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Taxonomy and evolution of putative thelytokous species of *Leptopilina* (Hymenoptera: Figitidae) from Japan, with description of two new species

Nakatada WACHI^{1,2,3}, Fumiaki Y. NOMANO⁴, Hideyuki MITSUI⁴, Nazuki KASUYA⁵ and Masahito T. KIMURA⁴

¹*Biosystematics Laboratory, Graduate School of Social and Cultural Studies, Kyushu University, Fukuoka, Japan,* ²*Center for Asian Conservation Ecology, Kyushu University, Fukuoka, Japan,* ³*JT Biohistory Research Hall, Takatsuki, Japan,* ⁴*Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan* and ⁵*Graduate School of Science and Engineering, Tokyo Metropolitan University, Hachioji, Japan*

Correspondence: Masahito T. Kimura, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan.

Email: mtk@ees.hokudai.ac.jp

Abstract

Wasps of the genus *Leptopilina* (Hymenoptera: Figitidae) are larvo-pupal parasitoids of *Drosophila* species. Here, we report three putative thelytokous species of this genus from Japan, with description of two new species *L. tokioensis* and *L. tsushimaensis* that have been recorded from central Japan and Tsushima, respectively. Another thelytokous species is *L. longipes* occurring in northern Japan, although its European populations are assumed to be arrhenotokous. Preliminary phylogenetic analyses suggest the present three species are diversified from each other and also from the other *Leptopilina* species. These three thelytokous species are infected by B-supergroup *Wolbachia* as in European thelytokous species, *L. clavipes* and *L. australis*. The evolution of these thelytokous species is discussed based on the present and previous results, and the occurrence and distributions of *Leptopilina* species in Japan and surrounding regions are briefly reviewed.

Key words: Asia, Cynipoidea, *Drosophila*, parasitism, parthenogenesis, *Wolbachia*.

INTRODUCTION

Parasitic wasps of the genus *Leptopilina* Förster, 1869 (Hymenoptera; Figitidae) are associated with *Drosophila* species, and used as model systems in the study of parasitoid-host interactions and coevolution (Fleury *et al.* 2009; Kraaijeveld & Godfray 2009; Lee *et al.* 2009; Eslin *et al.* 2009; Prévost *et al.* 2009). Furthermore, *Leptopilina* wasps are excellent materials for the study of coevolutionary interactions between insects and *Wolbachia* bacteria, which occur in up to 66% of insect species and often induce parthenogenesis, cytoplasmic incompatibility, or feminization in host insects (Werren *et al.* 1995; Stouthamer *et al.* 1999; Hilgenboecker *et al.* 2008; Vavre *et al.* 2009). To date, 27 *Leptopilina* species have been described from Europe, Africa, North America and Asia (Nordlander 1980; Quinlan 1988; Beardsley 1988; Nordlander & Grijpma 1991; van Alphen *et al.* 1991; Allemand *et al.* 2002; Novković *et al.* 2011), and at least two species, *L. clavipes* (Hartig) and *L. australis* (Belizin), reproduce thelytokously due to *Wolbachia* infection (Vet 1983; Carton *et al.* 1986; Driessen *et al.* 1991; van Alphen *et al.* 1991; Pannebakker *et al.* 2004; Vavre *et al.* 2009). However, faunal survey of this genus is still incomplete, especially in Asian regions (Nordlander 1980; Novković *et al.* 2011).

Here, we report three (putative) thelytokous species of *Leptopilina* from Japan, with description of two new species; one new species from Tokyo, central Japan, and the other from Tsushima located between Kyushu island of Japan and Korean peninsula. Thelytoky of *L. longipes* (Hartig) from Sapporo, northern Japan and the new *Leptopilina* species from Tsushima is indicated by the absence of males in their laboratory stocks (personal observation), while that of another new species from Tokyo is assumed by the absence of male in the field sample (Kasuya *et al.* 2013; cited as *Leptopilina* sp. TK1). We

further analysed their phylogenetic relationship with other *Leptopilina* species using partial nucleotide sequences of the mitochondrial *cytochrome oxidase subunit 1* (*CO1*) gene and ribosomal internal transcribed spacer 2 (ITS2). In addition, we preliminarily determined the infection with *Wolbachia* bacteria in the present putative thelytokous and some other *Leptopilina* species by checking the presence/absence of the *Wolbachia* surface protein gene (*wsp*) and the filamenting temperature sensitive gene Z (*ftsZ*), since B-supergroup *Wolbachia* have been reported to cause thelytoky in *L. clavipes* and *L. australis* (Vavre *et al.* 2009). We also give a brief review on the distributions of *Leptopilina* species in Asia.

