Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 34 (2005) 584–600
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular systematics of Eumolpinae and the relationships with Spilopyrinae (Coleoptera, Chrysomelidae)

Jesús Gómez-Zurita[a,b,*,1], Pierre Jolivet[c], Alfried P. Vogler[a,b]

[a] *Department of Entomology, The Natural History Museum, London, UK*
[b] *Department of Biological Sciences, Imperial College London, Silwood Park Campus, Ascot, Berkshire, UK*
[c] *67 Boulevard Soult, Paris, France*

Received 5 April 2004; revised 8 November 2004
Available online 8 January 2005

Abstract

The 3400 species of Eumolpinae constitute one of the largest subfamilies of leaf beetles (Chrysomelidae). Their systematics is still largely based on late 19th century monographs and remains highly unsatisfactory. Only recently, some plesiomorphic lineages have been split out as separate subfamilies, including the southern hemisphere Spilopyrinae and the ambiguously placed Synetinae. Here we provide insight into the internal systematics of the Eumolpinae based on molecular phylogenetic analyses of three ribosomal genes, including partial mitochondrial 16S and nuclear 28S and complete nuclear 18S rRNA gene sequences. Sixteen morphological characters considered important in the higher-level systematics of Eumolpinae were also included in a combined analysis with the molecular characters. All phylogenetic analyses were performed using parsimony by optimizing length variation directly on the tree, as implemented in the POY software. The data support the monophyly of the Spilopyrinae outside the clade including all sampled Eumolpinae, corroborating their treatment as a separate subfamily within the Chrysomelidae. The systematic placement of the Synetinae remains ambiguous but consistent with considering it a different subfamily as well, since the phylogenetic analyses using all the available evidence show the representative sequence of the subfamily also unrelated to the Eumolpinae. The Megascelini, traditionally considered a separate subfamily, falls within the Eumolpinae. Several recognized taxonomic groupings within Eumolpinae, including the tribes Adoxini or Typophorini, are not confirmed by molecular data; others like Eumolpini seem well supported. Among the morphological characters analyzed, the presence of a characteristic groove on the pygidium (a synapomorphy of the Eumolpini) and the shape of tarsal claws (simple, appendiculate or bifid) stand out as potentially useful characters for taxonomic classification in the Eumolpinae.

Keywords: Direct optimization; Leaf beetles; Phylogeny; Ribosomal markers; Southern hemisphere

1. Introduction

Leaf beetles in the Eumolpinae constitute one of the largest subfamilies within the species-rich Chrysomelidae, with over 400 genera and more than 3400 species worldwide, displaying their highest diversity in the tropics (Jolivet, 1997; Seeno and Wilcox, 1982). Several characters have been used to typify the Eumolpinae, including the generalized lack of apical spurs in tibiae, labrum not divided, abdominal segments without median constrictions, separated antennal insertions, lack of transversal constriction on pronotum, rounded front coxal cavities, presence of characteristic vaginal glands, structure of male genitalia, larval ecology and morphology, and very importantly the characteristics of hind wing venation, always with two cubital cells (lack of

* Corresponding author. Fax: +34 968 364906.
E-mail address: jgzurita@um.es (J. Gómez-Zurita).
[1] Present address: Área de Biología Animal, Departamento de Zoología y Antropología Física, Facultad de Biología, Universidad de Murcia, Murcia, Spain.

1055-7903/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2004.11.022

cubital binding patch mostly among primitive genera and tribes) (Flowers, 1996, 1999; Jolivet, 1954, 1957–1959, 1997; Jolivet and Verma, 2002; Reid, 1995). While the limits of the subfamily seem to be relatively well established, the suprageneric classification of the Eumolpinae is not satisfactory at all and has remained essentially untouched since the first major revisions of the group by Chapuis (1874) and Lefèvre (1885). The generic classification of the subfamily also demands a thorough revision. Various authors have referred to the taxonomy of Eumolpinae as"chaos" (Jacoby, 1888; Lea, 1915) or "mess"(Reid, 1995), and emphasized the difficulties of finding useful characters for establishing natural groups at any hierarchical level in this subfamily (Horn, 1892; Lea, 1915; Selman, 1965).

A few recent taxonomic changes have renewed the interest in higher classification of the Eumolpinae. Reid (1995), in a comprehensive review of the systematics and phylogeny of the Chrysomelidae based on adult and larval morphological characters, established the Spilopyrini from a group of several genera that had been associated with various tribes of the Eumolpinae (e.g., Seeno and Wilcox, 1982). The Spilopyrini *sensu* Reid (1995) have an intriguing distribution limited to the Southern hemisphere, from Chile (*Hornius* Fairmaire and *Stenomela* Erichson) to Australia (*Macrolema* Baly, *Cheiloxena* Baly, *Richmondia* Jacoby, and *Spilopyra* Baly), New Guinea (*Macrolema* and *Spilopyra*), and New Caledonia (*Bohumiljania* Monrós). The recognition of highly divergent larval characters, e.g., sighted surface-living spilopyrine larvae compared to blind root-feeding eumolpine larvae (Jerez, 1995; Jerez and Ibarra-Vidal, 1992; Reid, 2000), later resulted in the elevation of this group to subfamily, the Spilopyrinae (Reid, 2000). However, as Reid's grouping was mostly based on symplesiomorphic characters, this opinion has been debated recently by Verma and Jolivet (2002) who evaluated several characters of hind wing venation (similar to primitive eumolpines), female and male genitalia, and larval features. According to their view, the spilopyrines constitute a primitive lineage within the Eumolpinae, deserving tribal status.

A further conclusion from Reid's (1995) pioneering phylogenetic approach to the systematics of Chrysomelidae was the analysis of the taxonomically unstable Synetinae which have been difficult to place relative to other chrysomelids using morphological and ecological data (Reid, 1995, 2000; Verma and Jolivet, 2000, 2002; and references therein). Reid (1995) associated this group as the tribe Synetini within the Eumolpinae, together with the plesiomorphic eumolpine *Eupales* Lefèvre (Reid, 2000), in agreement with other published studies of immature stages (e.g., Cox, 1998; Lee, 1990). In contrast, Verma and Jolivet (2000) treated the synetines as a subfamily on the basis of an unusual character combination linking them to various well-recognized groups of diverse taxonomic ranks, including the Orsodacnidae, Galerucinae, Chrysomelinae, and Eumolpinae. A further important finding in the context of eumolpine relationships was the confirmation from cladistic analyses which placed the tribe Megascelini within or very close to the Eumolpinae (Bechyné and Bechyné, 1969; Jolivet, 1954, 1957–1959). This view has been ignored in the systematic arrangement of the Chrysomelidae in Seeno and Wilcox (1982) and in other recent works, and would benefit from further testing.

In this paper we present the results of a phylogenetic study based on mitochondrial and nuclear ribosomal RNA genes for a sample of Eumolpinae, using direct optimization analyses (Wheeler, 1996, 1999). Some recent molecular phylogenetic studies based on 18S rDNA sequences only and focusing on the higher-level systematics of the Chrysomelidae have included a small number of Eumolpinae (Duckett et al., 2004; Farrell, 1998; Farrell and Sequeira, 2004). These studies also included samples of *Syneta* Dejean and *Megascelis* Sturm, showing that the former did not and the latter did group within the monophyletic Eumolpinae. Simultaneously to the revision of our study, Farrell and Sequeira's (2004) recent study of the Chrysomelidae incorporated one sample of *Spilopyra sumptuosa*, which also did not cluster with the other Eumolpinae. Our study includes representatives of the main tribes, together with four genera of Spilopyrinae, and other taxa relevant to resolving basal eumolpine relationships, such as *Syneta*, *Eupales*, and *Megascelis*. We address the following questions: Is there molecular support for the proposition of a Spilopyrinae separate from the Eumolpinae, and do the molecular data confirm the monophyly of the proposed member taxa formerly included in disparate eumolpine tribes? What do the molecular phylogenetic analyses add to the controversy about the systematic placement of *Syneta* and *Eupales*, taxa with many symplesiomorphic characters and of unclear affinities with the Eumolpinae? Do the traditionally used morphological characters identify monophyletic lineages? How does the existing higher-level systematics of the Eumolpinae conform to the hypotheses generated from DNA markers? These studies will help to establish relationships of one of the least clearly defined of the ten or more subfamilies of the Chrysomelidae, and hence will contribute to resolve the phylogenetic relationships of basal groups in this large assembly of phytophagous beetles.

2. Materials and methods

2.1. Samples

Table 1 shows the list of specimens and taxa used in the molecular phylogenetic analyses. These include samples of 4 out of 7 genera of the subfamily Spilopyrinae, 2 genera of the Synetini (*sensu* Reid, 2000), a

