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Molecular phylogeny of the tribe Sphodrini (Coleoptera: Carabidae) based on mitochondrial and nuclear markers

Carlos Ruiz [a,*], Bjarte Jordal [b], José Serrano [a]

[a] Departamento de Zoología y Antropología Física (Biología Animal), Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, 30071 Murcia, Spain
[b] Museum of Natural History, University of Bergen. Museplass 3, NO-5007 Bergen, Norway

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ABSTRACT

A phylogenetic analysis of 6.4 kb of nucleotide sequence data from seven genes (mitochondrial cox1-cox2 and $tRNA_{leu}$ and nuclear Ef-1α C0, Ef-1α C1, 28S, and 18S) was done to reconstruct the phylogenetic relationships of the ground-beetle tribe Sphodrini. Gene regions of variable nucleotide length were aligned using both a secondary structure model, Clustal W, and a combination of the two. Sensitivity analysis was performed in order to explore the effect of alignment methods. The ribosomal and protein-coding genes were largely congruent based on the ILD test and partitioned Bremer support measures. MtDNA analysis provided high resolution and high support for most clades. The tribe Sphodrini and the related tribes Platynini, Pterostichini and Zabrini made up monophyletic clades, but the relationship between them was weakly resolved and sensitive to alignment strategy. Previously suggested relationships between subtribes of Sphodrini were not corroborated, and only the subtribe Atranopsina revealed high support as the sister clade to the other subtribes. The analyses clearly demonstrated the importance of exploring effects of alignment methods that may become particularly important in resolving polytomies and nodes with low support.

1. Introduction

The tribe Sphodrini is included in the subfamily Harpalinae of Erwin (1985), or the family Harpalidae of Deuve (1988), a large group of derived Carabidae that comprises around 19,500 species grouped in 37 (Lorenz, 2005) or 35 tribes (Erwin, 1985). Sphodrini includes about 823 species in 38 genera distributed mainly in the Holarctic region (Casale, 1988), with some additional species known from the East African region. According to the criteria used in the last Palearctic catalog (Hovorka and Sciaky, 2003) there are six subtribes: Atranopsina (101 spp), Calathina (179 spp), Dolichina (28 spp), Pristosiina (65 spp), Sphodrina (357 spp) and Synuchina (97 spp). These criteria are based on the classification proposed by Casale (1988).

The Sphodrini are distinguished from related tribes by the carinate posterior margin of prosternum, the gonostyli are usually armed by two or three spines (sensu Habu, 1978; Casale, 1988, etc.) but without a basal fringe of setae, and the asymmetric parameres of the male genitalia (the right one is often styloid). Members of the tribe are found in forests and open habitats of temperate areas but there are also a fair number of cave specialists. Life cycles vary notably (Matalin, 2007), but reproduction is more common in autumn (Luff, 1998).

The phylogenetic relationship of Sphodrini to other Harpalinae tribes is controversial. Jeannel (1942) suggested a close relationship between Sphodrini and Platynini, a hypothesis that has received general recognition on the basis of genitalia and external morphology (Lindroth, 1956; Liebherr, 1986; Casale, 1988). Recent catalogs (Hovorka and Sciaky, 2003; Lorenz, 2005) support also this relationship by admitting a subfamily Platyninae for both tribes. However, this hypothesis needs further assessment, as Arndt (1993) showed that platynines are polyphyletic with respect to larval characters and thus only a fraction of this tribe would be related to sphodrines. Recent analyses of 18SrDNA, 28SrDNA and *wingless* DNA sequences resulted in a polytomy comprising most representatives of Harpalinae (Sphodrini, Platynini, Pterostichini, Zabrini, etc.) (Maddison et al., 1999; Ober, 2002). Morphological data therefore provide the most reliable clues so far for Harpalinae relationships, indicating that Sphodrini and Platynini are likely related to Pterostichini and Zabrini (Erwin, 1985; Kryzhanovskij et al., 1995).

Relationships between the subtribes of Sphodrini are similarly controversial. Lindroth (1956) distinguished three lineages: Sphodri (Sphodrina, Calathina and *Dolichus*), Pristosiae (Pristosiina) and Synuchi (including Atranopsina), based mainly on characters in the male genitalia. Habu (1978) reclassified Sphodrini based on female genitalia, resulting in only two lineages—Sphodrina and Dolichina. Casale (1988) suggested a classification with six subtribes, Calathina, Sphodrina, Pristosiina, Dolichina, Synuchina,

* Corresponding author. Fax: +34 968 364906.
E-mail address: caruiz@um.es (C. Ruiz).

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and Atranopsina. He related Sphodrina and Calathina because both share a right styloid paramere and the gonostyli have a fovea with two short setae. He furthermore related Dolichina and Synuchina by sharing female gonostyli without fovea. Atranopsina and Pristosiina are supposedly more distantly related taxa, though Casale (1988, p. 126) considered the later closer to Calathina plus Sphodrina. Overall, the evolutionary history for Sphodrini is complicate and in need of new data to reconstruct the phylogeny and enable a more stable classification. We will therefore test the various hypotheses presented above using multiple sources of molecular data.

Previous molecular studies on Harpalinae (Maddison et al., 1999; Ober, 2002; Martinez-Navarro et al., 2005) have shown problems in solving their phylogenetic relationships. Some of them are possibly related to the low number of molecular markers applied. We are therefore increasing the number of mitochondrial and nuclear markers, and sample a large number of Sphodrini and outgroup taxa in the tribes Platynini, Pterostichini and Zabrini. The molecular data include partial sequences of two mitochondrial genes (cox1 and cox2), one nuclear protein-coding gene (Ef-1α), and two nuclear ribosomal genes (18S and 28S DNA). It is expected that the combination of mitochondrial and nuclear genes with different substitution rates will allow for a comprehensive phylogenetic analysis of Sphodrini.

In addition to increasing the amount of molecular data, we also consider several methodological problems, and particularly those associated with alignment of length variable regions. Morrison and Ellis (1997) showed that alternative alignments may result in alternative trees and concluded that phylogenies are often more sensitive to the alignment methods than to the tree reconstruction method used. Exploration of different alignment parameters is frequently neglected, e.g., by using default parameters in alignment software. Likewise, it may be also found that alignment ambiguous regions are simply excluded (Giribet and Ribera, 2000; Bleidorn et al., 2003; Kjer, 2004). However, these ambiguous regions can be aligned based on homologous secondary structures in the ribosomes which is more conservative than raw nucleotides, and that frequently yield more valuable phylogenetic information (Kjer, 1995; Morrison and Ellis, 1997; Xia et al., 2003; Kjer et al., 2007; Jordal et al., 2008). We are here exploring and comparing alignments based on rDNA secondary structure (Kjer et al., 2007) with a computer based alignment constructed in ClustalW. We are also exploring the impact of different parameter sets on the alignment by comparing the phylogenetic signal in the ambiguously aligned regions to unambiguous alignment regions, as previously emphasized by Wheeler (1995).

