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The separation of two hymenopteran parasitoids, *Tersilochus obscurator* and *Tersilochus microgaster* (Ichneumonidae), of stem-mining pests of winter oilseed rape using DNA, morphometric and ecological data

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Abstract

Tersilochus obscurator Aubert and *Tersilochus microgaster* (Szépligeti) are larval endoparasitoids of economically-important stem-mining pests of winter oilseed rape (*Brassica napus* L.) in Europe. They are difficult to separate morphologically. Their hosts are *Ceutorhynchus pallidactylus* (Marsham) and *Psylliodes chrysocephala* Linnaeus, respectively. The parasitoids' taxonomic status, identification, host range and phenology were studied using genetic, morphometric and ecological data. The study used 527 female parasitoids from the UK and Germany, either field-collected in emergence traps or reared from field-collected host larvae. Two morphometric characters, the ovipositor sheath to first metasomal tergite ratio and the percentage of the mesopleuron spanned by the sternaulus, were measured. A 440 bp section of mitochondrial DNA cytochrome oxidase subunit I (COI) gene was sequenced from 35 parasitoids reared from *C. pallidactylus*, 20 reared from *P. chrysocephala* and individuals from two outgroups, *Tersilochus heterocerus* Thomson and *Phradis interstitialis* Thomson. Distinct and invariable COI sequences corresponded exclusively to each parasitoid group, confirming that *T. obscurator* and *T. microgaster* are discrete species. Measurements of host-reared and COI-sequenced specimens indicated that the ranges of both morphometric characters overlapped between species. Using these ranges as criteria, all but 3.6% of UK specimens and 2% of German specimens were identifiable to species without reference to host or phenology. There were differences in emergence phenology in the UK, adult *T. microgaster* emerging from winter diapause by 29 March 2000, *T. obscurator* emerging between 12 April and 24 May 2000. The value of

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molecular techniques in the identification of closely-related parasitoid species is discussed.

Keywords: *Tersilochus obscurator*, *Tersilochus microgaster*, Ichneumonidae, Hymenoptera, molecular taxonomy, morphometrics, phenology, host range, DNA sequencing, COI sequences

Introduction

Hymenopteran parasitoids are a speciose and diverse group of insects (LaSalle, 1993; Quicke, 1997), whose larvae feed exclusively on the bodies of other arthropods, their hosts, eventually killing them. Most pest insects are preyed upon by one or more species of hymenopteran parasitoids, and applied entomologists seek to use parasitoids for biological control of pests (Eggleton & Belshaw, 1992; LaSalle, 1993). Although there is a wealth of literature on these Hymenoptera, there are still extensive gaps in our knowledge of their biology and taxonomy (LaSalle, 1993; LaSalle & Gauld, 1993; Vidal, 2003). Closely-related species can look identical even to the trained eye. In the past, the only means available for separating these species was comparison of morphological variation, phenological data and host preferences. Today these can be used in conjunction with molecular techniques to determine the relatedness of very similar species (Hillis, 1987; Unruh *et al*., 1989), though it ought to be noted that in some (possibly many) cases it might not be straightforward to find discriminating molecular differences between what might be good biological species (e.g. Alvarez & Hoy, 2003; Sperling, 2003; Quicke, 2004; Will & Rubinoff, 2004).

Several species of *Tersilochus* Holmgren (Hymenoptera: Ichneumonidae, Tersilochinae) are larval endoparasitoids of economically-important coleopteran pests of oilseed rape *Brassica napus* L. (Brassicaceae) and fly in spring and early summer (Horstmann, 1971, 1981; Ulber, 2003; Ulber & Williams, 2003). Separation of species on the basis of morphological characters without reference to host relationships and phenology is often difficult for females and impossible for males (Horstmann, 1971, 1981; Alford, 2003). In this study, a molecular technique has been developed to identify two *Tersilochus* species, *T. microgaster* (Szépligeti) and *T. obscurator* Aubert, and has been used to assess how well females of the two species can be separated on the basis of morphological characteristics.

Tersilochus microgaster and *T. obscurator* are both univoltine endoparasitoids of stem-mining larvae in winter oilseed rape in Europe (Horstmann, 1971, 1981; Ulber, 2003; Ulber & Williams, 2003). The host of *T. microgaster* is the cabbage stem flea beetle, *Psylliodes chrysocephala* Linnaeus (Coleoptera: Chrysomelidae), which mines leaf petioles and stems from November to May. The host of *T. obscurator* is the cabbage stem weevil, *Ceutorhynchus pallidactylus* (Marsham) (Coleoptera: Curculionidae) which also mines leaf petioles and stems but from April to June. In Germany, *T. microgaster* colonizes the crop in March and April (Nitzsche, 1998) and in France, adult *T. obscurator* were collected in winter oilseed rape from late April until beginning of June in 1952 (Jourdheuil, 1960; Nitzsche, 1998). In the UK, the adults of both species are active in the crop in April (Barari *et al*., 2005). Both species of parasitoid delay larval development until the host has reached full size and has entered the ground to pupate. Within a few weeks they develop to adulthood but emergence from the soil is delayed until the following spring.