Table 1
Studied taxa, sources, and GenBank accession numbers

Taxon[a]	Source	16S	18S	28S
Spilopyrinae [7][b]				
Hornius grandis (Philippi et Philippi)	Chile: Valdivia	AJ781507	AJ781561	AJ781624
Bohumiljania caledonica (Jolivet)	New Caledonia: La Foa	AJ781508	AJ781562	AJ781625
Stenomela pallida Erichson	Chile: Concepción, Hualpén	AJ781509	AJ781563	AJ781626
Spilopyra sumptuosa Baly	Australia: New South Wales, Murvillumbah	AJ781510	AJ781564	AJ781627
Eumolpinae [420]				
Synetini[c] [2]				
Eupales ulema (Germar)	Greece: Ipiros, Ioanina, env. Papingo	AJ781511	AJ781565	AJ781628
Syneta adamsi Baly	China: Hebei, Wulingshan	AJ781512	AJ781566	AJ781629
Megascelini[d] [2]				
Megascelis sp. [Sturm]	Nicaragua: Managua, Ticuantepe, R.N.P. Montibelli	AJ781513	AJ781567	AJ781630
Typophorini Chapuis [101]				
Nodostomites Chapuis [18]				
Basilepta multicostata Jacoby	Malaysia: Sabah, ca. 25 km SE Sapulut, Batu Punggul env.	AJ781514	AJ781568	AJ781631
Basilepta nr. *nitida* (Baly)	Malaysia: Sabah, ca. 25 km SE Sapulut, Batu Punggul env.	AJ781515	AJ781569	AJ781632
Basilepta nr. *wallacei* (Baly)	Malaysia: Sabah, ca. 25 km SE Sapulut, Batu Punggul env.	AJ781516	AJ781570	AJ781633
Undetermined genus 1	Australia: Queensland, Brisbane, Mt. Coot-tha	AJ781517	AJ781571	AJ781634
Pagriites Lefèvre [1]				
Pagria signata (Motschulsky)	India: Chattisgarh, Durg, Bhilai Steel Township, Sector 8 Pk.	AJ781518	AJ781572	AJ781635
Metachromites Chapuis [21]				
Rhyparida alleni Lea	Australia: Queensland, Brisbane, Mt. Coot-tha	AJ781519	AJ781573	AJ781636
Rhyparida dimidiata Baly	Australia: Queensland, Brisbane, Mt. Coot-tha	AJ781520	AJ781574	AJ781637
Typophorites Chapuis [59]				
Eulychius nr. *dentipes* Bechyné	Madagascar: Anjanaharibe	—	AJ781575	AJ781638
Paraivongius sp. [Pic]	Sudan: Mapuordit	—	AJ781576	AJ781639
Paria fragariae Wilcox	Canada: Ontario, Haldimand-Norfolk, Port Ryesse	AJ781521	AJ781577	AJ781640
Paria sellata (Horn)	USA: PA, Adams Co., Gettysburg	AJ781522	AJ781578	AJ781641
Pheloticus sp. [Harold]	Madagascar: Amparihibe	AJ781523	AJ781579	AJ781642
Phytorus dilatatus Jacoby	Malaysia: Sabah, ca. 25 km SE Sapulut, Batu Punggul env.	AJ781524	AJ781580	AJ781643
Phytorus sp. [Jacoby]	Sumatra: Gn Talamau, Ophir Mts., 17 km E Simpangempat	AJ781525	AJ781581	AJ781644
Pseudosyagrus grossepunctatus Fairm.	Madagascar: Amparihibe	AJ781526	AJ781582	AJ781645
Pseudosyagrus sp. [Fairmaire]	Madagascar: Anjanaharibe	—	AJ781583	AJ781646
Proliniscus sp. [Selman]	Tanzania: Tanga District, Muheza Village	—	AJ781584	AJ781647
Colasposomini Springlová [22]				
Colasposomites Wilcox [10]				
Colasposoma auripenne Motschulsky	India: Chattisgarh, Durg, Bhilai Steel Township, Sector 8 Pk.	AJ781527	AJ781585	AJ781648
Colasposoma pretiosum Baly	Nepal: Gandaki zone, Gorkha Distr., Gorkha env.	AJ781528	AJ781586	AJ781649
Colasposoma sp. [Laporte]	Malaysia: Pahang, Kuala Lipis env., Kenong Pumba Pk., Kenong river	AJ781529	AJ781587	AJ781650
Eumolpini Jacoby [171]				
Iphimeites Chapuis [133]				
Brachypnoea clypealis Horn	USA: NJ, Cape May Co., Cape May	—	AJ781588	AJ781651
Brachypnoea tristis (Olivier)	USA: WV, Greenbrier Co., Tuckahoe Lake	—	AJ781589	AJ781652
Chrysodinopsis curtula (Jacoby)	Mexico: Guerrero, Mezcala Bridge Overlock	AJ781530	AJ781590	AJ781653
Colaspis gr. *flavicornis* Fabricius	French Guyana: Cayenne Harbour, Mt. Rorota, 100m	AJ781531	AJ781591	AJ781654
Colaspis flavipes (Olivier)	French Guyana: Cayenne Harbour, Mt. Rorota, 100 m	AJ781532	AJ781592	AJ781655
Colaspis sp. 1 [Fabricius]	French Guyana: Tresor Mountains, Road to Kaw, 200 m	AJ781533	AJ781593	AJ781656
Colaspis sp. 2 [Fabricius]	Nicaragua: Selva Negra	—	AJ781594	AJ781657
Hermesia aurata (Olivier)	French Guyana: Cayenne Harbour, Mt. Rorota, 100 m	AJ781534	AJ781595	AJ781658
Lamprosphaerus sp. 1 [Baly]	French Guyana: Cayenne Harbour, Mt. Rorota, 100 m	AJ781535	AJ781596	AJ781659
Lamprosphaerus sp. 2 [Baly]	French Guyana: Tresor Mountains, Road to Kaw, 200 m, Camp Caiman	AJ781536	AJ781597	AJ781660
Nodonota sp. [Lefèvre]	French Guyana: Tresor Mountains, Road to Kaw, 200 m	AJ781537	AJ781598	AJ781661
Percolaspis nr. *gestroi* (Jacoby)	French Guyana: 30 km S Cayenne: Crossroad N1-D5	AJ781538	AJ781599	AJ781662
Percolaspis pulchella (Lefèvre)	French Guyana: Cayenne Harbour, Mt. Rorota, 100 m	AJ781539	AJ781600	AJ781663
Promecosoma viride Jacoby	Mexico: Oaxaca, road between Oaxaca and Ejutla	AJ781540	AJ781601	AJ781664
Rhabdopterus praetextus (Say)	Canada: Quebec, Quyon	AJ781541	AJ781602	AJ781665
Undetermined genus 2	New Caledonia: Ile des Pines, between Grotte Reine Hortense and Kwanyi	AJ781542	AJ781603	AJ781666

Table 1 (*continued*)

Taxon[a]	Source	16S	18S	28S
Edusites Chapuis [17]				
Edusella puberula Bohemann	Australia: Queensland, Brisbane, Mt. Coot-tha	—	AJ781604	AJ781667
Edusella sp. 1 [Chapuis]	Australia: Queensland, Brisbane, Mt. Coot-tha	AJ781543	AJ781605	AJ781668
Edusella sp. 2 [Chapuis]	Australia: Queensland, Brisbane, Mt. Coot-tha	AJ781544	AJ781606	AJ781669
Tymnes tricolor (Fabricius)	USA: LA, W. Feliciana Par., Feliciana Preserve	AJ781545	AJ781607	AJ781670
Corynodites Chapuis [5]				
Chrysochus auratus (Fabricius)	USA: NJ, Monmouth Co., Roosevelt	AJ781546	AJ781608	AJ781671
Platycorynus chalybaeus (Marshall)	Sumatra: Gn Talamau, Ophir Mts., 17 km E Simpangempat	AJ781547	AJ781609	AJ781672
Endocephalites Chapuis [14]				
Colaspoides nr. *simillima* Baly	Malaysia: Pahang, Kuala Lipis env., Kg. Malaka env.	AJ781548	AJ781610	AJ781673
Colaspoides sp. [Laporte]	Malaysia: Pahang, Cameron Highlands, Tahah Rata env.	AJ781549	AJ781611	AJ781674
Adoxini Jacoby [118]				
Scelodontites Chapuis [6]				
Scelodonta brevipilis Lea	Australia: Queensland, Brisbane, Mt. Coot-tha	AJ781550	AJ781612	AJ781675
Leprotites Chapuis [39]				
Lypesthes gracilicornis (Baly)	China: Hong Kong Is., Tai Tam Reg. Pk.	—	AJ781613	AJ781676
Bromiites Chapuis [4]				
Bromius obscurus (Linnaeus)	Russia: Tver region, Udomlya district, Kulikovo	AJ781551	AJ781614	AJ781677
Myochroites Chapuis [26]				
Myochrous sp. [Erichson]	French Guyana: 30 km S Cayenne, Crossroad N1-D5	AJ781552	AJ781615	AJ781678
Pachnephorus impressus Rosenhauer	India: Chattisgarh, Durg, Bhilai Steel Township, Sector 8 Pk.	AJ781553	AJ781616	AJ781679
Ebooina Reid [7]				
Parascela cribrata (Schaufuss)	China: Hong Kong Is., Tai Tam Reg. Pk.	AJ781554	AJ781617	AJ781680
Outgroup				
Galerucinae				
Diabrotica undecimpunctata howardi Barber	USA: VA, Rockingham Co., Harrisonburg	AJ781555	AJ781618	AJ781681
Chrysomelinae				
Linaeidea aenea (Linnaeus)	Germany: Upper Bavaria, Benediktbeuern	AJ781556	AJ781619	AJ781682
Bruchinae				
Bruchidius sp. [Schilsky]	Spain: Badajoz, Garbayuela	AJ781557	AJ781620	AJ781683
Criocerinae				
Crioceris asparagi (Linnaeus)	USA: VA, Rockingham Co., Harrisonburg	AJ781558	AJ781621	AJ781684
Donaciinae				
Donacia distincta LeConte	Canada: Ontario, Ottawa-Carleton RM, La Mer Bleue	AJ781559	AJ781622	AJ781685
Orsodacnidae				
Orsodacne atra Ahrens	Canada: Ontario, Renfrew, Sand Point	AJ781560	AJ781623	AJ781686

[a] The provisional classification follows Seeno and Wilcox (1982) with the modifications proposed by Reid (2000).

[b] The number in square brackets indicates the number of currently recognized genera in Spilopyrinae and Eumolpinae as an indicator of their diversification.

[c] The position of these taxa is unknown. We follow a provisional systematic placement recommended by Reid (2000) as a working hypothesis to test using molecular data.

[d] Megascelids are considered in a different subfamily in Seeno and Wilcox (1982), but they had been previously included within the Eumolpinae by Jolivet (1954, 1957–1959) and Bechyné and Bechyné (1969), a systematic placement that was phylogenetically demonstrated by Reid (1995).

representative of the supposedly primitive Megascelini, and a total of 51 species of Eumolpinae in 33 genera from 4 tribes, Typophorini, Colasposomini, Eumolpini, and Adoxini. This sample constitutes nearly 10% of the proposed genera in the Eumolpinae, and they represent around 60% of the suprageneric taxonomic units of this subfamily (*sensu* Seeno and Wilcox, 1982), including the largest and most widespread tribes and sections (Fig. 1). In addition, several representatives of other subfamilies of Chrysomelidae and *Orsodacne atra* (Orsodacnidae) were included as outgroups. Specimens were collected in the field or received from colleagues in absolute ethanol, and were preserved in the laboratory at −20 °C before processing. A few specimens, including *Hornius grandis*, *Eupales ulema*, *Syneta adamsi*, *Colasposoma pretiosum*, and *Colaspoides* sp. were studied from pinned collection samples, the oldest being *S. adamsi*, collected in 1994.

