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DNA Barcodes of Japanese Leafhoppers

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Abstract. Only five DNA barcodes of Japanese leafhoppers have been recorded, although the DNA barcoding method using of a part of the mitochondrial cytochrome oxidase subunit I (COI) gene is said to be one of new tools for taxonomic identification. In this study, 63 barcodes under 45 species (15 subfamilies and 37 genera) are newly added to the database of DNA barcodes. Barcodes of Japanese leafhoppers had different COI sequences, and none was shared between species. COI differences between most cicadellid species exceeded those within species.

Key words: DNA barcodes, COI, LCO1490, HCO2198, Cicadellidae.

The molecular identification method, DNA barcoding, is proposed by P. Hebert's research group (Herbert *et al*., 2003). This method is a new identification system of species using a short standardized region (648 base-pair, a region in the cytochrome oxidase I gene) of mitochondrial DNA, proving highly effective in identifying birds, butterflies, fish, flies and many other animal groups. The advantage of using COI is that it is short enough to be sequenced quickly and cheaply yet long enough to identify variations between species. Furthermore, DNA sequence can be used to identify different species in the same way and is very useful for non-taxonomists.

The Cicadellidae (Hemiptera, Auchenorrhyncha) comprise over 20,000 species globally and 500 species in Japan. Many of them are important agricultural pests, but nymphs and female adults are very difficult to identify using morphological features. Therefore, the DNA barcoding method is to be highly useful. However, now (4th Oct. 2010), 2,227 barcodes of leafhoppers are registered: 158 species with barcodes; 1,023 specimens with barcodes (iBOL, http://ibol.org/). Of them, about 1/3 of the specimens are collected from China and Australia, respectively, and only five specimens are from Japan. Therefore, the immediate purpose is to test whether identification using DNA barcoding is pratical. In this study, I test whether any DNA barcodes are found in two species and whether the differences within species are much less than those between species.

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Material and Methods

Cicadellid specimens were collected using the sweep method with an insect net and a light trap at several locations across Japan, Korea, Taiwan and Vietnam (Table 1). Under 15 subfamilies, 63 specimens of 45 species of 37 genera were used. Collected adult specimens were identified morphologically. Genitalia dissections were prepared and examined when necessary to validate the identification of a specimen. Specimens were collected directly into absolute ethanol.

DNA extraction, amplification and sequencing from abdominal segments I-VII of each adult Cicadellidae specimen were used for the extraction of total genomic DNA by DNeasy Blood & Tissue Kit (Qiagen: 69506) and the modified Takiya's protocol (Takiya *et al*., 2006).

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Table 1. Cicadellid specimens used for DNA analysis.

