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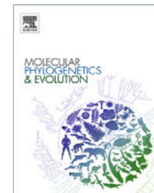

Phylogeny of ladybirds (Coleoptera: Coccinellidae): Are the subfamilies monophyletic?

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ABSTRACT

The Coccinellidae (ladybirds) is a highly speciose family of the Coleoptera. Ladybirds are well known because of their use as biocontrol agents, and are the subject of many ecological studies. However, little is known about phylogenetic relationships of the Coccinellidae, and a precise evolutionary framework is needed for the family. This paper provides the first phylogenetic reconstruction of the relationships within the Coccinellidae based on analysis of five genes: the 18S and 28S rRNA nuclear genes and the mitochondrial 12S, 16S rRNA and cytochrome oxidase subunit I (COI) genes. The phylogenetic relationships of 67 terminal taxa, representative of all the subfamilies of the Coccinellidae (61 species, 37 genera), and relevant outgroups, were reconstructed using multiple approaches, including Bayesian inference with partitioning strategies. The recovered phylogenies are congruent and show that the Coccinellinae is monophyletic but the Coccidulinae, Epilachninae, Scymninae and Chilocorinae are paraphyletic. The tribe Chilocorini is identified as the sister-group of the Coccinellinae for the first time.

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1. Introduction

The Coccinellidae, commonly called Ladybirds or Ladybugs, belong to the superfamily Cucujoidea and the Coleoptera suborder Polyphaga (Kovář, 1996; Hunt et al., 2007). This family comprises about 360 genera and nearly 6000 species (Vandenberg, 2002). It displays several characteristics which make it an interesting model, from a biological as well as economical point of view. Found worldwide, they are present in many diverse habitats and range widely from stenotopic to eurytopic species (Honk and Hodek, 1996). Food relationships are also highly diverse in the group: ladybirds are mainly predators but there is also a group of phytophagous species, including serious pests of economically important crops (Shaefer, 1983) and even some mycophagous species. The predatory species mainly eat either coccids (Coccoidea) or aphids (Aphidoidea), but some are predators of aleyrods (Aleyrodoidea), psyllids (Psylloidea), chrysomelids (Chrysomeloidea) or mites (Acari). The fact they mostly prey on Hemiptera often brings them into contact with ants, which exploit the same resources: some species are myrmecophilous (review in Majerus et al., 2007) and two myrmecophagous (Harris, 1921; Pope and Lawrence, 1990).

Ladybirds' predatory habits make them well-known biocontrol agents, mainly as a result of the success of the Australian ladybird *Rodolia cardinalis* (Mulsant) in controlling citrus infestation by the cottony-cushion scale *Icerya purchasi* (Maskell), at the end of the 19th century. Although there are many cases of successful control, mainly involving coccidophagous ladybirds (Dixon and Kindlmann, 1998), some of the introduced species have become invasive and are seen as a threat to native species. For example, *Coccinella septempunctata* L. and *Harmonia axyridis* (Pallas), both introduced in North America to control aphids, became extremely abundant and conspicuous in their new range, which has raised concern over their potential effects on local biological diversity (Horn, 1991; Howarth, 1991; Simberloff and Stiling, 1996; Koch et al., 2003, 2005). A growing number of studies on life history strategies, chemical defense, reproductive behavior, species interactions and other aspects of the ecology of ladybirds suffer from a lack of an evolutionary framework (e.g. Dixon, 2000). Such a framework will facilitate a more rigorous interpretation of ecological data and testing of hypotheses about Coccinellidae biology, including biogeography and the evolution of their morphology and food relationships. Therefore, a robust and comprehensive phylogeny of relationships within the family is needed.

Historically, the comparative studies of Coleoptera morphology placed the Coccinellidae among the Polyphaga (Cucujoidea)

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(Crowson, 1981). A recent large-scale molecular phylogeny confirms previous morphological studies: Coccinellidae were placed among the Cucujiformia in the Cerylonid series (Hunt et al., 2007). Moreover, together with a great number of morphological traits, a molecular phylogeny of the Cerylonid series (Robertson et al., 2008) confirms the monophyly of the Coccinellidae and suggests that the sister-group is a member of the current Endomychidae or Corylophidae. Thus, at present, the uncertainty is not about the position of the family among the Coleoptera but the phylogenetic relationships within the family, for which molecular analyses are lacking and classical morphological analyses are inconclusive.

Since Latreille (1807) first coined the family name "Coccinellidae" (Watt, 1975), the systematics of the whole group has been unstable: authors have suggested new classifications, created or redefined the subdivisions, transferred genera or tribes from one subfamily to another (review in Sasaji, 1968; Chazeau et al., 1989; Kovář, 1996; Fürsch, 1990, 1996; Ślipiński, 2007, this later only for Australian species) (see Supplementary Table 1). To date, few studies have attempted to reconstruct the phylogeny of the whole group rather than make intuitive reconstructions (Sasaji, 1968; Kovář, 1996: Fig. 1) or preliminary morphological cladistic analyses (Guoyue, 1994). The intuitive trees differ in the number of recognized subfamilies (six or seven) and the tribal or generic component of each subfamily, but agree about the relationships between the subfamilies Chilocorinae and Scymninae, and Coccinellinae and Epilachninae. Guoyue (1994) suggests, based on morphological characters, the paraphyly of the Coccidulinae and Scymninae.

A recent molecular phylogeny of the family Coccinellidae (20 species), based on the 18S and 28S rDNA genes, indicated that the Scymninae and Chilocorinae are paraphyletic, although there is incongruence between analyses and low statistical support (Robertson et al., 2008). Among the subfamilies, only the phytophagous Epilachninae have been subject to a molecular phylogenetic study (e.g. Ohta et al., 2004; Kobayashi et al., 1998). However, these analyses were done on beetles from a restricted geographical area and included only few genera of the 24 recognized; the monophyly of the Epilachninae thus remains to be adequately tested.

Despite the general interest in the family, the phylogenetic relationships of coccinellid taxa are poorly known. In the present study, molecular phylogenetic analyses are carried out using multi-locus molecular data with potential different evolutionary histories (e.g. transmission mode, recombination, evolutionary rate or introgression): the mitochondrial 12S rRNA (12S), 16S rRNA (16S) and cytochrome *c* oxidase subunit I (COI) genes, as well as nuclear 18S and 28S rRNA gene regions. To assess the reliability of the phylogenetic relationships across different methodologies, including the recent partitioning strategies and mixture models in Bayesian inference, taking into account different substitution models, were applied to the combined dataset. This study includes 61 species (37 genera) with representatives from all the subfamilies of the Coccinellidae. The aim of the study is to provide a large phylogenetic analysis of the family in order to: (1) test the monophyly of the subfamilies of Coccinellidae; (2) assess phylogenetic relationships at the inter- and intra-subfamily level; (3) test the current higher classification of the family and (4) analyse patterns of evolution for food preference.

2. Materials and methods

2.1. Taxon sampling and laboratory procedures

We obtained samples of species belonging to all the subfamilies of Coccinellidae: Coccinellinae, Scymninae, Coccidulinae, Chilochorinae, Epilachninae, Ortaliinae and Sticholotidinae (present taxonomic position from Kovář, 1996). A total of 37 genera and 61 species of Coccinellidae were analyzed (Tables 1 and 2). It should be noted however, that not all the tribes were included in the analysis (Supplementary Table 1). Other taxa of the infraorder Cucujiformia (Chrysomelidae, Cucujidae, Endomychidae, Cerylonidae, Oedemeridae and Cleroidea), to which Coccinellidae belong, were included as outgroups (Table 2). The choice of these latter taxa as valid outgroups was based on the results of a recent phylogenetic study of Coleoptera (Hunt et al., 2007).

Sequences were obtained from two specimens per species with the exception of *Subcoccinella*, for which only one individual was available. Most taxa are represented by sequences from a minimum of three or four genes. Chimeric data (i.e. different sequences derived from more than one species of a genus) were used only for the outgroup taxa and the Coccinellidae taxa that were only represented by sequences from Genbank (Table 2). The taxonomy of coccinellids suggested by Kovář (1996) was used, with three exceptions: we recognize the Noviini as belonging to the Coccidulinae, the Diomini as one of tribes of the Scymninae, and the genus *Crypolaemus* in the Scymnini (as Vandenberg, 2002). For the names of species we use those cited by Hodek and Honk (1996).

The specimens were field-collected mostly in the western part of the Paleartic region, either by the authors or numerous collaborators (Table 1). They were then preserved in 95% ethanol or frozen at −80 °C. In addition some taxa available in Genbank were added to our sample (Table 2).

Total genomic DNA was extracted from the entire individual (minus elytra) using the DNeasy Blood and Tissue Kit from QIAGEN (disposable pestle for grinding in a 1.5 ml microcentrifuge tube) with PBS protocol according to the manufacturer's instructions. Two nuclear genes (18S rDNA and 28S rDNA: D3 expansion segment of the 23 S-like ribosomal RNA segment) and three mitochondrial genes (12S rDNA, 16S rDNA and COI) were amplified using universal or specific primer sequences developed for this study (Table 3). Polymerase chain reactions were performed with 50 ng of DNA in 25 or 50 μl volumes containing a final concentration of 1X PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTPs, 1.5 mM of $MgCl_2$ and 1 U of Taq polymerase. PCR settings for amplifying COI sequences consisted of initial denaturing at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 40–52 °C for 1 min (depended on species), 72 °C for 1.5 min, a final extension at 72 °C for 10 min then

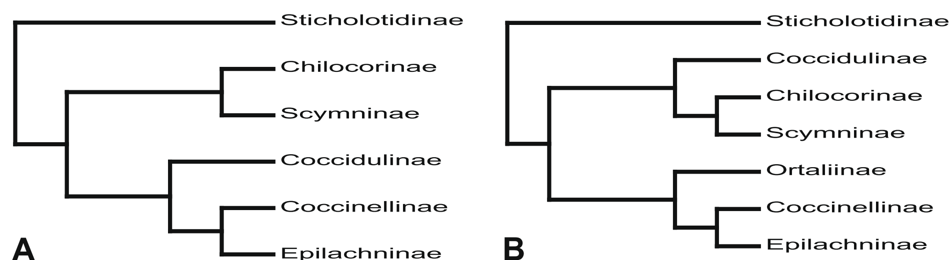

Fig. 1. Phylogenetic hypotheses of the relationships between the Coccinellidae subfamilies, interpreted from the dendrograms of (A) Sasaji (1968) and (B) Kovář (1996).