Tersilochus microgaster and *T. obscurator* are morphologically very similar. The genus *Tersilochus* has been divided into 21 species, most categorized into two groups (Horstmann, 1971, 1981). *Tersilochus microgaster* and *T. obscurator* are in the *jocator* group and are distinguished by a difference in the ratio between the length of the ovipositor sheaths and the length of the first metasomal tergite. This ratio, hereafter referred to as the 'sheath ratio' (fig. 1), is 1.2–1.3 in *T. microgaster* and about 1.7 in *T. obscurator* (Horstmann, 1971, 1981). Lectotype and paratype descriptions by Horstmann (1971) suggest that the length of the sternaulus which is spanned by a row of pits may be a simple-to-measure morphometric character useful for separating these species. However, our preliminary studies on parasitoids reared from host larvae or collected in emergence traps in the field, suggested that sheath ratio and sternaulus measurements are inadequate to reliably distinguish the two species. A third morphological feature, the transverse sectional shape of the petiole (Horstmann, 1971) is also useful to the trained eye but is difficult to see in many specimens and is not easily measured. It was therefore decided that a molecular technique would be valuable for the identification of *T. microgaster* and *T. obscurator* and to establish the limits of intraspecific morphological variation.

Primers for conserved regions of mitochondrial DNA (mtDNA) are valuable tools for the study of molecular systematics in insects (Simon *et al*., 1994) and are particularly suitable for studies of species for which sequence information does not exist (Moritz *et al*., 1987; Simon *et al*., 1994; Sperling & Hickey, 1994). Mitochondrial DNA is extracted and manipulated easily, and is inherited maternally with independent replication (Moritz *et al*., 1987; Simon, 1991; Dayhoff & Eck, 1994; Sperling & Harrison, 1994; Randi, 2000). Cytochrome oxidase subunit I (COI) is relatively conserved within insects (Simon *et al*., 1994). This region has been used in many phylogenetic studies and to investigate the variation in species groups within many insect families (Simon, 1991; Mitchell *et al*., 1993; Brown *et al*., 1994; Sperling & Hickey, 1994; Funk *et al*., 1995; Roehrdanz, 1997; Landry *et al*., 1999).

The aims of this study were: (i) to distinguish females of the two closely-related species, *T. microgaster* and *T. obscurator* and confirm their separate species status using mtDNA COI gene sequences; (ii) to compare DNA sequencing results with morphometric data, phenological data and host affinities; (iii) to test the value of sternaulus length as a second morphometric character for separating the two species. For comparison, mtDNA sequences were studied in two other species of Tersilochinae, *Tersilochus heterocerus* Thomson and *Phradis interstitialis* Thomson; these are endoparasitoids of larvae of the pollen beetle, *Meligethes aeneus* (Fabricius) (Coleoptera: Nitidulidae), another pest of oilseed rape.

