

DNA barcoding reveals 24 distinct lineages as cryptic bird species candidates in and around the Japanese Archipelago

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Abstract

DNA barcoding using a partial region (648 bp) of the cytochrome *c* oxidase I (COI) gene is a powerful tool for species identification and has revealed many cryptic species in various animal taxa. In birds, cryptic species are likely to occur in insular regions like the Japanese Archipelago due to the prevention of gene flow by sea barriers. Using COI sequences of 234 of the 251 Japanese-breeding bird species, we established a DNA barcoding library for species identification and estimated the number of cryptic species candidates. A total of 226 species (96.6%) had unique COI sequences with large genetic divergence among the closest species based on neighbour-joining clusters, genetic distance criterion and diagnostic substitutions. Eleven cryptic species candidates were detected, with distinct intraspecific deep genetic divergences, nine lineages of which were geographically separated by islands and straits within the Japanese Archipelago. To identify Japan-specific cryptic species from trans-Palaearctic birds, we investigated the genetic structure of 142 shared species over an extended region covering Japan and Eurasia; 19 of these species formed two or more clades with high bootstrap values. Excluding six duplicated species from the total of 11 species within the Japanese Archipelago and 19 trans-Palaearctic species, we identified 24 species that were cryptic species candidates within and surrounding the Japanese Archipelago. Repeated sea level changes during the glacial and interglacial periods may be responsible for the deep genetic divergences of Japanese birds in this insular region, which has led to inconsistencies in traditional taxonomies based on morphology.

Keywords: cryptic species, DNA barcoding, Japanese birds, Palaearctic region, taxonomy

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Introduction

DNA barcoding is a useful and proven tool for species identification. A partial region (648 bp) of the cytochrome *c* oxidase I (COI) gene in the mitochondrial genome is used as a standard DNA barcoding region for most animals (Hebert *et al.* 2003). In vertebrates, the DNA barcoding region has been very useful in identifying species due to its high interspecific and low intraspecific variation (Ward *et al.* 2005; Hernández-Dávila *et al.* 2012), and moreover, for identifying cryptic species (Clare *et al.* 2007; Lara *et al.* 2010).

The DNA barcoding library of bird DNA includes several geographical regions, such as the Nearctic

(Hebert *et al.* 2004), South Korea (Yoo *et al.* 2006), the Neotropics (Kerr *et al.* 2009a; Tavares *et al.* 2011), the eastern Palaearctic (Kerr *et al.* 2009b) and Scandinavia (Johnsen *et al.* 2010), and is continually expanding (Milá *et al.* 2012). DNA barcoding of distinct conspecific genetic divergences has revealed lineages of many cryptic species within continents (Hebert *et al.* 2004; Kerr *et al.* 2009b; Milá *et al.* 2012) and among trans-continents (Kerr *et al.* 2009a; Johnsen *et al.* 2010; Lijtmaer *et al.* 2011). Lohman *et al.* (2010) investigated the barcoding region of six resident birds in South-East Asia, which included many small islands, and found deep genetic divergence among the islands, suggesting that traditional taxonomy may have overlooked endemic species in the area.

Geographical barriers, such as mountain ranges and seas, prevent gene flow and form definite distribution boundaries for species and subspecies. The Japanese Archipelago is located off the eastern coast of the

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Eurasian continent, across the Sea of Japan and the East China Sea (Fig. 1). The avifauna of Japan consists of 633 bird species, including nonbreeding birds that largely share the eastern Eurasian continent despite the sea barrier; however, the Japanese Archipelago has 11 endemic resident bird species and six migratory bird species breeding only in that area (The Ornithological Society of Japan 2012). The archipelago is treated as a biodiversity hot spot (Conservation International 2012) and a new zoogeographic region based on the phylogenetic relationship of the birds and other vertebrates (Holt *et al.* 2013). The high biodiversity in Japan is due to a wide variety of climates and ecosystems, ranging from the humid subtropics in the Ryukyu and Ogasawara islands to the boreal zones in northern Japan, the alpine zone at over 3000 m above sea level, and more than 3000 islands (Conservation International 2012). The islands and the straits between them have separated avian species over geographical time. Traditional taxonomists have repeatedly disputed whether the populations isolated on islands and/or by the straits should be recognized as subspecies or as separate species (Ornithological Society of Japan 2012), as the birds form a morphologically well-defined species group. However, molecular analysis has determined that the straits and seas have split the Japanese birds into several distinct lineages, but these have currently only been revealed for a limited number of species such as the Arctic Warbler *Phylloscopus borealis* (Saitoh *et al.* 2010), the Eurasian Jay *Garrulus glandarius* (Akimova *et al.* 2007), the Varied Tit *Sittiparus varius* (Nishiumi *et al.* 2006) and the Ryukyu Robin *Erithacus kamodori* (Seki 2006; Seki *et al.* 2007).

