

The evolution of host use and unusual reproductive strategies in *Achrysocharoides* parasitoid wasps

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Abstract

We studied host selection and exploitation, two crucial aspects of parasite ecology, in *Achrysocharoides* parasitoid wasps, which show remarkable host specificity and unusual offspring sex allocation. We estimated a molecular phylogeny of 15 *Achrysocharoides* species and compared this with host (plant and insect) phylogenies. This tri-trophic phylogenetic comparison provides no evidence for cospeciation, but parasitoids do show phylogenetic conservatism of the use of plant genera. Patterns of sequence divergence also suggest that the parasitoids radiated more recently (or evolved much faster) than their insect hosts. Three main categories of brood production occur in parasitoids: (1) solitary offspring, (2) mixed sex broods and (3) separate (split) sex broods. Split sex broods are very rare and virtually restricted to *Achrysocharoides*, while the other types occur very widely. Our phylogeny suggests that split sex broods have evolved twice and provides evidence for a transition from solitary to mixed sex broods, via split sex broods, as predicted by theory.

Introduction

The reproductive strategy of an animal consists of a series of related decisions, which because of the close link between reproductive behaviour and fitness are likely to be under strong natural selection (Maynard Smith, 1978; Stearns, 1992). Insight into the way selection operates can be gained from cross-species comparisons, but only if account is taken of the phylogenetic relationships amongst species. The development of morphological and especially molecular techniques to construct phylogenies (Felsenstein, 2003), as well as the appropriate statistical techniques for their analysis (Pagel, 1999), has revolutionized the use of comparative approaches for understanding reproductive strategies (Mayhew & Pen, 2002).

Here we compare the reproductive strategies within a genus of parasitoid wasps, *Achrysocharoides* (Hymenop-

tera, Chalcidoidea, Eulophidae). We chose this group because of the variety and unusual nature of the reproductive behaviours it shows, and because it allows novel opportunities for testing evolutionary theory. All *Achrysocharoides* species attack leaf-mining Lepidoptera, that is micromoths whose larvae develop in 'mines' within the leaf lamina (the majority of hosts are in the genus *Phyllonorycter*, Gracillariidae). However, they differ considerably in their clutch size and sex allocation behaviour (Askew & Ruse, 1974; Bryan, 1983). Some species lay single male eggs in a host but clutches of typically two to three female eggs; others lay gregarious clutches of either males or females (i.e. split sex broods); while a further group lays gregarious mixed-sex clutches. A final category of species is parthenogenetic, producing no males. Split-sex broods are extremely rare in (non-polyembryonic) parasitoids (Pickering, 1980; Godfray, 1994) and prompted detailed studies of the reproductive behaviour of particular *Achrysocharoides* species (West *et al.*, 1996, 1999; West & Rivero, 2000; West *et al.*, 2001). Consequently, by mapping clutch size and sex allocation onto the phylogeny of this group we are able to test

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theoretical predictions for how evolutionary transitions are made between different reproductive strategies (Godfray, 1987; Rosenheim, 1993; Godfray, 1994; Pexton *et al.*, 2003).

Achrysocharoides are also unusual in their pattern of host specificity (Askew & Shaw, 1974). Their hosts are a species-rich lepidopteran group with the majority of species monophagous on different genera of trees in temperate regions. In Europe, members of common tree genera are typically attacked by a number of species of *Phyllonorycter*, often representing several independent host shifts and colonisations (Lopez-Vaamonde *et al.*, 2003). *Achrysocharoides* species usually attack all species on a tree genus, irrespective of their phylogenetic relationships, and thus appear to show host-plant specificity rather than host specificity. An exception to this occurs for species of moth that produce a mine just under the upper epidermis rather than in the more normal position just below the lower epidermis (termed upper and lower surface miners, respectively). The former are attacked by an *Achrysocharoides* species that specializes on mines in this position. While other examples of host plant taxonomy and ecology determining parasitoid host specificity exist, the pattern in this genus is unusually clear (Godfray, 1994).

Here we construct a phylogeny of 15 species of European *Achrysocharoides*, and use it to address questions in three areas. First, we examine the evolution of clutch size and sex allocation. Godfray (1987) showed theoretically that shifts from solitary to gregarious broods should be very difficult if larvae are aggressive, as in many solitary parasitoids. However, a subsequent model by Rosenheim (1993) showed that the transition could proceed more easily via an intermediate state of split sex broods. We can test this possibility because *Achrysocharoides* contains gregarious species with mixed and split sex broods, whilst species in closely related genera are solitary. We can also test whether the unusual trait of split-sex broods is a unique evolutionary event, or whether it has evolved several times. A single origin might reflect very unusual selection pressures that would be hard to reconstruct, while multiple origins are more likely to be due to explicable causes.

Second, we examine the evolution of host choice. Recently a phylogeny was constructed of the genus *Phyllonorycter* that includes all the (British) hosts of the *Achrysocharoides* included in our phylogeny (Lopez-Vaamonde *et al.*, 2003). A phylogeny of their host plants has also been constructed from published plant data (Lopez-Vaamonde *et al.*, 2003). With the parasitoid phylogeny described here we are in the hitherto unique position of having phylogenies for all three trophic levels. We use these to test a number of hypotheses. Specifically, we ask whether: (1) parasitoid phylogenies are correlated with host phylogenies, as might occur if parasitoids cospeciate with their hosts, or if host shifts are strongly determined by host phylogenies; (2) parasitoid

phylogenies are correlated with plant phylogenies for equivalent reasons – previous work has shown that host and host plant phylogenies are only weakly correlated (Lopez-Vaamonde *et al.*, 2003); (3) two parasitoid species that attack nonoverlapping sets of hosts with different ecology on the same plant species represent sister species or independent colonisations.

Finally, *Achrysocharoides* has been subject to a series of taxonomic revisions (Askew & Ruse, 1974; Bryan, 1983; Hansson, 1983, 1985), which have defined species boundaries and identified species groups. A subsidiary aim of this project was therefore to contribute towards creating a stable classification for this genus.

Natural history of *Achrysocharoides*

The genus *Achrysocharoides* Girault, 1913 (= *Enaysma* Delucchi, 1954) belongs to the subfamily Entedoninae of the chalcidoid family Eulophidae. The 48 species described are mostly from temperate regions, with 17 from Europe, 18 from north America (Yoshimoto, 1977; Kamijo, 1991), 11 from Asia (Bryan, 1983; Hansson, 1983, 1985; Kamijo, 1990a, b) and two from Australia (Boucek, 1988). Eleven *Achrysocharoides* species are known to occur in the British Isles (Askew & Ruse, 1974; Bryan, 1980) and these have been divided into two taxonomic groups (Graham, 1959; Bryan, 1980; Hansson, 1983). While the level and names of these two groups have been debated, all authors refer to the same sets of species. Graham (1959) regarded these groups as subgenera: *Enaysma* s.str. and *Pentanaysma* Graham, while Bryan (1983) referred to them as the *atys* and *latreillii* species groups. Finally, Hansson (1983) called them first and second group respectively. In this paper, we will refer to them as the *atys* and *latreillii* species groups.