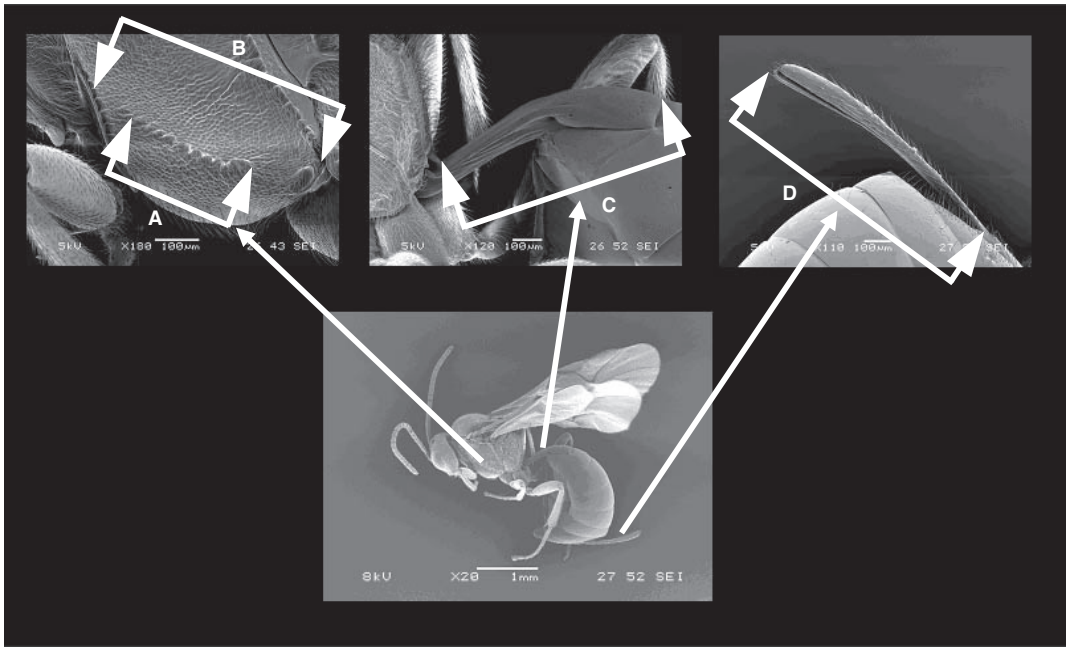

Fig. 1. Scanning electron micrographs of *Tersilochus microgaster*, indicating morphological characters measured. A: length of the row of pits running along the sternaulus; B: length of the sternaulus, a shallow indentation which takes an approximately diagonal path across the mesopleuron; sternaulus span = A/B × 100; C: length of the first metasomal tergite; D: length of the ovipositor sheaths; sheath ratio = D/C. Scale bar = 1 mm for whole insect and 0.1 mm for details.

Materials and methods

Parasitoids

Specimens from the UK

A total of 288 adult female *jocator* group parasitoids were collected between 8 March and 24 May 2000 from 40 emergence traps (0.5 m^2) set in a field of winter wheat at Rothamsted Research, UK, where winter oilseed rape had been grown in the previous year. In 2002 and 2003, 177 adult female *jocator* group parasitoids were reared from *P. chrysocephala* larvae collected from winter oilseed rape plants grown on Rothamsted farm. A further eight *jocator* group parasitoids were reared from *C. pallidactylus* larvae collected in the same way (see Barari *et al*., 2004). Outgroups, represented by six *T. heterocerus* and five *P. interstitialis* adult females, were field-collected by sweep net in May and June 2003 from winter oilseed rape crops within 5 km of Rothamsted. All parasitoids were killed in absolute ethanol and stored at −18°C.

Specimens from Germany

Fifty-four female *jocator* group specimens were made available for this study by Dr B. Ulber of the Georg-August University, Göttingen, Germany. Of these, 44 adults were reared from *C. pallidactylus* larvae and ten were reared from *P. chrysocephala* larvae, all collected from winter oilseed rape plants within 2 km of Göttingen on 9 May 2003. These specimens were stored in 70% ethanol and therefore were used for morphometric study only.

Morphometric measurements

Three morphometric measurements were made on each adult parasitoid: the length of the ovipositor sheaths, the length of the first metasomal tergite, and the span of the row of pits along the sternaulus (hereafter referred to as the 'sternaulus span'; fig. 1). The sternaulus is a shallow indentation taking an approximately diagonal path across the mesopleuron. The sternaulus span was defined as the percentage of the length of the sternaulus which was spanned by the row of pits and was estimated by eye to the nearest 5%. Ovipositor sheaths and tergites were measured with a graticule on a dissecting microscope at 50× magnification. 'Sheath ratio' was calculated as the length of ovipositor sheath/the length of first metasomal tergite.

Selection of parasitoids for DNA analysis

Four subsets of *jocator* group parasitoids were selected from the UK specimens for molecular study. Subset A consisted of five parasitoids reared from *C. pallidactylus* and with sheath ratios within the range 1.6–1.8 as recognized for *T. obscurator* (Horstmann, 1981); the sternaulus spans of these specimens were 0–20%. Subset B comprised ten parasitoids reared from *P. chrysocephala* larvae and with sheath ratios within the range 1.2–1.3 as recognized for *T. microgaster* (Horstmann, 1971); the sternaulus spans of these specimens were 60–85%. Subset C was ten parasitoids which were reared from *P. chrysocephala* larvae but which had sheath ratios outside the range recognized for *T. microgaster*. Subset D consisted of 30 parasitoids from field emergence traps which were unlike individuals from groups A or B, either because one or both of the sheath ratio and sternaulus

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span fell outside those in subsets A and B, or because one measurement fell within the range of subset A and the other within the range of subset B.

