ELSEVIER

Short communication

The origin of the East Asian *Erithacus* robin, *Erithacus komadori*, inferred from cytochrome *b* sequence data

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1. Introduction

The avian genus *Erithacus* is a member of the chat tribe, Saxicolini, which is grouped in the Old World flycatcher family, Muscicapidae (Sibley and Ahlquist, 1990; Voelker and Spellman, 2004). The genus presently comprises three species according to Sibley and Monroe (1990): *E. rubecula*, *E. akahige*, and *E. komadori*. *Erithacus rubecula* occurs in the western palearctic, from humid lowlands, wooded mountains to treeline, and breeds in various types of forests, parks and gardens with trees, and shrubs (Cramp, 1988). Contrary to this, the other two species are endemic to East Asia (Fig. 1). *Erithacus akahige* breeds in Sakhalin, the four main Japanese islands, and some other adjacent islands. Its preferred breeding habitat is coniferous and mixed forest in boreal montane and cool-temperate zones, except for some small islands in the southern part of Japan, where it breeds in evergreen broad-leaved forests (Kiyosu, 1965). *Erithacus komadori* breeds in lowland evergreen broad-leaved forests on small to middle-sized islands in the Ryukyu and Danjo Islands, located in the southwestern part of Japan (Kawaji and Higuchi, 1989).

The distribution of *E. komadori* is especially restricted among these three species (Kiyosu, 1965; Ornithological Society of Japan, 2000), and this species has been designated as a "national natural treasure" and "national endangered species of wild fauna and flora" of Japan. Two subspecies are described in its narrow distribution, *E. k. komadori* in the northern Ryukyu Islands and *E. k. namiyei* in the central Ryukyu Islands (Kawaji and Higuchi, 1989). The populations of both subspecies are recently declining in their major habitats, mainly because of the introduced predators (Kinjou et al., 2003; Sugimura et al., 2003), and the

two subspecies are, hence, also listed as vulnerable species in the Japanese Red List (Ministry of the Environment of Japan, 2002). To make an effective conservational decision about *E. komadori*, there must be an understanding of its present distribution, in relation to its origin and diversification history of subspecies.

The phylogenetic relationship among the *Erithacus* robins, however, is still a question under debate (Kajita, 1999; Ornithological Society of Japan, 2000). Some morphological similarity between *E. rubecula* and *E. akahige*, particularly the close similarity of feather coloration (Meinertzhagen, 1951), appears to be the main reason why the East Asian *Erithacus* robins and *E. rubecula* have been classified into the same genus by most authors (e.g., Cramp, 1988; Ornithological Society of Japan, 2000; Sibley and Monroe, 1990). On the other hand, some other authors classified the East Asian *Erithacus* robins into the genus *Luscinia*, leaving *E. rubecula* as a single species for the genus *Erithacus*, because their feather texture and the form of their tails are different from those of *E. rubecula*, and more closely resemble those of a certain group of *Luscinia* in East Asia, such as *L. cyane* and *L. sibilans* (Kiyosu, 1965; Ornithological Society of Japan, 1975; Yamashina, 1941). The genus *Erithacus* itself sometimes merged with *Luscinia* and *Tarsiger* in the past (reviewed in Cramp, 1988), but recent molecular studies revealed that western palearctic *Luscinia* forms a genetically distinguishable clade from *Erithacus* (Sibley and Ahlquist, 1990; Wink et al., 2002). To which group do the East Asian *Erithacus* robins belong? Is the discontinuous distribution of these similar looking robins a result of the wide distribution of a common ancestor, or just the convergence of feather coloration?