2.2. DNA isolation and genetic markers

Total DNA was extracted from the whole specimen using the DNeasy Tissue kit and following the manufacturer's instructions (Qiagen, West Sussex, UK). DNA was resuspended in 100–200 μl elution buffer. One microliter of the DNA solution was used in subsequent PCR amplifications of the selected markers. Three different phylogenetic markers were used for the study, including a partial sequence of the mitochondrial 16S rDNA (Simon et al., 1994; primers LR-N-13398 5′-CGC CTGTTTATCAAAAACAT-3′ and LR-J-12887 5′-CT

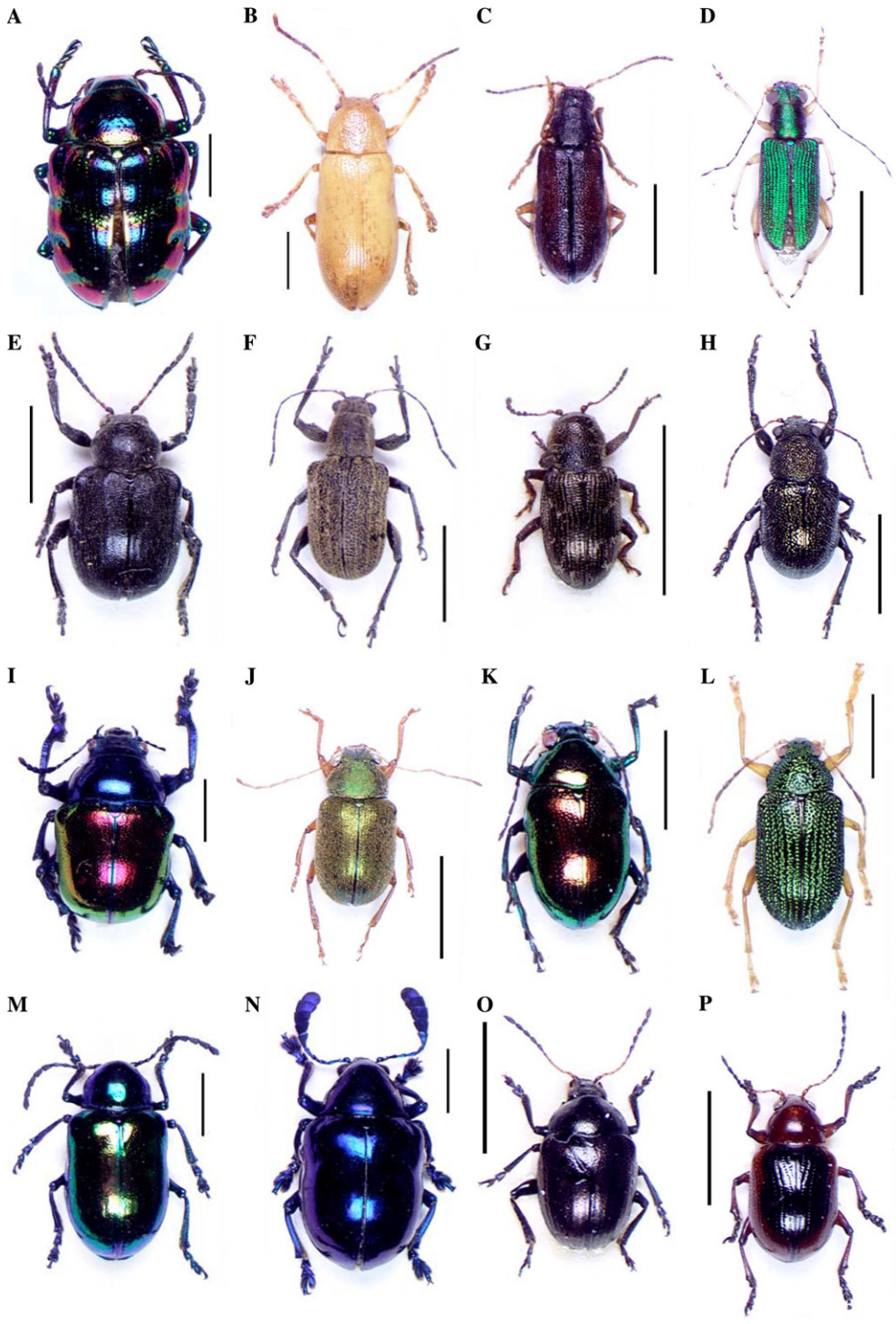

Fig. 1. Morphological diversity of the Eumolpinae and higher-taxa coverage examples in this study. (A) *Spilopyra sumptuosa* and (B) *Stenomela pallida* (Spilopyrinae); (C) *Syneta adamsi* (Synetinae); (D) *Megascelis* sp. (Megascelini); (E) *Bromius obscurus*, (F) *Lypesthes gracilicornis*, (G) *Pachnephorus impressus*, and (H) *Parascela cribrata* (Adoxini); (I) *Colasposoma pretiosum* (Colasposomini); (J) *Edusella* sp., (K) *Hermesia aurata*, (L) *Colaspis* sp., (M) *Chrysochus auratus*, (N) *Platycorynus chalybaeus*, and (O) *Brachypnoea clypealis* (Eumolpini); and (P) *Paria sellata* (Typophorini). Scale bars represent 3 mm.

CCGGTTTGAACTCAGATCA-3′), the nearly complete sequence of the nuclear 18S rDNA (using four primer combinations as described in Shull et al., 2001), and partial sequence of the nuclear 28S rDNA (primers 28SDD 5′-GGGACCCGTCTTGAAACAC-3′ and 28SFF 5′-TTACACACTCCTTAGCGGAT-3′). PCR consisted of 5 min at 96 °C, followed by 35 cycles of 30 s at 94 °C, 30 s 50 °C, 1 min at 72 °C, with a final extension

step of 10 min at 72 °C. PCR products were purified with the GeneClean II kit (Qbiogene, Livingston, UK) and sequenced in both directions with the same PCR primers and the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Cycle sequencing products were purified by ethanol/sodium acetate precipitation, prior to electrophoresis on an ABI 3700 automated sequencer (Applied Biosystems, Foster City, CA). Chromatograms were edited and contig-assembled using Sequencher version 4.1.2 (Gene Codes, Ann Arbor, MI). Sequences were deposited in GenBank under the accession numbers provided in Table 1.

2.3. Phylogenetic analyses

Character homology assessment and phylogenetic reconstruction, using each marker separately and in combination, were performed using 'direct optimization' analyses (Wheeler, 1996) as implemented in POY version 3.0.11 (Wheeler et al., 2002). Direct optimization was designed to conduct phylogenetic analyses on length variable sequence data, as is typically found in ribosomal RNA sequences. This method performs a parsimony analysis by optimizing nucleotide changes and insertion/deletions (indels) in a single step, and hence calculates the cost of character transformations on a tree based on a specified cost for indels relative to nucleotide substitutions. POY searches for the lowest cost tree, which is accepted as the optimal reconstruction of character variation, including putative insertions and deletions (Wheeler, 1996). The dynamic homology search maximizes global character congruence, which is a desirable property when analyzing characters from multiple sources as in the present study. The method does not produce an aligned matrix. However, a matrix of correspondences of homologous nucleotides (representing the character transformation path on a given tree) can be reconstructed from the inferred states at the hypothetical ancestors, to produce an 'implied alignment' (Wheeler, 2003).

The outcome of this analysis is dependent on the costs of indels applied to the transformation matrix. To test for the effects of different settings, a sensitivity analysis (*sensu* Wheeler, 1995) was performed using eight different weighting schemes: equal costs for substitutions and indels (111), indel cost twice (112), four (114), and eight times (118), and cost of transversions twice the weight of transitions, in combination with increasing the cost of indels (122, 124, 128, and 12[16]). Weighting schemes were chosen arbitrarily within a range of costs that usually produce meaningful results in similar phylogenetic studies (e.g., Gómez-Zurita, 2004). To speed up tree searches, sequences were subdivided into smaller portions for which homology is assumed to be unequivocal. Subdivision of the sequences was according to secondary structure for 16S rRNA (three fragments), and by separating conserved and variable domains in 18S (seven fragments) and 28S (five fragments) rRNA genes. The searches included a series of branch swapping routines using the following commands (Wheeler et al., 2002): `-noleading -norandomizeoutgroup -molecularmatrix` [matrix file] `-replicates 5 -stopat 3 -sprmaxtrees 1 -extensiongap 1 -tbrmaxtrees 2 -maxtrees 20 -holdmaxtrees l00 -fitchtrees -seed -l -slop 4 -checkslop 2 -buildsperreplicate 5 -buildspr -buildtbr -approxbuild -buildmaxtrees 1 -tree fuse -fuselimit l0 -fusemingroup 5 -numdriftchanges l5 -driftspr -numdriftspr 5 -drifttbr -numdrifttbr l0 -impliedalignment` [data files] > [output file]. More extensive searches were done under a selected set of parameters and consisted of 10 random sequence addition replicates, increasing the number of SPR and TBR rearrangements to 2 and 3, respectively, and the `-slop` and `-checkslop` parameters to 10 and 5. A simultaneous analysis of molecular and morphological data was also run in POY using equal cost for all characters under the same commands as those for molecular data given above.

Selection of preferred parameter values was based on the notion that interactions of diverse data partitions frequently bring out 'hidden support' in combined analyses, when a common historical signal emerges from the combined data (Gatesy et al., 1999). Hence the preferred assignments of character homologies in alignment variable regions are those where the data interactions are producing a signal of overall greatest consistency across all partitions. We therefore selected the alignment parameters of highest consistency with the signal from other partitions, roughly following the argumentation of Wheeler (1995), who proposed congruence as the optimality criterion to decide among competing phylogenetic hypotheses from length variable sequences. Congruence of the three gene markers was assessed estimating the incongruence length difference (ILD; Mickevich and Farris, 1981) calculated from tree lengths obtained in separate and combined analyses. This value was normalized according to the total number of steps in the combined data (ILD/tree length combined; Phillips et al., 2000). The meaning and interpretation of the ILD test have been recently discussed and criticized (e.g., Barker and Lutzoni, 2002; Dolphin et al., 2000; Hipp et al., 2004), including that the significance of the ILD test may be high simply due to the difference in the levels of sequence variation between partitions (see below) rather than true incongruence (Dolphin et al., 2000). Nodal support on the preferred trees was established calculating heuristic Bremer support values (Bremer, 1988) using searches implemented under the `-bremer` command in POY. Node recovery was also assessed using only the length conserved portions of each marker. An alternative measure of support was obtained by conducting bootstrap searches in PAUP* 4.0b10 (Swofford, 2002) using

the 'implied alignments' as input data, with heuristic searches using TBR branch swapping on 500 bootstrap pseudoreplicates (in the analysis of nuclear rRNA markers, the number of TBR rearrangements was limited to 10^7 because of the high number of equally parsimonious trees found in each replicate). In each case we bootstrapped a single possible alignment associated to a given tree topology. This procedure at best provides a measure of resilience to resampling for each particular homology assignment. In other words, it provides an estimate of the proportion of characters, aligned in the way they are, supporting the tree obtained with the full data.

Overall, direct optimization is a suitable approach for the phylogenetic investigation of rRNA data of the Eumolpinae, since it provides an objective treatment of length variation in the sequences and a maximization of character congruence and data interaction within and between several length variable data partitions.

2.4. Hypothesis testing

We used the data at hand to test several phylogenetic hypotheses based on the traditional systematic arrangement for the Eumolpinae as proposed by Seeno and Wilcox (1982). Scenarios were tested using POY by conducting searches for optimal tree alignments constrained to conform to these scenarios. Searches were conducted under equal weight of nucleotide substitutions and indels (the "preferred" parameter combination, see below). In addition, constrained scenarios were tested against unconstrained searches using the Kishino and Hasegawa (1989) test. Because the POY software currently does not implement these tests, we used the implied alignment as an input file for further parsimony searches in PAUP*. Implied alignments for the equally weighted searches in POY for the nuclear (18S and 28S rRNA) and for the combined molecular data sets were analyzed separately. Tree searches in PAUP* consisted of 20 replicates of random addition of taxa with TBR branch swapping and specifying the topological constraints.