Achrysocharoides species are larval endoparasitoids of leaf mining moths in the family Gracillariidae. Among the many parasitoid wasp genera that attack *Phyllonorycter* moth species, *Achrysocharoides* is the most host-specific (Askew, 1994). However, most *Achrysocharoides* species are also host plant specific, feeding on *Phyllonorycter* larvae from only one, or a few, plant genera (Askew & Ruse, 1974). This is an extremely rare habit amongst parasitoid Hymenoptera and suggests that this genus has divided up niche space under the influence of inter-specific competition (Godfray, 1994).

The ecology of *Achrysocharoides* species is reasonably well understood and there are host records for all but two species (*A. laticollaris* and *A. pannonica*). *Achrysocharoides* species attack *Phyllonorycter* larvae that mine lower surfaces of leaves, except for *A. suprafolius*, which feeds only on the polyphagous upper surface-mining *Phyllonorycter corylifoliella* (Askew & Ruse, 1974). Most *Achrysocharoides* attack tree or shrub leaf miners, but seven species (see electronic appendix) parasitise *Phyllonorycter* that mine herbs in the Fabaceae (Hansson, 1987; Kamijo, 1990b).

Methods

Parasitoid rearing and ecological data

Leaves with fully developed *Phyllonorycter* mines were collected in 1999 and 2000, mainly in the UK (Appendix 1). The mines were identified (Emmet *et al.*, 1985) and then placed in plastic boxes with ventilated lids. Emerging wasps were stored at -20°C . A few wasps from each collection (leaves from a single tree) were mounted for identification and voucher specimens have been deposited at the Natural History Museum, London. In order to test the monophyly of *Achrysocharoides*, we used two *Kratoysma* species and four *Chrysocharis* species as outgroups. A recent molecular phylogeny of eulophid genera (Gauthier *et al.*, 2000), identified *Chrysocharis* as the sister group of *Achrysocharoides*, but did not include *Kratoysma*, which is the other candidate sister genus (Bouceck, 1965; Hansson, 1983).

Sex ratio/clutch size strategy

Data on the clutch size and sex ratio strategies of British *Achrysocharoides* species were compiled (see Table 1) from a series of publications on this topic (Askew & Ruse, 1974; Bryan, 1983; West *et al.*, 1996, 1999, 2001). Species were placed into four brood categories: (1) solitary (*Kratoysma* and *Chrysocharis* species), (2) split sex ratios (six *Achrysocharoides* species), (3) mixed sex (three *Achrysocharoides* species), or (4) asexual (two *Achrysocharoides* species).

In addition, for *A. atys*, which has mixed gregarious broods, we analysed brood sex ratios to ascertain whether variance is less than binomial. This would provide evidence for a mating system with potentially high levels of inbreeding, termed local mate competition (LMC) (Hamilton, 1967), where males mate locally with females, including their sisters, before the females disperse. We did

Table 1 Combined sex ratio/clutch size strategies of British *Achrysocharoides* species.

Species	Strategy	References
<i>acerianus</i>	Mixed sex	Bryan (1983)
<i>butus</i>	Split sex	West <i>et al.</i> (1999)
<i>cilla</i>	Split sex	West <i>et al.</i> (1999)
<i>latreilli</i>	Split sex	West <i>et al.</i> (1999)
<i>splendens</i>	Split sex	West <i>et al.</i> (1999)
<i>atys</i>	Mixed sex	Bryan (1983)
<i>carpini</i>	Asexual	Bryan (1983)
<i>insignitellae</i>	Asexual*	Bryan (1983)
<i>niveipes</i>	Split sex	West <i>et al.</i> (1999)
<i>suprafolius</i>	Mixed sex	Bryan (1983)
<i>zwoelferi</i>	Split sex	West <i>et al.</i> (1996, 1999, 2001)

*Although *A. insignitellae* is regarded as an asexual species, we reared one male and about 50 females. However, many essentially asexual species produce the occasional male.

this following a standard method described in Green *et al.* (1982) and used in several similar studies (Morgan & Cook, 1994; Hardy & Cook, 1995).

Molecular techniques

DNA extraction, PCR, and sequencing were performed as described in Lopez-Vaamonde *et al.* (2001). We used only a single leg or the head from each wasp for DNA extraction and the bodies of sequenced individuals have been deposited at the Natural History Museum, London. For each wasp, we sequenced 1501 base pairs, comprising 473 bp of mitochondrial cytochrome *b* (Cyt *b*) and 1028 base pairs of nuclear 28S rDNA (28S). These regions often evolve at sufficiently high rates to provide phylogenetic resolution at lower taxonomic levels in the Hymenoptera (Stone & Cook, 1998; Kerdelhué *et al.*, 1999; Lopez-Vaamonde *et al.*, 2001). We sequenced one individual for nine species, two individuals for one species (*A. splendens*), three individuals for two species (*A. latreilli* and *A. cilla*) and five individuals for a single species (*A. zwoelferi*). New sequences were deposited in GenBank (accession numbers: AF477605–AF477622).

Estimating and comparing phylogenies

Cyt *b* sequences were all 473 bp in length and were aligned using Sequencher 4.1 (Genecodes Corp., Ann Arbor, MI). In contrast, 28S sequences varied in length from 1026–1035 bp and were therefore aligned using Clustal X (Aladdin Systems Inc., Heidelberg, Germany) with the default gap opening: gap extension costs. The automated alignment was then adjusted by eye where there were obvious mistakes. Both alignments are available from TreeBASE (<http://treebase.org/treebase/>) (study accession number = SN2131–7651). MacClade Version 4 was used to calculate the average nucleotide frequencies and the number of transitions (Ts) and transversions (Tv) at each Cyt *b* codon position.

We analysed each gene separately and then compared their phylogenetic signals using the incongruence length difference (ILD) test ('partition homogeneity test' option in PAUP*). This assigns data to two different partitions, one for each gene, and compares the number of steps in the phylogeny when data partitions are analysed separately or combined. The difference is then compared to that between the individual partition analyses and 1000 randomized data partitions.

