approximately 20 species distributed mainly in Australia and Asia (König et al. 2009). Members of this genus do not have uniform migratory behavior (Fig. 1), and the species diversity of some sedentary lineages is still underestimated (e.g., the recent discovery of *N. rumseyi* and *N. leventisi* by Rasmussen et al. 2012). Variation in migratory status also occurs at the conspecific level in some cases, such as in the *N. japonica* discussed in this study (König et al. 2009). The Taiwan population was initially regarded as a migrant (Hachisuka and Udagawa 1951), then changed to a resident based on its year-round presence (Mees 1970; King 2002; Dickinson 2003), and recently with more information reclassified as containing both sedentary and migratory elements (Brazil 2009; Severinghaus et al. 2010).

N. japonica (Temminck and Schlegel 1845) was previously classified as a subspecies of *N. scutulata* (Raffles 1822), i.e. *N. scutulata japonica*, and subsequently elevated to species (King 2002) based on its diagnostic vocal characters. According to the revised species definition, *N. japonica* has a distribution covering southeastern Siberia, Korea Peninsula, northeastern and central China, Japan, Taiwan, and Lanyu (Brazil 2009). King (2002) separated the species into two subspecies: *N. j. totogo* Momiyama 1930 distributed in Taiwan and Ryukyu Islands with type locality in Lanyu (an island located approximately 60 km to the southeast of Taiwan); and *N. j. japonica* distributed in the rest of the species' range. Brazil (2009) treated breeders in Korea and Japan as *N. j. japonica*, while breeders in Taiwan, Lanyu, and southern Ryukyus as *N. j. totogo* (Fig. 2a). Breeding of *N. japonica* in Taiwan was not formally described until 2012 (Lin et al. 2012), while breeding of *N. j. totogo* in Lanyu has been known for some time (Severinghaus et al. 2010). However, validity and residential status of these two taxa have never been discussed.

Analyzing genetic diversity can provide useful insight into population structure. According to current classification, there should be only one taxon occurring Taiwan in the breeding

