Geographical Variation in Mitochondrial DNA and Vocalizations in Two Resident Bird Species in the Ryukyu Archipelago, Japan

Shoji Hamao, Norimasa Sugita and Isao Nishiumi

Department of Zoology, National Museum of Nature and Science,
4–1–1 Amakubo, Tsukuba, Ibaraki, 305–0005 Japan
E-mail: hamao@kahaku.go.jp

Abstract   We investigated the geographic variation in mitochondrial DNA and vocalizations of two bird species, the brown-eared bulbul, *Hypsipetes amaurotis*, and the Japanese white-eye, *Zosterops japonicus*, among the islands of the Ryukyu Archipelago. The islands (except for the Daito Islands) contain five bulbul and two white-eye subspecies. DNA sequences of the bulbuls indicated a large variation among the islands, but the bulbuls on each island did not have specific songs, indicating that similar songs are sung by genetically diverged birds. The white-eyes displayed only minor genetic divergence among the islands. We found that the two white-eye subspecies had similar haplotypes, showing an inconsistency between taxonomy and genetic results. We also found that the white-eyes on the northern islands sang songs at lower pitch than those on the other islands despite there being no genetic divergence between them. This finding suggests that songs change more rapidly than genetic structure in a population. A difference in songs between populations is a potential force for reproductive isolation. Therefore, research on the geographic variation among birds in island areas requires comprehensive studies of morphology, genetics, and songs.

Key words : Brown-eared bulbul, COI, DNA barcoding region, genetic divergence, *Hypsipetes amaurotis*, Japanese white-eye, song, *Zosterops japonicus*.

Introduction

Molecular analyses have contributed to current avian taxonomy, for example, the discovery of cryptic species (Saitoh *et al.*, 2010; Alström *et al.*, 2011) and the construction of accurate phylogenies (Hackett *et al.*, 2008). Molecular studies have also contributed to an understanding of genetic structure in populations of a species, and have further determined evolutionarily significant units for conservation (Moritz, 1994).

Interspecific genetic distance between sister species in cytochrome c oxidase (COI) is more than 2% in most North American birds, and thus a criterion of approximately 2% is usually the value used to separate species (Hebert *et al.*, 2004). However, this criterion does not apply for many species in the eastern part of Asia. For example, in thrushes in East Asia, 0.7% of sequences differ between the eyebrowed thrush, *Turdus obscurus*, the pale thrush, *T. pallidus*. In addition the brown-headed thrush, *T. chrysolaus*, has only 0.15% (1 among 680 bp) sequence difference with the Izu thrush, *T. celaenops*, an island allospecies of the former (T. Saitoh *et al.*, in preparation). The variation in habitats among the islands of Japan provides a variety of selection pressures on avian populations, which is likely to accelerate speciation. Therefore, in this area, sequence variation may not be directly applicable for determining species boundaries.

Although many biologists have proposed various definitions of species with accordance to species concepts (de Queiroz, 1998, 2005), reproductive isolation is considered to be a key factor causing speciation (Mayr, 1942; Nishiumi,

2012). Birdsongs have an important species recognition role in reproductive behavior (Catchpole and Slater, 1995; Kroodsma and Miller, 1996). In closely related species that have a similar morphology, each species is often determined from acoustic differences in their songs (Hamao *et al.*, 2008; Alström *et al.*, 2011), and the responses to the playback of songs of allopatric populations (Irwin *et al.*, 2001; Hamao *et al.*, 2006). However, because songs change adaptively in response to the ecological and social environment of the populations, differences in songs produce reproductive barriers and can be a potential cause of speciation (Irwin *et al.*, 2001). Therefore, studies of the geographic variations in songs in a species are important to understand the distribution of species, evolutionarily significant units, and mechanisms of speciation.

The Ryukyu Archipelago (Nansei Islands) is a chain of continental islands in southern Japan, stretching southwest from Kyushu to Taiwan (24°02′–30°49′N, 122°56′–131°17′E). These islands have many endemic species owing to the long period of isolation created by the sea barrier. Despite their high flight ability, the bird populations of the islands also contain many endemic species and subspecies (Ornithological Society of Japan, 2012). The brown-eared bulbul, *Hypsipetes amaurotis*, and the Japanese white-eye, *Zosterops japonicus*, are the resident passerine birds in the Ryukyu Archipelago, although migratory populations also visit these islands in winter. Among the breeding birds on the islands, there are six subspecies of brown-eared bulbul and three subspecies of Japanese white-eye, as determined by their morphology (Fig. 1). A species with a large geographic variation across widely spaced islands is well suited to the investigation of genetic and acoustic variation. The purpose of this study was to describe geographical variation in mitochondrial DNA and vocalizations in the brown-eared bulbul and the Japanese white-eye in the Ryukyu Archipelago. We also discuss the implications of the taxonomy of subspecies and evolutionarily significant units of the species.

Materials and Methods

Sample collection. The variation in mitochondrial DNA and vocalization of brown-eared bulbuls was studied on six islands of the Ryukyu Archipelago: Yakushima, Tanegashima, Amami-Ohshima, Okinawajima, Ishigakijima, and Yohagunijima, which are occupied by five subspecies (Fig. 1a). We also studied Japanese white-eyes on similar islands [Kikaijima was chosen instead of Tanegashima because white-eyes are scarcely observed on Tanegashima (Hamao, personal observation)] (Fig. 1b). Two subspecies of the Japanese white-eye are distributed across the study islands.