2.5. Morphological data

Sixteen categorical morphological characters relevant in the tribal and suprageneric classification of the Eumolpinae were selected from the literature and their character states scored for the specimens used in the phylogenetic analyses. The list of morphological characters and their character states is given in Appendix. To test if these characters show significant hierarchical structure, we compared their distribution on the inferred molecular trees with a random distribution whereby the character states are shuffled between terminals. Only one representative was retained per genus where these represented monophyletic clades in the analyses, in order not to overestimate the significance of phylogenetic structure. Character optimization and permutation analyses of the data were done in Mesquite version 1.0 (Maddison and Maddison, 2003). Significance was assumed if the number of steps for a given character on the preferred topology was below the 5% threshold of the number of character steps obtained in 999 randomizations. Measures of character fit to the tree topologies were obtained determining the CI and RI for each character using MacClade version 3.0 (Maddison and Maddison, 1992). The potential of these characters as a guide for the systematic arrangement of the Eumolpinae was assessed based on their consistency with all data and their capability to diagnose monophyletic lineages.

3. Results

3.1. Sequence data and phylogenetic analysis

The amount of character variation and phylogenetic information in the three gene markers was compared using the implied alignment obtained in equal-cost POY searches (Table 2). The fragment length of the mitochondrial 16S rRNA marker is the shortest (505–515 bp) but produces an implied alignment 43% longer than the sequenced DNA fragment, compared to the 8 and 1% increases for the nuclear 28S and 18S rRNA genes, respectively. In 16S rRNA, 84.40% of the aligned positions are variable, indicating high divergence among the studied sequences with significantly higher degree of homoplasy than the other genes analyzed (CI = 0.398 vs. 0.696 and 0.591 for 18S and 28S rRNA genes, respectively). Accumulation of mutations for 16S rRNA is between one and two orders of magnitude higher than

Table 2
Characteristics of the markers used in this study

Marker	Sequence length	Homology lines	Variable sites	Informative sites	A (%)	C (%)	G (%)	CI	RI
Separate									
16S rRNA	505–515	737	622	356	34.20	9.37	16.72	0.398	0.613
18S rRNA	1825–1851	1875	215	99	24.17	24.13	27.82	0.696	0.795
28S rRNA	644–669	721	159	80	26.67	22.88	29.83	0.591	0.801
Combined									
Nuclear	2469–2543	2611	379	185	24.82	23.80	28.34	0.605	0.794
Total	2956–3053	3397	948	560	26.42	21.33	26.34	0.472	0.674

Table 3
Results of sensitivity analyses in POY

Cost scheme[a]	18S	28S	16S	Nuclear	ILD	ILD/nuclear	Total	ILD	ILD/total
111	393	349	2409	1030	288	0.279[b]	3288	137	0.042[b]
112	433	306	1981	1120	381	0.340	3428	708	0.207
114	481	242	1247	1173	450	0.384	3104	1134	0.365
118	561	175	218	1227	491	0.400	2316	1362	0.588
122	519	410	3272	1298	369	0.284	4900	699	0.143
124	576	318	2628	1384	490	0.354	4964	1442	0.290
128	669	354	1584	1427	404	0.283	4336	1729	0.399
12[16]	806	220	600	1458	432	0.296	3121	1495	0.479

[a] See main text for details (XYZ: transition:transversion:gap costs).
[b] Lower values and selected cost scheme.

for the other markers, as shown by the pairwise sequence divergences (*p*-distance) within Eumolpinae, which ranges between 0.0257 and 0.2592 (average: 0.1772 ± 0.0363) for 16S rRNA, 0.0000 and 0.0286 (0.0092 ± 0.0051) for 18S rRNA, and between 0.0000 and 0.0542 (0.0243 ± 0.0106) for 28S rRNA. The 16S rRNA marker also shows a high level of AT typical of mtDNA in insects, whereas base frequency is more balanced in the nuclear markers (Table 2). In the combined analysis the number of homology lines in the implied alignment is further increased, whereas the level of homoplasy assumes intermediate values (Table 2).

The study of different cost schemes in POY searches produced the lowest ILD between markers under equal costs of indels and nucleotide changes. This result was obtained in the analysis of incongruence between nuclear markers only, as well as in the combined analyses of all molecular data (Table 3). This tree is presented as the preferred phylogenetic hypothesis in Eumolpinae, although node recovery using the alternative weightings was also recorded. The equal-cost analysis of the nuclear data resulted in 50 parsimonious trees of 1030 steps (Fig. 2) and two trees of 3288 steps in the three combined ribosomal genes analysis (Fig. 3). Many of the nodes recovered under the equal-cost scheme were also obtained under alternative gap costs. The basal nodes were more sensitive to the variation in parameter values and also received weaker support in general. Thus the relationships represented by these basal nodes need to be considered with caution. The analysis of conserved (length invariable) regions for the two nuclear genes produced 50 trees of 580 steps, while the combined molecular analysis resulted in a single tree of 2174 steps. These analyses produced essentially the same phylogenetic hypotheses as those using all characters under equal costs, particularly for the nodes defining the main lineages that will be discussed below.

Finally, we performed a simultaneous analysis of all molecular and morphological data by direct optimization. This resulted in two equally parsimonious trees of 3401 steps, only differing in the nodes resolving three taxa in the Iphimeites (tribe Eumolpini) (Fig. 4). In the nuclear, nuclear plus mitochondrial, and the total evidence (all molecular plus morphological data) hypotheses, the Eumolpinae *sensu lato* constitute a monophyletic assemblage, although excluding *Syneta* in the analysis of all molecular data. The lineage identified as the Spilopyrinae appears as sister group to the remaining eumolpines, which are assembled in two major sister clades, roughly corresponding to the tribe Eumolpini (Clade I in Fig. 2) and the Typophorini + Colasposomini (Clade II), respectively. None of the trees shows the Adoxini as a monophyletic clade. The Megascelini, clearly appear in the clade with the other Eumolpinae, although in an unresolved basal position.

3.2. Testing for monophyly of key clades

Constrained searches were used to evaluate different hypotheses of monophyly for subclades in the Eumolpinae. We determined the extra length of trees obtained with POY under these constraints over the unconstrained topologies, and tested the statistical significance of the increase with the Kishino–Hasegawa test. The analyses were performed separately for the nuclear partitions and for all DNA data combined (Table 4). The phylogenetic scenarios tested in this way included the monophyly of the eumolpine tribes Typophorini (hypothesis TYP), Colasposomini (COL), Eumolpini (EUM), and Adoxini (ADO); and the suprageneric Sections Nodostomites (Nod), Typophorites (Typ), Iphimeites (Iph), Edusites (Edu), Corynodites (Cor), Endocephalites (End), and Myochroites (Myo). We also tested specific hypotheses recently proposed by Reid (2000) regarding newly raised and problematic groupings, including the monophyly of the Spilopyrinae (SPI), and their sister relationships with Eumolpinae (Spi); the monophyly of Synetini sensu Reid (2000) including *Syneta* and *Eupales* (SYN), and the inclusion of the latter within the Eumolpinae (Syn).

Based on the statistical significance in these tests (Table 4), the main conclusions from this analyses were: (i) the rejection of Typophorini, Adoxini, Typophorites, and Myochroites; (ii) full support for the monophyly of the Spilopyrinae, (iii) general support for Colasposo-

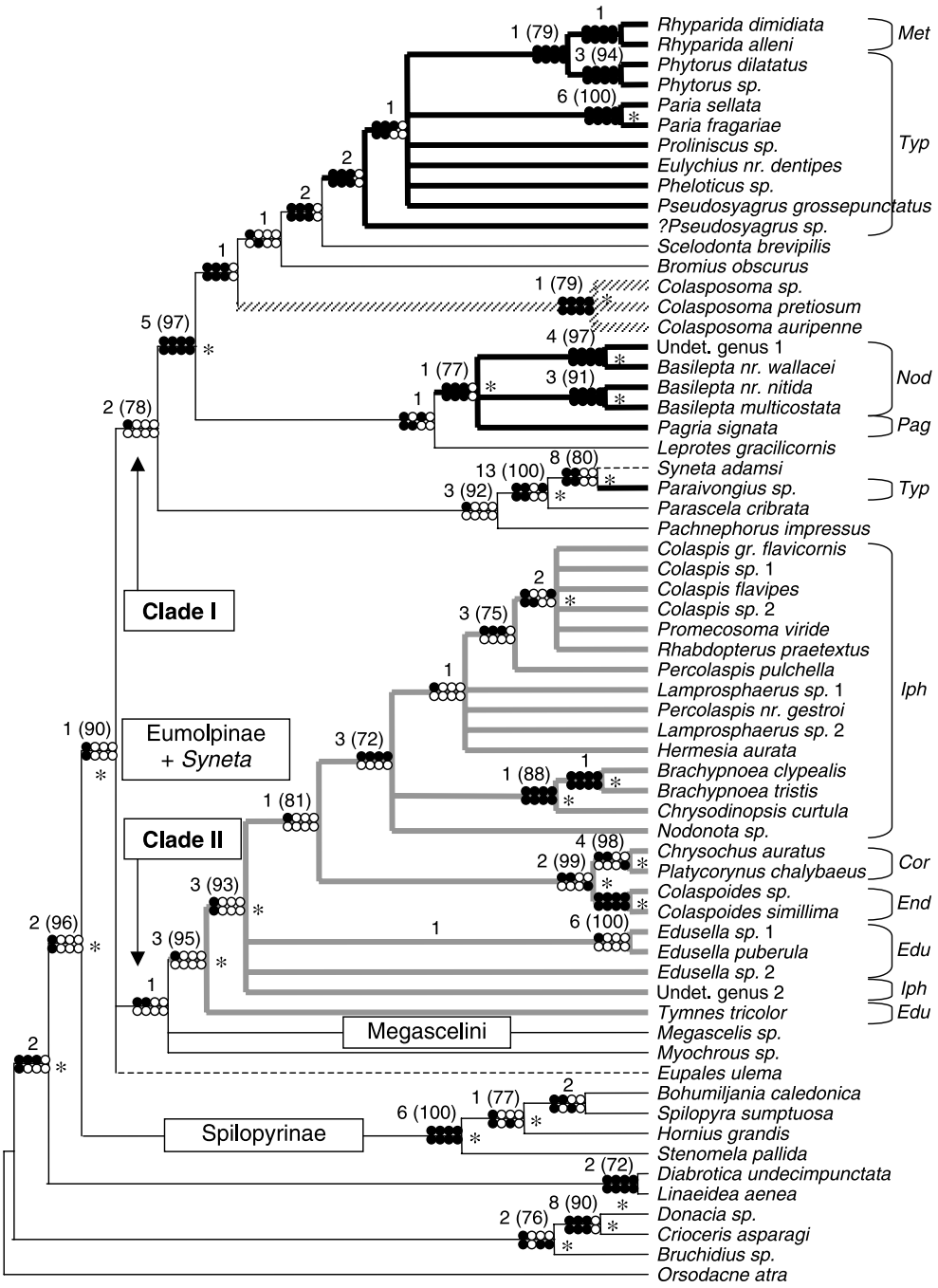

Fig. 2. Strict consensus of 50 trees of 1030 steps obtained using direct optimization analysis of 18S and 28S rRNA genes. The tree was rooted with *Orsodacne atra* (Orsodacnidae). The recovery of each node using alternative search parameters is indicated by circles as follows, from left to right, 111, 112, 114, and 118 (top row) and 122, 124, 128, and 12[16] (bottom row) for the weight of transitions, transversions, and indels; an open circle indicates that the particular node was not obtained under the set of parameters. Several Eumolpinae lineages discussed in the main text are identified by brackets or shaded branches (thick: Typophorini; thick hatched: Colasposomini; gray: Eumolpini; plain: Adoxini; dashed: Synetini *sensu* Reid, 2000). Numbers above the branches are Bremer and bootstrap (in brackets) supports. Asterisks identify those nodes recovered in the separate analysis of conserved gene regions.

mini, Nodostomites, Corynodites, and Endocephalites; (iv) sensitivity of conclusions to parameter values and the marker included in the analysis, regarding the monophyly of Eumolpini, Iphimeites, and Edusites; (v) support for monophyly of the Spilopyrinae and for their position as sister to Eumolpinae excluding Synetini (*sensu* Reid, 2000); (vi) rejection of the monophyly of Synetini *sensu* Reid (2000) proposed to include *Syneta* and *Eupales*, and rejection of the inclusion of *Syneta* in Eumolpinae.