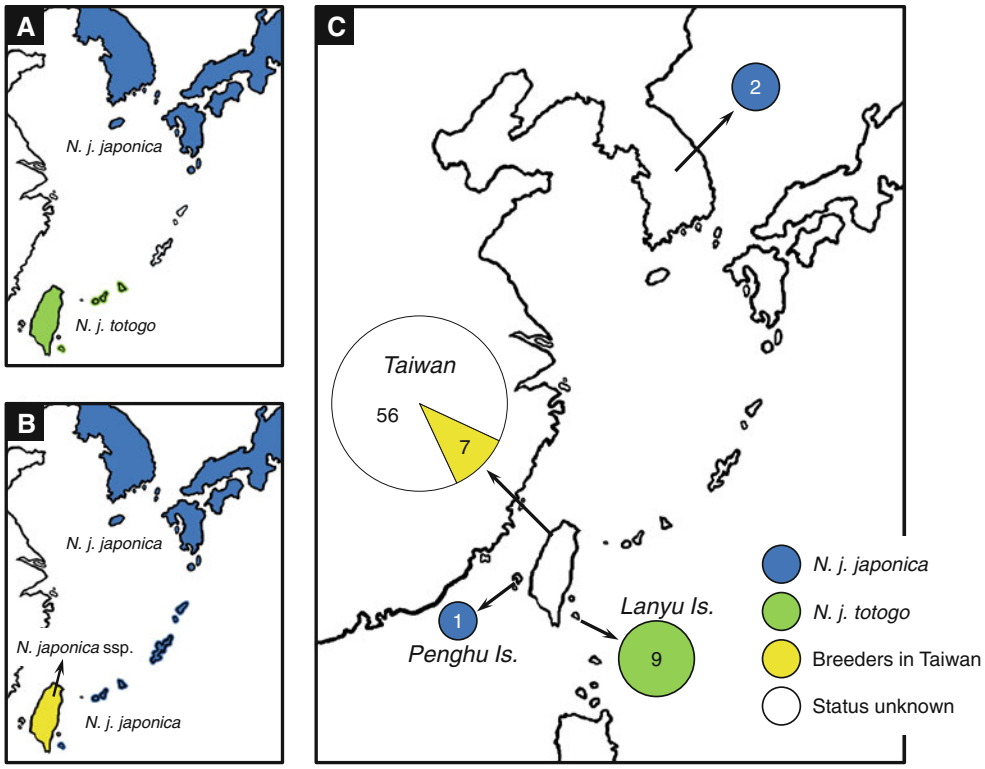

Fig. 2 **a** The taxonomy of Northern Boobook (*Ninox japonica*) as shown in the literature contains a migratory population (*N. j. japonica*) and a sedentary population (*N. j. totogo*). **b** This study invalidated *N. j. totogo*, and discovered a cryptic lineage of sedentary population in Taiwan. **c** Sampling location and sample size used in this study. The ascertained status of the samples were based on breeding or migratory evidences: *N. j. japonica* (*blue*), *N. j. totogo* (*green*), and ascertained breeders in Taiwan (*yellow*)

season, but two taxa coexisting in the nonbreeding seasons. The two subspecies have similar plumage patterns and colors, making them difficult to be identified by morphological characters alone. In several cases, the dispersal of fledglings into an urban park or school campus was mistaken by observers as migration and led to incorrect conclusion on the status of the bird (Wen-Loung Lin, unpublished radio tracking data). In this study, we aim to detect the genetic differentiation between migratory and sedentary lineages of this secretive nocturnal bird, and try to clarify their occurrence in space and time. We expect the result of the study to not only revise the current taxonomy of this owl but also provide insights into the evolution of migration in owls.

Methods

Sample collection and status definition

A total of 75 blood or tissue samples of *Ninox* were collected during 1994–2011, including 9 from Lanyu, 2 from South Korea, 1 from Penghu Island, and 63 from Taiwan (Fig. 2c). The Taiwan, Penghu, and South Korean tissues were from injured or dead owls provided by the wildlife rescue centers of Taichung Wildlife Conservation Group (TWCG) and Professor Kim Sooil of South Korea. Lanyu samples were bloods from owls caught in mist-nets and preserved in the Museum of Academia Sinica. All the samples were preserved in 70 % alcohol.

Owl samples obtained in autumn, winter, and spring could be from migrants or residents. We have five samples from known migrants, including N020 from Penghu where there are no resident owls (Severinghaus et al. 2010), KOR01 and KOR02 from South Korea, and two sequences from GenBank (AJ004008 and AY422981). These are classified as *N. j. japonica* according to current taxonomy. We considered samples collected in June, July and August as from ascertained breeders ($n = 7$, including 2 owlets), because the breeding season in Japan begins in May and eggs produced in early June would not give rise to independent young before August (http://www.avibirds.com/html/owls/Brown_Hawk-Owl.html). Birds collected from Lanyu ($n = 9$, all breeders) were classified as *N. j. totogo* following the original assignment that breeders on this islet belong to this specific subspecies (Momiyama 1930).

Molecular techniques

Total genomic DNA was isolated using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, 2009). DNA was suspended

in 1× TE buffer and stored at −20 °C. The anterior 5′ region of the mitochondrial cytochrome b sequence of 1,000 base pairs (bp) was amplified by polymerase chain reaction (PCR). Primers were then designed from the consensus sequences of several Strigids: F1 5′-ATGG CCCCCAAYATMCGHAARTC-3′, and R1 5′-TTAGTA GTTGAGTAGTTTGTTTTC-3′. Reactions were conducted in a 20-μL reaction volume containing 1× PCR buffer [10 mM Tris–HCl, pH 9.0; 50 mM KCl, 0.01 % (w/v) gelatine, and 0.1 % Triton X-100], 0.8 U Taq DNA polymerase (Amersham Biosciences), 0.2 μM each primer, 0.5 mM dNTP, and 50 ng template DNA. The PCR protocol was denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 52 °C for 40 s and 72 °C for 90 s, with a final extension at 72 °C for 10 min using an iCycler Thermal Cycler (Bio-Rad). PCR products were purified with a PCR Product Pre-Sequencing Kit (USB) and subsequently used as the template for the DNA sequencing reactions with a DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The same primers used for PCR were used for the sequencing reactions. Products were sequenced on a MegaBACE 1000 automated DNA sequencer (Amersham Biosciences). The sequences were determined in both directions, and the original signals were proofread using SEQUENCHER software v.4.9 (Gene Codes). The sequences obtained were compared to those of other *Ninox* species to ensure the accuracy of the PCR amplifications.

