Structural alignment of 18S and 28S rDNA sequences provides insights into phylogeny of Phytophaga (Coleoptera: Curculionoidea and Chrysomeloidea)

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We performed a comparative study of partial rDNA sequences from a variety of Coleoptera taxa to construct an annotated alignment based on secondary structure information, which in turn, provides improved rRNA structure models useful for phylogenetic reconstruction. Subsequent phylogenetic analysis was performed to test monophyly and interfamilial relationships of the megadiverse plant feeding beetle group known as 'Phytophaga' (Curculionoidea and Chrysomeloidea), as well as to discover their closest relatives among the Cucujiformia. Parsimony and Bayesian analyses were performed based on the structural alignment of segments of 18S rRNA (variable regions V4-V5, V7-V9) and 28S rRNA (expansion segment D2). A total of 104 terminal taxa of Coleoptera were included: 96 species of Cucujiformia beetles, representing the families and most 'subfamilies' of weevils and chrysomeloids (Phytophaga), as well as several families of Cleroidea, Tenebrionoidea and Cucujoidea, and eight outgroups from three other polyphagan series: Scarabaeiformia, Elateriformia and Bostrichiformia. The results from the different methods of analysis agree — recovering the monophyly of the 'Phytophaga', including Curculionoidea and Chrysomeloidea as sister groups. The curculionoid and chrysomeloid phylogeny recovered from the aligned 18S and 28S rDNA segments, which is independent of morphological data, is in agreement with recent hypotheses or concepts based on morphological evidence, particularly with respect to familial relationships. Our results provide clues about the evolutionary origin of the phytophagan beetles within the megaclade Cucujiformia, suggesting that the sister group of 'Curculionoidea + Chrysomeloidea' is a clade of the 'Cucujoidea', represented in this study by species in Boganiidae, Erotylidae, Nitidulidae, Cucujidae and Silvanidae. The Coccinellidae and Endomychidae are not grouped with the latter, and the remaining terminal taxa are nested in Tenebrionoidea and Cleroidea. We propose that the combination of structurally aligned ribosomal RNA gene regions 18S (V4-V5, V7-V9) and 28S (D2) are useful in testing monophyly and resolving relationships among beetle superfamilies and families.

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Introduction

The Chrysomeloidea (long-horned beetles and leaf beetles) and Curculionoidea (weevils) are collectively known as Coleoptera 'Phytophaga', with about 130 000 described species worldwide. They are classified in the Series Cucujiformia of the Suborder Polyphaga. Besides their plant feeding habits, the phytophagan beetles are defined by having pseudotetramerous tarsi (i.e., with four distinctly visible tarsomeres

and a reduced, usually hidden, penultimate one) and a particular 'cucujoid' aedeagus (Sharp & Muir 1912; Crowson 1955). These diagnostic features are usually provided in entomology texts to classify them as a group. However, they are not unique synapomorphies of Phytophaga, as they also occur in other cucujiformia beetles. Thus, the monophyly of Phytophaga, the largest group of herbivorous beetles, requires further evaluation.

Crowson (1955: 137) agreed with a 'tendency of authorities' to unite the Chrysomeloidea with Curculionoidea in beetle classifications, and considered both superfamilies as being 'certainly closely allied'. Conversely, Crowson (1981: 685) later recognized that their common origin was still an open question based on the distinctness shown by the oldest fossils of both groups (see also Kristensen 1999: 247). Within the Suborder Polyphaga and the Series Cucujiformia, morphological evidence suggests a classification scheme with the Tenebrionoidea and Cucujoidea closely aligned with the Chrysomeloidea and Curculionoidea (Crowson 1955, 1960, 1981; Lawrence & Newton 1995). Probably because the weevils and chrysomeloids are traditionally considered 'terminal' or most derived within Coleoptera, they are not included in the taxon sampling (or represented by only few species) in previous higher level phylogenetic studies based on morphology (e.g., Beutel & Haas 2000) or molecular evidence (e.g., Caterino *et al*. 2002; Hughes *et al*. 2006). Unfortunately, this leaves their position within the coleopteran tree poorly explored and ill supported. However, they were largely sampled (using 18S rDNA and two mitochondrial genes) in a recent molecular study of the Coleoptera (Hunt *et al*. 2007), showing weevils and chrysomeloids related to some cucujoid lineages within the Series Cucujiformia.

The superfamilies Curculionoidea and Chrysomeloidea in particular have been subjected to phylogenetic studies, with the aim of resolving relationships within weevils and/or chrysomeloids. Such studies were either based on morphological evidence (Napp 1994; Kuschel 1995; Reid 1995, 2000; Schmitt 1996; Marvaldi 1997; Svacha *et al*. 1997; Marvaldi & Morrone 2000; Marvaldi *et al*. 2002) or on 18S rDNA sequences in combination with morphology (Farrell 1998; Marvaldi *et al*. 2002; Duckett *et al*. 2004; Farrell & Sequeira 2004). As with many other molecular phylogenetic analyses on insect groups, the 18S rDNA was the dominant molecular marker used to infer higher level (i.e., familial) relationships in Curculionoidea and/or Chrysomeloidea, and the conclusions came from the combined analysis with morphological data. Studies based on molecular data alone, including 18S rDNA combined with other markers like partial 28S rDNA sequences, are mostly focused on lower-level relationships of some Phytophagan subfamilies or tribes (e.g., Farrell *et al*. 2001; Duckett *et al*. 2003; Gillespie *et al*. 2003, 2004a,b; Gómez-Zurita *et al*. 2005; Gillespie *et al*. 2008), and a recent study of Chrysomelidae was based exclusively on molecular data, using three rDNA markers (Gómez-Zurita *et al*. 2007). Aside from this, there are very few molecular studies dealing with Cucujiformia taxa outside Phytophaga (e.g., Robertson *et al*. 2004).

