

# Preliminary evolutionary relationships within the parasitoid wasp genus *Cotesia* (Hymenoptera: Braconidae: Microgastrinae): combined analysis of four genes

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**Abstract.** The braconid wasp genus *Cotesia* Cameron (Braconidae: Microgastrinae) is one of the largest genera of parasitoid wasps, and its species are employed frequently as biological control agents against pest insects. Several *Cotesia* species are also used as model organisms in physiology, ecology and population genetics studies. The genus thus has considerable importance from both applied and basic science perspectives. We investigated the phylogenetic relationships of twenty-five species of *Cotesia* commonly used in field and laboratory research, using the genes 16S, ND1, 28S and LW opsin and employing a range of phylogeny estimation methods including maximum parsimony, maximum likelihood, minimum evolution and Bayesian inference. Phylogenetic estimates differed little among methods, especially for the combined data analysis. The combined analysis of four genes identified four well-supported clades within *Cotesia*: the *melanoscela* group (containing *C. melanoscela*, the *C. flavipes* species complex and probably also *C. ruficrus*), the *kariyai* group (containing *C. kariyai*, *C. kazak*, *C. cyaniridis*, *C. flaviconchae* and probably also *C. anisotae* and *C. griffini*), the *rubecula* group (containing *C. congregata*, *C. electrae*, *C. eucaetis*, *C. marginiventris*, *C. obsuricornis* and *C. schizurae*), and the *glomerata* group (consisting of *C. glomerata*, *C. melitaeorum* and *C. plutellae*), plus a basal unresolved complex including *C. hyphantriae*, *C. diacrisiae* and *C. empretiae*. These groups correspond poorly with previous broad subgroups of *Cotesia* defined by Papp based on morphology. The current work constitutes the first real framework for comparative studies in systematics, ecology, physiology and population genetics of *Cotesia*. A preliminary analysis of the evolution of gregarious development from solitary is presented, in which it is apparent that solitary development is ancestral, and gregariousness has arisen several times independently within separate groups.

## Introduction

The order Lepidoptera includes a large percentage of the most important pests of field crops, shade and fruit trees, and forest resources throughout the world (Holloway *et al.*,

1987; Scoble, 1995). Virtually all of these are kept in check, at least partially, by parasitoid wasps which develop as larvae within living caterpillars and ultimately kill them (summarized in Waage & Greathead, 1986; LaSalle & Gauld, 1993; Godfray, 1994; Hawkins & Sheehan, 1994; Quicke, 1997; Hochberg & Ives, 2000). As a result, these wasps have been employed repeatedly as biological control agents against their host pests, and introduced into many parts of the world where they were originally not native (Greathead, 1986).

The Braconidae are the second most important family of parasitoid wasps in biological control, having been introduced in successful programmes on at least fifty-three separate occasions (Greathead, 1986). Within the Braconidae,

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the most abundant and diverse group attacking caterpillars is the Microgastrinae. The braconid wasp genus *Cotesia* is currently the largest microgastrine genus in North America (eighty-four Nearctic species recognized; Whitfield, 1995), on the basis of described species. This genus is an enormous group, with 400 species already described (Shaw & Huddleston, 1991) and an estimated total number of nearly 1000 species world-wide. *Cotesia* contains a number of species used throughout the world in the biological control of pest caterpillars, as well as many nonmanipulated, but important, species acting in their native settings to help regulate populations of important pests. Members of the genus generally attack large caterpillars, most commonly in temperate regions of the world. They are frequently encountered as natural enemies of conspicuous pest insects such as many Noctuidae (armyworms, cutworms, bollworms, etc.), Sphingidae (hornworms), Lymantriidae (gypsy and tussock moths), Arctiidae (salt marsh and other tiger moth caterpillars), Pieridae (cabbage and alfalfa caterpillars), and Geometridae (spanworms and inchworms). Moreover, several *Cotesia* species are also used as key model organisms in basic studies of the physiology and molecular biology of host-parasitoid interactions (*C. congregata*, *C. glomerata*, *C. kariyai*, *C. rubecula*), behavioural ecology (*C. rubecula*) and the ecology and genetics of metapopulation structure in fragmented habitats (*C. melitaearum*). There is thus considerable interest in the genus, from both applied and basic science perspectives.

#### Taxonomic history

*Cotesia* was erected originally as a genus by Cameron in the 19th century, but until the generic reclassification of the microgastrine Braconidae by Mason (1981), the name was listed in synonymy with many others under the familiar genus *Apanteles* Foerster. Under the name *Microgaster* (until 1865) and then the name *Apanteles*, many species were described by a variety of scientists throughout the world (e.g. W. A. Ashmead, H. L. Viereck, C. F. W. Muesebeck, D. S. Wilkinson, G. E. J. Nixon, J. Papp). The first taxonomic revision of the North American species of microgastrine Braconidae (including *Cotesia* as part of *Apanteles*) in the 1920s was provided by Carl Muesebeck (1920, 1922). This revision treated primarily those species common in the eastern U.S.A., so that subsequent fieldwork, especially in the western states, has turned up many undescribed species. In addition, a number of species have since been introduced from other parts of the world as biological control agents. Little descriptive work at the species level within *Cotesia* has been published recently, other than small papers dealing with species attacking a few important host species (e.g. Mohyuddin, 1971; Marsh, 1978, 1979), and reviews of the relatively well-known Palaearctic fauna (e.g. Nixon, 1974; Papp, 1986, 1987).

The main modern classificatory work (Mason, 1981), focused largely on the higher classification and phylogeny of the Microgastrinae, in particular on splitting up the

unmanageably large (more than 1300 described species) genus *Apanteles*. His generic reclassification of the microgastrine Braconidae produced most of the modern definitions of genera (including that of *Cotesia*) used today. Although Mason's work was somewhat controversial (especially for Old World faunas) and did not attempt to assign all known species to genera, it was widely adopted in North America and also stimulated further work on individual genera, e.g. on *Deuterixys* (Whitfield, 1985; Whitfield & Oltra, 2004), *Sathon* (Williams, 1985, 1988), *Bulukka* (Austin, 1989), *Miropotes* (Austin, 1990), *Micropplitis* and *Snellenius* (Austin & Dangerfield, 1993), *Alphomelon* (Deans *et al.*, 2003), *Pholetesor* (Whitfield, in press) and parts of the large genera *Apanteles* (Whitfield *et al.*, 2001) and *Glyptapanteles* (Whitfield *et al.*, 2002a).