DNA extraction

DNA was extracted from one leg of each specimen, using the 'salting out protocol' (Sunnucks & Hales, 1996). A leg was detached and placed in a 0.5 ml micro-tube (Sarstedt, Germany). Ethanol was evaporated from the leg by using a vacuum centrifuge (Eppendorf concentrator 5301, Germany) for 10 min. 5 µl TNES (50 mM Tris pH 7.5, 400 mM NaCl, 20 mM EDTA and 0.5% SDS) buffer and 3 µl proteinase K (10 mg ml^{-1}) were added to the tube. Each sample was then crushed using a specially-adapted electric screwdriver and incubated at 37°C for 18 h. Proteins were precipitated out by adding 85 µl 5 M NaCl, hard shaking for 15 s, and centrifuging at 14,000 rpm for 10 min. The supernatant was poured from the tube into a new 1.5 ml micro-tube. The DNA was precipitated by adding 400 µl of 100% ethanol, before cooling for 1 h at −20°C and then centrifuging the tube at 14,000 rpm for 20 min. Pelleted DNA was washed in 70% cold ethanol, dried by leaving in a vacuum centrifuge for 10 min, and re-suspended in 25 µl distilled water for 18 h.

mtDNA amplification and sequencing

The region of mtDNA containing the COI gene was amplified using the following primers: forward (C1-J-1718) 5′-GGA GGA TTT GGA AAT TGA TTA GTT CC-3′ (Simon *et al.*, 1994), reverse (C1-N-2191) 5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′ (Simon *et al.*, 1994). Polymerase chain reactions (PCR) were performed in a Hybaid Omnigene PCR machine (Hybaid, UK). 25 µl sample volumes were used containing: 2 µl DNA template, 2.5 µl PCR buffer, 2 µl $MgCl_2$, 0.25 µl dNTPs, 0.5 µl Taq polymerase (Promega, USA), 1 µl each respective forward and reverse primer and 15.75 µl of distilled water. The PCR programme started with an initial denaturation for 3 min at 94°C followed by 1 cycle of 30 s at 60°C, 45 s at 72°C and 15 s at 94°C; 1 cycle of 30 s at 58°C, 45 s at 72°C and 15 s at 94°C; 1 cycle of 30 s at 56°C, 45 s at 72°C and 15 s at 94°C; 1 cycle of 30 s at 55°C, 45 s at 72°C and 15 s at 94°C; 1 cycle of 30 s at 54°C, 74 s at 72°C and 15 s at 94°C; 29 elongation cycles of 30 s at 53°C, 45 s at 72°C and 15 s at 94°C. 5 µl of each PCR product was run on an agarose gel (1%) for 30 min at a voltage of 120 V. PCR products were purified using the materials and protocol supplied with a Qiaquick PCR Purification Kit (Qiagen, UK). Precipitation of purified DNA was achieved by adding 25 µl of 95% ethanol and 1 µl of 3 M sodium acetate to each. The solution was left at room temperature for 15 min, then transferred to a 0.5 ml micro-tube, and centrifuged at 13,000 rpm for 20 min. The supernatant was removed and the pellet washed with 150 µl of 70% ethanol, centrifuged for 5 min and supernatant again removed. The pelleted DNA was air-dried for 3 h, then prepared for the sequencing (Perkin Elmer Applied Biosystems) by re-suspending it with 10 µl formamide and centrifuging for a few seconds. A DNA segment of about 440 base pairs (bp; forward) and 410 bp (reverse) from the COI gene of *jocator* group, *T. heterocerus* and *P. interstitialis* specimens was sequenced.

Results

Nucleotide sequences and species identity

The percentage base composition of a 440 or 424 bp fragment of the COI gene from each of the six groups of parasitoids tested is given in table 1. Four distinct COI sequences were identified from the six parasitoid groups tested. Nucleotide sequences identical to that for *jocator* group subset A were designated 'COI sequence A' and nucleotide sequences identical to that for subset B were designated 'COI sequence B'. COI sequences derived from *T. heterocerus* and *P. interstitialis* were designated Th and Pi, respectively. Nucleotide sequences of the four COI sequences are illustrated in fig. 2, which indicates the sites of nucleotide substitutions by comparison with the sequence for COI sequence A.

There were no differences in nucleotide sequences amongst specimens within each of the six parasitoid groups tested. This suggests that: (i) each group contained a single species, (ii) specimens of subset A, reared from *C. pallidactylus*, and with sheath ratios as recognized for *T. obscurator*, were *T. obscurator*; and (iii) specimens of subset B, reared from *P. chrysocephala* and with sheath ratios as recognized for *T. microgaster*, were confirmed as *T. microgaster*. Sequence differences between *jocator* group subsets A and B were smaller than those between either of these subsets and *T. heterocerus* or *P. interstitialis* (table 2). The largest sequence differences were observed for inter-generic comparisons between *P. interstitialis* and the groups of *Tersilochus* specimens (table 2).