Exploration of meaningful markers of genealogical descent is fundamental for making progress in the phylogenetic systematics of Coleoptera, and this approach is essential towards having a better understanding of beetle evolution. The use of both small (18S) and large (28S) subunits of nuclear encoded ribosomal RNA (rRNA) sequences in phylogenetic studies is plagued by alignment difficulties. Studies based only on rDNA data frequently yield disappointing results (e.g., nonconcordance with well supported hypotheses based on morphology, uninformative conserved regions coupled with highly length-heterogeneous variable regions that are impossible to align with confidence). This is usually attributed to rRNA genes being bad choices as phylogenetic markers, without questioning the accuracy of the alignment or the missing contribution of structural information into the methods of tree estimation. In this study, we take the approach of using structural information to identify homologous positions of the most difficult to align (yet highly informative) fragments of nuclear rDNA. The rRNA genes have a core structure that is highly conserved across all life, as well as regions that are highly variable in nucleotide composition and sequence length even among closely related taxa (e.g., Hillis & Dixon 1991; Gutell *et al*. 1994; Gutell 1996; Schnare *et al*. 1996). These length heterogeneous regions make alignment of rDNA sequences exceedingly difficult. The rRNA molecule folds, bends and pairs with itself, forming both pairing regions (covarying, hydrogen-bonded stems) and nonpairing regions (i.e., loops of hairpin-stems and terminal and lateral bulges); and because such (secondary and tertiary) structure plays an important role in rRNA function, it is more conserved than the (primary) nucleotide sequence (Gutell *et al*. 1994). As an advantage, information from structural prediction can be used to objectively identify homologous positions within length heterogeneous alignments and to impartially delimit regions of ambiguous alignment (e.g., Kjer 1995, 2005; Gillespie 2004; Gillespie *et al*. 2004a; Kjer *et al*. 2007, 2008).

The objectives of this paper are twofold:

- To construct an annotated alignment based on secondary structure information, inferred through a comparative approach of the most difficult to align fragments of 18S and 28S rDNA sequences from a wide variety of beetle taxa, and in turn, to provide improved rRNA structure models useful for phylogenetic reconstruction in Coleoptera.
- To use the structural alignment to perform phylogenetic analyses to test the monophyly of Phytophaga, discover their closest relatives among the Cucujiformia beetles and examine hypotheses of familial relationships within Curculionoidea and Chrysomeloidea.

Materials and methods

Taxon sampling

The phylogenetic analysis was designed to include representatives of the major groups of weevils and chrysomeloids, and also several representatives of other Polyphagan beetles, with a major sampling of other cucujiformia (Cleroids, Tenebrionoids and Cucujoids). A total of 104 terminal polyphagan taxa were included (see Table 1): 96 species of Cucujiformia, representing the families and main 'subfamilies' of weevils and chrysomeloids (Phytophaga), as well as several families of Cleroidea, Tenebrionoidea and Cucujoidea, and eight outgroups from three other polyphagans, representing series: Scarabaeiformia, Elateriformia and Bostrichiformia. Beetle hierarchical classification in Table 1 follows Lawrence & Newton (1995); familial and subfamilial groupings of Chrysomeloidea are as in Reid (1995, 2000); the families of Curculionoidea are as in Marvaldi *et al.* (2002), with the genera of Curculionidae classified in subfamilies and tribes following the catalogue of Alonso-Zarazaga & Lyal (1999), and Wood (1986) for Scolytinae; subfamilies and tribes of Belidae as in Marvaldi *et al*. (2006).

Characters

The rDNA regions used in this study correspond mainly to the second and third domains of the 18S rRNA (namely variable regions V4-V5 and V7-V9), and the D2 expansion segment of the 28S rRNA. The estimated length of the sequences used ranges from 1470 to 1940 nucleotides (the 18S fragments have 500–660 bp and 490–495 bp, respectively, and the D2 region of 28S is 480–785 bp in length). The 18S rDNA fragments were chosen because they contain the majority of informative characters from the nuclear small subunit rRNA, and are also difficult to align by any means other than their conserved structure. Similarly, the D2 expansion segment of 28S rDNA is one of the most commonly used markers in insect molecular phylogenetic studies because of its sequence variation at different taxonomic levels (e.g., Gillespie *et al*. 2004a, 2006).

DNA extraction, PCR amplification and sequencing

Except for those sequences already available in GenBank, the 18S rDNA fragments were amplified in two PCR reactions, as in Duckett *et al*. (2004), and the expansion segment D2 of the 28S rDNA was amplified in one PCR reaction, as in Gillespie *et al*. (2003, 2004a,b). About 70% of the sequences are new from this study (see Table 1).

Alignment

The rDNA sequences were aligned with reference to secondary structure (e.g., Kjer 1995; Kjer *et al*. 2001; Gillespie *et al*. 2004a). This alignment method can be compared with the manner in which relative anatomical positions and connections are used to infer homologies among body structures, or the way in which organization of codons in triplets helps in aligning protein-coding genes. We used information of established secondary structure models (Gutell *et al*. 1994; Gillespie *et al*. 2004a, 2006; http://www.rna.icmb.utexas.edu) to first perform the alignment of conserved regions of the rRNA (converted from the sequenced rDNA) sequences. Then, paired regions that were more variable among taxa were aligned by searching for compensatory base changes, considering that a base change (mutation) on one strand of a stem is compensated, to maintain structure, by a change on the other strand, so that bases on partner strands are complementary (Gutell *et al*. 1994; Kjer 1995; Gillespie 2004; Gillespie *et al*. 2004a). After recognition of highly conserved unpaired (single-stranded) regions and paired (coevolving) stems, the interspersed regions of ambiguous alignment were delimited (Kjer 1995). The insertions and deletions (indels) were coded as in Kjer *et al*. (2001), using outgroup comparisons to distinguish between insertions and deletions. The 'ancestrally missing' condition was considered a fifth state (coded with asterisks *) and gaps were treated as missing data when representing deletions in the ingroup. Gaps within the bracketed alignment ambiguous regions were inserted to retain columnar homology assignment in the unbracketed regions (and to keep the NEXUS file executable), but they do not represent homologous columns as in the unambiguously aligned regions. The data file for analyses contains 1531 nucleotide positions (excluding unaligned regions and primer sites flanking D2), of which 479 were parsimony informative characters.

Phylogenetic analyses

Phylogeny reconstruction was done using parsimony and likelihood (Bayesian) analyses.

Maximum parsimony analyses were performed with TNT (Goloboff *et al*. 2003) and with PAUP* (Swofford 1999), using 1000 random addition sequences and TBR branch swapping, saving up to 10 trees per replicate. All characters had equal weights. Bremer support values (Bremer 1994) were calculated in TNT by saving suboptimal trees up to 10 steps longer than the most parsimonious solution. Bootstrap support values (Felsenstein 1985) were obtained with PAUP* using the fast heuristic search and 1000 bootstrap replicates.