This paper is the first in a series of studies on the phylogeography of the *E. komadori*. The aims of this study were: (1) to reveal the molecular phylogenetic relationships between the three species of *Erithacus* robins and some

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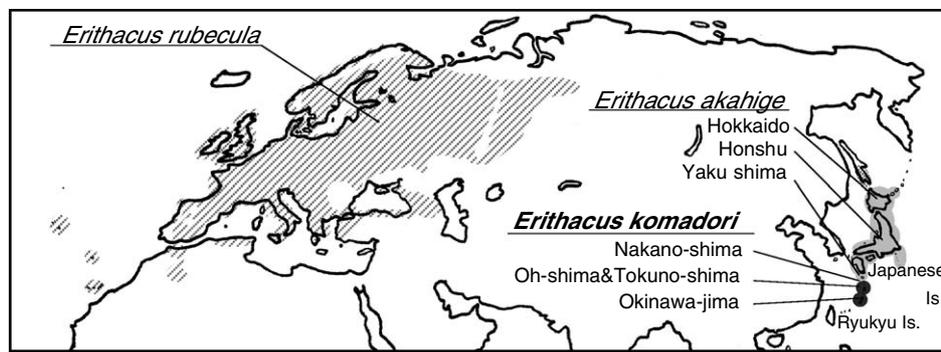 *E-mail address:* seki@ffpri.affrc.go.jp.

Fig. 1. Distribution of the species within the genus *Erithacus*, showing their approximate range (Cramp, 1988; Nakamura and Nakamura, 1995; Ornithological Society of Japan, 2000) and sampling locations.

related *Luscinia* species, and (2) to examine the origin and subspecies diversification history of *E. komadori* with such a restricted distribution, based on the mitochondrial cytochrome *b* (cyt *b*) sequence data.

2. Materials and methods

The sampling scheme was designed to identify the origin of *E. komadori*, and I used 18 samples from eight taxa in Saxicolini, collected from adult birds mostly during the breeding season. The sampled taxa with sampling location and sample size are as follows (see also Fig. 1 for sampling locations): *E. k. komadori* (Nakano-shima:1, Oh-shima:1, Tokuno-shima:1), *E. k. namiyei* (Okinawa-jima:3), *E. a. akahige* (Hokkaido:2, Honshu:1), *E. a. tanensis* (Yaku-shima:1), *L. calliope* (Hokkaido:2), *L. cyane* (Hokkaido:2), *Tarsiger cyanurus.* (Honshu:2, wintering individuals), and *Phoenicurus auroreus* (Honshu:2, wintering individuals). Although Kuroda (1923) described the third subspecies for *E. komadori*, *E. k. subrufus*, in the southern part of the Ryukyu Islands, I did not include samples of this doubtful subspecies in the present analysis; it was described based on the type captured in autumn, some individuals from the northern population of *E. k. komadori* is known to overwinter in the Southern Ryukyus, all morphological characters of that individual are well within the range of *E. k. komadori* (Kawaji and Higuchi, 1989), and there are no breeding records of this species in the southern Ryukyus. For the above samples, a large portion of the mitochondrial cyt *b* gene, 1007 base pairs (bp), was sequenced and analyzed. I also included published nucleotide sequences of *E. rubecula* (GenBank Direct Submission: Accession Nos. Y08058 and L78807) and *L. svecica* (Questiau et al., 1998: Accession Nos. Y10061 and Y08045) as ingroups, and *Muscicapa infuscata* (GenBank Direct Submission: Accession No. MST299690) and *M. striata* (Sætre et al., 2001: Accession No. AY206912) as outgroup taxa; Muscicapini which includes *Muscicapa* is known to be a sister taxa for the tribe Saxcolini (Voelker and Spellman, 2004). Of these, the 887–1007 bp segments of the cyt *b* gene were aligned to the present sequences.

