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Taxonomy and phylogeny of *Leptopilina* species (Hymenoptera: Cynipoidea, Figitidae) attacking frugivorous drosophilid flies in Japan, with description of three new species

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Abstract

Despite the intensive use of the *Leptopilina* genus and its drosophilid hosts as model-systems in the study of host-parasitoid interactions, the diversity and distribution of the species occurring in the Asian region remain elusive. Here we report the phylogeny of Japanese *Leptopilina* species attacking frugivorous drosophilid flies, based on CO1, ITS1 and ITS2 sequences. Consistent with molecular data, hybridization experiments and morphological examination, five species were recorded in Japan. *L. heterotoma, L. victoriae,* and three new species, two occurring in the Ryukyu archipelago, *L. ryukyuensis* and *L. pacifica*, and another species, *L. japonica*, distributed in Honshu and Hokkaido. *L. japonica* was further divided into two subspecies, *L. j. japonica* occurring in Japan, and *L. j. formosana* occurring in Taiwan. According to these results, we discuss the evolution, speciation and colonization history of Japanese *Leptopilina* species.

Key words: CO1, geometric morphometrics, hybridization experiments, ITS1, ITS2, nucleotide sequence, parasitoid.

INTRODUCTION

The astonishingly diverse group of parasitoid wasps potentially accounts for over 20 percent of all insect species (LaSalle & Gauld 1993). However, this remarkable speciation, resulting in behavioural, physiological and ecological differentiation, is often coupled with a low level of morphological variation among closely-related species. Thus, the study of this numerous insect group is frequently associated with difficult species identification and a high incidence of cryptic speciation (Baylac *et a*l. 2003; Kankare *et al*. 2005; Sha *et al*. 2006; Smith *et al*. 2008).

Although the *Leptopilina* genus, along with its drosophilid hosts, is a model organism in the study of host-parasitoid interactions (Dubuffet *et al*. 2009; Nappi *et al*. 2009; Dupas *et al*. 2009), the amount of information about this genus in Asia, including Japan, is fairly limited. So far *L. heterotoma* (Thomson) has been recorded from Sapporo, Sendai, Tokyo, Amami-oshima (Japan) and Los Baños (Philippines), *L. victoriae* Nordlander from Kagoshima Prefecture, Amami-oshima, Okinawa-jima and Iriomote-jima (Japan), *L. rufipes* (Cameron) from Kuching (Malaysia), *L. cupulifera* (Kieffer) from Luzon (Philippines) and an unidentified species from Tokyo (Nordlander 1980; Carton *et al.* 1986; Mitsui *et al*. 2007). Furthermore, the species identification in these studies was solely based on external morphology, and therefore required further verification.

In this paper, we assess the phylogeny of *Leptopilina* specimens from Japan, in relation to other specimens from Southeast Asia, based on nucleotide sequences of cytochrome oxidase subunit I (CO1) gene and intertranscribed spacer sequences I and II (ITS-1 and -2) of ribosomal RNA genes. The second ribosomal intertranscribed spacer (ITS2) sequence has been previously used to establish the phylogeny of various species

in this genus (Schilthuizen *et al*. 1998; Allemand *et al*. 2006). Hybridization experiments are performed to verify the species status of phylogenetically close groups. Finally, we assess the morphological differences between taxa, and additionally test the forewing differences by geometric morphometric analyses. Wing geometric morphometry has been commonly carried out on various insect species (Morales *et al.* 2004; Bischoff *et al*. 2009), but rarely on parasitoid wasps, due to the highly reduced wing venation in some taxa. According to these results, three new species, one of which contains two subspecies, are described. Furthermore, the validity of morphological characters in species identification is evaluated and the colonization history of this group is discussed.

MATERIALS AND METHODS

Parasitic wasps

Wasp specimens were collected in the period from 2003 to 2010, from eight localities in Japan and Southeast Asia (Fig. 1): Sapporo (SP: 43° 3' N, 141° 21' E), Tokyo (TK: 35° 40' N, 139° 46' E), Amami-oshima (AM: 28° 22' N, 129° 30' E), Naha (NH: 26° 13' N, 127° 42' E; 2009), Iriomote-jima (IR: 24° 19' N, 123° 49'E), Taipei (TP: 25° 2' N, 121° 38' E), Kota Kinabalu (KK: 5° 59' N, 116° 4' E) and Bogor (BG: 6° 35' S, 106° 47' E). Collection localities, collectors and collection dates for each sample are summarised in Table 1. To establish laboratory strains, traps containing banana were placed in the field for a period of 5-7 days, and then brought back to laboratory. When host (i.e., drosophilid) pupae were formed in the containers, they were placed in Petri dishes and

then examined for the emergence of host flies or wasps (*Leptopilina* individuals are solitary larvo-pupal parasitoids; i.e., parasitized host larvae grow up to pupae from which new adult wasps emerge). Two distinct morphological types of emerging wasps were obtained from Naha, Amami-oshima and Iriomote-jima, but only one from the other localities. These were reared in laboratory using *Drosophila simulans* Sturtevant (for strains BG, KK, TP, IR, AM, NH, TK, SP) and *D. sulfurigaster* Duda (for strains BGp, IRp, NHp) as hosts. Rearing and all consequent experiments were conducted at a constant temperature of 23 ºC under a 15 h light-9 h dark condition.

In addition to these species, *L. heterotoma* specimens from France were used in phylogenetic and morphological analyses. These specimens were reared at 20 ºC using *D. melanogaster* Meigen as host. Furthermore, ITS2 sequences of *L. victoriae* from Thailand (Accession No. AF015902) and Mauritius (Accession No. AY124554), and CO1 sequences of *L. heterotoma* from France (Accession No. AB456712) and *Ganaspis xanthopoda* (Ashmead) (Figitidae) from Japan (Accession No. AB456711) were obtained from the DNA database and used for constructing phylogenetic trees.

Molecular analyses

Genomic DNA was extracted from each specimen (several specimens in case of laboratory strains) by a modified phenol-chloroform protocol. All amplifications were preformed in 23μl reaction volumes containing 1.3 mM $MgCl_2$, 0.042 mM dNTP, 2.6 μM primers, 0.042 U Ampli *Taq* DNA polymerase, and 2.4 μL 10× PCR buffer. PCR profile consisted of one cycle of denaturation (94°C for 10 min), 35 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for

1.5min), followed by one cycle of final extension at 72°C for 12min. Amplified products were diluted to 1ng/μL, and used as sequencing templates.

Amplification of ITS1 and ITS2 fragments was performed by following primer pairs: 5'-GCTGCGTTCTTCATCGAC-3' (7246) and 5'-CGTAACAAGGTTTCCGTAGG-3' (7247) for ITS1 (412-647 bp); 5'-TGTCAACTGCAGGACACATG-3' and 5'-AATGCTTAAATTTAGGGGGTA-3' for ITS2 (448-564 bp). Amplification of COI fragments was preformed by 5'-GGTCAACAAATCATAAAGATATTGG-3' (LCO) and 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (HCO) (about 600 bp) for all except Iriomote-jima (2003), Tokyo (2007, 2008) samples and the SP and TK strains. Samples collected from Iriomote-jima in 2003 and Tokyo in 2007 and 2008 were amplified using 5'-CDTTYCCWCGWATAAATAATATAAG-3' and HCO (420 bp), because the amplification with HCO-LCO primers failed. In addition, in strains derived from Sapporo (SP) and Tokyo (TK), the primer pair HCO-LCO amplified a CO1 pseudogene sequence containing several stop codons and a one-base deletion, resulting in a frame-shift. In order to obtain the functional CO1 gene sequences for these two strains, first, mRNA was isolated from 20 individuals of each strain using RNA SV Total RNA isolation system (Promega). Subsequently, cDNA was obtained by reverse transcription PCR (RT-PCR), using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). In both procedures, manufacturer's protocol was followed. Finally, the CO1 gene sequence was amplified using a primer set of LCO and HCO3 (5'-TAAACTTCTGGATGACCAAAAAATCA-3').

For all sequence reactions, Big Dye Terminator Cycle Sequencing Kit (ABI) was used. Sequencing was carried out with a 3100 Genetic Analyzer (ABI), utilizing the same primers used for PCR amplification. Accession numbers of the obtained sequences

are shown in Table 1.

Phylogenetic analysis of sequence data was conducted using Mega4 software (Tamura *et al*. 2007). Sequences were aligned manually and used to construct phylogenetic trees using neighbor-joining (NJ) method (Saitou & Nei 1987). Nucleotide distances in NJ trees were estimated by Kimura's two-parameter method (Kimura, 1980). Bootstrap values were obtained after 1000 replications.

Hybridization experiments

Hybridization experiments were performed between the SP, TK, IR, TP, KK and BG strains. These strains differed in host use to some extent, but all of them successfully parasitized *D. simulans*, which was used as a common host for this experiment. Rearing all strains on the same host species enabled result comparison. Also, in order to take into account the possible effect of host species on mate choice and/or gender bias, controls were established using males and females of the same strain.