Bayesian inference under maximum likelihood was performed using MrBayes ver. 3.1 (Ronquist & Huelsenbeck 2003), running three analyses of varying sampling (3, 5, 10 million) generations, each with unique starting seeds. Samples of likelihood scores, tree lengths and all model parameters from the estimated posterior distribution were taken every 1000 iterations and assessed with Tracer ver. 1.2.1 (Rambaut & Drummond 2005) to ensure that stationarity was reached. The doublet model was enforced, which models RNA basepairs (16×16 Q matrix) separately from nonpairing RNA strands (standard DNA substitution matrices). Model

Table 1 The beetle taxa and accession numbers of sequences analysed in this study.

Series/**Superfamily**	Family/Subfamily	Species	18S rDNA	28S rDNA	Country	Extract code
Scarabaeiformia						
Scarabaeoidea						
	Scarabaeidae	*Phyllophaga* sp.	AY310601	AY310552		
	Geotrupidae	*Eucanthus lazarus*	FJ000484†	FJ000406†		
	Pleocomidae	*Pleocoma rubiginosa*	FJ000485†	FJ000407†		
Elateriformia						
Buprestoidea						
	Buprestidae	Buprestidae Gen. sp.	FJ000486†	FJ000408†		
		Agrilus sp.	FJ000487*	FJ000409*	Argentina	CND679
		Trachykele blondeli	FJ000488*	FJ000410*	USA	CND780
Bostrichiformia						
Bostrichoidea						
	Bostrichidae	*Xyloprista hexacantha*	FJ000489*	FJ000411*	Argentina	CND688
		Apatides sp.	AF423766	—		
Cucujiformia						
Cleroidea						
	Cleridae	Cleridae Gen. sp.	FJ000490†	FJ000412†		
		Blackburniella intricata	FJ000491*	FJ000413*	South Africa	CND690
	Melyridae	*Collops* sp.	FJ000492*	FJ000414*	Mexico	CND669
		Arthrobrachus nigromaculatus	FJ000493*	FJ000415*	Argentina	CND680
Tenebrionoidea						
	Tenebrionidae	*Tenebrio molitor*	X07801	FJ000416*	Argentina (28S)	CND761 (28S)
		Coelometopinae Gen. sp.	AY310607	AY310668		
		Diaperinae Gen. sp.	AY310610	AY310671		
		Bolitophagous corticola	FJ000494*	FJ000417*	USA	CND811
	Ciidae	*Cis* sp.	AY310605	AY310666		
	Meloidae	*Epicauta* sp.	FJ000495*	FJ000418*	Argentina	CND671
Cucujoidea						
	Boganiidae	*Paracucujus rostratus*	FJ000496*	FJ000419*	Australia	CND684 & 686
	Cucujidae	*Cucujus clavipes*	AF423767	AY310660		
	Silvanidae	*Uleiota* sp.	AY310604	AY310665		
	Nitidulidae	*Carpophilus* sp.	AY310603	AY310664		
		Neopocadius nitiduloides	FJ000497*	FJ000420*	Argentina	CND771
	Erotylidae	*Languria mozardi*	AY310559	AY310658		
		Erotylina jaspidea	AY310649	AY310710		
		Mycotretus scitulus	AY310621	AY310682		
		Xenocryptus tenebroides	FJ000498*	FJ0004421*	Australia	CND685
	Endomychidae	*Chondria armipes*	AY310609	AY310670		
		Encymon bipustulatus	AY310608	AY310669		
	Coccinellidae	*Olla v-nigrum*	AY310602	AY310663		
Chrysomeloidea						
	Cerambycidae					
	Parandrinae	*Parandra brunnea*	AF267414	FJ000422*	USA	CND641 (28S)
		Parandra sp. *(cf. longicollis)*	FJ000499*	FJ000423*	Panama	CND657
	Prioninae	Prioninae Gen. sp.	FJ000500†	FJ000424†		
		Prionus californicus	FJ000501*	FJ000425*	USA	CND770
	Anoplodermatinae	*Sypilus* sp. *(cf. orbignyi)*	FJ000502*	FJ000426*	Argentina	CND644
	Aseminae	*Arhopalus asperatus*	FJ000503*	FJ000427*	USA	CND651
	Cerambycinae	*Megacyllene robiniae*	FJ000504*	FJ000428*	USA	CND665
	Lamiinae	*Tetraopes tetraophtalmus*	FJ000505*	FJ000429*	USA	CND656
	Lepturinae	*Stictoleptura canadensis cribipennis*	FJ000506*	FJ000430*	USA	CND653
	Megalopodidae					
	Megalopodinae	*Agathomerus rufus*	FJ000507*	FJ000431*	Mexico	CND777
	Palophaginae	*Palophagoides vargasorum*	AF267418	FJ000432*	Argentina	CND646 (28S)
		Palophagus bunyae	FJ000508*	FJ000433*	Australia	CND682 & 694
	Zeugophorinae	*Zeugophora vittnea*	FJ000509*	FJ000434*	Australia	CND 664
		Zeugophora sp.	FJ000510*	FJ000435*	Madagascar	CND661