Table 1
Geographical origin and food preferences of the specimens used in this study. Samples were collected by the authors with the following exceptions: (1) Drs. R. Ware and M. Majerus, (2) Dr. J.-F. Godeau, (3) Dr. P. Oromi, (4) Dr. B. Fréchette, (5) Dr. N. Osawa, (6) Mr. E. Lombaert, (7) Mr. L. Saharaoui, (8) Dr. J.A.Qureshi, (9) Dr. J. Orivel and A. Vantaux, (10) Biobest material, (11) Dr. P. Milonas, (12) Mrs. I. Borges, (13) No specimen available, sequences obtained from Genbank database. A, Aphids; At, Ants (myrmecophagous); C, Coccids; Mt, Mites; F, Fungi; P, Plants; Ps, Psyllids; WF, White flies.

Taxon		Origin	Food
Coccinellinae			
Coccinellini			
Adalia	*decempunctata* (L.)	France (Toulouse)	A
	bipunctata (L.)	France (Toulouse)	A
Anatis	*ocelatta* (L.)	England (1)	A
Anisosticta	*novemdecimpunctata* (L.)	(13)	A
Calvia	*quatuordecimguttata* (L.)	England (1)	A
	muiri (Timberlake)	Japan (Fuchu) (1)	A
Cheilomenes	*sexmaculata* (Fabricius)	Japan (Yamagate) (1)	A
	lunata (Fabricius)	Madagascar	A
Coccinella	*undecimpunctata* L.	New Zeland	A
	magnifica Redtenbacher	Belgium (2)	A
	miranda Wallaston	Canary Islands (3)	A
	quinquepunctata L.	Wales (1)	A
	septempunctata L.	France (Toulouse)	A
Coccinula	*cf. sinensis* (Weise)	Japan (Kofu) (1)	A
	quatuordecimpustulata (L.)	France (Toulouse)	A
Coleomegilla	*maculata* (DeGeer)	Canada (4)	A
Harmonia	*quadripunctata* (Pontoppidan)	France (Toulouse)	A
	axyridis (Pallas)	Japan (Kyoto) (5)	A
	conformis (Boisduval)	France (Antibes) (6)	A
Hippodamia	*variegata* (Goeze)	Algeria (7)	A
	undecimnotata Schneider	France (Millau)	A
Myrrha	*octodecimguttata* (L.)	France (Toulouse)	A
Myzia	*oblongoguttata* (L.)	France (Toulouse)	A
Oenopia	*conglobata* (L.)	France (Toulouse)	A
	doubleri (Mulsant)	Algeria (7)	A
Olla	*v-nigrum* (Mulsant)	USA (Florida) (8)	A
Propylea	*quatuordecimpunctata* (L.)	France (Toulouse)	A
	japonica (Thunberg)	Japan (Yamagate) (1)	A
Psylloborini			
Halyzia	*sedecimguttata* (L.)	France (Toulouse)	F
Psyllobora	*vigintiduopunctata* (L.)	France (Toulouse)	F
Vibidia	*duodecimguttata* (Poda)	France (Toulouse)	F
Tytthaspidini			
Tytthaspis	*sedecimpunctata* (L.)	France (Toulouse)	F
Scymninae			
Diomini			
Diomus	*terminatus* (Say)	(13)	A
	sp.	French Guyana (9)	At
	thoracicus	French Guyana (9)	At
Hyperaspidini			
Hyperaspis	sp.	(13)	A
Scymnini			
Clitosthetus	*arcuatus* (Rossi)	Portugal (Azores)	WF
Cryptolaemus	*montrouzieri* Mulsant	(10)	C
Nephus	*includens* (Kirsch)	Greece (11)	C
	reunioni Fürsch	Portugal (Cascais)	C
	bisignatus Boheman	Greece (11)	C
Scymnus	*apetzi* Mulsant	Portugal (Algarve)	A
	interruptus (Goeze)	Portugal (Algarve)	A
	nubilus (Mulsant)	Portugal (Azores) (12)	A
	subvillosus (Goeze)	Portugal/Greece (11)	A
	rubromaculatus (Goeze)	Greece	A
Stethorini			
Stethorus	*punctillum* Weise	Portugal (Azores)	Mt
Chilocorinae			
Chilocorini			
Chilocorus	*bipustulatus* (L)	France (Toulouse)	C
	renipustulatus (L.G.Scriba)	France/England (1)	C
Exochomus	*quadripustulatus* (L)	England (1)	A/C
Halmus	sp.	(13)	C
Platynaspidini			
Platynaspis	*luteorubra* (Goeze)	Algeria (7)	A
Coccidulinae			
Coccidulini			
Rhyzobius	*chrysomeloides* (Herbst)	Portugal (Algarve)	A
	litura (Fabricius)	France (Toulouse)	A
	lophantae (Blaisdell)	Portugal (Algarve)	C
Noviini			
Rodolia	*cardinalis* (Mulsant)	Portugal (Algarve)	C
Epilachninae			
Epilachnini			
Epilachna	sp.	(13)	P
Henosepilachna	*elaterii* (Rossi)	Greece	P
Subcoccinella	*vigintiquatuorpunctata* (L)	Greece (11)	P
Ortaliinae			
Ortaliini			
Ortalia	sp.	(13)	Ps
Sticholotidinae			
Sthicholotidini			
Sthicolotis	sp.	(13)	C

finally held at 10 °C until removal from the machine. PCR conditions for 16S, 18S and 28S fragments involved an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 1 min at 50–52 °C (depended on gene), 60 s at 72 °C and 10 min extension at 72 °C. Cycles of amplification for 12S were programmed with the following profile (Touch Down PCR): 5 min at 95 °C, 5 cycles with 1 min at 95 °C, 1 min at 55–50 °C, 1 min at 72 °C, 30 cycles of 1 min at 95 °C, 1 min at 50 °C, 1 min at 72 °C and 10 min extension at 72 °C. Sequencing was carried out with an ABI 3730xl automated sequencer (Applied Biosystems) on both strands. The same primers used for amplification were used for the sequencing reactions. The new sequences are deposited in Genbank under the accession numbers listed in Table 2.

2.2. Alignment of sequences

The sequences of ribosomal genes varied in length in Coccinellidae: 15 bp for the 12S rDNA, 16 bp for the 16S rDNA, 10 bp for the 18S rDNA and 6 bp for the 28S rDNA genes. We aligned sequences using Clustal, with default options, and then reviewed and corrected alignments by eye: both the primary sequence and the secondary structure, estimated using RNAstructure v 4.06 (Mathews et al., 2004), were taken into account when aligning the sequences. As no model was available for the different rDNA genes for the family, we determined the stem and loop regions for several species in each subfamily, via the secondary structure obtained with the software RNAstructure v 4.06, following the parameters described by the authors (Mathews et al., 2004). These secondary structures were used as alignment profiles to check the absence of multiple gaps in the stem regions. A nexus file of the aligned sequences is available as Supplementary material. To test the effects of the alignment conditions, the ambiguous portions of the alignment were removed and the phylogenetic analyses conducted on the shortened dataset were compared with the results obtained using the complete dataset.

2.3. Phylogenetic analyses

The aligned sequences were analyzed assuming maximum parsimony (MP), maximum likelihood (ML) and Bayesian inferences

Table 2
Taxa studied and their Genbank accession numbers.

Taxon		Genbank accession no.					
		COI (part1)	COI (part2)	12S	16S	18S	28S
Coccinellinae							
Coccinellini							
Adalia	*bipunctata*	GU073919*	GU073889*	FJ621318*	GU073832*	GU073675*	FJ621325*
	decempunctata	AJ312061	GU073888*	FJ621317*	GU073831*	GU073674*	FJ621324*
Anatis	*ocelatta*	GU073920*	NA	GU073781*	GU073833*	GU073676*	GU073731*
Anisosticta	*novemdecimpunctata*	AJ429492	DQ155973	AM779606	AM779601	AY748146	NA
Calvia	*quatuordecimguttata*	GU073921*	NA	GU073782*	GU073834*	GU073677*	GU073732*
	muiri	GU073922*	GU073890*	GU073783*	GU073835*	GU073678*	GU073733*
Cheilomenes	*lunata*	GU073923*	GU073891*	GU073784*	GU073836*	GU073679*	GU073734*
	sexmaculatus	GU073924*	NA	GU073785*	GU073837*	GU073680*	GU073735*
Coccinella	*undecimpunctata*	GU073925*	GU073892*	FJ621319*	GU073838*	GU073681*	FJ621327*
	magnifica	GU073926*	NA	GU073786*	GU073839*	GU073682*	GU073736*
	miranda	GU073927*	NA	GU073787*	GU073840*	GU073683*	GU073737*
	quinquepunctata	GU073928*	NA	FJ621320*	GU073841*	GU073684*	FJ621326*
	septempunctata	GU073929*	GU073893*	FJ621321*	GU073842*	GU073685*	FJ621328*
Coccinula	*cf. sinensis*	NA	GU073894*	GU073788*	GU073843*	GU073686*	GU073738*
	quatuordecimpustulata	GU073930*	GU073895*	GU073789*	GU073844*	GU073687*	GU073739*
Coleomegilla	*maculata*	GU073931*	NA	GU073790*	GU073845*	GU073688*	GU073740*
Harmonia	*axyridis*	GU073932*	GU073896*	FJ621323*	GU073846*	GU073689*	FJ621330*
	conformis	GU073933*	NA	GU073791*	GU073847*	GU073690*	GU073741*
	quadripunctata	GU073934*	GU073897*	FJ621322*	GU073848*	GU073691*	FJ621329*
Hippodamia	*undecimnotata*	GU073935*	NA	GU073792*	GU073849*	GU073692*	GU073742*
	variegata	GU073936*	GU073898*	GU073793*	GU073850*	GU073693*	GU073743*
Myrrha	*octodecimguttata*	GU073937*	GU073899*	GU073794*	GU073851*	GU073694*	GU073744*
Myzia	*oblongoguttata*	GU073938*	NA	GU073795*	GU073852*	GU073695*	GU073745*
Oenopia	*doubleri*	GU073939*	GU073900*	GU073796*	GU073853*	GU073696*	GU073746*
	conglobata	NA	NA	GU073797*	GU073854*	GU073697*	GU073747*
Olla	*v-nigrum*	GU073940*	NA	GU073798*	GU073855*	GU073698*	GU073748*
Propylea	*quatuordecimpunctata*	GU073941*	GU073901*	GU073799*	GU073856*	GU073699*	GU073749*
	japonica	GU073942*	NA	GU073800*	GU073857*	GU073700*	GU073750*
Psylloborini							
Halyzia	*sedecimguttata*	GU073943*	GU073902*	GU073801*	GU073858*	GU073701*	GU073751*
Psyllobora	*vigintiduopunctata*	GU073944*	GU073903*	GU073802*	GU073859*	GU073702*	GU073752*
Vibidia	*duodecimguttata*	GU073945*	GU073904*	GU073803*	GU073860*	GU073703*	GU073753*
Tytthaspidini							
Tytthaspis	*sedecimpunctata*	GU073946*	GU073905*	GU073804*	GU073861*	GU073704*	GU073754*
Scymninae							
Diomini							
Diomus	sp.	GU073947*	GU073906*	GU073805*	GU073862*	GU073705*	GU073755*
	thoracicus	GU073948*	GU073907*	GU073806*	GU073863*	GU073706*	GU073756*
	terminatus	NA	NA	NA	NA	EU145618	EU145677
Hyperaspidini							
Hyperaspis	sp.	NA	NA	NA	NA	EU145611	EU145671
Scymnini							
Clitosthetus	*arcuatus*	GU073949*	NA	GU073807*	GU073864*	GU073707*	GU073757*
Cryptolaemus	*montrouzieri*	NA	GU073908*	GU073808*	GU073865*	GU073708*	GU073758*
Nephus	*bisignatus*	GU073950*	GU073909*	GU073809*	GU073866*	GU073709*	GU073759*
	includens	GU073951*	NA	GU073810*	GU073867*	GU073710*	GU073760*
	reunioni	GU073952*	NA	GU073811*	GU073868*	GU073711*	GU073761*
Scymnus	*apetzi*	GU073953*	GU073910*	GU073812*	GU073869*	GU073712*	GU073762*
	interruptus	GU073954*	GU073911*	GU073813*	GU073870	GU073713*	GU073763*
	nubilus	GU073955*	NA	GU073814*	GU073871*	GU073714*	GU073764*
	rubromaculatus	GU073956*	NA	GU073815*	GU073872*	GU073715*	GU073765*
	subvillosus	GU073957*	NA	GU073816*	GU073873*	GU073716*	GU073766*
Stethorini							
Stethorus	*punctillum*	GU073958*	NA	GU073817*	GU073874*	GU073717*	GU073767*
Chilocorinae							
Chilocorini							
Chilocorus	*bipustulatus*	GU073959	NA	GU073818*	GU073875*	GU073718*	GU073768*
	renipustulatus	GU073961*	NA	GU073820*	GU073877*	GU073720*	GU073770*
Exochomus	*quadripustulatus*	GU073962*	GU073912*	GU073821*	GU073878*	GU073721*	GU073771*
Halmus	sp.	NA	NA	NA	NA	EU145607	EU145669
Platynaspidini							
Platynaspis	*luteorubra*	GU073963*	GU073913*	GU073822*	GU073879*	GU073722*	GU073772*
Coccidulinae							
Coccidulini							
Rhyzobius	*chrysomeloides*	GU073964*	GU073914*	GU073823*	GU073880*	GU073723*	GU073773*
	litura	GU073965*	GU073915*	GU073824*	GU073881*	GU073724*	GU073774*
	lophantae	GU073966*	NA	GU073825*	GU073882*	GU073725*	GU073775*