MATERIALS AND METHODS

Sample collection

The wasps were collected from three localities in Japan; Sapporo (SP: 43°3' N, 141°21' E) in August, 2012 (4 times), Tokyo (TK: 35°40' N, 139°46' E) in September and October, 2009 (8 times), and Tsushima (TS: 34°12' N, 129°17' E) in June, 2011 (one time). In Sapporo and Tsushima, traps baited with the banana were set in forests and domestic environments (around areas) for a week, and banana in the traps was brought back to the laboratory and placed in plastic containers. When drosophilid larvae in banana pupated, they were collected and placed in Petri dishes. Parasitoid individuals that had emerged from drosophilid puparia were collected and reserved in 100% ethanol or reared in the laboratory. In the laboratory rearing, wasps were placed in vials containing *Drosophila* medium and 100-200 host larvae, and wasps that emerged from the vials were transferred into new vials with *Drosophila* medium and host larvae. As host in the laboratory rearing, *D. simulans* Sturtevant was used for *L. longipes*, and *D. auraria* Peng or *D. lutescens* Okada was used for the *Leptopilina*

species from Tsushima. In Tokyo, collections were conducted in forests and groves in the same way except that mushrooms were used as bait (see Kasuya *et al.* 2013 for details of collection methods). As above, adult parasitoid wasps emerged from the drosophilid puparia were immersed in 100% ethanol. We failed to rear this species in the laboratory.

Taxonomic analyses

The external structure of the dry-mounted wasp specimens was studied with binocular stereomicroscopes (model SZ60, Olympus, Tokyo, Japan; model MZ12, Leica, Solms, Germany, fitted with model DS-L1, Nikon, Tokyo, Japan), and the lengths of the forewing and hind tibia were measured using an ocular micrometer. Some specimens were gold-coated with a sputter coater and examined with a scanning electron microscope (model JSM-5600LV, JEOL, Tokyo, Japan). The forewing of a female wasp was slide-mounted in Euparal.

The following morphological abbreviations are used: ML, length of mesosoma; HH, head height; ED, diameter of compound eye; POL, the postocellar line (the distance between the inner edges of the two lateral ocelli); OOL, the ocular-ocellar line (the distance from the outer edge of a lateral ocellus to the compound eye); and LOL, the lateral-ocellar line (the distance between lateral and anterior ocelli). Morphological terminology follows Richards (1977), Ronquist & Nordlander (1989), Fontal-Cazalla *et al.* (2002), Forshage & Nordlander (2008), and Liljeblad *et al.* (2008), and cuticular surface terminology follows Harris (1979). Three carinae on the metapleuron are referred to as ridges I, II, and III following Nordlander (1980).

Molecular analyses

The phylogenetic position of the present three thelytokous species was determined based on the *CO1* and ITS2 sequences. In addition, *Wolbachia* infection was examined for these three species and some other *Leptopilina* species (*L. japonica japonica* Novković & Kimura from Tokyo, *L. japonica formosana* Novković & Kimura from Taipei, *L. ryukyuensis* Novković & Kimura and *L. pacifica* Novković & Kimura from Iriomote-jima) by checking the presence/absence of the two *Wolbachia* genes *wsp* and *ftsZ* in DNA samples from them (i.e., whether the specific primers for these genes successfully amplify these genes or not). Two individuals were examined for each species.

Extraction of genomic DNA and PCR were performed following Novković et al. (2011). Amplification of *CO1*, ITS2 and *wsp* fragments was performed using the following primer sets: 5'-GGTCAACAAATCATAAAGATATTGG-3' (LCO) and 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (HCO) for *CO1* (about 600 bp) (Folmer et al. 1994), 5'-TGTCAACTGCAGGACACATG-3' and 5'-AATGCTTAAATTTAGGGGGTA-3' for ITS2 (448–564 bp), and 5'-TGGTCCAATAAGTGATGAAGAAAC (81F), 5'-AAAAATTAAACGCTACTCCA (691R) for *wsp* (about 600 bp) (Braig et al. 1998). For samples for which the 81F-691R primer pair failed to amplify in PCR, the following primer pairs were used to judge whether the samples were infected by *Wolbachia*;