We aim to explore the phylogenetic relationships between Sphodrini and related tribes, and those within the tribe. More specifically, we are testing the hypotheses that (i) Platynini is the sister taxon of Sphodrini and (ii) the relationships between subtribes of Sphodrini are those depicted by Casale (1988). We also aim to test the effects of different alignment strategies on the reconstruction of phylogenetic relationships of these taxa.

2. Materials and methods

2.1. Taxa studied

Fifty-eight individuals belonging to 46 species and 29 genera representing the six subtribes of Sphodrini and related tribes have been sampled (Table 1). Available sequences in GenBank from six Sphodrini taxa were also included. The 14 studied genera of Sphodrini make up about 37% of described genera within the tribe. As initial outgroups we selected species from tribes currently accepted as putative closest relatives (Jeannel, 1942; Liebherr, 1986; Casale, 1988; Ober, 2002; Löbl and Smětana, 2003; Lorenz, 2005). Moreover, further analyses were made to search for related taxa within the subfamily Harpalinae using available 18S and 28S sequences from GenBank. Our preliminary phylogenetic analyses corroborated that Platynini, Pterostichini, Zabrini were the closest relatives to Sphodrini and have therefore been assigned as outgroups.

The individuals studied are kept in the collection of the Departamento de Zoología y Antropología Física (University of Murcia) at −20 °C in ethanol. Material of Nearctic Sphodrini were received on loan from the ESPM (UC-Berkeley) and will be subsequently returned.

2.2. Molecular markers

We studied a continuous fragment of the mitochondrial cytochrome oxidase I and II (cox1-cox2) genes (1675 bp) which includes part of cox1 gene (875 bp), the intervening tRNAleu (66 bp), and most of cox2 gene (734 bp). Within the ribosomal 28S rDNA we studied a partial sequence of about 974 bp length in the D2–D4 region. The most commonly amplified copy of the protein-coding gene Elongation Factor 1α (Ef-1α) lacked introns and consisted of 773 bp (named Ef-1α C0). A second paralogous copy included a single intron copy in position 753/754, similar to the Ef-1α C1 intron structure in other beetles (Jordal, 2002), and was studied in 19 taxa. EF-1α C0 is an intron free copy reported for the first time from Coleoptera, similar to that reported in Lepidoptera and Diptera (Jordal, 2002; Djernæs and Damgaard, 2006). The intron free copy of EF-1α is further distinguished from the intron-bearing copy in Carabidae by an average nucleotide sequence divergence of 9% similar to paralogous copies reported from other Coleoptera (Jordal, 2002). A nearly complete ribosomal 18S rDNA (2051 bp) sequence was studied in 16 taxa to assess outgroup relationships. All sequence data together comprised a total of 6.4 kb of nucleotide information. Sequences were deposited in GenBank under the accession numbers provided in Table 1.

2.3. Amplification and sequencing

DNA was extracted with QIAGEN Dneasy tissue kit (Qiagen, Hilden, Germany). Partial gene sequences were amplified by PCR using primers listed in Table 2. PCRs were made in a 25-μl volume. The mitochondrial cox1-cox2 fragment was amplified as described in Ruiz and Serrano (2006). The partial sequence of 28S rDNA (D2–D4 region) was amplified under standard PCR conditions (1 min at 50 °C annealing temperature). The nearly complete sequence of 18S rDNA (V1 to V9 domains) was obtained using four primer combinations as described in Shull et al. (2001). EF-1α sequences were amplified with a touchdown profile with primers Efs149 and Efa1043 (Normark et al., 1999), consisting of 31 cycles, each consisting of 94 °C denaturing for 30 s and 72 °C extension for 1 min. Annealing parameters in the first cycle were 58 °C during 1 min; temperature decreased by 2 °C in each of the next 6 cycles (it was then 46 °C in the seventh cycle), and was kept at 44 °C during the final 24 cycles. One microliter of the PCR product was used in a nested PCR with the primers Efs149 and Efa923 under standard PCR conditions (45 s at 50 °C annealing temperature). In some taxa it was found two bands of about 750 and 900 bp, corresponding to the two paralogous copies EF-1α C0 and EF-1α C1.

PCR products were purified with isopropanol and 5 M ammonium acetate. Double bands of EF-1α were purified directly from gel (MinElute Gel Extraction Kit, Qiagen, Hilden, Germany). Sequencing was performed in both directions using standard protocol for ABI BigDye(r) Terminator v3.1 Cycle sequencing kit (Applied Biosystems).

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Table 1
Species of the tribe Sphodrini and related taxa included in the molecular analysis, and accession numbers of the corresponding individual sequence. (CI: Canary Islands, CN: China, GE: Georgia, JP: Japan, MO: Morocco, RU: Russia, SP: Spain, TR: Turkey, US: USA).

