Journal of
Entomology

www.academicjournals.com

Mitochondrial Phylogenetics of UK Eurytomids

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Abstract: The aim of this study was to investigate the taxonomic limits of species within four Eurytomid genera, namely *Eurytoma* Illiger, *Tetramesa* Walker, *Ahtola* Claridge and *Sycophila* (Walker). In order to further clarify the taxonomic status of the genera, including *Tetramesa, Eurytoma, Ahtola* and *Sycophila*, mitochondrial DNA sequence analysis revealed differentiation between the above Eurytomid genera. With the sequence data, the monophyletic status of three of these genera is well supported by all methods of phylogenetic reconstruction. Only *Tetramesa*, which is by far the most specious taxon studied here, seems polyphyletic, but even in this case, there are certain relationships within the group that are interesting from both a morphological and host preference point of view. For example, *T. petiolata* (Walker) and *T. airae* (von Schlechtendal), which are both herbivores of the grass, *Deschampsia cespitosa* (L.) (Beauv.) group together and two clusters contained all species that use *Elymus repens* (L.), i.e., *T. linearis* (Walker), *T. hyalipenis* (Walker) and *T. cornuta* (Walker). Another result of this analysis supports the validity of *Ahtola* as a separate genus, which was previously uncertain.

Key words: *Tetramesa, Eurytoma, Sycophila, Ahtola*, DNA sequencing, Mitochondrial DNA, Cytochrome oxidase genes I and II

Introduction

Among all the chalcids, the systematics of the family Eurytomidae is particularly difficult since traditional taxonomic procedures based on morphology usually cannot identify of species group differences (Claridge, 1988). Within the family, both the classification at generic and sub-generic levels and the microtaxonomy of certain species have been a matter of disagreement among authors (Henneicke *et al.*, 1989). Examples concern are the validity of the genus *Ahtola* (Claridge, 1961; Fitton *et al.*, 1978), the limits of some genera such as *Eurytoma* and *Tetramesa* (Boucek, 1988) and the putative existence of aggregates of sibling species classified under single names (Claridge and Askew, 1960).

Acknowledging the difficulties associated with the morphological identification of Eurytomids, specialists in this research field have turned to methods of molecular systematics, but so far using only electrophoretic analysis protein (Dawah, 1988; Claridge and Dawah, 1994; Al-Barrak *et al.*, 2004) and random amplified polymorphic DNA markers (RAPDs) (Al-Barrak *et al.*, 2004). However, different

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molecular approaches, e.g., sequencing of mitochondrial DNA mtDNA have been used successfully in studies of other Chalcidoid wasps where morphological and behavioural traits are inadequate to resolve phylogenies (Gauthier *et al.*, 2000), to detect diversity within taxa (Taylor *et al.*, 1997), or to understand co-evolutionary processes (e.g., Agaoninae wasp pollinators of *Ficus* spp.) (Herre *et al.*, 1996; Weiblen, 2001).

Mitochondrial DNA is one of the most widely used genetic markers for the study of inter-and intra-specific evolution of animals (Posada and Crandall, 1998; Simon *et al.*, 1994; Taberlet *et al.*, 1996) and rapid screening of arthropod mithocondrial genomes for rearrangements (Roehrdanz, 2004). Several factors have contributed to this popularity. These include its compact size (15000-17000 bp), relatively conserved gene order and number, essentially non-recombining uniparental pattern of inheritance and few insertion/deletion events (Avise *et al.*, 1987; Harrison, 1987). Probably the most important reason for its widespread use is its rate and mode of evolution (Aquadro *et al.*, 1984; Moritz *et al.*, 1987). Different rates of nucleotide substitution in different gene segments and between evolutionary lineages are well-known features of mitochondrial genes and is probably due to gene-specific selection that limits divergence over evolutionary time (Lopez, 1997).

Population level studies have exploited the genome's rapid evolutionary rate, relying especially upon substitutions, which accumulate rapidly in third codon positions of coding genes (Palsbøll and Arctander, 1998). However, species and higher level relationships can be studied using the slower amino acid replacement changes (Moritz *et al.*, 1987) and protein coding genes may be very useful for both within and between species studies (Villablanca, 1994).

The relatively fast rate of nucleotide substitution and haploid (single copy) inheritance, which reduces the effective population size of this marker and increases its sensitivity to genetic drift, make mtDNA useful for revealing phylogenetic structure between populations of species (Moritz, 1994). MtDNA phylogenies can reveal patterns of genetic differentiation among cryptic species (Moritz *et al.*, 1993), such as may be the case in parasitic wasps.

Today, some mitochondrial genes have a universal status for certain applications and are now routinely applied in many insect molecular studies. For instance, the COI (Cytochrome Oxidase I) and COII (Cytochrome Oxidase II) genes have become the markers of choice in the study of intergeneric and interspecific relationships (Crozier *et al.*, 1989; Liu and Beckenbach, 1992; Willis *et al.*, 1992; Brown *et al.*, 1994; Danforth, 1999).