Despite the economic and scientific importance of *Cotesia*, little is known about its systematics. Whereas several recent phylogenies have been proposed at the subfamily level, either morphology based, molecular or combined (Mardulyn & Whitfield, 1999; Whitfield *et al.*, 2002b), none of them has focused on the genus *Cotesia*, except for one study of a small species complex (Smith & Kambhampati, 1999); a second study of species attacking melitaeine butterflies is in preparation (Maaria Kankare, unpublished data). There is no morphology-based hypothesis of relationships among the species of *Cotesia* beyond Papp's (1986, 1987) division of the Palaearctic species into three groups. An outdated, but relatively comprehensive, catalogue of the world species is provided by Shenefelt (1972); this work does not distinguish *Cotesia* from the other species in the older (pre-Mason) conception of *Apanteles* Foerster.

#### Rationale for this study

In this study we investigated the phylogenetic relationships of twenty-five economically important species of *Cotesia*, along with seven exemplar species of other related genera of braconids. The species are listed in Table 1. Although these species obviously represent a modest percentage of the *Cotesia* species which actually exist, they constitute an excellent sample of the species commonly featured in field and laboratory research. In addition, the outgroup exemplars were chosen to represent genera commonly used in applied research. Thus, the results of this study will be of disproportionate interest, if somewhat preliminary in scope. A more complete investigation of the relationships among microgastrine genera based on both morphological and molecular data is available in Whitfield *et al.* (2002b).

Four genes were chosen for their phylogenetic utility, demonstrated in previous studies: mt16S rDNA coding the large subunit of the mitochondrial ribosome, n28S rDNA coding the large subunit of the nuclear ribosome, NADH1 coding subunit 1 of NADH dehydrogenase, and the nuclear gene encoding the long-wavelength rhodopsin (LW Rh). The mt16S rDNA, n28S rDNA and NADH1 genes have been extensively used in phylogenetic analysis within

**Table 1.** The species analysed in this study with their sources and EMBL and GenBank accession numbers.

Taxon	Reference/origin	mt16S rDNA	n28S rDNA	NADHI	LW Rh
<i>Cotesia autographae</i> (Muesebeck)	Whitfield (1997)	U68156	–	AJ935957	–
<i>C. anisotae</i> (Muesebeck)	U.S.A.: AR: Wash. Co., Fayetteville, 1998 (H. Eichenseer)	AJ535915	AJ535934	AJ535958	–
<i>C. chilonis</i> (Munakata)	Smith & Kambhampati (1999)	AF110825	AJ535935	AF110828	AJ535979
<i>C. congregata</i> (Riley)	Whitfield (1997)	U68157	AJ535936	AF069198 <sup>c</sup>	AJ535980
<i>C. cyaniridis</i> (Riley)	U.S.A.: AZ: Tucson, 2002 (J. Weeks)	AJ535916	AJ535937	AJ535958	AJ535981
<i>C. diacrisae</i> (Gahan)	U.S.A.: MO: Shannon Co. Cardareva SF, 1992 (K. Stowe, J. Whitfield)	AJ535917	–	AJ535959	–
<i>C. electrae</i> (Viereck)	U.S.A.: CO: Estes Park, 1996 (J. Tuttle, M. Collins)	AJ535918	AJ535938	AJ535960	AJ535982
<i>C. empretriae</i> (Viereck)	U.S.A.: IL: Champaign Co., Allerton Park, 2001 (A. R. Deans)	AJ535919	AJ535939	AJ535961	AJ535983
<i>C. euchaetis</i> (Ashmead)	U.S.A.: WV: Morgantown, 2000, (J. Strazanac)	AJ535920	AJ535940	AJ535962	AJ535984
<i>C. flaviconchae</i> (Riley)	U.S.A.: AR: Wash. Co., Fayetteville, 1998 (H. Eichenseer)	AJ535921	AJ535941	AJ535963	AJ535985
<i>C. flavipes</i> (Cameron)	Smith & Kambhampati (1999)	AJ535922	AJ535942	AF110829	AJ535986
<i>C. glomerata</i> (Linnaeus)	Whitfield (1997)	U68158	AJ535944	AF110830 <sup>d</sup>	AJ535988
<i>C. griffini</i> (Viereck)	Mardulyn & Whitfield (1999)	AY044192 <sup>d</sup>	AF102729	–	–
<i>C. hyphantriae</i> (Riley)	U.S.A.: MO: St. Louis Co., Tyson Res. Sta, 2001 (J. T. Lill)	AJ535923	AJ535943	AJ535964	AJ535987
<i>C. kariyai</i> (Watanabe)	Japan: Hokkaido & Sapporo, 2002 (laboratory culture Y. Hayakawa)	AJ535924	AJ535945	AJ535965	AJ535989
<i>C. kazak</i> (Telenga)	New Zealand: Kintleth Forest, 1998 (T. Herman)	AJ535925	AJ535946	AJ535966	AJ535990
<i>C. marginiventris</i> (Cresson)	Mardulyn & Whitfield (1999)	AJ535926	AF102730	AJ535967	AJ535991
<i>C. melanoscela</i> (Ratzeburg)	Canada: NS: Halifax, 1996 (laboratory culture D. B. Stolz)	AJ535927	AJ535947	AJ535968	AJ535992
<i>C. melittae</i> (Cresson)	Finland: Ackland, 1998 (I. Hanski)	AJ535931	AJ535953	AJ535972	AJ535996
<i>C. obscuricornis</i> (Viereck)	U.S.A.: MD: Dueling Creek Nature Area, 1993 (P. Barbosa)	AJ535928	AJ535948	AJ535969	AJ535993
<i>C. orobanæ</i> (Forbes)	Whitfield (1997)	U68158	–	AJ535970	–
<i>C. plutellae</i> (Kurdjumov) <sup>e</sup>	England: Rothamstead Expt. Sta., 1997 (laboratory culture)	AJ535929	–	–	–
<i>C. rubecula</i> (Marshall)	Whitfield (1997)	U68159	AJ535949	AF110831 <sup>d</sup>	AJ535994
<i>C. ruficornis</i> (Haliday)	Whitfield (1997)	AF110826	AJ535950	AF110833	–
<i>C. schizurae</i> (Ashmead)	U.S.A.: AZ: Santa Cruz Co., Santa Rita Mts, 1999 (J. O. Stireman III)	AJ535930	AJ535951	AJ535971	–
<i>C. sesamiae</i> (Cameron)	Smith & Kambhampati (1999)	AF110827	AJ535952	AF110832	AJ535995
<i>Apanteles canarsiae</i> (Ashmead)	Mardulyn & Whitfield (1999)	AF102750	AF102728	AF069197 <sup>c</sup>	AJ535997
<i>Diologaster bakeri</i> (Muesebeck)	U.S.A.: AR: Wash. Co., Fayetteville, 1998, malaise trap (J. Whitfield)	AJ535932	AJ535954	AJ535973	–
<i>Pholetesor bedelliae</i> (Viereck)	Whitfield (1997)	U68153	AF102740 <sup>b</sup>	AJ535974	AJ535998
<i>Glyptapanteles portheiriae</i> (Muesebeck)	Mardulyn & Whitfield (1999)	AF102757	AF102739	AJ535975	AJ535999
<i>Microgaster canadensis</i> (Muesebeck)	Whitfield (1997)	U68154	AF102733 <sup>b</sup>	AJ535976	AJ536000
<i>Microplitis demolitor</i> (Wilkinson)	Whitfield <i>et al.</i> (2002b)	AY044196	AJ535955	AJ535977	AJ536001
<i>Chelonus inanius</i> (Linnaeus)	Switzerland: Berne, 2000 (laboratory culture B. Lanzrein)	AJ535933	AJ535956	AJ535978	AJ536002