Phylogenetic tree construction and haplotype genealogy

We calculated haplotype diversity and nucleotide diversity by using DnaSP 5.0 (Librado and Rozas 2009). In order to root the tree, six sequences comprising five other *Ninox* species from GenBank served as outgroups (accession number EU348984, EU348983, EU348981, AJ004007, AY309457, and EU348982). A hierarchical likelihood ratio test was implemented by using Modeltest 3.06 (Posada and Crandall 1998) to find the best fit model of *Ninox* mitochondrial sequences. The results identify the Hasegawa, Kishino, and Yano model with parameters for gamma distribution (HKY + *G*), with a nucleotide composition of $G = 0.1336$, $A = 0.2773$, $T = 0.2301$, $C = 0.3591$, a transition/transversion ratio of 9.9726, and a gamma shape parameter of 0.1123. We constructed sequence phylogeny by using PHYML (Guindon et al. 2010) under maximum likelihood (ML) criterion, with 1,000 bootstrap replicates to obtain the statistic supports on each node. To confirm the consistency in topology among different tree construction criteria, maximum parsimony (MP) and neighbor-joining (NJ) were also performed using PAUP* 4.0 (Swofford 2002) with 5,000 bootstrap replicates. Finally, the individual sequence dataset was transformed to the haplotype dataset using DnaSP 5.0 (Librado and Rozas 2009). The haplotype network was constructed by TCS (Clement et al. 2000).

Molecular sexing, measurements, and morphometrics

We sexed each specimen by PCR amplification of the CHD1 gene located on the sex chromosomes. PCR conditions followed the protocol provided by Fridolfsson and Ellegren (1999), with primers 2550F (5′-GTTACTGA TTCGTCTACGAGA-3′) and 2718R (5′-ATTGAAAT GATCCAGTGCTTG-3′). PCR products were visualized by 3 % agarose gel electrophoresis. Individuals with a single band were classified as males, while those with double bands were classified as females.

We measured the body length, bill length (bill tip to edge of cere), head length (bill tip to the end of the skull), wing length, tail length, and tarsus length of 43 owls from Taiwan and 17 owls from Lanyu using a digital dial caliper to the nearest 0.1 mm. To examine potential morphological differences among owls from different genetic lineages, principal component analysis (PCA) was conducted using these 6 measurements plus the wing–tail ratio (wing length divided by tail length) with SPSS 17.0 (SPSS). As no significant difference existed between the sexes, males and females were pooled together in analysis.

Results

Molecular phylogeny

The phylogenetic trees obtained under ML, MP, or NJ criteria were identical in shape, differing only in the probability of each node, and minor differences among outgroup species (Fig. 3). All the samples of *N. japonica* clustered to form a monophyletic group strongly supported by 100 % bootstrap values in all the three analyses. The samples then fell into two clades (Fig. 3). Clade A, which was strongly supported by bootstrap values, contained 11 haplotypes from 31 individuals including the 2 Korean birds, the ascertained migratory bird from Penghu Island, the 2 owl sequences from GenBank, and the 9 owls from Lanyu. All the 7 ascertained breeders from Taiwan fell under Clade B, which contains the remaining 10 haplotypes from 46 individuals. The support for the monophyly of Clade B was less strong, but still consistent under all three criteria. The genetic distance between these two clades is 0.0172 in *p*-distance and 0.0175 in the HKY model.

