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Phylogenetic Relationships of Japanese *Auritibicen* Species (Hemiptera: Cicadidae: Cryptotympanini) Inferred from Mitochondrial and Nuclear Gene Sequences

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We investigated the phylogenetic relationships and divergence times within the genus *Auritibicen* (Cicadidae: Cicadinae: Cryptotympanini), analyzing five Japanese species (*A. japonicus*, *A. bihamatus*, *A. kyushyuensis*, *A. esakii* and *A. flammatus*) and three species from East Asian mainland and Taiwan (*A. atrofasciatus*, *A. intermedius* and *A. chujoi*) using mitochondrial cytochrome oxidase subunit I (COI) and nuclear elongation factor 1-alpha (EF-1a) gene sequences. Although the EF-1a gene tree did not resolve the relationships among these *Auritibicen* species, the trees based on COI gene and the combined data set showed that Japanese taxa comprised three distinct lineages: the individual species *A. flammatus* and *A. bihamatus*, and the *A. japonicus* group, comprising *A. japonicus*, *A. esakii* and *A. kyushyuensis* from Japan and *A. intermedius* from Korea. In *A. kyushyuensis*, which comprises three populations in Kyushu, western Honshu and Shikoku, the specimens from western Honshu and Shikoku were closely related to each other, but not to the specimen from Kyushu; instead, they were sister to the Korean *A. intermedius*. The incongruence between the gene tree and species tree necessitates further population genetic and morphological studies to confirm the classification and species status of the western Honshu and Shikoku populations of *A. kyushyuensis*, which were originally described as two independent species. Divergence time estimation suggested that the most recent common ancestor of *Auritibicen* species studied dated back to the late Pliocene and that the species of the *A. japonicus* group diverged during the mid Pleistocene. Thus, the Pleistocene climatic fluctuation may have promoted the divergence of the *Auritibicen* species.

Key words: *Auritibicen*, elongation factor 1 alpha gene, mitochondrial COI gene, molecular phylogeny, Japanese islands

INTRODUCTION

Cicadas of the genus *Lyristes* Horváth, 1926 or *Tibicen* Latreille, 1825 in the tribe Cryptotympanini (Cicadidae: Cicadinae) from East Asia are now classified into the genus *Auritibicen* Lee, 2015. *Auritibicen* currently comprises 14 species from East Asia (Lee, 2015) and is most closely related to *Lyristes* or *Tibicen* in Europe, according to a recent molecular phylogenetic analysis of *Tibicen* and its allied species in the tribe Cryptotympanini by Hill et al. (2015). However, phylogenetic relationships among the *Auritibicen* species have not been studied by molecular phylogenetic methods. Five species of *Auritibicen* occur in the Japanese archipelago, which are not found in East Asian mainland (Hayashi and Saisho, 2011; note that *A. flammatus* (Distant, 1892) has once been recorded from China (Chou et al., 1997), but its species identity is uncertain.). It is unknown whether these species are of a monophyletic lineage or of multiple origins derived from different ancestors in East Asian mainland.

We conducted a phylogenetic study of the five Japanese *Auritibicen* species in relation to three congeneric species from other regions of East Asia, using sequences of mitochondrial cytochrome oxidase subunit I (COI) and nuclear elongation factor 1 alpha (EF-1a) gene. In particular, we aimed to clarify whether *A. kyushyuensis* (Kato, 1926) occurring in three main islands of Japan (Kyushu, Honshu and Shikoku) is closely related to *A. intermedius* (Mori, 1931) in Korea and northeastern China as expected from their similar morphological features (Hayashi and Saisho, 2011).

MATERIALS AND METHODS

Sampling and DNA sequencing

We used 17 specimens of the five Japanese *Auritibicen* species and four specimens of three *Auritibicen* species from other countries for DNA extraction (Table 1). Of the Japanese species,

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Table 1. List of specimens used in this study and DDBJ accession numbers of COI and EF-1a sequences.