Taxon	Locality	cox	28S	Ef-1α C0	Ef-1α C1	18S
Tribe Sphodrini						
Calathina						
Calathus mollis 120	Soria (SP)	FJ173193	FJ173071	FJ173133		FJ173117
Calathus fuscipes 126	Bab-Berret (MO)	AM410883	FJ173072	FJ173134		
Calathus fuscipes 315	Bab-Berret (MO)					FJ173118
Calathus rotundicollis 153	Soria (SP)		FJ173073	FJ173135	FJ173174	
Calathus rotundicollis 137	Soria (SP)	AM410879				
Calathus circumseptus 228	Córdoba (SP)	FJ173207	FJ173086	FJ173144	FJ173180	
Lindrothius caucasicus 271	Lebarde (GE)	FJ173220	FJ173100	FJ173157	FJ173182	
Lindrothius pseudopraestans 296	Caucasus (RU)	FJ173235		FJ173171		
Lindrothius sp. 297	Abkhazia (GE)	FJ173236	FJ173115	FJ173172		
Dolichina						
Xestopus sp. 194	Qingling mountains (CN)	FJ173200	FJ173080	FJ173140		
Anchomenidius astur 239	León (SP)	FJ173210	FJ173090	FJ173148	FJ173181	
Anchomenidius astur 277	León (SP)	FJ173221	FJ173101	FJ173158	FJ173183	
Dolichus halensis 282	Kōchi (JP)	FJ173226	FJ173105	FJ173162		FJ173129
Dolichus halensis 283	Kōchi (JP)	FJ173227	FJ173106			FJ173130
Pristosiina						
Pristosia aeneola 284	Shizuoka (JP)		FJ173107	FJ173163		FJ173131
Pristosia aeneola 285	Nagano (JP)	FJ173228	FJ173108	FJ173164		
Sphodrina						
Laemostenus terricola 227	Granada (SP)	FJ173206	FJ173085	FJ173143	FJ173179	
Laemostenus complanatus 256	Murcia (SP)	FJ173217	FJ173097	FJ173154		FJ173126
Laemostenus terricola 281	Málaga (SP)	FJ173225	FJ173104	FJ173161	FJ173186	
Licinopsis obliterata 254	La Gomera (CI)	FJ173215	FJ173095	FJ173152		
Calathidius accuminatus 322	Tenerife (CI)	AJ405004				
Synuchina						
Synuchus sp1 178	Westmoreland, Pennsylvania (US)	FJ173195	FJ173075			
Synuchus sp3 180	Qingling mountains (CN)	FJ173196	FJ173076	FJ173137		
Synuchus sp2 187	GSNP, North Carolina (US)	FJ173197	FJ173077	FJ173138		
Synuchus sp3 191	Qingling mountains (CN)	FJ173198	FJ173078	FJ173139		
Synuchus sp4 192	Qingling mountains (CN)	FJ173199	FJ173079		FJ173175	
Synuchus vivalis 202	Huesca (SP)	FJ173203	FJ173083	FJ173142		FJ173120
Synuchus melantho 286	Mt Taishaku. Fukushima (JP)	FJ173229	FJ173109	FJ173165	FJ173187	
Synuchus nitidus 289	Chiba (JP)	FJ173230	FJ173110	FJ173166	FJ173188	
Synuchus nitidus 290	Chiba (JP)	FJ173231	FJ173111	FJ173167		
Synuchus cycloderus 291	Fukui (JP)	FJ173232	FJ173112	FJ173168	FJ173189	
Synuchus angusticeps 293	Nara (JP)	FJ173233	FJ173113	FJ173169	FJ173190	
Atranopsina						
Platyderus varians 245	Madrid (SP)	FJ173211	FJ173091	FJ173149		FJ173122
Platyderus urbionensis 249	Soria (SP)	FJ173212	FJ173092			
Amaroschema gaudini 299	Tenerife (CI)	FJ173237	FJ173116	FJ173173	FJ173192	
Paraeutrichopus harpaloides 316	El Hierro (CI)	AM263032				
Paraeutrichopus harpaloides 317	El Hierro (CI)	AM263033				
Paraeutrichopus harpaloides 318	El Hierro (CI)	AM263034				
Paraeutrichopus pecoudi 319	La Gomera (CI)	AM262998				
Paraeutrichopus pecoudi 320	La Gomera (CI)	AM262999				
Gomerina calathiformis 321	La Gomera (CI)	AM263035				
Tribe Platynini						
Agonum muelleri 196	Asturias (SP)	FJ173201	FJ173081		FJ173176	FJ173119
Anchomenus dorsalis 197	Burgos (SP)	FJ173202	FJ173082	FJ173141	FJ173177	
Paranchus albipes 206	Málaga (SP)	FJ173204	FJ173084		FJ173178	
Paranchus albipes 278	Albacete (SP)	FJ173222	FJ173102		FJ173184	
Olisthopus fuscatus 259	Burgos (SP)	FJ173218	FJ173098	FJ173155		FJ173127
Tribe Pterostichini						
Percus politus 280	Málaga (SP)	FJ173224		FJ173160		FJ173128
Orthomus velocissimus 235	Málaga (SP)	FJ173209	FJ173088	FJ173146		
Steropus globosus 294	Málaga (SP)	FJ173234	FJ173114	FJ173170	FJ173191	FJ173132
Cryobius nemoralis 238	Madrid (SP)		FJ173089	FJ173147		
Poecilus purpurascens 279	Málaga (SP)	FJ173223	FJ173103	FJ173159	FJ173185	
Poecilus quadricollis 207	Málaga (SP)	FJ173205				
Ancholeus sp. 170	Lago Abant (TR)	FJ173194	FJ173074	FJ173136		
Tribe Zabrini						
Zabrus ignavus 255	Xauen (MO)	FJ173216	FJ173096	FJ173153		FJ173125
Amara aenea 250	León (SP)	FJ173213	FJ173093	FJ173150		FJ173123
Tribe Harpalini						
Stenolophus teutonus 252	Malaga (SP)	FJ173214	FJ173094	FJ173151		FJ173124
Pseudoophonus rufipes 260	Lérida (SP)	FJ173219	FJ173099	FJ173156		
Harpalus affinis 233	Asturias (SP)	FJ173208	FJ173087	FJ173145		FJ173121
Fifty-eight individuals of 46 spp.		54	46	41	19	16

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Table 2
Primers used to amplify the sequences studied.

Gene	Primer name	Other	F/R	Sequence (5′–3′)	Reference
mtDNA					
cox1	CALCOI	C1-J-2160	F	5′-TAA CAG ATC GAA ATT TAA ATA CTT-3′	Emerson et al. (2000a)
	UEA9CAL		F	5′-GTA AAT TTA ACA TTT TTT CCT CAA CA-3′	modified from Lunt et al. (1996)
tRNAleu	TEDCAL		F	5′-TAA TGT GGC AGA TTA GTG CAA TGA A-3′	Designed by Brent Emerson
cox2	CALCOII		F	5′-TTA AAA TCT ATT GGT CAT CAA TGA TA-3′	Designed by Brent Emerson
	EVA	TK-N-3782	R	5′-GAG ACC ATT ACT TGC TTT CAG TCA TCT-3′	Harrison Laboratory
Nuclear					
EF-1α	EFs149	Efs 149b	F	5′-ATC GAG AAG TTC GAG AAG GAG GCY CAR GAA ATG GG-3′	Normark et al. (1999)
	EFa923		R	5′-AGG TTC TTC ACG TTG AAR CAA A-3′	
	EFa1043		R	5′-GTA TAT CCA TTG GAA ATT TGA CCN GGR TGR TT-3′	
28S	28S 3690s		F	5′-GAG AGT TMA ASA GTA CGT GAA AAC-3′	modified from Sequeira et al. (2000)
	28Sb		R	5′-TCG GAA GGA ACC AGCTAC TA-3′	Cryan et al., 2001
18S	18S5′		F	5′-GAC AAC CTG GTT GAT CCT GCC AGT-3′	modified from Maddison et al. (1999)
	18Sb5.0		R	5′-TAA CCG CAA CAA CTT TAA T-3′	Whiting et al. (1997)
	18Sa.i		F	5′-CCT GAG AAA CGG CTA CCA CAT C-3′	Maddison et al. (1999)
	18Sb2.5		R	5́-TCT TTG GCA AAT GCT TTC GC-3′	Shull et al. (2001)
	18Sa1.0		F	5́-GGT GAA ATT CTT GGA CCG TC-3′	Whiting et al. (1997)
	18Sb.i		R	5′-GAG TCT CGT TCG TTA TCG GA-3′	Whiting et al. (1997)
	18Sa2.0		F	5′-ATG GTT GCA AAG CTG AAA C-3′	Whiting et al. (1997)
	18S3′I		R	5′-CAC CTA CGG AAA CCT TGT TAC GAC-3′	Shull et al. (2001)

2.4. Sequence alignment

Insertion of 3–8 bp in 4 sequences at the 3′ end of cox1 were unambiguously aligned by eye after translation to amino acids. Length in $tRNA_{leu}$ ranged from 60 to 66 bp, and was also readily aligned by eye. The cox2 sequence starts with methionine except for species in the subtribe Sphodrina where the coding region begins with isoleucine (ATT codon) as previously found in several other insects (Liu and Beckenbach, 1992; Gómez-Zurita et al., 2000). The 3′ end of cox2 also revealed length heterogeneity and was aligned according to translated amino acids: there was a 9-bp insertion in *Stenolophus teutonus* (Harpalini; outgroup) and 12 sequences showed a deletion of 3 bp.

