



Universiteit
Leiden
The Netherlands

Morphological and molecular phylogenetics in the genus *Leptopilina* (Hymenoptera: Cynipoidea: Eucoilidae)

Schilthuizen, M.; Nordlander, G.; Stouthamer, R.; Alphen, J.J.M. van

Citation

Schilthuizen, M., Nordlander, G., Stouthamer, R., & Alphen, J. J. M. van. (1998). Morphological and molecular phylogenetics in the genus *Leptopilina* (Hymenoptera: Cynipoidea: Eucoilidae). *Systematic Entomology*, 23(3), 253-264.
doi:10.1046/j.1365-3113.1998.00049.x

Version: Publisher's Version

License: [Licensed under Article 25fa Copyright Act/Law \(Amendment Taverne\)](#)

Downloaded from: <https://hdl.handle.net/1887/4310060>

Note: To cite this publication please use the final published version (if applicable).

Morphological and molecular phylogenetics in the genus *Leptopilina* (Hymenoptera: Cynipoidea: Eucoilidae)

MENNO SCHILTHUIZEN,^{1,3} GÖRAN NORDLANDER,²
RICHARD STOUTHAMER¹ and JACQUES J. M. VAN ALPHEN³

¹Department of Entomology, Wageningen Agricultural University, The Netherlands, ²Department of Entomology, Swedish University of Agricultural Sciences, Uppsala, Sweden, and ³Institute for Evolutionary and Ecological Sciences, Leiden University, The Netherlands.

Abstract. We have conducted cladistic analyses of the genus *Leptopilina*, a group of *Drosophila* parasitoids studied intensively by (behavioural) ecologists. Twenty-three morphological characters were scored in ten *Leptopilina* and two outgroup species. At the same time, DNA sequences for the second ribosomal internal transcribed spacer (ITS2) were gathered for eight *Leptopilina* and one outgroup species. Both data sets yielded phylogenetic trees which were largely compatible. A ‘total evidence’ analysis resulted in a single tree that provides a relatively robust phylogenetic reconstruction of the group, which may serve as a basis for historically interpreting the distribution of ecological and behavioural traits.

Introduction

Almost four decades ago, Walker (1959) used the parasitic wasp *Leptopilina heterotoma* (Fig. 1; then called *Pseudeucoila bochei*) to study the cellular immune response of its host *Drosophila melanogaster*. At the time, there was confusion about the taxonomic position of *L. heterotoma*, and no data were available on habitat and host use in the field. Since then, species of *Leptopilina* have become very popular for use in field and laboratory model systems to investigate host–parasitoid interactions. A taxonomic revision (Nordlander, 1980), behavioural studies by Vet & van Alphen (1985) and van Alphen *et al.* (1991), as well as the collection of species in the tropics by Chabora, Carton and others, characterized *Leptopilina* as a cosmopolitan genus, of which all known species are larval parasitoids of drosophilid flies. All the known tropical species have been collected from fermenting fruits, where they attack fly larvae of the *melanogaster* group of *Drosophila*. Other breeding sites of tropical *Drosophila*, i.e. fungi, flowers and decaying plant matter, have not been explored for their parasitoid fauna. In contrast, the six species occurring in Europe are known as specialists of either fermenting sap streams on wounded trees, fermenting fruits, fungi or decaying plants (van Alphen *et al.*, 1991). They are

adapted in many aspects of their searching behaviour, their life-history and physiology to attack a limited number of host species in a particular microhabitat.

Leptopilina species and their hosts are among the best studied host–parasitoid systems. There is a wealth of data already available on host-habitat location (Vet, 1983; Vet & van Opzeeland, 1985; Vet & Groenewold, 1990; van Alphen *et al.*, 1991; Vet & Dicke, 1992; Vet & Papaj, 1992), response to kairomones (Vet & van der Hoeven, 1984), mode of searching (Vet & van Alphen, 1985; van Alphen & Vet, 1986; van Dijken & van Alphen, 1997; van Alphen *et al.*, unpublished data), patch time allocation (van Lenteren & Bakker, 1978; Eijs & van Dijken, unpublished data), superparasitism (van Alphen *et al.*, 1987; van Alphen & Visser, 1990; Visser *et al.*, 1990; Visser, 1993; Roitberg *et al.*, 1992), timing of reproduction (Kopelman & Chabora, 1986; Eijs, unpublished data), diapause (Eijs, unpublished data), and on interactions with their hosts’ immune response (Bouletreau, 1986; Carton & Nappi, 1991; Rizki & Rizki, 1984a,b, 1990). Therefore, they form an ideal subject for comparative study of coevolution of parasitoids and hosts. Comparative studies, particularly those of closely related species, require a methodology based on the phylogeny of the species for two reasons. First, to distinguish between selectively neutral characters inherited from an ancestor and parallel adaptations. Secondly, characters in closely related species cannot be treated independently in statistical analyses due to possible historical dependencies (Brooks & McLennan, 1991; Harvey & Pagel, 1991).

Correspondence: Menno Schilthuisen, Institute for Evolutionary and Ecological Sciences, Leiden University, PO Box 9516, NL-2300 RA Leiden, The Netherlands. E-mail: Schilthuisen@rulsfb.leidenuniv.nl



Fig. 1. *Leptopilina heterotoma*, parasitising a *Drosophila* larva (photograph by Kees Hofker).

The taxonomic basis for such a comparative analysis is sufficient. *Leptopilina* was redescribed and revised by Nordlander (1980), who divided ten named and eleven additional species into three species groups: the *longipes* group, the *heterotoma* group and the *boulardi* group. The native European species *L. longipes*, *L. fimbriata* and *L. clavipes* were all placed in the *longipes* group. Later, an additional member of this group, *L. australis*, was discovered in Europe (van Alphen *et al.*, 1991; Nordlander & Grijpma, 1991). Also, the cosmopolitan species *L. heterotoma* and *L. boulardi* occur in Europe; however, other members of the *heterotoma* group are mainly found in the Oriental Region, and other species of the *boulardi* group appear to be confined to Africa (Nordlander, 1980). Species of the *longipes* group also occur in the Caribbean area and in South America (Nordlander, unpublished data). However, a phylogenetic framework is still lacking. What are available are Nordlander's (1980) species groups based on four morphological character complexes and a phylogeny of five European species (van Alphen *et al.*, 1991). The latter was also based on a small number of morphological characters. In the present study, we report a cladistic analysis of ten *Leptopilina* species previously or currently used for ecological studies. As the number of informative morphological characters is limited at this taxonomic level, we have supplemented them with DNA sequence data. Because of its easy amplification (Hillis & Dixon, 1991) and general applicability in intrageneric insect systematics (Wesson *et al.*, 1992; Campbell *et al.*, 1993; Sappal *et al.*, 1995; van Kan *et al.*, 1996; Schilthuizen & Stouthamer, 1997), the second internal transcribed spacer (ITS2) of the ribosomal DNA was our gene of choice. We have refrained from including the remaining eleven species which are not being studied ecologically because no live material was available for these and the inclusion of their morphological data would have resulted in large amounts of

missing data in the combined data set. Nevertheless, we think the resulting tree should provide a rigid support for interpreting previous and future ecological and behavioural data.

Materials and methods

Insect material

The species included in this study and the origin of the studied material are summarized in Table 1. Adult specimens from thirteen cultures of eight *Leptopilina* species and one *Ganaspis* species (outgroup) were used for the molecular study. The morphological data were gathered from the same species and, in addition, from a *Gronotoma* species (a more distant outgroup) and two undescribed Caribbean *Leptopilina* species, which have previously been included in a comparative study on host detection behaviour (Vet & van Alphen, 1985). No live material was available for molecular study of these additional species. Dry mounted specimens of all studied species are deposited in the collection of the Department of Entomology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

DNA extraction

Between one and five freshly frozen specimens from each culture were ground with a sterile, DNA-free glass rod in a mixture of 300 µl phenol and 300 µl extraction buffer (10 mM Tris; 2.5 mM MgCl₂; 50 mM KCl) and 15 µl SDS (20% solution). Subsequently, the aqueous phase was shaken with 300 µl chloroform/iso-amylalcohol (24:1), after which the DNA was precipitated at -20°C in 70% ethanol and 0.1 M sodium

Table 1. List of the material. IEES = Institute for Evolutionary and Ecological Sciences; WAU = Wageningen Agricultural University, Entomology Department.