Taxon	ID	Sex	Locality	Date (collector)	DDBJ accession no.	
					COI	EF-1a
Auritibicen atrofasciatus (Kirkaldy, 1909)	ez14	male	Linzhi, Tibet, China	July, 2005 (J. Li)	LC099991	NA
Auritibicen bihamatus (Motschulsky, 1861)	CD09	male	Karuizawa, Gunma, Honshu, Japan	Aug. 26, 2007 (J. Yoshimura)	LC099999	LC127308
"	ez01	male	Mt. Hachibuse, Hyogo, Hoshu, Japan	Aug. 4, 2012 (T. Kojima)	LC099980	LC127293
"	ez09	male	Mt. Hachibuse, Hyogo, Honshu, Japan	July 27, 2003 (T. Kojima)	LC099988	NA
"	ez17	female	Memuro, Hokkaido, Japan	Aug. 18, 2007 (T. Kojima)	LC099993	LC127303
"	ez18	male	Kumakogen, Ehime, Shikoku, Japan	Aug. 9, 2008 (T. Kojima)	LC099994	LC127304
Auritibicen chujoi (Esaki, 1935)	ez08	male	Lishan, Taiwan	July 23, 2010 (T. Kojima)	LC099987	LC127300
"	ez12	male	Lishan, Taiwan	July 23, 2010 (T. Kojima)	LC099990	LC127301
Auritibicen esakii (Kato, 1958)	ez04	male	Yakushima I., Kyushu, Japan	July 27, 2012 (T. Kojima)	LC099983	LC127296
"	ez21	male	Yakushima I., Kyushu, Japan	July 25, 2012 (T. Kojima)	LC099997	LC127306
Auritibicen flammatus (Distant, 1892)	ez03	male	Kumakogen, Ehime, Shikoku, Japan	Aug. 13, 2011 (T. Kojima)	LC099982	LC127295
"	ez10	male	Mt. Kongo, Osaka, Honshu, Japan	Aug. 14, 2013 (T. Kojima)	LC099989	NA
Auritibicen intermedius (Mori, 1931)	ez16	male	Mt. Godaesan, Yeoncheon-gun, Gyeonggi-do, Korea	Aug. 15, 2003 (Y.J. Lee)	LC099992	LC127302
Auritibicen japonicus (Kato, 1925)	CD12	female	Nobusawa, Nagano, Honshu, Japan	Aug. 25, 2007 (J. Yoshimura)	LC100000	LC127309
"	ez02	male	Mt. Rokko, Hyogo, Honshu, Japan	Aug. 12, 2012 (T. Kojima)	LC099981	LC127294
"	ez19	male	Memuro, Hokkaido, Japan	Aug. 18, 2007 (T. Kojima)	LC099995	NA
"	ez20	male	Mt. Sefuri, Fukuoka, Kyushu, Japan	Aug. 7, 2013 (Y. Yamauchi)	LC099996	LC127305
Auritibicen kyushyuensis (Kato, 1926)	ez05	male	Makinoto-toge, Kokonoe, Ohita, Kyushu, Japan	July 20, 2013 (H. Ono)	LC099984	LC127297
Auritibicen kyushyuensis = *A. shikokuanus* (Kato, 1959)	ez06	male	Kumakogen, Ehime, Shikoku, Japan	Aug. 13, 2011 (T. Kojima)	LC099985	LC127298
"	ez25	male	Kumakogen, Ehime, Shikoku, Japan	Aug. 13, 2011 (T. Kojima)	LC099998	LC127307
Auritibicen kyushyuensis = *A. ishiharai* (Kato, 1959)	ez07	male	Kitahiroshima, Hiroshima, Honshu, Japan	Aug. 7, 2013 (T. Kojima)	LC099986	LC127299

the current concept of *A. kyushyuensis* includes populations from Shikoku (Ehime Pref.), western Honshu (Hiroshima Pref.) and Kyushu (the type locality). The former two populations were described as *Tibicen shikokuanus* and *T. ishiharai*, respectively, by Kato (1959) but later synonymized with *A. kyushyuensis* by Nast (1972). For the present study, only pinned dry specimens containing degraded DNA were available, except for two specimens of *A. japonicus* (Kato, 1925) and *A. bihamatus* (Motschulsky, 1861). Total genomic DNA was extracted from the legs of the specimens using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) or the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). An approximately 1500-bp fragment of mitochondrial COI was PCR-amplified using the following primers. For the early half of the fragment, we newly designed COILyristes_F1 (forward, 5′-TCR GGR ATR ATT GGW ACW GC-3′) and COI_Lyristes_R3 (reverse, 5′-C AAC RTY TAT VCC AAC MGT A-3′). For the latter half, we used C1-J-2195 and TL2-N-3014 (Simon et al., 1994). We also sequenced an approximately 700-bp fragment of the nuclear EF-1a gene, which was used by Hill et al. (2015), using the primers EF1-PA-f650ambig (Lee and Hill, 2010) and EF-N-1419 (Sueur et al., 2007). PCR-products were sequenced using an ABI3130xl sequencer (Applied Biosystems, Foster City, CA, USA). Sequences used in this study were deposited at DNA Data Bank of Japan (DDBJ; accession numbers: COI, LC099980–LC100000; EF-1a, LC127293–LC127309; Table 1).