We estimated both maximum parsimony (MP) and maximum likelihood (ML) phylogenies in PAUP*4.0b10 (Swofford, 2000). MP trees were reconstructed using the branch-and-bound search method (Hendy & Penny, 1982), with gaps treated first as missing data and then as a fifth state. All character transitions were given equal weighting. For ML analyses, we selected the DNA substitution model using Modeltest3.0b6 (Posada & Crandall, 1998) and then conducted a heuristic search

(options: ASIS and TBR branch-swapping). In both MP and ML analyses, we evaluated support for individual nodes by nonparametric bootstrapping (Felsenstein, 1985) with 1000 replicates. We also used the Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) to determine whether MP and ML topologies were significantly different.

Cospeciation tests

We compared parasitoid, host and host plant phylogenies with three pairwise cospeciation analyses in Treemap 1.0 (Page, 1995). These analyses ask whether the maximum proportion of cospeciating nodes inferred is greater than the maximum proportion that can be inferred when one of the phylogenies is randomized (1000 times to obtain a null distribution). We used the *Achrysocharoides* ML phylogeny in Fig. 1, simplified so that each species appeared only once. This was achieved by pruning excess individuals from monophyletic species represented by multiple individuals. In addition, we treated *A. splendens*, which renders *A. cilla* paraphyletic, as its sister species. *Phyllonorycter* and host plant phylogenies were taken directly from Lopez-Vaamonde *et al.* (2003).

Host plant mapping

We used the pruned ML phylogeny described above for all trait mapping exercises. Traits were mapped onto the tree and the history of changes inferred using parsimony procedures in MacClade. We first mapped host plant taxonomy (see Appendix 1) with each *Achrysocharoides* species coded according to its host plant order/family/genus and treated as an unordered, multistate character. We then mapped host plant growth form (tree/shrub/herb), also as an unordered, multistate character.

We then investigated whether host plant switches tend to occur between related plant groups (i.e. phylogenetic conservation of host use) using Permutation Tail Probability tests (PTP utility in PAUP*). These compare the number of host change steps in the actual tree with the number of steps observed in 10 000 randomized trees. Each host taxon was treated as a binary character.

Mapping sex ratio strategies

Reproductive strategy was mapped onto the phylogeny as a trait with four unordered states: (i) solitary broods, (ii) gregarious broods, (iii) split sex broods and (iv)

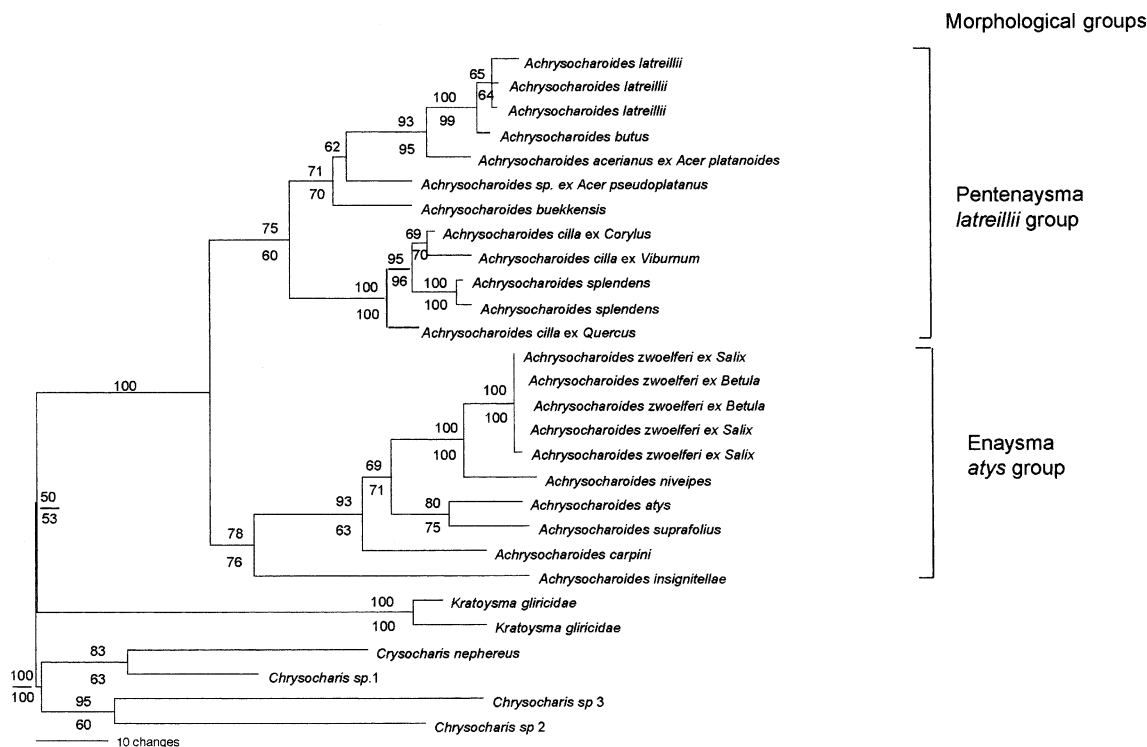


Fig. 1 ML tree based on combined 28S and Cyt *b* for 13 *Achrysocharoides* species $-\ln L = 4550.39606$. Model parameters: empirical base frequencies with rate heterogeneity, gamma shape parameter = 0.7469, proportion of invariable sites = 0.6762, six rate categories, GTR + I + G model with transformation parameters [A–C] = 7.8898, [A–G] = 25.2305, [A–T] = 48.3099, [C–G] = 2.1138, [C–T] = 81.5043 and [G–T] = 1.0000. Branch lengths are proportional to lengths estimated under the ML model; bootstrap values >50 are shown above branches for ML and below branches for MP.

parthenogenesis. The sequence of changes was then reconstructed using parsimony. Since two origins of split sex ratios are suggested, we tested whether there was statistical support for more than one origin by constraining all species with split sex ratios to form a monophyletic clade and then finding the ML tree. The likelihood of this tree was then compared to the best unconstrained ML tree using a Shimodaira-Hasegawa test.

Results

Phylogenies

Achrysocharoides was monophyletic in all analyses with *Kratoysma* as its sister group. There was also consistent support for monophyly of the two *Achrysocharoides* (*atys* and *latreillii*) species groups (Fig. 1). The position of *A. splendens* renders *A. cilla* paraphyletic, so we regarded these two as sister species for mapping purposes.

Achrysocharoides species show considerably lower levels of uncorrected nucleotide divergence than their *Phyllonorycter* hosts (data from Lopez-Vaamonde *et al.*, 2003). This applies to both 28S (*Achrysocharoides*: 0.09–0.8%; *Phyllonorycter*: 2.9–8.8%) and Cyt *b* (*Achrysocharoides*: 1.4–11.7%; *Phyllonorycter*: 6.9–15.4% unpublished data) and suggests that *Achrysocharoides* either evolve faster or represent a more recent radiation than their hosts. Uncorrected *p*-distances between *Achrysocharoides* species for Cyt *b* varied from essentially zero (*A. cilla/splendens*) to 11.7% (*A. insignitellae* and *A. zweelferi*).