To obtain virgin females for the experiments, parasitized host pupae were placed individually in small vials. Five virgin females obtained in this way were placed with 10 males in a single vial containing a Drosophila medium and more than 100 second instar host larvae. Wasps were left to mate and oviposit for two days, and then transferred to a new vial. The transfer was repeated after another two days. Thus, three vials were prepared for every cross. F_1 wasps were counted upon eclosion. In the case where both F_1 females and males eclosed, they were further crossed to examine the production of F_2 wasps. Finally, if both F_2 females and males were obtained, they were crossed to examine the production of F_3 wasps.

Morphometric analyses

Forewing size and shape were examined for 240 and 300 female specimens, respectively. Taking into account that the size of parasitoids differs depending on the size of their host, and that the size of the host additionally varies depending on the rearing temperature, size-related analyses were performed only on laboratory strains reared on known hosts and under controlled temperature conditions (i.e.. the SP, TK, AM, NH, IR, TP, KK and BG strains were reared on *D. simulans* on 23 ºC; the BGp, IRp and NHp strains on *D. sulfurigaster* on 23 ºC; the French specimens on *D. melanogaster* on 20 ºC).Given that wild caught specimens were not available for all analyzed populations, shape analyses were performed for both wild caught specimens and specimens obtained from laboratory strains.

The right forewing of each specimen was mounted on a slide in Hoyer's medium. Digitized forewings were assigned 7 landmarks, positioned at forewing vein intersections and terminations using TpsDig 2.12 software (Rohlf, 2008a) (Fig 2).

To examine forewing size differences among wasps, ANOVA with a post-hoc Tukey test were conducted on the centroid size parameter (the square root of the sum of squared distances between each landmark and the forewing centroid). In order to extract shape variables, raw coordinates were first superimposed by a generalized orthogonal least-square Procrustes (GPA) procedure, standardizing the size of landmark configurations and removing translational and rotational differences (Rohlf & Slice 1990). Next, the partial warps were calculated, and the obtained ‘weight matrix’ (*w*; Rohlf *et al*. 1996) was subjected to canonical discriminant analysis, resulting in a classification matrix. Centroid size and weight matrix were obtained utilizing TpsRelw 1.46 software (Rohlf, 2008b). Statistical analyses were preformed using Jmp ver. 6.1

(SAS Institute, Cary USA) and R ver. 2.9 (R Development Core Team, 2009).

RESULTS

Phylogenetic analyses

Figures 3 and 4 show phylogenetic relationships of the study specimens and experimental strains based on CO1 (322 bp), ITS1 (390 bp) and ITS2 (397 bp) nucleotide sequences. Six groups were recognized in all trees. Group I comprised BG and KK strains along with specimens from Bogor and Iriomote-jima. In the ITS2 tree, this group also included *L. victoriae* from Thailand and Mauritius. Group II comprised the TP strain with specimens from Taipei, Group III the SP and TK strains with specimens from Tokyo, Group IV the IR, AM and NH strains with specimens from Iriomote-jima, Taipei, and Bogor and Group V specimens from Sapporo, Tokyo, Iriomote-jima and a *L. heterotoma* specimen from France. Finally Group VI comprised the IRp, NHp and BGp strains with specimens from Iriomote-jima and Bogor.

High bootstrap support was obtained for all groups in the CO1 tree (99%) except Group V (49%), and all groups in the ITS2 tree (>96%) except Group III (88%). A moderate to high support was obtained for groups in the ITS1 tree (63%, 64%, 65% for Group II, Group I and Group III respectively, >98% for the other groups).

Longer CO1 sequences (about 600 bp) were obtained for all samples except those from Iriomote-jima (2003) and Tokyo (2007, 2008), and based on these sequences molecular divergence was calculated (the topology of the phylogenetic tree remained the same; figure not shown). The lowest CO1 molecular divergence was observed

between groups II and III (5.4%), and the highest between groups IV and VI (14.3%). Molecular divergence for ITS2 sequences ranged from 1% between groups II and III, to12.8% between groups I and V. The divergence for ITS1 spanned from 0.3% between groups I and II and between groups II and III, to 10.2% between groups I and VI.

CO1 pseudogenes of the SP and TK strains had exactly the same nucleotide sequences, forming a cluster with the functional CO1 genes of these strains. The divergence between the functional genes and their pseudogene copies was 7.7%.

Hybridization experiments

To establish the presence/absence of reproductive isolation, strains belonging to phylogenetically close groups I, II, III and IV (i.e., the BG, KK, TP, SP, TK and IR strains) were crossed in hybridization experiments. In wasps that are haplo-diploid organisms, females are only produced from fertilized eggs. If eggs are not fertilized, only males will emerge. If crosses are successful, the progeny will consist of both male and female offspring. Table 2 shows the percentage of females in first generation (F_1) offspring of the hybridization experiments, with notes on the production of subsequent generations (F_2 and F_3). F_1, F_2 and F_3 females were always produced in control crosses (crosses within strains), and in crosses between the SP and TK, and between the BG and KK strains (crosses within groups). The relative proportion of females was lower in high-latitude strains. In crosses between the BG and KK strains the percentage of females (24.5% and 33.7%) was much lower than in control crosses (61.6% and 64%).

The cross between SP females and TP males yielded a relatively high percentage of F_1 females (38.5%), higher than the control SP cross, also producing F_2

and F_3 females. The reciprocal cross between these strains produced no female offspring. The cross between TK and TP strains yielded no males in F_2, or no offspring in F_3. In other inter-group crosses, no or only few F_1 females were produced. In three of these crosses where F_1 females were produced there was no F_2 progeny (F_1 females might be sterile), and in another two crosses either females or males were not obtained in F_2 (Table 2).

Morphometric analyses

Forewing size. A significant difference (P<0.001, Tukey test after ANOVA) was observed in forewing centroid size among the strains: the BGp, IRp and NHp strains (Group VI) and French specimens (Group V) were significantly larger than the other strains (Fig. 5). This difference may be attributable to the difference in host species or, in the case of *L. heterotoma*, rearing temperature. In the SP, TK, TP, AM, NH, IR, KK and BG strains (Groups I to IV) that were reared on the same host at the same temperature, a cline was observed in the centroid size, with larger forewings in high-latitude strains.

Forewing shape. When shape analysis was performed for closely related groups I, II, III, and IV (Wilks' Λ=0.181, $F_{(30.52)}$=13.65, P<0.001; not shown), the correct classification rate for groups II and III was 66.7% and 78.7% respectively. By grouping these two groups into one, according to the results of the hybridization experiments, their joint classification rate rose to 89.1% (Wilks' Λ=0.233, $F_{(20.36)}$=19.08, P<0.001; Fig. 6a), with an overall classification rate of 82.6%. CV1, explaining 68.3% of the total variation, partially separated Group I from Group II-III cluster, and CV2 (31.7%) partially separated Group IV from the other two clusters.

Taking into account that there were no major morphological differences between Group I and Group IV, we tested their differences in wing shape and obtained a good classification rate of 92.3% for Group I and 90.6% for Group IV, overall 91.4% (Wilks' Λ=0.367, $F_{(10.11)}$=18.07, P<0.001)(Fig. 6b).

Finally, when the groups V and VI were included into the analyses, they had a correct classification rate of 60% and 98% respectively, with an overall correct classification rate of 85.7%(Wilks' Λ=0.072, $F_{(40.11)}$=26.62, P<0.001)(Fig. 6c). The low classification rate for Group V might be due to the fact that only five specimens were included in the analyses. Group VI was separated by CV1, which carried 67.2% of total variation.

TAXONOMY

Based on hybridization experiments, molecular and morphometric analyses and morphological examinations, the study strains and specimens were classified into six distinct groups. Group I was assigned as *L. victoriae* (Nordlander 1980), based on morphological characteristics and the sequence similarity with the Thailand and Mauritius reference specimens (Schilthuizen *et al.* 1998; Allemand *et al.* 2006). Groups II and III showed a clear difference in morphlology, but were only partially reproductively isolated (F_1, F_2 and F_3 were produced in the cross between SP females and TP males). Hence, these two groups were assigned as different subspecies of a new distinct species、herein described as *L. japonica*. Group IV was assigned as another new distinct species, *L. ryukyuensis*, reproductively isolated from the others. This species bears high morphological similarity to *L. victoriae*. Group VI was assigned as the third new distinct species *L. pacifica*. Finally, Group V was tentatively assigned as *L.*

heterotoma, based on morphology and sequence similarity with the French *L. heterotoma*. However, for some of the specimens in this group relatively high molecular divergence was observed (9.1% for CO1 between the specimens from Iriomote-jima and from France), suggesting possible inclusion of different species. Further analyses were not carried out for this group in this study, because the number of collected specimens was limited (3 specimens from Tokyo, 1 specimen from Iriomote-jima and 2 specimens from Sapporo).

Leptopilina ryukyuensis Novković&Kimura, sp. nov.

(Figs 7-12)

Type specimens. Holotype: ♀, Iriomote-jima, Japan, strain established in March 2005, leg. A. Fujita (SEHU, type no. 21051); *Paratypes*: same data as holotype, 3♀ and 3♂ (SEHU, type no. 21052-57); Naha, Okinawa, strain established in October 2009, leg. B. Novković, 3♀ and 3♂ (SEHU, type no. 21058-63); Amami-oshima, Japan, strain established in October 2009, leg. B. Novković, 1♀ and 4♂ (SEHU, type no. 21064-21068).