<sup>a</sup>Whitfield *et al.* (2002b).<sup>b</sup>Mardulyn & Whitfield (1999).<sup>c</sup>Smith *et al.* (1999).<sup>d</sup>Smith & Kambhampati (1999)<sup>e</sup>Should possibly be synonymized under *C. vestalis* (Haliday), according to M. R. Shaw (unpublished).

Hymenoptera, at both lower and higher taxonomic levels, especially in Microgastrine (Dowton & Austin, 1994; Belshaw & Quicke, 1997; Whitfield, 1997; Dowton & Austin, 1998; Dowton *et al.*, 1998; Mardulyn & Whitfield, 1999; Smith & Kambhampati, 1999; Smith *et al.*, 1999; Whitfield *et al.*, 2002b). LW Rh has been shown by several studies to be a promising gene to determine higher level phylogenies as well as intrafamily relationships (Carulli *et al.*, 1994; Mardulyn & Cameron, 1999; Briscoe, 2001; Cameron & Mardulyn, 2001; Lockhart & Cameron, 2001; Rokas *et al.*, 2002; Cameron & Mardulyn, 2003).

## Materials and methods

### *Obtaining specimens and extracting DNA*

The insects used in this study were collected in the field or reared in research laboratories. The origin of the twenty-five species of *Cotesia* and seven outgroups is provided in Table 1 along with the GenBank and EMBL accession numbers for the sequence data and the authors of the species. All specimens were placed in 95% ethanol and preserved at  $-20^{\circ}\text{C}$  until DNA was extracted. The insects were ground in an extraction buffer (0.05 M Tris HCl, 0.01 M ethylene diamine tetra acetic acid, 0.5% sodium dodecyl sulphate, 50 mM NaCl) and incubated with proteinase K (final concentration  $0.15\text{ mg ml}^{-1}$ ) for 4 h at  $40^{\circ}\text{C}$ . The incubation was followed by classical phenol–chloroform extraction and ethanol precipitation. The pellet was resuspended in  $30\ \mu\text{l}$  of ddH<sub>2</sub>O and conserved at  $-20^{\circ}\text{C}$ . Voucher material of specimen remnants and associated complete specimens from the same original series are deposited in the Illinois Natural History Survey.

### *Polymerase chain reaction (PCR) amplification and sequencing*

Double-stranded PCR products were amplified in an Eppendorf Mastercycler gradient (Eppendorf AG, Hamburg), using thirty-five cycles [first denaturation: 5 min at  $94^{\circ}\text{C}$  (denaturation: 1 min at  $94^{\circ}\text{C}$ , annealing: 1 min at  $47\text{--}50^{\circ}\text{C}$ , elongation: 1 min at  $70^{\circ}\text{C}$ )  $\times$  35, final elongation: 5 min at  $72^{\circ}\text{C}$ ]. The annealing temperatures were adjusted depending on the primers used for each gene. The partial mt16S rDNA was amplified using the 16SWb (5'-CACCTGTTTATCAAAAACAT-3') (Dowton & Austin, 1994) and 16S outer (5'-CTTATTCAACATCGAGGTC-3') (Whitfield, 1997) primers, with annealing steps at  $47^{\circ}\text{C}$ . The amplified fragment of  $\approx 500$  bp corresponds to the 3' end of the gene.

The NADH1 fragments were amplified with the primers used by Smith & Kambhampati (1999) (ND1-F: 5'-ACTAATTCAGATTCTCCTTCT-3', ND1-R: 5'-CAACCTTTA GTGATGC-3'; Smith *et al.*, 1999). The annealing temperature was  $48^{\circ}\text{C}$  for these primers and the amplified fragments were  $\approx 600$  bp in length.