Phylogenetic analysis and divergence time estimation

Hill et al. (2015) showed that the two *Tibicen* (= *Lyristes*) species from Europe (*T. plebejus* and *T. gemellus*) were the closest relatives to three *Auritibicen* species in their molecular phylogeny based on COI and EF-1a sequences. We used these *Tibicen* species as outgroups for phylogenetic analysis (GenBank accession numbers: *T. plebejus*, COI, KR674238, EF-1a, KR705860; *T. gemellus*, COI, KR674232, EF-1a, KR705854). The COI sequences were aligned unambiguously using MEGA version 6.06 (Tamura et al., 2013). The EF-1a gene sequences were aligned using MEGA and edited manually for obvious misalignment. Maximum-likelihood phylogenetic analyses were conducted using RAxML version 8.0.0 (Stamatakis, 2014). The COI sequence was partitioned according to the three codon positions. The EF-1a sequence containing a variable intron part between almost invariable exon parts was treated as a single partition. We applied the substitution model GTR + G (general time reversible model with gamma distribution for rate heterogeneity) for each partition because this model is the most general and versatile model, and we had no computational problem due to over-parameterization in our study. We conducted RAPID ML analyses with 1000 bootstrapping analysis to obtain ML trees and bootstrap percentages of nodes for COI, EF-1a and combined data sets.

We also conducted Bayesian inference (BI) of phylogeny using MrBayes version 3.2 (Ronquist et al., 2012) for the three data sets, with the same partitioning scheme and substitution models as in the RAxML analysis. Two runs of four Metropolis-coupled Markov chain Monte Carlo (MCMC) iterations were conducted for 10 million generations with a sampling frequency of every 1000 generations. We confirmed the convergence of runs, with the potential scale reduc-

tion factor (PSRF) approaching 1 for all parameters and the average standard deviation of split frequencies being less than 0.01. A 50% majority rule consensus tree was constructed after discarding data from the initial 25% generations as burn-in.

Divergence time estimation was conducted using BEAST version 1.8.0 (Drummond et al., 2012). We used a data set of 19 specimens (including two outgroup taxa) that contained both gene sequences. We used the strict clock model based on the result of model comparison by AICM (Baele et al., 2012), which showed a better fit for the strict clock model than uncorrelated lognormal relaxed clock model (AICM, 10908.694 vs. 10921.438). Partitioning scheme was the same as in the combined ML analysis (i.e., three COI and one EF-1a partitions), and substitution models (GTR + G) and clock models (strict clock) were unlinked among the four partitions, while the trees were linked. The clock rate of COI was set to 0.0177 (per site per million years [my]) referring to a divergence rate of 3.54% per my for insect mitochondrial genes, which was estimated based on a relatively ancient geographic event (9–12 million years ago [mya]) (Papadopoulou et al., 2010); the clock rate of EF-1a was estimated in the analysis. An exponential prior (mean = 1; offset = 0.0) was used for mutation rates of three codon positions of COI gene and the clock rate of EF-1a. Default settings were used for other parameters and priors. The MCMC run was conducted for 50 million generations with a sampling frequency of every 5000 generations. The stationarity and mixing of runs were checked using effective sample sizes in TRACER version 1.6.0 (Rambaut et al., 2014). A consensus tree was obtained from 9001 sample trees after removing the initial 1000 trees as burn-in using TreeAnnotator version 1.8.0 in BEAST.