We present haplotype genealogy network as shown in Fig. 4, where the area of the pie charts represent the sample size of each haplotype. The two clades differed in that Clade A had more segregating sites (17 vs. 9), higher haplotype

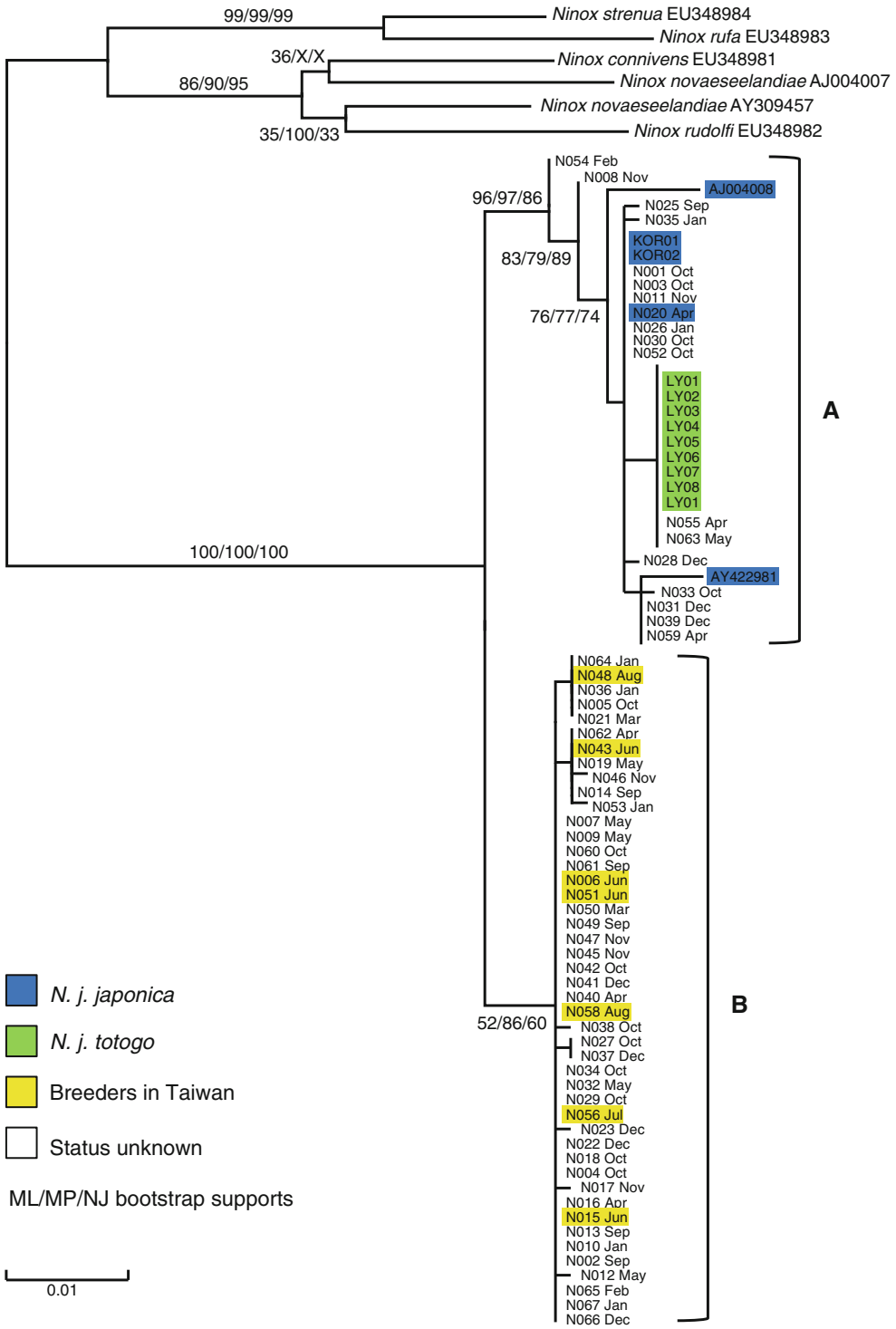

Fig. 3 Maximum likelihood tree of the *Ninox* mitochondrial cytochrome *b* sequences, with statistical support from bootstrapping of maximum likelihood (*ML*), maximum parsimony (*MP*), and neighbor-joining (*NJ*) criteria. Samples with ascertained status are highlighted: *N. j. japonica* (*blue*), *N. j. totogo* (*green*), and ascertained breeders in Taiwan (*yellow*)