Other specimens examined. 11♀, Iriomote-jima, Japan, December 2007, leg. H. Mitsui; 2♀, Iriomote-jima, Japan, December 2003, leg. H. Mitsui; 1♀, Iriomote-jima, May 2009, leg. H. Mitsui; 2♀, Taipei, Taiwan, June 2010, leg. B. Novković, 2♀, Bogor, Indonesia, June 2008, leg. A. Suwito.

*Diagnosis***.** *L. ryukyuensis* bears a high morphological similarity to *L. victoriae*. Propodeal carinae of *L. ryukyuensis* slightly diverging downwards, fusing into a dorsally more or less reticulate structure, that is more or less smooth in *L. victoriae* (Fig. 11). These two species differ in CO1, ITS1 and 2 sequences, and are completely

reproductively isolated. *L. ryukyuensis* differs from *L. rufipes* in the position and alignment of the metapleural ridges, ridge 2 starting lower down posteriorly and running more perpendicular to the posterior and anterior margins than in *L. rufipes* (Nordlander, 1980). In *L. ryukyuensis* females the hairy ring is rather dense, thinning out dorsally, while the hairy ring in *L. rufipes* is thin, and only present ventrally (Nordlander, 1980).

Description. Body length about 1.3 to 1.7mm in females, 1.1 to 1.5mm in males, presumably more variable depending on host species.

Head and eyes similar to those of *L. victoriae* in shape and size, temples short (head height/ thorax length HH/TL: 0.75-0.85; eye diameter/head height ED/HH: 0.5-0.6) (Fig. 7). Antennae in female around 0.6-0.7×body length; 3rd segment longer than the 4th; 5th and 6th segments shorter; 1st -6th flagellar segments yellow, the rest brown, with 6 to 7 club segments (Fig. 8). Antennae in male long, around 1.5-1.7×body length, with 15 segments; 4th antennal segment elongated, flattened on the outer side; first 3 segments yellowish, 4th somewhat darker, the rest dark brown.

Mesosoma dark brown. Pronotal plate with a broad median bridge connecting the anterior and posterior parts; not fused laterally, forming two laterally open cavities. Posterior part of pronotal plate protruding laterally. Scutellum rounded with rather dense reticulate disc sculpture. Scutellar plate oval, smooth and shiny on surface, with several hairs (3-4) arising from sockets, posterior pit round, medium sized (Fig. 9).

Metapleura with 3 ridges. Ridges 1 and 2 complete as in *L. victoriae* (Fig. 10). Propodeal carinae S shaped in lateral view, in posterodorsal view almost straight and parallel, slightly diverging downwards (Fig. 11).

Wings clear, ciliation on outer margin somewhat longer, radial cell of forewing closed (Fig. 12).

Legs slender, yellow.

Metasoma brown. Hairy ring in females rather dense ventrally, thinned out dorsally, sometimes with a narrow break (Fig. 10). In males, hairy ring present only ventrally.

Etymology. Referring to the first record of the species from the islands of Ryukyu archipelago, Japan.

Distribution. Japan (Ryukyu islands), Taiwan, Indonesia (Java).

Host. Natural host species *D. albomicans*. Under laboratory conditions reproduces well on *D. simulans*.

Leptopilina japonica japonica Novković&Kimura, sp. & subsp. nov.

(Figs 8-10)

Type specimens. *Holotype:* ♀, Sapporo, Japan, strain established in August 2005, leg. M. T. Kimura (SEHU, type no. 21069); *Paratypes*: same data as holotype , 5♀ and 5♂ (SEHU, type no. 21070-79).

Other specimens examined. 5♀ and 5♂, Sapporo, Japan, August 2005, leg. M. T. Kimura; 5♀ and 3♂, Mt. Takao, Tokyo, Japan, July 2008, leg. H. Mitsui; 5♀ and 1♂, Mt. Takao, Tokyo, Japan, October 2008, leg. H. Mitsui.

Diagnosis. Head relative to mesosoma somewhat smaller than in *L. victoriae* or *L. ryukyuensis*, (HH/TL: 0.7-0.83). Antennal segments darker, 5th and 6th antennal segments slender and longer compared to *L. victoriae* and *L. ryukyuensis* (Fig. 8). Scutellar plate wider, posterior pit larger than in *L. victoriae* or *L. ryukyuensis* (Fig. 9).

Description. Body length about 1.3-1.9 mm in females, 1.3-1.5mm in males, presumably more variable depending on host species.

Eyes large, temples short (ED/HH: 0.5-0.6) Antennae in female around 0.6-0.8×body length; 3rd and 4th segment almost equal in length; 5th and 6th segment

shorter, but more slender and longer than in *L. victoriae* or *L. ryukyuensis* (Fig. 8). 3rd and 4th segments somewhat lighter in colour, the first two and 5th -7th segments somewhat darker, the rest of the segments black, with 6-7 club segments. Antennae in males long, over 1.5×body length, with 15 segments. 3rd segment lightest in colour, 1st , 2nd and 4th somewhat darker, the rest dark. 4th segment elongated, flattened on the outer side.

Mesosoma dark brown to black. Pronotal plate with a broad median bridge connecting the anterior and posterior parts; not fused laterally, forming two laterally open cavities. Posterior part of pronotal plate protruding laterally. Scutellum with relatively dense reticulate disc sculpture. Scutellar plate widest in the middle in front of the posterior pit; smooth and shiny on surface, with several hairs arising from sockets (4-6). Posterior pit large in size (Fig. 9).

Metapleura with 3 ridges. Ridges 1 and 2 complete (Fig. 10). Propodeal carinae S shaped in lateral view, in posterodorsal view almost parallel, slightly widening in the middle.

Wings clear, ciliation on outer margin long, radial cell of forewing closed.

Legs slender, yellow.

Metasoma dark brown to black. Hairy ring rather dense ventrally, thinned out dorsally, only narrowly broken, if broken, dorsally (Fig. 10). In males, hairy ring present only ventrally.

Etymology. Referring to subspecies distribution.

Distribution. Japan (Hokkaido and Honshu).

Host. Host species recorded under natural conditions are *Drosophila biauraria* in Sapporo and *D. rufa* in Tokyo. Under laboratory conditions reproduces well on *D. simulans*.

Leptopilina japonica formosana Novković&Kimura, subsp. nov.

(Figs 8-10)

Type specimens. Holotype: ♀, Taipei, Taiwan, strain established in March 2008, leg. M. T. Kimura (SEHU, type no. 21080); *Paratypes*: same data as holotype, 5♀ and 5♂ (SEHU, type no. 21081-90).

Other specimens examined. 7♀, Taipei, Taiwan, June 2010, leg. B. Novković; 5♀and 5 ♂, Taipei, Taiwan, March 2009, leg. M. T. Kimura.

Diagnosis. Antennae darker than in *L. j. japonica* (Fig. 8), proportions of the segments in females 3^{rd}>4^{th} >5^{th} >6^{th}. Scutellar plate narrow and elongated compared to *L. j. japonica*, *L. victoriae* and *L. ryukyuensis* (Fig. 9).

Description. Length about 1.4-1.9mm in females, 1.2-1.6mm in males, presumably more variable depending on host species.

Head and eyes similar to those of *L. victoriae* in shape and size, temples short (HH/TL: 0.75-0.85; ED/HH: 0.45-0.6). Antennae in female about 0.6-0.8×body length; all segments dark, segment size as follows: 3^{rd}>4^{th} >5^{th} >6^{th} (Fig. 8). Antennae in males long, over 1.5×body length, with 15 segments. All segments dark, 4^{th} segment elongated, flattened on the outer side.

Mesosoma black. Pronotal plate with a broad median bridge connecting the anterior and posterior parts; not fused laterally, forming two laterally open cavities. Posterior part of pronotal plate protruding laterally. Scutellum with reticulate disc sculpture, reticulation more dense in the posterior part. Scutellar plate elongated, narrow, with several hairs arising from sockets (4-5); posterior pit medium in size (Fig. 9).

Metapleura with 3 ridges. Ridges 1 and 2 complete (Fig. 10). Propodeal carinae S shaped in lateral view, in posterodorsal view almost straight and parallel.

Wings clear, ciliation on outer margin somewhat long, radial cell of forewing closed.

Legs slender, yellow, coxae somewhat darker.

Metasoma black. Hairy ring rather dense ventrally, thinned out dorsally, sometimes with a narrow brake (Fig. 10). In males, hairy ring present only ventrally.

Etymology. Referring to the subspecies distribution.

Distribution. Taiwan.

Host. Natural host species *D. albomicans*. Under laboratory conditions reproduces well on *D. simulans*.

Leptopilina pacifica Novković&Kimura, sp. nov.

(Figs 8-10, 12)

Type specimen. *Holotype:* ♀, Iriomote-jima, Japan, strain established in December 2008, leg. H. Mitsui (SEHU, type no. 25615); *Paratypes*: same data as holotype, 5♀ and 5♂ (SEHU, type no. 25616-25).