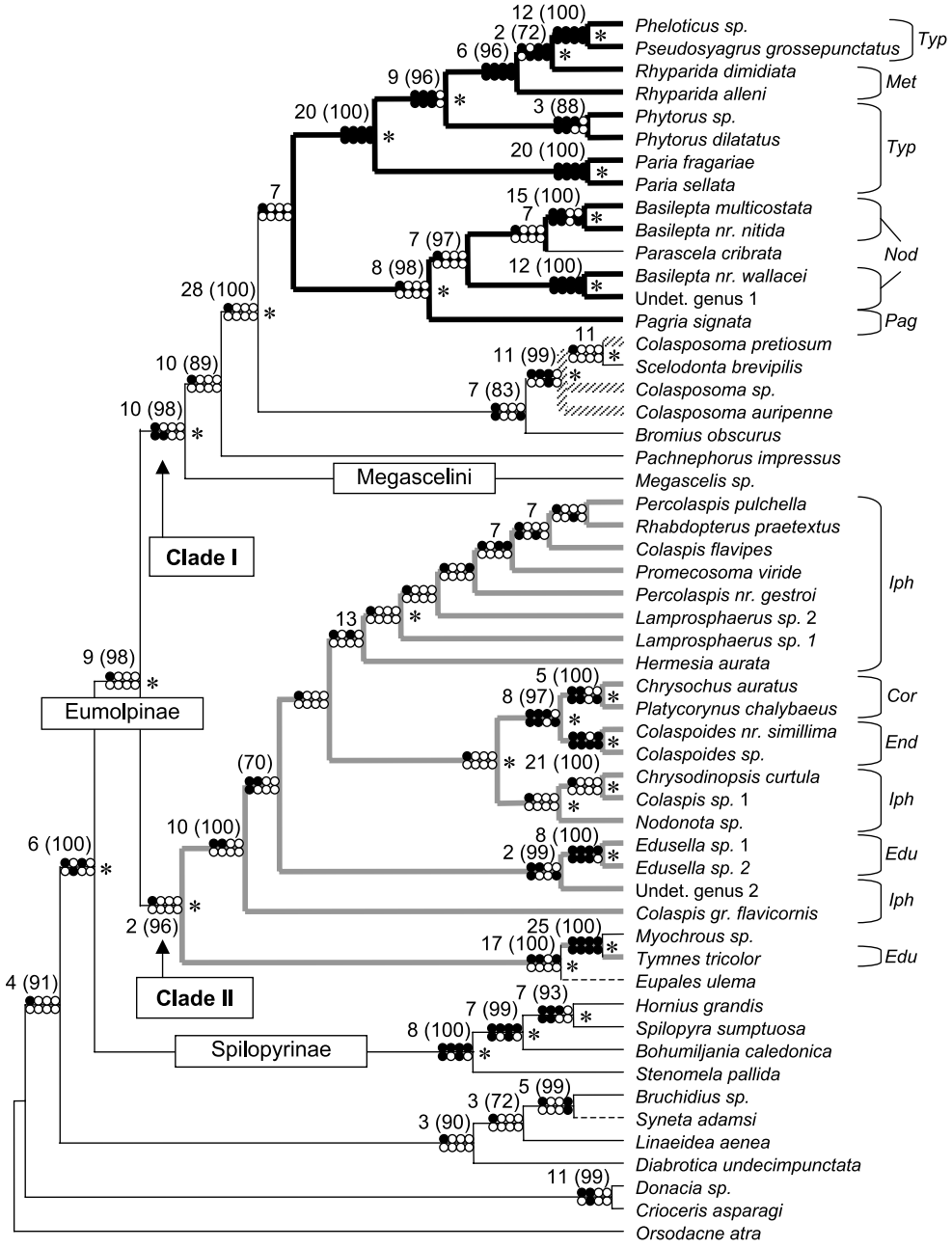

Fig. 3. Strict consensus of two shortest trees of 3288 steps obtained from combined analysis of 16S, 18S, 28S rRNA genes using direct optimization. All symbols as in Fig. 1.

3.3. Character permutation tests

Potential phylogenetic structure was assessed for 16 morphological characters testing for character variation consistent with the hierarchical arrangement of the tree, compared to a scenario of random evolution in simulated data (Table 5). The character distribution in several characters was not different from a randomized distribution, including the shape of the apical antennal segments (character H1), the shape of the eyes (H3) and the pronotum (T1), the shape of the lateral margin of the pronotum (T4), the development of elytral humeri (E1), and the existence of a profemoral tooth (L1). On the contrary, several other characters showed statistically significant structure on the trees tested. These were the presence of a characteristic groove above the eyes (H4), the relative width of the prothorax compared to the elytra (T2), the characteristics of elytral puncturation (E2), the presence of an apical emargination in tibiae (L2), the shape of claws (L3), and the existence of a median groove in the pygidium (A1). Other characters like dorsal pubescence (B1), the relative length of second antennomere (H2), the presence of margins in the pronotum (T3) or the shape of proepisterna (T5), only produced significant results for the topology based on nuclear markers.

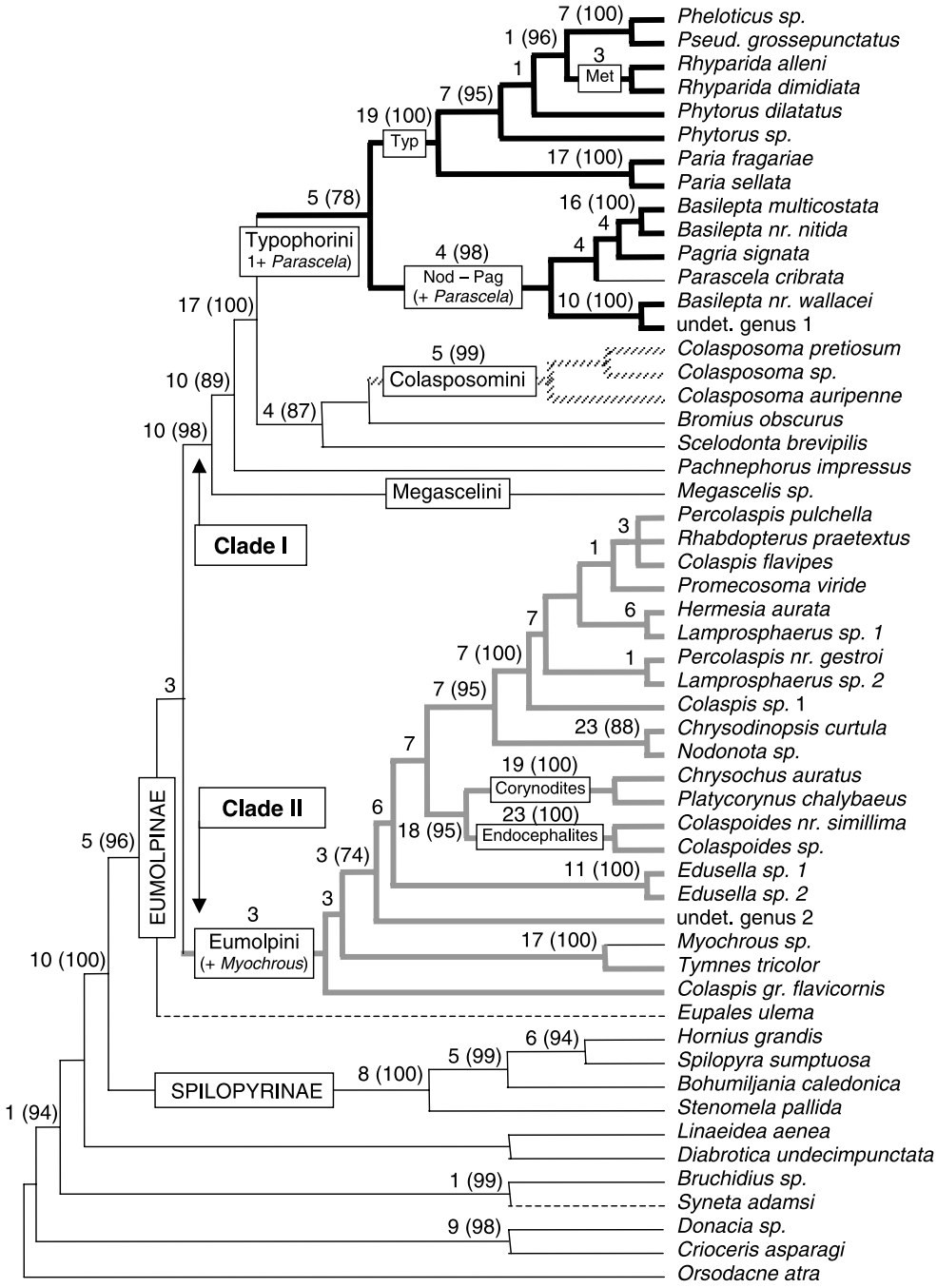

Fig. 4. Strict consensus of two shortest trees of 3401 steps from combined analysis of molecular and morphological data, obtained under equal weights using direct optimization. The analysis included other unrelated chrysomelids and was rooted with the Orsodacnidae *Orsodacne atra*. Major lineages of Eumolpinae are marked by different shading of branches (thick: Typophorini; thick hatched: Colasposomini; gray: Eumolpini; plain: Adoxini; dashed: Synetini *sensu* Reid, 2000). Abbreviations: Met (Metachromites), Typ (Typophorites), Nod (Nodostomites), Pag (Pagriites).