Species	Collected	Culture location
<i>Gronotoma allotriaieformis</i>	Stockholm, Sweden	Dry material only
<i>Ganaspis xanthopoda</i>	Thailand	IEES, Leiden
	The Philippines	IEES, Leiden
<i>Leptopilina longipes</i> Spier	The Netherlands	IEES, Leiden
<i>L. sp.</i> 'PR222-2'	Puerto Rico	Dry material only
<i>L. sp.</i> 'PB10-5'	Guadeloupe	Dry material only
<i>L. fimbriata</i>	Leiden, The Netherlands	IEES, Leiden
<i>L. clavipes</i>	Leiden, The Netherlands	IEES, Leiden
<i>L. sp. near clavipes</i>	U.S.A.	IEES, Leiden
<i>L. australis</i>	Emmeloord, The Netherlands	IEES, Leiden
<i>L. bouardi</i>	Fasogil, Greece	IEES, Leiden
	Gambia	IEES, Leiden
<i>L. heterotoma</i>	Rochester, New York, U.S.A.	IEES, Leiden
	Wageningen, The Netherlands	WAU
<i>L. victoriae</i>	Thailand	IEES, Leiden

Table 2. The morphological data set.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
<i>Gronotoma allotriaieformis</i>	0	0	0	–	–	–	0	0	0	0	0	–	–	0	0	0	0	0	0	3	0	0	
<i>Ganaspis xanthopoda</i>	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	–	0	
<i>Leptopilina longipes</i>	1	0	0	1	0	0	1	0	2	0	1	1	0	0	1	0	0	1	1	0	1	0	1
<i>L. sp.</i> PR222-2	?	?	1	1	1	2	1	0	2	0	1	1	0	0	1	0	0	1	1	0	1	0	1
<i>L. sp.</i> PB10-5	?	?	1	1	1	2	1	0	2	0	1	1	0	0	1	0	0	1	1	0	1	0	1
<i>L. fimbriata</i>	1	0	0	0	0	0	1	0	2	0	1	1	0	0	1	1	2	1	1	0	2	0	0
<i>L. clavipes</i>	1	0	0	0	0	0	0	0	2	0	2	2	0	0	1	1	2	1	1	1	2	0	0
<i>L. sp. near clavipes</i>	1	0	0	0	0	0	0	0	2	0	1	1	0	0	1	1	2	1	1	0	2	0	0
<i>L. australis</i>	1	0	0	0	0	0	0	1	2	0	1	1	0	0	1	1	2	1	1	0	2	0	0
<i>L. bouardi</i>	1	1	0	0	0	1	1	1	1/2	2	1	0	1	0	1	1	3	1	1	1	1	1	0
<i>L. heterotoma</i>	1	1	0	0	0	0	1	0	1	1	1	1	2	1	1	1	2	1	1	0	2	1	0
<i>L. victoriae</i>	1	1	0	0	0	0	1	0	1	1	1	1	2	1	1	1	2	1	1	0	2	1	0

acetate. Afterwards, the DNA was dissolved in 100 µl Tris/EDTA buffer. The material used is presented in Table 1.

PCR amplification

ITS2 in the ribosomal DNA (including portions of the flanking regions of the 5.8S and 28S genes) was amplified with the following PCR-primers: 5'TGT CAA CTG CAG GAC ACA TG3' (forward) and 5'AAT GCT TAA ATT TAG GGG GTA3' (reverse) (Campbell *et al.*, 1993). Each PCR reaction was carried out in a 50 µl reaction volume using, apart from standard conditions, 0.5 U SuperTth DNA-polymerase, 5 pmol of each primer and a final [Mg²⁺] of 2.8 mM.

Cloning and sequencing

PCR products were excised from agarose gels, extracted by the 'freeze-squeeze technique' (Sambrook *et al.*, 1989) and cloned in a pMOSBlue T-tailed vector (Amersham Life Science). Recombinants were checked for the presence of the

correct insert by rapid direct colony screening, as recommended by the manufacturer. Positive colonies were then grown overnight in liquid medium, and plasmid DNA was isolated using QIAprep spin columns (QIAGEN). The insert (generally ≈ 500 bp in length) was sequenced in one direction on an ABI automated sequencer. With the exception of *Ganaspis xanthopoda* from Thailand, from which two clones were sequenced, we analysed a single clone for each culture. Sequences are registered with GenBank under accession numbers AF015890–AF015902.

Phylogenetic analysis

ITS2 sequences were automatically aligned in the Sequence Navigator 1.0 (ABI, 1994), using a CLUSTAL (Higgins & Sharp, 1988) algorithm with settings of gap penalty = 3, gap extend penalty = 3, match penalty = 5, ktp = 3, top diagonals = 8, window size = 10. These settings were chosen from several different parameter settings because they produced an alignment in which the longest stretches of homology were observed.

	10	20	30	40
<i>G. xanthopoda</i> (Thailand)	GTCTCGCC-T	AAT-CTGAGG	TCGTCAAAT-	---TA----
<i>G. xanthopoda</i> (Thailand)	GTCTCGCC-T	AAT-CTGAGG	TCGTCAAAT-	---TA----
<i>G. xanthopoda</i> (Philippines)	GTCTCGCC-T	AAT-CTGAGG	TCGTCAAAT-	---TA----
<i>L. longipes</i>	GTCTCGCC-T	AAT-CTGAGG	TCGTCATATA	ATTTAA----
<i>L. fimbriata</i>	GTCTCGCC-T	AAT-CTGAGG	TCGTCATATA	AGA-AA----
<i>L. clavipes</i>	GTCTCGCC-T	AAT-CTGAGG	TCGTCATATA	-GA-AA----
<i>L. sp. near clavipes</i>	GTCTCGCC-T	AAT-CTGAGG	TCGTCATATA	-GA-AA----
<i>L. australis</i>	GTCTCGCC-T	AAT-CTGAGG	TCGTCATATA	GG-AA----
<i>L. boulandi</i> (Fasogil)	GTCTCCCCCT	AAT-CTGAGG	TCGNCA-ATA	AGA-ANNNNN
<i>L. boulandi</i> (Gambia)	GTCTCGCC-T	AAT-CTGAGG	TCGTCA-ATA	TG-----N
<i>L. heterotoma</i> (Rochester)	CTCTCNCC-T	AATGCTGAGG	TCGTCATA-A	Aa-----
<i>L. heterotoma</i> (Wageningen)	GTCTCGCC-T	AAT-CTGAGG	TCGTCATA-A	AG-----
<i>L. victoriae</i>	GTCTCGCC-T	AAT-CTGAGG	TCGTCATA-A	AG-----
	50	60	70	80
<i>G. xanthopoda</i> (Thailand)	-----AAA	ACTTTACACT	TTGCCTTTT	T-----
<i>G. xanthopoda</i> (Thailand)	-----AAA	ACTTTACACT	TTGC-TTTT	T-----
<i>G. xanthopoda</i> (Philippines)	-----AAA	ACTTTACACT	TT-----TT	T-----
<i>L. longipes</i>	-----AAAT	ATTATAAAAA	TAATATGACA	--ACAAC--A
<i>L. fimbriata</i>	-----GAAA	AA-AAAATAA	TACTAT----	
<i>L. clavipes</i>	-----GAAA	AA-ATAAAAA	TACTATTTAA	--ACAAC--A
<i>L. sp. near clavipes</i>	-----GAA-	A--ATAAAAA	TACTATTTAA	--ACAAC--A
<i>L. australis</i>	-----GAA-	A--ATAAAAA	TACTATTTAA	--ACAAC--A
<i>L. boulandi</i> (Fasogil)	GNNNNGGAAT	AATAANATAT	TTTTTTTTTT	TNATAACCCG
<i>L. boulandi</i> (Gambia)	GNNA---AAT	AATAA--TAA	TA-----	--ATAAC--A
<i>L. heterotoma</i> (Rochester)	-----AA	AAT-----	-----TTTAG	TCACAAAAAA
<i>L. heterotoma</i> (Wageningen)	-----AA	AAT-----	-----TTTAG	TCACAAAAAA
<i>L. victoriae</i>	-----AA	AAT-----	-----TTTAG	TCACAAAAAA
	90	100	110	120
<i>G. xanthopoda</i> (Thailand)	---TCTTTAT	T-TA--TAAC	---CACTtAT	GTGT-----
<i>G. xanthopoda</i> (Thailand)	---T-TTTAT	T-TA--TAAC	---CACTTAT	GTGT-----
<i>G. xanthopoda</i> (Philippines)	---T-TTTGT	T-TA--TAAC	---CACTTAT	GTGT-----
<i>L. longipes</i>	A---CCATAT	TATATATAAC	AC-CACTAAT	ATGTA-TCAA
<i>L. fimbriata</i>	-----CT	CATAGC-AAC	AC-CACTTAT	TTGTA-TCAA
<i>L. clavipes</i>	AAGTCAATCT	CATAGC-AAC	AC-CACTTAT	TTGTAATCAA
<i>L. sp. near clavipes</i>	AAGTCAATCT	CATAGC-AAC	AC-CACTTAT	TTGTAATCAA
<i>L. australis</i>	ATGTCAATCT	CATAGC-AAC	AC-CACTTAT	TTGTAATCAA
<i>L. boulandi</i> (Fasogil)	A---AATAT	CNAaCAAAAT	ATTCACCTAT	TTGTA-TCaA
<i>L. boulandi</i> (Gambia)	A---aAAAT	C-AaACAAAT	ATTCACCTAT	TTGTA-TCAA
<i>L. heterotoma</i> (Rochester)	AAATAAACAA	CAACATTA-	ATTCACCTAT	TTGTA-TCAA
<i>L. heterotoma</i> (Wageningen)	AAATAAACAA	CAACATTA-	ATTCACCTAT	TTGTA-TCAA
<i>L. victoriae</i>	AT-TACACAA	CAAA-----	ATTCACCTAT	TTGTA-TCAA
	130	140	150	160
<i>G. xanthopoda</i> (Thailand)	---G-TCAA-	---TT-----	TA-ACATATT	-tGTA---TA
<i>G. xanthopoda</i> (Thailand)	---G-TCAA-	---TT-----	TA-ACATATT	-TGTA---TA
<i>G. xanthopoda</i> (Philippines)	---G-TCAA-	---TT-----	TA-ACATATT	-TGTA---TA
<i>L. longipes</i>	A-AGATCACA	---TTC----	---AAA----	-----T-
<i>L. fimbriata</i>	ATAGA-CAAA	-CGTTC----	---AAAA--	-----A--T-
<i>L. clavipes</i>	ATAGA-CAAA	-CGTTC----	---AAAATT	C--CA--T-
<i>L. sp. near clavipes</i>	ATAGA-CAAA	-CGTTC----	---AAAATT	T--CA--T-
<i>L. australis</i>	ATAGA-CAAA	-CGTTC----	---AAAATT	T--CA--T-
<i>L. boulandi</i> (Fasogil)	ATAGA-CA-A	-CATTC----	-----AT-	-----A--T-
<i>L. boulandi</i> (Gambia)	ATAGA-CA-A	-CATTC----	TA-aAAAAAT	TTGCA---T-
<i>L. heterotoma</i> (Rochester)	TAAGA-CAAA	GCGTTCAAAA	TA---AAATC	TTGTACGATA
<i>L. heterotoma</i> (Wageningen)	TAAGA-CAAA	GCGTTCAAAA	TA---AAATC	TTGTACGATA
<i>L. victoriae</i>	TAAGA-CAAA	ACGTTCAAAA	AAGAAAAATC	TTGTATGATA