In this study, during 2001-2003, the limits and phylogenetic relationships of four Eurytomid genera in the UK (*Tetramesa, Eurytoma, Sycophila, Ahtola*) were investigated by phylogenetic analysis of a COI-COII DNA sequence fragment from several populations from South Wales and several isolated sites in England.

Materials and Methods

Specimen Preparation for Molecular Studies

After dissecting the stems, the larvae transferred to the gelatin capsule for rearing and then in early summer when they emerged, two-three days-old adults were collected and stored in small plastic tubes and deep-frozen at -70°C and labeled with a code number. Samples used in this study and their origins and plant hosts are listed in Table 1.

DNA Extraction

Purified DNA for the wasp species examined was obtained the QIAamp DNA Mini-kit (catalogue number 51306). All extractions followed t he manufacturer's recommendations QIAamp

Table 1: Hymenoptera species used in the molecular analysis, showing collection location and host grass species. The last two species in column on left (C. BIS and C.CAP) are reference species

Code	Species	Region	Grass
11	*T. linearis* (L.) 11.1	Caerphilly	*Elymus repens*
1	*T. petiolata* (Walker) 1.1	Caerphilly	*Descampsia cespitosa* (L.) (Beauv)
2 TLON 7.1	*T. longula* (Walker)	Kenfig Dunes	*Dactylis glomerata* (L.)
15 TAIR 1.1	*T. airae* (von Schlechtendal)	Caerphilly	*D. cespitosa*
36 TCAL 5	*T. calamagrostidis* (von Schlechtendal)	Kenfig Dunes	*C. epigejos*
8-37 TLON	*T. longula*	Rhoose	*D. glomerata*
38-40 TLON	*T. longula*	Cosmeston Lake	*D. glomerata*
23 TALB 6.1	*T. albomaculata* (Ashmead)	Rhoose	*Phleum pratense* (L.)
29 TPHL 6.2	*T. phleicola* (Hedicke)	Rhoose	*P. pratense*
13 TLNIS 8.2	*T. longicornis* (Walker)	Fairwater	*Phalaris arundinacea* (L.)
17 TBREV 4.4	*T. brevicollis* (Walker)	Aberthaw	*Festuca rubra* (L.)
21 TLIN	*T. linearis* 11.2	Wentlooge	*E. repens*
30 TLIN	*T. linearis* 12.1	Cosmeston Lake	*E. repens*
10 TFUL 8.1.1	*T. fulvicolis* (Walker)	Cosmeston Lake	*Brevicornis sylvaticum* (Huds)
25 TLIN 13.1	*T. linearis*	Rhoose	*E. repens*
26 TCOR 12.3	*T. cornuta* (Walker)	Cosmeston Lake	*E. repens*
22 TBNIS 4.1 4.3	*T. brevicornis* 4.1	Kenfig Dunes	*F. rubra*
	T. brevicornis 4.3	Aberthaw	*F. rubra*
24 THYA	*T. hyalipenis* 10.3	Wentlooge	*E. farctus*
31 THYA	*T. hyalipenis* 12.2	Cosmeston Lake	*E. repens*
28 TLIN 11.1	*T. linearis*	Caerphilly	*E. repens*
27 TLIN 13.2	*T. linearis*	Newport	*E. repens*
32 TEXI	*T. eximia* (Girauld) 3.1	Merthyr Mawr	*Ammophila arenaria* (L.)
	T. eximia 3.2	Kenfig Dunes	*A. arenaria*
	T. eximia 3.3	Ainsdale	*A. arenaria*
35 TEXI 3.3.1	*T. eximia*	Ainsdale	*A. arenaria*
3 SYC 4	*Sycophila mellea* (Curtis)	Kenfig Dunes	*A. arenaria*
4-6-33 SYC 15.1	*S. mellea*	Kenfig Dunes	*A. arenaria*
5 SYC 15.2	*S. mellea*	Southport	*A. arenaria*
7 EFLA	*Eurytoma flavimana* Boheman 30.2.1	Cosmeston Lake	*E. repens*
16 EFLA	*E. flavimana* 30.3	Rhoose	*E. repens*
12 EUR 23.3	*E.* sp.	Merthyr Mawr	*A. arenaria*
14 EAPP 20.1	*E. appendigaster* (Swederus)	Caerphilly	*D. cespitosa*
18 EROD 28	*E. erdoesi* Szelenyi	Rhoose	*D. glomerata*
20 EUR 21	*E.* sp.	Caerphilly	*D. cespitosa*
9 AHT 14.2	*Ahtola atra* Claridge	Mumbles	*F. rubra*
19 AHT 14.3	*Atra*	Cornwall	*F. rubra*
C.BIS AF200375	*Ceratosolen bisulcatus*	reference species	
C.CAP AF2003377	*C. capensis*	reference species	