Subfamily	Species	Locality	Collecting Date	Sample No.
Agalliinae	*Japanagallia pteridis*	Go-no-kawara, Mine, Yamaguchi	04-Jun-2008	SK012
Agalliinae	*Japanagallia pteridis*	Hegi, Minami-takaki, Nagasaki	23-May-2008	SK006
Aphrodinae	*Planaphrodes nigricans*	Washihara-Seto, Tsuwano, Shimane	04-Jun-2008	SK011
Aphrodinae	*Stroggylocephalus agrestis*	Hamajiri, Minami-Osumi, Sata Kagoshima	14-Jul-2008	SK101
Cicadellinae	*Bothrogonia ferruginea*	Mt. Dogo, Shobara, Hiroshima	07-Jun-2008	SK028
Cicadellinae	*Cicadella viridis*	Hongo, Osa, Kitahiroshima, Hiroshima	07-Jun-2008	SK004
Coelidiinae	*Thagria fuscovenosa*	Mt. Koba, Minami-Osumi, Sata, Kagoshima	14-Jul-2008	SK060
Deltocephalinae	*Albicostella albicosta*	Bessho, Bingo-Ochiai, Shobara, Hiroshima	07-Jun-2008	SK019
Deltocephalinae	*Albicostella kiusiuensis*	Take, Mt. Hoshu, Toho, Fukuoka	30-May-2008	SK008
Deltocephalinae	*Balclutha incisa*	Kyushu Univ., Motooka, Fukuoka, Fukuoka	10-Jul-2008	SK120
Deltocephalinae	*Doratulina producta*	Mt. Koba, Minami-Osumi, Sata, Kagoshima	14-Jul-2009	SK134
Deltocephalinae	*Hecalus okinawensis*	Shiramizu, Ishigaki Is., Okinawa	8-15-May-2008	SK129
Deltocephalinae	*Hecalus prasinus*	Hamajiri, Minami-Osumi, Sata, Kagoshima	15-Jul-2008	SK079
Deltocephalinae	*Hishimonus araii*	Torihama, Kinko-cho, Sata, Kagoshima	14-Jul-2008	SK115
Deltocephalinae	*Nephotettix cincticeps*	Kyushu Univ., Motooka, Fukuoka, Fukuoka	15-Jun-2009	SK172
Deltocephalinae	*Nephotettix cincticeps*	Kyushu Univ., Motooka, Fukuoka, Fukuoka	15-Jun-2009	SK193
Deltocephalinae	*Nephotettix nigropictus*	Nghe An Province, Nghia Dan Distric, VIETNAM	20-May-2009	SK196
Deltocephalinae	*Orosius orientalis*	Okitsu Seashore, Sanuki, Kagawa	15-Jun-2008	SK139
Deltocephalinae	*Phlogotettix cyclops*	Inokodani, Hamanose, Kobayashi, Miyazaki	15-Jul-2008	SK084
Deltocephalinae	*Psammotettix kurilensis*	Kitahama, Abashiri, Hokkaido	06-Jul-2009	SK221
Deltocephalinae	*Psammotettix kurilensis*	Minato-cho, Rankoshi, Hokkaido	12-Jul-2009	SK227
Deltocephalinae	*Psammotettix kurilensis*	Takeura, Shiraoi, Hokkaido	14-Jul-2009	SK229
Deltocephalinae	*Psammotettix kurilensis*	Toetoko, Yubetsu, Hokkaido	09-Jul-2009	SK222
Deltocephalinae	*Psammotettix kurilensis*	Uehira, Tomamae, Hokkaido	10-Jul-2009	SK223
Deltocephalinae	*Psammotettix striatus*	Sasabaru, Taku, Saga	05-Jun-2009	SK194
Deltocephalinae	*Psammotettix striatus*	Sasabaru, Taku, Saga	05-Jun-2009	SK199
Deltocephalinae	*Psammotettix striatus*	Tanezaki Seashore, Kochi, Kochi	23-Aug-2009	SK237
Deltocephalinae	*Psammotettix striatus*	Tanezaki Seashore, Kochi, Kochi	23-Aug-2009	SK239
Deltocephalinae	*Psammotettix striatus*	Tei Seashore, Ya-Sea Park, Kochi, Kochi	24-Aug-2009	SK238
Deltocephalinae	*Psammotettix striatus*	Tei Seashore, Ya-Sea Park, Konan, Kochi	24-Aug-2009	SK241
Deltocephalinae	*Psammotettix striatus*	Tsuda Pine Forest, Sanuki, Kagawa	15-Sep-2008	SK145
Deltocephalinae	*Psammotettix striatus*	Kure, Cheju Is., KOREA	30-May-2000	SK149
Deltocephalinae	*Psammotettix striatus*	Kure, Cheju Is., KOREA	30-May-2000	SK151
Deltocephalinae	*Scaphoideus festivus*	Kokose, Hamanose, Kobayashi, Miyazaki	15-Jul-2009	SK135
Deltocephalinae	*Scaphoideus* sp.	Hanase, Kinko-cho, Sata, Kagoshima	14-Jul-2009	SK099
Deltocephalinae	*Yamatotettix flavovittatus*	Kokose, Hamanose, Kobayashi, Miyazaki	15-Jul-2008	SK155
Evacanthinae	*Epiacanthus* sp.	Tatsukoma Pass, Ooro, Okuizumo, Shimane	16-Jun-2009	SK177
Evacanthinae	*Kurotsuyanus* sp.	Kiriishi, Mt. Shaka, Toho, Fukuoka	30-May-2008	SK010
Evacanthinae	*Pagaronia harpagonis*	Inokodani, Hamanose, Kobayashi, Miyazaki	18-Jun-2004	SK078
Evacanthinae	*Pagaronia protecta*	Mt. Dogo, Saijo, Shobara, Hiroshima	16-Jun-2009	SK192
Evacanthinae	*Pagaronia yabemurensis*	Omogo Vall., Kuma-kogen, Ehime	17-Jun-2009	SK178
Evacanthinae	*Sophonia orienatalis*	Hamajiri, Minami-Osumi, Sata, Kagoshima	14-Jul-2008	SK070
Iassinae	*Batracomorphus* sp.	Kyushu Univ., Hakozaki, Fukuoka, Fukuoka	02-Jul-2008	SK021
Idiocerinae	*Idioscopus yanonis*	Minami-kisashi, Obama, Nagasaki	23-May-2008	SK015
Ledrinae	*Ledra auditua*	Mt. Takanosu, Mts. Hiko, Soeda, Fukuoka	10-Jul-2008	SK058
Ledrinae	*Tituria angulata*	Sihjhongsi, Pingdong, TAIWAN	13-Mar-2002	SK032
Macropsinae	*Macropsis* sp.	Mt. Sanbe, Koyahara, Ota, Shimane	16-Jun-2009	SK197
Macropsinae	*Macropsis* sp.	Mt. Sefuri, Kanzaki, Saga	13-Jun-2009	SK003
Macropsinae	*Oncopsis omogonis*	Yoshiwa, Hatsukaichi, Hiroshima	07-Jun-2008	SK013
Penthimiinae	*Penthimia nitida*	Mt. Ge-zan, Shimonoseki, Yamaguchi	04-Jun-2008	SK035
Selenocephalinae	*Drabescus pallidus*	Mt. Takanosu, Mts. Hiko, Soeda, Fukuoka	10-Jul-2008	SK055
Selenocephalinae	*Waigara boninesis*	Kyushu Univ., Hakozaki, Fukuoka, Fukuoka	02-Jul-2008	SK009
Tartessinae	*Tartessus ferrugineus*	Shiramizu, Ishigaki Is., Okinawa	8-15-May-2008	SK056
Typhlocybinae	*Apheliona ferruginea*	Kyushu Univ., Motooka, Fukuoka, Fukuoka	10-Jul-2008	SK143
Typhlocybinae	*Dikraneura orientalis*	Ikenohara, Mt. Unzen, Nagasaki	23-May-2008	SK005
Typhlocybinae	*Limmasolla multipunctata*	Kametsuru, Sanuki, Kagawa	17-Apr-2008	SK158
Typhlocybinae	*Tautoneura mori*	Mt. Koba, Minami-Osumi, Sata, Kagoshima	14-Jul-2008	SK160
Typhlocybinae	*Tautoneura mori*	Kametsuru, Sanuki, Kagawa	17-Apr-2008	SK163
Typhlocybinae	*Tautoneura mori*	Kametsuru, Sanuki, Kagawa	17-Apr-2008	SK162
Xestocephalinae	*Xestocephalus iguchii*	Mt. Dakenotsuji, Gonoura, Iki Is., Nagasaki	27-May-2008	SK165
Xestocephalinae	*Xestocephalus japonicus*	Kokose, Hamanose, Kobayashi, Miyazaki	15-Jul-2008	SK113
Delphacinae	*Nilaparvata lugens*	Nghe An Province Nghia Dan Distric, VIETNAM	20-May-2009	SK205