5'-GGATCCGGGTCCAATAAGTGATGAAGAAAC-3'(wsp1) and 5'-GGATCCTTAAAACGCTACTCCAGCTTCTGC-3'(wsp2) for *wsp* (about 600 bp), and 5'-GTATGCCGATTGCAGAGCTTG-3' (fts1) and 5'-GCCATGAGTATTCACTTGGCT-3' (fts2) for *ftsZ* (about 750 bp) (Kondo et al. 1999). The DNA sequencing reaction was performed using an ABI BigDye Terminator Cycle version 3.1 sequencing kit (Applied

Biosystems, Foster City, CA, USA) following manufacturer's instructions and analyzed on an ABI PRISM 3100, using the same primers as for PCR amplification.

The obtained sequences were aligned and manually corrected using MEGA ver5.1 (Tamura *et al.* 2011). Neighbor-joining (NJ) trees (Saitou & Nei 1987) were constructed using MEGA based on Kimura's two-parameter distance (Kimura 1980). In the NJ analysis, indels were excluded by the partial deletion option with the site coverage cutoff value of 90% implemented in MEGA. On the other hand, maximum likelihood (ML) analysis and Bayesian inference (BI) were performed without exclusion of indels. The model selection for ML and BI was performed according to the Akaike information criterion (AIC) in JMODELTEST2 ver2.1.3 (Darriba *et al.* 2012). The best-fit substitution model was General time reversible model (GTR) with a heterogeneity of the rate among sites modeled using a gamma distribution (+ Γ) and a proportion of invariable sites (+I) for the *CO1* dataset, and transversional model (TVM) + Γ for the ITS2 dataset. The model parameters (*CO1*/ITS2) were as follows: nucleotide frequencies, A=0.3287/0.3733, C=0.1219/0.1254, G=0.1408/0.1389, T=0.4085/0.3624; substitution rates AC=0.082/0.8081, AG = 5.377/2.0375, AT=1.4664/1.7448, CG=0.8625/0.1538, CT=1.6135/2.0375. GT=1.00/1.00; P-inv=0.381/-; gamma shape =0.657/0.646. ML tree were constructed using PHYML ver3.0 (Guindon & Gascuel 2003) with NNI for tree search. For NJ and ML trees, bootstrapping was performed with 1000 replicates. BI analyses were performed using MRBAYES ver3.2.1 (Ronquist *et al.* 2012). The general GTR + Γ model (nst=6; rates=gamma) was used for the ITS2 dataset because the TVM model is not implemented in MRBAYES. BI analyses were executed for two replicates of 2000,000 generations each (four chains per replicate run) with trees sampled every 1000 generations. The first 25% of the trees were discarded as burn-in periods by default. The sequences of other *Leptopilina* species, *Ganaspis xanthopoda*

(Ashmead) (an outgroup species) and other *Wolbachia* strains were obtained from the NCBI database, and used for the reconstruction of phylogenetic trees (the accession numbers of the sequences are shown in Figs 14, 15). When multiple sequences were available for a species, two sequences were chosen for each species to avoid redundancy of the trees.

RESULTS

Taxonomy

***Leptopilina longipes* (Hartig, 1841) (Figs 1-13, A)**

Cothonapsis longipes Hartig, 1841, Z. Ent. (Germar), **3**: 356.

Leptopilina longipes – Förster, 1869, Verh. zool. -bot. Ges. Wien, **19**: 348.

Specimens examined. 8 ♀, Sapporo, Hokkaido, Japan, viii.2012, leg. M. T. Kimura.

Remarks. Individuals from Sapporo coincide with the description and illustration of European specimens of this species by Nordlander (1980). In Europe, males were frequently collected (Nordlander 1980), but no male was collected in Sapporo (personal observation), and no male was produced in our laboratory rearing of this species from Sapporo. This is the type species of *Leptopilina*.

Host. Natural host species *Drosophila nigromaculata* Kikkawa & Peng.

***Leptopilina tokioensis* Wachi & Kimura, sp. nov. (Figs 1-13, B)**

Type specimens. *Holotype*: ♀, Tokyo, Japan, in vi.2009, leg. N. Kasuya; *Paratypes*: 8 ♀, same data as holotype. The specimens are deposited in Systematic Entomology, Hokkaido University Museum, Hokkaido University, Sapporo, Japan (SEHU).