Table 2: List of the PCR primers for amplifying the COI and COII genes. First two primers are sited in the COI, the last two in the CO II gene used

Primer	Sequence		Reference
Jerry	5'-CAACATTTATTTTGATTTTTTGG-3'	Forward	Simon *et al.* (1994)
Pat	5'-TCCAATGCACTAATCTGCCATATTA-3'	Reverse	Simon *et al.* (1994)
Musid	5'-TCAATATCATTGATGACCAAT-3'	Reverse	Taylor *et al.* (1997)
S2792	5'-ATACCTCGACGTTATTCAGA-3'	Forward	Taylor *et al.* (1997)

DNA Mini Kit (Qiagen) protocol and specimens were homogenized in 180 μL of buffer ATL (Qiagen). Homogenization was performed in 1.5 mL plastic microcentrifuge tubes using a hand held pellet mixer followed by addition of 20 μL proteinase K (20 mg mL^{-1}). Samples were mixed by vortexing and incubated in a hybridization oven containing a rocking platform at 56°C overnight. Two hundred microliter of buffer AL (Qiagen) was added to the each sample and mixed by pulse-vortexing for 15 sec followed by incubation at 70°C for 10 min. Two hundred microliter of 100% absolute ethanol

was then added to each tube and mixed. The mixtures (including the precipitate), but no insect remains, were carefully applied to the QIAamp spin columns without wetting the rim. Thereafter, centrifugation was performed at 8000 rpm for 1 min. The spin columns were placed in clean collection tubes. Without wetting the rim, 500 µL of buffer AW1 (Qiagen) was added to the QIAamp spin columns and centrifuged at 8000 rpm for 1 min and the columns placed in clean 2 mL collection tubes. Again without wetting the rim, 500 µL of buffer AW2 (Qiagen) was added to the columns and these were centrifuged at 13,000 rpm for 3 min and the columns were placed in clean tubes. These were spun for 1 min at 13000 rpm to remove excess buffers and placed in clean collection tubes. Lastly, 200 µL of buffer AE (Qiagen) was added to the QIAamp spin columns and incubated at room temperature for 5 min and then centrifuged at 8000 rpm for 1 min. The last step was repeated and the DNA thus extracted as second sample.

Amplification of DNA

A 1500 bp fragment spanning COI to COII was amplified in a single reaction using the general insect primers 'Jerry' and 'Pat' (Simon *et al.*, 1994) and 'Muscid' and 'S2792' (41) (Table 2). PCR amplification was carried out in a Perkin-Elmer 9700 Automated Thermocycler.

Sequencing

For sequencing, PCR products were purified using the Genclean for PCR Turbo Kit (Bio101). Each sample was sequenced in both forward and reverse directions using the ABI prism ® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). This kit was diluted to produce a master mix containing two parts sequencing kit: 1 part 5x ABI Perkin-Elmer buffer ®: 1 part sterile water (Chippindale *et al.*, 1998). Two microlitres of DNA, 2 µL of the sequencing mix and 1 µL of either forward or reverse primers at 1.6 pmol µL^{-1} concentration were then used in the reaction. The sequencing PCR program was carried out as follows: 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Each individual was sequenced using the 'Jerry', 'Muscid' and the internal 'Pat' primers.

Sequence Alignment and Phylogenetic Analysis

Sequences were edited and aligned using the program Sequencer v3.1.2 (Gene codes) and translated into amino acid codons using the *Drosophila* genetic code option in the program. The reading frame of COI and COII was obtained by aligning existing Chalcidoid wasp sequences from GENBANK with the test sequences. The species chosen were *Ceratosolen capensis* (Grandi) (Hymenoptera: Agaonidae) (AF200377) and *C. bisulcatus* (AF200375) (Weiblen, 2001) and were used as outgroups for the subsequent analysis. In order to correctly align the tRNA$_{LEU}$ sequences, the secondary structure was determined (Fig. 1) and alignment gaps placed in some sequences to preserve base pairing and accommodate insertion/deletions which are common in RNA sequences (Kjer, 1995). The use of secondary structure models greatly improves the accuracy of alignment and allows a more precise determination of homologous characters for phylogenetic analysis (Medina and Walsh, 2000). An existing arthropod tRNA$_{LEU}$ structure was used as a template for alignment (Nardi *et al.*, 2001). Stems were required to contain a minimum of two paired bases.