4. Discussion

4.1. Phylogenetic support for the Spilopyrinae

The rRNA genes used here provided well-supported tree topologies resolving basal relationships in Eumolpinae and shed new light on the contentious proposition for separate subfamilial status of Spilopyrinae and Synetinae. The analyses strongly supported the monophyly of the four genera of spilopyrines studied here under a wide range of alignment parameters, which was backed up statistically in a Kishino–Hasegawa test constraining these genera to be not monophyletic (Table 4). This result corroborates recent hypotheses which consider these taxa as a discrete higher-level clade within the Chrysomelidae (Reid, 1995, 2000; Verma and Jolivet, 2002). The phylogenetic analysis revealed further that the spilopyrines are the sister group to the remainder of the Eumolpinae

Table 4
Results of topologically constrained searches

Null Hypothesis[a]	Nuclear data set				Total data set			
	POY	Tree length (PAUP)	*d*	*P*[b]	POY	Tree length (PAUP)	*d*	*P*
No constrain	1030	1040[c]			3288	3346[c]		
SPI	1058	1071	31	<0.0001	3300	3380	34	<0.0001
SPI*	1036	1046	6	0.0497–0.0833	3296	3360	14	0.0017
SYN	1058	1069	29	<0.0001	3302	3406	60	<0.0001
TYP	1066	1082	42	<0.0001	3295	3368	22	<0.0001
COL	1030	1040	—	1.0000	3294	3346	—	1.0000
EUM	1030	1040	—	1.0000	3308	3399	53	<0.0001
ADO	1069	1093	53	<0.0001	3380	3511	165	<0.0001
Nod	1030	1040	—	1.0000	3292	3346	—	1.0000
Typ	1067	1084	44	<0.0001	3296	3359	13	<0.0374
Iph	1031	1043	3	0.3658–0.4055	3315	3370	24	<0.0007
Edu	1036	1046	6	0.0578–0.1088	3328	3422	76	<0.0001
Cor	1030	1040	—	1.0000	3288	3346	—	1.0000
End	1030	1040	—	1.0000	3288	3346	—	1.0000
Myo	1039	1052	12	0.0073–0.0143	3336	3429	83	<0.0001
Syn	1030	1040	—	1.0000	3297	3377	31	<0.0003

[a] In SPI, this group is considered sister to Eumolpinae excluding Synetini sensu Reid (2000). In SPI* only the monophyly of Spilopyrinae is constrained. SYN is for Syneta and Eupales being monophyletic, while Syn is for these being included within Eumolpinae and excluding Spilopyrinae.

[b] Significance of tree comparisons according to the Kishino and Hasegawa (1989) test. When more than one tree was obtained in the constrained analyses, the range of values obtained in the KH tests is given.

[c] Discrepancies in tree length between POY and PAUP are the result of using the `-noleading` option in POY, which does not count the gaps in both ends of incomplete sequences; the effect is more marked in the dataset including 16S rDNA, where the sequences for a few taxa were shorter in one end.

Table 5
Character fit to the nuclear and total molecular analyses trees and results of permutation tests of morphological characters (the range of the null distribution is given in brackets)

Character[a]	Nuclear				Total molecular			
	CI	RI	No. steps	*P*	CI	RI	No. steps	*P*
B1 (12/57)	0.14	0.45	7	0.021 [6–10]	0.13	0.22	8	0.461 [5–9]
H1 (8/57)	0.14	0.14	7	1.000 [4–7]	0.14	0.00	7	1.000 [4–7]
H2 (11/57)	0.14	0.40	6	0.003 [5–10]	0.17	0.38	7	0.132 [5–9]
H3 (17/57)	0.08	0.31	12	0.294 [8–15]	0.08	0.20	13	0.573 [8–16]
H4 (14/57)	0.13	0.46	8	0.050 [7–11]	0.17	0.55	5	0.004 [4–10]
T1 (10/57)	0.13	0.22	7	0.074 [5–9]	0.13	0.00	7	1.000 [3–7]
T2 (25/57)	0.13	0.71	7	0.001 [9–19]	0.11	0.58	9	0.005 [9–18]
T3 (8/57)	0.17	0.29	6	0.043 [4–8]	0.14	0.00	7	1.000 [3–7]
T4 (8/57)	0.17	0.29	7	1.000 [5–7]	0.14	0.00	7	1.000 [5–7]
T5 (18/57)	0.10	0.47	8	0.008 [8–13]	0.13	0.46	8	0.074 [6–11]
E1 (4/57)	0.25	0.00	4	1.000 [2–4]	0.25	0.00	4	1.000 [2–4]
E2 (21/57)	0.13	0.65	8	0.002 [7–17]	0.14	0.63	6	0.001 [8–14]
L1 (15 + 1/57)	0.17	0.29	10	0.185 [7–12]	0.25	0.40	7	0.069 [5–9]
			9[b]	0.061 [7–12][b]			6[b]	0.018 [5–9][b]
L2 (21/57)	0.20	0.80	5	0.001 [9–17]	0.25	0.81	4	0.001 [7–14]
L3[c]	0.40	0.87	5	0.001 [12–20]	0.33	0.78	6	0.001 [11–18]
A1 (23/57)	1.00	1.00	1	0.001 [8–18]	0.5	0.95	2	0.001 [6–17]

[a] The proportion of taxa showing the less frequent character state is given as an indication of the upper threshold for the number of character steps on the trees. The comparison between this theoretical threshold and the actual score of the character on the tree is already an indication of hierarchical structure.

[b] Only two character states (0: absence of pro-femoral tooth; 1: presence of pro-femoral tooth).

[c] Three-state character: 6/57 state "0," 19/57 state "1," and 32/57 state "2."

(excluding *Syneta*), given our current outgroup sampling. This phylogenetic position would be consistent with a taxonomic status of the spilopyrines either as plesiomorphic tribe Spilopyrini subordinated in the Eumolpinae, or as separate subfamily Spilopyrinae. For practicality we prefer the latter, also acknowledging the apparently ancient origin of this group with its Gondwanian distribution perhaps dating back to the late Cretaceous (Verma and Jolivet, 2002) coincident with the age of other accepted subfamilies of the Chrysomelidae (Farrell, 1998).

Similar questions about taxonomic status have been raised for the Synetinae, represented here by *S. adamsi* which has been treated as subfamily (Seeno and Wilcox, 1982; Verma and Jolivet, 2000), or was included within Eumolpinae as the tribe Synetini (Reid, 2000). Our analyses clearly reject the affinities with the plesiomorphic eumolpine *Eupales*, but do not unequivocally resolve the phylogenetic placement of *Syneta*. Whereas in the analysis of nuclear genes *Syneta* groups in an unlikely position derived within the eumolpine Clade I, the combined analyses (Figs. 3 and 4) and the analysis of topological constraints (Table 4), clearly placed *Syneta* outside the eumolpines and spilopyrines (hypothesis "Syn") with the outgroups. This might suggest an altogether distant relationship with the Eumolpinae, and hence needs to be tested in the context of a wider sample of Chrysomelidae. As there is little evidence that the synetines would appear subordinated within the Eumolpinae, they should be assigned subfamilial rank, for similar reasons as given for the Spilopyrinae. This is corroborated by their great morphological and anatomical divergence (wings without the cubital cells, genitalia completely different from spilopyrines and true eumolpines, among others), with similarities of larval biology and morphology attributable to convergence possibly due to a common lifestyle.

4.2. Relationships within Eumolpinae sensu stricto

Excluding the Spilopyrinae and Synetinae, the Eumolpinae can be subdivided in two major Clades, roughly corresponding to Colasposomini and Typophorini (Clade I) and Eumolpini (Clade II), with taxa attributed to the clearly polyphyletic Adoxini grouped within each of them. Clade I also includes the Megascelini which is sister to all other taxa in this lineage. The Colasposomini has been sampled for a single genus only, *Colasposoma* Laporte, with representatives exclusive from South East Asia, and a more comprehensive taxonomic sampling is still needed to confirm their monophyly.

The Typophorini appeared monophyletic in the combined analysis, except for the presence of *Parascela* Baly (Adoxini) within the Nodostomites–Pagriites clade. The Typophorini was found to be divided into a paraphyletic Typophorites that are closely related to the genus *Rhyparida* Baly as representative of the Metachromites, and the Nodostomites and *Pagria* Lefèvre (Pagriites) in a loosely defined second clade. The Typophorini are mainly characterized by having the meso- and metatibiae emarginated at the apex (Chapuis, 1874; Lea, 1915; Selman, 1965), a character shown to have a significant phylogenetic structure despite being affected by homoplasy (Table 5).

Clade II is mostly composed of Eumolpini which are monophyletic except for the presence of *Myochrous* (Adoxini) which was strongly associated with *Tymnes* in the combined analysis. The Eumolpini were represented by a largely complete set of major taxa, mostly from the New World, where this lineage is particularly diverse, but also from Australia and South East Asia. The Eumolpini are diagnosed by a character of high systematic value, the presence of a characteristic longitudinal groove on the pygidium, which possibly helps to keep the elytra locked at rest, further reinforcing the validity of this clade. Additional phylogenetic structure in this tribe is partly recovered for the four Sections sampled, the Iphimeites, Edusites, Corynodites, and Endocephalites (represented by a single genus, *Colaspoides*), the two latter as sister taxa. The monophyly of Edusites is only contradicted in the three markers and total evidence hypotheses by the already mentioned placement of *Tymnes* outside the clade, but Iphimeites clearly appears as a paraphyletic lineage including the other sections, although with weak support.

Finally, the Adoxini is shown to be a polyphyletic group, confirming observations of authors like Selman (1965), who pointed out in his study of African Eumolpinae that Adoxini was the "less natural of the four tribes." These beetles have in common a combination of characters including the cylindrical pronotum usually lacking margins, and conspicuously pubescent or squamulose teguments (Selman, 1965). In the light of our results, and in agreement with previous studies based on male genitalia (Flowers, 1999), Adoxini cannot be considered a valid clade. In conclusion, the analysis revealed two major clades in which the large tribes of the Eumolpinae are subdivided. It will be interesting to examine the position of the seven minor tribes not investigated here, each of which is monogeneric and with restricted geographical ranges: Cubispini in Cuba, Merodini in the Amazonas, Pygomolpini in Argentina, Caryonodini in South America, Habrophorini, with two genera in central and South America, and Hemydacnini and Rosiroiini in Madagascar (Seeno and Wilcox, 1982).

4.3. Diagnostic characters for the classification of Eumolpinae

Treatises of the Eumolpinae frequently have drawn attention to the unsatisfactory morphological characters defining genera or groups of genera (e.g., Chapuis, 1874; Flowers, 1999; Horn, 1892; Lea, 1915; Selman, 1965). Subtleties in differences between character states and overlapping character combinations among most traditional taxonomic groups contribute to the difficulties with the systematics of Eumolpinae. We therefore assessed the utility of traditionally used morphological characters in our particular taxon sample for an improved classification of the Eumolpinae in the light of the new phylogenetic hypothesis.

