Avian Research

CrossMark

DNA reveals long-distance partial migratory behavior in a cryptic owl lineage

Keren R. Sadanandan[1], David J. X. Tan[1], Kolbjørn Schjølberg[2], Philip D. Round[3] and Frank E. Rheindt[1*]

Abstract

Background: The Brown Hawk Owl complex is said to consist of three species, the Brown Boobook (*Ninox scutulata*), the Chocolate Boobook (*Ninox randi*) and the Northern Boobook (*Ninox japonica*), which includes the nominate migratory lineage *Ninox japonica japonica* and a mitochondrially distinct lineage of taxonomically uncertain status that has been recorded year-round at least in Taiwan. Overlap in ranges during migration and morphological similarity have led to difficulties in distinguishing the Brown Boobook from the Northern Boobook.

Methods: PCR of cytochrome-*b* and Sanger sequencing of *Ninox* samples from Singapore and Brunei were used to determine sample identity.

Results: Two out of four Singaporean samples and the Brunei sample were identified as Northern Boobooks. This is the first official record of this species in Singapore and represents a considerable range extension for the species. Further, the samples belong to the mitochondrially distinct lineage previously characterized in resident Taiwanese populations rather than to the well-known nominate migratory lineage.

Conclusions: Our data show that the mitochondrial signature previously documented in resident Taiwanese populations of the Northern Boobook extends to migratory populations. This cryptic lineage may be more widespread in its breeding quarters, extending to the Chinese and Russian mainland, in which case the name *florensis* would apply to it. Further genetic and bioacoustic investigation is required to resolve the taxonomic status of this lineage.

Keywords: Migration, Cryptic species, Mitochondrial lineage, Brown hawk owl, Partial migrant

Background

Strigiformes, the bird order known commonly as owls, comprise cryptically colored nocturnal birds that can be notoriously hard to identify in the field. Their mysterious nature is typified by the Brown Hawk Owl or Brown Boobook complex (*Ninox* [*scutulata*] sp.), an assemblage widely distributed across Asia with populations in various regions exhibiting differentiation with respect to phenology, vocalizations and plumage. This differentiation has led to confusion about the taxonomic status of these populations. Some authors suggest that the complex is a single species comprising as many as 13 subspecies (König et al. 2009). However, King (2002) analyzed bioacoustic and morphometric data and determined
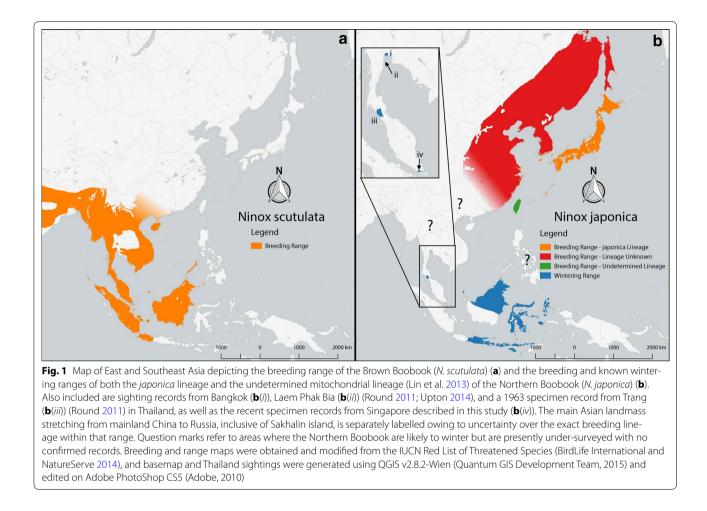
that the complex comprises three biological species: the Northern Boobook (*Ninox japonica*), which breeds from southeast Siberia to Taiwan and adjacent mainland China, and winters across Southeast Asia, the Chocolate Boobook (*Ninox randi*), which is endemic to the main Philippines (i.e. the Philippine archipelago with the exclusion of Palawan and satellite islands), and the Brown Boobook (*Ninox scutulata*), which is distributed across South and Southeast Asia to the Greater Sundas and Palawan (Fig. 1).