Diagnosis. This species is distinguished from other *Leptopilina* species by a combination of the following characteristics; elongated oval scutellum plate, the lack of ridge I on metapleuron, and the presence of projection at the posterior margin of metapleuron.

Holotype Female. Head dark brown to black except for light brown pedicel, scape, and flagellomeres 1-5; mesosoma light brown except for dark brown mesoscutum; metasoma brown anteroventrally, dark brown posterodorsally.

Head 1.1× as broad as high in anterior view (Fig. 1B), as broad as mesosoma in dorsal view. HH: ED: ML=3: 2: 4. POL: OOL: LOL=11: 4: 5. Area between antennal rims with sparse setae; distance between antennal rims as broad as distance between antennal rim and inner edge of compound eye, vertical distance between antennal rim and ventral margin of clypeus 2.8× as long as distance between antennal rim and anterior ocellus; vertical carina adjacent to ventral margin of antennal rim absent; malar sulcus present; ventral area of clypeus with long setae (Figs 1B, 2B). Antenna 13-segmented; relative lengths of flagellomeres 1-11: 0.8, 1, 0.8, 0.9, 0.9, 1, 1, 1, 1, 1, 1; flagellomeres 5-11 cylindrical, wider than flagellomeres 1-4 (Fig. 3B).

Pronotum with long setae anteriorly (Fig. 4B); pronotal plate 0.3× as wide as mesonotum in anterior view; anterior flange of pronotal plate protruding and transversely strigate; submedian pronotal depressions laterally open; dorsal margin of pronotal plate distinctly emarginate; ridges extending from lateral margin of pronotal plate distinct, but not extending to the dorsal margin of pronotum (Fig. 5B). Mesoscutum smooth and polished; notaulus absent; parascutal carina present; scutellum with two foveae at base, reticulate except for scutellar plate; scutellar plate elongated oval with four setae from sockets; posterior one-thirds of scutellar plate with a triangle glandular pit (Figs 4B, 6B). Metapleuron with one ridge, ridge I absent, ridge II present but indistinct, ridge III distinct

in posterior half; metapleural triangle indistinct laterally; posterior margin of metapleuron projecting posterolaterally in middle. (Figs 7B, 8B). Propodeum pubescent; propodeal carinae nearly parallel (Fig. 9B).

Marginal cell of forewing closed on anterior margin, 2.9× as long as broad, wing surface closely ciliated, ciliation on outer margin long (Fig. 10B).

Metasoma shiny; petiole with a posterior rim; metasomal tergum II with hairy ring having wide break dorsally (Figs 11B, 12B).

Length of forewing 1.45mm, of hind tibia 0.45mm.

Male. Unknown.

Variation. Length of forewing (mean $\pm$ SD): 1.25-1.45 (1.41 ± 0.079) mm (n=9); length of hind tibia: 0.38-0.45 (0.41 ± 0.037) mm.

Etymology. Named for its occurrence in Tokyo, Japan. Tokio is an alternative spelling for Tokyo.

Host. Natural host species *Drosophila bizonata* Kikkawa & Peng.

Remarks. This species has been treated as *Leptopilina* sp. TK1 by Kasuya et al. (2013).

***Leptopilina tsushimaensis* Wachi & Kimura, sp. nov.** (Figs 1-13, C)

Type specimens. *Holotype:* ♀, Tsushima, Japan, in vi.2011, leg. H. Mitsui; *Paratypes:* 5 ♀, same data as holotype. The specimens are deposited in SEHU.

Diagnosis. This species is distinguished from other *Leptopilina* species by a combination of the following characteristics; ridges I and II on metapleuron distinct in posterior half, posterior margin of metapleuron without process, and metasomal tergum II with hairy ring having wide break dorsally.

Holotype Female. Head dark brown to black except for brown antenna and brown mandible;

mesosoma dark brown to black except for brown legs; metasoma dark brown to black.

Head 1.1× as broad as high in anterior view (Fig 1C), as broad as mesosoma in dorsal view. HH: ED: ML=10: 7: 12. POL: OOL: LOL=5: 2: 3. Area between antennal rims with sparse setae; distance between antennal rims 0.7× as broad as distance between antennal rim and inner edge of compound eye, vertical distance between antennal rim and ventral margin of clypeus 3.4× as long as distance between antennal rim and anterior ocellus; vertical carina adjacent to ventral margin of antennal rim present; malar sulcus present; ventral area of clypeus with long setae (Figs 1C, 2C). Antenna 12-segmented; relative lengths of flagellomeres 1-10: 1.2, 0.9, 0.8, 0.9, 1, 1, 1, 0.9, 1, 1.1; flagellomeres 5-10 cylindrical, wider than flagellomeres 1-4 (Fig. 3C).