Fig. 2. Alignment of the DNA sequences for ITS2, plus flanking regions. Regions of ambiguous alignment have been indicated with bold toplines. These have been excluded from the phylogenetic analysis.

	170	180	190	200
<i>G. xanthopoda</i> (Thailand)	TGTG-TTGAT	ATTCAGTATG	--ACA-----	----T---AT
<i>G. xanthopoda</i> (Thailand)	TGTG-TTGAT	ATTCAGTATG	--ATA-----	----T---AT
<i>G. xanthopoda</i> (Philippines)	TGTG-TTGAT	ATTCAGTATG	--ATA-----	----T---AT
<i>L. longipes</i>	TG-----	ATGGTTTttG	--AAAAAATT	-----GT
<i>L. fimbriata</i>	TGTATTGA-	ATGGTCTTTT	--CTTTT---T	---TT---AT
<i>L. clavipes</i>	TGTGTT-GA-	ATGGT-TTTA	--AAATTT--	-----AT
<i>L. sp. near clavipes</i>	TGTGTT-GA-	ATGGT---T	--AAAAATT	-----AT
<i>L. australis</i>	TGTGTT-GA-	ATGGT---T	--AAAAATT	-----AT
<i>L. bouldardi</i> (Fasogil)	NNNANT----	---GATTTT-	-----A	---TT---AT
<i>L. bouldardi</i> (Gambia)	TTTATT--A-	ATTGATTTT-	-----T	---TT---AT
<i>L. heterotoma</i> (Rochester)	T--ATTT--T	TTTCTATATG	TCATTTAATT	CTGTTGGCAT
<i>L. heterotoma</i> (Wageningen)	T--ATTT--T	TTTCTATATG	TCATTTAATT	CTGTTGGCAT
<i>L. victoriae</i>	TTTATTG--T	TTTTTC-AAG	TCATTTAATT	CTGTTGGCAT
	210	220	230	240
<i>G. xanthopoda</i> (Thailand)	-----T--TC	ATCGGTC-G-	-GTGTG----	-CTTTAG---
<i>G. xanthopoda</i> (Thailand)	-----T--TC	ATCGGTC-G-	-GTGTG----	-CATTAG---
<i>G. xanthopoda</i> (Philippines)	-----T--TC	ATCGGTC-G-	-GTGTG----	-CTTTAG---
<i>L. longipes</i>	-----C-AT-	CTCGGTC-TA	AGTAA-ACGT	TC---AAACT
<i>L. fimbriata</i>	-----TGAA-	GTCGGTC-GA	AGTATGATTT	TTTTTAAACT
<i>L. clavipes</i>	-----TGAA-	GTCGGTC-GA	AGTATGATT-	-----AAACT
<i>L. sp. near clavipes</i>	-----TGAA-	GTCGGTC-GA	AGTAT---GA	TT---AAACT
<i>L. australis</i>	-----TGAA-	GTCGGTC-GA	AGTAT---GA	TT---AAACT
<i>L. bouldardi</i> (Fasogil)	-----TAATC	GTCGGTC-GA	AGTA-----	-----AAATT
<i>L. bouldardi</i> (Gambia)	-----TAATC	GTCGGTC-GA	AGTA-----	-----AAATT
<i>L. heterotoma</i> (Rochester)	ATTGATAATC	ATCGGTCTGA	AGTAT-----	-----AACT
<i>L. heterotoma</i> (Wageningen)	ATTGATAATC	ATCGGTCTGA	AGTAT-----	-----AACT
<i>L. victoriae</i>	AATGATAATC	ATCGGTCTGA	AGTAT-----	-----AACT
	250	260	270	280
<i>G. xanthopoda</i> (Thailand)	--CTCCATTG	ACTC---GAC	AAT-----CA	ATCA-A----
<i>G. xanthopoda</i> (Thailand)	--CTCCATTG	ACTC---GAC	AAT-----CA	ATCA-A----
<i>G. xanthopoda</i> (Philippines)	--CTCCATTG	ACTC---GAC	AAT-----CA	ATCA-A----
<i>L. longipes</i>	TATTTTATTG	ACACAAGGAC	AACAAAACCA	ATCA-ATA--
<i>L. fimbriata</i>	TACT-CATTG	ACACAACAAC	AACAA--CCA	AACA-AAATT
<i>L. clavipes</i>	TACT-CATTG	ACACAACGAC	AACAA--CCA	AACA-ATATT
<i>L. sp. near clavipes</i>	TACT-CATTG	ACACAACGAC	AACAA--CCA	AACA-ATATT
<i>L. australis</i>	TACT-CATTG	ACACAACGAC	AACAA--CCA	AACA-ATATT
<i>L. bouldardi</i> (Fasogil)	TACT-CATTG	ACACAGCGGC	AATA--GTCA	ATCA-A-AT-
<i>L. bouldardi</i> (Gambia)	TACT-CATTG	ACACAACGAC	AATA--GCCA	ATCA-A-ATG
<i>L. heterotoma</i> (Rochester)	TACT-CATTG	ACACAATAAC	AACA--ACCA	AACAGAAAAT
<i>L. heterotoma</i> (Wageningen)	TACT-CATTG	ACACAATAAC	AACA--ACCA	AACAGAAAAT
<i>L. victoriae</i>	TACT-CATTG	ACACAATGAC	AACACAACCA	AACGAAATTT
	290	300	310	320
<i>G. xanthopoda</i> (Thailand)	--CGAA--TA	CAACAG---C	TA-----CAA	TGT-----
<i>G. xanthopoda</i> (Thailand)	--CGAA--CA	CAACAG---C	TA-----CAA	TGT-----
<i>G. xanthopoda</i> (Philippines)	--CGAA--CA	CAACAG---C	TA-----CAA	TGT-----
<i>L. longipes</i>	----A-T-CA	-AA-----A	AAGAT--CA-	----TA----
<i>L. fimbriata</i>	CACAA-T-TA	-AA-AT---A	TA-----	T---ATGTC
<i>L. clavipes</i>	CACAA-T-TA	-AATAT---A	AA-----	T---TATGTC
<i>L. sp. near clavipes</i>	CACAA-T-TA	-AATAT---A	AATTT--CAA	T---TATGTC
<i>L. australis</i>	CACAA-T-TA	-AATAT---A	AATTT--CAA	T---TATGTC
<i>L. bouldardi</i> (Fasogil)	-ATAAAT-TT	NAA-AT----	T-----CA-	-----TGTC
<i>L. bouldardi</i> (Gambia)	CATAAAT-CA	CCACAT----	TATTA--CAA	TGTGTTTGTC
<i>L. heterotoma</i> (Rochester)	TTTAAATGCA	CAATAT-ATC	AATTAAACAA	-GTGTCT---
<i>L. heterotoma</i> (Wageningen)	TTTAAATGCA	CAATAT-ATC	AATTAAACAA	-GTGTCT---
<i>L. victoriae</i>	TTTAAATGCA	CAATATTATC	AATATTACAA	-GTGTCT---