The 28S and Cyt *b* data sets were congruent, since the ILD test was not significant with gaps treated as a 5th base (n.s.) or as missing data (n.s.). In addition, there were no incompatible clades that were strongly supported by the two data partitions. Furthermore, there was no significant difference between the combined (28S + Cyt *b*) MP and ML topologies, so we used only the fully resolved ML topology (Fig. 1) for cospeciation tests and character mapping. Summary statistics for nucleotide patterns and MP and ML analyses of each data set are given in Tables 2 and 3.

Table 2 Nucleotide and amino acid patterns.

	A	C	G	T	Ts/Tv	<i>n</i>	<i>nv</i>	<i>ic</i>
<i>Achrysocharoides</i>								
28S D1 + D2 + D3	20.58	26.63	32.37	20.38	n/a	1028	49	35
Cyt <i>b</i> Total sequence	34.74	13.88	9.86	41.52	0.92–0.94	473	172	116
Cyt <i>b</i> (1st)	34.50	13.77	16.93	34.79	0.86–0.90	158	53	27
Cyt <i>b</i> (2nd)	24.26	21.38	11.26	43.09	0.86	158	23	11
Cyt <i>b</i> (3rd)	45.09	5.81	1.08	48.01	0.94–0.96	158	132	104
Amino acids						157	58	33

A, C, G, T: average nucleotide frequencies; Ts/Tv: transition/transversion ratio; *n*: total number of positions; *nv*: number of variable positions (ingroup only); *ic*: number of parsimony informative characters (ingroup only).

Table 3 Summary of *Achrysocharoides* MP and ML analyses.

	Maximum parsimony				Maximum likelihood	
	Steps	Trees	CI	HI	Model	–ln <i>L</i>
28S rDNA	60	5	0.88	0.12	TrN + G	1811.5740
Cyt <i>b</i>	390	7	0.54	0.46	TVM + G	2467.4563
Cyt <i>b</i> + 28S	457	8	0.58	0.42	GTR + I + G	4554.6279

Steps: length of most parsimonious cladogram; trees: number of most parsimonious trees; CI: consistency index excluding uninformative characters; HI: homoplasy index excluding uninformative characters; Model: best-fit model selected by hierarchical likelihood ratio tests (hLRTs) in Modeltest Version 3.06; –ln *L*: score of best tree found; TrN: Tamura–Nei model (Tamura & Nei, 1993); I: proportion of invariable sites; G: shape parameter of the gamma distribution; TVM: submodel of the general-time-reversible model (Yang *et al.*, 1994); GTR: general time reversible model (Rodriguez *et al.*, 1990).

Cospeciation between wasps, moths and host plants

We found no evidence that any two of the three phylogenies were more similar than expected by chance (Table 4), ruling out a significant role for cospeciation (Figs 2 and 3).

Host plant use

Most *Achrysocharoides* species (45/54) attack moths feeding on one plant family (see electronic appendix), suggesting that host shifts are constrained by plant taxonomy. In agreement with this, PTP tests show that host plant use is phylogenetically conserved at the plant genus level ($P < 0.01$), although not at family (n.s.) and order (n.s.) levels. Mapping of host plant orders suggests that the ancestors of the extant European *Achrysocharoides* species may have attacked *Phyllonorycter* feeding on trees of the order Fagales (oaks, birch, alder, etc.) and colonized plants of the order Fabales, Rosales and Sapindales once each (Fig. 4).

Mapping of plant growth form indicates that the ancestor of *Achrysocharoides* parasitised moths on trees with a single colonisation of herb-feeding moths by *A. insignitellae*. A few other species also attack *Phyllonorycter* feeding on herbs and it would be interesting to

Table 4 Results of the Treemap cospeciation analysis using different datasets.

Pairwise comparisons between cladograms	N taxa	Max	Cosp	MPR	%	Observed <i>P</i> -value	Corrected <i>P</i> -value
<i>Achrysocharoides</i> / <i>Phyllonorycter</i>	15	14	3	3	21.4	0.069	0.248
<i>Achrysocharoides</i> /Host Plant	15	14	3	16	21.4	0.168	0.327
<i>Phyllonorycter</i> /Host Plant	29	28	9	20	32.1	0.123	0.198

Max: maximum possible number of cospeciation events (number of *Achrysocharoides* species-1); Cosp: observed number of cospeciation events; MPR: most parsimonious reconstructions; %: the percentage of cospeciating nodes detected ($\% = 100 \cdot \text{Cosp}/\text{Max}$); *P*-value: the 'corrected' *P*-values (see Lopez-Vaamonde *et al.*, 2001) obtained when randomizing both trees 1000 times using the proportional-to-distinguishable model.

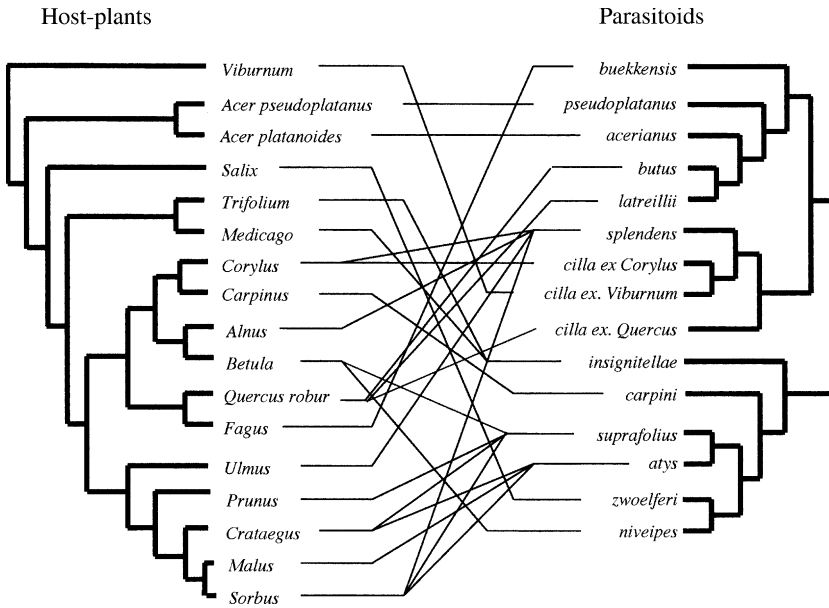


Fig. 2 Comparison of parasitoid and host plant phylogenies. The host plant phylogeny is based on Soltis *et al.* (1999), while parasitoid phylogeny is from Fig. 1. Lines connect host plants with their specific parasitoids.