To align the rDNA sequences (28S and 18S) three different strategies were followed. A manual alignment was based on a secondary structure (2S) modeled for other insects (Kjer, 1995). To develop the model more specifically for Sphodrini and relatives, we further characterized the expansion segments using the Mfold software (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi), which folds RNA sequences based on minimum free energy (ΔG) (Zuker, 2003). The base-pairing helices and hairpins were confirmed with compensatory base change across the majority of taxa. The final alignment resulted in conserved regions and variable-length regions of ambiguous alignment (RAA) (Gillespie, 2004). These RAA were excluded from the analysis. In a second strategy (CW), a computer-based alignment was produced in Clustal W (Thompson et al., 1994) version 1.6 as implemented in MEGA version 4 (Tamura et al., 2007) for the entire sequence. Two parameters were explored, the gap opening (GOP) and gap extension penalties (GEP). Transitions were weighted 0.5 related to transversions. We used different weighting regimes (GOP/GEP: 1/0.25, 1/0.5, 2/0.25, 2/0.5, 2/1, 4/0.5, 4/2, 8/1, 8/4, and default parameters 15/6.6 frequently used in phylogenetic analyses), resulting in 10 different matrices for each rDNA matrix. Third, although structural information can be used to improve alignment the exclusion of RAA remains questionable; we therefore conducted a mixed approach (named as MX) in which conserved and variable regions were defined based on secondary structure, and the variable regions were aligned with Clustal W using the same parameters. The secondary structure model of 18S followed the main domains of the model proposed for *Loricera foveata* (http://bioinformatics.psb.ugent.be/webtools/rRNA/sec-model/LfovSSU.html) (Figs. 1 and 2, Supplementary material). The alignment of 28S was carried out following the model of Gillespie et al. (2004) and refined using our new carabid specific corrections for the expansion segments (Figs. 3 and 4, Supplementary material).

The EF-1α C0 sequences were aligned unambiguously due to the lack of any insertion or deletion in the coding region. The EF-1α C1 intron was identified by the first two bases "GT" and the last two bases "AG" in position 753/754, and had a variable length between 177 and 188 bp. It was aligned in Clustal W using the same combination of gap penalties as above. We explored the influence from intron characters by including or deleting these nucleotides in the different analyses.

The quality of various alignments cannot be directly evaluated unless the phylogenetic history is known. Without any certain way of objectively measuring the accuracy of reconstruction, only precision (the agreement among data) can be used to arbitrate among competing hypotheses (Wheeler, 1995). We therefore performed a sensitivity analysis for each length variable marker by comparing the phylogenetic signal in the aligned region with the signal in length invariable data, using congruence as an optimality criterion. The alignment parameters that minimized the incongruence among partition were considered optimal for a given data set (Wheeler and Hayashi, 1998). A partition homogeneity test (Farris et al., 1994, 1995) as implemented in PAUP* was used to estimate incongruence length differences (ILD) (Mickevich and Farris, 1981). Heuristic searches (TBR, max. trees = 500) in each partition were done with 20-sequence addition replicates, with invariant characters removed (Cunningham, 1997). Congruence between coding genes (cox1-cox2 and EF1a C0) and the ribosomal genes was tested with the latter genes aligned with the different gap penalties described above for either the full sequence length (CW), or for the variable regions (MX) only.

Because empirical and simulated data have demonstrated the potential failure of the ILD test under certain conditions (Dolphin et al., 2000; Barker and Lutzoni, 2002; Darlu and Lecointre, 2002), we also measured the congruence between different markers using Bremer Support (Bremer, 1994). This approach gives the number of extra steps needed to construct a tree without a certain node. Partitioned Bremer support (PBS) was calculated with TreeRot v.3 (Sorenson and Franzosa, 2007), to assess the different contribution from each gene partition to the total branch support in the combined analysis (Baker and DeSalle, 1997). A tree was obtained using unambiguously aligned partitions (cox1-cox2, EF1a

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C0, EF1a C1 exon, and conserved regions of rDNA). Congruence between protein-coding genes and conserved rDNA regions vs. the intron and ambiguously aligned rDNA regions was then calculated. Default settings were used for the heuristic Bremer Support searches, with 1000 random addition sequences per constrained node. A positive PBS indicates support for the node by the gene partition, whereas a negative PBS indicates that the given data partition is incongruent with that node. PBS values were standardized for partitions of different length by dividing the PBS value by the minimum possible length of each node (DeBry, 2001). We also measured the relative PBS support from each partition in relation to the node length measured as the length accumulated from the tips to the root in the combined data topology.

Topological congruence (a measure of similarity between different topologies) was explored in more detail by studying the number and congruence of taxon groups (tribes and subtribes) recovered as monophyletic across different alignment strategies and markers, and also the variation in node support and stability (Giribet, 2003).

2.5. Phylogenetic analysis

Each data partition was analyzed separately as well as in combination. Combining different partitions more likely recovered the correct phylogeny through the interaction of concordant phylogenetic signals from different genes, signals that frequently are hidden in separate data partitions (Bremer, 1994; Gatesy et al., 1999). Protein coding genes were translated into amino acids to explore their phylogenetic signal in comparison to the nucleotide signal.

Different combinations of datasets were done to explore the effect of alignments and missing data. A complete dataset was built using a matrix of unambiguously aligned protein-coding genes (cox1-cox2 fragment, and the exons of EF-1α C0 and EF-1α C1) and the conserved region of 28S and 18S. The effect of the various alignments on the combined data topology was explored by adding the ambiguous data aligned under different parameters. To explore the effect of missing data two datasets of 16 and 19 taxa were constructed, the first included a pruned matrix of cox1-cox2, EF-1α C0, 28S, and 18S, the second was a pruned matrix of the former genes (except 18S) plus EF-1α C1.

Each of the data matrices was subjected to Bayesian analysis (BA) with MrBayes v3.1. (Ronquist and Huelsenbeck, 2003), and Maximum Parsimony (MP) analysis as implemented in PAUP* b4.0b10 (Swofford, 2002). All the data sets were analyzed with Modeltest (v.3.6) (Posada and Crandall, 1998) to find the best-fit model of sequence evolution (Table 3). The Akaike information criterion (AIC) was preferred over hLRTs to test the various models as recommended by Posada and Buckley (2004). The effect of data partition was tested under different partitioning strategies for separate and combined analysis under Bayesian analyses. Separated analysis was partitioned under three strategies: as whole genes (intron-exon for EF-1α C1) and partitioning by codon position (1st, 2nd and 3rd) and (1st + 2nd and 3rd). Bayesian analyses were performed with 1,250,000 generations, sampling trees every 100 generations. Likelihood values were observed with Tracer v.1.4 (Rambaut and Drummond, 2005), discarding all the trees before stability in likelihood values as a 'burn in' (first 1200 trees). Stationarity was also reassessed using a convergence diagnostic. An average standard deviation of the split frequencies <0.015 (combined analyses) and <0.03 (separate analyses) were used as criteria of convergence between both runs. Also convergence was assessed by the potential scale reduction factor (PSRF).