We collected DNA samples from 33 brown-eared bulbuls and 47 Japanese white-eyes in their breeding season (April to August) from 2002 to 2011. Although migratory bulbuls and white-eyes inhabited the study sites in winter, no birds belonging to wintering subspecies that could be identified by differences in plumage color (Kiyosu, 1965; Ornithological Society of Japan, 2012) were observed in the study periods. We also confirmed that the DNA-sampled birds belonged to the resident subspecies according to their morphology. The average number of birds sampled was 6.7 (range: 1–16) from each species on each island. For most birds blood samples were taken and placed in Queens' lysis buffer (Seutin *et al.*, 1991), but in some birds breast muscle was taken and placed in pure ethanol.

We also collected recordings of songs during the early breeding season (April to May) in 2008 and 2009 on each study island. Because no bulbuls or white-eyes belonging to wintering subspecies were observed in the study periods, our recordings were assumed to be obtained from resident birds. Brown-eared bulbuls produce several kinds of vocalizations, and typical songs have not been identified. However, breeding territories can be determined using a single uttering bird (Tateno, 2006; Yamaguchi and Saito, 2009). We collected recordings of repeated loud vocalizations from solitary birds in the breeding seasons (Fig. 2a), which were similar to the sounds

(a)

amaurotis

Tanegashima
Yakushima

Ishigakijima

ogawae

Amami-Ohshima

Yonagunijima

borodinonis

pryeri

Okinawajima

nagamichii

stejnegeri

(b)