Host separation

The COI nucleotide sequence of subset C was identical to that of subset B (fig. 2), suggesting that all parasitoids reared from *P. chrysocephala* were *T. microgaster*. Sequences from all subset D specimens were identical to those of subset A (fig. 2), suggesting that all the specimens, selected from

Table 1. Base composition of a portion of the COI gene in six groups of female parasitoids.

Parasitoid group	Source	No. specimens	COI sequence	Number (% of total)				Total no. of base pairs
				T	A	C	G	
jocator group subset A	ex *Ceutorhynchus pallidactylus*	5	A	179 (40.7)	145 (33.0)	62 (14.1)	54 (12.3)	440
jocator group subset D	field-collected	30	A					
jocator group subset B	ex *Psylliodes chrysocephala*	10	B	184 (41.8)	143 (32.5)	60 (13.6)	53 (12.1)	440
jocator group subset C	ex *Psylliodes chrysocephala*	10	B					
Tersilochus heterocerus	field-collected	6	Th	188 (42.7)	137 (31.1)	64 (14.6)	51 (11.6)	440
Phradis interstitialis	field-collected	5	Pi	171 (40.3)	141 (33.3)	63 (14.9)	49 (11.6)	424

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                      10        20        30        40        50        60        70        80        90       100       110
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
COI sequence A   AATAAATAATATAAGATTCTGATTATTACCCCCATCATTATTATTTTTAATTTTTAGATCTGTTATTAATCAAGGAGTAGGAACTGGATGAACTGTTTATCCTCCTTTAT
COI sequence B   ......C...........T.....................................A.....................G.....................
COI sequence Th  ..................T...........T..T..C.........C..C.A..A......T.A...........T..T........T.....A..A..C..A.......
COI sequence Pi                A.T.TGA..C....T..C.......C.......T.AA.....AGAA.....................A.............C..C..A....

                     120       130       140       150       160       170       180       190       200       210       220
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
COI sequence A   CATTAAATATTAATCATGAAGGGATATCAGTAGATTTAGCTATTTTTTCTCTTCATTTAGCAGGAATATCATCAATTATAGGAGCAATTAATTTTATTACTACTATTATA
COI sequence B   ..............................T.........................C.............C.....................................
COI sequence Th  ................C.....T........T........A.....................T.....................................T
COI sequence Pi  .T.............C....A........T........A.........T.A...A.............T..T...........T............T.A......T..

                     230       240       250       260       270       280       290       300       310       320       330
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
COI sequence A   AATATACGAGCATTAAATATTTCATTAGAAAAAATATCATTATTTGTTTGATCAATTTTAATCACTACAATTCTACTTTTATTGGCTGTTCCAGTATTTGCAGGAGCATT
COI sequence B   ..............................................................T.........T.........A.........T..............
COI sequence Th  ........TT....T........T...........TC.T..............T...C.T..T..A..TG.AT..T.A.....A..A..............T..T..T..
COI sequence Pi  .........AT.AAT..A...A..C.T......T..C.T..................C.T..T.........T..T.A.....A.....A...........T.....T..

                     340       350       360       370       380       390       400       410       420       430       440
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
COI sequence A   AACTATATTATTAACAGATCGTAATTTAAATACTTCATTTTTTGACCCATCAGGAGGAGGTGACCCAATTCTTTATCAACATTTATTTTGATTTTTTGGTCACCCTGAAG
COI sequence B   ..............................................................T.....................................T.......
COI sequence Th  ..............T..C..A...........C.....................T..A..T..T...T.A..C.....................A..T.......
COI sequence Pi  ..........C.T..T.....A...A.T.....A..T.....................A.....................................A..T..A....
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Fig. 2. The four partial nucleotide sequences of the COI gene (forwards). Dots indicate the same nucleotide as COI sequence A. COI sequence A = *jocator* group subset A, COI sequence B = *jocator* group subset B, COI sequence Th = *Tersilochus heterocerus* and COI sequence Pi = *Phradis interstitialis*.

emergence trap samples because of their variable morphometric characters, were *T. obscurator*.

Morphometric measurements and species resolution

The frequency distribution of the sheath ratio amongst all UK specimens was bimodal and specimens reared from *C. pallidactylus* and *P. chrysocephala* partitioned between the two peaks (fig. 3a). However, the distribution was continuous, providing no clear division amongst specimens from emergence traps. Moreover, the range of sheath ratio measurements for all those chosen for identification by mtDNA analysis overlapped between species (*T. obscurator*, 1.39–1.91 and *T. microgaster* 1.1–1.48; fig. 3b). The frequency distribution of the sternaulus span amongst all UK specimens was again continuous, showing only weak evidence of bimodality, although its range amongst specimens reared from the two hosts again did not overlap (fig. 4a). The range of sternaulus span measurements for those chosen for identification by mtDNA analysis overlapped considerably between species (*T. obscurator* 0–70%; *T. microgaster* 50–85%; fig. 4b). Of all 473 UK specimens, 32 (6.8%) had sheath ratios and 197 (41.6%) had sternaulus spans within the ranges of

Table 2. Numbers of nucleotide differences between pairs of parasitoid groups over aligned sequences of 440 or 424 base pairs.