Other specimens examined. 3♀, Iriomote-jima, Japan, May 2009, leg. H. Mitsui; 1♀, Iriomote-jima, Japan, December 2007, leg. H. Mitsui; 3♀ and 3♂, Iriomote-jima, Japan, December 2008, leg. H. Mitsui; 3♀, Naha, Japan, October 2009, B. Novković; 3♀ and 3♂, Bogor, Indonesia, June 2008, leg. M. T. Kimura, 4♀, Bogor, Indonesia November 2009, leg. Y. Murata.

Diagnosis. Scuttelar plate widest in the upper part, narrowing towards the posterior pit, which is narrow compared to that of *L. heterotoma*. Hairy ring on metasoma present only ventrally, completely absent dorsally. Rs vein of the radial cell longer than in *L. ryukyuensis* or *L. victoriae*. M Vein clearly visible (Fig. 12).

Description. Body length about 1.7-2mm in females, 1.5-1.8mm in males, might be

more variable depending on host species.

Head relatively large compared to mesosoma, eyes large, temples short (HH/TL: 0.75-0.85; ED/HH: 0.5-0.6). Antennae in female about 0.7×body length; 3rd segment slightly longer than the 4th, 5th and 6th of approximately equal length (Fig. 8). First 6 segments lighter, yellowish, the rest brown to dark brown, with 6-7 club segments. Antennae in males long, around 1.7×body length, with 15 segments. First 3 segments lighter in colour, 4th brown, the rest dark brown; 4th segment somewhat elongated, not as flattened on the outer side as in *L. victoriae*.

Mesosoma reddish-brown to dark brown in females, dark brown to black in males. Pronotal plate with a broad median bridge connecting the anterior and posterior parts; not fused laterally, forming two laterally open cavities. Posterior part of pronotal plate protruding laterally. Scutellum with relatively dense reticulate, laterally obliterated disc sculpture. Scutellar plate widest in the upper third, narrowing down towards the posterior pit, smooth and shiny on surface, with several hairs arising from sockets (4). Posterior pit rather narrow, somewhat triangular in appearance (Fig. 9).

Metapleura with 3 ridges, ridges 1 and 2 fully developed (Fig. 10). Propodeal carinae S shaped in lateral view, in posterodorsal view almost straight and parallel, wider and sometimes reticulate.

Wings clear, ciliation on outer margin long, radial cell of forewing closed.

Legs slender, yellow.

Metasoma reddish to light brown in females, reddish-brown to black in males. Hairy ring present only ventrally, completely absent dorsally (Fig. 10).

Etymology. Referring to species distribution, in the western pacific area.

*Distribution.*Japan (Ryukyu islands), Indonesia (Bogor).

Host. Natural host species *Drosophila albomicans* in Iriomote-jima and *D. sulfrigaster,*

D. albostrigata, D. keplauana in Bogor.

Leptopilina victoriae Nordlander, 1980

Specimens examined. 5♀ and 5♂, Kota-Kinabalu, Malesia, March 2008, leg. M. Kondo; 6♀, Iriomote-jima, Japan, December 2003, leg. H. Mitsui; 10♀ and 10♂, Bogor, Indonesia, Jun 2008, leg. M. T. Kimura;10♀ and 4♂, Bogor, Indonesia, November 2009, leg. Y. Murata.

Distribution and hosts. This species has a wide distribution ranging from Ivory Coast, Kenya, Madagascar, Seychells, over the islands in the Indian Ocean, to Thailand (Allemand et al., 2002; Nordlander, 1980). We additionally report findings from Japan (Ryukyu archipelago, Iriomote-jima 2003) **new record**, Indonesia (Bogor) **new record** and Malaysia (Kota-Kinabalu) **new record**. The host recorded under natural conditions is *D. malerkotliana*, and in the case of Iriomote-jima *D. bipectinata*.

Leptopilina heterotoma (Thomson, 1862)

Specimens examined. 10♀ and 10♂, strain from Antibes, France, established in 1995, kindly provided by F. Vavre; 1♂, Tokyo, Japan, June 2008, leg. H. Mitsui; 1♂, Tokyo, Japan, September 2009, leg. N. Kasuya; 2♂, Sapporo, Japan, August 2008, leg. Y. Murata.

Distribution and hosts. This species has a wide distribution ranging from Europe to Tunisia, Canary Islands and USA. In Asia it has been recorded in Philippines (Los Baños) and Japan (Sapporo, Sendai, Amami-oshima) (Nordlander, 1980; Mitsui *et al*, 2007). A wide range of hosts have been reported for this species.

DISCUSSION

According to molecular analyses and hybridization experiments, morphology and morphometric data, the Japanese *Leptopilina* specimens examined in this paper belong to at least five distinct species: *L. victoriae*, *L.heterotoma, L. ryukyuensis* sp. nov., *L. japonica* sp. nov. and *L. pacifica* sp. nov. In a previous paper, based on morphological characteristics, *Leptopilina* specimens from the Ryukyu archipelago (Iriomote-jima, Okinawa-jima and Amami-oshima) were assigned as *L. victoriae*; specimens from Sapporo were assigned as *L. heterotoma* and those from Tokyo were assigned as a new unknown species (Mitsui *et al.* 2007). Our results partially verify this species assignment, but there is still a possibility that the specimens identified as *L. victoriae* and *L. heterotoma* in Mitsui *et al.* (2007) included *L. ryukyuensi*s and *L. j. japonica*, respectively. The two other *Leptopilina* species recorded in Southeast Asia, *L. rufipes* and *L. cupulifera* (Nordlander, 1980), were not encountered in this study. The host of these species is unknown and therefore, it is possible that these species are not parasitoids of frugivorous drosophilids.

We cannot determine at present, whether all specimens in Group V belong to a single species (*L. heterotoma*). The relatively high molecular divergence between the examined specimens and the low bootstrap support for this group in the CO1 tree would suggest otherwise, especially in the case of the specimen from Iriomote-jima. However, only few specimens belonging to this group have been obtained so far, thus limiting further analyses. Additional samples need to be studied, and laboratory strains should be established to test the reproductive isolation between specimens belonging to different branches of this group.

Morphological characters previously relied on for species delimitation, e.g. the

structure of scutellum and female antennae, were found to show intra-specific variation in some cases (e.g. *L. japonica*) and failed to show inter-specific differences in others (*L. victoriae* and *L. ryukyuensis*). These cases call for caution when basing species identification in this group on morphological characters alone. For accurate species identification, especially in instances like *L. victoriae* and *L. ryukyuensis*, the use of molecular methods and/or hybridization experiments in conjunction with morphological analysis would be preferable.

The examined *Leptopilina* species were differentiated to some extent by forewing geometric morphometry, with an overall classification rate of 85.7%. Additionally, females of *L. victoriae* and *L. ryukyuensis* were separated with a 91% overall accuracy. Thus, despite relying on a low number of landmarks, the geometric morphometry used in this paper showed to be more reliable than most of the traditional morphological characters in the case of *L. victoriae* and *L. ryukyuensis*. Nonetheless, because we used both wild-caught samples and samples obtained from laboratory strains, some of the naturally occurring variation may have been overlooked, and an analysed of a large number of wild-caught specimens would be necessary to establish the usefulness of geometric morphometry in delimitation of these particular species.

Parasitoid wasps have a faster rate of molecular evolution, possibly associated with their increased speciation rate due to small effective population size and founder effects (Downton & Austin, 1995; Castro *et al*. 2002). This kind of rapid speciation could, on the other hand, leave insufficient time for the accumulation of morphological changes between different species. The generally low interspecific morphological divergence that was frequently associated with parasitoid wasps (Kankare *et al*. 2005; Sha *et al*. 2006; Smith *et al*. 2008) was also observed in this study.

Based on the fact that *L. victoriae* , *L. ryukyuensis* and *L. pacifica* are found in

Ryukyu islands, Taiwan, Indonesia and/or other tropical and subtropical Asian countries (Schilthuizen *et al*. 1998; Allemand *et al*. 2006), it is probable that the ancestor of this group occurred in the tropical and/or subtropical region. Additionally, taking in account the high relatedness of *L. victoriae* and *L. japonica*, *L. japonica* may have originated from *L. victoriae* or a common ancestral species. The pseudogene of SP and TK strains of this species formed a cluster with their functional CO1 genes, suggesting that this pseudogene evolved after the divergence of northern populations (*L. j. japonica*) from the southern ones (*L. j. formosana*). Finally, *L. victoriae* may have fairly recently spread over Southeast Asia, Indian Ocean islands and Africa, based on the fact that the divergence of examined nucleotide sequences was low among specimens from these areas. For further understanding of the evolutionary and colonization history of this group, sampling from mainland Asia is required. In addition, one of the main parasitoid speciation mechanisms - the formation of host races, and further host related ecology need to be investigated.