A fragment of about 700 bp of the n28S rDNA was amplified with the primers designed by Mardulyn & Whitfield (1999) (28S: 5'-AAGAGAGAGTTCAAGAG-TACGTG-3' and 28S-PM: 5'-TAGTTCACCATCTTTC GGGTCCC-3'), with annealing temperatures of  $48^{\circ}\text{C}$ . The amplified fragment includes the D2 and D3 expansion segments. Some LW Rh fragments were initially amplified with the primers designed for bees and used by Mardulyn & Cameron (1999) (LWRhF: 5'-AATTGCTATTAYGAR-ACNTGGGT-3' and LWRhR: 5'-ATATGGAGTC-CANGCCATRAACCA-3'); we later designed new specific primers from slightly internal consensus regions among wasp sequences. The new primers are 5'-GTGCTGGTT-CCCTGTTCGGATG-3' and 5'-GCAAGTTTGCAYT-CAGCGCTTTG-3'. The annealing temperature used was  $49^{\circ}\text{C}$  for both pairs of primers. This gene encodes the long-wavelength visual pigment also known as the green or major opsin (Chang *et al.*, 1995; Crandall & Cronin, 1997; Townson *et al.*, 1998) and has been studied in *Apis* (Chang *et al.*, 1996) and *Mantis* (Towner & Gärtner, 1994). Our amplified region comprises two exons and one intron, and corresponds to nucleotide positions 421–920 in the *Apis mellifera* long-wavelength opsin sequence (introns excluded) (Chang *et al.*, 1996). For each specimen and gene, at least two independent PCR products were obtained and sequenced to check accuracy.

PCR products were purified using the kit Qiaquick (QIAGEN Genomics Inc., Germantown, U.S.A.) according to the manufacturer's protocol and sequenced in both directions, using the PCR primers, with BIGDYE version 3.0 (Applied Biosystems, Foster City, U.S.A.), according to the manufacturer's protocol. The sequences were run on an ABI 377 DNA sequencer (Applied Biosystems) in the Keck Biotechnology Centre at the University of Illinois at Urbana-Champaign.

All new sequences are deposited in the EMBL database (Table 1). Some additional sequences were retrieved from GenBank and added to our dataset (Table 1). All trees and aligned datasets have been deposited in TreeBASE (Sanderson *et al.*, 1996).

### *Alignment and phylogenetic reconstruction*

Sequences were edited using BIOEDIT version 5.0.9 (Hall, 1999) and aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994) using default parameters (gap opening = 10; gap extension = 0.2). At this low taxonomic level, alignment was generally straightforward and not parameter specific. The 16S rDNA alignment was then improved by eye and by comparison with the known secondary structure model (Gutell *et al.*, 1994). The protein coding genes (NADH1 and LW Rh) were translated to amino acids using MEGA version 2.1 (Kumar *et al.*, 2001), to assist in manual adjustments and proof-reading. Distances (with Tamura–Nei correction; Tamura & Nei, 1993) and nucleotide composition were calculated with MEGA version 2.1. The homogeneity of

base frequencies among sequences was tested with the BaseFreqs option of PAUP\* (Swofford, 2001). The datasets were tested for heterogeneity of substitution pattern to assess whether combining data might be straightforward using pairwise applications of the partition homogeneity test (Farris *et al.*, 1994; 1000 iterations) implemented in PAUP\* (option HomPart).

Maximum parsimony (MP), minimum evolution (ME) and maximum likelihood (ML) analyses were performed using PAUP\*, under the heuristic search option with a stepwise addition with 100 random taxon addition sequence replicates, with gaps treated as missing data. The MP analyses were conducted using equal weights for all positions. The model of substitution for ME and ML analyses was determined using MODELTEST version 3.04 (Posada & Crandall, 1998). Support values (BV) were determined by bootstrap analyses (Felsenstein, 1985; 1000 replicates for MP and ME, 100 replicates for ML). The Bayesian approach to phylogenetic reconstruction (Yang & Rannala, 1997; Huelsenbeck *et al.*, 2001) was implemented using MR BAYES 2.01 (Huelsenbeck & Ronquist, 2001). Each run was performed using default starting parameters and comprised 500 000 generations. Bayesian posterior probabilities ( $P_{\text{bay}}$ ) were calculated from the majority-rule consensus of trees sampled every ten generations once the Markov chain reached stationarity (determined by empirical checking of likelihood values).

## Results and discussion

### Sequence variation and alignments

For each dataset the numbers of characters (total, variable and parsimony informative), the genetic distances (calculated with the Tamura–Nei correction), the base composition and the model chosen for ML and ME analyses are reported in Table 2. Complete aligned datasets are available in TreeBASE (Sanderson *et al.*, 1996).

Alignment of the mt16S rDNA revealed several variable regions corresponding to RNA loops in secondary structures, according to the model published by Gutell *et al.* (1994). These small regions were highly variable in length

and composition, with many gaps and thus difficult to align. Forty-six positions of the initial alignment (corresponding to the highly AT-rich regions highlighted by Whitfield & Cameron, 1998) were therefore removed from the analyses. The final dataset thus comprised 323 sites for thirty-four taxa. No significant difference in base frequencies across taxa was detected ( $\chi^2 = 16.83$ , d.f. = 99,  $P = 1.00$ ).

The alignment of the n28S rDNA showed several short variable regions with numerous insertions and deletions. After removal of these regions the dataset was 569 bases in length for twenty-eight taxa. No significant difference in base frequencies across taxa was detected ( $\chi^2 = 8.70$ , d.f. = 87,  $P = 1.00$ ). The distances and base frequencies observed for both 16S rDNA and 28S rDNA are similar to those previously observed in braconid wasps (Dowton & Austin, 1998; Mardulyn & Whitfield, 1999; Dowton *et al.*, 2002).

The NADH1 dataset comprised 389 easily aligned sites and twenty-seven taxa and no significant difference in base frequencies across taxa was detected ( $\chi^2 = 23.06$ , d.f. = 87,  $P = 1.00$ ). The nucleotide sequences were translated to amino acids using the invertebrate mitochondrial genetic code. The base composition was calculated for each position in the codons and revealed a high AT bias for the first and third positions (83.4 and 97.1%, respectively). This could influence the level of homoplasy observed in these positions.