In order to correctly align the different genes between species and genera, alignment gaps were inserted between the 3' end of COI and the 5' end of the tRNA to accommodate a variable length non-transcribed region occurring between these two genes in some species. This phenomenon has also been described in other arthropod species (Stauffer, 1997; Weiblen, 2001). This non-transcribed region accounted for 91 bp and was excluded from the subsequent analyses.
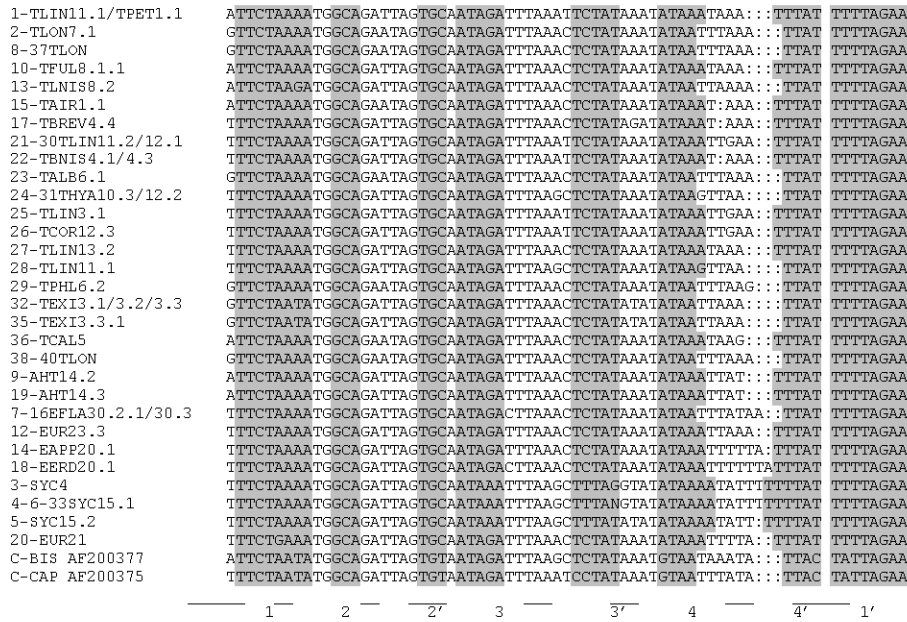
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1-TLIN11.1/TPET1.1    ATTCTAAAATGGCAGATTAGTGCAATAGATTTAAATTCTATAAATATAAATAAA:::TTTAT  TTTTAGAA
2-TLON7.1             GTTCTAAAATGGCAGAATAGTGCAATAGATTTAAACTCTATAAATATAATTTAAA:::TTAT  TTTTAGAA
8-37TLON              GTTCTAAAATGGCAGAATAGTGCAATAGATTTAAACTCTATAAATATAATTTAAA:::TTAT  TTTTAGAA
10-TFUL8.1.1          ATTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATAAA:::TTTAT  TTTTAGAA
13-TLNIS8.2           ATTCTAAGATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAATTAAA:::TTTAT  TTTTAGAA
15-TAIR1.1            ATTCTAAAATGGCAGAATAGTGCAATAGATTTAAACTCTATAAATATAAAT:AAA:TTTAT  TTTTAGAA
17-TBREV4.4           TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAGATATAAAT:AAA:TTTAT  TTTTAGAA
21-30TLIN11.2/12.1    TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATTGAA:TTTAT  TTTTAGAA
22-TBNIS4.1/4.3       TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAATTCTATAAATATAAAT:AAA:TTTAT  TTTTAGAA
23-TALB6.1            GTTCTAAAATGGCAGAATAGTGCAATAGATTTAAACTCTATAAATATAATTTAAA:::TTAT  TTTTAGAA
24-31THYA10.3/12.2    TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAGCTCTATAAATATAAGTTAA::::TTTAT  TTTTAGAA
25-TLIN3.1            TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAATTCTATAAATATAAATTGAA::TTTAT  TTTTAGAA
26-TCOR12.3           TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAATTCTATAAATATAAATTGAA::TTTAT  TTTTAGAA
27-TLIN13.2           TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATAAA:::TTTAT  TTTTAGAA
28-TLIN11.1           TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAGCTCTATAAATATAAGTTAA::::TTAT  TTTTAGAA
29-TPHL6.2            GTTCTAAAATGGCAGAATAGTGCAATAGATTTAAACTCTATAAATATAATTTAAG:::TTAT  TTTTAGAA
32-TEXI3.1/3.2/3.3    GTTCTAATATGGCAGATTAGTGCAATAGATTTAAACTCTATATATATAATTAAA::::TTAT  TTTTAGAA
35-TEXI3.3.1          GTTCTAATATGGCAGATTAGTGCAATAGATTTAAACTCTATATATATAATTAAA::::TTAT  TTTTAGAA
36-TCAL5              ATTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATAAG:::TTAT  TTTTAGAA
38-40TLON             GTTCTAAAATGGCAGAATAGTGCAATAGATTTAAACTCTATAAATATAATTTAAA:::TTAT  TTTTAGAA
9-AHT14.2             ATTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATTAT:::TTTAT  TTTTAGAA
19-AHT14.3            ATTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATTAT::TTTAT  TTTTAGAA
7-16EFLA30.2.1/30.3   TTTCTAAAATGGCAGATTAGTGCAATAGACTTAAACTCTATAAATATAATTTATAA::TTAT  TTTTAGAA
12-EUR23.3            TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATTAAA:::TTAT  TTTTAGAA
14-EAPP20.1           TTTCTAAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATTTTTA:TTAT  TTTTAGAA
18-EERD20.1           TTTCTAAAATGGCAGATTAGTGCAATAGACTTAAACTCTATAAATATAAATTTTTTATTTAT  TTTTAGAA
3-SYC4                TTTCTAAAATGGCAGATTAGTGCAATAAATTTAAGCTTTAGGTATATAAAATATTTTTTTAT  TTTTAGAA
4-6-33SYC15.1         TTTCTAAAATGGCAGATTAGTGCAATAAATTTAAGCTTTANGTATATAAAATATTTTTTTAT  TTTTAGAA
5-SYC15.2             TTTCTAAAATGGCAGATTAGTGCAATAAATTTAAGCTTTATATATATAAAATATT:TTTTAT  TTTTAGAA
20-EUR21              TTTCTGAAATGGCAGATTAGTGCAATAGATTTAAACTCTATAAATATAAATTTTA::TTAT  TTTTAGAA
C-BIS AF200377        ATTCTAATATGGCAGATTAGTGTAATAGATTTAAATGTAATAAAATGTAATAAATA:::TTAC  TATTAGAA
C-CAP AF200375        TTTCTAATATGGCAGATTAGTGTAATAGATTTAAATCCTATAAATGTAATTTATA:::TTAC  TATTAGAA
                       ‾‾   2    ‾‾2'   3  ‾‾   ‾‾3'   4    ‾‾4'   1'
                        1
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Fig. 1: Alignment of tRNA$_{LEU}$ to show secondary structure. Shaded area indicate areas of complimentary base pairing in strems