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Table 1. Collection localities, collectors, collection dates and accession numbers of samples and experimental strains

Species				Accession No.*		
Sample	Collection locality	Collector	Collect.ion date	CO1	ITS1	ITS2
L. japonica						
SP strain	Sapporo, Japan	Kimura M.T.	August 2005	AB546874, AB546877**	AB546879	AB546888
TK strain	Tokyo, Japan	Mitsui H.	August 2006	AB546875, AB546878**	AB546880	AB546889
Tokyo 1-5, 13-15	Tokyo, Japan	Mitsui H.	July 2008	AB583569-71	AB583629-33, AB583640-42	AB583702-06, AB583712-13
Tokyo 6-12	Tokyo, Japan	Mitsui H.	October 2008	AB583672-73	AB583634-39	AB583707-11
TP strain	Taipei, Taiwan	Kimura M.T.	March 2009	AB546876	AB546881	AB546890
Taipei 1-7	Taipei, Taiwan	Novković B.	June 2010	AB583599-605	AB583648-54	AB583718-23
L. ryukyuensis						
IR strain	Iriomote-jima, Japan	Fujita A.	March 2005	AB546869	AB546884	AB546893
AM strain	Amami-oshima, Japan	Novković B.	October 2009	AB546871	AB546885	AB546895
NH strain	Naha, Japan	Novković B.	October 2009	AB546870	AB546886	AB546894
Iriomote 26-27	Iriomote-jima, Japan	Mitsui H.	December 2003	AB583597, AB583627		
Iriomote 1-9, 11-12	Iriomote-jima, Japan	Mitsui H.	December 2007	AB583576-84, AB583586-87	AB583645-46	AB583714-16
Bogor 17-18	Bogor, Indonesia	Kimura M.T.	June 2008		AB583668-69	
Iriomote 13	Iriomote-jima, Japan	Mitsui H.	May 2009	AB583588		
Taipei 8-9	Taipei, Taiwan	Novković B.	June 2010	AB583606	AB583655-56	AB583724
L. pacifica						
IRp strain	Iriomote-jima, Japan	Mitsui H.	December 2008	AB583623	AB583672	AB583740
BGp strain	Bogor, Indonesia	Kimura M.T.	June 2008	AB583624	AB583673	AB583741
NHp strain	Naha, Japan	Novković B.	October 2009	AB583625	AB583674	AB583742
Iriomote 10	Iriomote-jima, Japan	Mitsui H.	December 2007	AB583585		
Iriomote 14-16	Iriomote-jima, Japan	Mitsui H.	May 2009	AB583589-91	AB583647	AB583717
Bogor 16, 21-22	Bogor, Indonesia	Murata Y.	November 2009	AB583621-22	AB583667, AB583670-71	AB583738-39
L. victoriae						
KK strain	Kota-Kinabalu, Maleysa	Kondo M.	March 2008	AB546872	AB546882	AB546891
BG strain	Bogor, Indonesia	Kimura M.T.	June 2008	AB546873	AB546883	AB546892
Iriomote 21-25, 28	Iriomote-jima, Japan	Mitsui H.	December 2003	AB583592-96, AB583598		
Bogor 1-13,15	Bogor, Indonesia	Murata Y.	November 2009	AB583607-20	AB583657-66	AB583725-37
L. heterotoma						
France h	Antibes, France	Rolland A.	1995		AB546887	AB546896
Sapporo 1	Sapporo, Japan	Murata Y.	August 2008	AB583568	AB583628	
Tokyo h 2	Tokyo, Japan	Kasuya N.	September 2009	AB583574		
Tokyo h 3	Tokyo, Japan	Mitsui H.	June 2008	AB583575		
Iriomote h	Iriomote-jima, Japan	Mitsui H.	December 2007	AB583626	AB583675	AB583743

Table 2. Percentage of females in the first generation offspring of hybridization experiments

		♂					
		SP	TK	TP	IR	KK	BG
♀	SP	21.2(104)	37.4(131)	38.5(195)	0(66)	0(101)	3.4*(89)
	TK	15.0(40)	28.6(69)	6.7***(45)	0(51)	0(10)	12.5**(24)
	TP	0(284)	14.8****(61)	43.2(266)	0(190)	0(109)	18.8*(133)
	IR	0(247)	0(207)	6.7*(284)	48.5(251)	0(195)	0(188)
	KK	0(215)	0(302)	0(143)	0(253)	61.6(203)	33.7(199)
	BG	0(327)	0(230)	8.9****(224)	0(227)	24.5(229)	64(89)

Numbers in parenthesis refer to the total number of F_1 progeny produced.

*no progeny produced in F_2; **no F_2 females produced; ***no F_3 progeny produced; ****no F_2 males produced

Captions for Figures

Figure 1 Original localities of the experimental strains and study specimens.

Figure 2 Position of the 7 landmarks used in geometric morphometric analyses on the right forewing of a *Leptopilina* specimen.

Figure 3 Neighbor-joining tree based on CO1 nucleotide sequences. Bootstrap values are given above the branches supporting groups (values below 50% are omitted).

Figure 4 Neighbor-joining trees based on nucleotide sequences of ITS1 (a) and ITS2 (b) regions. Bootstrap values are given above the branches supporting groups (values below 50% are omitted).

Figure 5 Boxplot of the wing centroid sizes of the studied strains, with Tukey test results (a-f). Same letters indicate no significant difference (P>0.05).

Figure 6 (a) Distribution of canonical values among CV1 and CV2; (▲), Group II-III – *L. japonica*; (□), Group IV – *L. ryukyuensis*; (●), Group I – *L. victoriae*. (b) Distribution of canonical values along CV1, (grey), Group I – *L. victoriae*; (white), Group IV – *L. ryukyuensis*. (c) Distribution of canonical values among CV1 and CV2 (▲), Group II-III – *L. japonica*; (□) Group IV – *L. ryukyuensis*; (●) Group I – *L. victoriae*. (X), Group V – *L. heterotoma*, (■), Group VI – *L. pacifica*.

Figure 7 Female specimen of *L. ryukyuensis* (Iriomote-jima). Arrows indicate the

distances measured: Thorax Length (TL), Head Height (HH) and Eye Diameter(ED).

Figure 8 Female antennae of the studied species.

Figure 9 Scutellum in dorsal view. (1) *L. victoriae* (Kota-kinabalu); (2) *L. ryukyuensis* sp. nov. (Iriomote-jima); (3) *L. j. formosana* sp. & ssp. nov. (Taipei); (4) *L. j. japonica* sp. & ssp. nov. (Sapporo); (5) *L. heterotoma* (Antibes); (6) *L. pacifica* sp. nov. (Iriomote-jima).

Figure 10 Metapleural ridges and metasoma with the hairy ring. Ridges 1, 2 and 3 are indicated by arrows. (1) *L. pacifica* sp. nov. (Iriomote-jima); (2) *L. victoriae* (Kota-kinabalu); (3) *L. ryukyuensis* sp. nov. (Iriomote-jima); (4) *L. j. formosana* sp. & ssp. nov. (Taipei); (5) *L. j. japonica* sp. & ssp. nov. (Sapporo)

Figure 11 Propodeal carinae in *L. ryukyuensis* (Iriomote-jima) and *L. victoriae* (Bogor).

Figure 12 Forewing venation in *L. ryukyuensis* and *L. pacifica* from Iriomote-jima.