Total DNA was extracted from muscle tissue of frozen specimens using QIAamp Tissue Kits (Qiagen), and also from plucked feathers of live individuals following the protocol described by Baba et al. (2001) using the extraction kit IsoQuick (ORCA). These samples show relatively high concentrations of mitochondrial DNA compared with those from blood, which reduce the possibility of isolating nuclear pseudogenes (Lee et al., 1997). Specific fragments were amplified using the polymerase chain reaction (PCR) and one set of primers: AXL149 (5′-ACT CCA TCA AAC ATC TCA GCT TGA TGA A-3′) which was modified from L14841 (Kocher et al., 1989) and AXH160 (5′-AGT CTT CAA TCT TTG GCT TAC AAG ACC-3′) which was modified from H15915 (Irwin et al., 1991). All PCRs were performed in 25 μl volumes on a Perkin Elmer 9600 Thermal Cycler, using *Taq* DNA polymerase (TaKaRa *Ex Taq*, Takara). Each reaction mixture included 1 μl of purified total DNA template, 2 μl of dNTP mixture (2.5 mM each), 2.5 μl of 10× *Ex Taq* Buffer (Takara), 0.25 μl of bovine serum albumin (20 mg/ml), 1.25 μl of each primer (2 pmol/μl), and 0.5 μl of *Taq* (1.25 U/μl). The thermal profile comprised an initial denaturing step of 94 °C for 2 min, followed by a "touch-down" scheme where the annealing temperature was lowered by 1 °C per cycle, starting from 65 °C until a temperature of 55 °C was reached. Then 25 additional cycles were run at a constant annealing temperature of 55 °C. Denaturation was done at 94 °C for 15 s, annealing for 30 s, and extension at 72 °C for 90 s, and a final extension step of 5 min was added after the last cycle. The resulting products were sequenced using Big Dye Terminator ver3.1 Cycle Sequencing Kit (Applied Biosystems) and visualized on an ABI PRISM 310 Automated sequencer. All sequences are available from DDBJ nucleotide Databank with Accession Nos. AB236370–AB236376, and AB244005–AB244015.

Sequences were aligned by eye, because no gaps occurred in the target region. All phylogenetic analyses were performed using the computer program PAUP* 4.0b10 (Swofford, 2003). Maximum-parsimony (MP) analysis in the form of heuristic search was performed with 1000 random stepwise-addition replicates. The robustness of the phylogeny was tested by bootstrapping with 1000 replicates with

100 random stepwise-addition replicates. In maximum-likelihood (ML) analysis, I used Modeltest 3.06 Posada and Crandall, 1998) with PAUP* to determine the model of evolution and parameter estimates by Akaike Information Criterion (AIC) value. I applied the likelihood ratchet algorithm as a tree searching strategy (Vos, 2003), using the software PAUPRat (Sikes and Lewis, 2001), consisted of 500 replicates with 600 s of time limit per iteration. Maximum-likelihood bootstrapping was performed with fast-stepwise addition option for 1000 replicates.

Although some rate heterogeniety was detected for the present data set, I speculatively used the branch height ($h \pm SE$), the mean branch length of two sister lineages in the maximum-likelihood tree, to date the divergence times between the subspecies of *E. komadori*, between the two Asian *Erithacus* robins, and also between the Asian *Erithacus* and its sister clade, since I do not have any known calibration point in the studied group. The rate of mtDNA divergence is known to differ among genes, taxa, body size, and calibration date (Ho et al., 2005). I, therefor, examined three estimates of the mtDNA divergence rate, 1.6%/million years (mys) estimated for the cyt *b* gene of Drepanidinae, small passerine birds, with calibration points at 0.43–3.7 million years before the present (mybp) (Fleischer et al., 1998), 5.63%/mys estimated for the cyt *b* region of Nectariniidae, again small passerines, with a calibration point at

0.5 mybp (Warren et al., 2003), and divergence rate curve for mitochondrial coding region estimated by Ho et al. (2005) using data from variety of avian lineage and also age of calibration point ($d = 1/\lambda(-\mu \exp(-\lambda t_d) + k t_d + \mu)$), where t_d is the divergence date of two sequences, d is their sequence divergence, and three other constants are $\lambda = 0.445$, $\mu = 0.0400$, $k = 0.0054$).

3. Results

The aligned sequences totaled 1007 bp in length and corresponded to the chicken, *Gallus gallus*, mitochondrial genome position 15029–16035 (Desjardins and Morais, 1990). Mean base compositions of taxa were 28.1% A, 34.1% C, 13.3% G, 24.5% T, and the difference among taxa was small in this region and no significant departure from homogeneity was found ($\chi^2 = 17.525559$, df = 69, $P > 0.999$). A total of 308 characters (30.5%) were variable, and 262 characters (26.0%) were parsimony-informative. The transition/transversion ratio ranged from 1.16 to 3.92 averaging 1.87, and there was some tendency of transition saturation in comparisons between divergent taxa.