Table 2 (*continued*)

Taxon		Genbank accession no.					
		COI (part1)	COI (part2)	12S	16S	18S	28S
Noviini							
Rodolia	*cardinalis*	GU073967*	GU073916*	GU073826*	GU073883*	GU073726*	GU073776*
Epilachninae							
Epilachnini							
Epilachna	*admirabilis*/sp.	AB002178	NA	NA	NA	EU145616	EU145675
Henosepilachna	*elaterii*	GU073968*	NA	GU073827*	GU073884*	GU073727*	GU073777*
Subcoccinella	*vigintiquatuorpunctata*	GU073969*	NA	GU073828*	GU073885*	GU073728*	GU073778*
Ortaliinae							
Ortaliini							
Ortalia	sp.	NA	NA	NA	NA	EU145621	EU145680
Sticholotidinae							
Sticholotidini							
Sticholotidis	sp.	NA	NA	NA	NA	EU145613	EU145673
Oedemeridae							
Oedemera	sp.	GU073917*	NA	GU073779*	GU073829*	GU073672*	GU073729*
Oedemera	*nubilis*	NA	DQ221991	NA	NA	NA	NA
Cleroidea							
Clerus	*alveorius*	GU073918*	NA	GU073780*	GU073830*	GU073673*	GU073730*
Anthocomus	*rufus*	NA	DQ221960	NA	NA	NA	NA
Cerylonidae							
Cerylon	spp.	NA	DQ156021	NA	EF512341	EF363010	EU145660
Chrysomelidae							
Bruchidius	spp.	AY390689	DQ351974	DQ524351	AJ841299	AJ841415	AJ841542
Cucujidae							
Cucujus	*clavipes*	NA	DQ222036	NA	DQ202569	AF423767	AY310660
Endomychidae							
Phymaphora	*californica*	NA	DQ222033	NA	DQ202565	AY748160	DQ202678

* Newly acquired sequences. NA: sequence not available.

(BI). Best-fit models of evolution for each dataset were determined using the Akaike information criterion (AIC), as implemented in MODELTEST version 3.7 (Posada and Crandall, 1998). The evolutionary model selected for each dataset and the associated parameters are listed in Table 4.

The MP analyses were performed using PAUP v4.0b10 (Swofford, 2001). Heuristic searches were conducted using tree-bisection-reconnection (TBR) branch swapping, 1000 random-addition replicates and a MaxTree's value of 1000. Graphical analyses of saturation of the COI gene was realized by plotting the patristic distance against the uncorrected pairwise distance. It appears that the third codon positions and, to a lesser extent the first positions, are saturated. Thus, we performed weighted analyses with two differential weightings of the COI codon positions to minimize the effect of substitutions that may accumulate at a high frequency due to the degeneracy of the genetic code at the third positions (4:4:1, as Kergoat et al., 2004) and, to a lesser extent, at the first positions (2:4:1). Moreover, gaps constitute a valuable source of phylogenetic information (Giribet and Wheeler, 1999) and thus were treated as a fifth character in the parsimony analyses. Then, the

Table 3
Names, sequences and references of the primers used.

Gene	Primer	Sequence 5′–3′	Reference
COI	LCO 1490*	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO 2198*	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
	C1-J-2183-b	CAACAYTTATTTTGATTTTTTGG	This study
	TL2-N-3014*	TCCAATGCACTAATCTGCCATATTA	Simon et al. (1994)
	COCC-A	CTAACCATAAAGATATTGGAACATT	This study
	COCC-B	AAACTTCTGGATGACCAAAAA	This study
	COCC-1	GGATCCAGTTTTATACCAACA	This study
	COCC-C	GGAGGAGGGGATCCAGTTT	This study
	COCC-D	CCAATGCACTAATCTGCCATA	This study
12S rRNA	SR-J-14233 *	AAGAGCGACGGGCGATGTGT	Kergoat et al. (2004)
	SR-N-14588 *	AAACTAGGATTAGATACCCTATTAT	Kergoat et al. (2004)
16S rRNA	16S coleoptF	ATGTCTTTTTGAKWATAATWTAA	This study
	16Sr (luisa) *	ACGCTGTTATCCCTAAGGTAATTT	Orsini et al. (2007)
18S rRNA	18S ai*	CCTGAGAAACGGCTACCACATC	Whiting et al. (1997)
	18S bi*	GAGTCTCGTTCGTTATCGGA	Whiting et al. (1997)
28S rRNA	28S sa*	GACCCGTCTTGAAACACGGA	Whiting et al. (1997)
	28S sb*	TCGGAGGGAACCAGCTACTA	Whiting et al. (1997)

* Most commonly used primers (the other primers were used for individual taxa that did not amplify with commonly used primers).

Table 4
Best model and estimated substitution parameter values estimated from maximum likelihood analyses of each gene separately and from combined-gene datasets (all genes together) using AIC with MODELTEST.

Gene	COI (part 1)	COI (part 2)	12S	16S	18S	28S	Combined data
Length (bp)	614	764	345	336	933	323	3315
Best model	TVM + I + G	GTR + I + G	TIM + I + G	TVM + I + G	GTR + I + G	GTR + I + G	GTR + I + G
πA	0.377	0.425	0.467	0.429	0.255	0.293	0.340
πC	0.116	0.096	0.075	0.017	0.227	0.219	0.120
πG	0.039	0.046	0.019	0.057	0.284	0.313	0.154
πT	0.468	0.433	0.439	0.496	0.234	0.175	0.385
rA–C	0.082	2.318	1.000	2.401	1.862	0.849	2.923
rA–G	8.512	17.822	9.384	9.187	2.568	2.267	8.130
rA–T	0.504	2.102	1.458	2.308	1.779	2.421	9.497
rC–G	2.219	9.451	1.458	0.000	0.421	0.218	2.353
rC–T	8.512	29.318	3.651	9.187	8.132	8.927	25.152
rG–T	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pinv	0.447	0.352	0.156	0.316	0.658	0.510	0.493
alpha	0.460	0.446	0.543	0.488	0.596	0.374	0.686

topologies obtained are compared to those found with the gaps treated as missing data (default parameter). The robustness of nodes was estimated by bootstrap procedure (Felsenstein, 1985) with 1000 replicates (full heuristic search) of 100 random-addition replicates each, for all analyses.

Congruence among all pairs of genes studied was assessed by the incongruence length difference test (ILD; Farris et al., 1994) as implemented in PAUP, with all uninformative characters excluded (Cunningham, 1997) using 1000 replicates and a MaxTree's value of 200. Since the result of the partition-homogeneity test was not significant ($P > 0.05$), we concatenated the different loci and the combined dataset was analyzed. The latter was preferred over separate analyses due to the presence of missing data resulting from PCR failures: the combined matrix allowed us to broaden the scope of the separate analyses in order to obtain more synthetic and direct comparisons. The PCR failures were a recurrent problem for the COI gene despite several trials using different DNA concentrations and PCR conditions, while using the same DNA extracts the other genes were amplified successfully. Moreover, in the absence of heterogeneity in the data, adding more data from distinct sources generally increase the accuracy of phylogenetic estimates (Bull et al., 1993; Huelsenbeck et al., 1996), even if several sequences are missing as the benefits of including taxa with missing data in a phylogenetic analyses usually overcomes the associated disadvantages (Wiens, 2003, 2005, 2006).

Bayesian inference was carried out using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) and PhyloBayes 3.2 (Lartillot et al., 2009). In MrBayes analyses, four partitioning strategies were defined *a priori*: strategy P1, which corresponds to an analysis without partition; strategy P2 (2 partitions), which implements a partition for the mitochondrial genes and one for the nuclear genes; strategy P5 (5 partitions), which implements a partition for each gene; strategy P6 (6 partitions), which implements a partition for each gene, with he two non-contiguous regions of COI gene being treated as different genes; strategy P7 (7 partitions), which uses one partition for each ribosomal gene and three partitions for the mitochondrial gene (the two non-contiguous regions of COI gene being treated as one gene and one partition per codon position was used); strategy P10 (10 partitions), which uses one partition for each ribosomal gene and six partitions for the mitochondrial gene (the two non-contiguous regions of COI gene being treated as different genes with one partition per codon position).