Table 1 *Continued.*

Series/**Superfamily**	Family/Subfamily	Species	18S rDNA	28S rDNA	Country	Extract code
	Orsodacnidae					
	Orsodacninae	*Orsodacne cerasi*	FJ000511*	FJ000436*	France	CND609
	Aulacoscelinae	*Aulacoscelis* sp.	AF267419	FJ000437*	USA	JJG497 (28S)
	Chrysomelidae					
	Bruchinae/Bruchini	*Callosbruchus maculatus*	FJ000512*	FJ000438*		JJG509
	/'primitive'	Bruchinae Gen. sp.	FJ000513*	FJ000439*	South Africa	CND756
	/Pachymerini	*Caryobruchus gleditiae*	FJ000514*	FJ000440*		JJG524
	Sagrinae/Sagrini	*Sagra femorata*	FJ000515*	FJ000441*	Indonesia	CND542
	/Diaphanopsidini	*Diaphanops* sp. *(cf. westermani)*	FJ000516*	FJ000442*	Australia	CND687
	/Megamerini	*Mecynodera coxalgica*	FJ000517*	FJ000443*	Australia	JJG520
	Donaciinae	*Donacia rufescens*	FJ000518*	FJ000444*	USA	JJG486
		Plateumaris flavipes	FJ000519*	FJ000445*	USA	JJG521
	Criocerinae/Criocerini	*Crioceris duodecempunctata*	FJ000520*	FJ000446*		JJG217
	Cassidinae/Hispini	*Microrhopala vittata*	AF276434	AY243650		JJG218 (28S)
	/Notosacanthini	*Notosacantha* sp. *(cf. circumminata)*	FJ000521*	FJ000447*	Madagascar	JJG504
	Spilopyrinae	*Spilopyra sumptuosa*	FJ000522*	FJ000448*	Australia	CND551
		Hornius sulcifrons	FJ000523*	FJ000449*	Chile	CND698 & 752
	Lamprosomatinae	*Lamprosoma* sp.	AY244878	AY243651		JJG516 & 215
	Cryptocephalinae/Cryptocephalini	*Cryptocephalus aulicus*	FJ000524*	FJ000450*	USA	JJG480
	/Clytrini	*Saxinus saucia*	AF267456	FJ000451*	USA	CND625 (28S)
	Eumolpinae/Eumolpini	*Metaxyonycha panamensis*	FJ000525*	AY646288		JJG311
	/Megascelidini	*Megascelis* sp.	AF267463	AY243652	Costa Rica	JJG244
	Synetinae	*Syneta adamsi*	FJ000526*	AY171441	Korea	CND543
	Chrysomelinae/Chrysomelini	*Chrysomela tremulae*	FJ000527*	AY171423	France	SJK705
	/Timarchini	*Timarcha tenebricosa*	FJ000528*	FJ000452*	France	CND521
	Galerucinae/Galerucini	*Oides decempunctata*	AY244868	AY243674		
	/Alticini	*Podagrica malvae*	FJ000529*	FJ000453*	Spain	CND460
Curculionoidea						
	Nemonychidae					
	Rhinorhynchinae	*Mecomacer* sp.	FJ000530*	FJ000454*	Chile	CND681
		Rhynchitomacerinus kuscheli	FJ000531*	FJ000455*	Argentina	CND673
		Basiliorhinus sp. *(cf. araucariae)*	FJ000532*	FJ000456*	Australia	CND683
	Anthribidae					
	Anthribinae	Ptychoderes rugicollis	FJ000533*	FJ000457*	Panama	CND753
		Toxonotus sp.	FJ000534*	FJ000458*	USA	CND762
	Belidae					
	Belinae/Belini	*Rhinotia* sp.	FJ000535*	FJ000459*	Australia	CND692
		Rhinotiodes spinipennis	FJ000536*	FJ000460*	Australia	CND643
	Oxycoryninae/Metrioxenini	*Metrioxena* sp1 *(cf. serricollis)*	FJ000537*	FJ000461*	Indonesia	CND830
		Mertrioxena sp2	FJ000538*	FJ000462*	Indonesia	CND832, 833 & 831
	Attelabidae					
	Attelabinae/Attelabini	*Attelabus nigripes*	FJ000539*	FJ000463*	USA	CND655
	/Hoplapoderini	*Phymatopoderus latipennis*	FJ000540*	FJ000464*	Korea	CND755
	Rhynchitinae/Auletini	*Auletobius* sp.	FJ000541*	FJ000465*	Dom. Rep.	CND668
	Caridae					
	Carinae	*Car condensatus*	FJ000542*	FJ000466*	Australia	CND 562
	Brentidae					
	Brentinae	*Paratrachelizus dispar*	FJ000543*	FJ000467*	Panama	CND699
	Apioninae	*Rhinorhynchidius* sp.	FJ000544*	FJ000468*	Australia	CND691
	Curculionidae					
	Erirhininae/Erirhinini	*Notaris acridulus*	FJ000545*	FJ000469*	Poland	CND760
	/Stenopelmini	*Cyrtobagous salviniae*	FJ000546*	FJ000470*	Australia	CND768
	/Tanysphirini	*Tanysphirus lemnae*	FJ000547*	FJ000471*	Poland	CND766
	Dryophthorinae/Sphenophorini	*Sphenophorus* sp.	FJ000548*	FJ000472*	Panama	CND751
		Cactophagus sp.	FJ000549*	FJ000473*	Panama	CND767
	/Rhynchophorini	*Rhynchophorus palmarum*	AJ850018	AY131092		
	Cossoninae/Araucariini	*Araucarius minor*	AF308304	AF308351		
	/Cossonini	*Cossonus* sp.	AJ850000	AF308349		

Table 1 *Continued.*

Series/**Superfamily**	Family/Subfamily	Species	18S rDNA	28S rDNA	Country	Extract code
	Scolytinae/Hylesinini/Hylastina	*Hylastes porculus*	AF308339	AF308387		
	/Hylesinini/Tomicina	*Hylurgonotus tuberculatus*	AF308328	AF308375		
	Molytinae/Hylobiini	*Heilipodus argentinicus*	FJ000550*	FJ000474*	Argentina	CND678
	/Tranes group of genera	*Tranes vigorsii*	FJ000551*	FJ000475*	Australia	CND774
		Melanotranes roei	FJ000552*	FJ000476*	Australia	CND775
	Cryptorhynchinae/Cryptorhynchini	*Rhyephenes goureaui*	FJ000553*	FJ000477*	Argentina	CND672
	Entiminae/Ophryastini	*Ophryastes* sp.	FJ000554*	FJ000478*	USA	CND754
	/Cneorhinini	*Protostrophus* sp.	FJ000555*	FJ000479*	S. Africa	CND758
	/Naupactini	*Naupactus xanthographus*	FJ000556*	FJ000480*	Argentina	CND675
	Eugnominae	*Meriphus* sp.	FJ000557*	FJ000481*	Australia	CND689
	Ceutorhynchinae/Ceutorhynchini	*Ceutorhynchus* sp.	FJ000558*	FJ000482*	Turkey	CND794
	Baridinae/Baridini	*Melanobaris* sp.	FJ000559*	FJ000483*	Turkey	CND798

*New sequences from this study, with geographic procedence and DNA extraction codes for these taxa listed as recorded on vouchered specimens.
†Sequences provided by David Hawks. Identifications for the following specimens in Table 1 are provided in brackets: Buprestidae Gen. sp. (*Chrysobothris* sp.); Cleridae Gen. sp. (*Enoclerus* sp.); Prioninae Gen. sp. (*Xixuthrus heros*).

selection, MCMC parameter settings and burn-in criterion follow Gillespie *et al*. (2008).