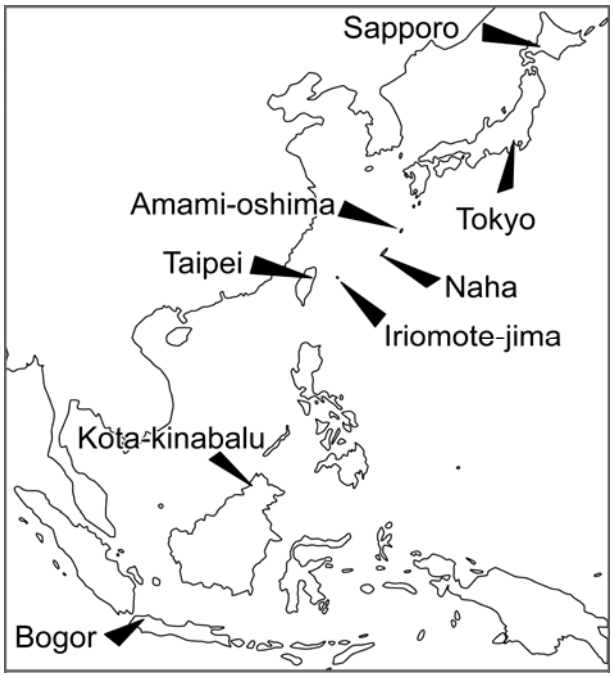

Sapporo
Amami-oshima
Tokyo
Taipei
Naha
Iriomote-jima
Kota-kinabalu
Bogor

Figure 1

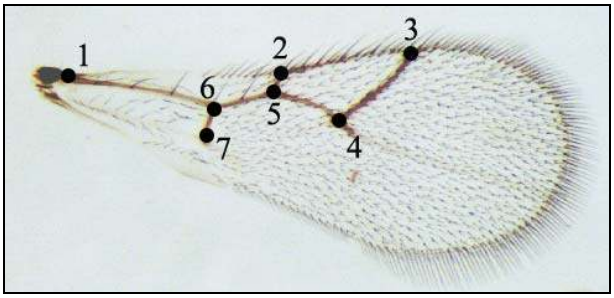


Figure 2

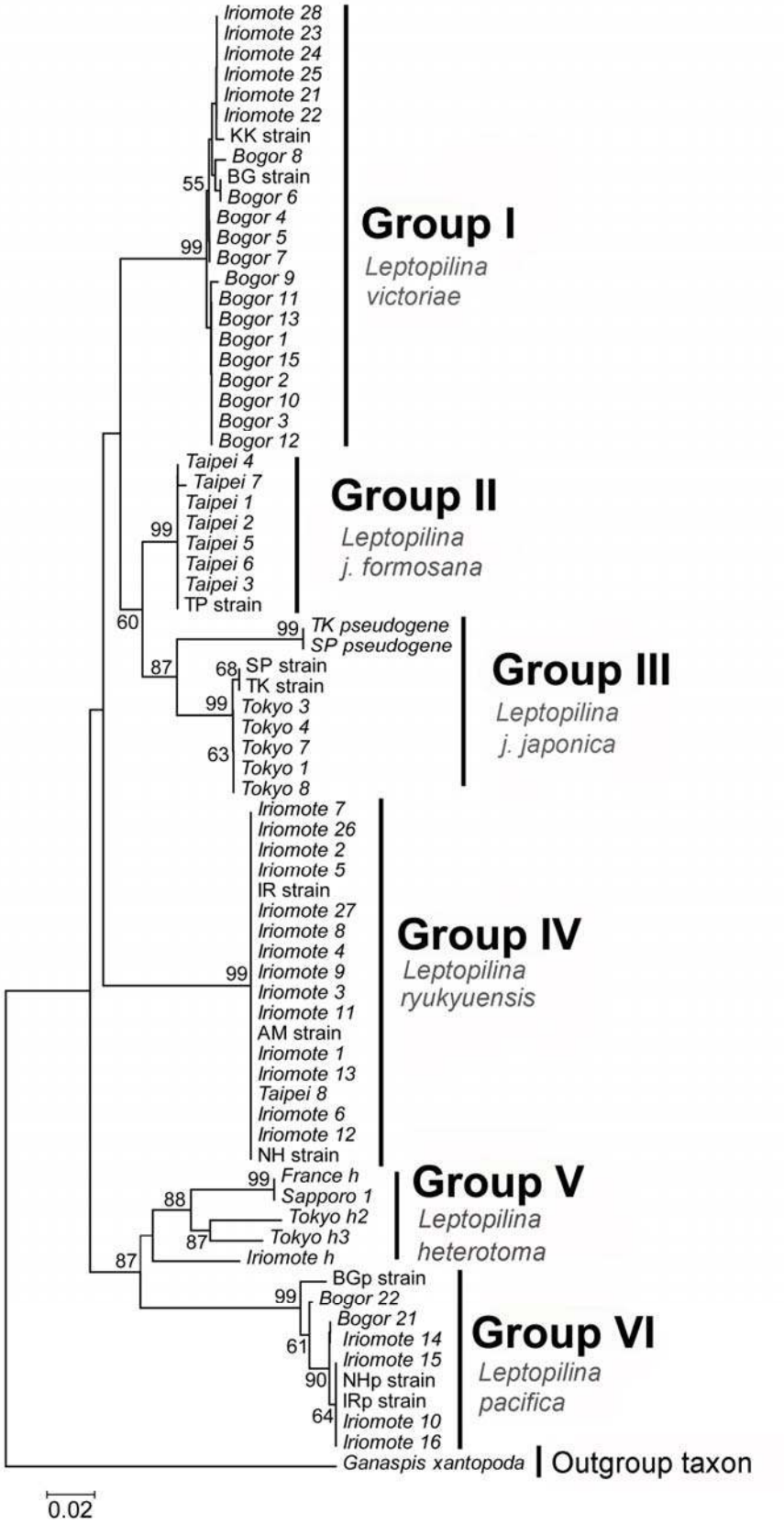

Iriomote 28
Iriomote 23
Iriomote 24
Iriomote 25
Iriomote 21
Iriomote 22
KK strain
Bogor 8
BG strain
Bogor 6
Bogor 4
Bogor 5
Bogor 7
Bogor 9
Bogor 11
Bogor 13
Bogor 1
Bogor 15
Bogor 2
Bogor 10
Bogor 3
Bogor 12
Group I
Leptopilina victoriae
Taipei 4
Taipei 7
Taipei 1
Taipei 2
Taipei 5
Taipei 6
Taipei 3
TP strain
Group II
Leptopilina j. formosana
TK pseudogene
SP pseudogene
SP strain
TK strain
Tokyo 3
Tokyo 4
Tokyo 7
Tokyo 1
Tokyo 8
Group III
Leptopilina j. japonica
Iriomote 7
Iriomote 26
Iriomote 2
Iriomote 5
IR strain
Iriomote 27
Iriomote 8
Iriomote 4
Iriomote 9
Iriomote 3
Iriomote 11
AM strain
Iriomote 1
Iriomote 13
Taipei 8
Iriomote 6
Iriomote 12
NH strain
Group IV
Leptopilina ryukyuensis
France h
Sapporo 1
Tokyo h2
Tokyo h3
Iriomote h
Group V
Leptopilina heterotoma
BGp strain
Bogor 22
Bogor 21
Iriomote 14
Iriomote 15
NHp strain
IRp strain
Iriomote 10
Iriomote 16
Group VI
Leptopilina pacifica
Ganaspis xantopoda
Outgroup taxon
0.02