The aim of this barcoding study was to examine whether the diversification and phylogeographic structure of Japanese birds is related to the complex arrangement of islands, which extend northeast and southwest of Japan. We focused on current Japanese-breeding bird species. We generated a DNA barcoding library of bird species to test whether DNA barcoding was a suitable method for species identification and to identify the prevalence of cryptic species. However, the region covered by our survey was not sufficient to fully establish a relationship between genetic diversification and the Japanese Archipelago because many Japanese birds are common throughout Eurasia. A wider-scale investigation was needed, and accordingly, we conducted a comprehensive survey to assess the genetic divergence of bird species breeding in the east Eurasian Continent and the Japanese Archipelago using published barcoding libraries.

Materials and methods

In total, 1367 voucher specimens representing 234 Japanese bird species collected throughout the Japanese

Archipelago (Appendix S1, Supporting information) were included in this research. Blood and frozen tissue samples (pectoral muscle) were taken from voucher specimens held at the National Museum of Nature and Science, Tokyo (NSMT) and the Yamashina Institute for Ornithology (YIO). All the samples were linked to voucher specimens from the NSMT (49.3%), the YIO (40.1%), the Higashi Taisetsu Museum of Natural History (6.8%), the Botanical Garden at the Field Science Centre for Northern Biosphere, Hokkaido University (1.9%), the Kushiro-Shitsugen Wildlife Centre (1%) and other institutions (0.8%). Detailed information about these samples is accessible via 'Birds of Japan, NSMT' and 'Birds of Japan, YIO' projects on the Barcode of Life Data Systems (BOLD) website (<http://www.boldsystems.org/>).

To estimate the full genetic divergence of birds in East Asia, including the insular region of the Japanese Archipelago, we also used 1737 sequences representing 142 bird species from the intersection of 437 Eurasian species (Yoo *et al.* 2006; Kerr *et al.* 2009b) and 234 Japanese species. The taxonomy followed Clements (2007) as recommended by the 'All Birds Barcoding Initiative' (<http://www.barcodingbirds.org/>).

DNA extraction, PCR amplification and DNA sequencing of the COI barcoding region was conducted at the NSMT and YIO laboratories. DNA was extracted from blood or tissue samples using a standard phenol-chloroform procedure at NSMT and using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) at YIO. Several pairs of primers were used as standard primers for amplification of the COI barcoding region; Bird F1 (5'-TTCTCCAACCACAAAGACATTGGCAC-3'), Bird R1 (5'-ACGTGGGAGATAATTCCAAATCCTG-3') and Bird R2 (5'-ACTACATGTGAGATGATTCCGAATCCAG-3'), alongside newly designed primers, L6697Bird (5'-TCAACYAACCACAAAGAYATCGGYAC-3') and H7390Thrush (5'-ACGTGGGARATRATTCCAAATCCTG-3') for passerine birds. If this approach was unsuccessful, an alternative forward primer, FalcoFA (5'-TCAACAACCACAAAGACATCGGCAC-3'), or a reverse primer, VertebrateR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'), was used (Kerr *et al.* 2007). The 25 μ L polymerase chain reaction (PCR) reaction mix comprised 19.2 μ L ultrapure water, 1.0 U Taq polymerase (Ex Taq, TaKaRa, Shiga, Japan), 2.5 μ L PCR buffer (Mg^{2+} free), 0.3 μ L of each primer (0.24 mM), 2.5 μ L of each dNTP (2.5 mM) and 0.4–1.0 μ L of DNA. The amplification protocol was as follows: 94 °C for 3 min followed by five cycles at 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min, then 30 cycles at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min and a final 72 °C for 5 min. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide and purified using