The DNA was resuspended in 50 µl of fresh Buffer AE solution and stored at -25 °C. The 'barcode' region of COI was amplified using the universal primer combination (Folmer *et al*., 1994), LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3'). Each 15 µl reaction mixture contained 1.50 µl sample DNA template, 1.50 µl 10×Ex Taq Buffer, 1.50 µl 2.5M dNTP Mixture, 4.5 µl 1.6 pmol/µl primers (LCO1490 and HCO2198), 0.04 µl TaKaRa Ex Taq (TaKaRa: RR001A) and 1.50 µl miriWater. The PCR temperature cycles consisted of an initial 60 s denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 60 s, primer annealing across a 48°C temperature gradient for 90 s and elongation for 90 s at 72°C. A final 5 min incubation at 72 °C allowed for the completion of any partially synthesized strands. These amplifications were treated with the ExoSAP-IT treatment by one cycle of 37 °C for 31 min and 80 °C for 15 min. The cycle sequence reaction used BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems: 0905075) by an initial 30 s at 94°C, followed by 25 cycles of 96 °C for 30 s and 50 °C for 30 s, and 50°C for 4 min. Individual sequencing products were mixed with 2.7 µl 3µlM Sodium acetate and 25 µl 99.5 % ethanol. These were incubated at room temperature for 15 min and then centrifuged at 15,000×g for 10 min. The resulting supernatant was removed, and the pellet was washed with 250 µl of ice-cold 70% ethanol and centrifuged for a further 5 min at 15,000×g. Again the supernatant was discarded, the pellet allowed to air-dry and stored at −20°C. The sequencing products were separated using 3100 Genetic Analyzer (Applied Biosystems).

Alignment of the sequences was carried out using MEGA 4.0.2 (Tamura *et al*., 2007). Construction of neighbor-joining (NJ) trees under the Kimura 2-Parameter model was also analyzed by MEGA 4.0.2. A bootstrap (500 replicates) NJ analysis was also performed. A semi-strict consensus tree of the 500 bootstrap most-parsimonious (MP) trees was generated with the close-neighbor-interchange method also by MEGA 4.0.2.