Table 3: Percentage base composition between British Chalcid wasp genera

Species	Gene	T	C	A	G
Tetramesa	COI	46.3	9.4	33.6	10.7
	COII	46.7	6.5	37.4	9.3
	TRNA	38.7	7.1	42.4	11.8
	INS	41.4	1.6	53.4	3.7
	ALL	43.3	7.7	36.9	10.2
Eurytoma	COI	44.2	9.0	36.2	10.6
	COII	43.3	6.1	39.8	10.8
	TRNA	41.7	7.9	39.9	10.5
	INS	43.8	0.0	50.0	6.3
	ALL	43.3	7.4	38.7	10.5
Sycophila	COI	44.4	9.9	30.1	15.6
	COII	46.8	6.8	31.1	15.3
	TRNA	44.7	5.8	38.0	11.5
	INS	0.0	0.0	0.0	0.0
	ALL	45.5	8.0	31.7	14.8
Ahtola	COI	44.6	9.2	35.9	10.3
	COII	43.9	7.1	39.5	9.5
	TRNA	40.3	7.5	41.8	10.4
	INS	46.2	0.0	53.8	0.0
	ALL	43.8	7.8	38.7	9.7

Base composition (Table 3) and transition: transversion ratios (Table 4) were calculated in MEGAv2 (Kumar *et al.*, 2001).

Phylogenetic analysis was conducted using PAUP 4.0b10 (Swofford, 2002). Gaps were treated as a 5th character state in all analyses. Neighbour-joining (NJ) and Maximum Likelihood (ML) methods

Table 4: Transition:transversion ratios (R) and the number of transitions (s) and transversions (v) in pairwise comparisons of British Chalcid wasps

Species		All genes	All coding	Non-coding	1ST	2ND	3RD
All species	R	0.705±0.075	0.702±0.078	0.630±0.213	0.692±0.156	0.59±0.138	0.588±0.094
	s	29.123±2.070	26.421±1.924	2.702±0.619	9.978±1.201	4.815±0.827	11.629±1.162
	v	60.294±3.457	54.321±3.150	5.974±1.241	17.048±1.792	9.484±1.535	27.788±1.903
Tetramesa	R	1.018±0.172	0.985±0.161	0.786±0.268	0.726±0.197	0.673±0.227	0.793±0.146
	s	25.321±2.265	23.437±2.151	1.884±0.583	7.995±1.327	3.711±0.974	11.732±1.373
	v	45.000±3.503	41.116±3.070	3.884±1.146	12.574±1.869	6.853±1.393	21.689±2.132
Sycophila	R	6.000±3.003	n/c	1.000±0.787	n/c	n/c	n/c
	s	4.667±1.676	4.000±1.537	0.667±0.584	0.000±0.000	0.667±0.705	3.333±1.432
	v	0.333±0.315	0.000±0.000	0.333±0.326	0.000±0.000	0.000±0.000	0.000±0.000
Eurytoma	R	1.019±0.223	1.548±0.524	0.245±0.189	2.138±1.146	0.926±0.582	1.397±0.625
	s	23.400±2.857	21.800±2.950	1.600±0.817	7.700±1.768	1.200±0.662	12.900±2.152
	v	25.000±3.354	16.800±2.791	8.200±1.894	4.200±1.337	1.400±0.788	11.200±2.103
Ahtola	R	n/c	n/c	n/c	n/c	n/c	n/c
	s	1.000±0.964	1.000±0.986	0.000±0.000	0.000±0.000	1.000±0.929	0.000±0.000
	v	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000