Parasitoid group	*jocator* group subset B[1]	*Tersilochus heterocerus*[1]	*Phradis interstitialis*[2]
jocator group subset A[1]	13 (3.0%)	59 (13.4%)	68 (16.0%)
jocator group subset B[1]		57 (13.0%)	64 (15.1%)
T. heterocerus[1]			74 (17.5%)

[1] 440 base pairs; [2] 425 base pairs.

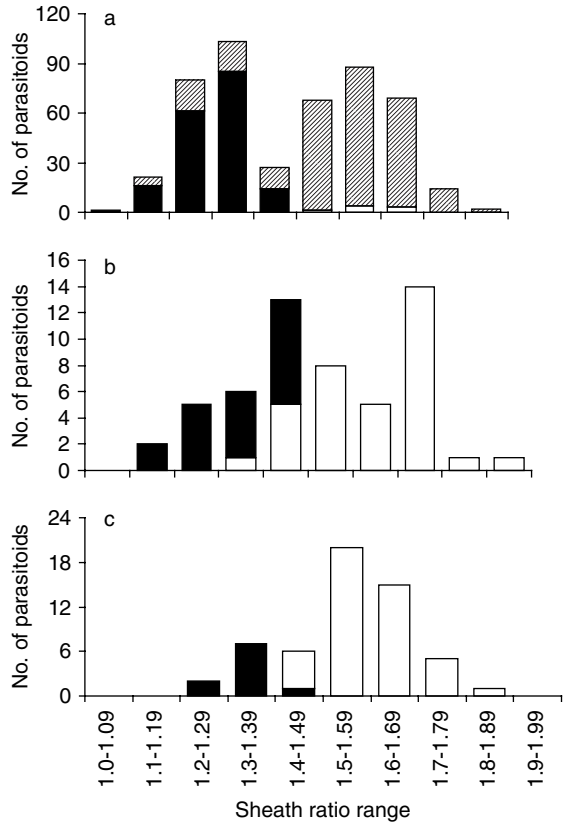

Fig. 3. Frequency distributions of values of the sheath ratio in *jocator* group specimens: (a) All 473 specimens from the UK: ■, reared from *Psylliodes chrysocephala* larvae; □, reared from *Ceutorhynchus pallidactylus* larvae; ▨, collected in emergence traps. (b) All mtDNA tested specimens: □, COI sequence A (identified as *Tersilochus obscurator*); ■, COI sequence B (identified as *T. microgaster*). (c) All 54 specimens from Germany: ■, reared from *P. chrysocephala* larvae; □, reared from *C. pallidactylus* larvae.

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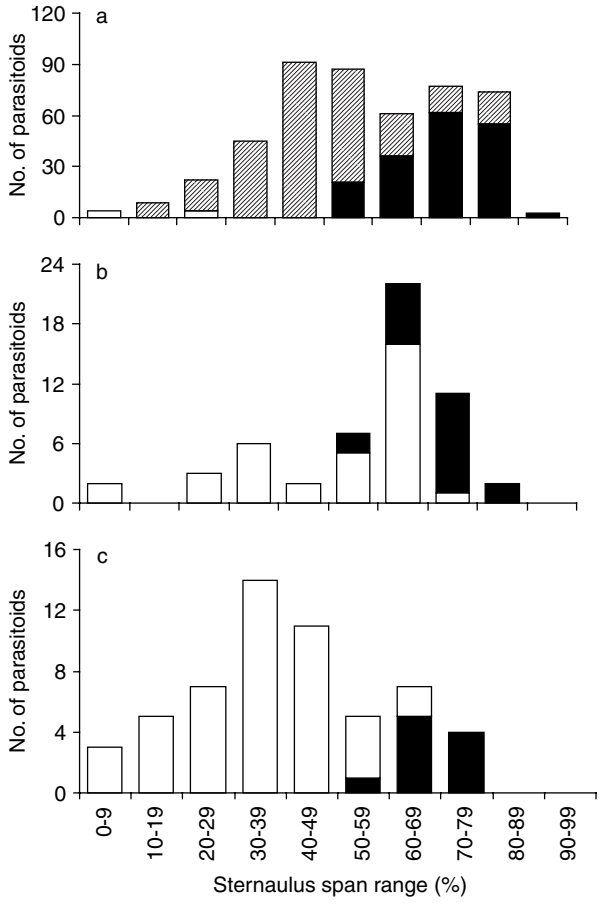

Fig. 4. Frequency distributions of values of the sternaulus span in *jocator* group specimens. Legend as fig. 3.

overlap between species; in 17 (3.6%) specimens both characters were within the range of overlap. Ten of the latter were identified by mtDNA analysis as *T. obscurator* (COI sequence A) and seven as *T. microgaster* (COI sequence B).