Two independent BI runs were carried out, each one with four chains (with incremental heating) of 10,000,000–15,000,000 generations, with random starting trees, default priors (but with variable rates) and trees sampled every 1000 generations, applying a model with six substitutions types and a gamma distributed rates with shape parameter and a proportion of invariant site estimated from the data, as suggested by the best-fit model estimated for all datasets. We allowed each partition to have its own set of parameters and to evolve at different rates. Stationarity was assessed graphically by plotting likelihood scores against chain generation and verifying that the standard deviation of split frequencies was under 0.01 (Ronquist and Huelsenbeck, 2003). For each run, the first 25% of sampled trees were discarded as burn-in and the remaining trees used to construct a 50% majority-rule consensus tree. The robustness of clades was assessed by clade posterior probabilities (PP).

As suggested by Brandley et al. (2005), Bayes factor (BF)-based statistics (2lnBF) were used to choose among the different partitioning strategies. However, instead of using a fixed threshold of 10, following Kergoat et al. (2007), a more conservative threshold that takes into account the increase number of parameters for each competing partitioning strategy was used.

Phylogenetic relationships were also inferred through the use of mixture models, as implemented in the program PhyloBayes 3.2 (Lartillot et al., 2009). This software uses a Bayesian Monte Carlo Markov Chain (MCMC) sampler and a combination of two independent Dirichlet processes: one for modeling site-specific rates (Huelsenbeck and Suchard, 2007), and one for describing site-specific profiles (Lartillot and Philippe, 2004). Unlike traditional partitioning strategies, mixture models account for data heterogeneity without requirement of *a priori* knowledge of within-data differences in evolutionary pattern. Two independent chains were run in parallel, using the CAT model, for at least 5,000,000 generations starting from random trees, until convergence (maximum discrepancy across all bipartions <0.1). The first 5000 cycles were then discarded as the burn-in and the remaining ones were sampled every ten generations to calculate majority-rule posterior consensus trees with bipartition frequencies at each node.

We tested the significance of topological differences in phylogenetic trees using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999). The SH test compares the likelihood score ($-\ln L$) of a given data set across its ML tree with the $-\ln L$ of that data set across alternative topology. The differences in the $-\ln L$ values are evaluated for statistical significance using bootstrap (1000 replicates) based on re-estimated log likelihoods (RELL) method (Kishino et al., 1990), as implemented in PAUP v4.0b10 (Swofford, 2001).

The ML analyses were performed using PhyML (Guindon and Gascuel, 2003), with the model and parameters estimated from the AIC criterion in MODELTEST (cf. Table 4), to identify the optimal tree. This tree was re-used for a new round of parameter esti-

mation/branch swapping. This procedure was repeated until both the topologies and parameters stabilized. The robustness of the nodes was determined by using 1000 bootstrap replicates and the recent method of the approximate likelihood-ratio test (aLRT) for branches (Anisimova and Gascuel, 2006). ML analyses were also conducted with RAxML 7.0.4 web-servers (Stamatakis, 2006; Stamatakis et al., 2008) considering each codon position and each gene as an independent partition (10 partitions). The GTR + I + G model was applied to all partitions: individual alpha-shape parameters, substitution rates, and base frequencies were estimated and optimized separately for each partition. Bootstrap support was determined by using 100 pseudo-replicates.

2.4. Hypotheses testing

A priori hypotheses (i.e. the monophyly of all subfamilies) were compared statistically with the *a posteriori* phylogenetic hypothesis (i.e. the unconstrained tree obtained through partitioned BI analyses). The monophyly of all subfamilies and of each subfamily independently were compared with the unconstrained tree. Comparisons were made using Bayes factor (BF)-based statistics (2lnBF) and the likelihood-based nonparametric SH test (Shimodaira and Hasegawa, 1999). The constrained tree (in which subfamilies were monophyletic) was reconstructed using the same parameters as those used in the construction of the unconstrained tree using partitioned BI analyses, applying the best strategy defined and constraints on monophyly for all subfamilies (strategy C-all) or for each subfamily independently. Alternatively, for both *a priori* and *a posteriori* hypotheses, branch lengths were further re-estimated in PAUP using the best-fitting evolutionary model used in the ML analyses. The re-estimated logaritmic likelihood (RELL) method (Kishino et al., 1990), as implemented in PAUP v4.0b10 (Swofford, 2001), was used to resample the logarithmic likelihoods (1000 replicates) in the SH tests.

2.5. Evolution of food preferences

Food preference was treated as a discrete and unordered character and mapped on the inferred phylogeny by maximum parsimony ancestral reconstruction using Mesquite version 2.5 (Maddison and Maddison, 2005). In addition, a maximum likelihood model was used to infer ancestral states of food preference because likelihood-based optimizations can take into account branch lengths and they allow the assessment of uncertainty in ancestral trait reconstruction (Schluter et al., 1997; Pagel, 1999). We conducted global ML optimizations, using the one-parameter Markov k-state model (Mk1; Lewis, 2001) as implemented in Mesquite version 2.5 (Maddison and Maddison, 2005).

3. Results

3.1. Sequence variation

Sequences were obtained from 30 genera and 54 species of Coccinellidae plus two outgroup species. These new results were combined with previously published sequences and provided information on a total of 37 genera and 61 species of Coccinellidae and five outgroup taxa belonging to the infraorder Cucujiformia.

Of the species of Coccinellidae all were successfully sequenced for the rDNA genes; the success was lower for the COI gene: 90.7% for the first part and 55.5% for the second (Table 2). In the final matrix, due to the poor availability of sequences in the database, the missing data increased with the sequences of Coccinellidae more represented for the rDNA genes (98.4% for the 18S and 28S, 90.2% for 12S and 16S) than for the COI gene (83.6% for the first part and 49.2% for second part) (Table 2). Within the Coccinellidae, 42.6% of the species were sampled for all 6 gene fragments. After alignment, the combined dataset was 3315 bp in length, with 1190 parsimony-informative characters (35.9%), when gaps were treated as a fifth state: 633 (45.9%) for the COI, 196 (56.8%) for the 12S, 166 (49.4%) for the 16S, 115 (12.3%) for the 18S and 80 (24.8%) for the 28S. The mitochondrial genes were the most variable: 53.2% of characters were variable for the COI, 70.4% for 12S, and 56.8% for 16S, in contrast to the 22.8% and 37.5% of the autosomal 18S and 28S variable, respectively.

Across all datasets, no significant base composition heterogeneity was detected among taxa ($P = 1.0$; Chi-2 test implemented in PAUP v4.0b10, Swofford, 2001). According to the ILD test, partitions of the data into COI, 12S rRNA, 16S rRNA, 18S rRNA and 28S rRNA, were homogeneous ($P = 0.935$). Thus, all the datasets were combined for the phylogenetic analyses.

3.2. Phylogenetic analyses

3.2.1. A priori partitioning strategies and mixture models

Bayes factor-based statistics indicate that among the different partitioning strategies determined *a priori* (the codon positions and the genes), the most complex model (i.e. involving the greatest number of partitions) was optimal ($P = 0.00$; Table 5). The partition-rich strategies were always found to be the best ones ($P = 0.00$), even in those cases where the COI gene was partitioned into two fragments (i.e. P5 vs. P6 and P7 vs. P10; cf. Table 5). The tree derived from the analysis with the most complex partitioning strategy (10 partitions) is shown in Fig. 2 and used to discuss support values.

The comparison between analyses using mixture models and *a priori* partitioning strategy indicates that mixture models did not perform better (SH test, $P < 0.001$).

3.2.2. Test of alignment conditions and gap coding

After deletion of ambiguous sections in the alignments, the combined dataset was 3056 bp in length, with 964 parsimony-informative characters (31.5%). The phylogenetic analysis was conducted using the best partitioning strategy (P10) by Bayesian inference. The tree obtained is completely congruent with that reconstructed from the complete dataset (Supplementary Fig. 1), despite a lower resolution of the most basal nodes, which were also poorly supported in the tree derived from the complete dataset. However, the support is higher for the association of *Clitosthetus* with the clade Chilocorini–Coccinellinae, and the relationships among Coccinellinae.

In addition, some parsimony analyses were conducted to assess the effect of gap coding on the phylogenetic relationships among the family. The comparisons between the MP analyses with gap coded as missing data or as 5th character state show small differ-

Table 5
Comparisons of all partitioning strategies using Bayes factors (2lnBF) (below the diagonal) with the associated critical values of the chi-2 distribution (above diagonal). Bold values indicate the 2lnBF comparisons used in determining the optimal partitioning strategy.

H_0	H_1						
	P1	P2	P5	P6	P7	P10	C-all
P1	–	21.02	65.17	79.08	92.80	133.25	133.25
P2	1746.38	–	50.99	65.17	79.08	106.39	106.39
P5	3037.24	1290.86	–	21.02	50.99	79.08	79.08
P6	3097.06	1350.68	59.82	–	21.02	65.17	65.17
P7	3705.34	1958.96	668.1	608.28	–	50.99	50.99
P10	**5130.34**	**3383.96**	**2093.1**	**2033.28**	**1425.0**	–	3.84
C-all	4680.92	2934.54	1643.68	1583.86	975.58	−449.42	–