Pronotum with long setae anteriorly (Fig. 4C); pronotal plate 0.3× as wide as mesonotum in anterior view; anterior flange of pronotal plate protruding, central area transversely strigate; submedian pronotal depressions laterally open; dorsal margin of pronotal plate almost flattened; ridges extending from lateral margin of pronotal plate distinct, but not extending to the dorsal margin of pronotum (Fig. 5C). Mesoscutum smooth and polished; notaulus absent; parascutal carina present; scutellum with two foveae at base, reticulate except for scutellar plate; scutellar plate oval with 3 setae from sockets; posterior half of scutellar plate with a large granular pit (Figs 4C, 6C). Metapleuron with three ridges, ridge III complete, ridges I and II distinct in posterior half; metapleural triangle present (Figs 7C, 8C). Propodeum pubescent; propodeal carinae nearly parallel (Fig. 9C).

Marginal cell of forewing closed on anterior margin, 2.6× as long as broad, wing surface closely ciliated, ciliation on outer margin long (Fig. 10C).

Metasoma shiny; petiole with a posterior rim; metasomal tergum II with hairy ring having wide break dorsally (Figs 11C, 12C).

Length of forewing 1.13mm, of hind tibia 0.50mm.

Male. Unknown.

Variation. Length of forewing (mean $\pm$ SD): 1.13-1.75 (1.48 ± 0.20) mm (n=6); length of hind tibia: 0.45-0.60 (0.51 ± 0.045) mm.

Etymology. Named for its occurrence in Tsushima Island, Nagasaki Prefecture, Japan.

Host. Natural host species *Drosophila lutescens* and *D. auraria* Peng.

Remarks. Yorozya (2006) collected several individuals of Figitidae from Tokamokai, Hokkaido, northern Japan, and assigned them as *Ganaspis* sp. Our morphological examination of his samples indicates that two of them are probably *L. tsushimaensis*, although they are smaller in size and lighter in body color than individuals from Tsushima. In a previous paper (Kasuya *et al.* 2013), the authors assigned these two individuals as *Leptopilina* sp. TK1 (i.e., *L. tokioensis*), but this might be miss assignment. It is noticeable that all specimens from Tsushima and Tomakomai had 12-segmented antenna in the female (females of the other *Leptopilina* species have 13-segmented antenna).

Phylogeny and Wolbachia infection

Figure 14 shows the phylogenetic trees for *Leptopilina* species based on the partial *CO1* sequence (520 bp), and Figure 15 shows those based on the ITS2 sequence (324-596 bp). The species tree topology differed by genes (*CO1* and ITS2) and by analysis methods (NJ or ML/Bi), but the *L. victoriae*–*L. japonica*–*L. ryukyuensis* and *L. heterotoma*–*L. spp. affi. heterotoma* clades were supported in all of the four trees. The present three species *L. longipes*, *L. tokioensis* and *L. tsushimaensis* were diverged from each other and also from other *Leptopilina* species in all of the four trees. *Leptopilina longipes* from Sapporo and Netherlands had similar ITS2 sequences.

The present three thelytokous species were infected by *Wolbachia* bacteria having the *wsp* sequences of B-supergroup; i.e., the *wsp* sequences of *Wolbachia* from them were clustered together with those of *Wolbachia* from not only *L. clavipes*, *L. australis* and *L. victoriae* Nordlander but also *Diplolepis rosae* (Linnaeus) (Hymenoptera: Cynipoidea: Cynipidae; *wRos*), *Tagosodes orizicolus* (Muir) (Homoptera: Delphacidae; *wOri*) and *Ephestia cautella* (Walker) (Lepidoptera: Pyralidae; *wCauB*) (Fig. 16). The *wsp* sequences from the present species were diverged to some extent from each other and also from that of *Wolbachia* from *L. clavipes* or *L. australis*. In the other study species *L. j. japonica* from Tokyo, *L. j. formosana* from Taipei, *L. ryukyuensis* from Iriomote-jima and *L. pacifica* from Iriomote-jima, *Wolbachia* was not detected by the present three primer sets; i.e., no amplification occurred in PCR for DNA samples from these *Leptopilina* species.