One species of Delphacidae (Hemiptera, *Nilaparvata lugens* Stål) was included as the out-group for the analysis with all cicadellid species. The cicadelline species, *Bothrogonia ferruginea* (Fabricius), was used as the out-group for the analyses of Deltocephalinae, Evacanthinae and Typhlocybinae.

Results

All cicadellid and one delphacid species were successfully amplified. The sequences were aligned to 498 base pairs (bp), although the basic sequences of a DNA barcode is 648 bp.

Bootstraps of clades in the NJ tree (Fig. 1) were very low except those of *Psammotettix striatus* (Linnaeus) (99%), *Psammotettix kurilensis* Anufriev (99%), *Psammotettix* Haupt (99%), *Scaphoideus* Uhler (99%), *Tautoneura mori* (Matsumura) (99%), *Japanagallia pteridis* (Matsumura) (99%) and *Xestocephalus* Van Duzee (98%). Other highly supported clades coincided with taxonomic relationships: *Albicostella albicosta* (Matsumura) + *Hishimonus araii* Okada (83%), *Albicostella kiushiuensis* Vilbaste + *Nephotettix cincticeps* (Uhler) (99%) and *Tartessus ferrugineus* (Walker) + *Tautoneura mori* (Matsumura) (99%). In contrast, bootsraps of most clades in semi-strict MP tree (Fig. 2) are highly supported. However, the phylogenetic relationships by the NJ and MP trees are very similar to each other.

COI sequences in the four species represented by two or more individuals were identical (Figs. 1-3, 5): *Japanagallia pteridis, Psammotettix kurilensis, Psammotettix striatus* and *Tautoneura mori*. However, only the sequences of *Nephotettix cinticeps* were not similar to each other.

The sequences in the five genera represented by two or three species were most similar to other sequences of the same species (Figs. 1-4): *Hecalus* Stål, *Psammotettix*, *Scaphoideus*, *Pagaronia* Ball and *Xestocephalus* Van Duzee. *Psammotettix*, *Scaphoideus*, *Pagaronia* and *Xestocephalus* were monophyletic and highly supported (NJ/MP: 99%/98% in Fig. 3, 100%/99% in Fig. 3, 99%/93% in Fig. 4, 98%/100% in Fig. 1, respectively). *Hecalus* was also monophyletic but weakly supported both in the NJ and semi-strict MP trees (70%/100% in Figs. 1-2, less than 70%/50% in Fig. 3). In contrast, two sequences of *Nephotettix* Matsumura and *Macropsis* Lewis were polyphyletic and not similar to each other (Figs. 1-3).

The subfamilies Xestocephalinae and Macropsinae represented by one or two genera in this study were monophyletic (98%/100% in Figs. 1-2, less than 70%/100% in Figs. 1-2, respectively). But, most subfamilies represented by several genera were polyphyletic: Aphrodinae, Deltocephalinae, Evacanthinae, Ledrinae, Selenocephalinae and Typhlocybinae.

Discussion

The test of identification for the Japanese cicadellid species by DNA barcoding is whether or not any sequences are found in two species; none was in this study. The next test is whether or not the differences within species are