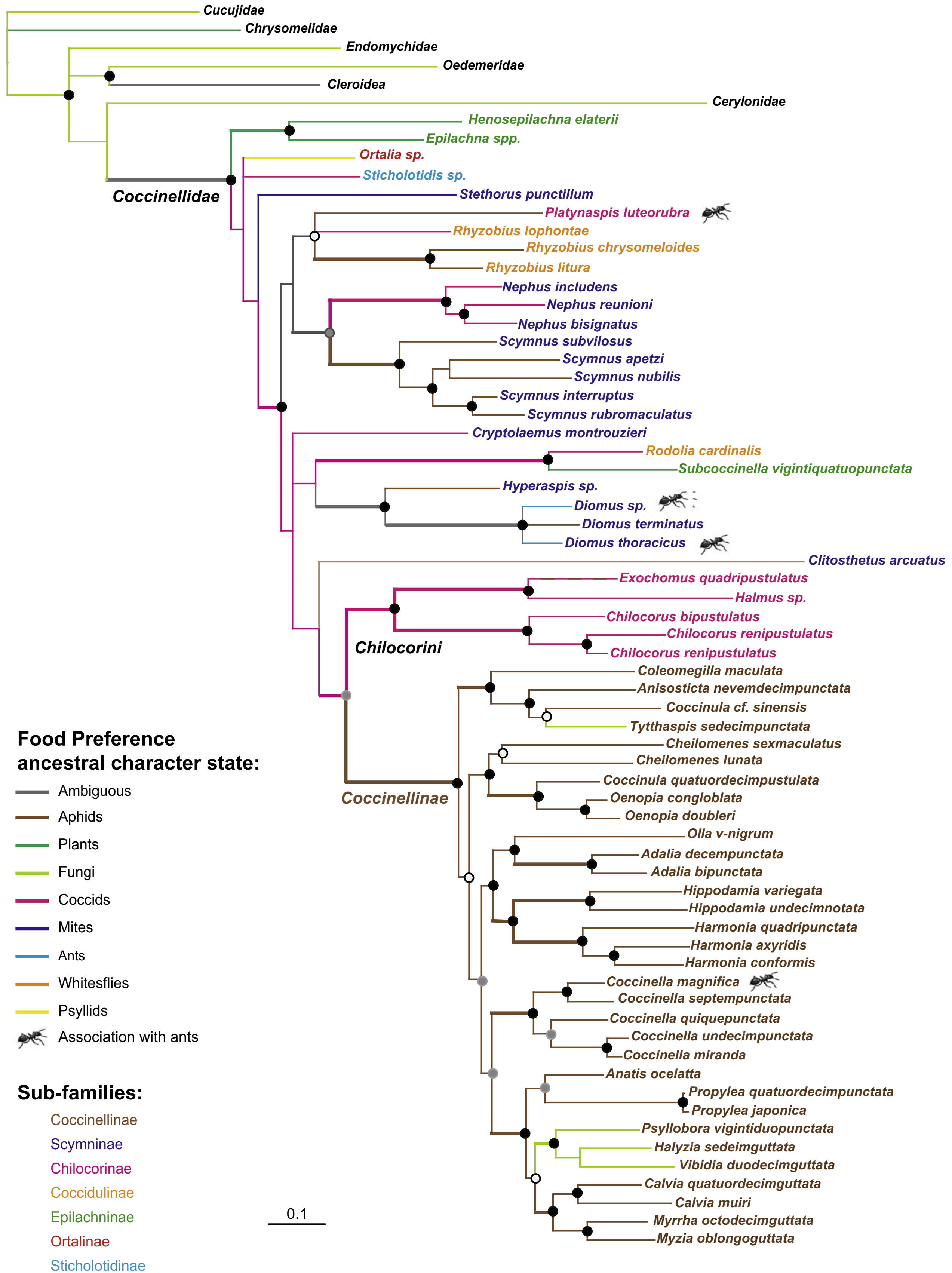

Fig. 2. Phylogenetic tree from the Bayesian inference of the combined dataset using the *a priori* partitioning strategy P10 (six partitions corresponding to 1st, 2nd and 3rd codon positions of the two COI gene fragments and four partitions corresponding to the ribosomal genes: 12S, 16S, 18S and 28S rDNA). A bold branch indicates a branch identified both in the ML and weighted MP analyses. A black dot indicates a PP > 0.90, a grey dot 0.80 < PP < 0.90 and a white dot 0.70 < PP < 0.80, otherwise the posterior probability is less than 0.70. Food preferences are indicated by the color of the branch. The membership of each subfamily is indicated by the color of taxa names.

ences and always concern poorly supported nodes (<50% BP). The gap coding did not modify the robustness of nodes. In the unweighted and 2:4:1 analyses, the strict consensus of the most parsimonious trees shows a large polytomy at the base of the entire family and the Coccinellinae subfamily: only some terminal clades are resolved, already identified and supported in the other MP analyses. In the 4:4:1 weighted analysis, the topological differences concern the basal relationships among the Coccinellinae subfamily, the relative position of the genus *Rhyzobius* and the clade *Hyperaspis–Diomus*, as well as the position of the genus *Stethorus*.

3.2.3. Hypothesis testing

Constrained trees were built to specifically test the monophyly of the subfamilies. SH tests significantly rejected the alternative hypotheses of the monophyly of all the Coccinellidae subfamilies ($P < 0{:}001$) as well as the monophyly of each subfamily ($0.001 < P < 0.049$). Bayes factors indicate that the unconstrained tree (with 10 partitions) was better than the constrained trees for the monophyly of all subfamilies (C-all, $P = 0.00$; cf. Table 5), as well as for each subfamily independently ($-46.26 < BF < -386.88$; $P < 0.001$).

3.2.4. Congruence and reliability

Among the analyses, the topologies are quite similar: some clades are found in all analyses, despite the incongruent basal branching. Most incongruence is due to conflict between the parsimony and the bayesian and likelihood analyses. The unweighted MP analysis of the combined data set yielded 4 more parsimonious trees (9375 steps; CI = 0.268; RI = 0.447). In the strict consensus of the four trees, most clades are resolved but few are supported (BP < 50%). Most well-supported clades are terminal, with the exception of the family Coccinellidae and the subfamily Coccinellinae (Supplementary Fig. 2). Two weighting schemes were used based on the codon positions, to assess the effect of COI gene saturation on the phylogenetic relationships within the family. The 2:4:1 weighted analysis yielded three trees (6787.25 steps; CI = 0.271; RI = 0.468) and the 4:4:1 analysis resulted in a single tree (7177.25 steps; CI = 0.271, RI = 0.468). The use of weighting schemes for the COI gene does not modify the bootstrap support of the nodes. The topological incongruence between parsimony analyses concerns mainly the basal branching within the family, but none of the incongruent nodes are well-supported (<50% BP). In all MP analyses, terminal clades, as well as two internal nodes (monophyly of the family and Coccinellinae subfamily), are generally congruent and highly supported. In the weighted analyses, the Coccinellinae and the tribe Chilocorini are sister-groups, whereas the affiliation was different in the unweighted MP analysis. Moreover, some taxa associations are recovered in the 2:4:1 weighted MP analysis and probabilistic analyses (the basal position of the genera *Ortalia*, *Stethorus*, *Sticholotidis* and the cluster *Henoepilachna–Epilachna*) but not in the MP analyses without downweighting of the 1st codon position.

Bayesian inferences conducted using an *a priori* partitioned strategy and mixture models provide similar topologies, although resolution at the basal level is higher for the *a priori* partitioned strategy (see Fig. 2 and Supplementary Fig. 3). The only incongruence between the two analyses is among basal relationships within the Coccinellinae subfamily.

The ML analyses provided slightly different topologies depending on whether or not a partitioned strategy was used. The partitioned analysis (RAxML tree) provided a tree identical to the 10-partitions Bayesian tree, while the ML analysis conducted without partitioning strategy (PhyML tree) yielded a topology similar to the Bayesian without partitions and that differs the relationships among some basal taxa in the Coccinellidae (Fig. 3).

The support for the nodes was examined in each analysis to assess confidence in the recovered relationships. The overall level of support of the BI topology was greater than the bootstrap support in the parsimony and ML analyses (Huelsenbeck et al., 2002; Miller et al., 2002), although the same model was used in both BI and ML (partitioned or not). Some studies suggest that Bayesian posterior probabilities overestimate phylogenetic support (Cummings et al., 2003; Douady et al., 2003; Suzuki et al., 2002). Caution should be exercised when considering Bayesian posterior probability values and especially low ones (i.e. <0.80). However, most nodes with moderate to high support in BI were also resolved in ML and MP analyses despite possible lower support in those analyses. Moreover, the aLRT supports obtained in ML (without partitions) were generally close to the Bayesian posterior probabilities (see Figs. 2 and 3). However, because this method was only recently developed there is little hindsight about the aLRT values. Overall, the aLRT values provide congruent but higher support than ML bootstrap values, with few significant exceptions: some nodes, consistently recovered in all analyses, have a relatively high support from Bayesian posterior probabilities and ML bootstrap values, but a low aLRT value (e.g. the cluster *Nephus–Scymnus* and *Coccinula* cf. *sinensis–Tytthaspis*). In contrast, some nodes well supported by aLRT values (e.g. the cluster *Cryptolaemus–Rhyzobius–Platynaspis–Hyperaspis–Diomus*; aLRT = 0.93) were not supported by bootstrap values (BP < 50%) or incongruent among analyses, as the node was not recovered in both MP and partitioned BI analyses (Figs. 2 and 3). Thus, the nodes that are recovered in all the different analyses are considered to be reliable and those that are not, even though highly supported when recovered, less reliable. The congruent nodes among the weighted analysis (2:4:1) MP, ML and BI analyses, with and without partitioning, are represented in the trees as bold branches.

3.3. Phylogenetic relationships within Coccinellidae

The monophyly of the Coccinellinae was demonstrated, and received strong support from all analyses and datasets (PP = 1.0; BP = 100%). The relationships described are always annotated with the Bayesian posterior probabilities (PP) of the analysis with the most complex partitioning strategy (10 partitions) and the aLRT and bootstrap (BP) values of the ML analysis without partitions.

3.3.1. Relationships between subfamilies

Our data do not support any clear pattern regarding the order of emergence among lineages. However, the cluster *Henosepilachna–Epilachna*, the subfamilies Ortaliinae and Sticholotidinae, and the genus *Stethorus* (Scymninae) are basal among the Coccinellidae (PP = 0.99, aLRT = 0.87, BP = 52%). The cluster *Henosepilachna–Epilachna* is the first taxa to diverge but this has little support (PP = 0.52, aLRT = 0.75, BP = 56%). The order of emergence of the other lineages is uncertain but there is clearly a large mixture of lineages belonging to the Epilachninae, Chilochorinae, Scymninae and Coccidulinae, which makes them polyphyletic. These basal relationships are generally poorly supported and incongruent between analyses, although some relationships were identified by all the methods.

In the subfamily Scymninae, the genera *Nephus*, *Scymnus* and *Diomus* are monophyletic (PP = 1.0; BP > 99%). However, in this subfamily there are different lineages (Fig. 2). The genera *Nephus* and *Scymnus* are sister taxa in all analyses (PP = 0.83, aLRT = 0.71, BP = 78%). A second lineage consists of the cluster *Hyperaspis – Diomus* (PP = 0.95, aLRT = 0.34, BP = 73%). The positions of these lineages and the genera *Clitosthetus* and *Cryptolaemus* are ambiguous, as analyses yielded incongruent relationships with low support values.