DISCUSSION

Of 29 described species of *Leptopilina* (including the present two species), the five species *L. clavipes*, *L. australis*, *L. longipes*, *L. tsushimaensis* and *L. tokioensis* have been indicated to reproduce thelytokously by the laboratory rearing or the absence of males in the field samples (Vet 1983; Carton *et al.* 1986; Driessen *et al.* 1991; van Alphen *et al.* 1991; Pannebakker *et al.* 2004; Vavre *et al.* 2009; Kasuya *et al.* 2013). Among them, *L. clavipes* has been reported to show geographic variation in the mode of reproduction; i.e., populations from Netherlands and mid-France are thelytokous, whereas those from Pyrenees and further south are arrhenotokous (Pannebakker *et al.* 2004). The mode of reproduction also seems to vary in *L. longipes*; individuals from Sapporo reproduce thelytokously,

whereas samples from Europe have been reported to include both females and males (Nordlander 1980).

According to the present and previous molecular phylogenetic analyses using nucleotide sequences of the *CO1* gene and ITS2 (Schilthuizen *et al.* 1998), *L. clavipes* and *L. australis* are closely related with each other, while the other three thelytokous species are rather distantly related from each other and from the other *Leptopilina* species, suggesting that the four thelytokous lineages (i.e., *longipes*, *tokioensis*, *tsushimaensis*, and *clavipes-australis*) split off at an early phase of the *Leptopilina* diversification.

All of the five thelytokous *Leptopilina* species were infected by B-supergroup *Wolbachia*, a group most commonly found among arthropod species as well as A-supergroup (Werren *et al.* 1995). In *L. clavipes*, in addition, thelytokous populations are infected by B-supergroup, whereas arrhenotokous populations are not (Pannebakker *et al.* 2004; Vavre *et al.* 2009). Thus, infection of B-supergroup *Wolbachia* plays an important role in the evolution of thelytoky in the *Leptopilina* species. However, an arrhenotokous species, *L. victoriae*, is also infected by B-supergroup *Wolbachia* (Gueguen *et al.* 2012), revealing that infection of B-supergroup *Wolbachia* does not always cause thelytoky.

Except for *L. victoriae*, infection with B-supergroup *Wolbachia* has not been reported in arrhenotokous *Leptopilina* species. In the *heterotoma* species group to which *L. victoriae* belongs, infection of A-supergroup *Wolbachia* is known in *L. heterotoma* (Thomson) and *L. guineaensis* Allemand & Nordlander (Vavre *et al.* 2009), whereas infection of *Wolbachia* has not been detected in *L. japonica*, *L. ryukyuensis*, and *L. pacifica*. These results may suggest that *L. victoriae* has acquired B-supergroup *Wolbachia* horizontally from other insect species not vertically inherited from the ancestor.

Wolbachia strains from the closely related species *L. clavipes* and *L. australis* are also closely related (Vavre *et al.*, 2009), suggesting that the infection occurred before these two *Leptopilina* species speciated. On the other hand, it is not certain when the four major thelytokous lineages (*longipes*, *tokioensis*, *tsushimaensis*, and *clavipes-australis*) were infected. In the present study, it is revealed that *Wolbachia* strains from the four major thelytokous lineages are diversified from each other as well as the host *Leptopilina* lineages. This may suggest that the infection have occurred before the diversification of the four lineages.

Through our survey in Japan, eight described have been recorded in *Leptopilina*, *L. japonica*, *L. ryukyuensis*, *L. victoriae*, *L. heterotoma*, *L. pacifica*, *L. longipes*, *L. tokioensis* and *L. tsushimaensis* (Mitsui *et al.* 2007; Novković *et al.* 2011; Kasuya *et al.* 2013; the present study). The first three species form a monophyletic lineage in the phylogenetic trees based on the *CO1* gene and ITS2. Among them, *L. japonica* is adapted to the temperate climates, while *L. ryukyuensis* and *L. victoriae* are adapted to the tropical and subtropical climates (Murata *et al.* 2013). *Leptopilina pacifica* is an oriental species occurring in the subtropical and tropical regions of Asia as well as *L. ryukyuensis* (Novković *et al.* 2011). *Leptopilina longipes* has been recorded only from Europe and northern Japan, and therefore assigned as a Palearctic species. The present two new species have been recorded only from Japan; *L. tokioensis* occurs in Tokyo (Kasuya *et al.* 2013), and *L. tsushimaensis* in Tsushima, islands located between Kyushu island of Japan and Korean peninsula, and probably in Sapporo (see *Taxonomy*).