japonicus

insularis

Yakushima

loochooensis

Kikaijima

Amami-Ohshima

Ishigakijima
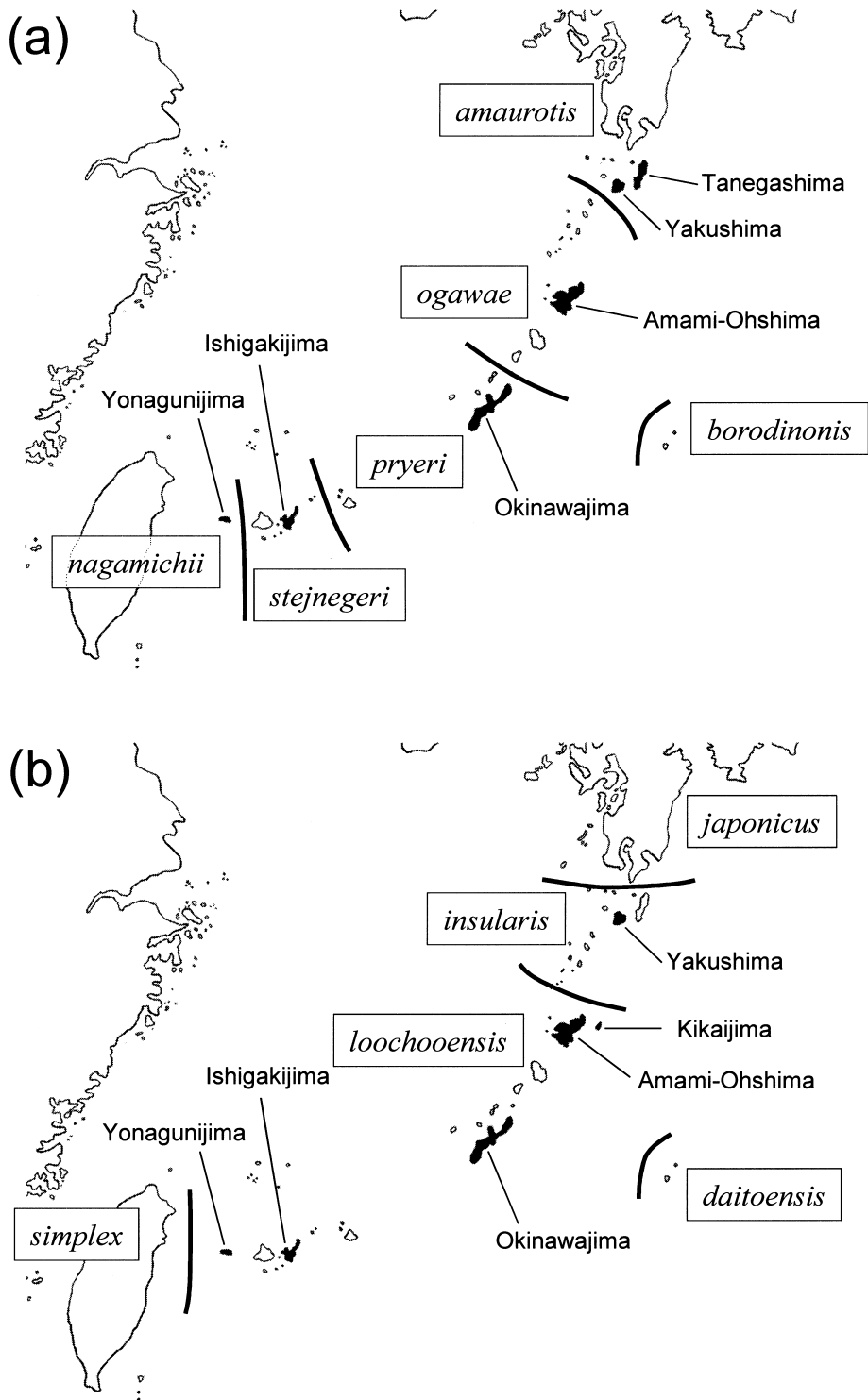
Yonagunijima

daitoensis

Okinawajima

simplex

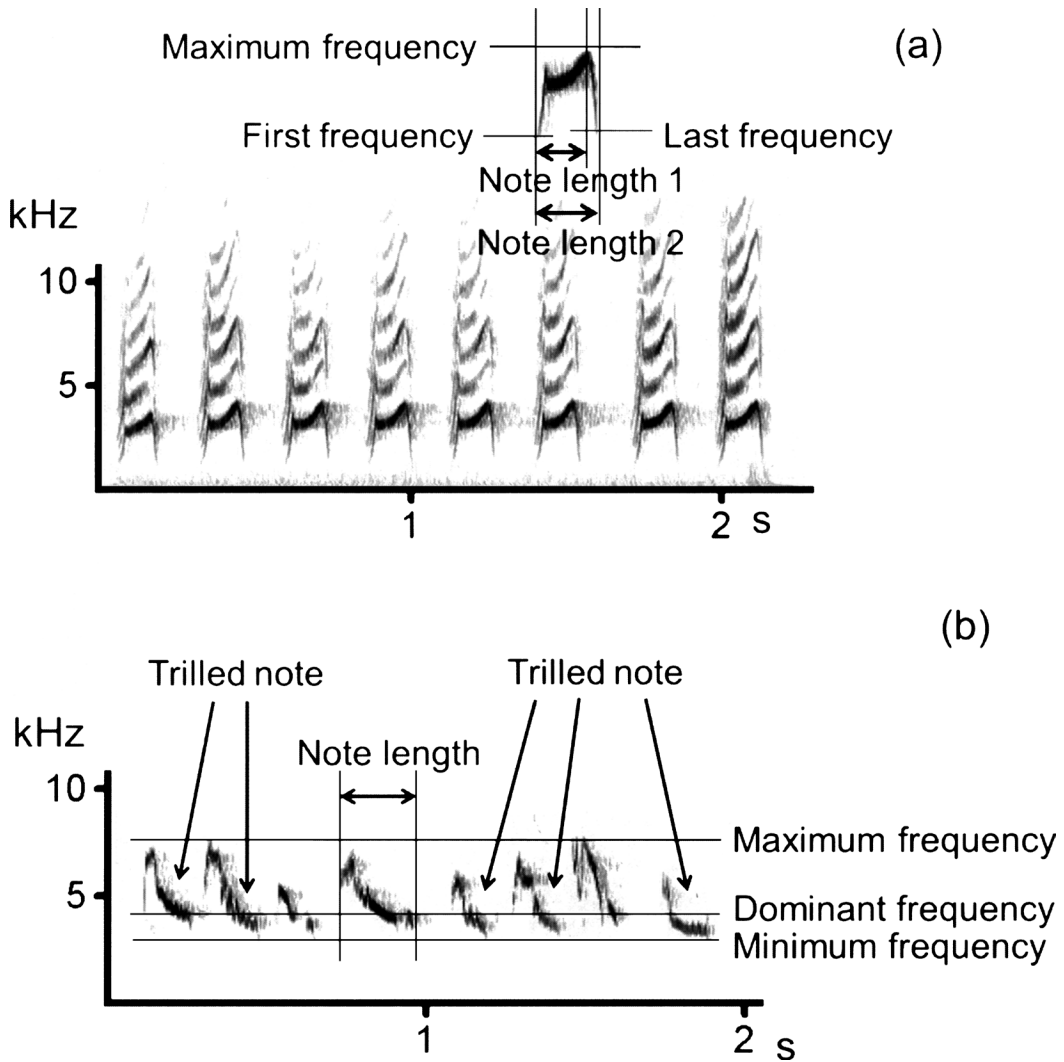Fig. 1. Study sites and distribution of subspecies of (a) the brown-eared bulbul and (b) the Japanese white-eye.

Fig. 2.   Spectrograms of the songs showing the parameters analyzed. — (a) the brown-eared bulbul and (b) the Japanese white-eye. The dominant frequency is the frequency at the highest sound pressure level in the song.

shown in the figures by Tateno (2006). Because the vocalizations may be connected with the reproductive behavior of the bulbuls, we regarded the vocalization as a song. In both species, songs from a singing bird were recorded for at least 3 min. We used a digital recorder (PCM-D1: Sony, Tokyo, Japan) equipped with a directional microphone (ECM-G3M: Sony). We obtained recordings of bulbul songs from 14 males on Yakushima, 22 on Tanegashima, 18 on Amami-Ohshima, 30 on Okinawajima, 43 on Ishigakijima, and 23 on Yonagunijima. We also collected recordings of white-eye songs from 37 males on Yakushima, 40 on Kikaijima, 36 on Amami-Ohshima, 41 on Okinawajima, 22 on Ishigakijima, and 5 on Yonagunijima. Although we did not individually mark the birds, we avoided duplicate sampling of the same males by walking along roads and recording new birds when we encountered them. Each route was used only once.

Genetic analysis. DNA was extracted from tissue samples using the standard phenol/chloroform procedure in a laboratory. A set of newly

designed primers modified from Hebert *et al.* (2004), L6697Bird (5′-TCAACYAACCACA-AAGAYATCGGYAC-3′) and H7390Thrush (5′-ACGTGGGARATRATTCCAAATCCTG-3′), was used in the PCR amplification of the COI gene. The 25 µl PCR reaction mixes contained 19.2 µl of ultrapure water, 1.0 U of Taq polymerase (TaKaRa Ex Taq, Takara), 2.5 µl of PCR buffer, 0.3 µl of each primer (0.24 mM), 2.5 µl of each dNTP (2.5 mM), and 0.4–1.0 µl of DNA (ca. 20 ng/µl). The amplification regime was 94°C for 3 min followed by five cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min, followed in turn by 30 cycles of 94°C for 30 sec, 51°C for 30 sec, 72°C for 1 min, and finally 72°C for 5 min. PCR products of the target size (ca. 700 bp) were visualized in a 1.5% agarose gel. PCR products were purified using ExoSAP-IT (Amersham Bioscience, Amersham, UK) to remove the remaining primers and other short fragments. Sequencing reactions were carried out using BigDye v1.1 and analyzed on an ABI 3130 sequencer (Applied Biosystems, California, USA). The electropherogram (Trace files) and sequences for each specimen were deposited in the "Birds of Japan" (NSMT) at the Barcode of Life Database (BOLD) (http://www.boldsystems. org/) or in GenBank (Accession Numbers: AB765881-AB765907). Phylogenetic network estimation using statistical parsimony was done by TCS ver.1.21 (Clement *et al.*, 2000). Genetic distance in percentage was calculated by the Kimura 2 Parameter method using BOLD Systems.