The subfamily Coccidulinae was not recovered in our analyses, which strongly (PP = 1.0; aLRT = 1.0, BP = 100%) indicates that the genus *Rodolia* is closely related to *Subcoccinella* (Epilachninae) and calls into the presumed monophyly of the Epilachninae. In

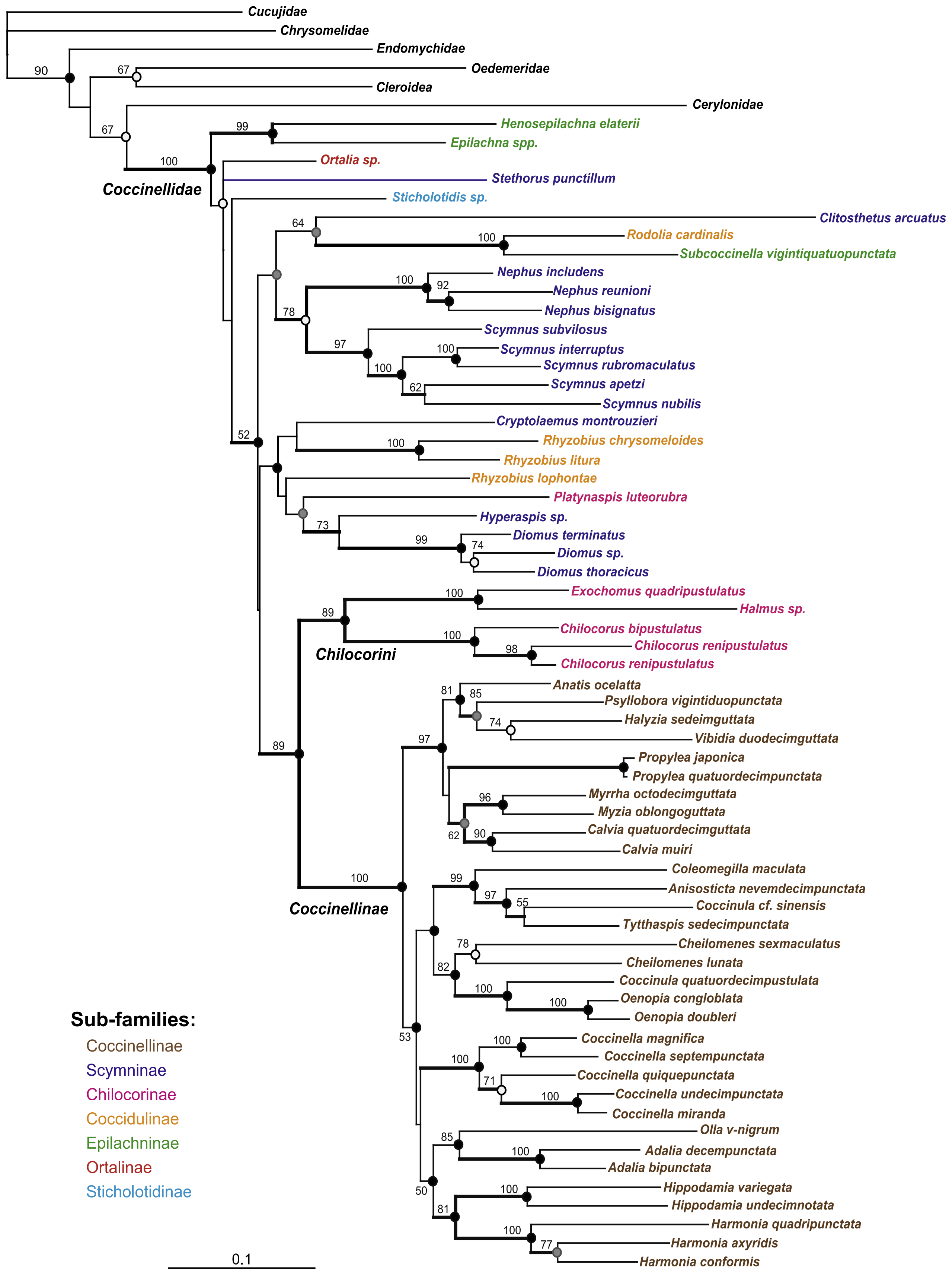

Fig. 3. Phylogenetic tree from the maximum likelihood analysis of the combined dataset under the GTR + I + G model without partitioning strategy. A bold branch indicates a branch identified both in the BI and MP weighted analyses. A black dot indicates an aLRT > 0.90, a grey dot 0.80 < aLRT < 0.90 and a white dot 0.50 < aLRT < 0.80, otherwise the aLRT is less than 0.50. Numbers on the branches are bootstrap support (>50%). The membership of each subfamily is indicated by the color of taxa names.

the ML analysis, the Coccidulinae species *Rhyzobius lophantae* (previously attributed to the genus *Lindorus*) is not associated with the other two *Rhyzobius* species, but all three *Rhyzobius* species belong to a clade that also includes *Platynaspis* (Chilocorinae) and the Scymninae genera *Cryptolaemus, Hyperaspis* and *Diomus* (aLRT = 0.93; BP < 50%). In the BI analyses *Rhyzobius lophantae* belongs, despite unresolved relationships, to a clade comprising only the other two *Rhyzobius* species and *Platynaspis* (PP = 0.70) (Fig. 2 and Fig. 3). Moreover, the relative position of these Coccidulinae lineages remains ambiguous.

The subfamily Chilocorinae, represented in this study by four genera, splits into two lineages. The first has high support (PP = 1.0; aLRT = 0.92, BP = 89%) and includes members of the Chilocorini, *Exochomus*, *Halmus* and *Chilocorus*. All our analyses strongly (PP = 0.82; aLRT = 1.0; BP = 89%) identified this clade as the sister-group of Coccinellinae. In the second lineage, the position of the genus *Platynaspis* (Platynaspidini tribe) differs among analyses and is poorly supported, but clearly unrelated to the Chilocorini (Fig. 2).

Among the monophyletic Coccinellinae, the basal nodes are both poorly supported by bootstrap and posterior probability values and incongruent among analyses. However, overall relationships between Coccinellinae taxa were relatively well resolved, with some terminal clades consistently found. The MP, ML and BI phylogenetic analyses resulted in highly similar topologies for these terminal clades with the exception of the position of the genus *Anatis*.

3.3.2. Relationships among the subfamily Coccinellinae

The analyses reveal that the monophyly of the genera represented by several species is often highly robust (PP = 1.0; BP = 100%: see Figs. 2 and 3), despite the low support value for the genus *Cheilomenes* (PP = 0.74; aLRT = 0.55, BP = 78%). In contrast the genus *Coccinula* is clearly paraphyletic: *Coccinula quatuordecimpustulata* clusters with the genus *Oenopia* (PP = 1.0; aLRT = 1.0, BP = 99%), while *Coccinula cf. sinensis* appears to be the sister-group of *Tytthaspis* (PP = 0.70; aLRT < 0.50, BP = 55%). Our analyses clearly indicate an association of *Cheilomenes* with the clade *Coccinula quatuordecimpustulata–Oenopia* (PP = 1.0; aLRT = 0.94, BP = 82%).

Moreover, all of our analyses recovered a strongly supported (PP = 1.0; BP = 99%) clade comprising 10 taxa with four distinct lineages, although the relationship among them remains unresolved. The first lineage consists of the genus *Propylea* (PP = 1.0; BP = 100%). The second includes the cluster *Myrrha–Myzia* (PP = 1.0; aLRT = 0.97, BP = 96%), which appears to be the sister-clade of the genus *Calvia* (PP = 0.98; aLRT = 0.85, BP = 62%). The third corresponds to the tribe Psylloborini (PP = 1.0; aLRT = 0.89, BP = 85%) and within which the genus *Psyllobora* is the first to emerge, followed by the cluster *Halyzia–Vibidia* (PP = 1.0; aLRT = 0.63, BP = 74%). The last lineage consists of the genus *Anatis*, the position of which is unstable, either clustering with the genus *Propylea* or the tribe Psylloborini.

Among *Coccinella*, the species *C. miranda* and *C. undecimpunctata* are sister species (PP = 1.0; aLRT = 1.0, BP = 100%), as are *C. septempunctata* and *C. magnifica* (PP = 1.0; aLRT = 0.99, BP = 100%), while the association of *C. quinquepunctata* with the cluster *C. septempunctata–C. magnifica* remains uncertain (PP = 0.74; aLRT = 0.58, BP = 71%).

The genus *Harmonia* is closely related to *Hippodamia* (PP = 1.0; aLRT = 0.93, BP = 81%), represented here by the two subgenera *Semiadalia* and *Adonia*. Within *Harmonia*, the species *H. axyridis* and *H. conformis* are the closest.

The analyses identified another well-supported cluster (PP = 1.0; aLRT = 0.99, BP = 99%), which includes the genus *Coleomegilla* that diverges first, followed by the genus *Anisosticta* (PP = 1.0; aLRT = 0.96, BP = 97%) and then the cluster *C. cf. sinensis–Tytthaspis* (PP = 0.70; aLRT < 0.50, BP = 55%).

Finally, the genus *Olla* strongly (PP = 1.0, aLRT = 0.92, BP = 85%) clusters with *Adalia* in all BI and ML analyses.

3.3.3. Evolution of food preference

The mapping of food preference onto the phylogeny does not reveal any clear pattern at the root of the Coccinellidae tree. The parsimony analysis indicates that the ancestral state can either be "coccidophagy", "mycophagy" or "phytophagy" (Fig. 2), while the ML character optimization suggests two alternatives: "coccidophagy" (probability $P = 0.45$ and statistical support) or, alternatively, "phytophagy", "aphidophagy" or "mycophagy" ($P = 0.22$, $P = 0.15$ and $P = 0.10$, respectively) is ancestral. The reconstruction of ancestral states under parsimony along the spine of the tree suggests that the coccidophagy is ancestral (Fig. 2), while the ML reconstruction is less confident, estimating that coccidophagy condition is more probable (from $P = 0.57$ to $P = 0.51$, Fig. 4) than aphidophagy (from $P = 0.19$, at the base of the tree, to $P = 0.49$ at the base of the clade Chilocorini–Coccinellinae, Fig. 4).

Although the ancestral state is ambiguous, some general conclusions about the evolution of food preference in ladybirds can be made. The character reconstruction under parsimony indicates that "aphidophagy" has evolved at least three times and ML analysis considers it to be probable at the basal levels in the tree. Both methods agree that "mycophagy" and "herbivory" have evolved at least twice and that "myrmecophily" (association with ants resulting from feeding on ant-tended Hemiptera), facultative, obligate, or even "myrmecophagy", appeared at least three times. Predation on mites (*Stethorus*), whiteflies (*Clitosthetus*) and psyllids (*Ortalia*) each represent direct transitions from an ancestral feeding mode. There is no evidence of a sequence in the evolution of different food preferences.

4. Discussion

4.1. A priori partitioning strategies and mixture models

The analysis partitioned across loci and across codon positions of COI (P7, P10) performed better in comparison to other *a priori* partitioning strategies. Studies based on real and simulated datasets have strengthened the idea that partitioning data according to the expected differences in patterns of evolution increases accuracy of phylogenetic reconstructions (e.g. Brandley et al., 2005; Brown and Lemmon, 2007). Although no statistical comparison of the likelihood scores was possible between the ML analyses, with and without *a priori* partitioning (see RAxML user manual), it appears that the topology based on the *a priori* partitioned dataset is identical to the one issued from the best *a priori* partitioning strategy under BI. Similarly, the tree derived from the ML analyses without partition is identical to the one yielded by the BI without partitioning strategy. This result suggests that it is more informative to take into account the *a priori* partitioning strategy when comparing BI with ML analyses.