Fig. 2. Continued.

	330	340	350	360
<i>G. xanthopoda</i> (Thailand)	TTTATAACAT	TGACACGTGG	-----TATC	GTTCAACAAT
<i>G. xanthopoda</i> (Thailand)	TCTATAACAT	TGACACGTGG	-----TATC	GTTCAACAAT
<i>G. xanthopoda</i> (Philippines)	TTTATAACAT	TGACACGTGG	-----TATC	GTTCAACAAT
<i>L. longipes</i>	---ATAT-AT	TGATACGTGG	CT---G---C	GTTCAA----
<i>L. fimbriata</i>	TTTATAT-AT	TGATACGTGG	AT---TTATC	GTTCAAAAAT
<i>L. clavipes</i>	TTTATAT-AT	TGATACGTGG	AT---ATATC	GTTCAAAA--
<i>L. sp. near clavipes</i>	TTTATAT-AT	TGATACGTGG	AT---ATATC	GTTCAAAA--
<i>L. australis</i>	TTTATAT-AT	TGATACGTGG	AT---ATATT	GTTCAAAA--
<i>L. bouldardi</i> (Fasogil)	TTTATTTT-TT	TGANACGTGN	ATTAAATC--	GTTCAA--AT
<i>L. bouldardi</i> (Gambia)	TTTATTTT-AT	TGATACGTGA	ATTAAATCTC	GTTCAA--AT
<i>L. heterotoma</i> (Rochester)	TT-ATAT-AT	TGATACGTGA	ATT---TATC	GTTCAA----
<i>L. heterotoma</i> (Wageningen)	TT-ATAT-AT	TGATACGTGA	ATT---TATC	GTTCAA----
<i>L. victoriae</i>	TTTATAT-AT	TGATACGTGA	ATT---AGTC	GTTCAA----
	370	380	390	400
<i>G. xanthopoda</i> (Thailand)	--TT-----	-TA-TAAATT	TG---TGT--	TCA-GTCG--
<i>G. xanthopoda</i> (Thailand)	--TT-----	-TA-TAAATT	TG---TGT--	CCA-GTCG--
<i>G. xanthopoda</i> (Philippines)	--TT-----	-TA-TAAATT	TG---TGT--	TCA-GTCG--
<i>L. longipes</i>	--TT--A--A	GTAAGTTC	TG--TTATAG	TCAT--ATTA
<i>L. fimbriata</i>	GATTGCATGA	G-----	-----	-----TG
<i>L. clavipes</i>	GATTGCATGA	G-AGTTCATG	TGACTTGTCG	TCA---ATTG
<i>L. sp. near clavipes</i>	GATTGCACGA	G-AGTTCATG	TGACTTGTCG	TCA---ATtG
<i>L. australis</i>	GATTGCACGA	G-AGTTCATG	TGACTTGTCG	TCA---ATTG
<i>L. bouldardi</i> (Fasogil)	AATA-----	--ATTG-TT	TGACTTGCCA	TTTTGTATTA
<i>L. bouldardi</i> (Gambia)	AATT-----	-TATTTG-TT	TGACATGTCA	TTCTGTAT--
<i>L. heterotoma</i> (Rochester)	-----	-TAG--AAGT	TGT-TTGT-G	TTATTTCTT-
<i>L. heterotoma</i> (Wageningen)	-----	-TAG--AAGT	TGT-TTGT-G	TTATTTCTT-
<i>L. victoriae</i>	-----	-TAGTTAAGT	TGT-TTGT-G	T-ATTT--T-
	410	420	430	440
<i>G. xanthopoda</i> (Thailand)	-AT----TTA	T-----	AA-----AA	TTCAACTTGT
<i>G. xanthopoda</i> (Thailand)	-AT----TTA	T-----	AA-----AA	TTCAACTTGT
<i>G. xanthopoda</i> (Philippines)	-AT----TTA	T-----	AA-----AA	TTCAACTTGT
<i>L. longipes</i>	TATAATATTA	TAATAT----	-AT--ATCAT	T-CAACTTAT
<i>L. fimbriata</i>	TAT---ATT-	TTATTTT-CT	AATA-AGCAA	TTCAACTTGT
<i>L. clavipes</i>	TATTTTATTA	TTATTTT-CT	aATA-AGCAA	TTCAACTTGT
<i>L. sp. near clavipes</i>	TATTTTATTA	TTATTTT-CT	AATA-AGCAT	TTCAACTTGT
<i>L. australis</i>	TATTTTATTA	TTATTTT-CT	AATC-AGCAT	TTCAACTTGT
<i>L. bouldardi</i> (Fasogil)	TATA-TATTA	TTTTTTT-AT	AATATAATAA	TTCAACTTAT
<i>L. bouldardi</i> (Gambia)	-----ATTA	TTATTTT-AT	AATATATAAA	TTCAACTTAT
<i>L. heterotoma</i> (Rochester)	TTTCTT-TCA	-AATGTGACT	GAAATAT--A	TTCAACTTGT
<i>L. heterotoma</i> (Wageningen)	TTTCTT-TCA	-AATGTGACT	GAAATAT--A	TTCAACTTGT
<i>L. victoriae</i>	TTTCTT-TCA	-AATGTGACT	GAAATAT--A	TTCAACTTGT
	450	460	470	480
<i>G. xanthopoda</i> (Thailand)	TTTAAACGAC	ACCTTTTCAA	AGTAAAAAAG	AATT----TC
<i>G. xanthopoda</i> (Thailand)	TTTAAACGAC	ACCTTTTCAA	AGTAAAAAAG	AATT----TC
<i>G. xanthopoda</i> (Philippines)	TTTAAACGAC	ACCTTTTCAA	AGTAAAAAAG	AATT----TC
<i>L. longipes</i>	TTCAAACAAC	A-----TCAA	-----	--T---AAC
<i>L. fimbriata</i>	TTCAAACGAC	A-----TCAA	-AT-----	--TT---ATC
<i>L. clavipes</i>	TTCAAACGAC	A-----TTAA	-AT-----	--TT---ATC
<i>L. sp. near clavipes</i>	TTCAAACGAC	A-----TTAA	-AT-----	--TT---ATC
<i>L. australis</i>	TTCAAACGAC	A-----TTAA	-AT-----	--TT---ATC
<i>L. bouldardi</i> (Fasogil)	TTCAAACGAC	A-----TCAA	TATATATTTT	TTTT---TTT
<i>L. bouldardi</i> (Gambia)	TTCAANCGAC	A-----TCAA	TATATATATA	TATT---AT-
<i>L. heterotoma</i> (Rochester)	TTCAAATGAC	ACC-----AA	TAT---TATA	TTTTTCGAA--
<i>L. heterotoma</i> (Wageningen)	TTCAAATGAC	ACC-----AA	TAT---TATA	TTTTTCGAA--
<i>L. victoriae</i>	TTCAAATGAC	ACC-----AA	T-----TG	TTTTCTAAAC

Fig. 2. Continued.