diversity (0.7883 vs. 0.5913), and higher nucleotide diversity (0.00288 vs. 0.00087). It had two major haplotypes, A01 and A09, comprising 9 and 12 individuals, respectively (Fig. 4). Three ascertained migratory birds had a haplotype of A01, while all the Lanyu birds represented A09. In contrast, a single haplotype B01 dominated Clade B, found on 29 owls from Taiwan. The two clades had a fixed divergence of 9 base pairs.

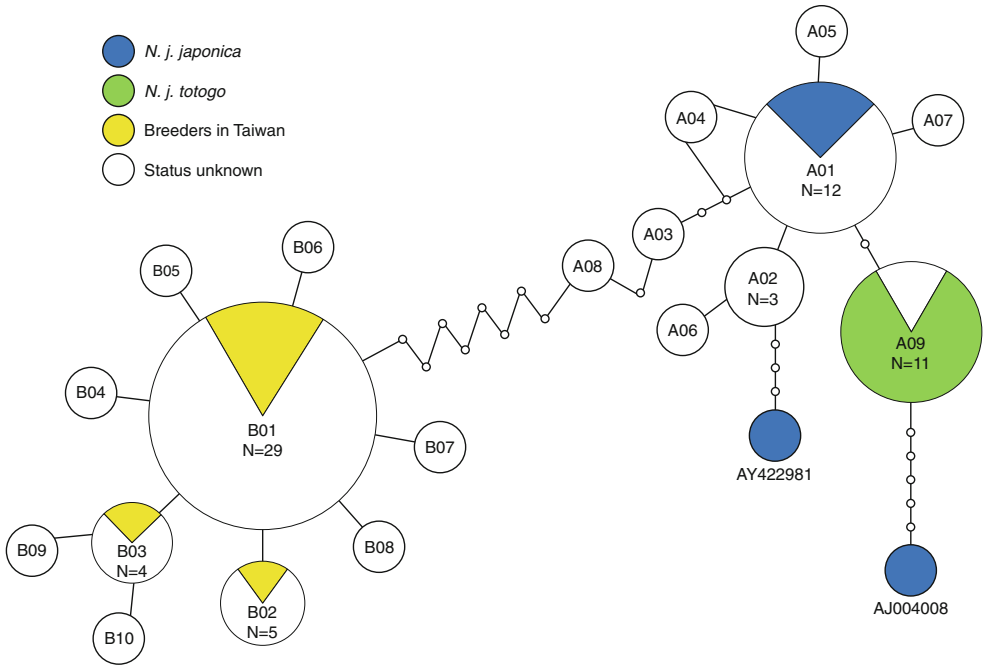

Fig. 4 The haplotype network of *Ninox japonica* obtained in this study. Sample size and population composition of each haplotype are given in the *pie charts* (*N. j. japonica* in *blue*, *N. j. totogo* in *green*, ascertained breeders from Taiwan in *yellow*)

The 63 Taiwan owls fell into either of the 2 clades. Clade B contained the 7 known residents, thus the remaining 39 are "suspected residents", while the 17 owls that fall under Clade A are "suspected migrants" (Supplementary Material, ESM Table 1).

Principal component analysis among different groups

We compared the body sizes of the 17 Lanyu owls, 11 Taiwan owls from Clade A, and 32 Taiwan owls from Clade B with PCA. The first three axes explained 67.2, 15.8, and 12.6 % of total variation, respectively. Owls of the three groups could not be differentiated by size or shape (Fig. 5).

Discussion

This study uncovered two genetic clades within *N. japonica* in Taiwan, with striking differentiation between the clades (1.72 % in *p*-distance and 1.75 % in HKY distance). The migratory clade (Clade A) included *N. j. japonica*, ascertained migrants, and some other birds found outside the breeding season. In contrast, all the ascertained breeders as well as birds received in June, July, and August were grouped into the sedentary clade (Clade B). During the breeding season, only Clade B exists; while outside the breeding season, both clades coexist in Taiwan. This feature suggested that both migratory and sedentary populations winter on the island.