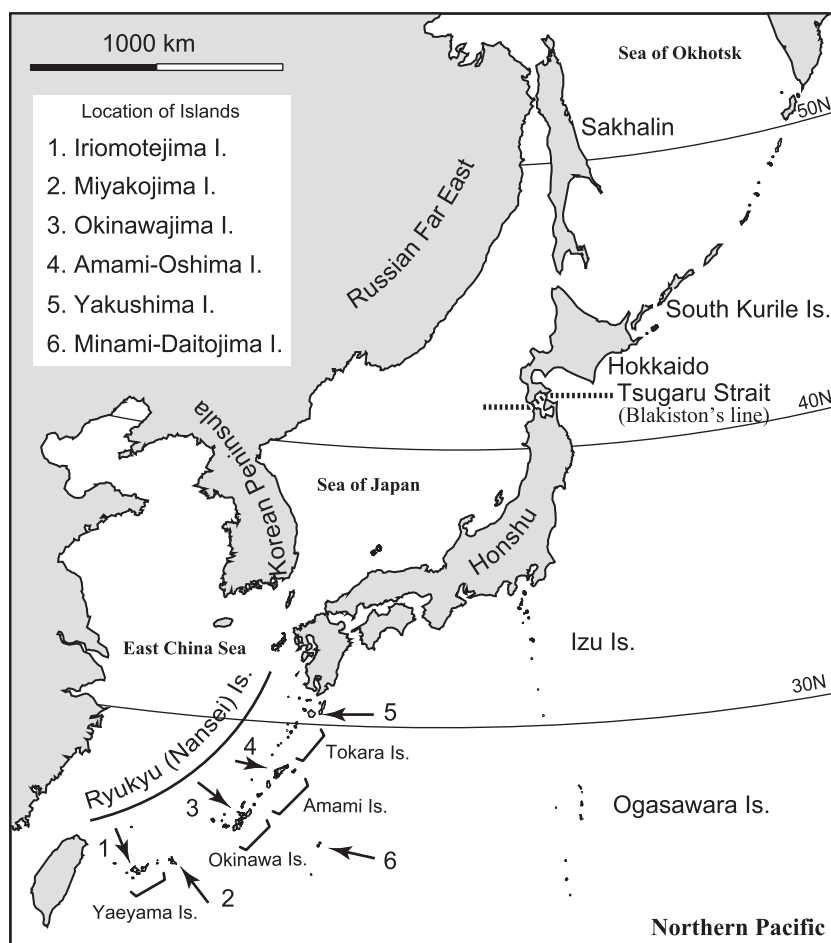


Fig. 1 Map of the Japanese Archipelago and surrounding regions, showing the location and the names of islands and areas. The so-called Blakiston's Line is a biogeographical boundary that separates the Japanese bird and mammal fauna between Hokkaido and the other areas.

ExoSAP-IT (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Sequencing reactions were carried out using BigDye Terminator v1.1 (ABI, Paisley, UK), and analysis was performed on an ABI 3130 Genetic Analyser (ABI) at NSMT, and BigDye Terminator v3.1 (ABI) and an ABI 3100 at YIO. COI sequences were re-covered for all 234 bird species and did not contain insertions, deletions, or nonsense- or stop codons, supporting the absence of nuclear pseudogene amplification (Song *et al.* 2008).

Genetic distances of breeding birds in Japan were ascertained using the Distance Summary and the Barcode Gap Analysis applications of the BOLD website, with the Kimura 2-parameter model (K2P) (Ratnasingham & Hebert 2007). Neighbour-joining (NJ) trees with bootstrap values (1000 replications) were constructed from the COI data using the K2P model and Mega 6.05 (Tamura *et al.* 2013). In cases of very low nearest NJ distances (<1%), the Diagnostic Character

application on the BOLD website was used for a molecular diagnostic of species assignment. We applied >1.6% intraspecific variation as the criterion to find candidates of cryptic species (Kerr *et al.* 2009b). Similarly, genetic distances of 142 common birds from the Japanese Archipelago and the Eurasian continents were calculated using the applications on the BOLD website. The mean genetic distances among populations and bootstrap values of the NJ tree (1000 replications) were ascertained using MEGA 6.05 (Tamura *et al.* 2013). Bird species with a high bootstrap value (>95%) were grouped to estimate the phylogeographic structure.

Results

Species identification in Japanese birds

Sequence data from the COI barcoding region were obtained for 1367 specimens of 234 bird species from the

Japanese Archipelago. Of these species, 200 were represented by multiple specimens. The average K2P genetic distances within species and genus were 0.49% (range 0–6.13) and 8.82% (0–16.39), respectively (Fig. 2). Average K2P intraspecific distance was 0.21% based on 200 species from multiple specimens (0–6.13%). Of the 234 species, 226 (96.6%) had unique DNA sequences that did not overlap with any other species. Ninety-six percentage of the species were >1.0% divergent from their nearest neighbour, and 92.7% were >1.5% divergent. The NJ tree of K2P genetic distances formed 192 monophyletic clusters (96% of the species represented by multiple specimens) that were well supported by bootstrap values (>95%). The bootstrap value of *Erithacus* (94%) was slightly lower than the reliable bootstrap value (95%) for phylogenetic structure.

Of the 234 bird species, five pairs of sister species showed relatively low interspecific genetic variation (<1.0%) (Table 1). Five pairs were exhibited on the NJ tree with unreliable bootstrap values (30–72) or on non-monophyletic clades. For example, the Pleske's Warbler *Locustella pleskei* and the Middendorff's Warbler *Locustella ochotensis* had bootstrap values of 55% and 65%, respectively, and were unable to be identified by diagnostic

substitutions due to small sample sizes ($n = 2$ and 6 , respectively). Only one diagnostic substitution was observed between the Izu Thrush *Turdus celanops* and the Brown-headed Thrush *T. chrysolaus*. Two Guillemot species (*Cepphus carbo* and *C. columba*) could not be discriminated based on the diagnostic sequences due to small sample sizes ($n = 1$ and 1 , respectively). The Common and Oriental cuckoos were paraphyletic and could not be perfectly discriminated. Some Spot-billed Ducks *Anas poecilorhyncha* and Mallards *A. platyrhynchos* shared the same sequences, although the majority of *A. poecilorhyncha* had diverged with a cluster supported by 96% bootstrap values (0.79% genetic distance). Thus, 226 Japanese bird species (96.6%) were identified by their COI barcoding regions using either a distance-based criterion or a NJ tree with bootstrap values and diagnostic sequences in cases of low interspecific genetic distances (<1.0%).