Our dataset comprises four ribosomal RNA genes (either mitochondrial or autosomal) characterized by secondary structures (stem-loop), evolving at different mutation rates. In order to take this into account in the reconstruction of the phylogeny, the best approach is to supplement a partitioning strategy by applying different evolutionary models and parameters for the stem and the loop regions. However, after alignment of the rRNA gene sequences, it appears that the position of the secondary structure is not conserved in the mitochondrial genes (12S and 16S) and only a few loop positions are conserved in the nuclear genes (18S and 28S). All the rRNA genes were treated homogeneously and not partitioned. Moreover, if the few nuclear loops were partitioned, the length of the sequences would be too small to allow a precise

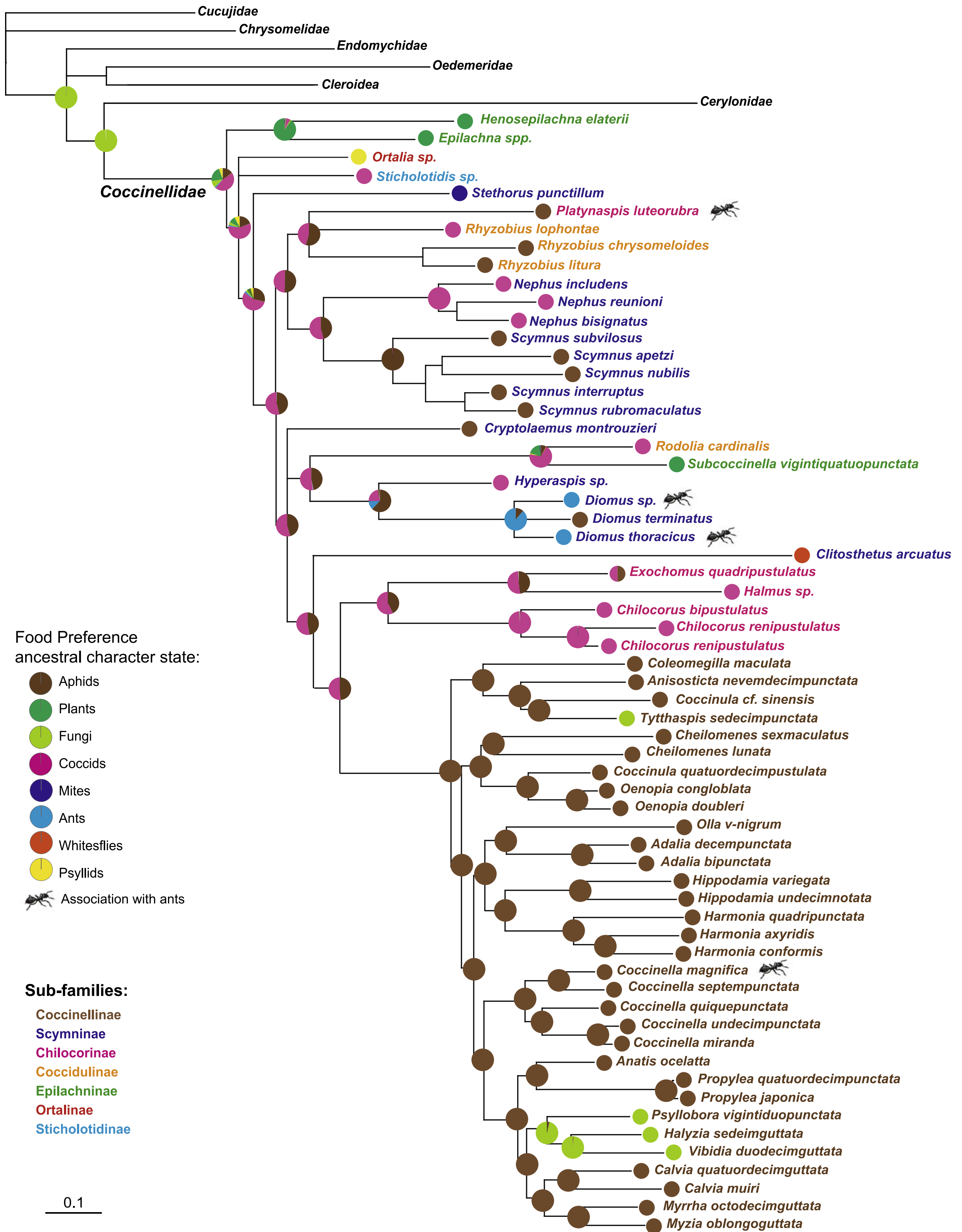

Fig. 4. Phylogenetic tree with ancestral character states of food preference, reconstructed under Mesquite using global optimizations and an Mk1 model. Probabilities of character states are figured at the nodes with pie diagrams.

and reliable estimate of the model parameters. The work of Kergoat et al. (2007) confirms this approach, as they found that partitioning based only on codon positions leads to a significant increase in the mean likelihood scores (and presumably also of phylogenetic accuracy). In contrast, using only the secondary structure of ribosomal genes did not lead to such an increase and yielded a presumably suboptimal topology, as previously observed (Brandley et al., 2005). Pagel and Meade (2004) recommend the use of mixture models, principally in the absence of a clear means of partitioning the dataset. As our dataset was not partitioned *a priori* according to the stem and loop positions, pattern heterogeneity within ribosomal genes have only been take into account by mixture models. When comparing the best *a priori* partitioning strategy (P10) to the mixture models, these later did not perform better, as shown by the SH test. This result indicates that the *a priori* partitioning strategy P10 accounts for most of the pattern heterogeneity in our dataset. Moreover, the use of mixture models did not change the general tree topology, suggesting that heterogeneity among ribosomal genes is not important. Overall, these empirical results suggest that partitioned analyses based on codon positions may outperform those that use standard "one partition per gene" or "secondary structure-based" strategies. Our analyses confirm the pertinence of codon position partitioning since a major increase in the mean likelihood scores was observed with a partition per gene and per COI codon position (cf. Table 5). Surprisingly, an increase in the mean likelihood scores was observed when a partition per COI gene fragment was defined (P5 *vs* P6 and P7 *vs* P10: cf. Table 5). This can be attributed to the large amount of missing data in the COI dataset.

4.2. Phylogeny of the Coccinellidae

The Coccinellidae comprise approximately 360 genera and 6000 species (Vandenberg, 2002). Since Mulsant (1850), several different subdivisions of the family have been proposed based on morphological characters as well as several phylogenetic hypotheses about the relationships between subfamilies (see Sasaji, 1968 and Kovář, 1996 for review). However, only two formal phylogenetic analyses have been performed: a cladistic analysis by Guoyue (1994), based on morphological characters, and a molecular phylogeny proposed by Robertson et al. (2008). The family is generally accepted to be monophyletic on morphological grounds and more recently this was supported by molecular phylogeny of the Cerylonid Series, based on the 18S and 28S rDNA genes (Robertson et al., 2008). This last study included 20 species from six subfamilies of Coccinellidae and provided a preliminary exploration of the internal relationships at higher-levels. However, the distribution of the species among the higher taxa of Coccinellidae was insufficient for testing the monophyly of Coccidulinae, a very diverse subfamily. Moreover, the relationships identified among Coccinellidae are incongruent between partitions and analyses, and the majority of nodes are poorly supported, making the affiliations questionable. Thus, the phylogenetic relationships of the coccinellid taxa are still poorly understood.

The present study is based on a larger sample of Coccinellidae (61 species; 37 genera) and includes species from several tribes of five subfamilies: Coccinellinae (3 of the 5 tribes), Scymninae (4 of 9 tribes, as we included Diomini in the Scymninae), Chilocorinae (2 of 3 tribes), Epilachninae (1 of 4 tribes) and Coccidulinae (2 of 7 tribes, as we included Noviini in the Coccidulinae) (see Table 1 and Kovář, 1996 in Supplementary Table 1). The last two subfamilies – Sticholotidinae and Ortaliinae – are each represented by one species. Our study confirms the monophyly of the family and for the first time tests the monophyly of main subfamilies. This study also provides a first reconstruction of the inter-generic relationships within the Coccinellinae. It is noteworthy, however, that the Coccinellinae is undoubtedly the best represented subfamily in our sample (over 50% of the ingroup taxa), and that the relationships of the less well sampled groups need to be confirmed by further studies.

4.3. Relationships between subfamilies

The order of emergence of lineages is not clearly defined; however the Sticholotidinae, Ortaliinae, *Henosepilachna–Epilachna* cluster (Epilachninae) and the genus *Stethorus* appear to occupy basal positions. It is suggested that the Sticholotidinae is the most basal coccinellid subfamily, on the basis of both intuitive reconstructions (Sasaji, 1968; Kovář, 1996; Fig. 1) and a morphological cladistic analysis (Guoyue, 1994), although in the Robertson et al. (2008) study, *Sticholotis* is nested deeply within the coccinellid clade. Our analysis supports the basal position of Sticholotidinae, *Sticholotis* being among the first lineages to diverge among the Coccinellidae (Fig. 2). Further, our results place the Ortaliinae at the base of the Coccinellidae together with the Sticholotidinae, the clade *Henosepilachna–Epilachna* (Epilachninae) and the genus *Stethorus* (Scymninae), despite low support (PP = 0.54; aLRT = 0.92, BP = 52%) and missing data for *Sticholotis* and *Ortalia* (only 18S and 28S are available). The other Coccinellidae have evolved in a variety of lineages comprising a mixture of Scymninae, Coccidulinae, the genera *Platynaspis* (Chilocorinae) and *Subcoccinella* (Epilachninae) in addition to the most recent lineage to diverge, which is composed of the Coccinellinae and the tribe Chilocorini (Chilocorinae). This does not accord with the general consensus based on morphology (Fig. 1).

Interestingly, the beetles in the terminal clade Chilocorini–Coccinellinae are glabrous dorsally, while the species at the base of our tree are hairy. This observation accords with Mulsant's (1850) classification based on the presence or absence of pubescence on the dorsal surface (respectively the Trichosomides and Gymnosomides), which was considered as artificial and soon abandoned (Sasaji, 1968). Further analyses are needed of a larger sample to test the hypothesis that pubescence on the dorsal face is a primitive character.

The taxonomic composition of the different subfamilies is the subject of much controversy, and there have been numerous rearrangements of tribes between subfamilies (see Sasaji, 1968 for review and Supplementary Table 1). Guoyue (1994) overcame this problem by using the tribal level as the unit in his morphological cladistic analyses, which indicates that Coccidulinae and Scymninae are polyphyletic. A recent molecular phylogenetic analysis (Robertson et al., 2008) confirms the polyphyly of the Scymninae but not the monophyly of Chilocorinae, while the other subfamilies are either monophyletic or represented by a single taxon. However, this analysis failed to robustly resolve the internal relationships of the Coccinellidae, as the Bayesian and parsimony analyses of the results revealed different relationships. Our analyses support the polyphyly of Scymninae, Coccidulinae and Chilocorinae, and provide for the first time evidence of the polyphyly of Epilachninae. The existence of non-natural subfamilies probably accounts for the different views concerning the subdivision of the family. Thus, a redefinition of the subdivisions within Coccinellidae is clearly necessary.