At present knowledge, *Ninox japonica* occurs as two mitochondrially distinct populations—a nominate population that occurs in both Japan (including Ryukyu) and little islands off of Taiwan (e.g. Lanyu), and a resident population on the Taiwanese mainland said to be nonmigratory due to year-round records of the taxon on the island (Lin et al. 2013). While the latter lineage has been identified based on DNA samples from the island

*Correspondence: dbsrfe@nus.edu.sg
[1] Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117543 Singapore, Singapore
Full list of author information is available at the end of the article

Sadanandan *et al. Avian Res* (2015) 6:25

Page 2 of 7

Fig. 1 Map of East and Southeast Asia depicting the breeding range of the Brown Boobook (*N. scutulata*) (**a**) and the breeding and known wintering ranges of both the *japonica* lineage and the undetermined mitochondrial lineage (Lin et al. 2013) of the Northern Boobook (*N. japonica*) (**b**). Also included are sighting records from Bangkok (**b**(*i*)), Laem Phak Bia (**b**(*ii*)) (Round 2011; Upton 2014), and a 1963 specimen record from Trang (**b**(*iii*)) (Round 2011) in Thailand, as well as the recent specimen records from Singapore described in this study (**b**(*iv*)). The main Asian landmass stretching from mainland China to Russia, inclusive of Sakhalin island, is separately labelled owing to uncertainty over the exact breeding lineage within that range. Question marks refer to areas where the Northern Boobook are likely to winter but are presently under-surveyed with no confirmed records. Breeding and range maps were obtained and modified from the IUCN Red List of Threatened Species (BirdLife International and NatureServe 2014), and basemap and Thailand sightings were generated using QGIS v2.8.2-Wien (Quantum GIS Development Team, 2015) and edited on Adobe PhotoShop CS5 (Adobe, 2010)

of Taiwan only, unsampled breeding populations in mainland China and Russia may well be closely related or identical to this lineage. Lin et al. (2013) refer to this as an unnamed population, but it may well have a taxonomic name already if mainland populations are shown to belong with it: based on morphological considerations, many treatises nowadays refer to mainland populations as *N. j. florensis* (del Hoyo et al. 2015), a name based on type material from the wintering range in East Indonesia that may extend to Taiwan if future research can verify the connection between the type of *florensis* and mainland Chinese breeding populations. For the purposes of clarity and brevity, we will refer to this mitochondrial lineage as the "undetermined lineage" henceforth.

The wintering range of the migratory nominate form is unclear; there have been confirmed records from Wallacea, Borneo and Java, but the limits of its winter range further west remain unknown (Fig. 1). Additionally, morphological similarity between Northern and Brown Boobooks makes it difficult to confirm identification in the field where their ranges potentially overlap.

Recent advances in identification between Brown and Northern Boobooks based on wing: tail ratios, wing formulas (King 2002) and ventral markings (Round 2011) have led to new Northern Boobook winter records in mainland Southeast Asia, with several reports of the species in Thailand and a single new record from Kedah in peninsular Malaysia (Table 1). However, the range of variation in ventral patterning is not fully understood, which has led to some confusion with regards to distinguishing the two species in the field. For instance, individuals displaying ventral markings typical of Brown Boobooks (sensu Round 2011) have been photographed breeding across Japan (e.g., Morlan 2012), over 3000 km away from the nearest confirmed breeding locality of Brown Boobooks, indicating that Northern Boobooks, especially from Japan, may display similar ventral markings.

On 23 March 2014, the carcass of a *Ninox* owl (ID CR059) was found near the Tanglin Halt estate in Singapore (01.3009500°N, 103.7930667°E). Close inspection of the bird revealed its ventral patterning to be indicative of the Northern Boobook (sensu Round 2011). This