were used for phylogenetic reconstruction. Distance trees were derived using uncorrected p-values with the heuristic search and 1000 bootstrap replications. As an extreme A-T bias and saturated transitions have been reported in several species of fig wasp (family Agaonidae and Toryminae) mtDNA (Machado, 1998; Weiblen, 2001), neighbour-joining trees were also constructed using all substitutions and with transversions only, to determine whether saturation had an effect on tree topology.

The evolutionary model of 'best fit' for ML analysis was ascertained by running likelihood ratio test scores through Model test v3.06 (Posada and Crandall, 1998) with and without the non-transcribed sequence between COI and tRNA$_{LEU}$. In both cases, the best evolutionary model chosen was the 'General Time Reversible' (GTR)+G model. The shape of the gamma parameter (0.29) and the rate matrix were determined using PAUP and were employed using this model in subsequent heuristic searches to obtain the best ML trees for the data set.

Results

Sequence Data

Complete DNA sequences for the region used in the analysis (551 bp) were obtained for 39 individuals from the four genera under study (Table 1). Base composition is shown in Table 3. Of the 546 bp sequence examined, 366 sites were potentially parsimony informative, although when the non-transcribed region was removed, this figure was reduced to 158 of 455 sites. Overall, the transition: transversion ratios were low (<1.0); this significant transversion bias possibly indicates saturation of multiple substitutions at the same site (Table 4).

Distance based trees using all substitutions (Fig. 2) and those considering transversions only (Fig. 3), exhibited similar topologies. Using only transversions resulted in a tree which had better supported basal nodes than when all substitutions were considered, but support for internal branches was strong using both methods. The groupings of *Ahtola*, *Sycophila* and *Eurytoma* were strongly supported and distinct from each other and from *Tetramesa*. The types of substitution used for generating the tree did not affect this separation.

Using the GTR+G model, the -log likelihood score was 4325.2. Bootstrap analysis of the ML tree indicated good support for the *Sycophila* (100%), *Ahtola* (86%), but was less strong for *Eurytoma* (87%). *Tetramesa* clades were also well supported, although the relative position in the tree of three of the clades was not resolved (Fig. 4).
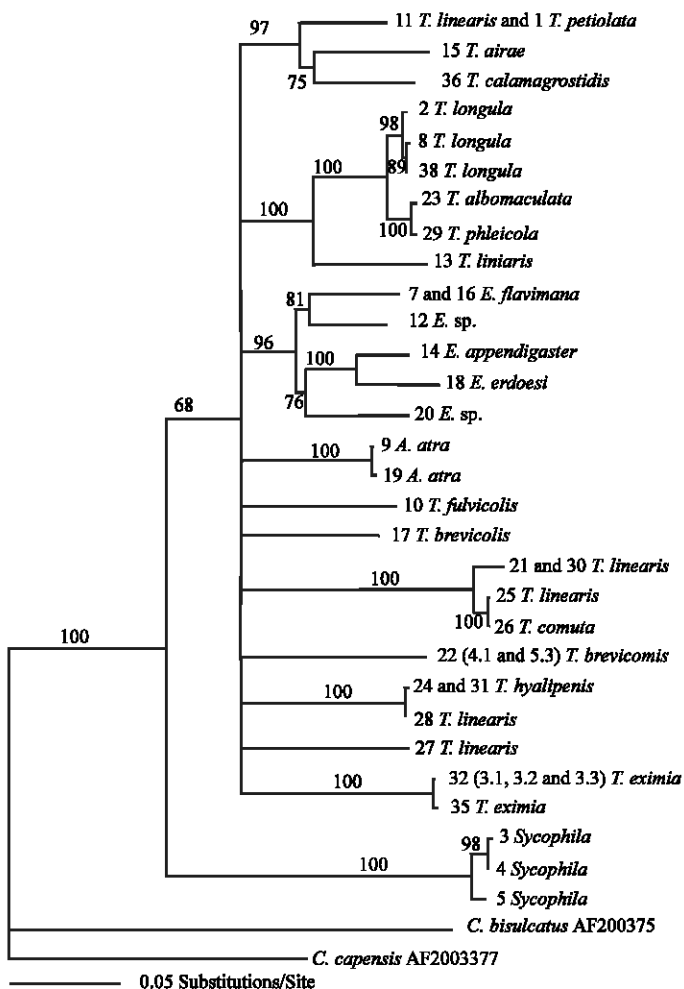
Fig. 2: NJ tree using all substitutions with 1000 bootstrap replications