The LW Rh sequences were aligned with other sequences of insects to determine the position of the exons and the intron. The intron was identified between positions 164 and 500 of our first alignment. It was variable in length from 52 to 271 bp. Very long introns (from 232 to 271 nucleotides) were identified in five *Cotesia* species (*C. congregata*, *C. electrae*, *C. glomerata*, *C. hyphantriae* and *C. schizurae*). The intron was removed from the analyses except for fifty-seven nucleotides, which were less variable in length and well aligned across taxa. The resulting dataset contained 469 sites for twenty-six taxa. No significant difference in base frequencies across taxa was detected ( $\chi^2 = 19.17$ , d.f. = 75,  $P = 1.00$ ). The nucleotide frequencies were calculated by position in the codon, and no significant compositional bias was observed.

**Table 2.** Number of taxa and characters, sequence divergence (Tamura–Nei correction), base composition, and best models for maximum likelihood (ML) and minimum evolution (ME) approaches (as determined with MODELTEST 3.04). Short-name descriptions of the ML models are according to Posada & Crandall (1998).

	mt16S rDNA	n28S rDNA	NADH1	LW Rh	Combined data
Number of taxa (total/ingroup)	34/27	28/21	27/20	26/19	26/19
Number of characters (total/variable/parsimony informative)	323/147/80	569/223/71	389/169/112	469/223/127	1750/762/390
Sequence divergence (%; total/ingroup)	11.0/5.9	5.8/1.8	12.1/8.5	11.9/5.7	9.2/4.9
Nucleotide frequencies (%; A/C/T/G)	46.4/6.5/9.2/37.9	25.4/18.2/22.5/33.8	35.5/8.9/6.9/48.7	30.3/20.2/19.4/30.1	32.8/14.5/15.8/36.9
Model for ML and ME analyses/alpha	TVM + G	HKY + G	K81uf + G	HKY + G	TVM + I + G
	0.3311	0.6618	0.3731	0.3708	0.6775

**Table 3.** Support values for different groups in the genus *Cotesia*. For each dataset, the bootstrap values above 50% and the Bayesian posterior probabilities above 0.50 are indicated.

Clade	mt16S rDNA	n28S rDNA	NADHI	LW Rh	Combined data
<i>melanoscela</i> group					67(MP)/73(ME)/51(ML)/1.00(bay)
<i>flavipes</i> complex				98(MP)/94(ME)/1.00(bay)	94(MP)/96(ME)/90(ML)/1.00(bay)
<i>chilonis</i> + <i>flavipes</i>			61(MP)/69(ME)/1.00(bay)		66(MP)/86(ME)/82(ML)/0.98(bay)
<i>kariyai</i> group					0.84(bay)
<i>kazak</i> + <i>cyaniridis</i> + <i>flaviconchae</i>			92(MP)/97(ME)/1.00(bay)	0.97(bay)	100(MP)/100(ME)/100(ML)/1.00(bay)
<i>cyaniridis</i> + <i>flaviconchae</i>		61(MP)/0.95(bay)	51(MP)/71(ME)	67(MP)/67(ME)/0.99(bay)	92(MP)/96(ME)/95(ML)/1.00(bay)
<i>rubecula</i> group				68(MP)/58(ME)/0.66(bay)	51(ML)/1.00(bay)
<i>marginiventris</i> + <i>euchaetis</i> + <i>obscuricornis</i>				81(MP)/54(ME)/0.92(bay)	0.99(bay)
<i>euchaetis</i> + <i>obscuricornis</i>				85(MP)/75(ME)/0.96(bay)	62(ML)/1.00(bay)
<i>schizurae</i> + <i>electrae</i> + <i>congregata</i>	53(MP)/0.90(bay)			97(MP)/52(ME)/0.90(bay)	1.00(bay)
<i>electrae</i> + <i>congregata</i>				77(ME)	
<i>rubecula</i> + <i>electrae</i> + <i>congregata</i>		85(MP)	93(MP)/91(ME)/0.73(bay)		80(MP)/99(ME)/83(ML)/1.00(bay)
<i>glomerata</i> group			52(MP)/60(ME)/0.76(bay)		90(ME)
			100(MP)/100(ME)/1.00(bay)		92(MP)/100(ME)/97(ML)/1.00(bay)

MP, maximum parsimony; ME, minimum evolution; ML, maximum likelihood; bay, Bayesian approach.

*Phylogenetic analysis*

*mt16S rDNA sequence data.* MP analysis resulted in three equally parsimonious trees, with a length of 416, consistency index (CI) = 0.474, retention index (RI) = 0.488. However, few nodes were supported by high bootstrap values. The clades recovered with bootstrap values >50% are described in Table 3. The ML and ME analyses revealed that the internal branches are very short compared with external ones. That phenomenon explains the low bootstrap values obtained in both MP and ME analyses. The Bayesian approach generally provided only low branch support ( $P_{\text{bay}} < 70$ ).

16S rDNA has been extensively used at different levels of phylogeny in insects, especially in hymenopterans. It often provides poor resolution at deep levels of relationships (among superfamilies – Whitfield & Cameron, 1998), but is a useful molecular marker for resolution among closely related subfamilies (Whitfield, 1997) and genera (Mardulyn & Whitfield, 1999; Whitfield *et al.*, 2002b). It has been used to study the relationships among braconids (Whitfield, 1997; Dowton & Austin, 1998; Dowton *et al.*, 1998, 2002; Mardulyn & Whitfield, 1999; Belshaw *et al.*, 2000; Whitfield *et al.*, 2002b). However, it has been shown that this gene sometimes exhibits too much homoplasy to be very useful at higher levels, and our results confirmed that at the species level it alone cannot provide enough phylogenetic information.

*n28S rDNA sequence data.* The MP analysis resulted in 451 equally parsimonious trees of 356 steps, with CI = 0.753 and RI = 0.514.

The strongest bootstrap values were higher than those observed with 16S, but few clades were well supported. ME and ML approaches resulted in trees with the same pattern as 16S rDNA, with very short internal branches. The Bayesian analysis resulted in few well-supported clades, corresponding to those well supported with the other methods (Table 3).