Sadanandan *et al. Avian Res* (2015) 6:25

Page 3 of 7

Table 1 Winter records of *Ninox japonica* in mainland Southeast Asia

Location	Date	Record type and status	Source
Chiang Mai: Mae Rim:Ban Khi Lek	24/11/1970	Specimen: TISTR53-1848; wing measurements and ventral plumage intermediate between *scutulata* and *japonica*; identification unconfirmed	Round (2011)
Samut Prakan: Suan Sri Nakhon Kheaun Khan, Phra Pradaeng	2/10/2008	Photograph; unconfirmed identification merely based on ventral spotting (see text)	Round (2011)
Bangkok: Suan Rotfai (Railway Park; Suan Venchirabhas)	5/4/2010	Photograph; unconfirmed identification merely based on ventral spotting (see text)	http://bangkokcitybirding.blogspot.com/2010_04_01_archive.html
Malaysia: Kedah State: coast between Kuala Kedah and Kuala Sanglang	5/12/2010	Photograph; unconfirmed identification merely based on ventral spotting (see text)	David Bakewell (pers. comm.)
Nakhon Pathom: Phutthamonthol, Salaya	7/2/2011	Photograph; unconfirmed identification merely based on ventral spotting (see text)	Round (2011)
Bangkok: Suan Rotfai (Railway Park; Suan Venchirabhas)	9/1/2014–8/2/2014	Photograph; unconfirmed identification merely based on ventral spotting (see text)	Limparungpattanakij (pers. comm.)
Bangkok: Sammakorn Village, Ramkhamhaeng	11/10/2014	Photograph; unconfirmed identification merely based on ventral spotting (see text)	https://www.facebook.com/photo.php?fbid=10205116649379611&set=a.119610546563.2030022.1345440622&type=1&fref=nf
Phetchaburi: Laem Phak Bia, Ban Laem	*30/3/2015*	*Trapped; ring no 8A01825; measurements confirm identification*	*Round (2011)*

Confirmed identifications in italic. All records are from Thailand unless otherwise specified

Sadanandan *et al. Avian Res* (2015) 6:25

Page 4 of 7

potentially represents the first country record of the Northern Boobook and extends the mainland wintering range of the species considerably further south. If substantiated with molecular methods, this record would constitute the first corroboration that Northern Boobooks winter on or just off the southern part of mainland Southeast Asia. We here present morphological measurements and cytochrome-*b* (cyt*b*) sequences of this bird and additional specimens collected throughout the region to confirm their identity and to discuss *Ninox* taxonomy and field identification.

Methods

Tissue was sampled from the carcass as well as three presumable Brown Boobooks collected in Singapore that had been deposited in the Lee Kong Chian Natural History Museum (Additional file 1: Table S1). DNA extractions were carried out with GeneAll® DNA Purification kits as per the protocol for animal tissue. Polymerase chain reactions (PCR) were carried out in a C1000™ Thermal Cycler and cyt*b* was amplified as per Cibois et al. (1999) and Dong et al. (2010) using the conditions stated in Sorenson et al. (1999). PCR amplifications were done in 20 μL reaction volumes, which comprised 2 μL 10 × Taq PCR buffer, 1.2 μL MgCl$_2$ (25 mM), 1 μL of each primer (10 μm), 0.4 μL Fermentas *Taq* polymeras, 2 μL mtDNA template and 11.6 μL MilliQ water. PCR product clean up was carried out using ExoSAP-IT® and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) was used to cycle-sequence samples. Sequences were obtained by capillary electrophoresis using an Applied Biosystems 3130 × l Genetic Analyzer.

DNA sequences were assembled with CodonCode Aligner version 4.1 (CodonCode Corporation, http://www.codoncode.com) and aligned with MEGA 6.06 using ClustalW (Larkin et al. 2007; Tamura et al. 2013). The final alignment was 923 base pairs in length and contained no indels. We compared our four novel sequences with those of 32 individuals of the nominate form of *Ninox japonica* from Japan and Lanyu, 48 individuals of the presumably resident undetermined lineage from the main island of Taiwan and a single novel sequence of *Ninox japonica* from Brunei for a total sample size of 85 (Additional file 1: Table S1; Lin et al. 2013). Sequences were easily aligned, therefore unlikely to be paralogs. MEGA 6.06 was employed to construct phylogenetic trees using Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML). For each analysis, 1000 bootstrap replicates were run. MP was run with all sites used for gap treatment, Tree-Bisection-Reconnection as the tree search method and other settings in default mode. The program jModelTest 2.1.4 (Darriba

et al. 2012) was used to determine the best evolutionary model for ML. The results identified the Hasegawa, Kishino, and Yano model with parameters for gamma distribution (HKY + *G*) as the most suitable model for ML analysis. The outgroup for tree analyses was a cyt*b* sequence of the Powerful Owl (*Ninox strenua*) downloaded from GenBank. DnaSP v5.10.01 (Librado and Rozas 2009) was used to calculate raw sequence divergence and nucleotide diversity between and within taxa.