Discussion

Molecular Evolution

By reporting the presence of a variable length non-transcribed region between the 3' end of COI and the 5' end of the tRNA$_{LEU}$ in the four genera analysed here, this study confirms previous work in chalcid wasps (i.e., Agaonid fig wasps; Weiblen, 2001). To extend COI-COII analysis to other Chalcidoidea families is important in order to determine if this characteristic is common to the entire superfamily. A similar trait was described in a scolytid beetle (Stauffer, 1997), but the fact that such a feature is absent in the majority of the insect orders screened to date for this sequence fragment argues for a scenario of independent and incidental acquisition. A low transition bias or a tranversion bias in among-species comparisons suggests either that species are ancient or that rapid molecular evolution is taking place. Since a transversion bias is typical of parasitic hymenoptera (Dawton and
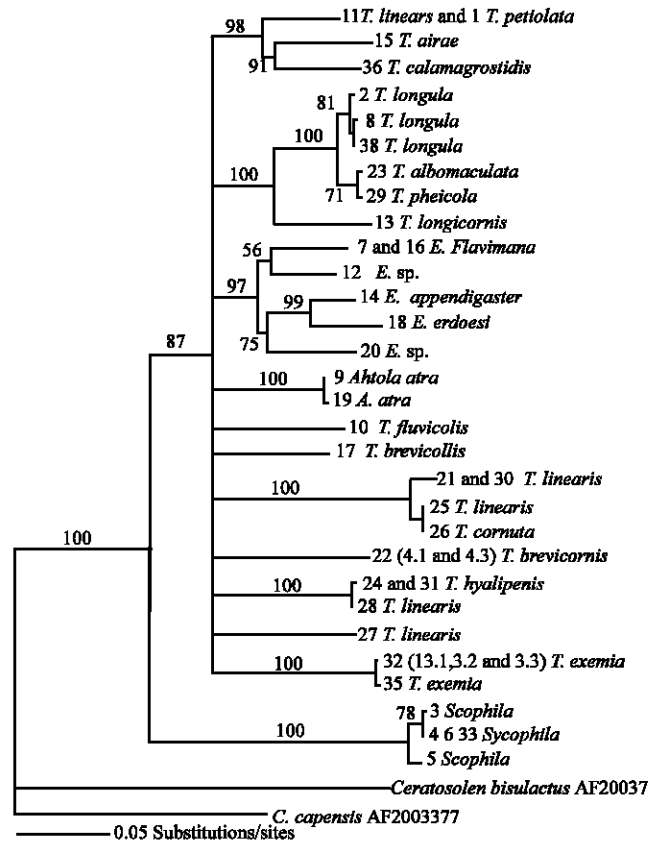
Fig. 3: NJ tree using only transversions with 1000 bootstrap replications

Austin, 1997) and not for parasitic dipterans (Castero *et al.*, 2002), this feature does not seem to be associated with lifestyle nor probably with evolutionary age, but rather, with an overall accelerated evolutionary rate in Hymenoptera.

Phylogenetics and Taxonomy

The number of unambiguous or good quality sequences retrieved was some what limited compared with the total number of samples amplified for the target DNA fragment. The reason for this low success rate may be the existence of copies of COI-COII inserted in the nuclear genome (Numts) (Bensasson *et al.*, 2001) that prevent straightforward sequencing of the true mitochondrial version of the gene. It was not possible to solve this problem by extensive cloning in the time available. However, the problem of Numts leading to spurious PCR amplifications will be one of the priorities of any extension of the research initiated here.

Although less of a problem for *Tetramesa* sp., from which sequences were obtained were obtained from fourteen different species, even so the sequence data was hindered by the possible presence of Numts in all the genera studied. This was particularly true for *Sycophila* to such an extent in fact that sequencing problems prevented the acquisition of significant data at a population level for the *S. mellea* complex.
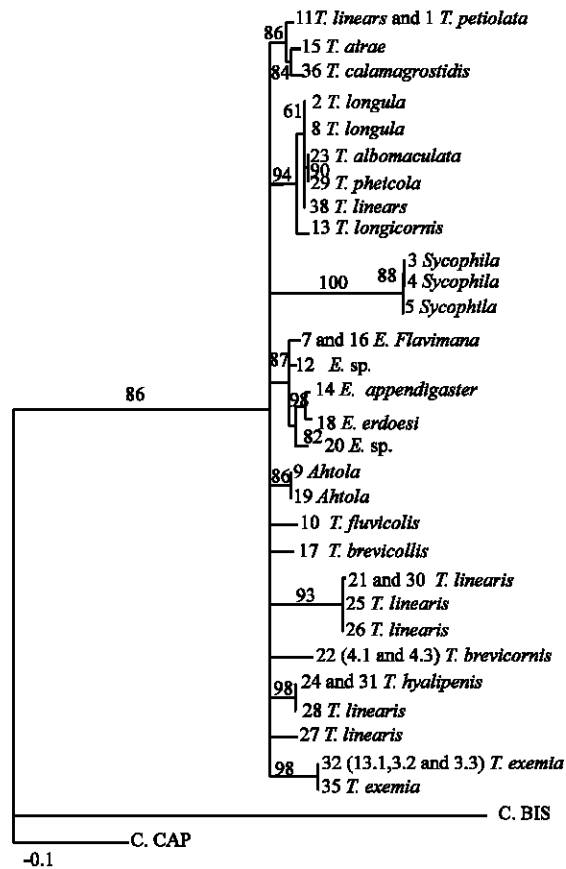
Fig. 4: Maximum likehood tree using GTR+G following 100 bootstrap replications