The combined analysis was partitioned by gene (7 partitions) with 4 million generations and sampling trees every 100 generations.

Table 3
Data set and the estimated models of sequence evolution by the Akaike information criterion (AIC). Normal type denotes codon partition, bold type the main partitions.

Partition	AIC
cox_1st	GTR + I + G
cox1_2nd	TVM + I
cox1_3rd	TVM + G
cox1_tot	**GTR + I + G**
tRNAleu	**TIM + G**
cox2_1st	GTR + G
cox2_2nd	TVM + I + G
cox2_3rd	TVM + G
cox2_tot	**GTR + I + G**
cox1-cox2	**GTR + I + G**
EFc1_1st	TrN + I
EFc1_2nd	TVM
EFc1_3rd	HKY + G
EFc1_intron	**TrN + I + G**
EFc1_exon	**SYM + I + G**
EFc1_tot	**GTR + I + G**
Efc0_1st	TIM + I
EFc0_2nd	GTR + I
EFc0_3rd	TVM + G
EFc0_tot	**GTR + I + G**
18S_tot	**TVM + I + G**
28S_tot	**GTR + I + G**

All the partitioned analyses were done following Marshall et al. (2006) by unlinking branch lengths and accommodating among partition rate variation (APRV) with option "prset ratepr = variable" in MrBayes. Parsimony analyses were run in PAUP* using heuristic searches with TBR branch swapping and 10,000 random addition sequences. Confidence in each node was assessed by bootstrapping (500 pseudo-replicates, heuristic search of 20 random addition replicates with TBR option).

2.6. Evolutionary rates and age estimation

We used the mtDNA to estimate approximate divergence times for Sphodrini. A Bayesian Markov Chain Monte Carlo (MCMC) approach was used with uncorrelated Lognormal relax molecular clock analysis (Drummond et al., 2006), as implemented in BEAST v.1.4.7 (Drummond and Rambaut, 2007). Input files were generated with BEAUti (Rambaut and Drummond, 2008). Mutation rate was not fixed and an uncorrelated lognormal relaxed molecular clock was selected. The data set was treated as two partitions by codon position 1 + 2 and 3 under the GTR + I + Γ model of nucleotide substitution. The substitution model, rate heterogeneity, and base frequencies across codon positions were unlinked. A Bayesian tree was used as user defined starting tree and considering the Yule process tree prior.

No suitable fossil record for Sphodrini tribe was available to be used as "minimum age" for a node. However two external calibration points (nodes a and b; Fig. 6) were used. A normal distribution was used to provide for uncertainties of fossil calibration and fossil identifications of both calibration points. For node a we used a normally distributed estimate prior of 100 mya, standard deviation: 20 (95% range: 132 to 67 mya) based on the first Harpalinae fossils (Ponomarenko, 1992), and node b was calibrated to 35 mya, SD 10 (95% range: 51 to 18 mya) based on a pterostichini–platynini-like fossil in Baltic amber dated to 35 mya (Lindroth, 1974). The origin of the Macaronesian islands was used as "maximum age" for three Canarian clades (nodes c $\leqslant$ 11.6 Myr; d $\leqslant$ 10 Myr and e $\leqslant$ 1 Myr in Fig. 6; Guillou et al., 2004) using a prior uniform distribution. This approach has been successfully applied in several recent studies

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(Emerson et al., 2000b; Emerson and Oromí, 2005; Contreras-Díaz et al., 2007). Mutation rates per lineage were constrained under uniform distribution between 0.0025 and 0.0175 values adjusted in the majority of previously published studies on Coleoptera. Two independent runs consisting on 10 million generations each were performed sampling every 100 generations and a burn-in was set to 10% of the samples. Results were displayed in Tracer v.1.4 (Rambaut and Drummond, 2005) to check for stationarity. Both runs were combined in LogCombiner v.1.4.7 (Rambaut and Drummond, 2007). Tree information was annotated with TreeAnnotator v.1.4.7 (Rambaut and Drummond, 2008a) and visualized in FigTree v.1.1.2 (Rambaut, 2008).

3. Results

3.1. Sequence alignment

Sequences of 28S varied notably in length. CW approach produced longer alignments than MX and length range obtained under different gap penalties was higher in CW (from 974 to 1487 bp) than in MX (from 1001 to 1136 bp) approach. There is no markedly variation in 18S due to the small length of the variable regions compared to the conserved ones.

The secondary structure of the 18S sequences were conservative, with only two variable regions in the expansion segments V4 and V7 (Figs. 1 and 2, Supplementary material). The secondary structure of 28S had 11 variable-length regions (about 28% of the total length). The unambiguously aligned regions in the conserved domains had 148 phylogenetically informative sites across all taxa. The proposed model for D2 and D3 domains are shown in Figs. 3 and 4, respectively (Supplementary material).

3.2. Sensitivity analyses

Matrices of cox1-cox2 and EF-1α C0 were congruent (ILD: $P > 0.05$). Therefore we combined these two partitions to measure the incongruence with the variously aligned data partitions.

The ILD test revealed that 18S was most congruent when using the lowest values of the weighting range (GOP: 1, GEP: 0.25, $P = 0.49$; Table 4). There was incongruence between 28S and protein coding genes for all parameter settings (P value = 0.001) when full sequence lengths were aligned with CW. With MX alignment the sensitivity curve showed a peak of congruence at low gap cost: GOP/GEP = 1/0.5 (P value = 0.105). For the EF-1α C1 intron the higher level of congruence was obtained when GOP was set to 2, although when comparing congruence between exon vs. intron the ILD lowest value was GOP/GEP: 8/4.

The Partition Bremer Support (PBS) was calculated for the various alignments (CW and MX). The PBS landscape of 18S using CW alignment revealed a relatively even and low values but with a much higher contribution at 15/6.6 (Fig. 5a, Supplementary material). For the MX approach, alignment parameters lower than 2/0.5 produced low PBS values (Fig. 5b, Supplementary material), while all higher parameter values showed higher contribution to node support. The highest peak was found with a weighting scheme of 4/0.5. For 28S the PBS values from the CW alignment showed two clear peaks (Fig. 6a, Supplementary material), at 2/0.5 and 8/4, with the first one having the highest value. For the MX alignment of 28S, there was a peak of PBS values when the GOP:GEP was set to 8/1 (Fig. 6b, Supplementary material). It must be noted that default parameters showed the lowest values of total congruence. The PBS contribution from the EF-1α C1 intron increased with the increase of the GOP/GEP cost, with the highest value under default parameters (both ends anchored, Fig. 7, Supplementary material).

The PBS contribution of each partition at various levels in the phylogeny was plotted in Fig. 1. MtDNA showed highest contribution to the combined data tree at median depth (distance about 0.05) and at tip levels. Negative contribution was found in EF-1α C1 and with certain alignment parameters of 18S at the tips level

Table 4
P values of the partition homogeneity test between protein-coding genes (cox1-cox2 and EF-1α C0) and ambiguous aligned genes (rDNA and intron of EF-1α C1). Bold numbers indicate ILD test significance at $P < 0.05$. CW, entire gene aligned with Clustal; MX, aligning only variable regions with Clustal.