Maximum parsimony analysis using the equally weighted 1007 sites yielded two most parsimonious trees and a consensus tree as shown in Fig. 2A (tree length = 645, consistency index = 0.616, retention index (RI) = 0.791,
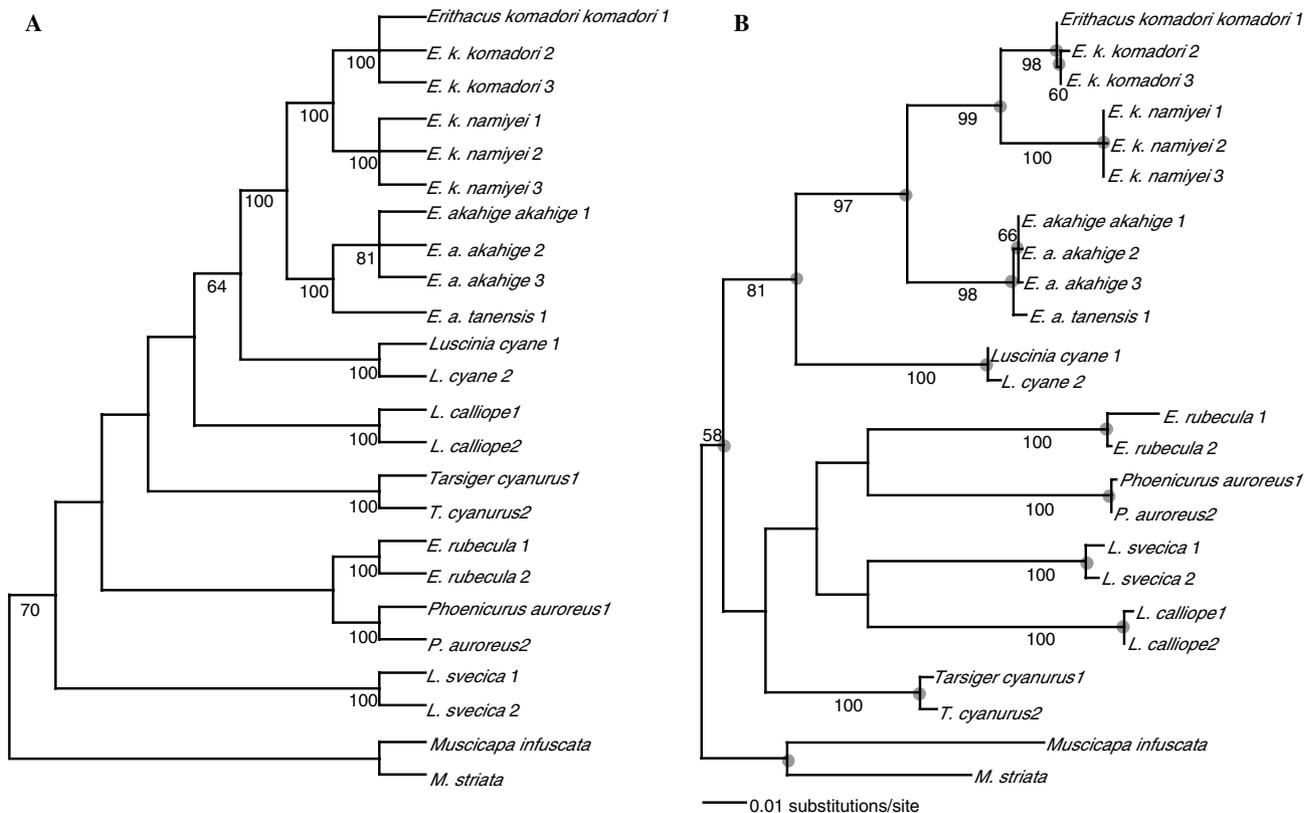

Fig. 2. A molecular phylogeny of the *Erithacus* robins and some Saxcolini species based on cyt *b* sequence data. Numbers at each node indicate the bootstrap percentages, if over 50%. (A) The consensus of two most parsimonious trees using all sites equally weighted; (B) the maximum likelihood tree using the general time reversible model with proportion of invariable sites and gamma rate (GTR + I + G). The gray circles indicate the nodes that were maintained in the consensus tree of the three local peaks found in the likelihood landscape by likelihood ratchet algorithm.