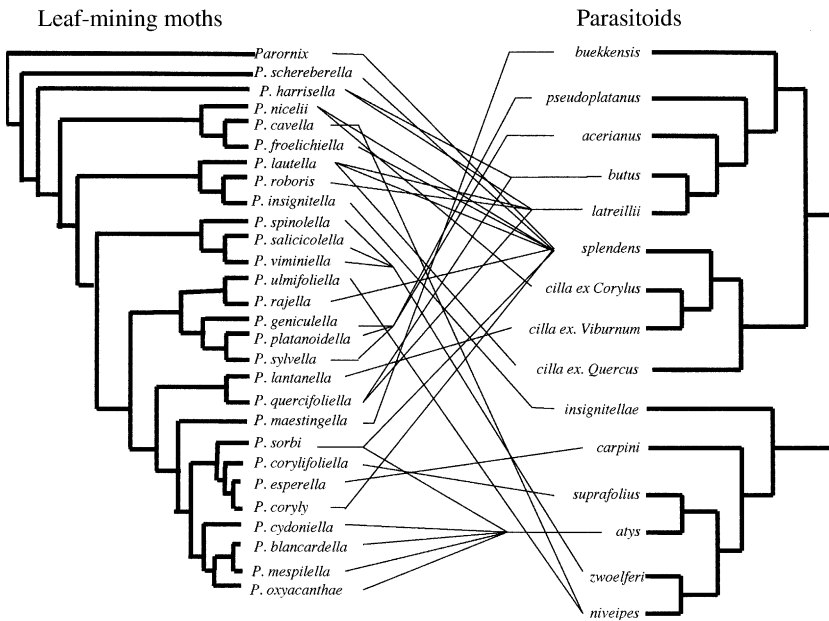


Fig. 3 Comparison of parasitoid and host (moth) phylogenies. The host phylogeny is from Lopez-Vaamonde *et al.* (2003) and the parasitoid phylogeny from Fig. 1. Lines connect hosts with their specific parasitoids.

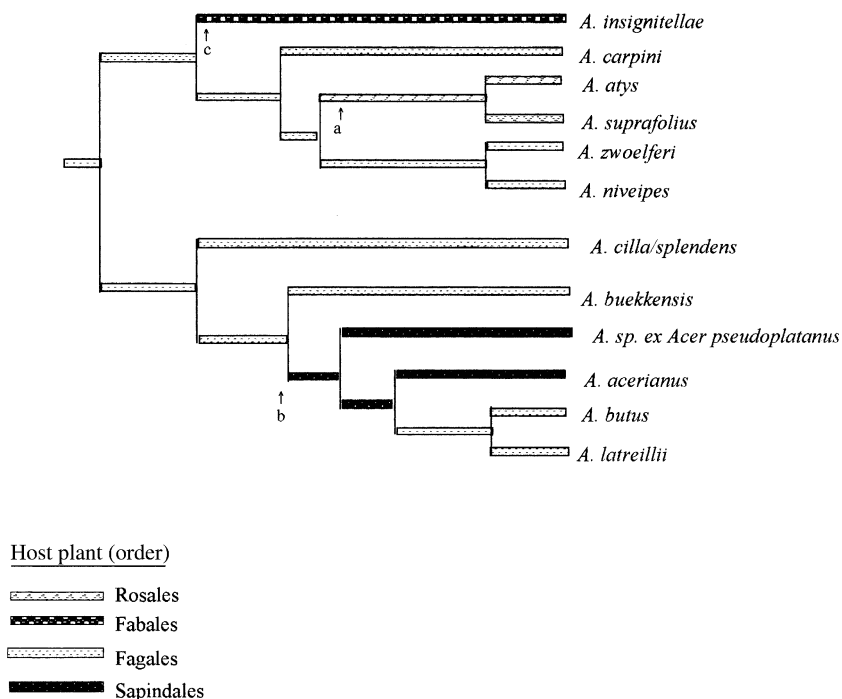


Fig. 4 Mapping of host-plant orders onto a parasitoid phylogeny from Fig. 1. Major host shifts occurred to Rosales (a), Sapindales (b) and Fabaceae (c).

include these in a future study to investigate the number of colonisations of herbs by these wasps. Interestingly, the only upper surface leaf miner parasitoid (*A. suprafolius*) is most closely related to the lower surface leaf miner parasitoid (*A. atys*) on the same host plants (*Crataegus* and *Sorbus*), suggesting ecological speciation to reduce competition for resources or enemy-free space on the same host plants.

Clutch size and sex ratio

The mapping indicates several changes in reproductive strategy with apparently parallel evolution of the unusual split sex broods (and of mixed sex broods) in the *latreillii* and *atys* species groups (Fig. 5). Two origins of split sex broods were also supported by a significant difference ($P < 0.001$) between the likelihood of the ML phylogeny ($-\ln L = 4550.39606$) and the ML tree constrained ($-\ln L = 4618.99673$) to have a single origin. The large number of changes relative to the number of taxa hinders reconstruction of ancestral states and changes. However, since the close outgroups have solitary broods, it is clear that the gregarious and split sex habits arose in our focal genus. In addition, in the *latreillii* group, it appears that gregarious broods may have arisen from split sex broods, as predicted by Rosenheim (1993). In the *atys* group the order of changes cannot be resolved unequivocally as taxa with mixed and split sex broods appear as sister groups (Fig. 5). The two parthenogenetic species appear basal in the *atys* group, and we cannot yet identify their closest sexual relatives.

We also examined the pattern of sex allocation in one of the species that laid mixed sex gregarious broods. In that species, *A. atys*, sex allocation was highly precise (less than binomial variation), with a significant tendency to produce one male and $n-1$ females in a brood of size n (Table 5). This suggests that LMC occurs in this species, with males mating the females before the females disperse, which may lead to high levels of inbreeding (Green *et al.*, 1982; Morgan & Cook, 1994; West & Herre, 1998).

Discussion

Radiation of *Achrysocharoides* parasitoids

Achrysocharoides provides an interesting case of ‘ecological specificity’, because most species attack *Phyllonorycter* moths confined to single host plant genera. For instance, *A. zwoelferi* only attacks closely related *Phyllonorycter* species feeding on willows (*Salix*), while *A. latreillii* only attacks a number of *Phyllonorycter* species that all feed on oaks (*Quercus*). Despite this ecological specificity, we found no evidence for cospeciation of *Achrysocharoides* with either host insects or host plants. In addition, patterns of *Cyt b* and 28S sequence divergence suggest that the parasitoids either evolve much faster at the DNA level, or, more likely, are a more recent radiation than their leaf-miner hosts. Similarly, *Phyllonorycter* moths are younger than the host plants that they feed upon (Lopez-Vaamonde *et al.* unpublished data).