Song analysis. All recorded sounds were digitized (16-bit resolution and 32-kHz sample rate) and analyzed using a computer-based analysis system (Avisoft-SASLab Pro, version 5.1: Avisoft Bioacoustics, Berlin, Germany). Sounds were displayed as sound spectrograms. Fast Fourier transform (FFT) lengths of 256 and 1024 were used to produce plots for temporal and frequency measurement, respectively. We selected one clear song from each recording, and obtained the following measurements from the song.

In brown-eared bulbuls, we measured the length of a song (duration) and the number of notes in the song. As the bulbul songs contained similar sound elements (i.e., notes), we selected a clear note that had the highest sound pressure in the song and measured the length of the increasing frequency part (note length 1), the length of the note (note length 2), the frequency of the initial part (first frequency), the highest frequency (maximum frequency), and the frequency of the end part (last frequency; Fig. 2a). A note is a discrete song element (Catchpole, 1980; Baker, 1996) and was defined as any continuous trace on a spectrogram. In Japanese white-eyes, we measured the song length (duration), the number of notes, and the number of trilled notes in the song. A trilled note is a note with repeated vibrations (the length of one vibration is less than 0.05 s). We also obtained the maximum frequency in the song, the minimum frequency in the song, the frequency at the highest sound pressure level (dominant frequency) in the song, and the length of the longest note (maximum note length; Fig. 2b).

To compare acoustic variables of the songs among the six study sites, we used generalized linear models (GLMs). The number of notes and trilled notes were analyzed using a GLM with a log-link and Poisson error distribution. The other measurements, i.e., duration and frequency variables, were analyzed using a GLM with an identity-link and Gaussian error distribution. To test for significance, we compared the song variables between two study sites (all combinations among the six sites) using likelihood ratio tests with sequential Bonferroni correction (Rice, 1989). We report P values that have been corrected by the Bonferroni methods. In all analyses, the significance levels were set at 0.05, and analyses were conducted using R software (version 2.15.1; R Core Team, 2012).

Results

Genetic variation. A part of the COI gene sequences (591 bp) for brown-eared bulbuls displayed large variations among the study sites. In

the two northern islands of the Ryukyu Archipelago, a single haplotype was shared with all individuals on Tanegashima and a unique haplotype was found in one individual on Yakushima, which differed from each other by five substitutions despite the close geographic distance. The sequences on the southern islands (i.e., Ishigakijima and Yonagunijima) were linked to the Yakushima haplotype by a minimum of four substitutions. The populations on the central islands (i.e., Okinawajima and Amami-Ohshima), especially Amami-Ohshima, diverged substantially from the Ishigakijima population by at least 14 substitutions (2.30% of the sequence), except for one individual on Okinawajima (Fig. 3a). Thus, the brown-eared bulbul populations in the
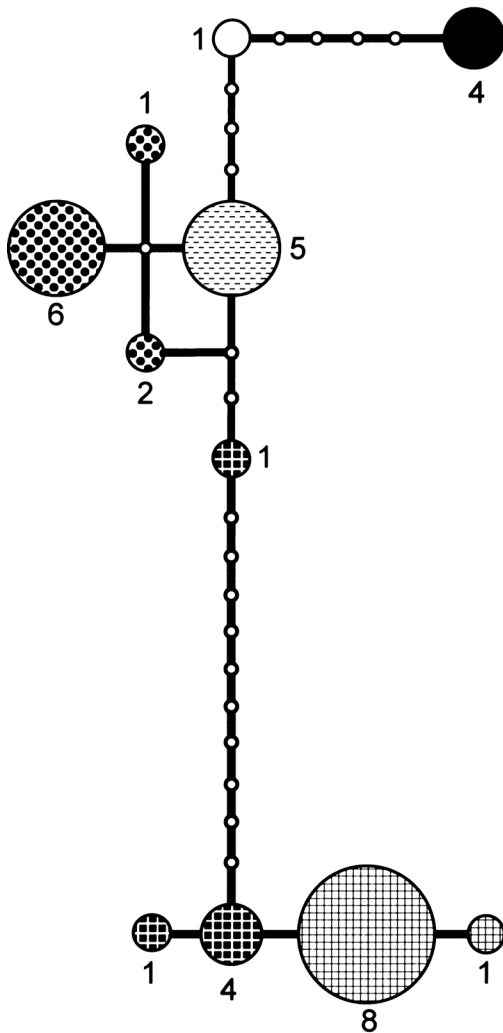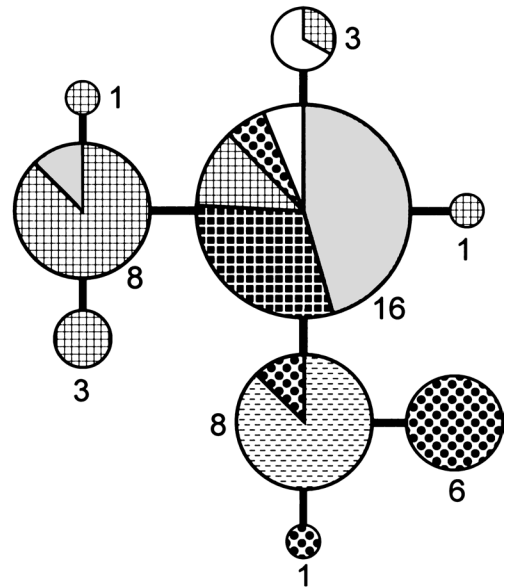
Fig. 3. Genetic networks of COI haplotypes in (a) the brown-eared bulbul and (b) the Japanese white-eye. Small white circles in (a) indicate putative extinct (or unobserved) haplotypes connecting the observed haplotypes. Circle sizes and attendant numerals show the number of individuals.