Results and discussion

Structural alignments and models

Multiple sequence alignments spanning variable regions V4-V5 and V7-V9 of 18S rRNA and expansion segment D2 of 28S rRNA were generated across 104 beetle taxa. The complete alignments are posted at the jRNA web site (*http://hymenoptera.tamu.edu/rna/models.php*) and as supplementary material for this article on line. Figures 1 and 2 show the annotated structural alignments of the analysed regions of 18S and 28S rRNA, respectively, for a sample of nine beetle taxa. Additionally, the annotation is extended for the entire 18S rRNA across three beetles (Fig. 1), and based on comparison with several existing 18S rDNA sequences of Coleoptera, we suggest that the model presented here is applicable throughout the order. The comparative approach undertaken in this paper yielded improved annotated structural models of coleopteran 18S and 28S (D2 expansion segment only) rRNA, and is concordant with the current structural model of arthropod rRNA (Gillespie *et al*. 2006).

Phylogenetic relationships

Maximum parsimony analysis of the aligned 18S and 28S rDNA data resulted in 34 trees of length 4786, the strict consensus of which is presented in Fig. 3. Results from the Bayesian analyses are shown in Fig. 4, and the model parameters, model parameter summaries and revised plot of likelihood are provided as supplementary material. The parsimony and Bayesian trees are highly concordant, with minimal differences.

The results recover the monophyly of Phytophaga, including Curculionoidea and Chrysomeloidea as sister groups. The curculionoid and chrysomeloid phylogeny based on the aligned 18S and 28S rDNA segments, which is independent of morphological data, is in agreement with recent hypotheses or ideas based on morphological evidence, particularly with respect to familial relationships. The results provide clues about the evolutionary origin of the phytophagan beetles, suggesting that the sister group of Curculionoidea plus Chrysomeloidea is a clade of Cucujoidea, represented in this study by species in Boganiidae, Erotylidae, Nitidulidae, Cucujidae and Silvanidae. Consistently separated from such phytophagan-cucujoid clade result the Coccinellidae and Endomychidae (representing the cerylonid group in cucujoid classification). Some hypotheses on cucujoid relatedness based on morphology are concordant with our molecular results, such as Silvanidae being close to Cucujidae (Leschen *et al*. 2005), as well as Languriinae belonging to Erotylidae (Leschen & Buckley 2007), while evidence from male genitalia (Wanat 2007) also suggests the association of part of the Cucujoidea with weevils and chrysomeloids.

Within Curculionoidea, we recover the relationships between the 'seven' weevil families, in concordance with current hypotheses based on morphology (Marvaldi & Morrone 2000; Marvaldi *et al*. 2002; Oberprieler *et al*. 2007). The Nemonychidae plus Anthribidae are the sister clade to the remaining curculionoids, the Belidae are the sister clade to the rest of Curculionoidea (excluding Nemonychidae plus Anthribidae), the Attelabidae are the sister clade of Caridae plus Brentidae and Curculionidae, and Caridae is the taxon most closely related to the clade Brentidae plus Curculionidae. Monophyly of the major weevil lineages (families) is recovered, yet the Brentidae was paraphyletic in the MP tree (Fig. 3) but monophyletic in the Bayesian tree (Fig. 4).

Within Chrysomeloidea, the results provide additional support for the growingly popular concept of Chrysomeloidea

Fig. 1 Multiple sequence alignment of primary and secondary structure of regions of 18S rDNA analysed in this study from nine diverse beetle species (*Trachykele blondeli* (Buprestoidea), *Blackburniella intricata* (Cleroidea), *Chondria armipes* (Cucujoidea: Endomychidae), *Tenebrio molitor* (Tenebrionoidea), *Paracucujus rostratus* (Cucujoidea: Boganiidae), *Cucujus clavipes* (Cucujoidea: Cucujidae), *Palophagoides vargasorum* (Chrysomeloidea: Megalopodidae), *Rhynchitomacerinus kuscheli* (Curculionoidea: Nemonychidae), *Cossonus* sp. (Curculionoidea: Curculionidae)). The annotation is extended to the entire 18S rRNA for three of these beetle species (in bold). Complementary strands are indicated with a prime (e.g., strand X hydrogen bonds with strand X′ to form helix X). Regions of alignment ambiguity (RAA) are placed within square brackets. Nucleotide positions within helices involved in hydrogen-bonding are depicted by parentheses in the mask. Single insertions (*) and deletions (–) are noted as in Kjer *et al*. (2001). Missing nucleotides are represented with question marks (?). Lower-case letters correspond to nucleotides confirmed by one strand only in sequencing. Note: this alignment has not been amended for these nine taxa from the original alignment of 104 beetle sequences, and thus gaps and insertions may correspond to taxa not presented in this figure.