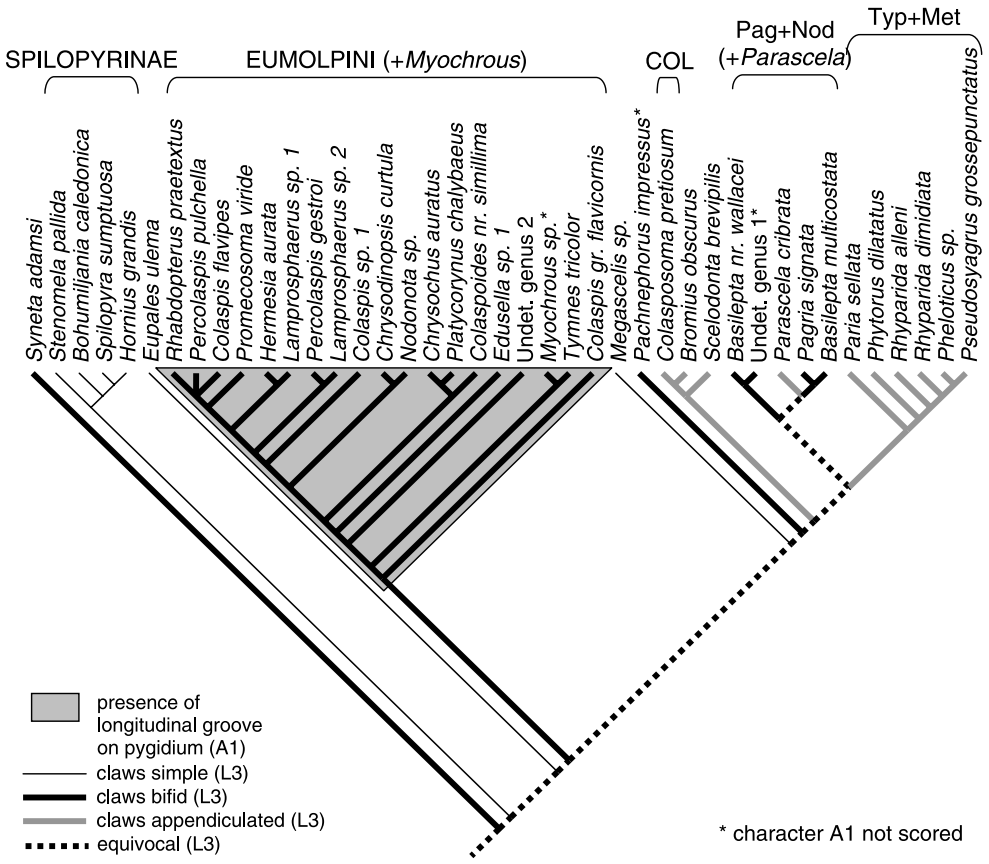

Fig. 5. Character transformations of selected morphological traits on the tree topology using all characters available. Character reconstructions for two taxonomically important traits are labeled with different branch shading, as indicated. Abbreviations: COL, Colasposomini; Pag + Nod, Pagriites + Nodostomites [Typophorini]; Typ + Met, Typophorites + Metachromites [Typophorini].

Several characters showed no significant phylogenetic association relative to a randomized distribution on the phylogenetic trees (Table 5). These include the characteristics of apical antennal segments, the shape of the internal margin of the eyes, the general shape of the prothorax, the profile of lateral margins in the pronotum, the development of the elytral humeri, and the presence of a profemoral tooth. These characters may be of importance in the delimitation of species or groups of species, but according to the phylogenetic hypotheses presented here, the character variation is not better than a random distribution and hence unlikely to be useful for higher-level classification.

A second group of characters showed non-random variation on the tree from nuclear genes, but not on the combined analysis tree. The relevance of these characters is difficult to assess, since the character state changes seem to be restricted to the less stable portions of the trees. Dorsal pubescence (B1), presence of lateral margins in the pronotum (T3), and the shape of proepisterna (T5), for instance, exhibit changes mostly at the basal poorly supported basal nodes in the Eumolpinae clade. The character T5 was one of the key elements in Chapuis' (1874) and many subsequent classifications of Eumolpinae, even if considered 'highly unsatisfactory' by some (Lea, 1915). Our results showed it to be a rather homoplastic character, present in several lineages, although it was consistent with certain groups, such as the clade of the 'Corynodites + Endocephalites' within Eumolpini. The value of these characters hence is restricted to the diagnosis of particular lineages within Eumolpinae, but not useful for the higher-level systematics in the subfamily.

A last group of characters, with greatest utility for taxonomy, allows the diagnosis of major clades established in this study (Fig. 5). Specifically, the presence of a dorsal median groove on the pygidium (character A1) separates the Eumolpini from all other lineages in the vast majority of cases, although might be absent in some species considered to belong to this tribe (e.g., some *Edusella* spp.; C. Reid and R. Wills Flowers, pers. comm.). This character was the basis, together with biogeographical considerations, for the separation of Colasposomini from Eumolpini (Springlová, 1960), but clearly it is useful for the diagnosis of Eumolpini against the remainder of the subfamily. Within the Eumolpini, the 'Corynodites' are characterized by the presence of a conspicuous groove above the eyes (character H4) and a

pronotum narrower than the elytra (character T2), characters that are not present in any of the other Eumolpini included in this study. The shape of tarsal claws (character L3) has been neglected as a diagnostic character for deeper relationships within the Eumolpinae (Chapuis, 1874; Lea, 1915). However, the three character states are largely consistent with the combined analysis tree, where basal groups exhibit simple claws giving rise to bifid and appendiculated claws in a limited number of lineages (Fig. 5). The character appears to be a very promising feature for identifying further subdivisions once the Eumolpini are split out, such as the association of the Pagriites and the Nodostomites, characterized by bifid tarsal claws, as opposed to appendiculate claws present in the other studied 'Typophorini' (Fig. 5). Interestingly, both pygidial groove and tarsal claws were deemed useful for eumolpine classification by Crowson (1955). However, even some of these conservative characters show reversals, and the precise conclusions about the extent of groups they diagnose depends on whether the trees are derived from nuclear data alone or the simultaneous analyses with morphological data.

5. Conclusions

The application of molecular analyses in systematics often confirms ideas that were proposed based on intuition and experience of classical taxonomists. In the case of the present work, we refined ideas about the classification of Eumolpinae that were already anticipated in the literature, such as the separation of Spilopyrinae from Eumolpinae as a valid subfamily, the controversial placement of the plesiomorphic genus *Eupales* or that of the synetines (retaining their interpretation as a different subfamily), the subordination of *Megascelis* within Eumolpinae, the diagnostic value of several characters, or the Adoxini as an invalid taxon from a phylogenetic perspective. This study offers what we think is a valuable mix of traditional and morphological aspects combined with modern phylogenetic and molecular analyses in support of the classification and, thus, filling a gap recently criticized in the discussion of the higher classification of the Chrysomelidae. Moreover, the objectivity of molecular data provides the means to test these hypotheses rigorously based on an independent and unprejudiced source of characters. The finer details of Eumolpinae systematics still remains largely unsolved, but we have opened the path for future work that will include additional taxa and guide the interpretation of morphological characters for a full understanding of the evolution of this fascinating group of beetles.

Acknowledgments

We are indebted to several colleagues who helped collecting samples for this study: T. Anthony, M. Balke, M. Barclay, L. Bocak, M. Bocakova, A. Cardoso, D. Carpenter, F. Čiampor, A. Cline, C. Duckett, D. Duran, K. Jackson, V. Jerez, T. Kalaichelvan, S. Kim, J. Maté, J.-M. Maes, Y. Peiyu, and K.K. Verma. Michael Cox (NHM, London) assisted in the identification of many specimens. Chris Reid (Australian Museum, Sydney) and R. Wills Flowers (Florida A&M Univ., Tallahassee) helped with advice on nomenclature and systematic interpretations. We thank F. Kopliku for technical assistance and F. Wright for automated sequencing. Three anonymous reviewers provided very useful comments for improving the manuscript. This work was funded by a Marie Curie postdoctoral fellowship (Contract No. HPMF-CT-2000-00744).

Appendix. Matrix of morphological characters

List of characters:
B1, dorsal pubescence: glabrous (0), pubescent or squamulate (1)
H1, apical antennal segments: elongated (0), thickened (1)
H2, second antennomere: globulous/shorther than third (0), at least as long as third (1)
H3, eye emargination: absent (0), present (1)
H4, supraocular groove: absent (0), present (1)
T1, shape of prothorax: cylindrical (0), transverse (1)
T2, width of prothorax: narrower than elytra (0), as broad as elytra (1)
T3, pronotal lateral margin: absent (0), present (1)
T4, sides of pronotum: regular (0), irregular or toothed (1)
T5, shape of proepisterna: straight or concave (0), convex (1)
E1, development of humeri: prominent (0), obliterated (1)
E2, elytral puncturation: regular (0), irregular (1)
L1, profemoral tooth: absent (0), minute (1), large (2) [also analysed as two categories: absent (0), present (1)]
L2, tibial preapical emargination: absent (0), present (1)
L3, claws: simple (0), appendiculate (1), bifid (2)
A1, pygidium median groove: absent (0), present (1)