Ryukyu Archipelago displayed large variations, with the populations in the central Ryukyu Archipelago being substantially diverged from the others.

The results of the sequence analysis on a part of the COI gene (534 bp) indicated that the divergence of Japanese white-eyes was much smaller than that observed for the bulbuls. The populations in the southern islands (i.e., Ishigakijima and Yonagunijima) were narrowly diverged from the other islands (i.e., Yakushima, Kikaijima, Amami-Ohshima, and Okinawajima). However, the closest haplotypes were linked by only one substitution, and one individual on Yonagunijima had the same haplotypes as the birds on the four northern and central islands (Fig. 3b). Thus, the Japanese white-eye populations in the Ryukyu Archipelago varied only slightly, but populations in the southern islands were likely diverged from the other populations.

Vocal variation. In brown-eared bulbuls, the duration of songs was shorter on Yakushima and Tanegashima than on Okinawajima, Ishigakijima, and Yonagunijima (Fig. 4a). This was related to the songs on Yakushima, Tanegashima, and Amami-Ohshima having fewer notes than the songs on other islands (Fig. 4b). The length of each note did not clearly differ among populations, but note length 1 on Ishigakijima was slightly longer than the notes on other islands (Fig. 4c, d). Frequencies, especially the first and last frequencies of the notes, were lower in the populations on Ishigakijima and Yonagunijima (Fig. 4e–g).

In Japanese white-eyes, song duration was slightly shorter on Ishigakijima than on other islands (Fig. 5a). This was a consequence of the songs having fewer notes on Ishigakijima (Fig. 5b); note length did not differ among the populations (Fig. 5d). The number of trilled notes was also smaller on Ishigakijima (Fig. 5c). The maximum frequencies of songs were lower on Yakushima and Kikaijima (Fig. 5e), but the minimum or dominant frequencies were not obviously different among the islands (Fig. 5f, g).

Discussion

The brown-eared bulbul. This species displayed enormous genetic divergence among the populations in the Ryukyu Archipelago (Fig. 3a). All birds sampled on the central islands (except for one individual) were substantially diverged from those of the northern and southern islands by at least 2.3%, i.e., more than the 2% frequently used as the genetic criterion for a species boundary (Hebert *et al.*, 2004). Each bulbul population did not share the same haplotype among islands. Therefore, the populations must have been isolated for long periods and have attained clear genetic differentiation, suggesting that the populations can be considered as evolutionarily significant units. If the differences in mitochondrial DNA among subspecies fitted with the differences in morphological traits, each subspecies should not have distinct haplotypes. However, the populations on Yakushima and Tanegashima displayed relatively large divergence despite belonging to the same subspecies, *H. a. amaurotis*.