The wing of the carcass retrieved in Singapore was measured by determining the distance between the foremost extremity of the carpus to the tip of the longest primary feather using the closed, flattened wing. Wing point measurements were determined by closing the wing and placing the tips of the primary feathers in order. The feathers were then splayed slightly and the primaries were measured in relation to the longest primary. The tail was measured by sliding a ruler between the retrices and the undertail coverts up to the base of the tail, and then flattening and measuring the longest tail feather. Wing and tail measurements were compared against those obtained for vouchered Brown and Northern Boobook specimens (King 2002).

Results

NJ, MP and ML analyses all yielded trees with identical topology. Three similar clades emerged from the phylogenetic analysis, with the first two clades representing the two genetically distinct Northern Boobook taxa (Lin et al. 2013). The first clade contained known Taiwanese breeders [the undetermined lineage identified by Lin et al. (2013)] and the second contained individuals from the migratory main population (Fig. 2). The Singaporean carcass and the Brunei sample were nested within the first clade, confirming that they are Northern Boobooks. Of the three tissue samples analyzed from the Lee Kong Chian Museum of Natural History, one sample (November 2000) also emerged within the undetermined lineage whilst the other two samples (July 2003; June 2004) formed a separate clade (Fig. 2). These latter two samples represent Brown Boobooks resident in Singapore as they were collected in summer months when no migrants are present.

Analysis of raw sequence divergence between clades showed that *Ninox scutulata* exhibited a divergence of 2.1–2.6 % from the two *Ninox japonica* clades, whilst the nominate clade of *Ninox japonica* showed a smaller divergence of 1.6 % from the undetermined lineage (Table 2).

The wing and tail measurements of the Singaporean carcass were 217 mm and 114 mm, respectively, giving a wing:tail ratio of 1.90 (Table 3). These results, combined with wing morphology measurements (Table 4) provided
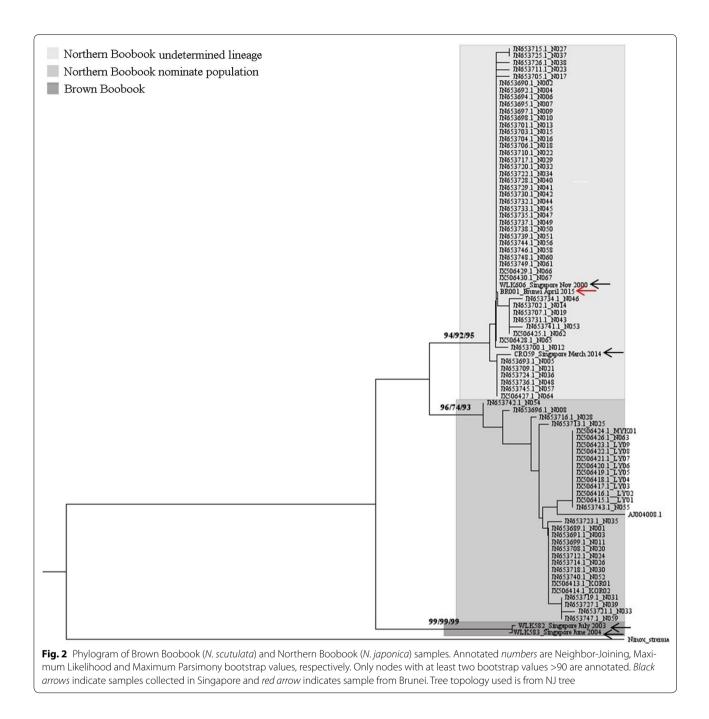
Sadanandan *et al. Avian Res* (2015) 6:25

Page 5 of 7

Fig. 2 Phylogram of Brown Boobook (*N. scutulata*) and Northern Boobook (*N. japonica*) samples. Annotated *numbers* are Neighbor-Joining, Maximum Likelihood and Maximum Parsimony bootstrap values, respectively. Only nodes with at least two bootstrap values >90 are annotated. *Black arrows* indicate samples collected in Singapore and *red arrow* indicates sample from Brunei. Tree topology used is from NJ tree