rescaled consistency index = 0.487), in which the East Asian *Erithacus* robins formed a strongly supported monophyletic clade (100% bootstrap support value). Each of the two subspecies of *E. komadori* formed a genetically distinctive clade, with bootstrap support values of both 100%. Among the *E. akahige* clade, three samples of *E. a. akahige* also formed a distinct clade from a single sample of *E. a. tanensis* supported by a bootstrap support value of 81%, although the genetic distance between them was small compared to that of the pair between the two subspecies of *E. komadori* (see also Fig. 2B). The East Asian *Erithacus* robins' clade was placed as a sister of *L. cyane*, not as a sister of *E. rubecula*. The clade including the East Asian *Erithacus* robins and *L. cyane* received a bootstrap support of 64%. However, the monophyly of the genus *Luscinia* was not supported either; *L. svecica* was placed at the root of all the examined Sxicolini, although the tree topology between genera only was supported with low bootstrap values far below 50%.

Using Modeltest with AIC criterion, the general time reversible model with a proportion of invariable sites and gamma rate (GTR + I + G) was selected as an optimal for the data (base frequencies: A = 0.2774, C = 0.3764, G = 0.1308, T = 0.2154; rate matrix: [A−C] = 6.2388, [A−G] = 16.3604, [A−T] = 2.8948, [C−G] = 0.6933, [C−T] = 28.5609, [G−T] = 1.0000; proportion of invariable sites = 0.5531; Gamma distribution shape parameter = 0.2786). Maximum-likelihood analysis using this model and likelihood ratchet algorithm resulted in a single tree (Fig. 2B) with $-\ln L$ value of 4338.0. However, two other local peaks in the likelihood landscape that had slightly lower likelihood values ($-\ln L = 4339.9$ and 4340.0) were found by the likelihood ratchet analysis (trees not shown), and the resulting trees were not significantly different from the best tree (Shimodaira and Hasegawa (1999) test, $P = 0.59$ for each pair). The nodes that were maintained in the strict consensus tree in the three-tree topology are indicated by the shaded circles in Fig. 2B. Although those three trees differed greatly, the topology within the clade including *E. komadori*, *E. akahige*, and *L. cyane* was the same, supported by a bootstrap value of 81%. Furthermore, by the ML analysis, neither the monophyly of the *Erithacus* nor the *Luscinia* was suggested in either tree topology.

The branch height and the estimated divergence time for each pair of lineages, *E. k. komadori* versus *E. k. namiyei*, *E.*

komadori versus *E. akahige*, and the East Asian *Erithacus* robins and *L. cyane*, under three different divergence rates are listed in Table 1. The range of the divergence time estimates was 0.28–1.91 mybp for the pair between the two subspecies of *E. komadori* including standard errors, 0.59–3.75 mybp for *E. komadori* and *E. akahige*, and 1.01–6.63 mybp for the East Asian *Erithacus* lineage and *L. cyane*.

4. Discussion

4.1. Phylogenetic relationship

The East Asian *Erithacus* robins, *E. komadori* and *E. akahige*, were consistently classified as a sister group to *L. cyane*, a representative of the East Asian *Luscinia* robins. The nodes including those three species received bootstrap support values of 64 and 81% in MP and ML analyses, respectively. Judging from the results of the present molecular phylogenetic analysis, the morphological similarity between *E. rubecula* and *E. akahige* merely derived from convergence, and not from a common ancestor with wide distribution. The East Asian *Erithacus* robins should be classified into the same genus with East Asian *Luscinia*, as suggested by Yamashina (1941), rather than *E. rubecula*.