The songs of the bulbuls had lower frequencies on the southern islands, Ishigakijima and Yonagunijima (Fig. 4e–f). Body size affects the frequency of vocalization, as smaller birds produce songs with higher frequencies (Ryan and Brenowitz, 1985; Hamao *et al.*, 2008). However, the body sizes of the bulbuls do not explain the differences in the frequency of their songs because the size of the birds on the different islands did not differ (Kiyosu, 1965; Nishiumi, unpublished data). Because birds inhabiting forests with dense foliage use pure tone-like, low-frequency sounds that attenuate less in their habitats (Morton, 1975; Richard and Wiley, 1980), the low frequency of songs might be an adaptation to the sound transmission properties on the southern islands. Bulbul songs had a smaller number of notes and a shorter duration on the northern islands (Fig. 4a–b). If the sound transmission conditions are not good on the southern islands, it is plausible that the bulbuls on these islands have developed longer songs with more notes. However, the factors responsible for the
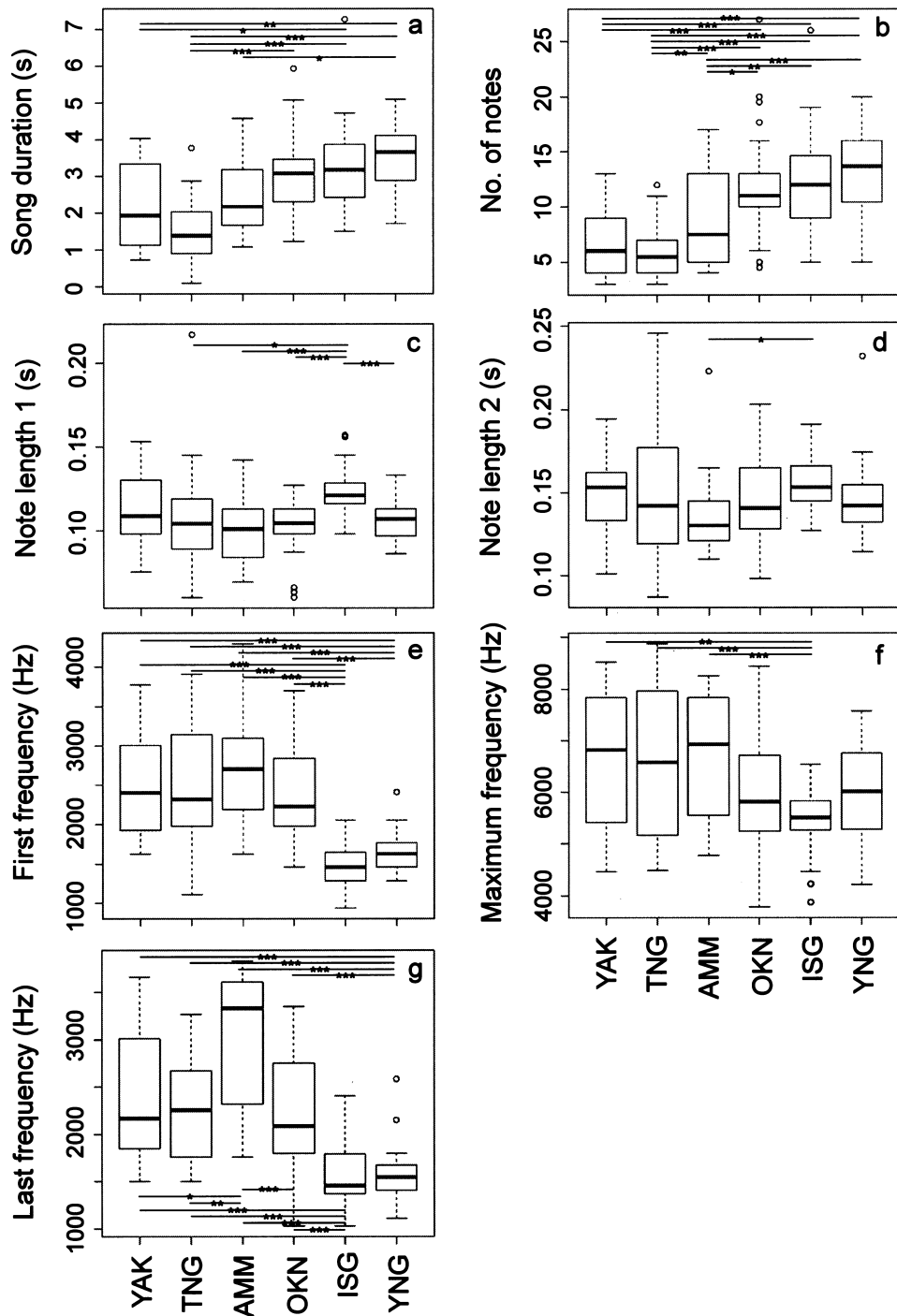
Fig. 4. Differences in the song structure of the brown-eared bulbul among six islands in the Ryukyu Archipelago. — (a) duration, (b) number of notes, (c) note length 1, (d) note length 2, (e) first frequency, (f) maximum frequency, (g) last frequency. See details in the Methods section. Box plots show the 25th and 75th percentiles (boxes), medians (thick lines within boxes), and ±1.5 interquartile ranges (whiskers). Outlying points are shown as open circles. Horizontal lines indicate significant differences between two populations. *P* values (***P＜0.001, **P＜0.01, *P＜0.05) have been adjusted by Bonferroni correction.
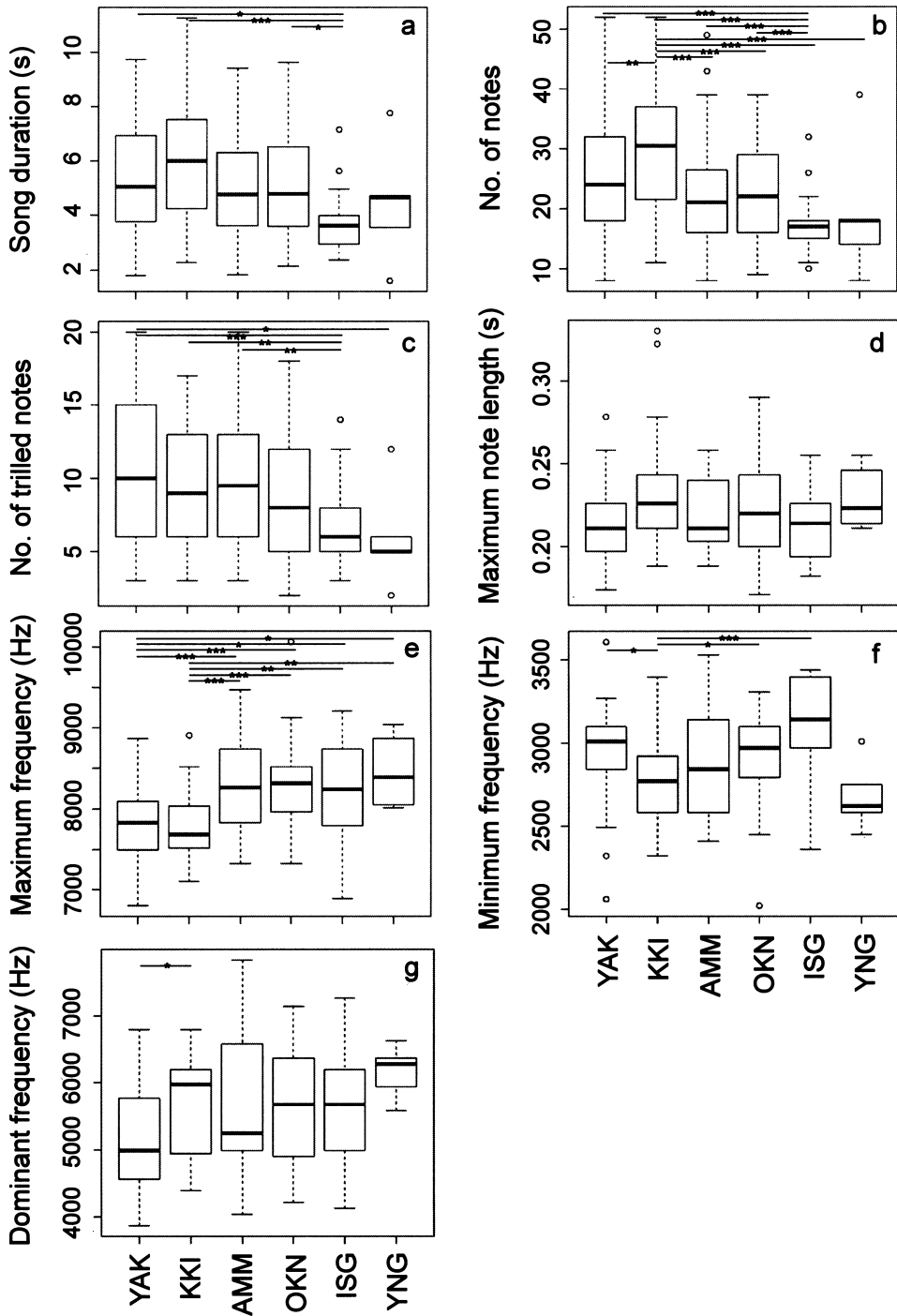
Fig. 5. Differences in the song structure of the Japanese white-eye among six islands in the Ryukyu Archipelago. — (a) duration, (b) number of notes, (c) note of trilled notes, (d) note length, (e) maximum frequency, (f) minimum frequency, (g) dominant frequency. See details in the Methods section. Also, see Fig. 4 for box plots and horizontal lines.