GOP	GEP	18S CW *P*	28S CW *P*	28S MX *P*	Intron EF-1α C1 *P*
1	**0.25**	0.492	**0.001**	0.061	0.640
1	**0.5**	0.477	**0.001**	0.105	0.649
2	**0.25**	0.425	**0.001**	**0.017**	0.95
2	**0.5**	0.455	**0.001**	**0.034**	0.939
2	**1**	0.453	**0.001**	**0.011**	0.949
4	**0.5**	0.077	**0.001**	0.051	0.819
4	**2**	0.230	**0.001**	**0.007**	0.582
8	**1**	0.288	**0.001**	**0.019**	0.887
8	**4**	0.247	**0.001**	**0.005**	0.924
15	**6.6**	0.008	**0.001**	**0.001**	0.617

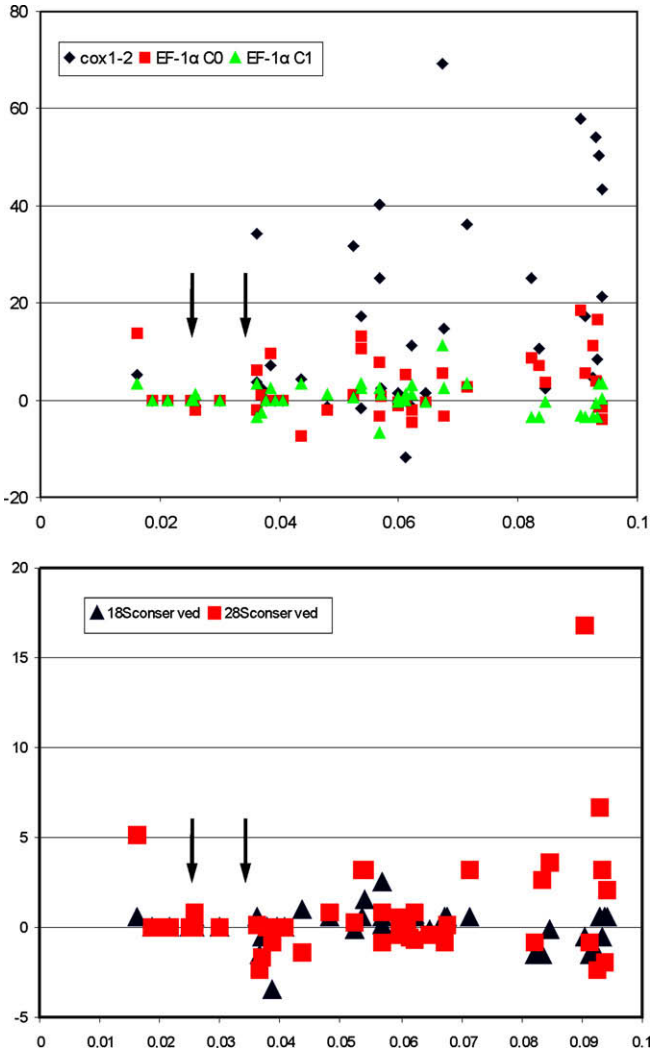

Fig. 1. Distribution of partitioned Bremer support values in relation to the distance of each node from the root (all molecular data, molecular clock enforced). Line at value 0 shows the transition between positive and negative contribution to the combined topology by the unambiguously aligned data. Arrows are discussed in the text.

(data not shown). Low PBS values were found at basal level for all the partitions (distance about 0.02, corresponding at nodes A, B and C).

3.3. Phylogenetic analysis

3.3.1. Separate analysis

The best model to account for substitution rates was in most cases the general time reversible model (GTR + I + Γ) or some derivatives (Table 3).

EF-1α C1 and rDNA genes showed the highest values of retention index (RI) and consistency index (CI) and cox1-cox2 the lowest under maximum parsimony (Table 5). Each individual gene generated an overall similar topology with low resolution for basal clades. Most subtribes and tribes were recovered as monophyletic groups with high support but the relationships between these clades varied depending on the gene and the method (Fig. 2).

MtDNA analysis provided high resolution and support for most clades. In the Bayesian analyses the resolution was improved in the basal clades when data were partitioned by codon. Subtribes Calathina and Dolichina were recovered with high support in all the partitioned strategies (pp: 0.98–1, Fig. 2). Unweighted parsimony analyses occasionally revealed alternative relationships (e.g., between *Dolichus* and the Platynini *Olisthopus fuscatus*).

Trees resulting from the analyses of nuclear protein-coding genes generally agreed with the mitochondrial ones. Basal nodes were better resolved although there was no increase in the resolution when these data were partitioned by codon position. EF-1α C0 grouped Calathina + Synuchina + Dolichina + Pristosiina (Fig. 2) with high support (pp = 1.0), with Sphodrina as sister taxon. The basal position of Sphodrina within clade P was incongruent with the relationships derived from the other protein-coding genes (cox1-cox2 and EF-1α C1), that placed Synuchina as basal.

The 18S trees showed limited resolution and only a few clades (subtribes of Sphodrini except Pristosiina) received node support. In the 28S analyses the conserved regions alone recovered most clades corresponding to higher taxa (tribes and subtribes), but not their interrelationships. There was an increase in support and resolution when variable regions were included and aligned with the full CW alignment approach using default parameters (Fig. 2). In contrast, the MX alignment approach resulted in poor resolution and low support for basal clades, and the topology was more sensitive to parameter choice. Likewise did the CI and RI values decrease relative to the CW approach (Table 6). The relationship between certain clades was sensitive to alignment parameters. In all Bayesian and some parsimony analyses a number of clades were obtained with high support only under certain alignment parameters; e.g., a clade formed by Pterostichini + Platynini + Zabrini was recovered with high support with parameters 2/0.5 in CW (pp = 0.92) and 8/4 in MX (pp = 0.98). These parameters were close to those resulting in the highest values in the PBS sensitivity analysis (CW: 2/0.5 and MX: 8/1). Clade 1 (Calathina) + 4 (Pristosiina) was recovered with high support with CW using default parameters (pp = 1, bootstrap = 91).

Analyses comparing both copies of EF-1α showed parallel evolution and congruence between both copies (corrected PBS = 3.6 and 0.3, respectively). The Bayesian analysis grouped each copy in separate clades, and also generally recovered the same topology for each copy (Fig. 8, Supplementary material).