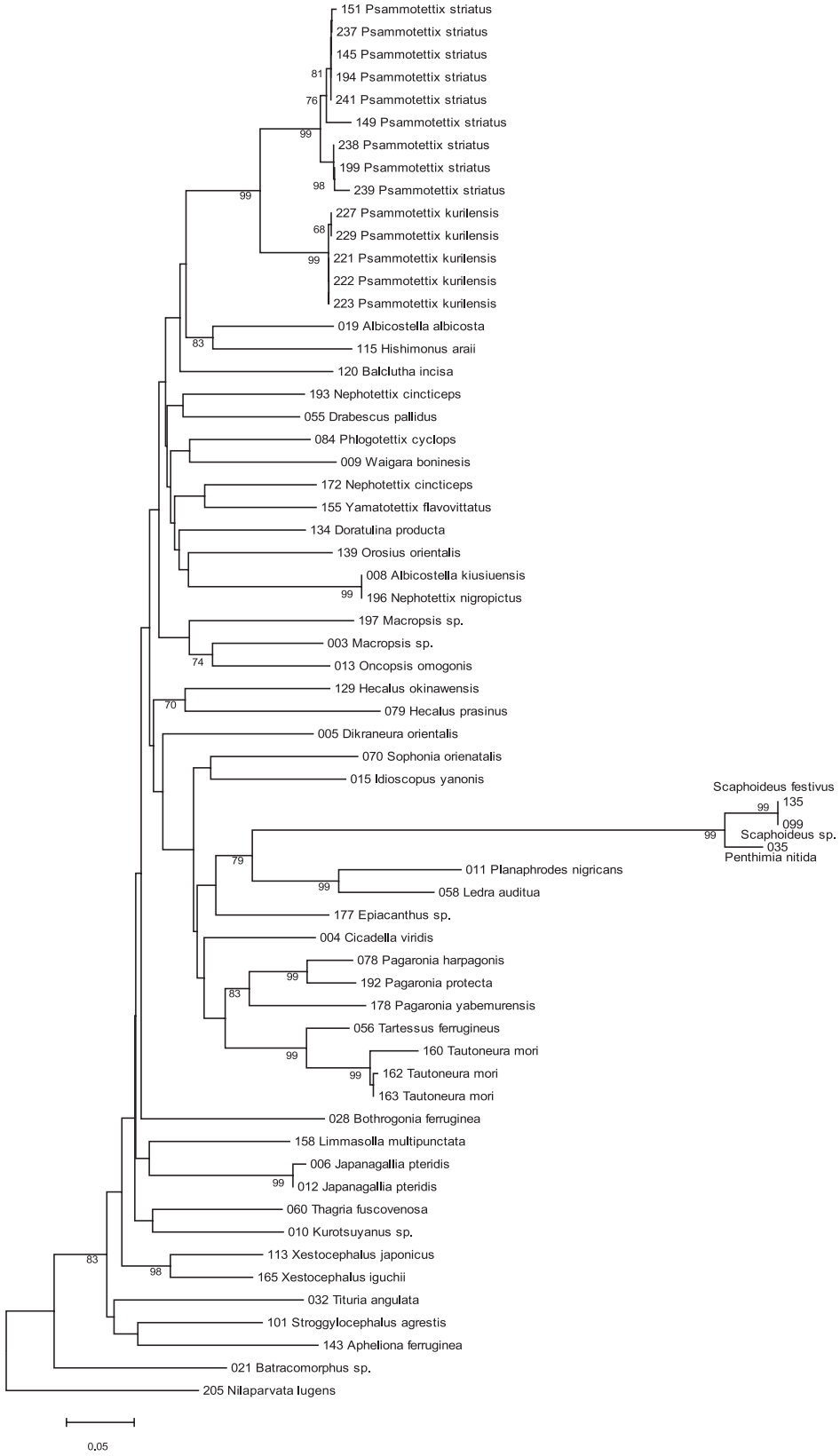

Fig. 1. Neighbour-joining (NJ) tree of Kimura-two-parameter (K2P) distances for 63 cytochrome oxidase subunit I (COI) gene sequences from 45 species of Japanese Cicadellidae. Numbers given at branches refer to bootstrap proportions among 500 bootstrap replicates. Out-group consists of one species of Delphacidae (*Nilaparvata lugens*). Evolutionary distance divergence scale bar is 0.05.