	490	500	510	520
<i>G. xanthopoda</i> (Thailand)	TTCT--TTTC	---ACA-AAT	CA--A----	-----GATGA
<i>G. xanthopoda</i> (Thailand)	TTCT--TTTC	---ACA-AAT	CA--A----	-----GATGA
<i>G. xanthopoda</i> (Philippines)	TTCTCTTTTA	---ACA-AAT	CA--A----	-----GATGA
<i>L. longipes</i>	ATAAATAT-A	---AAA-AAT	A-----TGT-	T-TATGATGA
<i>L. fimbriata</i>	ATTTTTTTT-A	---AAA-AAA	AA--AATGT-	T-T-TGATGA
<i>L. clavipes</i>	ATTTTAT----	-----A	AA--AATGT-	N-T-TAATGA
<i>L. sp. near clavipes</i>	ATT--TAT-A	---AAA-A--	-----TGT-	t-T-TAATGA
<i>L. australis</i>	ATT--TAT-A	---AAA-A--	-----TGT-	T-T-TAATGA
<i>L. boulandi</i> (Fasogil)	ATTATTATTA	---TAATAAT	AA--AATAT-	TATTTGATGA
<i>L. boulandi</i> (Gambia)	AT-ATTA--A	---AAATATA	AA--AATAT-	TATTTGATGA
<i>L. heterotoma</i> (Rochester)	----TTTCTA	-ATAAAGAAA	AAGAAATATA	TAT-TGATGA
<i>L. heterotoma</i> (Wageningen)	----TTTCTA	-ATAAAGAAA	AAGAAATATA	TAT-TGATGA
<i>L. victoriae</i>	TTTTTTTTTTA	CAAAAAGAAG	TAGAAATGTA	T---TGATGA
	530	540	550	560
<i>G. xanthopoda</i> (Thailand)	ACGTTTATTT	CTTTCGCTCG	TATC-----	---TCTCC--
<i>G. xanthopoda</i> (Thailand)	ACGTTTATTT	CTTTCGCTCG	TATC-----	---TCTCC--
<i>G. xanthopoda</i> (Philippines)	ACGTTTATTT	CTTTCGCTCG	TATC-----	---TCTCC--
<i>L. longipes</i>	ATGTTCAATA	-TTTCGCTCG	TATCAGT-AT	GTGTGTGTAA
<i>L. fimbriata</i>	ACGTTTCATTA	-TTTCGCTA-	TATCA-----	-----CAA
<i>L. clavipes</i>	ACGTTTCATTA	-TTTCGCTA-	AATCT-----	-----TAA
<i>L. sp. near clavipes</i>	ACGTNCATTA	-TTTCGCNAA	-ATCT-T-A-	-----AA
<i>L. australis</i>	ACGTCCATTA	-TTTCGCTAA	-ATCT-T-A-	-----AA
<i>L. boulandi</i> (Fasogil)	ACGTTTCATTA	-TTTCGCT-T	AAAT--T-AT	-----TCAAA
<i>L. boulandi</i> (Gambia)	ACGTTTCATTA	-TTTCGCTAT	AATT--T-AT	-----TCAAA
<i>L. heterotoma</i> (Rochester)	ACATTCATTA	-TTTCGCTTG	AATCTTTT--	TT-TCTC---
<i>L. heterotoma</i> (Wageningen)	ACATTCATTA	-TTTCGCTTG	AATCTTTT--	TT-TCTC---
<i>L. victoriae</i>	ACATTCATTA	-TTTCGCTTA	AATCTTTTAT	TT-TTTC---
	570	580	590	600
<i>G. xanthopoda</i> (Thailand)	-TAATG----	-----	AAA-GGAGAG	ATAAGCAGTT
<i>G. xanthopoda</i> (Thailand)	-TAATG----	-----	AAA-GGAGAG	ATAAGCAGTT
<i>G. xanthopoda</i> (Philippines)	-TAATA----	-----	AAAAGGAGAG	ATAAGCAGTT
<i>L. longipes</i>	TTT-TATAAA	TTACAACAAC	AACAATAAAG	ATAAGCAGTT
<i>L. fimbriata</i>	T----ATACA	T-----	-----ATG	ATAAGCAGTT
<i>L. clavipes</i>	----ATTCT	TTTCGGANA	-----GAAAG	ATAAGCAGTT
<i>L. sp. near clavipes</i>	TTC-T-----	TTTCGA----	---AAGAAAG	ATNNGCNGTT
<i>L. australis</i>	TTC-T-----	TTTCGA----	---AAGAAAG	ATAAGCAGTT
<i>L. boulandi</i> (Fasogil)	TTA-----	---TGA----	---ATAAA-	--AAGCAGTT
<i>L. boulandi</i> (Gambia)	ATAATA-AAA	TTTTTGA----	---ATaAA-	--AAGCAGTT
<i>L. heterotoma</i> (Rochester)	----TATAGA	T-----AG	AGAGA-AA-G	ATAAGCAGTT
<i>L. heterotoma</i> (Wageningen)	----TATAAA	T-----AG	AGAGA-AA-G	ATAAGCAGTT
<i>L. victoriae</i>	----TGTATT	A-----AG	AAAAA-AAAG	ATAAGCAGTT
	610	620	630	640
<i>G. xanthopoda</i> (Thailand)	TTTATATTAA	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>G. xanthopoda</i> (Thailand)	TTTATATTAA	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>G. xanthopoda</i> (Philippines)	TTTATATTAA	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. longipes</i>	TTTAAATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. fimbriata</i>	TTTATATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. clavipes</i>	TTTATNTTAT	TAACGACCCT	CAGNCAGNG	TNGCCCNNGA
<i>L. sp. near clavipes</i>	TTTATATTAT	AAACGTCCCN	CACCCAGGCG	TGGTCCGGGA
<i>L. australis</i>	TTTATATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. boulandi</i> (Fasogil)	TTTATATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. boulandi</i> (Gambia)	TTTATATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. heterotoma</i> (Rochester)	TTTATATTAT	AAACGACCCT	CNNNNNNNNN	NNNNNNNNNN
<i>L. heterotoma</i> (Wageningen)	TTTATATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA
<i>L. victoriae</i>	TTTATATTAT	AAACGACCCT	CAGCCAGGCG	TGGTCCGGGA

Fig. 2. Continued.

	650	660	670	680
<i>G. xanthopoda</i> (Thailand)	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTNG
<i>G. xanthopoda</i> (Thailand)	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>G. xanthopoda</i> (Philippines)	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. longipes</i>	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. fimbriata</i>	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. clavipes</i>	ATTGTNTCCN	TGGACCGCAA	TGTGCGGTNC	GCAAATGTTCG
<i>L. sp. near clavipes</i>	ATTGTATCCG	TGGACCGCAA	CGTGCG-TTC	G-AAATGTC-
<i>L. australis</i>	ATCGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. bouleari</i> (Fasogil)	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. bouleari</i> (Gambia)	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. heterotoma</i> (Rochester)	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN
<i>L. heterotoma</i> (Wageningen)	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
<i>L. victorae</i>	ATTGTATCCG	TGGACCGCAA	TGTGCG-TTC	G-AAATGTTCG
	690	700	710	720
<i>G. xanthopoda</i> (Thailand)	A-TGTT			
<i>G. xanthopoda</i> (Thailand)	A-TGTT			
<i>G. xanthopoda</i> (Philippines)	A-TGTT			
<i>L. longipes</i>	A-TGTT			
<i>L. fimbriata</i>	A-TGTT			
<i>L. clavipes</i>	GNTGTT			
<i>L. sp. near clavipes</i>	A-TGTT			
<i>L. australis</i>	A-TGTT			
<i>L. bouleari</i> (Fasogil)	A-TGTT			
<i>L. bouleari</i> (Gambia)	A-TGtT			
<i>L. heterotoma</i> (Rochester)	NNNNNN			
<i>L. heterotoma</i> (Wageningen)	A-TGTT			
<i>L. victorae</i>	A-TGTT			

Fig. 2. Continued.