Taxon	Character state															
	B1	H1	H2	H3	H4	T1	T2	T3	T4	T5	E1	E2	L1	L2	L3	A1
Hornius grandis	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0
Bohumiljania caledonica	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0
Stenomela pallida	0	0	0	1	0	1	0	1	0	0	1	1	0	0	0	0
Spilopyra sumptuosa	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Syneta adamsi	1	0	1	0	0	0	0	0	1	0	0	1	0	0	2	0
Eupales ulema	1	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0
Megascelis sp.	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Basilepta multicostata	0	0	0	1	1	1	0	1	0	0	0	1	1	1	2	0
Basilepta nr. *nitida*	0	0	0	1	1	0	0	1	0	0	0	0	1	1	2	0
Basilepta nr. *wallacei*	0	0	1	1	0	1	0	1	0	0	0	0	0	1	2	0
Undetermined genus 1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	2	?
Pagria signata	0	0	1	1	1	1	0	1	1	0	0	0	1	1	2	0
Rhyparida alleni	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1	0
Rhyparida dimidiata	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1	0
Eulychius nr. *dentipes*	0	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0
Paraivongius sp.	0	0	1	1	1	1	0	1	0	1	0	0	1	1	1	0
Paria fragariae	0	0	0	1	0	1	0	1	0	1	0	0	0	1	1	0
Paria sellata	0	0	0	1	0	1	0	1	0	1	0	0	0	1	1	0
Pheloticus sp.	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0
Phytorus dilatatus	0	0	0	1	0	1	1	1	0	1	1	0	0	1	1	0
Phytorus sp.	0	0	0	1	1	1	0	1	0	0	0	0	1	1	1	0
Pseudosyagrus grossepunctatus	0	1	0	1	1	1	0	1	0	1	0	0	2	1	1	0
Pseudosyagrus sp.	0	0	0	1	1	1	1	1	0	1	0	0	1	1	1	0
Proliniscus sp.	0	0	0	1	0	0	0	1	0	1	0	0	1	1	1	0
Colasposoma auripenne	0	0	1	0	0	1	1	1	0	0	0	1	0	0	1	0
Colasposoma pretiosum	0	1	0	0	0	1	1	1	0	0	0	1	1	0	1	0
Colasposoma sp.	0	1	0	1	0	1	1	1	0	0	0	1	0	0	1	0
Brachypnoea clypealis	0	0	0	1	0	1	1	1	0	0	0	1	0	0	2	1
Brachypnoea tristis	0	0	0	1	0	1	1	1	0	0	0	1	0	0	2	1
Chrysodinopsis curtula	0	0	0	1	0	1	1	1	0	0	0	1	0	0	2	1
Colaspis gr. *flavicornis*	0	0	0	0	0	1	1	1	1	0	0	1	0	0	2	1
Colaspis flavipes	0	0	0	1	0	1	0	1	0	0	0	1	1	0	2	0
Colaspis sp. 1	0	0	0	0	0	1	1	1	1	0	0	1	0	0	2	1
Colaspis sp. 2	0	0	0	1	0	1	1	1	1	0	0	1	0	0	2	1
Hermesia aurata	0	0	0	1	0	1	1	1	0	0	0	1	0	0	2	1
Lamprosphaerus sp. 1	0	0	0	1	0	1	1	1	0	0	0	1	0	0	2	1
Lamprosphaerus sp. 2	0	0	0	1	0	1	1	1	0	0	0	1	0	0	2	1
Nodonota sp.	0	0	1	1	0	1	1	1	0	0	0	1	0	0	2	1
Percolaspis nr. *gestroi*	0	0	0	0	0	1	1	1	1	0	0	1	0	0	2	1
Percolaspis pulchella	0	0	0	0	0	1	1	1	1	0	0	1	0	0	2	1
Promecosoma viride	0	0	0	0	0	1	1	1	0	0	0	1	0	0	2	1
Rhabdopterus praetextus	0	0	0	1	0	1	1	1	0	0	0	0	0	0	2	1
undetermined genus 2	0	0	0	1	0	1	1	1	0	0	1	1	0	0	2	1
Edusella puberula	1	0	0	1	0	1	0	1	0	1	0	1	0	0	2	1
Edusella sp. 1	1	0	0	1	0	1	0	1	0	1	0	1	0	0	2	1
Edusella sp. 2	1	0	0	1	0	1	0	1	0	1	0	1	0	0	2	1
Tymnes tricolor	0	0	0	1	0	1	0	1	0	1	0	1	0	0	2	1
Chrysochus auratus	0	0	0	1	1	1	0	1	0	1	0	1	0	0	2	1
Platycorynus chalybaeus	0	1	0	1	1	1	0	1	0	1	0	1	0	0	2	1
Colaspoides simillima	0	0	0	1	0	1	1	1	0	1	0	1	1	0	2	1
Colaspoides sp.	0	0	0	1	0	1	1	1	0	1	0	1	1	0	2	1
Scelodonta brevipilis Lea	1	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0
Lypesthes gracilicornis	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0
Bromius obscurus	1	0	1	0	0	0	0	0	0	1	0	1	0	0	1	0
Myochrous sp.	1	1	0	0	0	0	0	0	0	1	0	1	0	0	2	?
Pachnephorus impressus	1	1	1	1	0	0	0	0	0	1	0	0	0	1	2	?
Parascela cribrata	1	0	1	0	1	0	0	0	0	0	0	1	1	1	1	0

References

Barker, F.K., Lutzoni, F.M., 2002. The utility of the incongruence length difference test. Syst. Biol. 51, 625–637.

Bechyné, J., Bechyné, B.S., 1969. La posición sistemática de *Megascelis* Chevrolat (Col., Phytophaga). Rev. Fac. Agr., Univ. Central de Venezuela 5, 65–76.

Bremer, K., 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42, 795–803.

Chapuis, F., 1874. Famille des Phytophages. In: Lacordaire, T., Chapuis, F. (Eds.), Histoire naturelle des Insectes. In: Genera des Coléoptéres, vol. 11. Libraire Encyclopédique de Roret, Paris.

Cox, M.L., 1998. The pupae of Chrysomeloidea and their use in phylogeny (Coleoptera). Mus. reg. Sci. Nat. Torino 1998, 73–90.

Crowson, R.A., 1955. The Natural Classification of the Families of Coleoptera. Nathaniel Lloyd, London pp. 187.

Dolphin, K., Belshaw, R., Orme, C.D.L., Quicke, D.L.J., 2000. Noise and incongruence: interpreting results of the incongruence length difference test. Mol. Phylogenet. Evol. 17, 401–406.

Duckett, C.N., Gillespie, J.J., Kjer, K.M., 2004. Relationships among the subfamilies of Chrysomelidae inferred from small subunit ribosomal DNA and morphology, with special emphasis on the relationship among the flea beetles and the Galerucinae. In: Jolivet, P., Santiago-Blay, J.A., Schmitt, M. (Eds.), New Developments in the Biology of Chrysomelidae. SPB Academic Publ. bv, The Hague, The Netherlands, pp. 3–18.

Farrell, B.D., 1998. 'Inordinate Fondness' explained: Why are there so many beetles?. Science 281, 555–559.

Farrell, B.D., Sequeira, A.S., 2004. Evolutionary rates in the adaptive radiation of beetles on plants. Evolution 58, 1984–2001.

Flowers, R.W., 1996. La subfamilia Eumolpinae (Chrysomelidae: Coleoptera) en América Central. Rev. Biol. Trop. (special publication) 2, 1–60.

Flowers, R.W., 1999. Internal structure and phylogenetic importance of male genitalia in the Eumolpinae. In: Cox, M.L. (Ed.), Advances in Chrysomelidae Biology. I.. Backhuys Publishers, Leiden, The Netherlands, pp. 71–93.

Gatesy, J., O'Grady, P., Baker, R.H., 1999. Corroboration among data sets in simultaneous analysis: hidden support for phylogenetic relationships among higher level Artiodactyl taxa. Cladistics 15, 271–313.

Gómez-Zurita, J., 2004. Molecular systematics and time-scale for the evolution of *Timarcha*, a leaf-beetle genus with a disjunct Holarctic distribution. Mol. Phylogenet. Evol. 32, 647–665.

Hipp, A.L., Hall, J.C., Sytsma, K.J., 2004. Congruence versus phylogenetic accuracy: revisiting the incongruence length difference test. Syst. Biol. 53, 81–89.

Horn, G.H., 1892. The Eumolpini of Boreal America. Trans. Am. Entomol. Soc. 19, 195–234.

Jacoby, M., 1888. Biologia Centrali-Americana, Insecta, Coleoptera, Supplement to Phytophaga, vol. 6, pt. 1, Suppl., 1–80.

Jerez, V., 1995. *Stenomela pallida* Erichson, 1847. Redescripcion, ontogenia y afinidad con el genero *Hornius* (Chrys. Eumolpinae). Gayana Zool. 59, 1–12.

Jerez, V., Ibarra-Vidal, H., 1992. Morfologia y bionomia de *Hornius grandis* (Phil., 1864) (Chrys. Eumol.). Bol. Soc. Biol. Concepcion, Chile 63, 93–100.

Jolivet, P., 1954. L'aile des Chrysomeloidea (Coleoptera). Misc. Entom. 48, 85–101.

Jolivet, P., 1957–1959. Recherches sur l'aile des Chrysomeloidea. Mem. Inst. R. Sc. Nat. Belgique 2° sér. 51, 1–180; 58, 1–252.

Jolivet, P., 1997. Biologie des Coléoptères Chrysomélides. Boubée Publishers, Paris.

Jolivet, P., Verma, K.K., 2002. Biology of Leaf Beetles. Intercept Publishers, Andover, UK.

Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29, 170–179.

Lea, A.M., 1915. Notes on Australian Eumolpides (Coleoptera Chrysomelidae), with descriptions of new species. Trans. Proc. R. Soc. South Australia 39, 102–339.

Lee, J.E., 1990. Description of first-instar larva of *Syneta adamsi* Baly from Japan, with notes on the systematic position of Synetinae (Coleoptera: Chrysomelidae). Esakia 29, 77–81.

Lefèvre, E., 1885. Eumolpidarum Hujusque Cognitarum Catalogus. Hayez, Bruxelles.

Maddison, W.P., Maddison, D.R., 1992. MacClade: Analysis of Phylogeny and Character Evolution, Version 3.0. Sinauer Associates, Sunderland, MA.

Maddison, W.P., Maddison, D.R., 2003. Mesquite: A Modular System for Evolutionary Analysis. Version 1.0. Available from: <http://mesquiteproject.org>.

Mickevich, M.F., Farris, J.S., 1981. The implications of congruence in *Menidia*. Syst. Zool. 30, 351–370.

Phillips, A., Janies, D., Wheeler, W.C., 2000. Multiple sequence alignment in phylogenetic analysis. Mol. Phylogenet. Evol. 16, 317–330.

Reid, C.A.M., 1995. A cladistic analysis of subfamilial relationships in the Chrysomelidae *sensu lato* (Chrysomeloidea). In: Pakaluk, J., Slipinski, S.A. (Eds.), Biology, Phylogeny and Classification of Coleoptera. Papers Celebrating the 80th Birthday of Roy A. Crowson. Muzeum i Instytut Zoologii PAN, Warszawa, pp. 559–631.

Reid, C.A.M., 2000. Spilopyrinae Chapuis: a new subfamily in the Chrysomelidae and its systematic placement. Inv. Tax. 14, 837–862.

Seeno, T.N., Wilcox, J.A., 1982. Leaf beetle genera. Entomography 1, 1–221.

Selman, B.J., 1965. A revision of the Nodini and a key to the genera of Eumolpidae of Africa (Col. Eumolpidae). Bull. Brit. Mus. (Nat. Hist.) Ent. 16, 143–174.

Shull, V.L., Vogler, A.P., Baker, M.D., Maddison, D.R., Hammond, P.M., 2001. Sequence alignment of 18S ribosomal RNA and the basal relationships of Adephagan beetles: evidence for monophyly of aquatic families and the placement of the Trachypachidae. Syst. Biol. 50, 945–969.

Simon, C., Frati, F., Beckenbach, A.T., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Springlová, B., 1960. Essai monographique du genre *Eumolpus* (Coleoptera Phytophaga). Mem. Inst. R. Sci. Nat. Belgique 60, 1–79.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Verma, K.K., Jolivet, P., 2000. Phylogeny of Synetinae—reconsidered. Nouv. Revue Ent. (N.S.) 17, 35–49.

Verma, K.K., Jolivet, P., 2002. Comments on Spilopyrinae (Col. Chrys.). Nouv. Revue Ent. (N.S.) 19, 99–110.

Wheeler, W.C., 1995. Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. Syst. Biol. 44, 321–331.

Wheeler, W.C., 1996. Optimization alignment: the end of multiple sequence alignment in phylogenetics?. Cladistics 12, 1–9.

Wheeler, W.C., 1999. Measuring topological congruence by extending character techniques. Cladistics 15, 131–135.

Wheeler, W.C., 2003. Implied alignment: a synapomorphy-based multiple-sequence alignment method and its use in cladogram search. Cladistics 19, 261–268.

Wheeler, W.C., Gladstein, D., DeLaet, J., 2002. POY version 3.0. Phylogeny reconstruction via Direct Optimization of DNA and other data. American Museum of Natural History, New York.