The frequency distributions of the sheath ratio and sternaulus span amongst the specimens of *jocator* group from Germany both showed only weak evidence of bimodality and there was overlap in the range of each between specimens reared from *C. pallidactylus* and from *P. chrysocephala* (figs 3c and 4c). Of the 54 specimens from Germany, six had sheath ratios and 16 had sternaulus spans within the ranges of overlap between species for UK specimens; one had both morphometric measurements within the ranges of overlap.

Phenology

The phenology of emergence of *jocator* group females into emergence traps in 2000 was clearly bimodal (fig. 5). All those emerging by 29 March had sheath ratios and sternaulus spans within the ranges found for specimens identified by mtDNA analysis as *T. microgaster*. Those emerging after 12 April included the 30 specimens of subset D identified by mtDNA as *T. obscurator*, and the remainder had measurements consistent with all *T. obscurator* mtDNA-type, except for one specimen with an even longer sheath ratio of 1.97.

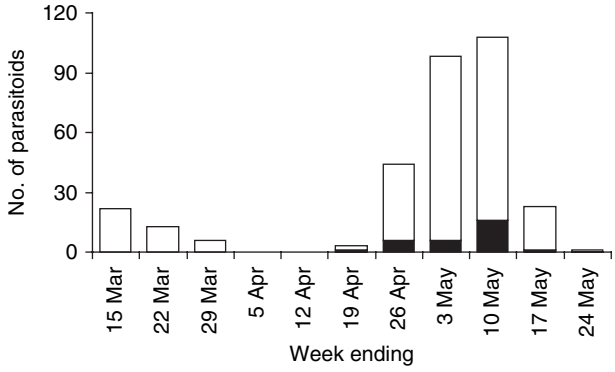

Fig. 5. Phenology of female *jocator* group parasitoids collected in 40 emergence traps at Rothamsted in 2000. □, Specimens not DNA-tested; ■, DNA tested specimens, all are COI sequence A, *Tersilochus obscurator*.

Discussion

This study has provided the first information on molecular genetic variation among four species of Tersilochinae, *T. microgaster*, *T. obscurator*, *T. heterocerus* and *P. interstitialis*, and demonstrates the value of the COI gene for distinguishing species of this subfamily.

mtDNA sequence and species identity

DNA sequence analysis of the mtDNA COI gene has confirmed the species-level separation of *T. microgaster* from *T. obscurator*. These two species are problematic to distinguish morphologically but mtDNA divergences indicated genetic differences between them. The differences that were observed between COI sequences for *T. microgaster* and *T. obscurator* were 3% but the observed substitutions were consistently in the same positions involving the same bases. Had the substitutions proved inconsistent, they could simply have been considered as intra-specific genetic variation and the splitting of *T. microgaster* and *T. obscurator* into two discrete species might have been called into question.

The mtDNA's of these parasitoids were very rich in A/T bases. This result concurs with other studies on the mitochondrial genome of many insects such as *Anopheles quadrimaculatus* Say (Diptera: Culicidae) (Mitchell *et al.*, 1993), blackflies (Diptera: Simuliidae) (Xiong & Kocher, 1991), *Ophraella* (Coleoptera: Chrysomelidae) (Funk *et al.*, 1995), the honeybee and *Drosophila* (Crozier & Crozier, 1993).

Morphometric measurements and species resolution

The range of sheath ratio measurements in specimens of each species identified by mtDNA encompassed that reported by Horstmann (1971, 1981) but was wider. The range showed a considerable range of overlap between species and measurements from nearly 7% of UK and 9% of German specimens fell into the region of uncertainty (sheath ratio 1.39–1.48). The sternaulus span was even more variable, especially in *T. obscurator*, 42% of UK and 28% of German *jocator* group specimens falling into the range of overlap (sternaulus span 50–65%). However, if the two characters were taken together, and the ranges measured in mtDNA-typed specimens were taken as species-definitive, then it was

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possible to reduce the number of specimens not identifiable morphologically to 3.6% in UK specimens and 2% in German specimens. Thus it is proposed that morphological identification of *T. obscurator* and *T. microgaster* would be improved if sheath ratios of 1.4–1.9 and 1.1–1.5, respectively, and sternaulus spans of 0–70% and 50–85%, respectively, were adopted as key distinguishing characters.