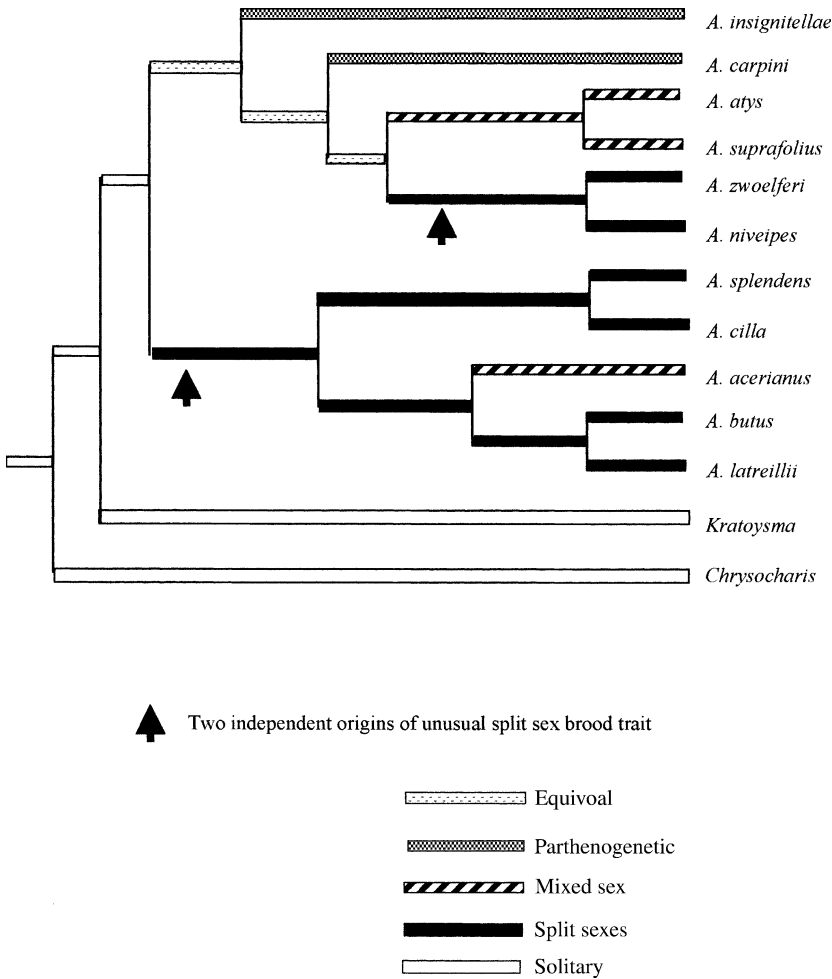


Fig. 5 Changes in combined clutch size/sex ratio strategy.

Table 5 Precise sex allocation in *A. atys*.

Brood size	Frequency	One-male broods (proportion)	Expected one-male broods (if binomial)	P-value
1	172	47	–	–
2	209	151 (0.72)	104.5	4.84×10^{-11}
3	122	89 (0.73)	54.2	1.65×10^{-10}
4	34	23 (0.68)	14.3	0.00241
5	5	3 (0.60)	2.0	0.334

The table shows, for each brood size: the number of broods observed; the number of those broods that contained only one male; the number of broods expected to have only one male if sex allocation showed binomial variance; and the significance level of the difference between observed and expected number of broods containing only one male. For brood sizes 2–4, sex allocation is precise, showing a significant tendency to produce only one male.

Taken together, these results suggest that the parasitoids have radiated partly through host-plant switching and they have, indeed, colonized, several different plant orders (Fig. 4). Nevertheless, significant conservation of host plant use at the genus level suggests that some parasitoid

speciation also occurs without recourse to host plant switches. An intriguing example is provided by *A. suprafolius*, the only species studied here that attacks upper surface leaf miners, which is the sister species of the lower surface leaf miner parasitoid (*A. atys*) on the same host plants. This suggests a role for competition in the radiation of this genus (Godfray, 1994) and supports a speciation event that did not involve a host plant switch.

Does leaf miner phylogeny play any role in explaining patterns of parasitoid radiation? In many cases, it may be at best minor. For example, although *A. zwoelferi* attacks several related leafminer species (Fig. 3), the crucial aspect may be that they all feed upon *Salix* (Fig. 2). Certainly, this would seem to be the key for the polyphyletic group of leafminer species that are hosts for *A. latreillii* (Fig. 3), but all feed on oak (*Quercus*) (Fig. 2). Nevertheless, we discuss a case below where a parasitoid attacks a polyphagous leafminer that occurs on several host plant taxa.

In summary, this is to our knowledge the first co-phylogenetic study of a tri-trophic plant-herbivore-parasite interaction and it supports a greater role for plant (than herbivore) traits in parasitoid radiation.

Host specificity and speciation

Most parasitoid taxa that attack *Phyllonorycter* species are polyphagous with broad host ranges (Askew & Shaw, 1979), so *Achrysocharoides* is a striking exception, with each species feeding on a few species of *Phyllonorycter* from only a few related plant genera. However, there are exceptions to the generally impressive host plant specificity of *Achrysocharoides* species (see electronic appendix). For instance, *A. atys* has been reared from different *Phyllonorycter* species feeding on several host plant genera from the family Rosaceae. In addition, *A. suprafolius* attacks the polyphagous moth species *P. corylifoliella* on all its host-plants: *Betula* and some Rosaceae (*Crataegus*, *Prunus* and *Sorbus*). In this case the parasitoid appears to track the moth, regardless of the host plant.

Mistakes/changes in host plant choice are the raw material for novel evolutionary associations. We found one case (four insects) of *A. zwoelferi* in mines on *Betula* (Betulaceae), which is not closely related to its normal host plant *Salix* (Salicaceae). Such mistakes can provide the ecological opportunity for new host races and subsequent speciation. A mistake can be defined as a rare event where a species is associated with a host plant upon which it does not usually occur. However, a few *Achrysocharoides* species occur commonly on a small range of host plants. For example, *A. cilla* was reared from five different moth species on five plant genera belonging to four plant families (see electronic appendix) and was only very rare on two of these. Given that most *Achrysocharoides* species are very host-specific, such a case is interesting. It could be a genuine case of a more generalist species, or represent incipient speciation or even cryptic species. Such issues require further study and would be best investigated using a combination of population genetics and experiments on oviposition preferences and larval performance on different hosts.