RESULTS

The COI sequences showed approximately nine times greater divergence than EF-1a (mean pairwise uncorrected p distance, 0.0664 vs. 0.0074). Both gene trees showed a deep divergence between *Auritibicen* and *Tibicen* (mean uncorrected p distance: 0.1556 and 0.0236 for COI and EF-1a, respectively). However, EF-1a gene showed low sequence divergences within *Auritibicen* with an unresolved topology regarding the species relationships (mean pairwise uncorrected p distance within *Auritibicen*: 0.0034 and 0.0483 for EF-1a and COI, respectively).

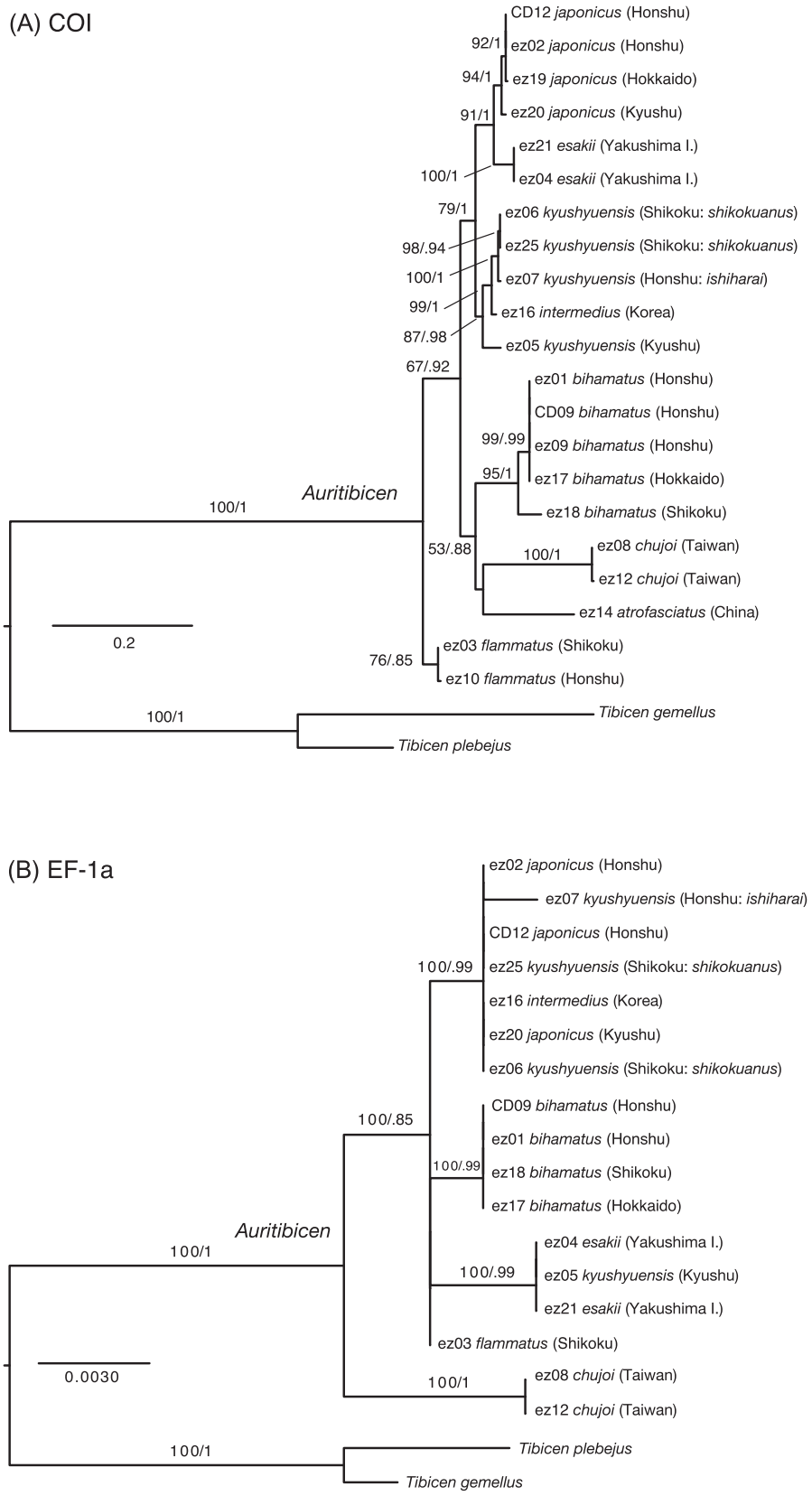

Fig. 1. Maximum-likelihood tree of *Auritibicen* species reconstructed using mitochondrial COI **(A)** and nuclear EF-1a **(B)** gene sequences. Numerals above the branches or near the nodes are bootstrap percentages (shown when > 50%) followed by posterior probability of Bayesian inference tree (shown when > 0.5).