Our findings showed that the current taxonomic treatment (Fig. 2a) of the two subspecies is obviously problematic. Genetic sequences of topotypes of *N. j. totogo* from Lanyu were grouped into Clade A, which also contained specimens representing migratory *N. j. japonica*. This result suggests an extremely low genetic differentiation between *N. j. japonica* and *N. j. totogo* under their current definition (divergence = 0.0032 in *p*-distance). We suggest treating *N. j. totogo* as an invalid subspecies (Fig. 2b). In contrast, the breeding *N. japonica* in Taiwan has diverged from the known *N. j. japonica* as much as 1.7 %, but has not been described (Fig. 2b). Lack of obvious morphological difference between the Taiwan residents from the migrants no doubt obscured its existence. Compared to other taxa in *Ninox* (Norman et al. 1998), such a high genetic divergence has reached the level of subspecies (1.5–2.3 %). From the perspective of conservation genetics, this cryptic lineage already qualified as a management unit (MU) or evolutionarily significant unit (ESU) (Ryder 1986; Moritz 1994; Funk et al. 2008). Future work combining genetic analyses with song comparisons (e.g., King 2002; Rasmussen et al. 2012) or careful evaluation of the color patterns of known individuals might produce external cues for delineation between the two groups.

The newly discovered lineage of breeding *N. japonica* in Taiwan provides an interesting example of the evolution of avian migration. First, although the Taiwanese and north Asian breeders are sympatric in their wintering sites, the

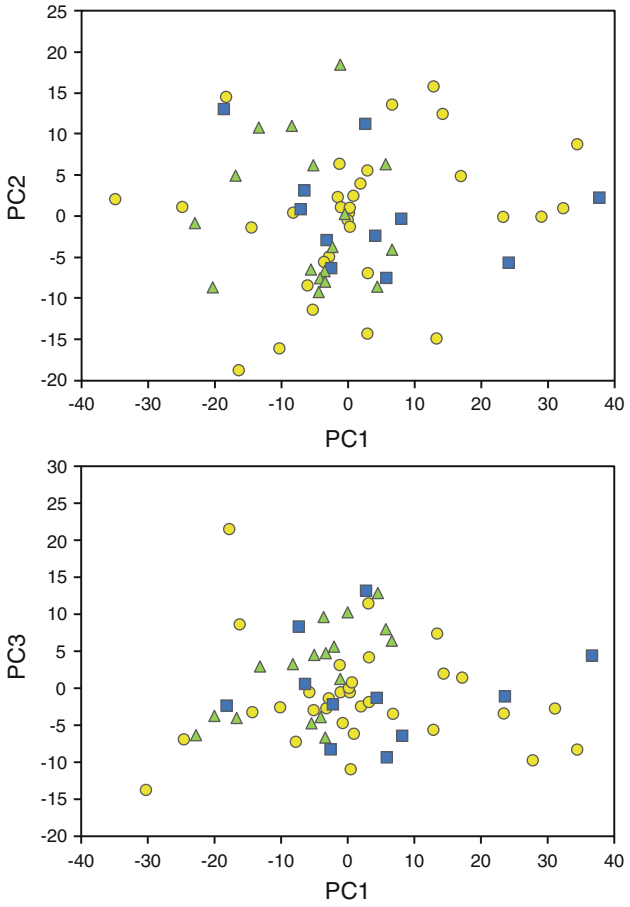

Fig. 5 Principal component analysis (PCA) of 60 owls showed no morphological differentiation among the migratory clade (11 *blue rectangles*), the sedentary clade (32 *yellow circles*), and topotypes of *Ninox japonica totogo* from Lanyu (17 *green triangles*)