There is extensive literature on the use of 28S rDNA in insect phylogeny and it has been used frequently in hymenopteran systematics (Caterino *et al.*, 2000). However, it shows low levels of divergence at subfamily levels (Rokas *et al.*, 2002) and is limited to providing good resolution and rooting of the tree at lower levels as in braconids (Belshaw & Quicke, 1997; Belshaw *et al.*, 1998). As shown in Table 2, the nucleotide distances observed are very low, especially in the ingroup (1.8%) which could explain the lack of phylogenetic resolution obtained from 28S rDNA alone.

*NADHI sequence data.* The MP analysis resulted in thirty-three equally parsimonious trees, with a length of 444, CI = 0.556, RI = 0.637. The bootstrap analysis resulted in more well-supported clades than the other two genes. The well-supported clades were recovered in all analyses (MP, ME and ML) and were supported by high Bayesian posterior probabilities (Table 3).

NADH1 has been used less frequently than the two previous markers in insect systematics (for examples see Vogler *et al.*, 1993; Weller *et al.*, 1996). It has also been useful in resolving phylogenetic relationships at the species and generic levels in the aphidiine braconid wasps (Smith *et al.* 1999) and clarifying the status of the *C. flavipes* complex in braconids (Smith & Kambhampati, 1999). By itself, in our study, NADH1 proved useful for the recovery of some closely related clades reported in Table 3.

*Long-wavelength rhodopsin sequence data.* The MP analysis resulted in six equally parsimonious trees with a length of 511, CI = 0.618, RI = 0.526. High bootstrap values provided strong support for many clades in the ingroup and outgroup. The Bayesian approach resulted in a tree supported by high posterior probabilities. The same clades were well supported by high bootstrap values with all methods and by high posterior probabilities using the Bayesian approach; they are reported in Table 3.

LW Rh has been used only recently in phylogenetic reconstructions in insects, with a study at the generic and tribal levels in bees (Mardulyn & Cameron, 1999; Cameron & Mardulyn, 2001, 2003). Moreover, Rokas *et al.* (2002), in exploring the usefulness of eight different phylogenetic molecular markers in hymenopterans, stressed that this gene is relatively useful at the family level and for intrageneric relationships in gall wasps. Our results confirm the usefulness of the gene in resolving intrageneric relationships in hymenopterans.

#### Combining data

We performed incongruence length difference tests (ILD) to determine whether the datasets could be considered as congruent. The ILD tests as described by Farris *et al.* (1994) indicated that two of the data partitions were marginally incongruent with NADH1 ( $P < 0.01$ ); all other pairwise comparisons were nonsignificant. However, there is no clear indication when data should not be combined if congruence is not demonstrated. Several authors have provided some support for combining data even if tests judge them incongruent (Baker *et al.*, 1998; Davis *et al.*, 1998; Downton *et al.*, 2002). Furthermore, our datasets considered separately are insufficiently informative, but the results of the combined analysis appear to be additive in support. Thus, we concatenated the datasets in a 'total evidence' approach designed to maximize the number of characters analysed. Several taxa for which data from several genes were missing were deleted from this combined analysis (Fig. 1), although their tentative placement based on available data is indicated in Fig. 2.

The combined dataset comprising 1750 characters and twenty-six taxa showed no significant difference in base frequencies across taxa ( $\chi^2 = 17.98$ , d.f. = 75,  $P = 1.00$ ). The model selected by MODELTEST for the ML and ME analysis was TVM + I + G, with the parameter of the gamma distribution ( $\alpha$ ) = 0.6775. MP, ME and ML

analyses, as well as the Bayesian approach, were applied to the combined dataset.

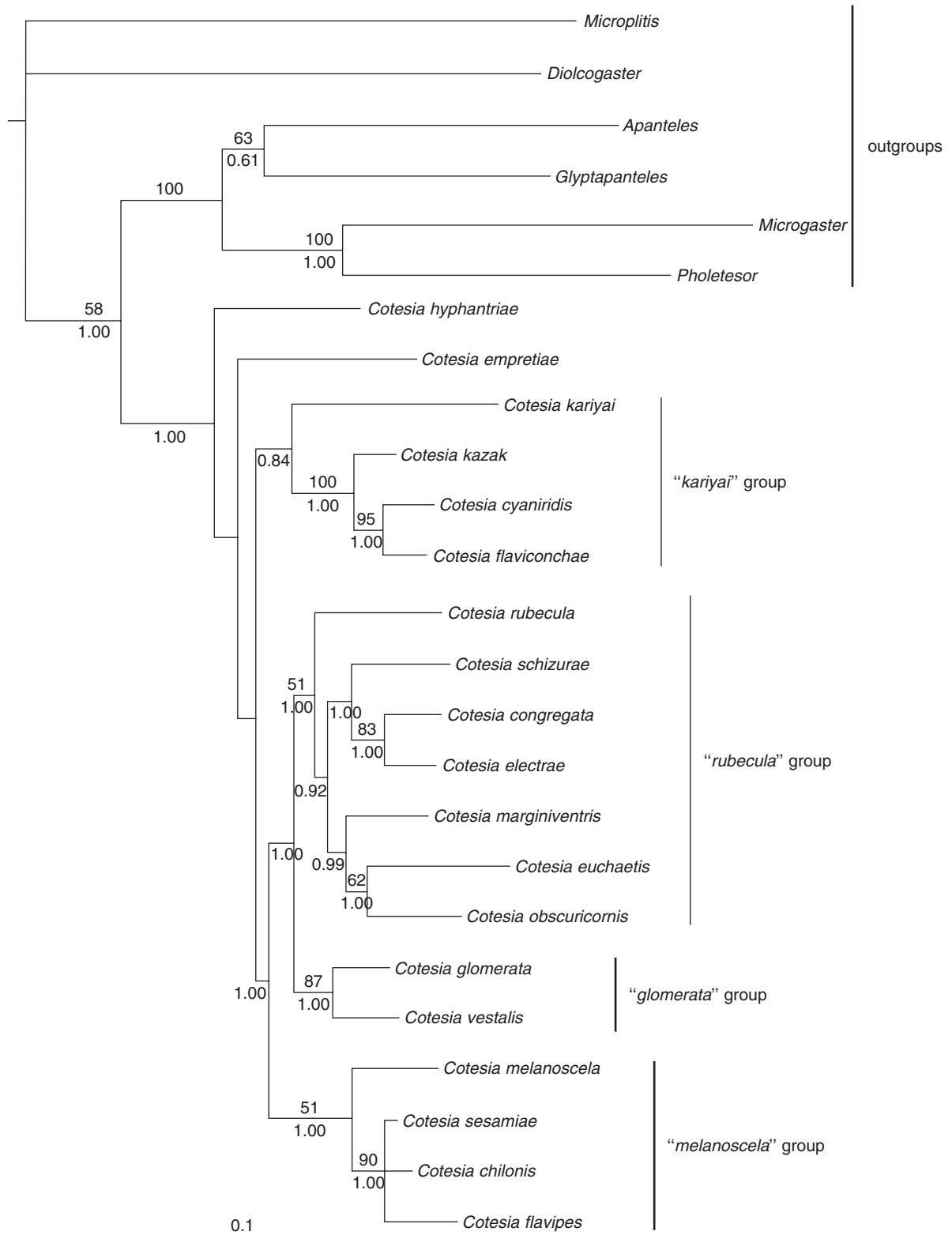
The MP analysis resulted in relatively poorly supported trees, in which few clades were supported by bootstrap values above 50%. ME, ML and Bayesian approaches resulted in trees very similar to one another. The three equally parsimonious trees (length = 1677, CI = 0.575, RI = 0.470) resulting from MP analysis were more different from the others, but no incongruent node was supported by bootstrap values >50%.