acoustic characteristics of bulbul songs are unclear because songs have been formed not only in response to the sound transmission properties of the habitat but also due to the existence of competitive species (Doutrelant and Lambrechts, 2001; Grant and Grant, 2010) and sexual selection pressure in each species (Hamao and Ueda, 2000; Hamao, 2013). These factors have not been investigated on the study islands.

The Japanese white-eye. In contrast to the brown-eared bulbul, this species displayed only slight genetic divergence among the islands in the Ryukyu Archipelago (Fig. 3b). Although the southern populations on Ishigakijima and Yonagunijima were narrowly diverged from other populations, all island populations shared haplotypes with other island populations. Therefore, an evolutionarily significant unit was not determined in the white-eyes. In the studied populations, birds on Yakushima belonged to the two subspecies *Z. j. insularis* and *Z. j. loochooensis*. However, the Yakushima population did not show genetically unique characteristics. The most common haplotype that was found from the five island populations is also present on the Japanese mainland and the Izu Islands (Nishiumi, unpublished data), where another subspecies, *Z. j. japonicus*, is distributed. Thus, the Japanese white-eye displays little genetic divergence in Japan.

In the white-eyes, the songs produced on Ishigakijima had a shorter duration and a smaller number of notes. Songs on Yonagunijima showed a similar tendency, although statistically significant effects were not found because of the small sample size (five males; Fig. 5a–c). These acoustic features on the southern islands are consistent with populations that have genetically diverged from other populations, although the factor that produced the shorter songs is unclear. The white-eye songs on Yakushima and Kikaijima also had lower maximum frequencies. The haplotypes found on these islands were shared with birds on other islands. This indicates that birds with similar mitochondrial DNA can produce different songs.

Songs and population structure. In the present study, we compared genetic and behavioral differences of bulbuls and white-eyes among islands in the Ryukyu Archipelago. Furthermore, we compared the differences in these traits with differences in morphology (i.e., subspecies). Genetically diverged populations are expected to display different behavior and morphology because they have been isolated for a long time. We also identified two examples of genetically similar populations having different songs and morphology. First was the Japanese white-eye, which was divided into two subspecies (i.e., *insularis* on Yakushima and *loochooensis* on other islands), although they were not genetically diverged from each other. To understand this inconsistency, more detailed investigations of both their morphology and DNA are required. The second and more important finding was that Japanese white-eyes on Yakushima and Kikaijima produced songs with a lower pitch than those of birds on other islands, although they were not genetically diverged from other populations. This suggests that songs can change more rapidly than the genetic structure in a population. The populations on Yakushima and Kikaijima may have been established fairly recently as they had a restricted number of haplotypes (Fig. 3b). Because songbirds acquire their specific song patterns by social learning (Kroodsma and Miller, 1996), songs can change without genetic change. Therefore, songs potentially change more rapidly than genetic structure in a population. Furthermore, the divergence of songs between populations will accelerate reproductive isolation (Irwin *et al.*, 2001), which will cause genetic divergence.

The pattern of geographic variation in the mitochondrial DNA differed between the brown-eared bulbul and the Japanese white-eye. Bulbuls displayed a large divergence among the islands, but white-eyes displayed only one small divergence. Factors that caused these differences were equivocal, but the bulbul young, for example, might have a very small distance in their natal dispersal, which has resulted in an accumulation of mutation on a particular island. The white-

eyes might have experienced a bottle neck in the past, which has resulted in minimal genetic diversity. The ecology and history of each species would affect the pattern of geographic variation.