Deep genetic divergence within the Japanese Archipelago

Eleven bird species showed deep intraspecific divergence (>1.6% K2P) and had two or more clusters supported by high bootstrap values (>95%) (Table 2). Nine of 11 birds had three distinct geographical splits, separating the lineages within the Japanese Archipelago. The Tsugaru Strait splits Hokkaido and Honshu in the south and phylogeographically separates *Garrulus glandarius*, *Phylloscopus borealis* and the Ural Owl *Strix uralensis*. The Narcissus Flycatcher *Ficedula narcissina* and several Brown-eared Bulbul *Ixos amaurotis* populations collected from the Ryukyu Islands had different lineages from those collected at Kyushu in the north. *Erithacus komadori* split into two distinct lineages between the Okinawa Islands and the Amami Islands in the north. Certain populations of the Oriental Greenfinch *Carduelis sinica*, *Sittiparus varius* and the Scaly Thrush *Zosterops dauma*, which occupy a specific island and its neighbouring small islands, had sequences that had deeply diverged from sequences from other areas, although the three species are widely distributed throughout the Japanese Archipelago. Distinct lineages were found for *C. sinica* on the Ogasawara Islands, *S. varius* on the Iriomote Island and *Z. dauma* on the Amami-Oshima island. No evidence of a geographical split was detected between the two distinct lineages of both the Oriental Turtle-Dove *Streptopelia orientalis* and the Ryukyu Scops-Owl *Otus elegans*. The Japanese Scops-Owl *O. semitorques* formed two clusters, one of which could reliably be discerned as originating from the Ryukyu Islands (>95% bootstrap value), and the other, with a moderate cluster value (87%), from Honshu, despite having relatively low maximum intraspecific distances (0.9%).

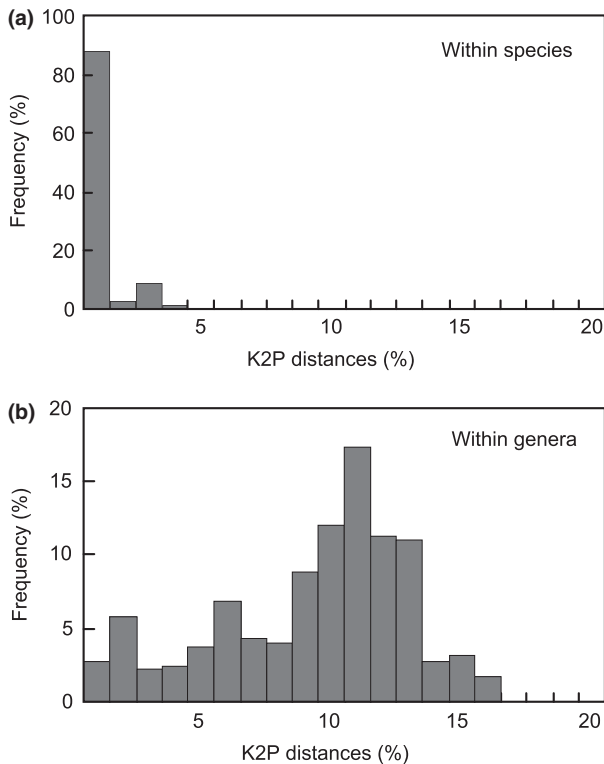


Fig. 2 Frequency histogram of COI among Japanese-breeding birds showing (a) the distribution of the average distance within species (200 species) and (b) the average congeneric distance (47 genus).

Table 1 Japanese bird species with small (<1.0%) interspecific genetic COI sequence distances

	Order	Common name	Scientific name	<i>n</i>	Genetic distance (%)
1	Anseriformes	Mallard	<i>Anas platyrhynchos</i>	4	0
		Spot-billed Duck	<i>Anas poecilorhyncha</i>	7	
2	Passeriformes	Izu Thrush	<i>Turdus celaenops</i>	4	0.15
		Brown-headed Thrush	<i>Turdus chrysolaus</i>	12	
3	Cuculiformes	Common Cuckoo	<i>Cuculus canorus</i>	5	0.3
		Oriental Cuckoo	<i>Cuculus optatus</i>	6	
4	Passeriformes	Middendorff's Warbler	<i>Locustella ochotensis</i>	6	0.63
		Pleske's Warbler	<i>Locustella pleskei</i>	2	
5	Charadriiformes	Spectacled Guillemot	<i>Cephus carbo</i>	1	0.85
		Pigeon Guillemot	<i>Cephus columba</i>	1	

Comparison of the Eurasian and Japanese birds

COI barcoding sequences of 1437 individuals were collected from 142 species that represented multiple specimens from the Japanese Archipelago and the eastern Eurasian continent (Appendix S2, Supporting information). The average intraspecific genetic distance (K2P) was 0.46% (0–6.13%). The NJ tree of K2P genetic distances described 139 monophyletically distinct species supported by high bootstrap values (>95%). *Cuculus optatus* and *C. canorus* were not reciprocally monophyletic. *Motacilla flava* was part of a paraphyletic relationship between a sample from western Eurasia and samples from Japan and eastern Eurasia.