In all analyses the outgroups exhibited very long branches and the relationships among them were poorly supported. Using the Bayesian approach, we reduced the dataset by removing some outgroups (see Table 2) to maximize the sequence data available per taxon and to obtain reliable results. The differences between the ML, ME and Bayesian trees essentially consisted of the position of the species *C. empretiae* (only incongruence between ML and Bayesian trees) and *C. rubecula*, whose position is slightly different in the ME tree than in the two others. In all methods, the same groups were well supported by high bootstrap values or high posterior probabilities. The species *Chelonus inanitus*, belonging to another subfamily (Cheloniinae) than the other outgroup species (Microgastrinae), was used to root the trees. As expected, it exhibited a very long branch compared with the rest of the tree.

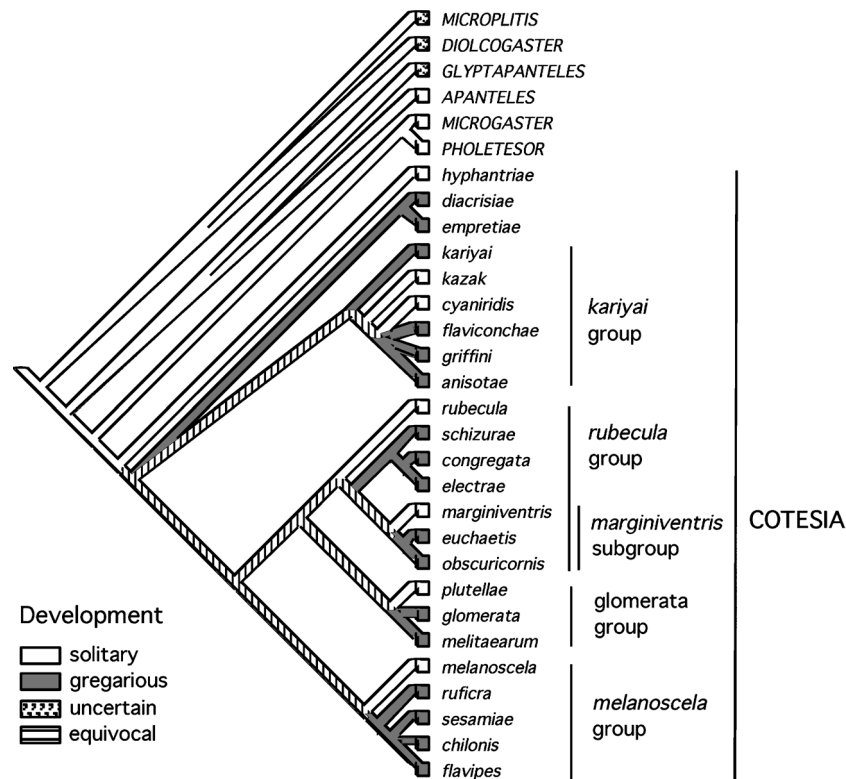
Monophyly of the genus *Cotesia* (Fig. 1) was supported by high bootstrap values (>80%) and posterior probability (1.00). Within the genus, several groups summarized in Table 3 could be distinguished: the *kariyai* group, with *C. kariyai*, *C. kazak*, *C. cyaniridis*, *C. flaviconchae*; the *melanoscela* group with *C. melanoscela*, *C. flavipes*, *C. sesamiae* and *C. chilonis*; the *rubecula* group, with *C. rubecula*, *C. schizurae*, *C. congregata*, *C. electrae*, *C. marginiventris*, *C. euchaetis* and *C. obscuricornis*; and the *glomerata* group with *C. glomerata* and *C. vestalis*. *Cotesia hyphantriae* seems to be the most basal species of *Cotesia* represented in our dataset. The position of *C. empretiae* is variable – either closely related to *C. hyphantriae* or emerging near the *kazak* group. The composition of the four main groups identified is summarized in Fig. 1; the tentative positions of several additional taxa (based on more limited sequence data) are shown in Fig. 2.

#### The melanoscela group

The group was supported by bootstrap values between 51 and 96% and a posterior probability of 0.98. *Cotesia melanoscela* is in a basal position compared with *C. flavipes*, *C. sesamiae* and *C. chilonis*. These latter three species are often used as biological control agents and correspond to the *C. flavipes* complex (Polaszek & Walker, 1991). The status of the *flavipes* complex has been investigated by Smith & Kambhampati (1999) using molecular data, but the analysis of mt16S rDNA and NADH1 sequences did not resolve fully the relationships among the three species. Our data suggest that *C. sesamiae* is the most basal, with



**Fig. 1.** Maximum likelihood tree from the analysis of the combined data, with bootstrap values (100 replicates; above the nodes) and Bayesian posterior probabilities (below the nodes) above 50%. The tree has been rooted with *Chelonus inanitus*.