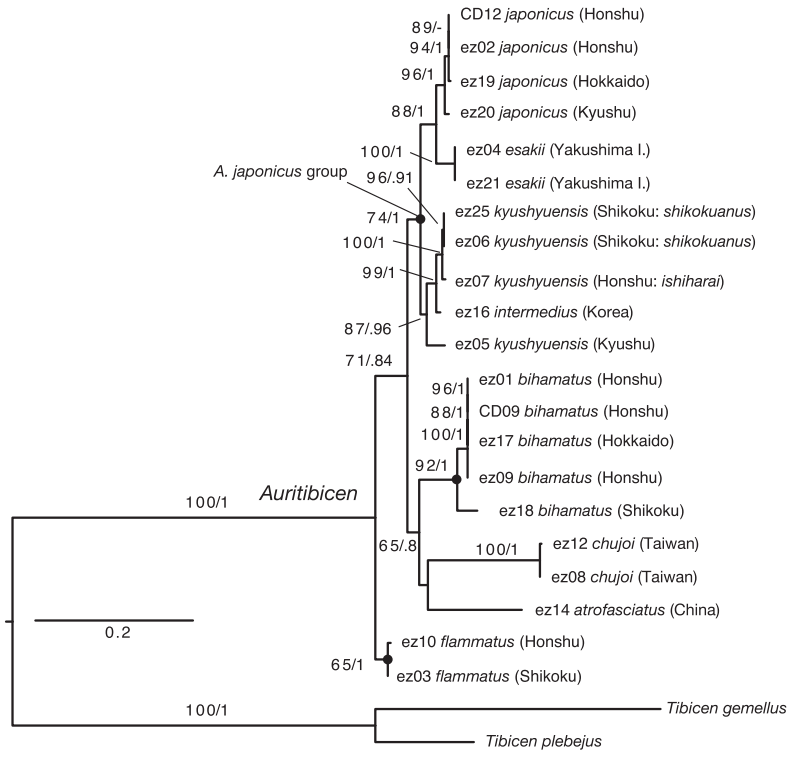

Fig. 2. Maximum-likelihood tree of *Auritibicen* species reconstructed using combined matrix of mitochondrial COI and nuclear EF-1a gene sequences. Numerals above the branches or near the nodes are bootstrap percentages (shown when > 50%) followed by posterior probability of Bayesian inference tree (shown when > 0.5).

In the COI gene tree (Fig. 1A), *A. flammatus* was sister to all the other species. *Auritibicen bihamatus* was related to *A. atrofasciatus* (Kirkaldy, 1909) from China and *A. chujoi* (Esaki, 1935) from Taiwan, but not to the Japanese *Auritibicen* species. The five specimens of *A. bihamatus* from Honshu and Hokkaido had an identical COI haplotype, which differed from the haplotype of the specimen from Shikoku. *Auritibicen esakii* (Kato, 1958) and *A. japonicus* were sister to each other, and these two species were sister to the clade containing *A. kyushyuensis* and *A. intermedius*. The three populations of *A. kyushyuensis* from Kyushu, Honshu, and Shikoku were closely related to *A. intermedius* from Korea, with the Kyushu population being sister to the others. The Honshu and Shikoku populations were closely related to each other and sister to *A. intermedius*. PCR amplification of EF-1a gene was not successful for four specimens of *A. atrofasciatus*, *A. bihamatus*, *A. flammatus* and *A. japonicus* (Table 1) due to highly degraded DNA; thus, no EF-1a sequence was available for *A. atrofasciatus*. The topology of EF-1a gene tree (Fig. 1B) differed from that of COI gene tree, where *A. chujoi* was sister to all the other species. The EF-1a sequence of *A. kyushyuensis* from Kyushu was similar to those of *A. esakii*, and the sequences of *A. kyushyuensis* from Honshu and Shikoku and *A. intermedius* were similar to those of *A. japonicus*. The topology of ML tree resulting from a combined analysis of COI and EF-1a sequences was identical to that of the COI tree (Fig. 2).