Birds can move for long distances owing to their flight ability. Their population structure cannot be simply understood from geographic barriers. Moreover, culturally transmitted songs are related to their reproductive isolation. Because changes in songs do not require genetic changes (Kroodsma and Miller, 1996), the variation in songs does not always coincide with population structure. Thus, research on the geographic variation in birds in island areas requires comprehensive studies of morphology, genetics, and songs.

Acknowledgments

References

Alström, P., T. Saitoh, D. Williams, I. Nishiumi, Y. Shigeta, K. Ueda, M. Irestedt, M. Björklund and U. Olsson 2011. The arctic warbler *Phylloscopus borealis* — three anciently separated cryptic species revealed. Ibis, 153: 395–410.

Baker, M. C. 1996. Depauperate meme pool of vocal signals in an island population of singing honeyeaters. Animal Behaviour, 51: 853–858.

Catchpole, C. K. 1980. Sexual selection and the evolution of complex songs among European warblers of the genus *Acrocephalus*. Behaviour, 74: 149–165.

Catchpole, C. K. and P. B. Slater 1995. Bird song: biological themes and variations. 248 pp. Cambridge University Press, Cambridge.

Clement M., D. Posada and K. Crandall 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology, 9: 1657–1660.

de Queiroz, K. 1998. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In D. J. Howard and S. H. Berlocher (eds.): Endless forms: species and speciation, pp. 57–75. Oxford University Press, Oxford.

de Queiroz, K. 2005. Ernst Mayr and the modern concept of species. Proceedings of the National Academy of Sciences, USA, 102: 6600–6607.

Doutrelant, C. and M. M. Lambrechts 2001. Macrogeographic variation in song — a test of competition and habitat effects in blue tits. Ethology, 107: 533–544.

Grant, B.R. and P. R. Grant 2010. Songs of Darwin's finches diverge when a new species enters the community. Proceedings of the National Academy of Sciences, USA, 107: 20156–20163.

Hackett, S. J., R. T. Kimball, S. Reddy, R. C. K. Bowie, E. L. Braun, M. J. Braun, J. L. Chojnowski, W. A. Cox, K-L Han, J. Harshman, C. J. Huddleston, B. D. Marks, K. J. Miglia, W. S. Moore, F. H. Sheldon, D. W. Steadman, C. C. Witt and T. Yuri 2008. A phylogenomic study of birds reveals their evolutionary history. Science, 320: 1763–1768.

Hamao, S. 2013. Acoustic structure of songs in island populations of the Japanese bush warbler, *Cettia diphone*, in relation to sexual selection. Journal of Ethology, 31: 9–15.

Hamao, S. and K. Ueda 2000. Simplified song in an island population of the bush warbler *Cettia diphone*. Journal of Ethology, 18: 53–57.

Hamao, S., M. J. S. Veluz and I. Nishiumi 2006. Species recognition by song between the Luzon Bush Warbler, *Cettia seebohmi*, and the Japanese Bush Warbler, *C. diphone*. Memoirs of the National Science Museum, (44): 21–29.

Hamao, S., M. J. S. Veluz, T. Saitoh and I. Nishiumi 2008. Phylogenetic relationship and song differences between closely related Bush Warblers *Cettia seebohmi* and *C. diphone*. Wilson Journal of Ornithology, 120: 268–276.

Hebert P. D. N., M. Y. Stoeckle, T. S. Zemlak and C. M. Francis 2004. Identification of birds through DNA barcodes. PLoS Biology, 2(10): e312.

Irwin, D. E., S. Bensch and T. D. Price 2001. Speciation in a ring. Nature, 409: 333–337.

Kiyosu, Y. 1965. The birds of Japan I. 444 pp. Kodansya,

Tokyo. (In Japanese.)

Kroodsma, D. E. and E. H. Miller (eds.) 1996. Ecology and evolution of acoustic communication in birds. 587 pp. Cornell University Press, New York.

Mayr, E. 1942. Systematics and the origin of species. 334 pp. Dover, New York.

Moritz, C. 1994. Defining 'evolutionarily significant units' for conservation. Trends in Ecology and Evolution, 9: 373–375.

Morton, E. S. 1975. Ecological sources of selection on avian sounds. American Naturalist, 109: 17–34.

Nishiumi, I. 2012. DNA barcoding and species classification of Japanese birds. Japanese Journal of Ornithology, 61: 223–237. (In Japanese.)

Ornithological Society of Japan 2012. Check-list of Japanese birds, 7th revised edition. 438 pp. Ornithological Society of Japan, Sanda.

R Core Team 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051–07–0, URL http://www.R-project.org/.

Richard, D. G. and R. H. Wiley 1980. Reverberations and amplitude fluctuations in the propagation of sound in a forest: implications for animal communication. American Naturalist, 115: 381–399.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution, 43: 223–225.

Ryan, M. J. and E. A. Brenowitz 1985. The role of body size, phylogeny, and ambient noise in the evolution of bird song. American Naturalist, 126: 87–100.

Saitoh T., P. Alström, I. Nishiumi, Y. Shigeta, D. Williams, U. Olsson and K. Ueda 2010. Old divergences in a boreal bird supports long-term survival through the Ice Ages. BMC Evolutionary Biology, 10: 35.

Seutin G., B. N. White and P. T. Boag 1991. Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology, 69: 82–90.

Tateno, K. 2006. Estimation of breeding territories of brown-eared bulbuls by the territory mapping method. Urban Birds, 23: 41–49. (In Japanese.)

Yamaguchi, Y. and M. Saito 2009. Estimation of the breeding density of Brown-eared Bulbuls *Hypsipetes amaurotis* in southern Ibaraki Prefecture. Japanese Journal of Ornithology, 58: 179–186.