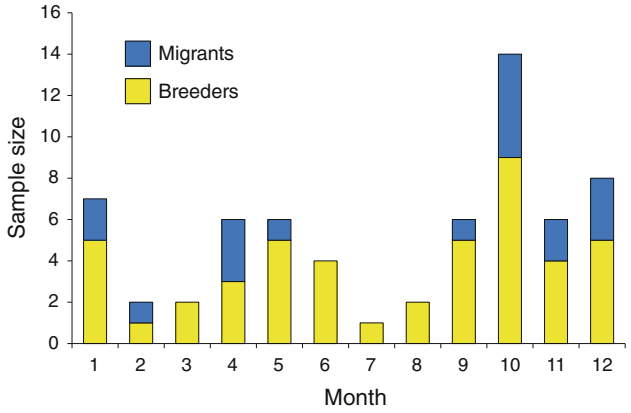

Fig. 6 Occurrence of sedentary clade (*yellow bars*, $n = 46$) and migratory clade (*blue bars*, $n = 18$) by month in Taiwan and Penghu. The migratory clade occurs in Taiwan between September and mid-May, while the sedentary clade appears all year round (ESM Table 1)

genetic differentiation between them is high, whereas the differentiation between breeders on Lanyu and their north Asian relatives is not prominent. Second, although they have good dispersal ability (see Fig. 1), breeders on the two adjacent islands (Taiwan and Lanyu. only 60 km apart) belong to separate lineages with notable genetic differentiation. Genetic differentiation among conspecific birds with different migratory behavior has been known. European Blackcaps *Sylvia atricapilla* using different migration routes showed genetic and behavioral differentiation within a short time span (Bearhop et al. 2005; Rolshausen et al. 2009). Migratory Willow Warblers *Phylloscopus trochilus* from different breeding sites formed geographic clines in accordance with their genetic and morphological traits (Bensch et al. 2009). *N. japonica* provides another example of differentiation between migratory and sedentary populations. Assortative mating of birds with the same migratory status, such as based on mating calls, might be an essential factor leading to such genetic differentiation.

Based on the samples we collected in Taiwan (Supplementary Material, ESM Table 1), owls in the migratory clade appeared between September and May, with the earliest bird arriving in early September and the last bird leaving on 10 May (Fig. 6). These dates approximately correspond to the beginning and end of the migration season. All owls collected after mid-May in Taiwan genetically belonged to Clade B, indicating that only resident birds remained then. This result is consistent with the reports that the breeding season for northeastern Asian populations begins in May (Brazil and Yabuuchi 1991; Oba 1996). The fact that Taiwan breeders start egg laying between March and April (Lin et al. 2012) suggests that temporal differentiation might also contribute to the reproductive isolation between the two clades. The rescue center of TWCG receives most injured birds of the breeding population (Clade B) in October, suggesting that owls might encounter higher risks at this period owing to extra stress related to first year dispersal or food shortage.

Molecular differentiation observed in this study not only provides an insight into the genetic biodiversity of an owl but also provides clues to their breeding and migratory habits. We believe the discovery of this cryptic lineage contains great potential for future research, which will not only facilitate a better understanding of this owl in terms of its genetic diversity, the evolution of its migratory behavior, and its conservation but also stimulate a closer examination of the genetic diversity of other species lacking overt morphological differentiation.

Acknowledgments We are grateful to Dr. Chi-Li Tsai and Miss Shu-Ping Tseng for their assistance in molecular work and data analyses. Miss Shue-Ju Wu assisted in the handling of injured owls. Members of the Ornithology Lab, Biodiversity Research Center, Academia Sinica, assisted with trapping, measuring and sample collection on Lanyu. Professor Kim Sooil, Korean National University of

Education, provided tissue samples of Korean owls. Mr. Cheng-Te Yao and Mr. Fang-Tse Chan of the Taiwan Endemic Species Research Institute provided some tissue samples of Taiwan owls. Miss Yen-Jean Chen of the National Museum of Natural Science and Miss Meng-Min Hsueh of the Biodiversity Research Museum, Academia Sinica, allowed us access to the museum sample collections. This study complies with the current Taiwanese laws and was supported by the Forestry Bureau, National Science Council, and Biodiversity Research Center, Academia Sinica of Taiwan.

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