The placement of East Asian *Erithacus* robins in the traditional *Luscinia*, however, needs further consideration; the molecular phylogenetic relationships of the traditional *Luscinia*, which includes 11 species (Sibley and Monroe, 1990), has never been examined thoroughly, and the monophyly of this group is highly doubtful as was suggested by the present result. Among the three *Luscinia* robins investigated, *L. svecica*, which I had included as a representative of the monophyletic Western Palearctic *Luscinia* group (*L. luscinia*, *L. megarhynchos*, and *L. svecica*; Wink et al., 2002; Fig. 1), branched off far earlier than *L. cyane* in the MP tree, and, in the ML tree, it formed a clade with *L. calliope* independent of *L. cyane*. However, the topology at this part of the tree is inconsistent depending on the tree reconstruction method employed, each node had a low bootstrap support value, and, therefore, the present data set solely from the cyt *b* region appear to be insufficient for denying or accepting the monophyly of the three *Luscinia* species examined. Six other species of *Luscinia*, mostly inhabiting Asia, have not been investigated either. Accordingly, I could not reveal the overall phylogeny of *Luscinia*, lacking

Table 1
The divergence time estimates based on the branch height ($h \pm$ SE), the mean branch length of two sister lineages in the maximum-likelihood tree, using three rate estimates of divergence

Divergence	$h \pm$ SE	Rate = 1.6%/mys[a] Time (mybp) $\pm$ SE	Rate = 5.63%/mys[b] Time (mybp) $\pm$ SE	Rate curve[c] Time (mybp) (interval)
E. k. komadri–E. k. namiyei	0.0231 ± 0.0074	1.45 ± 0.46	0.41 ± 0.13	0.57 (0.37–0.78)
E. komadori–E. akahige	0.0465 ± 0.0137	2.90 ± 0.85	0.83 ± 0.24	1.30 (0.85–1.84)
Asian *Erithacus*–Asian *Luscinia*	0.0830 ± 0.0230	5.19 ± 1.44	1.47 ± 0.41	3.03 (1.83–4.87)

Each divergence rate estimate refer to [a]Fleischer et al. (1998), [b]Warren et al. (2003), and [c]Ho et al. (2005), respectively.

in the samples and available data set, unfortunately, and molecular phylogenetic consequences have to wait for the results of more comprehensive studies.

4.2. The origin of Erithacus komadori

The estimates of the divergence time for the three pairs of groups, between the two subspecies of *E. komadori*, between *E. komadori* and *E. akahige*, and between East Asian *Erithacus* robins and *L. cyane*, all had wide error ranges (Table 1), due to the variation of probable estimates of the molecular clock. To practically qualify the estimates, I examined the historical geographic events that might have promoted the divergence between *E. komadori* and *E. akahige*, because there appears to be a large ecological difference between them; apart from the mostly migratory *L. cyane* and *E. akahige* breeding in coniferous and deciduous forests of cool-temperate or mountain region, *E. komadori* is the primarily resident species inhabiting lowland evergreen broad-leaved forest in the subtropic region.

During the last 3.75 myr, from the maximum aging of the *E. komadori* lineage to the present, there have been two kinds of historical geographic events that might have affected the speciation. The first is the repetitive formation and submergence of the land–bridges connecting the Ryukyu Islands, the Japanese Islands, and the Asian continent, along with the crustal movement and glacial cycles (Kimura, 2000). After the Ryukyu and Japanese Islands first separated from the Asian continent in the Miocene, there were three major land connection periods (Kawamura, 1991; Kimura, 2000, 2002; Otsuka and Takahashi, 2000): late Pliocene to early Pleistocene land connection relating with the dry up of the East China Sea by sedimentation (2.0–1.3 mybp), middle Pleistocene land connection related to the uplifting crustal movement in the Ryukyu Islands and also the sea-level decline during Mindel and Riss glaciation (0.40–0.13 mybp), and late Pleistocene land connection related to the sea-level changes during Würm glaciation (0.03–0.01 mybp). The glaciation during the land connection period also means global cooling, and, therefore, expansion of the cool-temperate forest and mountain forest, the preferred breeding habitat for *E. akahige* and *L. cyane*, southward and downward. The easy dispersal through land–bridges followed by the isolation during sea-level rise might have gave the robin a chance of differentiation, like the cases reported in other endemic terrestrial animals in the Ryukyu Islands (e.g., Masuda and Yoshida, 1995; Ota, 1998; Yamada et al., 2002). Isolation in the Ryukyu Islands, however, would be incomplete for the ancestor of the robin; *E. akahige* is a primarily migratory species and migrate through the Japanese and Ryukyu Islands, and probably the ancestor of the East Asian *Erithacus* robins might have been. Migration, in general, is considered to counter differentiation, promoting the gene flow among geographically distant populations. In the case of present day *E. akahige*, for example, the samples from three distant populations in three different islands, Hokkaido, Honshu, and Yaku-shima, showed only a low level of differentiation (Figs. 1 and 2B). Furthermore, the information on the timing of the repeated formation and submergence of landbirdge alone is insufficient for qualifying the timing of divergence to a single probable period.