We refrained from any subsequent manual improvements, to prevent further subjectivity. Gaps were treated as 'fifth base' and were weighted as the inverse of the length of the deletion. Regions with ambiguous alignment (positions 29–103, 115–205, 226–245, 277–288, 297–320, 357–428, 454–515, 546–584; see Fig. 2) were excluded from the data set, leaving seventy informative sites. The morphological characters were weighted equally. Their states were unordered, with the exception of character 13 (see *List of morphological characters* below), which was ordered due to the obvious intermediacy of one of the three states.

The molecular and morphological data sets were analysed separately and in conjunction. In all cases the branch-and-bound option in the computer program PAUP 3.1 (Swofford, 1993) was used, with the option 'collapse zero-length branches' not in effect. *Ganaspis* and (where the morphological data were included) *Gronotoma* were taken as outgroups. Numerous characters showed that *Gronotoma* is phylogenetically more distant from *Leptopilina* than *Ganaspis* (Nordlander, unpublished data). Characters informative about outgroup relationships were, however, not included in the analysis. Instead, these relationships were constrained during tree searches. (In the absence of such constraints, the two outgroup genera would form a monophyletic rather than a paraphyletic group.) In the combined data set, the weight of each morphological character was the same as the base weight for each nucleotide. It should be noted that this data set included three taxa (*Gronotoma* sp., 'PR222-2' and 'PB10-5') for which

only morphological data were known. Support for the clades in a tree was assessed by bootstrapping (Felsenstein, 1985) 100 times, using a branch-and-bound search in which the characters were sampled with equal probability, with their weighting applied, and excluding invariant characters.

List of morphological characters

1. *Size, shape and structure of flagellomere 1 of male antenna:* (0) longer than following flagellomeres, distinctly curved outwards, outer side flattened, and flattened surface without placodeal sensillae; (1) shorter than following flagellomeres, almost straight, outer side more or less flattened, placodeal sensillae present also on flattened surface.
2. *Size and shape of flagellomere 2 of male antenna:* (0) about equal in length to flagellomere 3, equal in shape to following flagellomeres, i.e. symmetrical; (1) longer than flagellomere 3, thickened distally, slightly curved outwards, outer side flattened.
3. *Colour of club-forming flagellomeres of female antenna:* (0) all dark; (1) last or last two flagellomeres contrastively white.
4. *Shape of dorsal margin of posterior part of pronotal plate:* (0) flat or slightly convex medially; (1) distinctly concave medially.

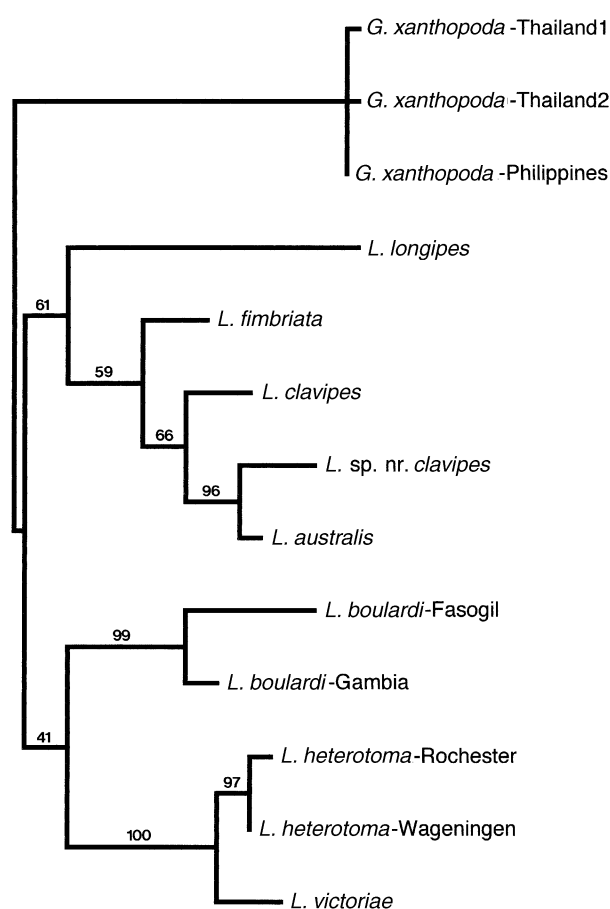


Fig. 3. A strict consensus phylogram over the three most parsimonious phylogenetic trees, based on the DNA data (length = 157, RI = 0.86). Bootstrap values are given above the branches that support clades.

5. *Pubescence on lateral ends of anterior part of pronotal plate*: (0) with rather few, not exceptionally long hairs; (1) with a dense tuft of very long hairs, reaching almost to mesoscutum.
6. *Pubescence along anteroventral margin of pronotum*: (0) dense; (1) weak, but with some long hairs; (2) with only very short, almost invisible pubescence.
7. *Hairs on mesoscutum*: (0) a row of long hairs laterally and usually a few submedially; (1) no long hairs except one or two near posterolateral corners.
8. *Shape of scutellar plate in dorsal view*: (0) not very narrow, broadest at about middle; (1) very narrow, widest near posterior end.
9. *Surface of scutellar plate*: (0) with many small hair-punctures along entire margin; (1) with relatively few (< 7) hair-punctures inside margin anterior to posterior fovea; (2) with hair-punctures and irregular cavities of various size scattered over surface anterior to posterior fovea.
10. *Sculpture on dorsal surface of scutellum (outside scutellar plate)*: (0) punctate reticulate to reticulate rugose, usually coarser near rim; (1) reticulate sculpture modified with ridges from rim towards scutellar plate dominating; (2)

almost smooth except for ridges from rim towards scutellar plate.

11. *Lateral keel on scutellum*: (0) absent; (1) present, distinct from other sculpture; (2) present basally, upper part not distinguishable from reticulate rugose sculpture covering the area.
12. *Shape of area enclosed by ridges anteroventrally on mesopleuron, just above forecoxa*: (0) area not tilted inwards in lateral view, slightly longer than twice its width; (1) area tilted inwards in lateral view, elongate, distinctly longer than twice its width; (2) area tilted inwards in lateral view, rectangular, about twice as long as wide.
13. *Shape of area below ventral ridge on mesopleuron, immediately above midcoxa*: (0) area continuous, elongate triangular, reaching to anterior of midcoxa; (1) area indistinctly subdivided into an anterior and a posterior triangular area, the latter reaching to about middle of midcoxa; (2) only posterior triangular area distinct, reaching to about middle of midcoxa. Ordered.
14. *Ridges on metapleuron*: (0) varying number of short ridges extending from hindmargin; (1) two distinct ridges extending from hindmargin and more than halfway across metapleuron (usually across entire metapleuron).
15. *Pubescence on backward-facing area behind postero-ventral corner of metapleuron, just above hindcoxa*: (0) densely pubescent; (1) nude.
16. *Metanotum posteriorly*: (0) clearly visible under scutellum; (1) concealed under scutellum.
17. *Shape of propodeal carinae*: (0) short, widely separated, almost parallel or slightly curved outwards at the middle; (1) short, very widely separated at base, strongly converging anteriorly; (2) long, more narrowly separated, almost straight and parallel; (3) long, strongly converging from base to middle, very narrowly separated in anterior half, curved at middle in lateral view (in contrast to states 0–2).
18. *Dorsolateral patch of hairs on midcoxa*: (0) present; (1) absent.
19. *Pubescence posteriorly on hind coxa*: (0) a band of hairs along upper half; (1) a small tuft of hairs posterodorsally, sometimes with a few hairs below.
20. *Pubescence of hindwing*: (0) almost entirely pubescent, although less densely on inner third; (1) inner third to half almost without hairs, outer part mainly with hair bases only.
21. *Shape of petiole*: (0) not abruptly widened posteriorly; (1) posterior part abruptly widened ventrally and laterally; (2) posterior part abruptly widened ventrally and laterally, but to a lesser extent also dorsally; (3) posterior part abruptly and very strongly widened in all directions.
22. *Anterior margin of widened part of petiole*: (0) straight, not curved forwards ventrally; (1) curved forwards ventrally.
23. *Narrow, longitudinal depression dorsolaterally on posterior part of petiole*: (0) absent; (1) present.