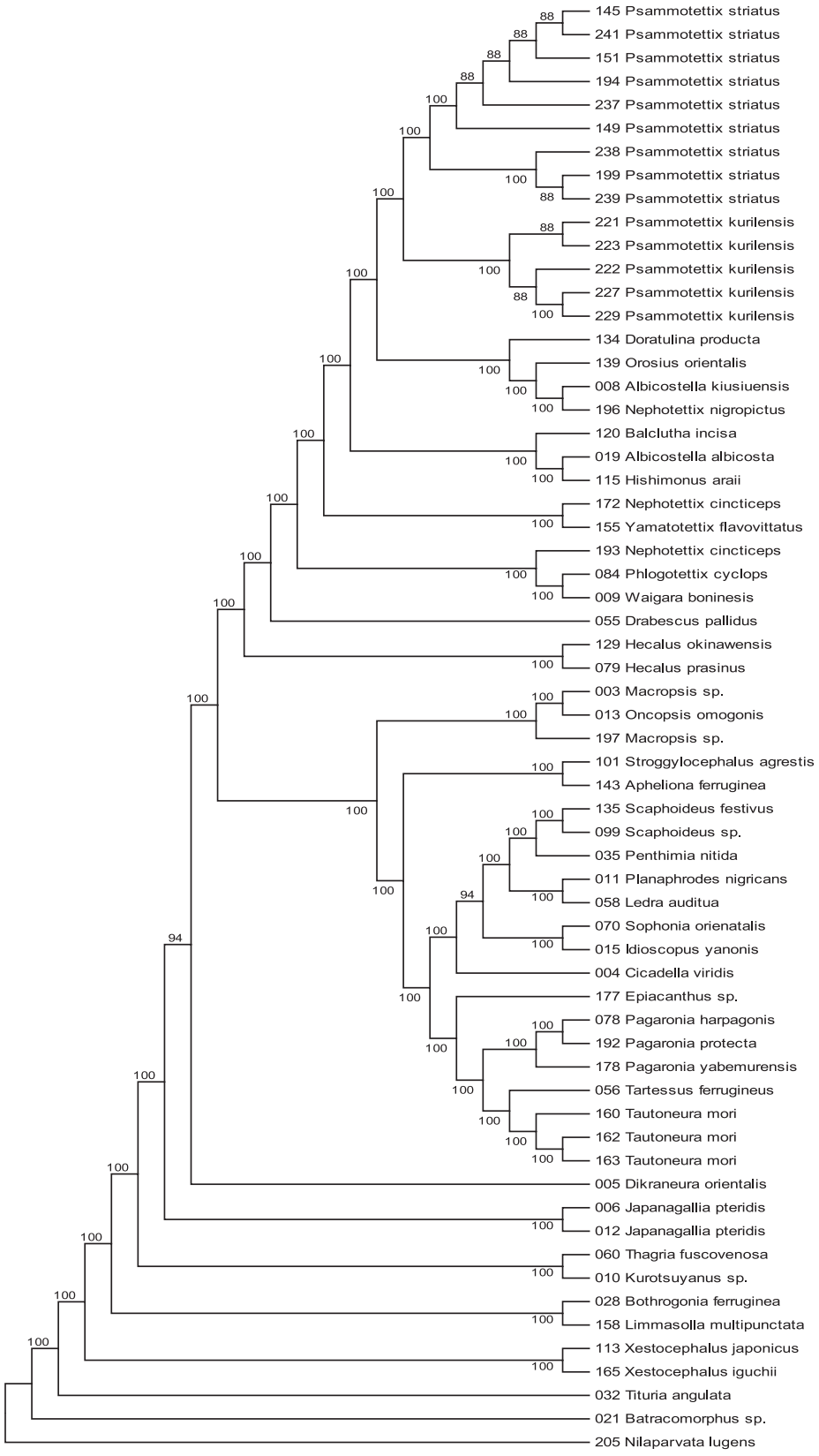

Fig. 2. Semi-strict most-parsimonious (MP) tree for 63 cytochrome oxidase subunit I (COI) gene sequences from 45 species of Japanese Cicadellidae. Numbers given at branches refer to bootstrap proportions among 500 bootstrap replicates. Out-group consists of one species of Delphacidae (*Nilaparvata lugens*).