**Fig. 2.** Optimization, using MACCLADE 4.05 (Maddison & Maddison, 2000), of solitary vs gregarious development onto the phylogeny from Fig. 1, with some additional taxa provisionally added based on more limited genetic data, as detailed in the text.

*C. chilonis* and *C. flavipes* sister species ( $66\% < BV < 86\%$ ;  $P_{\text{bay}} = 0.98$ ). Preliminary data from 16S rDNA and NADH1 suggest that *C. ruficra* might also belong in the larger *melanoscela* group, perhaps as sister species to the *C. flavipes* complex.

#### The kariyai group

The *kariyai* group is formed by *C. kariyai* as the most basal species, then *C. kazak*, and *C. cyaniridis* + *C. flaviconchae*, sister species ( $92 < BV < 96$ ;  $P_{\text{bay}} = 1.00$ ). The clade formed by *C. kazak*, *C. cyaniridis* and *C. flaviconchae* was very stable across all analyses ( $BV = 100\%$  in all approaches;  $P_{\text{bay}} = 1.00$ ).

#### The rubecula group

The *rubecula* group is the largest. *Cotesia rubecula* forms the basal branch of the group, which is then divided into two sister subgroups, one with *C. marginiventris*, *C. euchaetis* and *C. obscuricornis*, and the other with *C. schizurae*, *C. electrae* and *C. congregata*. The clades (*C. euchaetis* + *C. obscuricornis*) and (*C. electrae* + *C. congregata*) were recovered in separate and combined analyses, with high bootstrap values and posterior probabilities (Table 3). The

position of *C. rubecula* might be less reliable, as it appears to be included in the first subgroup in the ME tree and in some separate analyses.

#### The glomerata group

The small group formed by *C. glomerata* and *C. melitaeorum* was recovered constantly with high bootstrap values (92–100%) and posterior probability ( $P_{\text{bay}} = 1.00$ ). It was also recovered in most of the separate analyses (Table 3). The *glomerata* and *rubecula* groups were found to be sister groups with ME, ML and Bayesian approaches but the node was only supported by high posterior probability ( $P_{\text{bay}} = 1.00$ ). Preliminary 16S data from *C. plutellae* indicate that it also belongs to this group, but this requires confirmation with data from the other genes.

Some species were removed from the combined dataset when one or several sequences were missing. The results of the separate analyses might give some useful information on their relationships. *Cotesia anisotae* was identified as closely related to *C. cyaniridis*, and *C. plutellae* as belonging to the *glomerata* group from mt16S rDNA ( $92\% < BV < 93\%$ ;  $P_{\text{bay}} = 0.85$ ); from the results with n28S rDNA, *C. griffini* was grouped with *C. cyaniridis* and *C. flaviconchae* ( $BV = 61\%$ ;  $P_{\text{bay}} = 0.95$ ). Therefore, these two species could belong to the *kariyai* group. From the NADH1

trees, *C. diacrisae* and *C. empetriae* were sister species ( $90\% < BV < 94\%$ ;  $P_{\text{bay}} = 1.00$ ). Despite some strong bootstrap values and Bayesian posterior probabilities, further analyses are necessary to assess the phylogenetic relationships of these species.

## Conclusions

Each of the four molecular markers contained too little phylogenetic information on its own to allow strong resolution of the relationships between the *Cotesia* species. However, the combination of the data provided enough information to determine four well-supported groups within the genus *Cotesia*. These clades were recovered by every analysis performed – in itself a good indication of their repeatability with these data, if not their ultimate accuracy. Moreover, the bootstrap values and Bayesian posterior probabilities were high for these groups. Uncertainties remain concerning the relationships of *C. griffini*, *C. diacrisae*, *C. empetriae*, *C. anisotae* and *C. plutellae* with respect to the four groups defined. The reliable information obtained is summarized in Fig. 1, whereas the possible relationships of the additional taxa are included in Fig. 2.

The well-supported species groups identified here (albeit preliminary in composition due to the sparse taxon sampling), appear not to correspond to the three broad subgroups (*tibialis*, *brevicornis* and *glomeratus*) designated by Papp (1987, 1987) based on morphology. For instance, Papp placed *C. glomerata* in a different subgroup (*glomeratus*) than *C. plutellae* and *C. melitaeorum* (*tibialis*), and *C. ruficra* and *C. melanoscela* in different subgroups (*tibialis* and *glomeratus*, respectively). It is not possible to compare completely the classifications, as Papp's subgroups were based primarily on the European fauna, whereas our species come from all over the world. Nixon (1974) conjectured rather loosely that at least some of the solitary species might be relatively basal, but produced no explicit phylogenetic hypothesis.

Figure 2 shows how solitary vs gregarious parasitism by these wasps is distributed across the phylogeny. A suggestive, repeated pattern of basal solitary species and terminal gregarious species appears within several of the species groups (solitary behaviour appears to be ancestral among the outgroups as well). It will be of considerable interest for the study of solitariness vs gregariousness (e.g. Le Masurier, 1987; Mayhew, 1998) and of the forms of complementary sex determination (Godfray, 1994), if this pattern continues to hold up with additional taxon sampling as well as additional genetic data. These two aspects of wasp biology may well be linked, especially as gregarious species often habitually inbreed yet lack diploid males (Godfray, 1994).

This study of *Cotesia* phylogenetics has resulted in the first hypothesis of evolutionary relationships for this group. However, additional work is necessary to enlarge the sampling of *Cotesia* species and thus extend the applicability of the dataset. We should ideally also choose at least one additional molecular marker variable enough to provide

useful phylogenetic content. The ribosomal genes have been shown to be of limited utility, thus we could more profitably add a second nuclear protein-encoding gene such as EF1 alpha, demonstrated to be informative for the resolution of insect within-family divergences (Rokas *et al.*, 2002).

## Note added in proof

While this manuscript was in press, Shaw (2003) validated the name *C. vestalis* (Haliday) as a senior synonym of *C. plutellae* (Kurdjumov).

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