Figure 3

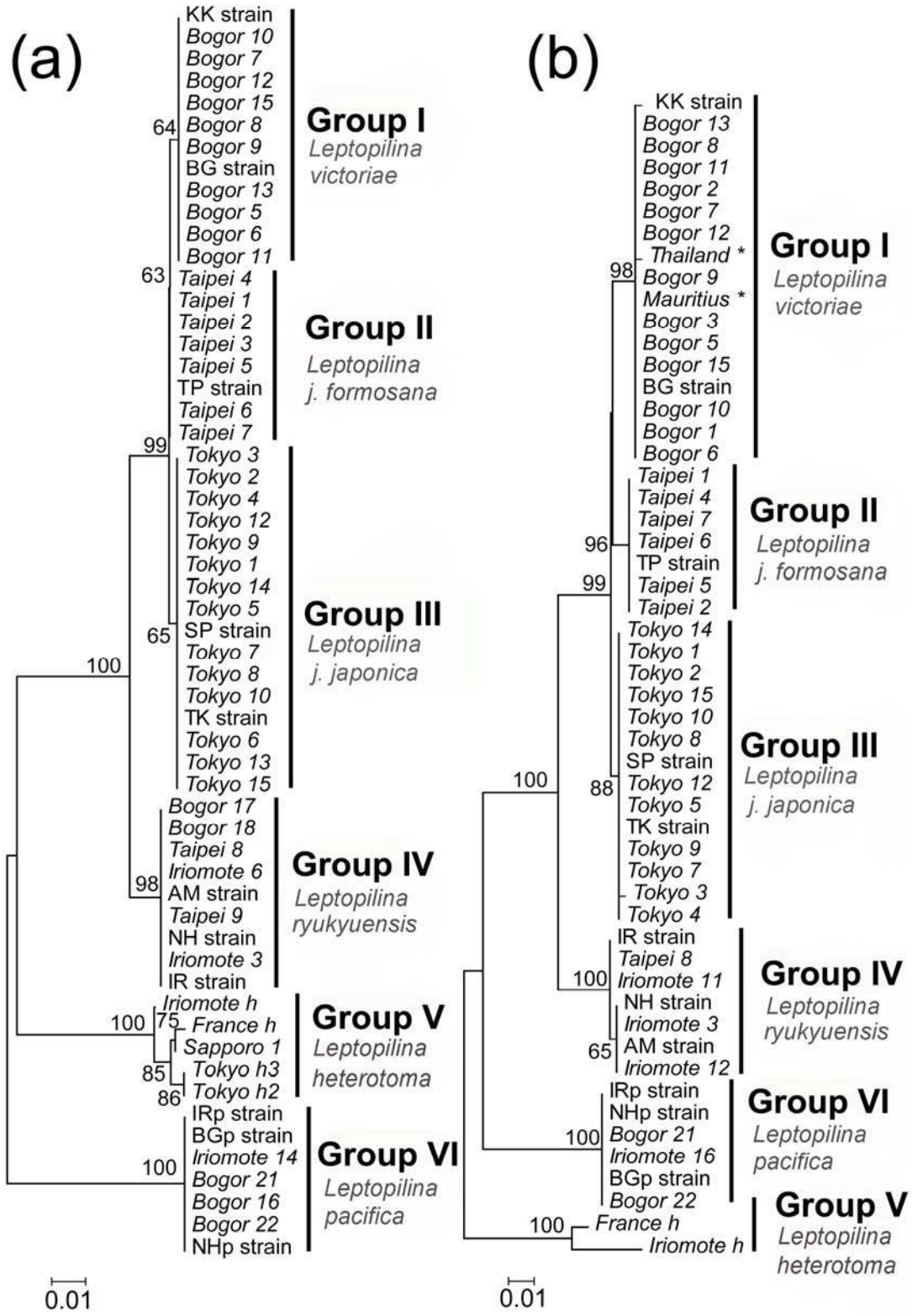

(a)
KK strain
Bogor 10
Bogor 7
Bogor 12
Bogor 15
Bogor 8
Bogor 9
BG strain
Bogor 13
Bogor 5
Bogor 6
Bogor 11
Group I
Leptopilina victoriae
Taipei 4
Taipei 1
Taipei 2
Taipei 3
Taipei 5
TP strain
Taipei 6
Taipei 7
Group II
Leptopilina j. formosana
Tokyo 3
Tokyo 2
Tokyo 4
Tokyo 12
Tokyo 9
Tokyo 1
Tokyo 14
Tokyo 5
SP strain
Tokyo 7
Tokyo 8
Tokyo 10
TK strain
Tokyo 6
Tokyo 13
Tokyo 15
Group III
Leptopilina j. japonica
Bogor 17
Bogor 18
Taipei 8
Iriomote 6
AM strain
Taipei 9
NH strain
Iriomote 3
IR strain
Group IV
Leptopilina ryukyuensis
Iriomote h
France h
Sapporo 1
Tokyo h3
Tokyo h2
Group V
Leptopilina heterotoma
IRp strain
BGp strain
Iriomote 14
Bogor 21
Bogor 16
Bogor 22
NHp strain
Group VI
Leptopilina pacifica
64
63
99
65
100
98
100
75
85
86
100
0.01
(b)
KK strain
Bogor 13
Bogor 8
Bogor 11
Bogor 2
Bogor 7
Bogor 12
Thailand *
Bogor 9
Mauritius *
Bogor 3
Bogor 5
Bogor 15
BG strain
Bogor 10
Bogor 1
Bogor 6
Group I
Leptopilina victoriae
Taipei 1
Taipei 4
Taipei 7
Taipei 6
TP strain
Taipei 5
Taipei 2
Group II
Leptopilina j. formosana
Tokyo 14
Tokyo 1
Tokyo 2
Tokyo 15
Tokyo 10
Tokyo 8
SP strain
Tokyo 12
Tokyo 5
TK strain
Tokyo 9
Tokyo 7
Tokyo 3
Tokyo 4
Group III
Leptopilina j. japonica
IR strain
Taipei 8
Iriomote 11
NH strain
Iriomote 3
AM strain
Iriomote 12
Group IV
Leptopilina ryukyuensis
IRp strain
NHp strain
Bogor 21
Iriomote 16
BGp strain
Bogor 22
Group VI
Leptopilina pacifica
France h
Iriomote h
Group V
Leptopilina heterotoma
98
96
99
100
88
100
65
100
100
0.01

Figure 4

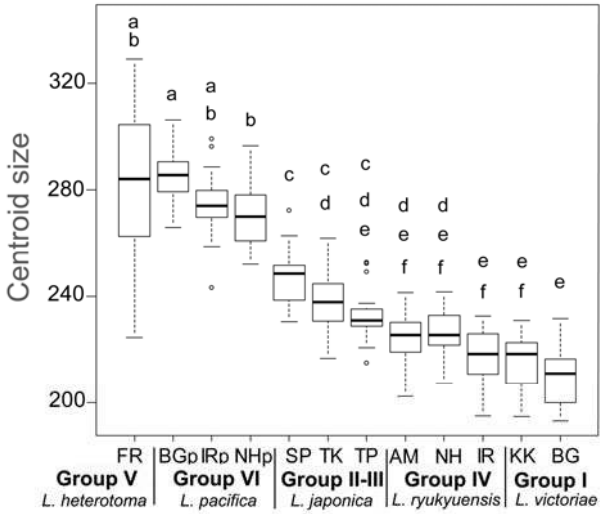

Centroid size
320
280
240
200
a
b
a
a
b
b
c
c
d
c
d
e
d
e
f
d
e
f
e
f
e
f
e
FR
BGp
IRp
NHp
SP
TK
TP
AM
NH
IR
KK
BG
Group V
L. heterotoma
Group VI
L. pacifica
Group II-III
L. japonica
Group IV
L. ryukyuensis
Group I
L. victoriae

Figure 5

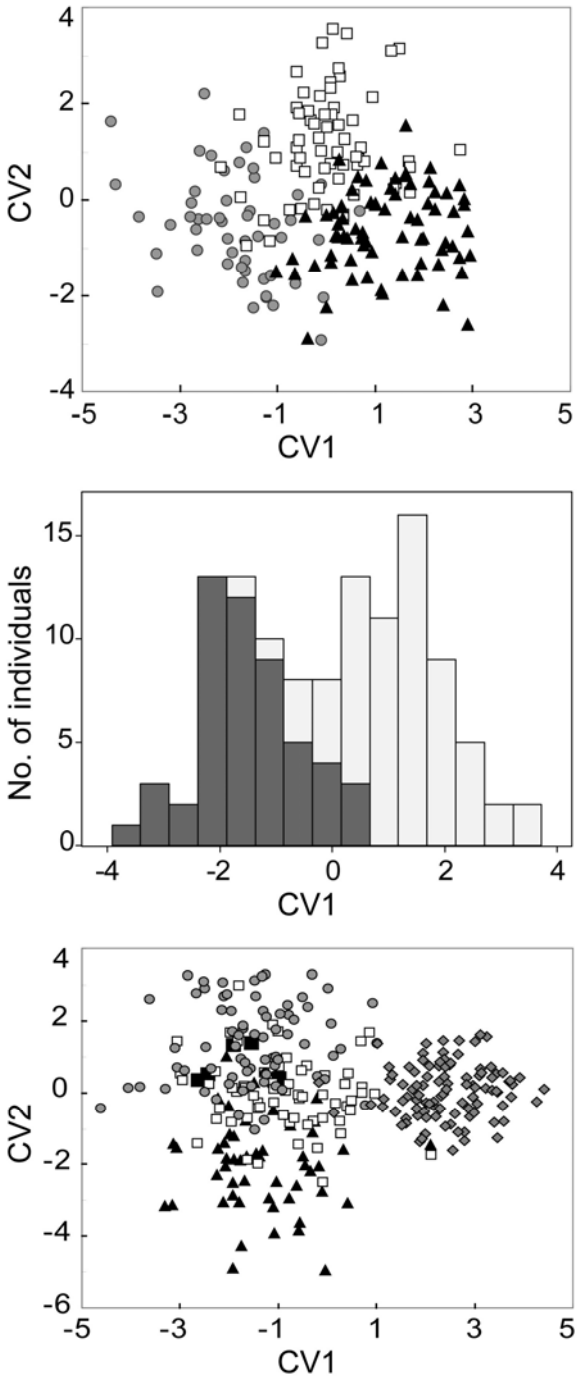


Figure 6

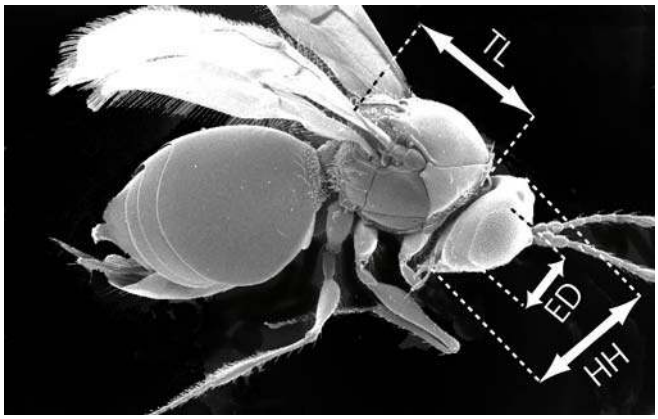


Figure 7

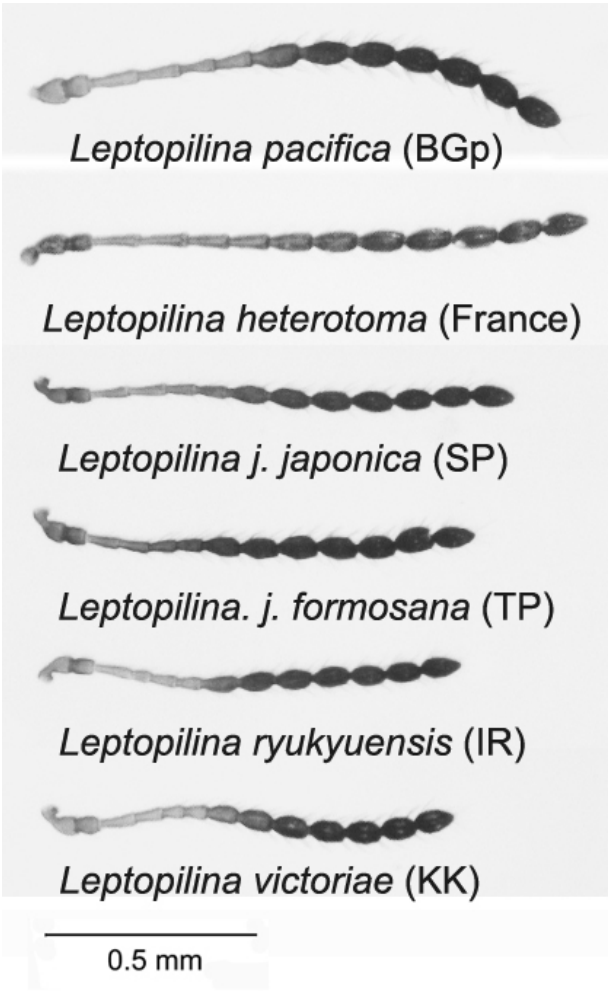

Leptopilina pacifica (BGp)
Leptopilina heterotoma (France)
Leptopilina j. japonica (SP)
Leptopilina. j. formosana (TP)
Leptopilina ryukyuensis (IR)
Leptopilina victoriae (KK)
0.5 mm