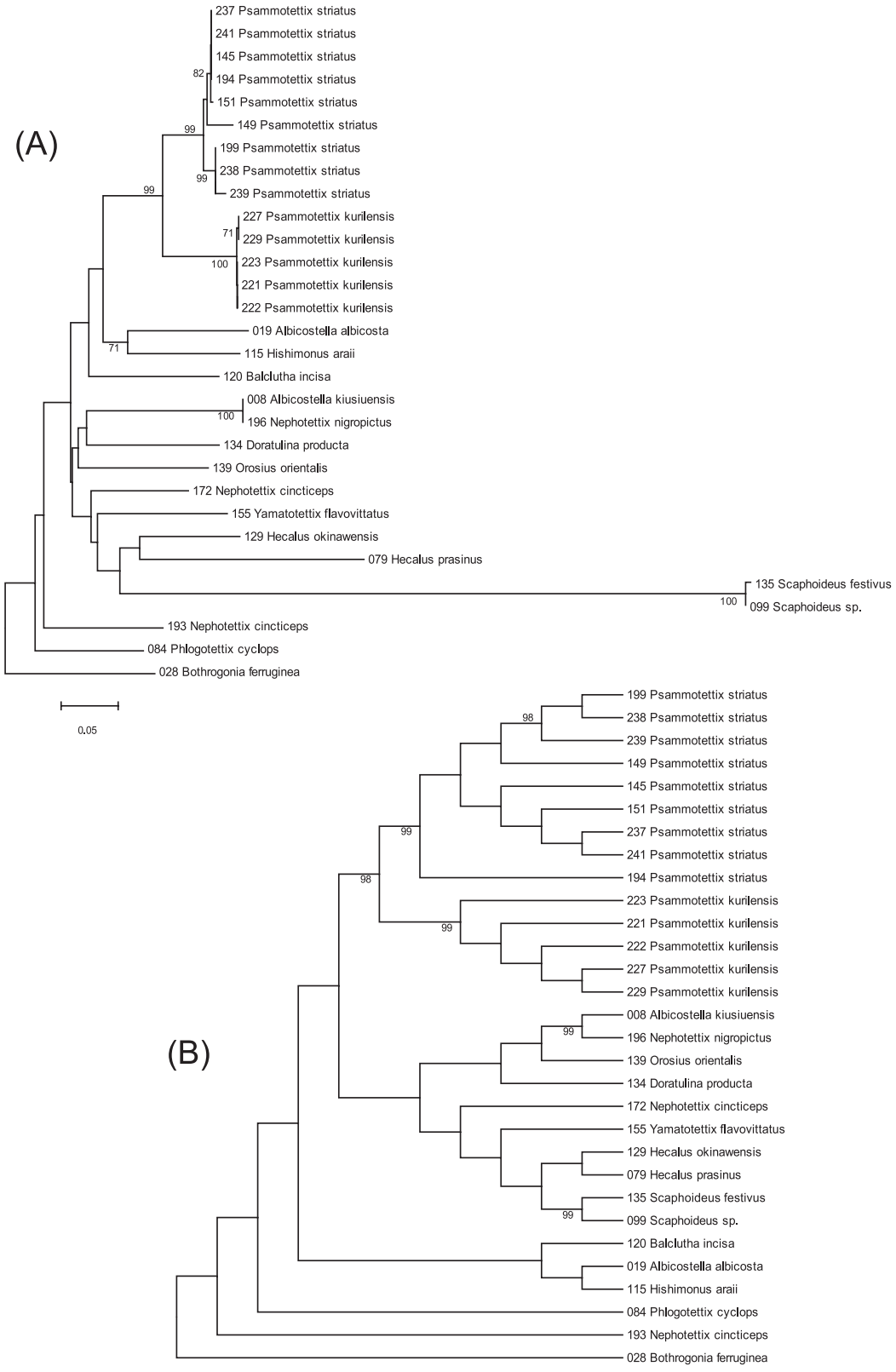

Fig. 3. NJ (A) and semi-strict MP (B) trees from 15 species of Japanese Deltocephalinae. Numbers given at branches refer to bootstrap proportions among 500 bootstrap replicates. Out-group consists of one species of Cicadellinae (*Bothrogonia ferruginea*). Evolutionary distance divergence scale bar in the NJ tree is 0.05.

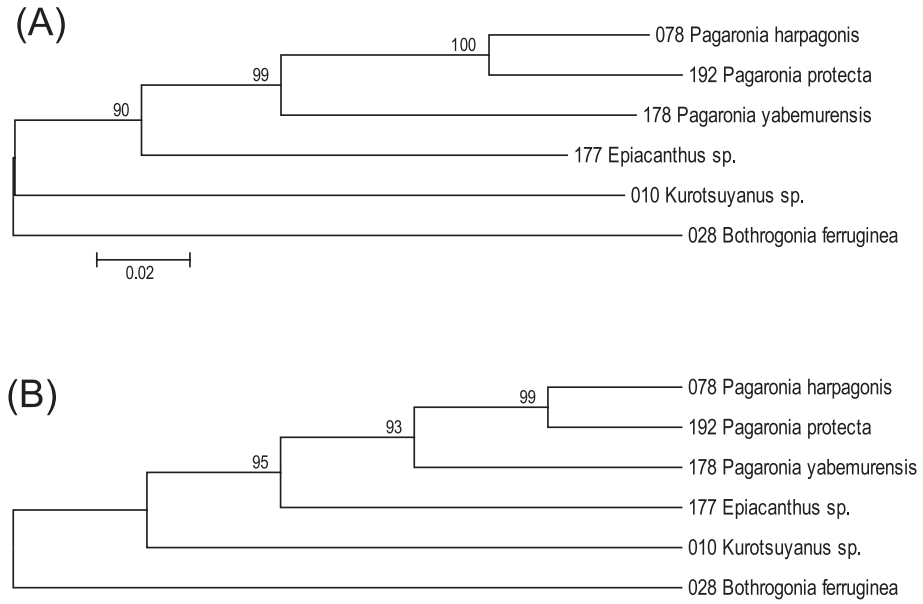

Fig. 4. NJ (A) and semi-strict MP (B) trees from five species of Japanese Evacanthinae. Numbers given at branches refer to bootstrap proportions among 500 bootstrap replicates. Out-group consists of one species of Cicadellinae (*Bothrogonia ferruginea*). Evolutionary distance divergence scale bar in the NJ tree is 0.02.