Fig. 1 *Continued.*

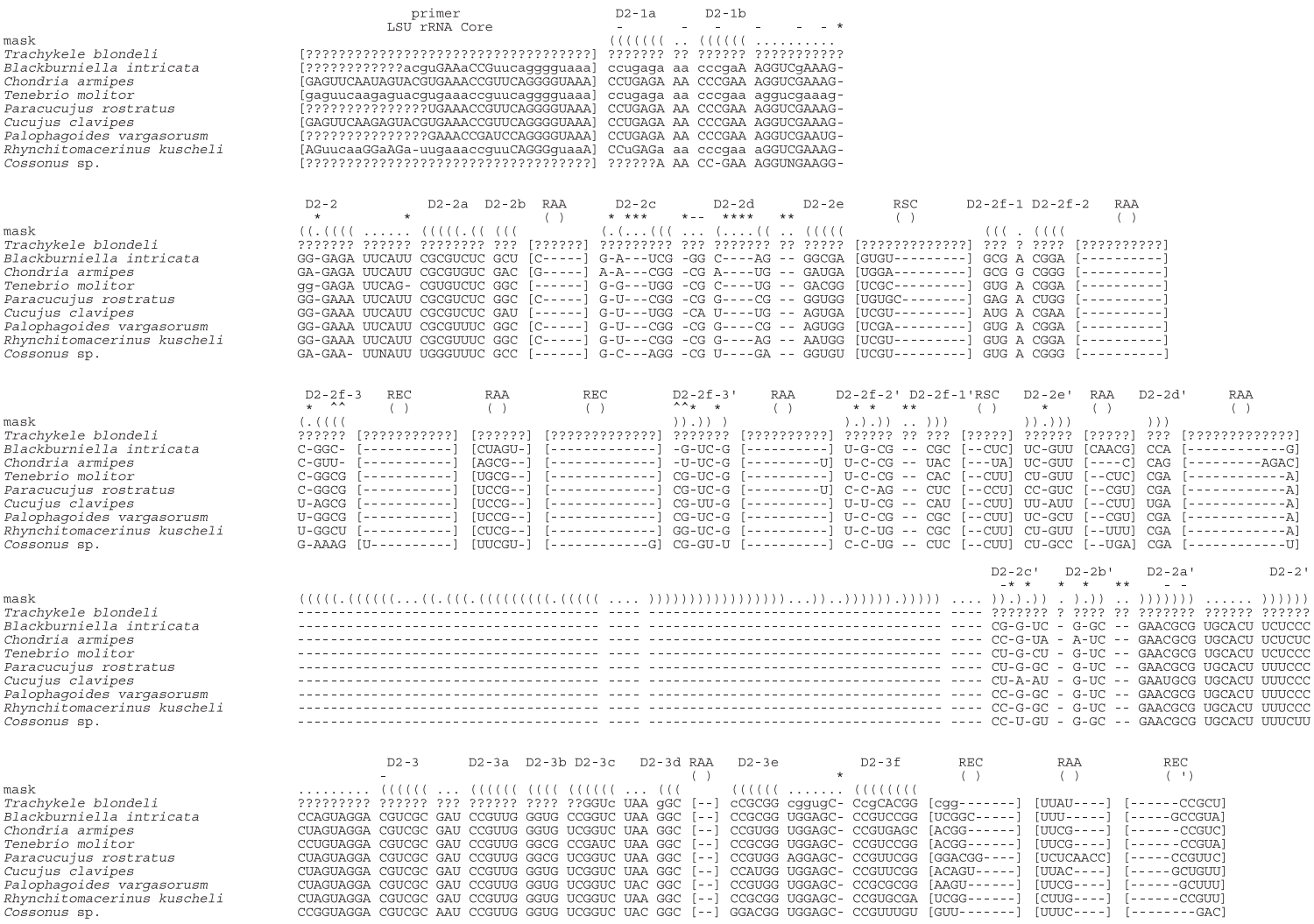

Fig. 2 Multiple sequence alignment of primary and secondary structure of the expansion segment D2 of the 28S rRNA gene region from nine beetle species (as in Fig. 1). Complementary strands are indicated with a prime (e.g., strand X hydrogen bonds with strand X′ to form helix X). Regions of alignment ambiguity (RAA), slipped-strand compensation (RSC) and expansion and contraction (REC) are placed within square brackets. Nucleotide positions within helices involved in hydrogen-bonding are depicted by parentheses in the mask. Single insertions (*) and deletions (–) are noted as in Kjer *et al.* (2001). Positions that can form an expansion of a helix across some but not all taxa are labelled with a caret (^). Missing nucleotides are represented with question marks (?). Lower-case letters correspond to nucleotides confirmed by one strand only in sequencing. Note: this alignment has not been amended for these nine taxa from the original alignment of 104 beetle sequences, and thus gaps and insertions may correspond to taxa not presented in this figure.