Bayesian inference of divergence time between *Auritibicen* and *Tibicen* was approximately 18.2 my (95% highest probability density interval [HPDI], 13.8–23.4 my; Fig. 3). The age of the most recent common ancestor (MRCA) of *Auritibicen* was estimated at 3.6 my (95% HPDI, 2.9–4.4 my). The MRCA age of the Japanese species was 2.6 my (95% HPDI, 2.6–3.2 my), and the divergence into three lineages, *A. flam-*

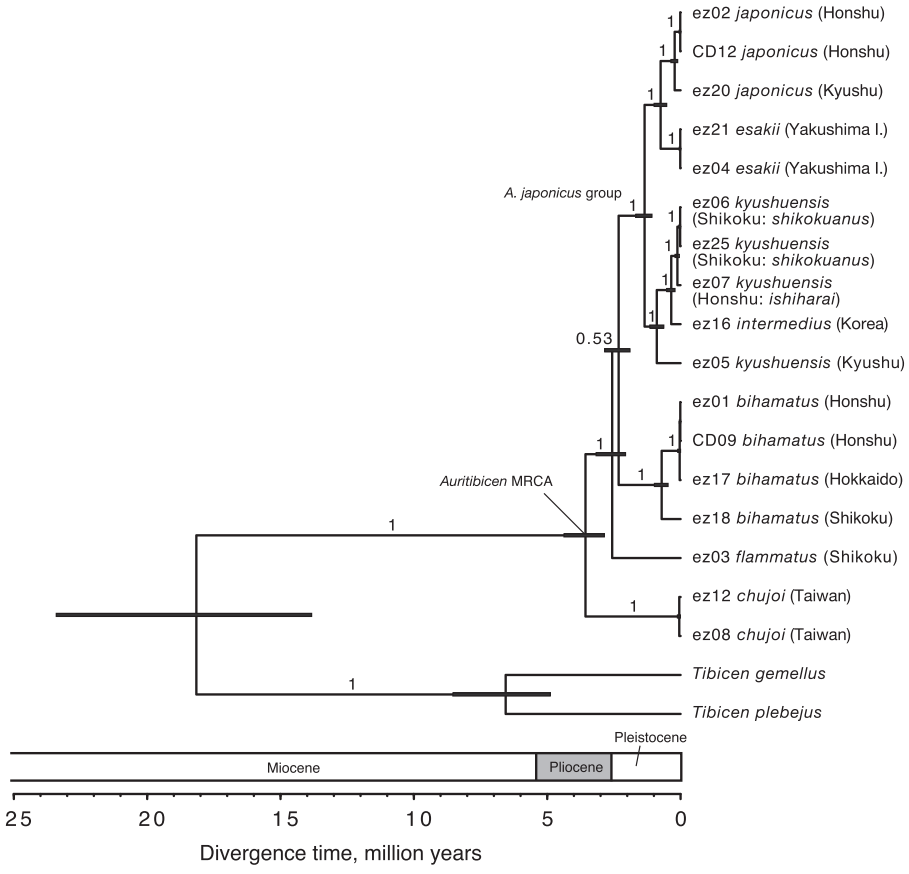

Fig. 3. Divergence times of *Auritibicen* species estimated using a Bayesian inference analysis of COI and EF-1a gene sequences with a strict clock model. Thick bars indicate 95% highest probability density intervals of node ages. Numerals above the branches or near the nodes are posterior probabilities (shown when > 0.5).

matus, *A. bihamatus*, and a lineage containing *A. japonicus*, *A. esakii* and *A. kyushyuensis* occurred during the early Pleistocene (2.3–2.6 mya; 95% HPDI, 1.9–3.2 mya). The last lineage, which also contained *A. intermedius* from Korea, diverged into two lineages 1.4 mya (95% HPDI, 1.1–1.7 mya). Further, *A. esakii* and *A. japonicus* diverged 0.8 mya (95% HPDI, 0.5–1.0 mya). The MRCA age of *A. kyushyuensis* from Kyushu and *A. intermedius* was 0.9 my (95% HPDI, 0.6–1.2 my), and that of *A. intermedius* and the Honshu and Shikoku populations of *A. kyushyuensis* 0.35 my (95% HPDI, 0.05–0.21 my).