Even with the recognition that the data should be enlarged, both in terms of the total number of specimens screened and of *Eurytoma* and *Sycophila* species/populations studied, the monophyly of three of the four eurytomid genera is well supported by the different methods of phylogenetic reconstruction here applied to the sequence data. Consequently, the validity of *Ahtola* as a separate taxa is also shown from this analysis. Only *Tetramesa*, for which we have many more sequences than for other species, appears to be paraphyletic. In the case of the ML tree reconstructions, *Eurytoma*, *Sycophila* and *Ahtola* are monophyletic within the paraphyletic structure of *Tetramesa*, whereas in the NJ trees *Sycophila* is basal to the three other genera.

Several taxonomists have studied *Tetramesa* species (Al-Barrak *et al.*, 2004; Claridge and Dawah, 1994; Dawah *et al.*, 1995; Dawah, 1987). Despite the efforts of such researches, many *Tetramesa* species remain difficult to identify because of their uniform morphology. Molecular techniques could be useful tools to clear up their taxonomic status. For example, Al-Barrak *et al.* (2004) could successfully discriminated closely-related species of *Tetramesa* by using RAPD-PCR.

In terms of the within-genus taxonomy of *Tetramesa*, it is possible to draw some conclusions from the available results. It is interesting that the clades produced from the molecular phylogenies show some parallels with the trophic clusters of *Tetramesa* species associated with host grasses in the

eurytomid food web (Dawah *et al.*, 1995). A cluster is present with *T. petiolata* and *T. airae* (both herbivores of *D. cespitosa*) and two clusters occur containing all species that use *E. repens*. Furthermore, with the exception of *T. linearis*, all *Tetramesa* species included in the study seem to be paraphyletic and are therefore likely to be valid species, a result that extends previous findings using protein electrophoresis (Dawah, 1987). Al-Barrak *et al.*, (2004) could discrimination between three closely-related species of *T. calamagrostidis*, *T. longicornis* and *T. petiolata* using RAPD-PCR. They reported that two forms of *T. hyalipennis* (ex: *E. repens* and *E. farctus*) were the most closely-related of any of the species investigated and probably diverged the most recently. They believed that some degree of sympatric evolution has occurred, most obviously in the case of the host adapted forms of *T. hyalipennis*. Dawah (1986) used electrophoresis for phylogenetic analysis of certain *Tetramesa* spp. And reported that *T. hyalipennis* reared from *E. repens* and *E. farctus* (Viv) represented a single species. He also stated that *T. exemia* reared from *Calamagrostidis epigejos* and *Ammophila arenaria* represented a single species which showed that there was a restriction of gene flow between these particular populations. Henneicke *et al.* (1992) used a comparative morphological study on final-instar larval stage of 33 species of grass-inhabiting wasps belonging to four genera: *Eurytoma. Tetramesa*, *Sycophila* and *Ahtola*. They found that *Ahtola* was intermediate between *Eurytoma* and *Tetramesa* and *S. mellea* was confirmed as a distinct genus.

DNA sequencing has been shown in this study to be a powerful tool which has allowed successful discrimination between the four eurtytomid genera, *Tetramesa*, *Eurytoma*, *Ahtola* and *Sycophila*. With the sequence data, the monophyletic status of three of the four genera is well supported by different methods of phylogenetic reconstruction. Only *Tetramesa*, which is by far the most numerous taxon here studied, seems paraphyletic but even in this case, there are certain phylogenetic relationships within the group that are interesting from both a morphological and host preference point of view. Thus for example, the *T. petiolata* and *T. airae* group together, which are both herbivores of *D. cespitosa* and two clusters occurred containing all species that use *E. repens*, i.e., *T. linearis*, *T. hyalipennis* and *T. cornuta*. Another interesting result of this analysis is the support for the validity of *Ahtola* as a separate taxon. Thus, these results clearly support the taxonomic separation of these four genera of eurytomids.

Acknowledgement

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