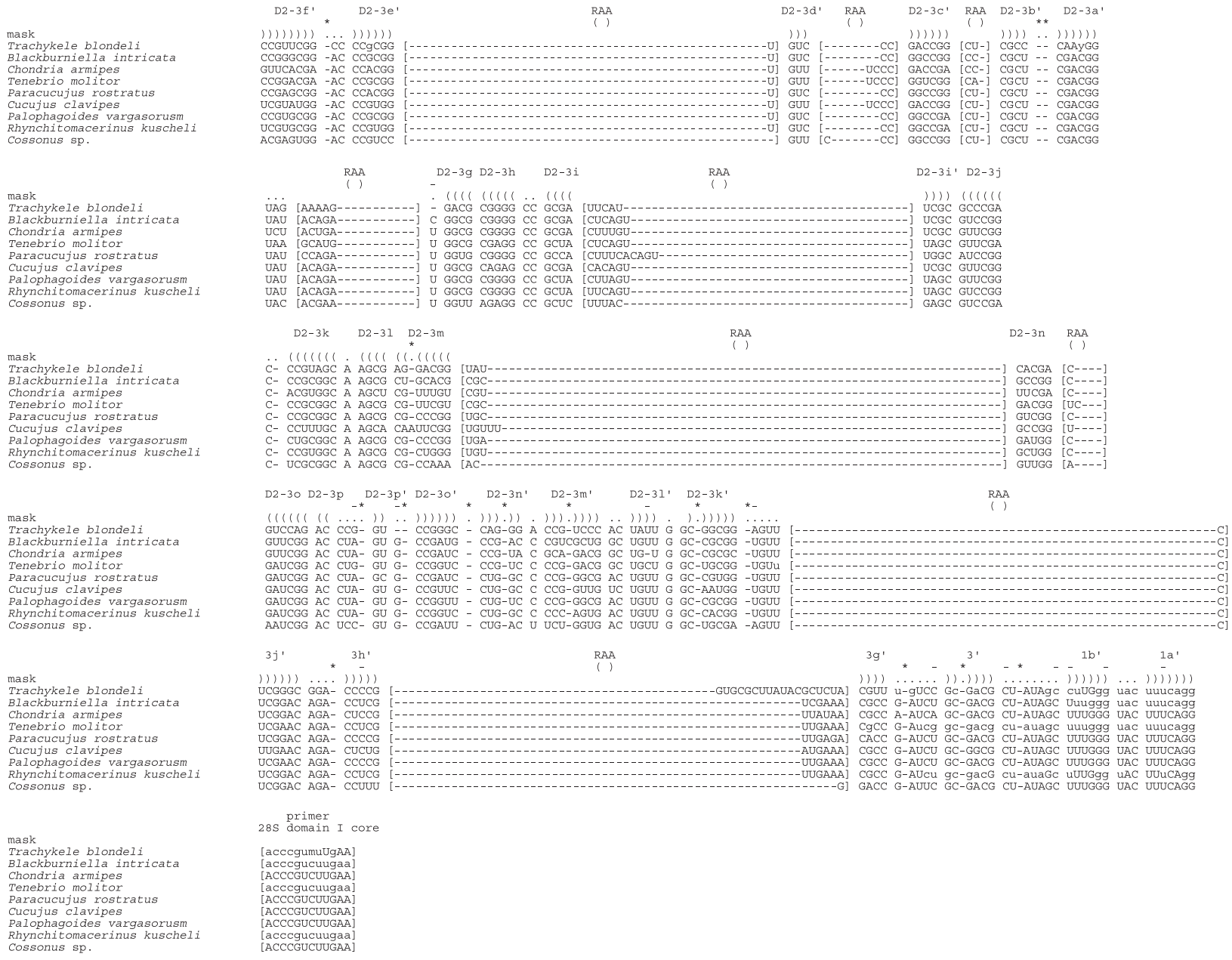

Fig. 2 *Continued.*

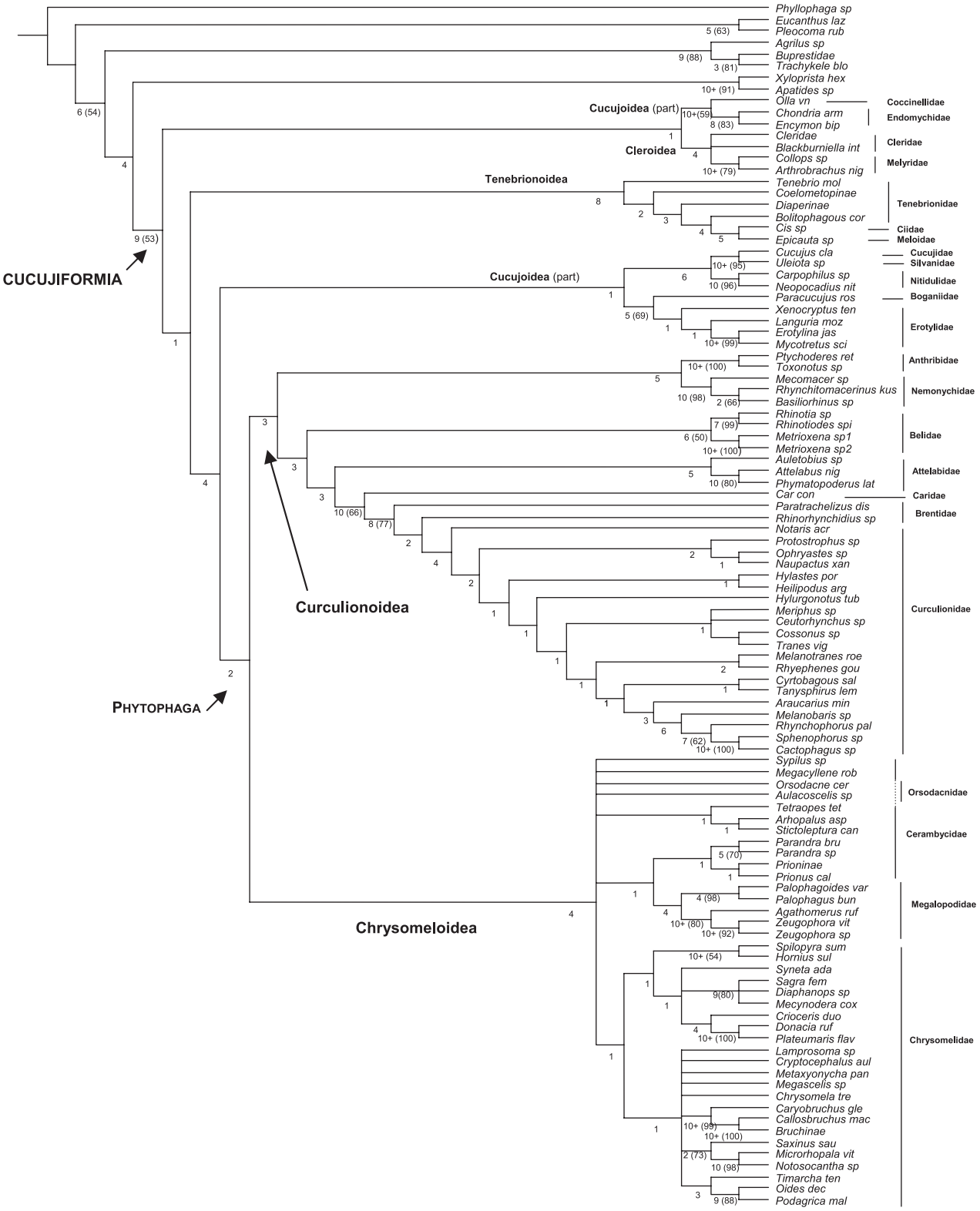

Fig. 3 Phylogenetic relationships of Coleoptera Cucujiformia based on the parsimony analysis of the structurally aligned 18S and 28S rDNA sequences. This is the strict consensus of 34 MPTs of length = 4786. Numbers below branches indicate Bremer support and bootstrap values over 50% (in brackets).