Results and discussion

The phylogenetic analysis of the molecular data resulted in three most parsimonious trees of length 157, and a retention

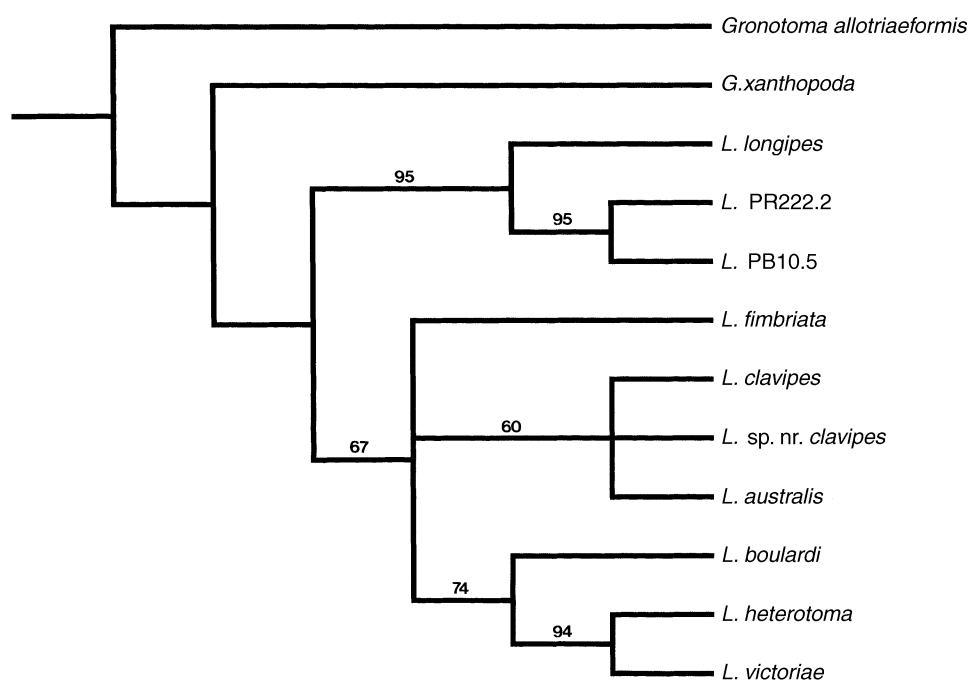


Fig. 4. A strict consensus cladogram over the nine most parsimonious trees (length = 39; RI = 0.85), resulting from the analysis of the morphological data. Bootstrap values are given above the branches that support clades.

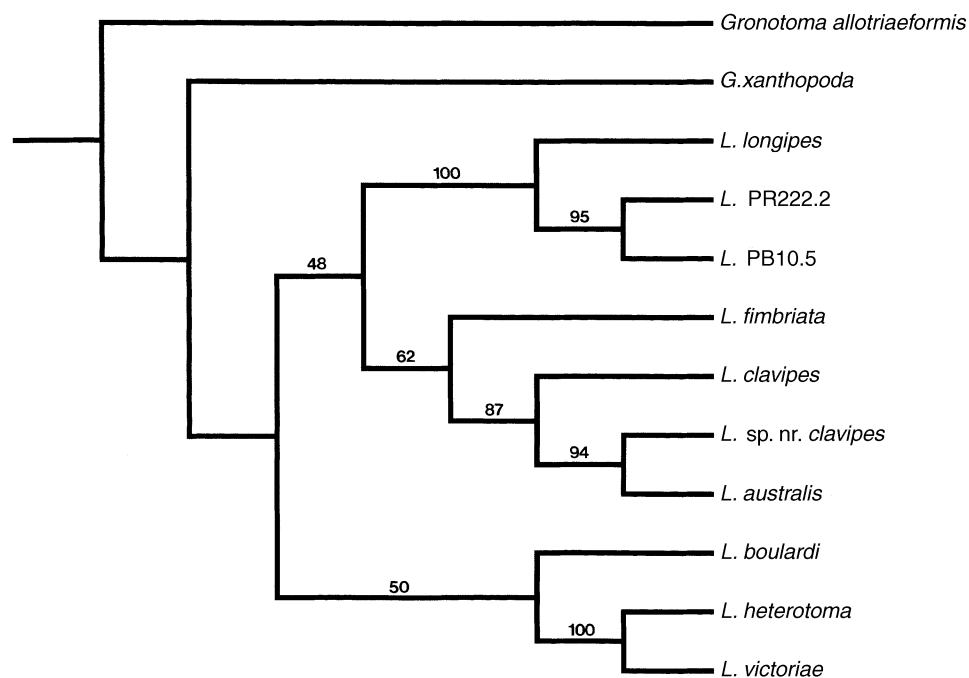


Fig. 5. The most parsimonious tree (length = 203; RI = 0.87) resulting from phylogenetic analysis of the combined data sets. For clarity, each species is represented by a single branch. Bootstrap values are given above the branches that support clades.

index (RI, Archie, 1989; Farris, 1989) of 0.86 (Fig. 3). The differences in topology were restricted to the three outgroup sequences. The topology of the ingroup was identical in all three trees. Most groups were supported by moderate to high bootstrap values, with the exception of the clade composed of

L. bouldardi, *L. heterotoma* and *L. victoriae*, which had a support of only 41%. There is, however, one caveat: a number of ITS studies (Paskewitz *et al.*, 1993; Buckler *et al.*, 1997; Fenton *et al.*, 1997) have reported sequence variation among multiple copies of the ribosomal gene. Such variation will

build up if gene conversion (which normally homogenises the variation among multiple copies within a single genome) proceeds slower than speciation, thus erroneous phylogenies may result. As we used mostly single sequences to represent the various species, we cannot exclude this possibility, although the concordance between morphology and DNA (see below) suggests that such problems will be of minimal importance, if at all.

The analysis of the morphological data yielded nine equally parsimonious trees (length = 39; RI = 0.85), differing only in the position of *L. fimbriata*, and the branching sequence among the very similar species *L. clavipes*, *L. sp. near clavipes*, and *L. australis*. These trees were combined into the strict consensus tree shown in Fig. 4. Here too, bootstrap values of the fully resolved parts of the tree were moderate to high.

The main difference between the molecular and the morphological trees is the position of *L. longipes*. In the molecular tree, this species forms a monophyletic group with *L. fimbriata*, *L. clavipes*, *L. sp. near clavipes* and *L. australis*. In the morphological tree, a clade including *L. longipes* and the two Caribbean species is placed closest to the root of the tree. This clade is strongly supported by the morphological data (bootstrap value 95%). The widely disjunct distribution of *L. longipes* and the Caribbean species may indicate that the separation of this clade is a relatively old event.

When the two data sets were combined for a 'total evidence' analysis, three most parsimonious trees resulted (length = 203; RI = 0.87), with the difference between the trees again restricted to the three *Ganaspis* branches (not shown in the figure). The position of *L. longipes* follows the molecular tree, but bootstrap values for the deeper nodes in the tree are relatively low (Fig. 5). Nevertheless, the species groups that were provisionally distinguished by Nordlander (1980), viz. the *longipes*, *heterotoma* and *boulardi* groups, remain intact, although the over-representation of species from the *longipes* group makes this statement less impressive. Within the *longipes* group, the position of *L. fimbriata* differs from that of a previously presented phylogeny (van Alphen *et al.*, 1991), where *L. fimbriata* was more closely related to *L. longipes* than to *L. clavipes* and *L. australis*. The change in position in the present analysis is due to the inclusion of some newly discovered characters as well as the deletion of a few characters with ambiguously defined states.

Our analyses show a large degree of correspondence between the molecular and the morphological data sets. This encourages us to believe that the tree depicted in Fig. 5 is close to the true phylogeny, although some bootstrap values are low. Thus, we hope that the cladogram presented will provide the phylogenetic framework that is necessary for interpreting the multitude of ecological and ethological observations in this group of drosophilid parasitoids.

Acknowledgements

We thank Jack Werren for continuous support and interest during the development of this study. John LaSalle and John Heraty are acknowledged for acting as referees and giving

very helpful suggestions for improvement. Irene Eijs and Henk Snellen took care of the laboratory cultures in Leiden and Wageningen, respectively. Tony van Kampen operated the automated DNA sequencer. This work was funded by the research school Functional Ecology.