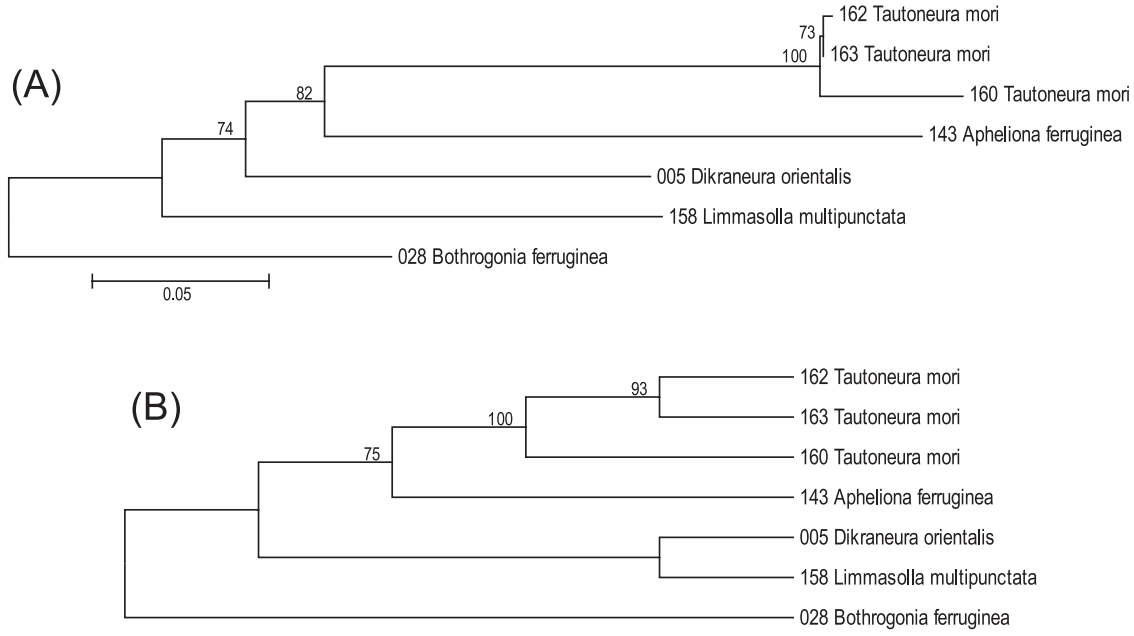

Fig. 5. NJ (A) and semi-strict MP (B) trees from four species of Japanese Typhlocybinae. Numbers given at branches refer to bootstrap proportions among 500 bootstrap replicates. Out-group consists of one species of Cicadellinae (*Bothrogonia ferruginea*). Evolutionary distance divergence scale bar in the NJ tree is 0.05.

much less than those among species. In this study, COI differences among most of the cicadellid species far exceeded those within species. In *Psammotettix striatus*, the DNA barcodes of Korean specimens (SK149 and SK151) were almost the same as those of Japanese specimens (SK145, SK194, SK237, SK241). The two barcodes of *Nephotettix cincticeps* (SK172 and SK193) were quite different, although these were collected at the same locality and date. This may be caused by bad sequences. However, most DNA barcodes of the Japanese leafhoppers should be available to identify the species.

To identify Japanese cicadellid genera, the species and genera with DNA barcodes are too few. Therefore, at the present, it is difficult to identify a genus with the BOLD Identification System (IDS) for All Barcode Records on BOLD (http://www.boldsystems.org/views/login.php). For example, if the barcode of *Nephotettix cincticeps* (SK172) were searched with IDS for All Barcode Records on BOLD, the highest search result is *Orosius canberrensis* (Hemiptera, Auchenorrhyncha, Cicadellidae, Deltocephalinae, 84.75% of specimen similarity). This result shows that IDS is available at the subfamily level. However, the highest search result of *Pagaronia yabemurensis* Okada was *Oncometopia dispar* Fowler (Hemiptera, Auchenorrhyncha, Cicadellidae, Cicadellinae, 83.97% of specimen similarity). This result shows that IDS is not available at the subfamily level.

In the phylogenetic relationship among *Tautoneura mori* (SK160, SK162 and SK163, Fig. 5), the genetic distance between SK160 and SK162+163 was very large, comparing barcode differences within species such as *Japanagallia pteridis* and *Psammotettix kurilensis*. Two of the three specimens of *T. mori* were collected at the same locality on the same date, but their host plants were different. The host plant of SK162 and SK163 was *Morus* sp. (Moraceae), which is one of its ordinary hosts. The host plant of SK160 was *Aphananthe aspera* (Thunb.) Planch (Cannabaceae), which is a new host tree for this species. Furthermore, the coloration and some morphological features of male genitalia of SK160 were slightly different from the typical *T. mori* including SK162 and SK163. This might suggest that SK160 is a sibling species of *T. mori*.

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