The second historical geographic event is the subsidence of high ancient mountains in the central Ryukyu Islands caused by the tectonic movement: in the late Pliocene and early Pleistocene strata of the central Ryukyu Islands, there appeared a mixed pollen assemblage containing the warm and cool-temperate trees, such as *Cryptomeria*, *Abies*, and *Tsuga*, and the tropical–subtropical trees including *Dacrydium*, *Liquidambar*, *Lagerstroemia*, and *Sapium*, suggesting a paleolandscape with lowland subtropical forest attended by high mountain forest behind it (Kuroda and Ozawa, 1996). Although such a mountain forest in the subtropics might have been a preferable breeding habitat for the ancestor of the East Asian *Erithacus* robins, as in the case of some South Asian traditional *Luscinia* species (Sibley and Monroe, 1990), the Central Ryukyu Mountains disappeared by the middle Pleistocene, about 0.8 mybp, probably due to the downward tectonic movement around the Okinawa Trough. It appears quite probable to expect that the ancestral population of *E. komadori* breeding in such a mountain forest had changed its breeding habitat to the lowland subtropical forest as the mountains disappeared; the relatively loose bird community, as is often the case in small isolated islands, might also have advantaged such habitat shift. The suppression of migratory habit, relatively short dispersal distance, and the more than two-month earlier breeding observed in *E. komadori* (Seki, 2002; Kinjou, M., 2002, pers. comm.) may have evolved successively, and these traits might have restricted the gene flow between the two ancestral groups of East Asian *Erithacus*. The timing of this tectonic event coincided with the submergence period of the land–bridge, and the isolation of the central Ryukyu Islands from the Japanese Islands might also have reduced the chance of the gene flow between those two populations. Based on these assumptions, I suggest that the divergence time between *E. komadori* and *E. akahige* was about 0.8 mybp, when the mountain forest of central Ryukyu disappeared.

The time estimate of 0.83 ± 0.24 mybp based on the divergence rate of 5.63‰/mys (Table 1) appeared to best suit the above hypothesis among the three rates examined, probably because it was based on the data solely from small passerine birds and single calibration point at 0.5 mybp (Warren et al., 2003), relatively close to the present conditions. According to this rate estimate, the divergence time between the East Asian *Erithacus* lineage and *L. cyane* would be 1.47 ± 0.41 mybp, near the end of the first land connection period between the Japanese Islands and the Asian continent. Although the separation between the Japanese Islands and the Asian continent might have had some effects on this lineage separation, comprehensive studies on molecular phylogeny of *Luscinia*, at least covering Asian species, is again required to discuss this problem; the *L. cyane* is not yet defined as a closest sister of East Asian *Erithacus* lineage.

The divergence time estimate between the two subspecies of *E. komadori* would be 0.41 ± 0.13 mybp, which coincided with the second land connection period. This estimate, coinciding with the beginning not the end of the land connection period, appears odd, if I assume that the differentiation had been caused by the isolation between islands. On the contrary, the differentiation between the two subspecies might have been related to the population expansion through the chain of islands in the Ryukyu area. I will continue studies to examine the validity of this time estimate and the effect of population expansion on subspecies differentiation through the intraspecific phylogeographical studies of *E. komadori*.

In conclusion, the restricted distribution of *E. komadori* might mainly be due to the narrow distribution range of the ancestral population which originated in the central Ryukyu Islands for about 0.8 mybp, not a result of local extinction in its adjacent areas unlike the view of Kawaji and Higuchi (1989), and it might have even expanded its range northward along with the later land–bridge formation.

Acknowledgments

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