References

- van Alphen, J.J.M. & Vet, L.E.M. (1996) An evolutionary approach to host finding and selection. *Insect Parasitoids* (ed. by J. K. Waage and D. Greathead), pp. 23–61. Academic Press, London.
- van Alphen, J.J.M., Nordlander, G. & Eijs, I. (1991) Host habitat finding and host selection of the *Drosophila* parasitoid *Leptopilina australis* (Hymenoptera, Eucoilidae), with a comparison of the niches of European *Leptopilina* species. *Oecologia*, **87**, 324–329.
- van Alphen, J.J.M., van Dijken, M.J. & Waage, J.K. (1987) A functional approach to superparasitism: host discrimination needs not to be learnt. *Journal of Animal Ecology*, **57**, 167–179.
- van Alphen, J.J.M. & Visser, M.E. (1990) Superparasitism as an adaptive strategy for insect parasitoids. *Annual Review of Entomology*, **35**, 59–79.
- Archie, J.W. (1989) Homoplasy excess ratios: new indices for measuring levels of homoplasy in phylogenetic systematics and a critique of the consistency index. *Systematic Zoology*, **38**, 253–269.
- Bouleureau, M. (1986) The genetic and evolutionary interaction between parasitoids and their hosts. *Insect Parasitoids* (ed. by J. K. Waage and D. Greathead), pp. 169–199. Academic Press, London.
- Brooks, D.R. & McLennan, D.A. (1991) *Phylogeny, Ecology and Behavior. A Research Program in Comparative Biology*. University of Chicago Press, London.
- Buckler, E.S., Ippolito, A. & Holtsford, T.P. (1997) The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics*, **145**, 821–832.
- Campbell, B.C., Steffen-Campbell, J.D. & Werren, J.H. (1993) Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Molecular Biology*, **2**, 225–237.
- Carton, Y. & Nappi, A.J. (1991) The *Drosophila* immune reaction and the parasitoid capacity to evade it: genetic and evolutionary aspects. *Acta Oecologica*, **12**, 89–104.
- van Dijken, M.J. & van Alphen, J.J.M. (1998) The ecological significance of differences in coexisting parasitoid species. *Functional Ecology*, in press.
- Farris, J.S. (1989) The retention index and the rescaled consistency index. *Cladistics*, **5**, 417–419.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.
- Fenton, B., Malloch, G. & Moxey, E. (1997) Analysis of eriophyid mite rDNA internal transcribed spacer sequences reveals variable simple sequence repeats. *Insect Molecular Biology*, **6**, 23–32.
- Harvey, P.H. & Pagel, M.D. (1991) *The Comparative Method in Evolutionary Biology*. Oxford University Press, Oxford.
- Higgins, D.G. & Sharp, P.M. (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene*, **73**, 237–244.
- Hillis, D.M. & Dixon, M.T. (1991) Ribosomal DNA: molecular evolution and reliability in molecular phylogenetic analyses. *Quarterly Review of Biology*, **66**, 411–453.
- van Kan, F.J.P.M., Silva, I.M.M.S., Schilthuizen, M., Pinto, J.D. & Stouthamer, R. (1996) Use of DNA-based methods for the identification of minute wasps of the genus *Trichogramma*.

- Proceedings of the Section Experimental and Applied Entomology of the Netherlands Entomological Society (NEV)*, **7**, 233–237.
- Kopelman, A.H. & Chabora, P.C. (1986) Aspects of the reproductive biology of *Leptopilina boulardi* (Hymenoptera: Eucoilidae). *Annals of the Entomological Society of America*, **79**, 808–813.
- van Lenteren, J.C. & Bakker, K. (1978) Behavioural aspects of the functional responses of a parasite (*Pseudocoila bachei* Weld) to its host (*Drosophila melanogaster*). *Netherlands Journal of Zoology*, **28**, 213–233.
- Nordlander, G. (1980) Revision of the genus *Leptopilina* Förster, 1869, with notes on the status of some other genera (Hymenoptera, Cynipoidea: Eucoilidae). *Entomologica scandinavica*, **11**, 428–453.
- Nordlander, G. & Grijpma, P. (1991) Systematics and biology of *Rhoptromeris strobigena* sp.n., a parasitoid of chloropids inhabiting conifer cones (Hymenoptera: Cynipoidea: Eucoilidae). *Entomologica scandinavica*, **22**, 209–218.
- Paskewitz, S.M., Wesson, D.M. & Collins, F.H. (1993) The internal transcribed spacer of ribosomal DNA in five members of the *Anopheles gambiae* species complex. *Insect Molecular Biology*, **2**, 247–257.
- Rizki, R.M. & Rizki, T.M. (1984a) Selective destruction of a host blood cell type by a parasitoid wasp. *Proceedings of the National Academy of Sciences of the USA*, **87**, 6154–6158.
- Rizki, T.M. & Rizki, R.M. (1984b) The cellular defence system of *Drosophila melanogaster*. *Insect Ultrastructure* (ed. by R. C. King and H. Akai), pp. 579–603. Plenum, New York.
- Rizki, R.M. & Rizki, T.M. (1990) Parasitoid virus-like particles destroy *Drosophila* cellular immune system. *Proceedings of the National Academy of Sciences of the USA*, **87**, 8388–8392.
- Roitberg, B.D., Mangel, M., Lalonde, R.G., Roitberg, C.A., van Alphen, J.J.M. & Vet, L.E.M. (1992) Seasonal dynamic shifts in patch exploitation by parasitic wasps. *Behavioural Ecology*, **3**, 156–165.
- Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- Sappal, N.P., Jeng, R.S., Hubbes, M. & Liu, F.H. (1995) Restriction fragment length polymorphisms in polymerase chain reaction amplified ribosomal DNAs of three *Trichogramma* (Hymenoptera: Trichogrammatidae) species. *Genome*, **38**, 419–425.
- Schilthuizen, M. & Stouthamer, R. (1997) Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proceedings of the Royal Society of London, Series B*, **264**, 361–366.
- Swofford, D.L. (1993) *PAUP V3.1.1*. Computer program distributed by the Smithsonian Institution, Washington, DC.
- Vet, L.E.M. (1983) Host-habitat location through olfactory cues by *Leptopilina clavipes* (Hartig) (Hymenoptera: Eucoilidae), a parasitoid of frugivorous *Drosophila*: the influence of conditioning. *Netherlands Journal of Zoology*, **33**, 225–248.
- Vet, L.E.M. & van Alphen, J.J.M. (1985) A comparative functional approach to the host detection behaviour of parasitic wasps. I. A qualitative study on Eucoilidae and Alysiinae. *Oikos*, **44**, 478–486.
- Vet, L.E.M. & Dicke, M. (1992) Ecology of infochemical use by natural enemies in a tritrophic context. *Annual Review of Entomology*, **17**, 141–172.
- Vet, L.E.M. & Groenewold, A.W. (1990) Semiochemicals and learning in parasitoids. *Journal of Chemical Ecology*, **16**, 3119–3135.
- Vet, L.E.M. & van der Hoeven, R. (1984) Comparison of the behavioural response of two *Leptopilina* species (Hymenoptera: Eucoilidae), living in different microhabitats, to kairomone of their host (*Drosophilidae*). *Netherlands Journal of Zoology*, **34**, 220–227.
- Vet, L.E.M. & van Opzeeland, K. (1985) Olfactory microhabitat selection in *Leptopilina heterotoma* (Thomson) (Hym.: Eucoilidae), a parasitoid of *Drosophilidae*. *Netherlands Journal of Zoology*, **35**, 497–504.
- Vet, L.E.M. & Papaj, D.R. (1992) Effect of experience on parasitoid movement in odour plumes. *Physiological Entomology*, **17**, 90–96.
- Visser, M.E. (1993) Adaptive self- and conspecific superparasitism in the solitary parasitoid *Leptopilina heterotoma* (Hymenoptera: Eucoilidae). *Behavioural Ecology*, **4**, 22–28.
- Visser, M.E., van Alphen, J.J.M. & Nell, H.W. (1990) Adaptive superparasitism and patch time allocation in solitary parasitoids: the influence of the number of parasitoids depleting the patch. *Behaviour*, **114**, 21–36.
- Walker, I. (1959) Die Abwehrreaktion des Wirtes *Drosophila melanogaster* gegen die Zoophage Cynipidae *Pseudeucoila bochei* Weld. *Revue Suisse de Zoologie*, **66**, 569–632.
- Wesson, D.M., Porter, C.H. & Collins, F.H. (1992) Sequence and secondary structure of ITS-rDNA in mosquitoes (Diptera: Culicidae). *Molecular Phylogenetics and Evolution*, **1**, 253–269.

Accepted 25 September 1997