Table 2 Matrix of p-divergences between taxa (below diagonal) and p-divergences within each sampled taxon (along diagonal, italic)

	Ninox japonica [nominate form]	*Ninox japonica* [undetermined lineage]	*Ninox scutulata*
Ninox japonica [nominate form]	*0.000–0.009*		
Ninox japonica [undetermined lineage]	0.016	*0.000–0.004*	
Ninox scutulata	0.026	0.021	*0.000*

Sadanandan *et al. Avian Res* (2015) 6:25

Page 6 of 7

Table 3 Comparison of morphological measurements across *Ninox* sp. and the Singaporean carcass (in italic)

	Wing	Tail	Wing/tail ratio
Ninox japonica	220.8 (214.0–226.8)	115.8 (107.5–122.6)	1.91 (1.75–2.03)
Ninox scutulata	195.7 (182.4–213.3)	109.5 (100.3–115.0)	1.78 (1.69–1.87)
Singapore carcass CR059	*217.0*	*114.0*	*1.90*

Average values are provided in mm with range given in brackets. Values highlighted show instances where measurement range overlaps with values from Singapore carcass

Table 4 Wing morphology; comparative length differences among five longest primary feathers in the following order: P9, P8, P7, P6, P5

	P9	P8	P7	P6	P5
Ninox japonica	15.6	0	3.9	19.6	46.1
Ninox scutulata	16.7	1.6	0	5.3	23.3
Singapore carcass CR059	16.0	0	1.0	15.0	43.0

Measurements start from wing tip, so zero stands for longest primary. Data for *Ninox japonica* and *Ninox scutulata* adapted from King (2002)

morphological confirmation that our specimen was a Northern Boobook as diagnosed by King (2002).

Discussion

All previous records of Northern Boobooks in mainland Southeast Asia have relied on visual identification based on the ventral markings or measurements based on wing:tail ratios (Round 2011). However the full extent of intra-specific variation in ventral markings within Northern and Brown Boobooks is not well understood, making it difficult to distinguish the two species visually. Our molecular results constitute a considerable southward extension of the wintering range of >1400 km from a previous winter record (Phetchaburi; Table 1) confirmed by measurements. It is possible that the wintering range of Northern Boobooks may even include Sumatra based on its proximity to Singapore. This discovery represents a new avian country record for Singapore. We show here that in cases where visual identification is challenging, genetic analysis is a handy tool for clarifying species identity.

Of the three additional Singaporean tissue specimens used as a comparison, two were Brown Boobooks as expected, but surprisingly the third also emerged as a Northern Boobook. The fact that two out of four randomly collected boobooks in Singapore refer to Northern rather than Brown Boobooks suggests that the recently procured carcass is not an accidental stray, but

that Northern Boobooks may be regular annual visitors to the island. Given that Singapore has one of the best-known national avifaunas in the world (Wang and Hails 2007; Chisholm et al. 2015), it seems that this seasonal visitor may have been overlooked due to confusion with the island's resident Brown Boobook. The procurement date of one specimen in November suggests that Northern Boobooks are not mere migrants but may overwinter on the island.

The Northern Boobooks identified from Singapore and Brunei were not of the migratory Japanese population but of the undetermined mitochondrial lineage. This lineage has been reported year-round in Taiwan including breeding in March (Lin et al. 2013)—the month of procurement of the carcass in Singapore. Our discovery suggests that some populations of the undetermined mitochondrial lineage are long-distance migratory, whereas others are not. While we cannot rule out that Taiwan harbors both migratory and non-migratory populations, we consider it more likely that the undetermined mitochondrial lineage extends to genetically unsampled parts of the breeding range of the Northern Boobook across the East Asian mainland from South China north all the way to Russia. If true, it is likely that the provenance of the migratory populations of this lineage lies in the more northerly parts of their distribution, such as northeast China (Heilongjiang Province) and eastern Siberian Russia.