Among the Scymninae, the tribe Scymnini is polyphyletic, as the genera *Clitosthetus* and *Cryptolaemus*, although occupying an ambiguous position within the family, were never associated with the genera *Scymnus* and *Nephus*. *Cryptolaemus* are usually placed in the Scymninae; however, Kovář (1996) and Ślipiński (2007) contest this position. Our results do not place them clearly in either subfamily, and they may constitute a distinct lineage. In contrast, the genera *Diomus* (Diomini) and *Hyperaspis* (Hyperaspidini) appear to be closely related. The genus *Stethorus* (Sthetorini) seems

to occupy a basal position in the family. Thus, among Scymninae there are at least five highly divergent lineages.

The four species of Coccidulinae are, in some way, associated with those of Scymninae despite incongruence and poor support values. Additionally, *Rodolia* appears closely related to *Subcoccinella* (Epilachninae), which is unexpected. All of this supports Pope's opinion (1988) that the recognition of the Coccidulinae in the higher classification of the family is questionable. Our analyses suggest that *R. lophantae* is related to the other *Rhyzobius* species, despite the absence of a resolution in some analyses and low support values. This might favor the recognition of two distinct genera, as *R. lophantae* previously belonged to the genus *Lindorus*. To confirm such an affiliation and clarify the taxonomic status of *Rhyzobius lophantae*, other taxa, especially related Coccidulinae genera need to be included in the analysis.

The subfamily Epilachninae was always considered as homogeneous, mainly on the basis of the morphology and the organization of mouth-parts associated with phytophagy (e.g. Kovář, 1996). In previous phylogenetic analyses, the monophyly of the subfamily was either not tested (Guoyue, 1994; Kobayashi et al., 1998) or recovered based on only two taxa (Robertson et al., 2008). Our analysis, including *Epilachna* and *Henosepilachna* (Epilachnini) and the genus *Subcoccinella*, clearly indicates a close relationship between *Subcoccinella* and *Rodolia* (Coccidulinae), which would make the Epilachninae and Coccidulinae polyphyletic.

The polyphyly of Chilocorinae is also confirmed, as previously suggested by Robertson et al. (2008), and the existence of two distinct lineages corresponding to the Chilocorini (*Exochomus*, *Halmus* and *Chilocorus*) and Platynaspidini (*Platynaspis*) is indicated. Analysis of the combined dataset, revealed, for the first time and with strong support (PP = 1.0, BP = 92%), that the tribe Chilochorini is the sister-group of Coccinellinae, whereas in a recent molecular phylogeny (Robertson et al., 2008) the sister-group of Coccinellinae was not identified due to incongruence between analyses and low support values. The consistent proximity of Chilocorini and Coccinellinae found in this study is therefore quite unexpected as traditionally Chilocorinae are considered to be related to Scymninae and Coccinellinae to Epilachninae (see Sasaji, 1968; Kovář, 1996, Fig. 1). The combination of the genus *Platynaspis* with *Chilocorus* and other species of Chilocorinae dates from Crotch (1874). Although the very different structure of the larvae of *Platynaspis* led to the creation of the Platynaspidini (Iablokoff-khnzorian, 1982), it is generally accepted that this tribe, along with the Chilocorini and Telsimiini belong to the Chilocorinae, as they all have a sideways expansion of the head below the eyes and the number of antennal segments is reduced from 11 to 10 or less. However, some genera of Sticholotidinae also share these characters. Ślipiński et al. (2005) argued that these characters are anti-molestation devices, which develop in response to environmental pressures and therefore do not necessarily indicate a common origin. Our results support Ślipiński et al. (2005) and Ślipiński and Giorgi (2006), who claim that the constitution of the Chilocorinae is questionable. Our analyses clearly indicate that the resolution the subdivisions within the family can only be achieved by including other genera and tribes in phylogenetic analyses.

Finally, our analyses based on five genes and the largest sampling to date, indicated that the monophyly of the Coccinellinae has strong support, and confirm the results of preliminary molecular analyses (Robertson et al., 2008) and a morphological phylogeny (Guoyue, 1994).

4.4. Relationships within the Coccinellinae

Our relatively large sample of Coccinellinae (3 tribes, 19 genera and 32 species) provides, for the first time, strong support for the relationships between tribes, genera and species. The Coccinellinae includes the largest number of genera (Fürsch, 1996). For a long time, the classification of this highly diverse subfamily was based exclusively on external characters. Later, the structure of the genitalia of both sexes was more commonly used in taxonomy.

Iablokoff-khnzorian (1982) considers the shape of the genitalia as a highly valuable feature for studying the phylogeny of this subfamily and argues that *Coleomegilla*, *Anisosticta*, *Tytthaspis* and *Coccinula* genera have the most primitive type of genitalia. Our results clearly support the clustering of these species and the value of such a character for Coccinellinae taxonomy. Moreover, our results are consistent with the Iablokoff-Khnzorian scenario, with this lineage basal in the Coccinellinae clade. Despite the incongruence among analyses concerning the basal branching, the partitioned analyses (BI or ML) indicate this clade as the first lineage to diverge (Fig. 2). In some analyses without partitions (BI: P2, P5, P6, P7 and ML), this clade is related to the one including *Cheilomenes, Oenopia* and *Coccinula quatuordecimpustulata* (Fig. 3). If this is the case, the status of *Coccinula* has to be redefined, as the genus is split between two lineages. *Cheilomenes* is essentially a tropical genus and the simple structure of its sipho places it close to the aforementioned *Coleomegilla* + *Anisosticta* + *Tytthaspis* + *Coccinula* group (Iablokoff-khnzorian, 1982), and therefore it is not surprising to find it in a nearby position, either as its sister-group (ML, Fig. 3) or as the second lineage emerging among the Coccinellinae (BI, Fig. 2). Its strongly supported affiliation to *Oenopia* is quite unexpected, as *Oenopia* differs morphologically and in the structure of its genitalia and is usually placed close to the genus *Coccinella*.

Also, there is strong support for a clade of eight genera including *Anatis*, *Propylea*, *Psyllobora*, *Halyzia*, *Vibidia*, *Calvia*, *Myrrha* and *Myzia*. To our knowledge, Raimundo and Alves (1986) are alone in considering these genera as a separate group based on the size and shape of the antenna and head, which is either completely or partially covered by the anterior margin of the pronotum. *Psyllobora*, *Halyzia* and *Vibidia* are all members of the Psylloborini, a group first proposed by Casey in 1899 (cit Sasaji, 1968). Here, the monophyly of this tribe is recovered with strong support values. The clade *Myrrha*–*Myzia* shares the same kind of sipho. Based on the morphology of the sipho and spermatheca and shape of the infundibulum, Iablokoff-khnzorian (1982) considers *Anatis* to be closely related to *Calvia* and *Psyllobora*. Additionally, *Anati*s, like *Myrrha* and *Myzia*, is associated with coniferous trees.

The genus *Olla*, which used to be placed in a separate tribe, is now widely accepted to belong to the Coccinellini (Vandenberg, 1992). Our data show indeed that the genus is deeply nested within Coccinellini and related to *Adalia*. We found, for the first time, with strong support, that *Harmonia* is the sister-group of *Hippodamia*. The affiliations of the genus *Coccinella* remains unresolved.

Finally, the Coccinellini, as currently defined, was not recovered in our trees due to the inclusion of Psylloborini and Tytthaspidini into the clade. Therefore, in order to redefine the subdivisions within the subfamily a larger sample, including members of other tribes of Coccinellinae needs to be analyzed.

4.5. Evolution of food preference

Higher-level phylogenetic studies have recovered Coccinellidae emerging from mycophagous clades (Hunt et al., 2007; Robertson et al., 2008). Therefore, at least one transition from mycophagy to predation occurs in the Coccinellidae history. Our reconstruction of the ancestral states of food preference within the family did not reveal a clear pattern but seems nevertheless to suggest that "coccidophagy" is probably more primitive than aphidophagy, and several hypotheses have been proposed to account for its more basal position. The first is that Coccids emerged before aphids, and therefore ladybirds specialized first on coccids. However, Coccoidea and Aphidoidea emerged together with other Hemiptera in the

Permian (Dohlen and Moran, 1995) and both groups were well established before ladybirds emerged in the Cretaceous (Hunt et al., 2007). Alternatively, the different geographical distributions in species richness of coccids and aphids when ladybirds diversified may account for coccidophagy being the ancestral condition. Currently, 80% of aphid species occur in temperate regions while 67% of the coccid species occur in the tropics (Eastop, 1978). This suggestion is acceptable if we assume a similar situation in the past.

Within the family, a trend toward taxonomic conservatism at the terminal levels is suggested by our results. Also, the polyphyletic taxa are characterized by different food preferences: as is the case for the two Chilocorinae lineages (Platynaspidini *vs* Chilochorini), the Scymninae lineages (e.g. *Stethorus*, *Clitostethus*, *Hyperaspis*, *Diomus* and *Nephus*) and the *Rhyzobius* species group (Table 1, Fig. 2 and Fig. 4). Similar food preference, however, is also present in paraphyletic taxa (e.g. the herbivorous Epilachninae or the aphidophagous Coccinellini), implying that morphological characters related to the mode of feeding, namely the structure of the mandibles (Samways et al., 1997), arose through evolutionary convergence. Finally, robust clade also display variation in food preference (e.g. the clade *Nephus–Scymnus* or the Coccinellinae subfamily). That is, no clear pattern of food preference evolution comes out of our results (conservation, convergence nor independent evolution), although the importance of this trait in the ecological studies.

Within the Coccinellinae, an association with aphids living on conifers appears at least three times, and mainly in recent members of the subfamily (i.e. *Anatis ocelatta, Myrrha octodecimguttata/Myzia oblongoguttata, Harmonia quadripunctata)*. The first aphids lived on coniferous trees (Heie, 2004), so we would expect their predators to be basal among the subfamily. Several alternative hypotheses may be considered to explain this observation. One is that the ancestral prey (coniferous aphids) was only conserved in a few taxa while most of Coccinellinae switched to other prey. The second interpretation is that the Coccinellinae diversified later, after the angiosperm aphid emergence. In that case, the predation on coniferous aphids appears independently within Coccinellinae. Blackman and Eastop (1984) suggest that most of the Aphididae that currently feed on Coniferae have acquired these host plants relatively recently, which seems to support this second hypothesis. To elucidate this point, it is essential to include other predators of coniferous aphids from the other subfamilies, as they are absent from our sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.10.022.

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