Figure 8

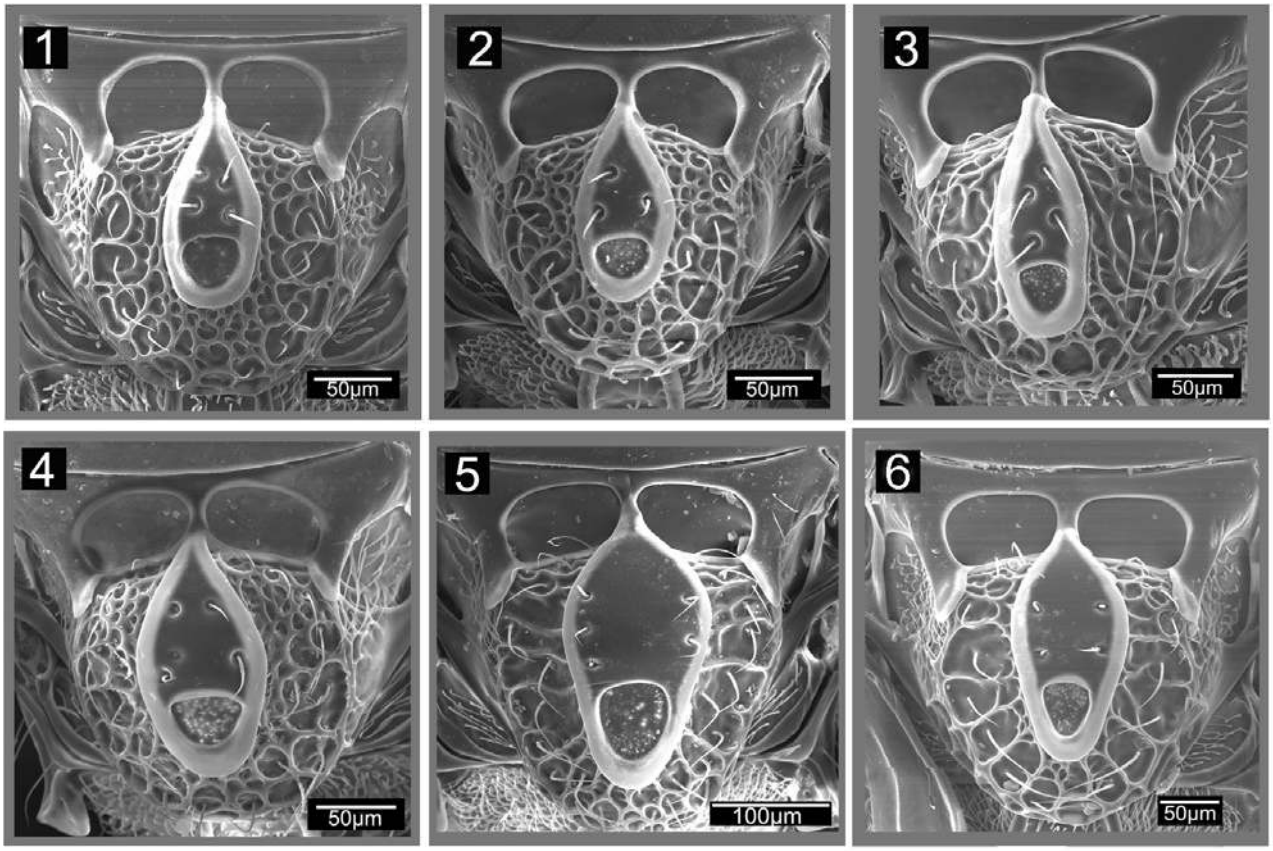


Figure 9

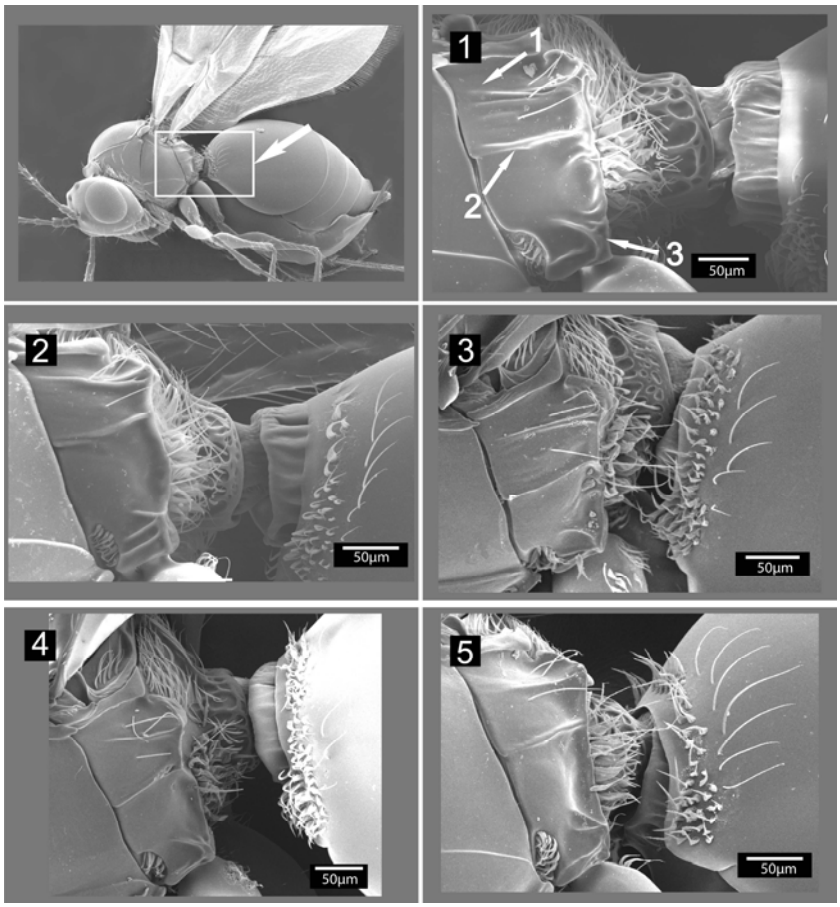


Figure 10

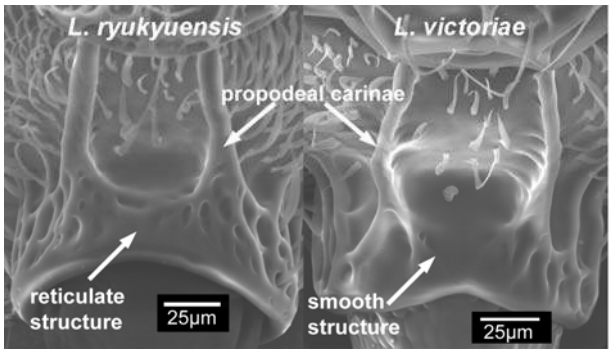
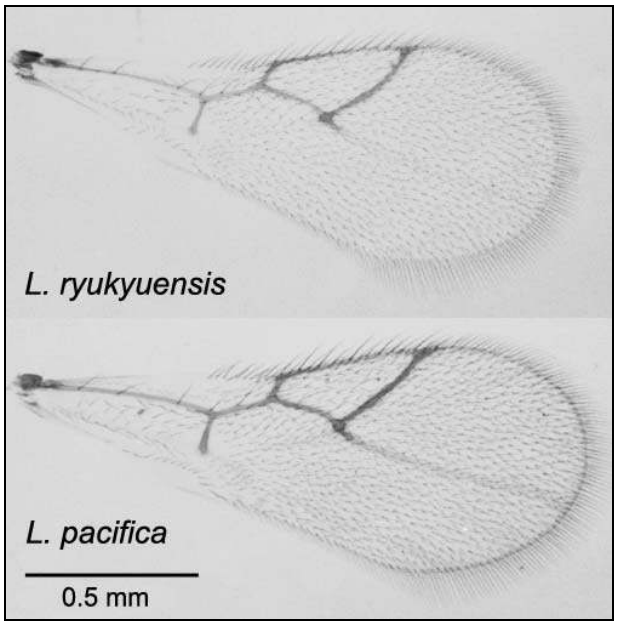

L. ryukyuensis
L. victoriae
propodeal carinae
reticulate structure
25μm
smooth structure
25μm

Figure 11


L. ryukyuensis
L. pacifica
0.5 mm