The internal taxonomy of the Northern Boobook *N. japonica* is confused: subspecies *florensis* was described on the basis of a wintering specimen from the island of Flores (eastern Indonesia); it has been attributed to breeding populations from northern mainland Asia (such as eastern Siberian Russia and northeastern China) presumably because of its large size. If true, this name would apply to the undetermined mitochondrial lineage of Lin et al. (2013), of which Taiwanese birds would then be a resident subset. Additional genetic research including mainland breeding populations of Northern Boobooks is urgently needed to confirm this hypothesis. Further, the use of high resolution genome-wide SNP data will help provide a more comprehensive understanding of the taxonomy of these boobooks.

Conclusions

Analysis of mitochondrial DNA has extended the wintering range of Northern Boobooks in Southeast Asia and suggests that most winterers in the Sundaic region may refer to a mitochondrial lineage of uncertain taxonomic status that has previously been documented from Taiwan but may breed over large parts of mainland Eastern Asia. A genetic research into mainland breeders is urgently required to characterize the geographic distribution of

Sadanandan *et al. Avian Res* (2015) 6:25

Page 7 of 7

this undetermined mitochondrial lineage. Future taxonomic research may show that the name *florensis*, based on a wintering individual from the island of Flores, refers to this lineage.

Additional file

Additional file 1: Table S1. *Ninox* sp. samples used in study.

Author details
[1] Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117543 Singapore, Singapore. [2] TSG/31, BSP HQ, Jalan Utara, KB3534, Seria, Brunei Darussalam. [3] Department of Biology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand.

References
BirdLife International and NatureServe. Bird Species Distribution Maps of the World. 2014. The IUCN Red List of Threatened Species. Version 2014.3. 2014. (http://www.iucnredlist.org).

Chisholm RA, Giam X, Sadanandan KR, Fung T, Rheindt FE. A robust nonparametric method for quantifying undetected extinctions. Conserv Biol. 2015. doi:10.1111/cobi.12640.

Cibois A, Pasquet E, Schulenberg TS. Molecular Systematics of the Malagasy Babblers (Passeriformes: Timaliidae) and Warblers (Passeriformes: Sylviidae), Based on Cytochrome b and 16S rRNA Sequences. Mol Phylogent Evol. 1999;13:581–95.

Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772.

del Hoyo J, Collar N, Marks JS. Northern Boobook (*Ninox japonica*). In: del Hoyo J, Elliott A, Sargatal J, Christie DA, de Juana E (editors). Handbook of the Birds of the World Alive. Lynx Edicions, Barcelona. 2015. (http://www.hbw.com/node/467319).

Dong F, Li SH, Yang XJ. Molecular systematics and diversification of the Asian scimitar Babblers (Timaliidae, Aves) based on mitochondrial and nuclear DNA sequences. Mol Phylogenet Evol. 2010;57:1268–75.

King B. Species limits in the Brown Boobook *Ninox scutulata* complex. Bull Br Ornithol Club. 2002;122:250–6.

König C, Weick F, Becking JH. Owls of the World. New Haven: Yale University; 2009.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. ClustalW and ClustalX version 2. Bioinformatics. 2007;23:2947–8.

Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009;25:1451–2.

Lin WL, Severinghaus LL, Tseng HY, Lin SM. Genetic differentiation between migratory and sedentary populations of the Northern Boobook (Ninox japonica), with the discovery of a novel cryptic sedentary lineage. J Ornithol. 2013;154:987–94.

Morlan J. Brown Hawk-Owl (*Ninox scutulata japonica*) aka Northern Boobook (*Ninox japonica japonica*). 2012. (http://fog.ccsf.edu/~jmorlan/Japan/BrownHawkOwlCP1020017.htm).

Round PD. Brown and Northern Boobooks both occur in Thailand. BCST Bulletin. 2011;28(3):17–9.

Sorenson MD, Ast JC, Dimcheff DE, Yuri T, Mindell DP. Primers for a PCR-based approach to mitochondrial genome sequencing in birds and other vertebrates. Mol Phylogenet Evol. 1999;12:105–14.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.

Upton N. Additions to the Thai bird checklist. 2014. (http://www.thaibirding.com/news/latestadditions.htm).

Wang LK, Hails CJ. An annotated checklist of the birds of Singapore. Raffles B Zool Supplement. 2007;15:1–179.