Forty-one of the 142 species had two or more distinct clusters supported by high bootstrap values (>95%). Of these 41 species, 23 displayed phylogeographic patterns that suggested genetic splits between the Eurasian continent and the Japanese Archipelago (Table 3). Of these 23 species, 19 had two or more clusters differing by at least 1.6% K2P. Some species formed clades that

included multiple samples derived from Japanese birds, and one sample from Eurasia (the Eurasian Skylark *Alauda arvensis*, the Carrion Crow *Corvus corone*, the Large-billed Crow *C. macrorhynchos*, the Grey-faced Woodpecker *Picus canus* and the Eurasian Magpie *Pica pica*), for which it was not possible to ascertain any geographical splits between Eurasia and the Japanese Archipelago. In addition to those mentioned above, three species formed Japanese-specific monophyletic clusters with moderate to relatively high bootstrap support (77–94%). A clade for the Siberian Blue Robin *Luscinia cyane*, which included specimens from Hokkaido and the South Kuril Islands (77% bootstrap value), was separated from another clade from Eurasia and Honshu (91%), differing by 1.06%. A clade for the Asian Brown Flycatcher *Muscicapa dauurica* from the Japanese Archipelago (94%) differed by 0.88% from a Eurasian continent clade (88%). The Rock Ptarmigan *Lagopus muta* formed a Japan-specific monophyletic clade (82%) that differed by 0.3% from another monophyletic clade formed of Eurasian samples (68%).

Table 2 Japanese bird species with large (>1.6%) intraspecific COI divergence, treated as candidates of cryptic species

Common name	Scientific name	<i>n</i>	Bootstrap	Max intraspecific distance (%)	Collection area*	Genetic break
1 Ryukyu Robin	<i>Erithacus komadori</i>	3/4	100/100	6.13	O/A, Yk	Ryukyu Is.
2 Arctic Warbler	<i>Phylloscopus borealis</i>	1/4/3	–/89/100	5.06	Hn/H/Hn	Tsugaru Strit
3 Eurasian Jay	<i>Garrulus glandarius</i>	4/3	100/100	4.43	H/Hn	Tsugaru Strit
4 Scaly Thrush	<i>Zoothera dauma</i>	2/18	100/99	3.73	A/Y, Og, I, Hn, H	Isolated Islands
5 Narcissus Flycatcher	<i>Ficedula narcissina</i>	7/10	99/100	3.71	Y, O, A/Hn, H	Ryukyu Is.
6 Brown-eared Bulbul	<i>Ixos amaurotis</i>	1/10/33	1/99/99	3.57	Mi/M, O, A/Y, Og, I, Hn, H	Ryukyu Is.
7 Oriental Greenfinch	<i>Carduelis sinica</i>	9/1	99/–	3.37	Tk, I, Hn, H/Og	Isolated Islands
8 Ryukyu Scops-Owl	<i>Otus elegans</i>	7/6	100/100	2.90	Y, O/O, A, T	Sympatric
9 Varied Tit	<i>Sittiparus varius</i>	4/18	100/100	2.82	Y/O, A, T, Yk, I, Hn, H	Isolated Islands
10 Ural Owl	<i>Strix uralensis</i>	8/13	100/99	2.81	H/Hn	Tsugaru Strit
11 Oriental Turtle-Dove	<i>Streptopelia orientalis</i>	3/5	100/99	2.42	Hn, H/I, Hn, Y	Sympatric

*A = Amami Is., H = Hokkaido, Hn = Honshu (including Kyushu and Shikoku), I = Izu Is., M = Miyakojima I., Mi = Minami-Daito-ima I., O = Okinawa Is., Og = Ogasawara Is., T = Tokara Is., Y = Yaeyama Is., and Yk = Yakushima I.

Discussion

The COI barcoding region enabled identification of 226 species from 234 Japanese-breeding birds, with distance threshold criteria, a NJ tree with high bootstrap values and diagnostic substitution analyses. The average congeneric difference (8.8%) was 18-fold that of the average conspecific genetic difference (0.49%) (Fig. 2). The COI barcoding region has a high resolution for identification of Japanese bird species, making it an effective tool for the identification of bird species inhabiting insular regions, such as the Japanese Archipelago. DNA barcodes have identified 24 cryptic species candidates in the Japanese Archipelago, suggesting that the sea and the straits around the Japanese Archipelago act as effective genetic barriers, despite their relatively small area.