The taxonomic status and distributions of *L. heterotoma* and its relatives are still uncertain. As shown in Fig. 14, specimens collected from Sapporo and identified as *L. heterotoma* by their external morphology have almost identical *CO1* nucleotide sequence

with *L. heterotoma* from France, indicating they are conspecific (Novković et al. 2011). On the other hand, Nordlander (1980) assigned a specimen collected from the Philippines and described as *Erisphagia philippinensis* by Kieffer in 1916 as *L. heterotoma*. In Iriomote-jima, a subtropical island of Japan, we have collected a specimen that is morphologically close to *L. heterotoma* but shows an 8.9% difference in the *CO1* nucleotide sequence from the Sapporo specimens of *L. heterotoma* (Fig. 14; cited as “Iriomote-h” in Novković et al. 2011). Except for these specimens from Iriomote-jima, no other individual that is close to *L. heterotoma* in morphology or the *CO1* nucleotide sequence has not been collected from subtropical or tropical Asia. This Iriomote specimen may be conspecific or closely related to the individual collected by Kieffer from the Philippines.

From Tokyo, we have also collected two different groups of *Leptopilina* individuals that are morphologically close to *L. heterotoma* but show 6.7–10.2% differences from the Sapporo and Iriomote-jima specimens in the *CO1* nucleotide sequence (Fig. 14, cited as Tokyo h2 and Tokyo h3 in Novković et al. 2011). The two groups show also a 5.6% difference from each other in the *CO1* nucleotide sequences. To date, however, only a few individuals of “Tokyo h3” and “Iriomote-h” have been collected. Further sampling is needed to clarify their taxonomic status.

From tropical Asia, *L. rufipes* (Cameron) and *L. cupulifera* (Kieffer) have been recorded (Nordlander 1980), but these species have not been collected after the type specimens were collected in the beginning of the 20th century. Except for the study by Kimura and Suwito (2012) in Java (Indonesia), faunal survey of *Leptopilina* species has not been carried out in tropical Asia. Further survey is needed in this region to understand the diversity and evolution in the genus *Leptopilina*.

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Figure Legends

Figures 1-2 Head (scale bar = 100µm). **1** Anterior view; **2** Dorsal view. (A) *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figure 3 Antenna (scale bar = 200µm). *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figures 4-6 **4** Mesosoma, dorsal view (scale bar = 100µm); **5** Pronotum, anterodorsal view (scale bar = 100µm); **6** Scutellum, dorsal view (scale bar = 50 µm). (A) *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figures 7-9 **7** Mesosoma, lateral view (scale bar = 100µm); **8** Melapleuron, lateral view (scale bar = 100 µm); **9** Mesosoma, posterodorsal view (scale bar = 100µm). (A) *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figure 10 Forewing (scale bar = 500 µm). (A) *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figures 11-12 **11** Metasoma, lateral view (scale bar = 100µm); **12** Petiole and metasomal base (scale bar = 100µm). (A) *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figure 13 Lateral habitus of female. (A) *Leptopilina longipes*, (B) *L. tokioensis*, (C) *L. tsushimaensis*.

Figure 14 Phylongenetic trees for *Leptopilina* species based on the partial *CO1* sequences (A: NJ, B: ML and BI). NJ bootstrap values (>80) and ML bootstrap values (>80)/BI posterior probabilities (>0.8) are given at node for each tree. The sequences of *Leptopilina longipes* from Sapporo, *L. tokioensis* and *L. tsushimaensis* are indicated by arrows.

Figure 15 Phylongenetic trees for *Leptopilina* species based on the ITS2 sequences. (A: NJ, B: ML and BI). NJ bootstrap values (>80) and ML bootstrap values (>80)/BI posterior probabilities (>0.8) are given at node for each tree. The sequences of *Leptopilina longipes* from Sapporo, *L. tokioensis* and *L. tsushimaensis* are indicated by arrows.

Figure 16 NJ tree for *Wolbachia* bacteria based on the partial *wsp* sequence. Bootstrap values (>80) are given at node. The sequences obtained from *Leptopilina longipes*, *L. tokioensis* and *L. tsushimaensis* are indicated by arrows.