There is no doubt that plants have a major influence on the interactions between parasitoids and herbivorous insects (Godfray, 1994). Nothing is known about the host location mechanisms used by *Achrysocharoides* and in particular whether they use volatile chemicals emanating from the plants to locate where *Phyllonorycter* larvae may be found. More studies on host location would assist our understanding of macroevolutionary patterns of host use.

Systematics

The molecular phylogeny provides an independent evaluation of *Achrysocharoides* taxonomy. The traditional species groupings have been considered problematic (Hansson, 1983), but our molecular results support Hansson's (1983), classification. This suggests that the morphological characters (shape of petiolus in both sexes, coloration and segmentation of flagellum in males) used to define the two species groups (*atys* and *latreillii*)

are indeed good synapomorphies. Our results also support inclusion of the previously unplaced *A. insignitellae* in the *atys* group. This suggests that purple coloration of the scutellum, which *A. insignitellae* shares with *A. atys* and *A. cruentus* (Hansson, 1983) is a good synapomorphy.

Two specimens identified as *A. splendens* render *A. cilla* paraphyletic (Fig. 1) and this result is consistent with the idea that these two species should be synonymized (Hansson, 1983). However, recently separated species may not show reciprocal monophyly of particular genetic loci, so this is insufficient evidence in itself to justify a nomenclatural change. Morphological and genetic studies of further specimens of these species would be most interesting.

Sex ratio and clutch size

The mapping exercise indicates that reproductive strategy is quite labile, with several changes of brood type. The most notable result is the parallel evolution of split sex broods in the *latreillii* and *atys* species groups (Fig. 5). Split sex broods are extremely rare in general, but appear to have two independent origins in this genus. Further sampling of *Achrysocharoides* species with split broods will help us to confirm this result and to determine with a higher level of accuracy the number of independent origins of this reproductive strategy. Examples of adaptive parallel evolution are known from other taxa; for example, different lineages of stickleback fish invading post-glacial lakes have evolved pelagic and demersal species in parallel (Schluter, 2000). In a more closely related example, male wing loss has evolved in parallel in different fig wasp lineages in response to the availability of large numbers of potential mates in the local patch (Cook *et al.*, 1997).

The distribution of brood sizes across parasitoid species shows a dichotomy, with species tending to have either solitary or relatively large broods, and a lack of species with relatively small gregarious broods (Godfray, 1994). Godfray (1987) provided a possible explanation for this, by pointing out that shifts from solitary to mixed sex broods should be very difficult if larvae are aggressive, as in many solitary parasitoids, and so the solitary state can act as an evolutionary absorbing state. A possible solution to this problem was provided by Rosenheim (1993), who showed that the transition could proceed more easily via an intermediate state of split sex broods (see also Pexton *et al.*, 2003). Our study provides the first test of this idea. Solitary broods provide the common state in most eulophids, including the close relatives of *Achrysocharoides* (Fig. 5). Both mixed sex and split sex broods arose within *Achrysocharoides* and both also appear to have arisen twice (Fig. 5). Our data suggest that in the *latreillii* species group the transition from solitary broods to mixed sex broods has proceeded via an intermediate state of split sex broods, as predicted by Rosenheim (1993). Our data are also consistent with this having happened in the *atys*

group, although lack of resolution prevents any strong inference.

We detected precise sex allocation in *A. atys*, where the variance in brood sex ratios is significantly less than binomial (Table 5). This is interesting because it means that different members of this genus show very overdispersed (split) or very underdispersed (precise) sex ratios, depending upon the prevailing selective regime. In addition, at least in the *latreillii* group, the transition between these scenarios completes the link between two extremes of parasitoid mating systems: (1) solitary larvae and outbreeding and (2) gregarious larvae with strong local mating and inbreeding.

It is also notable that, despite many changes within the genus, there are four cases where sister species share the same reproductive strategy (Fig. 5). This could suggest a degree of phylogenetic inertia. However, we favour the alternative explanation that there is a degree of conservation of selective regime, since there is (1) such overwhelming evidence for strong selection upon, and adaptation of, brood production tactics (Godfray, 1994); (2) evidence from this and other studies (Herre *et al.*, 2001; Mayhew & Pen, 2002) of considerable lability in the traits.

Taxon sampling

Sequencing multiple specimens per species helps to increase confidence in the data, and most importantly test the hypothesis that the species under study represent natural (monophyletic) groups (Barraclough & Nee, 2001). However, most studies are limited by time and expense as to how many individuals can be sequenced. In our study, we decided to sequence multiple individuals in three species that showed some level of taxonomic uncertainty (i.e. *A. latreillii*, *A. cilla*, *A. splendens*) or were reared from unusual hosts (i.e. *A. zwoelferi* on *Betula*).

The density of taxon sampling is important for both an accurate estimation of species phylogenetic interrelationships and reconstruction of ancestral host use and reproductive strategies. Indeed, a poor and biased taxon sampling can lead to spurious ancestral character state reconstructions. In our study, we included 15 *Achrysocharoides* species, which comprise a third of known species of this genus. Regarding the effect of taxa sampling on the reconstruction of ancestral host use, most of our species are European, reflecting the most detailed host data, but we included species that attack half of the plant families known to be used by these parasitoids (see electronic appendix). Our taxa sampling does not include Japanese or Northamerican species from several interesting plant families (i.e. Juglandaceae, Malvaceae, Celtidaceae). Further studies of *Achrysocharoides* from these regions would be very valuable to determine with higher degree of certainty whether *Phyllonorycter* that fed on Fagales (Fig. 4) is indeed the ancestral host of *Achryso-*

chroides. Regarding the effect of taxa sampling on the reconstruction of reproductive strategies, although the biology of most species in other parts of the world is less well-known, it is clear that in Japan there are species with split sex ratios and others with mixed sex broods (Sato Hiroaki, personal communication). Incorporation of a wider range of species into the phylogenetic and brood composition data sets would allow further testing of the number and pattern of changes in brood production strategies.

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Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/JEB/JEB900/JEB900sm.htm>

Table S1 Host–moth–plant affiliations of *Achrysocharoides* species.

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Appendix 1 Specimens used in this study.