There were five pairs of nearest-neighbour species that had small interspecific genetic differences (<1.0%) in the COI barcoding region (Table 1). Identical sequences were shared between *Anas platyrhynchos* and *Anas poecilorhyncha*, which has also been found for other geographical regions in previous studies (Yoo *et al.* 2006; Park *et al.* 2011). *Anas platyrhynchos* is closest to *A. poecilorhyncha* based on cytochrome *b* and ND2 (Johnson & Sorenson 1999), as well as on COI; thus, further taxonomic re-evaluation of these species should be performed. Two scenarios were proposed for the sequence similarities of these species. An ancestral duck species could have split relatively recently into the two species of duck. Alternatively, the two species of duck may have hybridized in the Russian Far East, resulting in their sharing of the barcoding region (Kulikova *et al.* 2004). *Cuculus canorus* and *C. optatus* also had a very low interspecific distance (0.3%) and formed paraphyletic clades; this pair was previously grouped together as a single clade (Kerr *et al.* 2009b), but hybrids have not been documented to date (Sorenson & Payne 2005). The interspecific genetic difference between *Cephus carbo* and *C. columba* was 0.85%, which is similar to results based on the cytochrome *b* gene (1.4%) (Friesen *et al.* 1996). Only a very small genetic distance (0.15%) separated *T. chrysolais*, a migrant breeder in northern Japan, and *Turdus celaenops*, a resident bird living in several islands off the main islands of Japan (Ornithological Society of Japan 2012), with only a single substitution enabling the species to be individually identified. In addition to those mentioned above, the sequence of the Slaty-backed Gull *Larus schistisagus* that breeds in Japan completely matched those of other gulls recorded in previous studies (Yoo *et al.* 2006; Kerr *et al.* 2009b; Lijtmaer *et al.* 2011) that do not breed in Japan, but migrate to Japan as winter visitors (Ornithological Society of Japan 2012). The COI barcoding region could not distinguish between *L. schistisagus* breeding in Japan and the winter visitor gulls.

We found 11 species with deep intraspecific divergences (>1.6%) (Table 2) that were supported by high bootstrap values (>95%). The patterns of these deep intraspecific divergences were consistent with some of the biogeographical boundaries across the Japanese Archipelago, and these were classified into three groups according to the sea barrier: (i) the Tsugaru Strait, (ii) the Ryukyu Islands and (iii) other specific islands. *Streptopelia orientalis* and *O. elegans*, however, exhibited an intricate pattern of haplotypic distribution. Although haplotypes of the dove were clearly split into two clades with high bootstrap values, no geographical structure was observed across the Japanese Archipelago, including the Ryukyu Islands. *Otus elegans* only partially separated into two clades between Okinawa and Amami-Oshima because two Okinawa birds belonged to the Amami-Oshima clade. Takagi (2013) reported that the owl had a vocal divergence among Amami-Oshima, Okinawa Islands and Yaeyama Islands. The Okinawa population could be an interbred population between the two clades. Sympatric divergence of *O. elegans* and *S. orientalis* would reflect the admixture of multiple lineages isolated over a geographical timescale (Webb *et al.* 2011; Hogner *et al.* 2012).

The Tsugaru Strait lies between Hokkaido and Honshu and divides the lineages of *S. uralensis*, *Garrulus glandarius* and *Phylloscopus borealis* into two clades (Table 2). In recent studies, *P. borealis* has been split into two species, the Kamchatka Leaf Warbler *Phylloscopus examinandus* on Hokkaido and the Japanese Leaf Warbler *Phylloscopus xanthodryas* on Honshu, based on morphological, behavioural and genetic assessments (Saitoh *et al.* 2010; Alström *et al.* 2011). *Strix uralensis* and *G. glandarius* have also been split in different subspecies in the Tsugaru Strait (Ornithological Society of Japan 2012). The Tsugaru Strait (so-called Blakiston's Line) represents one of the biogeographical boundaries that separate Japanese avifauna south of Honshu from typical Eurasian and Hokkaido avifauna (Blakiston 1883).

The Ryukyu Islands have also formed boundaries that separate some bird species and have led to deep genetic divergences. *Ficedula narcissina* is classified into two subspecies according to the breeding populations on the main islands of Japan and the Ryukyu Islands (Ornithological Society of Japan 2012). In the Narcissus Flycatcher, the *narcissina* and *owstoni* subspecies are sometimes treated as separate species, with *F. narcissina* present on the main islands of Japan and *F. owstoni* on the Ryukyu Islands (Brazil 2009). The *I. amaurotis* population from middle Ryukyu (Okinawa and Amami Islands) was formed as a single unique clade, supported by high bootstrap values, but individuals on the south Ryukyu (the Yaeyama and Miyakojima islands) belong to the same clade as those on the Japanese main islands.

Table 3 Mean genetic distance and distinguishable lineages for birds that breed in the Palearctic region including the Japanese Archipelago. We listed the bird species with robust divergent clusters with high bootstrap values (>95%). Superscripts show groups arranged in the NJ tree