DISCUSSION

The EF-1a gene tree differed from the COI gene tree especially in the position of *A. flammatus*. In the COI gene tree, *Tibicen* (outgroup) and *Auritibicen* exhibit larger sequence divergence due to a faster evolutionary rate, and this may have resulted in incorrect rooting of *Auritibicen* (thus, the EF-1a gene tree may show correct rooting). On the other hand, EF-1a gene sequences showed only limited sequence variation within introns, and did not resolve the species relationships within *Auritibicen*. Based on the tree resulted from the combined analysis of both gene sequences, the Japanese *Auritibicen* species comprised three distinct lineages. One of the lineages, referred to here as "the *A. japonicus* group", includes *A. japonicus*, *A. esakii*, *A. kyushyuensis*, and *A. intermedius*. The species relationships within the *A. japonicus* group indicated by COI and EF-1a gene trees differed from each other. However, both gene trees suggested that *A. kyushyuensis* from Honshu and Shikoku is not directly related to *A. kyushyuensis* from Kyushu but closely to *A. intermedius* from Korea. The species status of the Honshu and Shikoku populations, which were originally described as two independent species by Kato (1959), may require reconsideration. The incongruence between gene tree and species tree and also among gene trees can occur due to stochastic or incomplete lineage sorting, especially when speciation interval is short and ancestral population size is large, in which randomly fixed haplotypes from ancestral polymorphic haplotypes may reveal incongruence between gene tree and species tree (Ballard and Whitelock, 2004; Maddison and Knowles, 2006). Introgressive hybridization between species can also cause gene tree–species tree incongruence. However, no hybrid is known for *Auritibicen* species, and it is unlikely that currently allopatric populations of *A. kyushyuensis* and *A. intermedius* hybridized in the past. Because the *A. japonicus* group appears to have diverged within a relatively short time interval, we cannot rule out the possibility that the three *A. kyushyuensis* populations are in fact monophyletic; stochastic lineage sorting resulted in a gene tree–species tree incongruence. To resolve the relationships among *A. intermedius* and the three populations of *A. kyushyuensis*, we need to study multiple loci from both mitochondrial and nuclear DNA in combination with a detailed morphological study of these populations.

The estimated divergence times suggest that the East Asian *Auritibicen* species diverged from the European *Tibicen* approximately 18 mya (mid Miocene), and the diversification within *Auritibicen* occurred more recently, mainly during the late Pliocene and Pleistocene. Note that the evolutionary rate of COI gene used in this study (0.0177 per my; Papadopoulou et al., 2010) is among one of the fastest in insects; this rate was estimated based on a relatively ancient geographic event with a substantial genetic distance correction, and thus may be more plausible than traditional slower rates (e.g., 0.0115 from 2.3% sequence divergence per my; Brower, 1994) estimated using uncorrected genetic distance. Employing a slower rate in our data set would result in more ancient divergence times for the *Auritibicen* species. Also, our use of a constant clock rate for COI gene may have resulted in moderate confidence intervals (95% HPDIs) of divergence times compared to other calibration methods.

The five Japanese *Auritibicen* species were estimated to have diverged during the Pleistocene; this result suggests the influence of the Pleistocene climatic fluctuation on allopatric divergence of *Auritibicen* species. The *Auritibicen* species are generally restricted to high altitudes and are likely adapted to a cool climate. Thus, allopatric divergence among segregated mountain regions may have occurred following wide-range dispersal during cooler periods (i.e., glacial periods). The divergence among *A. flammatus*, *A. bihamatus* and the *A. japonicus* group likely occurred in the early Pleistocene, followed by the divergence within the *A. japonicus* group in the mid Pleistocene; allopatric differentiation of the *A. japonicus* group among the Korean Peninsula, Yakushima Island, Kyushu, western Honshu and Shikoku and eastern Honshu may have resulted in the present species diversity of this group. *Auritibicen japonicus* has overlapping ranges with the *A. kyushyuensis* populations in western Honshu, Kyushu and Shikoku; this overlapping may have resulted from morphological, behavioral and/or ecological divergences among these species (e.g., larger bodies of *A. japonicus* than the other species). Although we did not study mainland species other than *A. atrofasciatus*, there may be mainland species closely related to the *A. japonicus* group. A molecular phylogenetic study including all the East Asian mainland species is needed to understand the diversification processes of *Auritibicen*.

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