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

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

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. biahmatus* (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 COILytistes_F1 (forward, 5′-GGR ATR ATT GGW ACW GC-3′) and COILytistes_R3 (reverse, 5′-C AAC RTY TAT TCC 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-
Phylogeny of *Auritibicen* cicadas

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).

(A) COI

![Maximum-likelihood tree of COI](image)

(B) EF-1a

![Maximum-likelihood tree of EF-1a](image)
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. flammatus*...
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 mya (95% HPDI, 0.6–1.2 mya), and that of A. intermedius and the Honshu and Shikoku populations of A. kyushyuensis 0.35 mya (95% HPDI, 0.05–0.21 mya).

**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 divergence among closely related lineages 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). Intрогressive 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% HPDs) 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. atrofuscatus, 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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**REFERENCES**


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