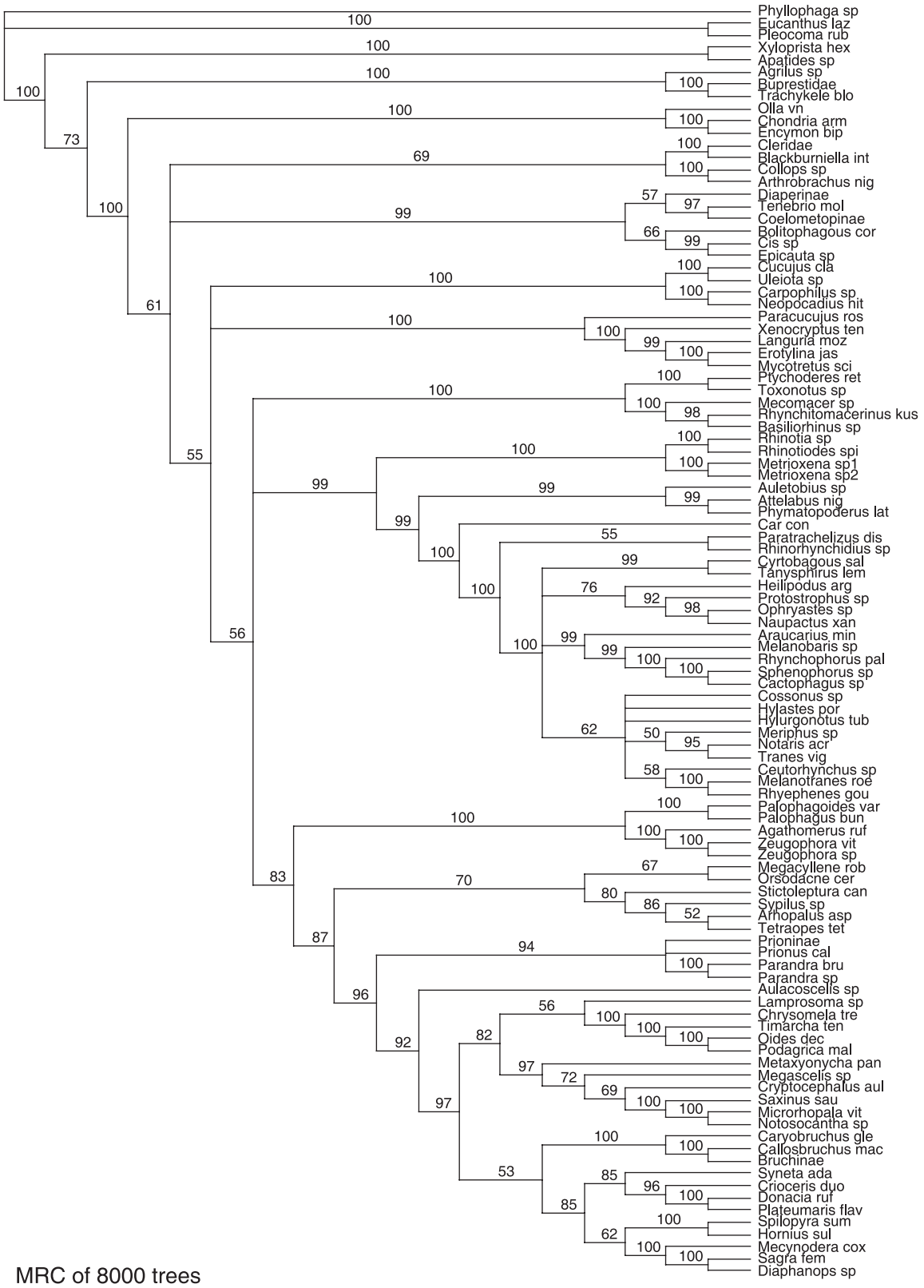

Fig. 4 Phylogenetic relationships of Coleoptera Cucujiformia based on the Bayesian analysis of the structurally aligned 18S and 28S rDNA sequences. The posterior probability values from the majority rule consensus tree (from 8000 trees) are given above the nodes.

inclusive of Cerambycidae. Our phylogeny recovers the monophyly of two of the four chrysomeloid families. The family Chrysomelidae (with all currently recognized subfamilies sampled in our study) is monophyletic, including Bruchinae and excluding Orsodacninae and Aulacoscelinae (as in Reid 1995, 2000). Among the 'novel' subfamilial relationships proposed here, it is worth to note the placement of the Spilopyrinae in a clade with the Sagrinae, Synetinae, Donaciinae and Criocerinae. The affinities and classificatory rank of the spilopyrines have been controversial, with the genera having been described in different chrysomeloid taxa (Reid 2000: 855). Our results support their subfamily status as proposed by Reid (2000), but suggest their affinity to sagrines. In agreement with Verma & Jolivet (2002) is the placement of the Synetini outside the Eumolpinae. Our result concerning the position of the Cassidinae *s. l.* (incl. hispines) in a 'eumolpine' clade near Cryptocephalinae and separate from other chrysomelids with bifid tarsal setae (Donaciinae, Criocerinae, Sagrinae, Bruchinae) for which they typically have been associated (e.g., Reid 1995, see also Duckett *et al*. 2004) agrees with Gómez-Zurita *et al.* (2007). The clade Megalopodidae includes Palophaginae, Megalopodinae and Zeugophorinae, in agreement with Kuschel & May (1990).

Both the 18S and 28S rRNA fragments (characters 1–1030 and 1031–1531 in the analysed data file) contribute to resolving beetle higher level relationships, as can be noticed by examining the unambiguous apomorphies defining each node. For example, of the 20 apomorphies supporting the clade Cucujiformia, seven are from 18S (four are unique changes, with CI = 1) and 13 are from 28S (four are unique changes). The important phytophagan – cucujoid clade is supported by eight unambiguous apomorphies, of which three come from 18S and five (two unique) from 28S. The Phytophaga is defined by six unambiguous apomorphies, of which two come from 18S and four from the 28S fragment. Both markers also provide characters supporting interfamilial relationships. For instance, in Curculionoidea, the clade Caridae plus Brentidae and Curculionidae is supported by 12 unambiguous apomorphies, of which four (one unique) are contributed by 18S and eight (one unique) come from the 28S fragment.

Conclusions

We constructed multiple sequence alignments for regions of 18S and 28S rDNA from a wide sample of beetles (Coleoptera: Polyphaga). The comparative approach undertaken in this study yielded improved annotated structural models of the 18S and 28S (D2 expansion segment only) rRNAs of Coleoptera, and contribute to the growing knowledge-base of arthropod and arthropod taxon-specific rRNA structure. These structures will be useful as homology templates for future comparative studies utilizing rDNA sequences of insects and related arthropods (see Morrison 2006).

This study represents a formal test of the monophyly of Phytophaga, and a preliminary approach to place them in the beetle tree, in relation to other cucujiform beetles. The expansion of the taxon sampling and the use of additional molecular markers, as well as the combination with morphological data, are further steps toward the larger goal of reconstructing a robust phylogeny of Phytophaga and clarifying details of their relationship with cucujoid beetles. Because results of this paper come from two structurally aligned rDNA markers and are largely concordant (or not in conflict) with hypotheses based on morphology, we have renewed confidence in our hypothesis, and our approach towards incorporating structural information into the process of alignment and phylogeny estimation.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Phyt_18S_Nov07.doc
Alignment of 18S rRNA sequences of the 104 beetle species.

28SD2Phyt_Nov07.doc
Alignment of D2 28S rRNA sequences of the 104 beetle species.

Phyt_104taxa_1aem.Sep07.txt
Nexus file with the combined aligned data.

Tracer_all_final.doc
Model parameters.

PSRFs_all.doc
Model parameter summaries.

phyt_Tracer.pdf
Revised plot of likelihoods.

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