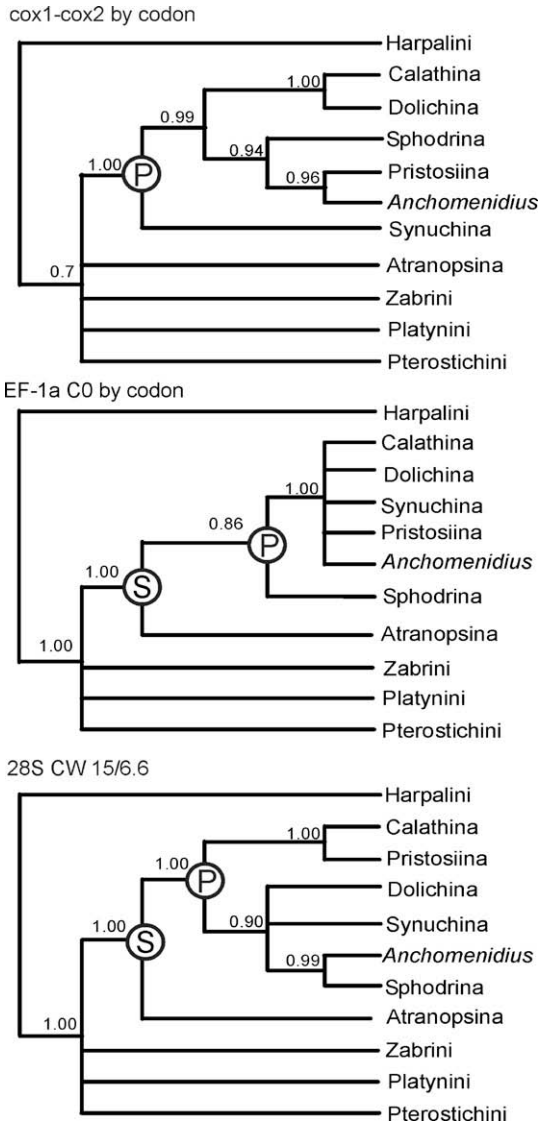

Fig. 2. Simplified cladogram with the main tribes of the 50% majority consensus tree resulting from the BA of the cox1-cox2 data partitioned by codon (1st, 2nd and 3rd), EF-1α C0 partitioned by codon (1st, 2nd and 3rd) and 28S aligned with CW approach (GOP/GEP selected: default parameters 15/6.6). Number above node shows posterior probability ⩾0.90. Circle letters are discussed in the text.

Table 5
Tree statistics for each data set. Ti/Tv ratio, transition/transversion ratio; CI, consistency index and RI, retention index under maximum parsimony. Results of ribosomal genes were obtained under default parameters of Clustal alignment (CW approach).

	cox1-cox2	EF-1α C0	EF-1α C1 exon	28S	18S
No. of taxa	47	46	19	41	16
Length	1675	773	738	974	2051
Conserved sites	914	533	552	531	1886
Variable sites	761	240	186	443	165
% Variable	45	31	25	45	8
Informative sites	600	197	141	346	80
Pairwise divergence	15.2	9	9	9	2
Mean base frequency					
A	33.7	25.3	26.2	25.5	25.8
C	13.5	27.0	25.5	18.3	21.7
G	13.3	25.1	23.7	25.6	26.4
Ti/Tv ratio	0.8	2.6	3.7	1.1	1.9
Tree length	3558	833	378	1429	318
No. of trees	6	91	9	4	12
CI	0.325	0.415	0.584	0.483	0.73
RI	0.484	0.595	0.6729	0.701	0.517

3.3.2. Combined analysis

The ILD test showed no incongruence between any of the protein-coding genes. Significant incongruence ($P < 0.05$) was found

Table 6
Tree statistics for ribosomal data set, under different alignment strategy. Conserved, only conserved region; CW, aligning the entire gene with Clustal; MX, aligning only variable regions with Clustal; CI, consistency index and RI, retention index under maximum parsimony.

Gene GOP/GEP	Length	C	Pi	Tree length	No. of trees	CI	RI
28S conserved	689	481	148	532	1932	0.494	0.690
28S CW2/05	1230	797	317	1097	4	0.522	0.701
28S CW 8/1	998	560	340	1356	6	0.486	0.698
28S CW15/6.6	974	531	346	1429	4	0.483	0.701
28S MX 8/1	1023	572	337	1372	68	0.484	0.658
28S MX 15/6.6	1001	558	341	1539	16	0.453	0.653
18S conserved	1908	1784	44	182	1180	0.753	0.408
18s CW 15/6.6	2051	1886	80	318	12	0.730	0.517
18s MX 4/0.5	2064	1889	74	279	90	0.731	0.460
18S MX 15/6.6	2052	1866	84	330	32	0.724	0.431

between combined protein-coding genes and 28S with CW alignment, and with most of the MX alignments (Table 4). Analyses of PBS showed that mitochondrial data contributed two times or more than the combined nuclear genes to the combined tree topology. Among the nuclear data, the protein coding EF-1α genes contributed twice the amount of ribosomal genes (Fig. 9, Supplementary material). Likewise the ambiguously aligned regions in the ribosomal genes contributed more than the conserved regions to the combined tree topology (Fig. 10, Supplementary material).

Bayesian analyses of the five markers under different alignment conditions revealed very similar tree topologies (Figs. 3–5). All topologies recovered the monophyly of tribes Sphodrini, Pterostichini, Platynini and Zabrini, each with high posterior probability (pp = 1.0). The relationships between Sphodrini (clade S) and the related tribes within clade A were not fully resolved and varied depending on the alignment strategy. Alignment under the full ClustalW approach using the selected parameters from the sensitivity analysis (18S: 15/6.6; 28S: 2/0.5) places Zabrini as the sister group to Pterostichini, Platynini and Sphodrini (clade B) with high support (1.0) (Fig. 11, Supplementary material). However, when default parameters were used in the full Clustal alignment, Zabrini, Platynini and Pterostichini (clade C) made up a sister clade to Sphodrini (clade S) with a support of 0.94. (Fig. 4). When the secondary structure was implemented in the rDNA alignments together with Clustal alignment of variable regions (MX procedure), a polytomy of the four tribes was recovered (Fig. 5). Pruned analyses recovered similar topologies showing no effects of missing data in the combined analysis of 18S and EF-1α C1.

Within Sphodrini the subtribe Atranopsina was always placed as the sister group to the remaining subtribes (clade P) with high node support (1.0). Support and stability of relationships within clade P were also sensitive to alignment strategy. Using CW approach with default parameters the relationships between subtribes were almost fully resolved (Fig. 4): Calathina (clade 1) was related to Pristosiina (clade 4) with high support (pp = 1, bootstrap = node not present) and both tribes were related (pp = 0.99, bootstrap = node not present) to the clade made up by Sphodrina (clade 5) plus *Anchomenidius*. Relationships with other alignment parameters tended to place Calathina as sister taxa of Dolichina, and the Sphodrina (clade 5) as basal to the remaining taxa but with low support (Figs. 3 and 5, and Fig. 11, Supplementary material).

Parsimony analyses of the combined data showed overall similar topologies with respect to the Bayesian analyses. Clades well supported in the Bayesian analyses were also recovered in the MP analyses although some of the nodes received lower support. For instance, Sphodrini (clade S) was always recovered as monophyletic but showed modest bootstrap support (62–70). Within clade P, Synuchina was always recovered as the sister taxon to the remaining subtribes but with low bootstrap support (50–71).