The power of morphology to resolve these species might be further improved if other morphological measurements or characters were tested using mtDNA-typed specimens. Samples should be tested from as wide a range of European locations as possible to allow for geographical variation between populations.

Host separation

This study supported the host separation of *T. microgaster* and *T. obscurator*. Parasitoids with COI sequence A, identified as *T. obscurator*, were reared only from *C. pallidactylus* and parasitoids with COI sequence B, identified as *T. microgaster*, were reared only from *P. chrysocephala*. This is in accord with previous rearing records. *Tersilochus microgaster* has been reared from *P. chrysocephala* larvae in Germany (Klingenberg & Ulber, 1994) and *T. obscurator* has been reared from *C. pallidactylus* larvae in the former Czechoslovakia, Austria, France, Germany and Switzerland (Ulber, 2003). Host records therefore provide key information for the separation of these species but are only available for reared specimens.

Phenology

Both mtDNA and morphological evidence supported the partitioning of the two species between the two peaks of post-diapause emergence phenology, *T. microgaster* emerging by 29 March and *T. obscurator* emerging between 12 April and 24 May 2000 at Rothamsted. This timing is consistent with the earlier availability of host larvae for *T. microgaster* and concurs with previous studies. In 1991 in Germany, *T. microgaster* emerged from mid March to the beginning of April and *T. obscurator* emerged from mid April to the end of May (Klingenberg & Ulber, 1994). In the UK, *T. microgaster* was caught in flight traps in April and *T. obscurator* was caught from mid April to early June 2002 (Barari *et al*., 2005). Thus phenological information is of potential value in distinguishing these species but, whereas their emergence phenologies may be discrete, their periods of flight activity are likely to overlap.

Phylogenetic relationships

The observed genetic divergence between *T. microgaster* and *T. obscurator* was considerably less than that between either species and *T. heterocerus* or *P. interstitialis*. This is supported by a classification based on morphological characters (Horstmann, 1971, 1981). These results suggest that the divergence of *T. microgaster* and *T. obscurator* along two distinct evolutionary trajectories is a relatively recent event. It is interesting to speculate why *T. obscurator* exhibits greater intraspecific variation in sternaulus span. Possibly *T. obscurator* has a wider host range with the concomitant selection pressures maintaining more variable morphological characters. Alternatively, this species could be older than *T. microgaster*; the gene pool of *T. obscurator* having a greater period of time in which to accrue mutations that manifest as morphological variation. Comparing more variable DNA regions between populations of the two species could enable the latter postulation to be tested.

It is possible to hypothesize the mechanisms by which species of the *jocator* group became adapted to different hosts occupying overlapping ecological niches. The *jocator* group is now known to contain at least seven species (K. Horstmann, personal communication). DNA sequences only exist for *T. obscurator* and *T. microgaster* so the evolutionary relationships of the group as a whole are unknown. Moreover, rearing records exist for only three species, *T. obscurator*, *T. microgaster* and *T. fulvipes* Gravenhorst. However, each of these three has been reared from different species of stem-mining coleopteran larvae in oilseed rape in Europe (Ulber, 2003; Ulber & Williams, 2003). It is possible that a species ancestral to these three parasitized a single stem-mining host species in Brassicaceae. Chance changes in phenology of part of the parasitoid population or the successive arrival of new potential hosts within their geographic range could lead to opportunistic parasitism of the different hosts thus made available. Speciation would be accelerated by the necessity for endoparasitoids to adapt to the immune defences of hosts from different families of Coleoptera. Mitochondrial DNA analysis has shown that mutations occur at a known, predictable rate, elucidating not only evolutionary relationships, but also an approximate time frame for the process (Moritz *et al*., 1987; Arbogast *et al*., 2002).

This study has shown that two parasitoids of important crop pests, which are difficult to differentiate on the basis of their morphological characteristics, are clearly separate genetically, even in a portion of the genome that is recognized as being conserved. DNA sequence analysis, morphometric data, host-rearing and phenological records were combined successfully to support the conventional view that *T. microgaster* and *T. obscurator* are distinct species and to suggest improved morphometric criteria for separating them. Using the results from this study it should be possible to design PCR markers specific to *T. microgaster* and *T. obscurator*. These could be used in a simple test to identify morphologically indeterminate specimens where phenological and rearing data are lacking. This would overcome the particular difficulty in the identification of males. The taxonomy of the parasitic Hymenoptera is often problematic and this study has shown that species determination should make use of a range of evidence. With the advent of relatively straightforward molecular approaches and their use in conjunction with conventional methods, the recognition and separation of difficult species can be more readily assured.

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