Species	n	Bootstrap	Mean K2P (%) [*]	Max K2P (%)		Collection area [†]	Japan-specific lineages [‡]	Genetic split within Japan	Cypic species in relation to Japan
				Eurasia vs. Japan	Japan				
1 <i>Alauda arvensis</i>	1 ^a /6 ^b /10 ^c	- ^a /99 ^b /99 ^c	7.95 ^{a-b} , 4.80 ^{a-c}	8.38	E ^a /E, S ^b /S, H, Hn ^c	Yes		Yes	
2 <i>Delichon dasypus</i>	1 ^a /1 ^b /9 ^c	- ^a / ⁻ / ⁻	6.40 ^{a-b} , 3.85 ^{a-c}	6.4	E ^a /S ^b /Hn, H ^c	Yes	Yes	Yes	
3 <i>Caprimulgus indicus</i>	4/5	99/99	5.9	6.04	E/H, Hn	Yes		Yes	
4 <i>Phylloscopus borealis</i>	8 ^a /9 ^b /4 ^c /1 ^d	99 ^a /99 ^b /90 ^c / ⁻ / ^d	3.60 ^{a-b} , 1.78 ^{a-c} , 3.57 ^{a-d}	5.15	E ^a /S, H ^b /Hn ^c /Hn ^d	Yes	Yes	Yes§	
5 <i>Pica pica</i>	9/5	99/99	3.8	4.73	E/E, H	Yes		Yes	
6 <i>Garrulus glandarius</i>	3 ^a /12 ^b /3 ^c	99 ^a /99 ^b /99 ^c	2.72 ^{a-b} , 3.59 ^{a-c} , 4.32 ^{b-c}	4.53	E ^a /E, H ^b /Hn ^c	Yes	Yes	Yes§	
7 <i>Emberiza spodocephala</i>	8/17	99/99	3.55	3.95	E/Hn, H, S, Ss	Yes		Yes	
8 <i>Zootherna dauma</i>	2/25	99/99	3.63	3.73	A/E, Y, Og, I, Hn, H	Yes	Yes	Yes§	
9 <i>Ficedula narsissina</i>	7/12	99/99	2.88	3.71	Y, O, A/Hn, H, S	Yes	Yes	Yes§	
10 <i>Ixos amaurotis</i>	10 ^a /37 ^b /1 ^c	99 ^a /99 ^b / ⁻ / ^c	2.53 ^{a-b} , 3.19 ^{a-c}	3.57	E, Y, Mi, Og, I, Hn, H ^a /O, A ^b /Mi ^c	Yes	Yes	Yes§	
11 <i>Cettia diphone</i>	2/35	96/99	2.93	3.43	E/Y, O, A, Og, I, Hn, H, S, Ss	Yes		Yes	
12 <i>Muscicapa sibirica</i>	6/3	99/99	2.66	2.92	E/Y, Hn, S	Yes		Yes	
13 <i>Dendrocopos major</i>	4/11	99/99	2.69	2.82	E/Hn, H, S	Yes		Yes	
14 <i>Strix uralensis</i>	10/13	99/99	2.81	2.81	E, H/Hn	Yes	Yes	Yes§	
15 <i>Corvus corone</i>	7/7	85/98	2.12	2.45	E/E, Hn, H	Yes		Yes	
16 <i>Urosphena squameiceps</i>	4/5	99/99	2.1	2.21	E/Hn, H, S	Yes		Yes	
17 <i>Corvus macrorhynchos</i>	2/23	99/78	1.55	2.13	E/E, Y, O, A, Yk, Hn, H, S	Yes		Yes	
18 <i>Lanius cristatus</i>	3/1	99/ ⁻	1.91	2.01	E/Y	Yes		Yes	
19 <i>Uragus sibiricus</i>	5/12	97/99	1.49	1.82	E/Hn, H, S, Ss	Yes		Yes	
20 <i>Picus canus</i>	7/14	99/88	1.25	1.44	E/E, H	Yes		Yes	
21 <i>Luscinia calliope</i>	6/9	98/68	1.03	1.34	E/Hn, H	Yes		Yes	
22 <i>Tachybaptus ruficollis</i>	1/4	-/96	1.05	1.05	E/Y, M, A, Hn	Yes		Yes	
23 <i>Mergus merganser</i>	1/2	-/98	0.59	0.59	E/Hn	Yes		Yes	

^{*}Mean population genetic distance (K2P) between clades.

[†]E = Eurasian continent, S = Sakhalin, Ss = South Kril Is., Y = Yaeyama Is., O = Okinawa Is., M = Miyakojima I., Mi = Minami-Daitojima, A = Amami Is., Yk = Yakushima I., Og = Ogasawara Is., I = Izu Is., H = Hokkaido, and Hn = Honshu (including Kyushu and Shikoku).

[‡]Including the area of Sakhalin and S Kuril Is.

[§]Duplication of cypic species candidates in Table 2.

Hamao *et al.* (2013) also reported the complex genetic structure of the bulbuls among the Ryukyu Islands. Climate change has caused repeated emergences and disappearances of land-bridges between the Ryukyu Islands, temporarily connecting some of the islands, and even the continent. These islands biogeographically form the boundary between the Palearctic and the Oriental regions.