Host plant	Gracillarid host	Species name	Collection site	Voucher number	Collec.	GenBank accession numbers 28S/Cyt b
Asterids						
<i>Euasterid II</i>						
Order dipsacales						
Family Valerianaceae						
<i>Viburnumx carlcephalum</i>	<i>Phyllonorycter lantanella</i> (Schrank, 1802)	<i>Achrysocharoides cilla</i> (Walker, 1839)	Kew Gardens, UK	303	CLV	AF477594/AF477612
Rosids						
<i>Eurosoid II</i>						
Order sapindales						
Family sapindaceae						
<i>Acer pseudoplatanus</i>	<i>Phyllonorycter geniculella</i> (Ragonot, 1874)	<i>Achrysocharoides</i> sp.	Silwood Park, Ascot, Berkshire, UK	298	CLV	AF477590/AF477608
<i>Acer platanoides</i>	<i>Phyllonorycter platanoidella</i> (Joannis, 1920)	<i>Achrysocharoides acerianus</i> (Askew, 1974)	Silwood Park, Ascot, Berkshire, UK	296	CLV	AF477588/AF477606
<i>Eurosoid I</i>						
Order Malpighiales						
Family Salicaceae						
<i>Salix caprea</i>	<i>Phyllonorycter</i> sp.	<i>Achrysocharoides zwoelferi</i> (Delucchi, 1954)	Cirencester Park, UK	300 (197)	CLV	AF477592/AF477610
<i>Salix caprea</i>	<i>Phyllonorycter viminiella</i> (Sircom, 1848)	<i>Achrysocharoides zwoelferi</i> (Delucchi, 1954)	Silwood Park, Ascot, Berkshire, UK	329 (114)	CLV	AY756572/AY756583
<i>Salix caprea</i>	<i>Phyllonorycter viminiella</i> (Sircom, 1848)	<i>Achrysocharoides zwoelferi</i> (Delucchi, 1954)	Silwood Park, Ascot, Berkshire, UK	330 (114)	CLV	AY756573/AY756584
Order fabales						
Family fabaceae						
<i>Trifolium</i> sp.	<i>Phyllonorycter insignitella</i> (Zeller, 1846)	<i>Achrysocharoides insignitellae</i> (Erdos, 1966)	La Gachere, Brem sur Mer, France	93	CLV	AF477587/AF477605
Order fagales						
Family betulaceae						
<i>Betula</i> sp.	<i>Phyllonorycter ulmifoliella</i> (Hübner, 1817)	<i>Achrysocharoides niveipes</i> (Thomson, 1878)	Silwood Park, Ascot, Berkshire, UK	01	CLV	AY756575/AY756586
<i>Betula</i> sp.	<i>Phyllonorycter ulmifoliella</i> (Hübner, 1817)	<i>Achrysocharoides zwoelferi</i> (Delucchi, 1954)	Osterley Park, Middlesex, UK	324	CLV	AY756571/AY756581
<i>Betula</i> sp.	<i>Phyllonorycter ulmifoliella</i> (Hübner, 1817)	<i>Achrysocharoides zwoelferi</i> (Delucchi, 1954)	Osterley Park, Middlesex, UK	325	CLV	AF477592/AY756582
<i>Alnus glutinosa</i>	<i>Phyllonorycter rajella</i> (Linnaeus, 1758)	<i>Achrysocharoides splendens</i> (Delucchi, 1954)	Silwood Park, Ascot, Berkshire, UK	305 (113)	CLV	AF477595/AF477613
<i>Corylus avellana</i>	<i>Phyllonorycter nicellii</i> (Stainton, 1851)	<i>Achrysocharoides cilla</i> (Walker, 1839)	Silwood Park, Ascot, Berkshire, UK	299	CLV	AF477591/AF477609
<i>Carpinus betulus</i>	<i>Phyllonorycter esperella</i> (Goeze, 1783) = <i>quinnata</i> (Geoffroy, 1785)	<i>Achrysocharoides carpini</i> (Bryan, 1980)	Osterley Park, Middlesex, UK	297	CLV	AF477589/AF477607

Appendix 1 Continued.

Host plant	Gracillarid host	Species name	Collection site	Voucher number	Collec.	GenBank accession numbers 28S/Cyt b
Family fagaceae						
<i>Quercus robur</i>	<i>Phyllonorycter lautella</i> (Zeller, 1846)	<i>Achrysocharoides cilla</i> (Walker, 1839)	Pett's Wood, Kent, UK	302 (178)	DO	AF477593/AF477611
<i>Quercus robur</i>	<i>Phyllonorycter roboris</i> (Zeller, 1839)	<i>Achrysocharoides butus</i> (Walker, 1839)	Queen's wood, Dymock, Worcestershire, UK	308 (196)	CLV	AF477596/AF477614
<i>Quercus robur</i>	<i>Phyllonorycter</i> sp.	<i>Achrysocharoides latreillii</i> (Curtis, 1826)	Raigadas, Lugo, Spain	328	CLV	AY756576/AY756587
<i>Quercus robur</i>	<i>Phyllonorycter</i> sp.	<i>Achrysocharoides latreillii</i> (Curtis, 1826)	Osterley Park, Middlesex, UK	327	CLV	AY756577/AY756588
<i>Quercus robur</i>	<i>Phyllonorycter</i> sp.	<i>Achrysocharoides latreillii</i> (Curtis, 1826)	Silwood Park, Ascot, Berkshire, UK	326	CLV	AY756578/AY756589
<i>Fagus sylvatica</i>	<i>Phyllonorycter maestingella</i> (Muller, 1764)	<i>Achrysocharoides buekkensis</i> (Erdos, 1958)	Silwood Park, Ascot, Berkshire, UK	311 (7)	CLV	AF477597/AF477615
Host unknown		<i>Achrysocharoides atys atys</i> (Walker, 1839)	UK	Fw12		AF477598/AF477616
Host unknown		<i>Achrysocharoides splendens</i> (Delucchi, 1954)	UK	84 (S)		AF477599/AF477617
Order rosales						
Family rosaceae						
<i>Crataegus monogyna</i>	<i>Phyllonorycter corylifoliella</i> (Hübner, 1796)	<i>Achrysocharoides suprafolius</i> (Askew, 1974)	Silwood Park, Ascot, Berkshire, UK	315 (93)	CLV	AY756574/AY756585
Outgroups						
Superfamily chaicidoidea						
Family eulophidae						
Subfamily entedoninae						
Tribe entedonini						
		<i>Kratoysma gliricidiae</i> (Hansson & Cave, 1993)	Costa Rica, Guanacaste, Bosque Diria	320	IJ	AY756569/AY756579
		<i>Kratoysma gliricidiae</i> (Hansson & Cave, 1993)	Costa Rica, Guanacaste, Bosque Diria	321	IJ	AY756570/AY756580
		<i>Chrysocharis nopherus</i> (Walker)	UK	Fw20		AF477600/AF477618
	<i>Parornix petiolella</i>	<i>Chrysocharis</i> sp. 1	Sofia, Bulgary	290	PL	AF477603/AF477621
	<i>Phyllonorycter anderidae</i>	<i>Chrysocharis</i> sp. 2	Reading, UK	310	IS	AF477602/AF477620
	<i>Stigmella</i> sp.	<i>Chrysocharis</i> sp. 3	Bulgary	293	PL	AF477601/AF477619

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