Four species showed large genetic differences on other islands (Table 2). For example, the sequence of *C. sinica* on the Ogasawara Islands was clearly separated from a clade clustered by other subspecies of *C. sinica* on the main islands of Japan and the continental regions. The Ogasawara Islands are oceanic islands located 1000 km south of the main islands of Japan. They contain some endemic bird species and subspecies, including some species which are now extinct (Ornithological Society of Japan 2012). *Sittiparus varius* shows deep genetic divergence between Iriomote Island and the other islands. The Iriomote population belongs to the same clade as the Taiwanese population (McKay *et al.* 2014). On Amami-Oshima, *Z. dauma* forms a separable clade to that on the Japanese islands. The Amami-Oshima *Z. dauma* is often treated as a separate species of *Zoothera major*, although this was not supported by genetic evidence (Dickinson 2003; Brazil 2009). *Erithacus komadori* is split into two clades with deep intraspecific divergence (6.13%): one clade, *E. k. namiyei*, originates from Okinawa, and the other clade, *E. k. komadori*, comes from Amami-Oshima and Yakushima Islands. Our findings closely resemble those of Seki (2006) and Seki *et al.* (2007). These deep divergences between the middle/southern Ryukyu Islands and the main islands of Japan may have occurred during the Pliocene, when the middle Ryukyu Islands finally separated from the main island (Kizaki & Oshiro 1980).

In total, 142 of 398 bird species that breed in the Japanese Archipelago and the eastern Eurasian Continent were analysed to reveal the genetic structure of trans-Japanese and Eastern Eurasian birds (Appendix S2, Supporting information). The average intraspecific distance was 0.46% (0–3.32%). Ten percentage of 234 species exhibit deep intraspecific divergence in relation to the Japanese Archipelago. This percentage is higher than for species in the Nearctic (2%) (Hebert *et al.* 2004; Kerr *et al.* 2007), Korea (1%) (Yoo *et al.* 2006), Scandinavia (1%) (Johnsen *et al.* 2010), and approximately equal to the Palearctic (10%) (Kerr *et al.* 2009b). Geographical barriers, such as mountains and oceans, have divided populations and prevented gene flow. For example, the birds inhabiting both regions of Scandinavia and the Nearctic had deep intraspecific divergence in 24% of species (Kerr *et al.* 2007) and the birds of the Nearctic and

Argentina in 24% (Kerr *et al.* 2009a). Relatively high levels of divergence were reported between Palearctic and Nearctic populations of some Holarctic birds (e.g. Drovetski *et al.* 2004; Koopman *et al.* 2005; Zink *et al.* 2006). The avifauna of the Japanese Archipelago includes many possible cryptic species, despite the differences in size and geographical history of the region.

Japan has a relatively high proportion of cryptic bird species compared with South Korea and Scandinavia, which are similar in size to Japan, and a similar proportion as Eurasia, which is geographically much larger. Sea barriers would have led to the isolation of bird populations around Japan. The repeated southward spread of the ice sheets to 40°N in North America during the quaternary (Hewitt 2004) would have caused shallow genetic divergence (Ball & Avise 1992; Seutin *et al.* 1995; Zink 1996; Weir & Schluter 2004) and low genetic diversity (Hewitt 1996; Soltis *et al.* 1997; Conroy & Cook 2000) among the various taxa in the northern regions, while the ice sheets that were limited to 50°N in the east Palearctic region allowed bird populations to speciate. The southward spread of ice did not affect the Japanese Archipelago equally, and some areas would have been linked to the Eurasian Continental by land-bridges. The fluctuating sea levels during the quaternary would have led to repeated periods of connection, isolation and submergence of the islands of the Japanese Archipelago, which could have isolated bird populations for lengthy periods. Such populations would have retained geographical and temporal isolation without admixture of other populations and extinction for glacial and interglacial periods. These isolated populations may lead to the deep intraspecific genetic divergence observed in 11 Japanese bird species. The distinct intraspecific divergence observed between 23 trans-Palearctic species may reflect the history of isolation and gene flow of these bird populations with respect to the changes in the land-bridges over the straits between the Japanese Archipelago and the Eurasian continent.

In this study, we constructed a barcoding library comprising 93.2% of Japanese-breeding bird species and demonstrated an effective tool for the identification of these bird species. The relatively deep genetic divergence was related to the periodic occurrence and disappearance of sea barriers. We found 24 cryptic species candidates in this study, which suggests that traditional taxonomic methods for identification of East Asian birds do not fully reflect the divergences. For example, *P. borealis* has been split into three species in recent studies (Saitoh *et al.* 2010; Alström *et al.* 2011). Our study contributes additional Japanese bird data to the global DNA barcoding library and provides valuable data for future investigation of the taxonomy of East Asian birds.

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T.S. collected the specimens, analysed the data and prepared the draft. N.S. provided the sequences, analysed the data and wrote the manuscript. S.S. performed the genetic analysis. Y.I. and S.K. collected the specimens. H.K., A.H., S.A. and Y.Y. provided many of the sequences and organized the sequence data. I.N. designed and coordinated the project.

Data accessibility

DNA sequences: DDBJ accessions AB842491–AB843857. Sequence alignment and final neighbour-joining tree uploaded as Online Supporting Information. BOLD project BJNSM and YIO, accessions BJNSM001-08–BJNSM842-12 and YIO001-08–YIO558-12, respectively.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 List of Japanese-breeding bird species analysed in this study.

Appendix S2 List of the bird species analysed in this study (breeding in both Eurasia and the Japanese Archipelago).

Appendix S3 A K2P neighbour-joining tree of COI made with 1367 voucher specimens from 234 species breeding in the Japanese Archipelago.