3.4. Evolutionary rate and age estimation

According to the relaxed molecular clock analysis of the mtDNA data, with the implemented calibration constraints described in the methods, the origin of tribe Sphodrini is estimated of about 36 mya with a 95% confidence interval ranging between 19.3 and 44.3 mya (Fig. 6). The divergence between Atranopsina started about 33 mya with a 95% confidence interval of 16.9–40.1 mya. The divergences between the subtribes of clade P started around 20–25 mya. (95% confidence intervals: 9–29 mya). Mean value for the estimated mutation rate for all taxa was 0.0046 mutations per site per million year per lineage (standard deviation = 2.5×10^{-5}).

4. Discussion

The seven genes analysed in this study agreed in many of the same relationships in Sphodrini and close relatives. The low levels of conflict in these data were well illustrated particularly by the high correlation in node specific PBS scores. Mitochondrial data contributed more than half of the node support in the combined analyses, which seems slightly at odds with many previous studies on insect phylogeny where mitochondrial genes usually perform less well in resolving higher taxon relationships (Baker et al., 2001; Lin and Danforth, 2004; Danforth et al., 2005). However, the highly congruent signal between mitochondrial and nuclear markers reject the possibility for strongly supported false relationships due to the higher number of variable characters in mtDNA; hence, these data contribute to higher confidence in our phylogenetic hypotheses.

4.1. Phylogenetic relationships

4.1.1. Relationships between Sphodrini and related tribes

The tribes Sphodrini, Platynini, Pterostichini, and Zabrini were all monophyletic and together they made up the sister clade to the tribe Harpalini. This result corroborates some current ideas about the relationship between these tribes (Erwin, 1985; Kryzhanovskij et al., 1995). However, the close relationship between Sphodrini and Platynini put forward by authors (Lindroth, 1956; Liebherr, 1986; Casale, 1988) has not received the expected strong support, though it can not be completely dismissed. The finding that that Platynini and Sphodrini are more distant to Harpalini than to Pterostichini and Zabrini also contradicts the hypothesis suggested by Liebherr (1986) on the basis of the structure of defensive glands and their secretory products. The puzzling relationships of the large group of 'modern' carabids (the Conchifera of Jeannel, 1941; or the subfamily Harpalinae of Erwin, 1985) is nevertheless not fully resolved in this study, and a more comprehensive study will require samples of representatives from tribes not

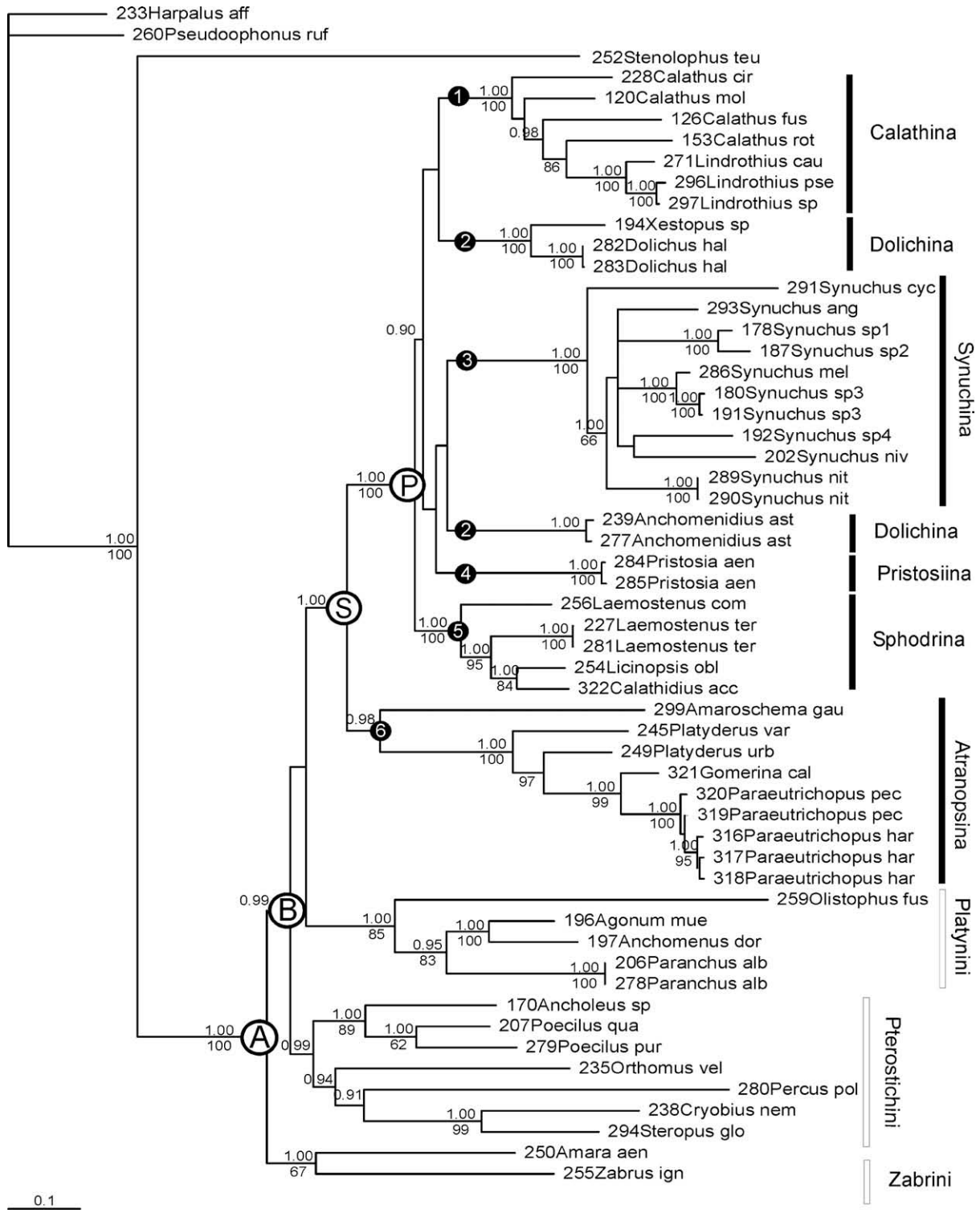

Fig. 3. The 50% majority consensus tree resulting from the BA of the combined data without variable region (2S approach). Number above node shows posterior probability ⩾0.90. Bootstrap support values are shown above the nodes. Circle letters are discussed in the text. Circle numbers and black bars represent the Sphodrini subtribes, white bars represent related tribes.

included here, and perhaps also sequence data from additional genes than those considered here.

4.1.2. Relationships within Sphodrini

All analyses demonstrated the monophyly of all subtribes in Sphodrini and thus corroborate the current taxonomy of the group. The only exception was the Dolichina species *Anchomenidius astur* that was found closer to either Sphodrina or Pristosiina. The systematic position of the genus *Anchomenidius* must therefore be reassessed.

The relationship between most subtribes was not well resolved and only the position of the subtribe Atranopsina as the sister clade to all other subtribes (clade P) was well supported. The basal position of Atranopsina within Sphodrini suggests the direction of the

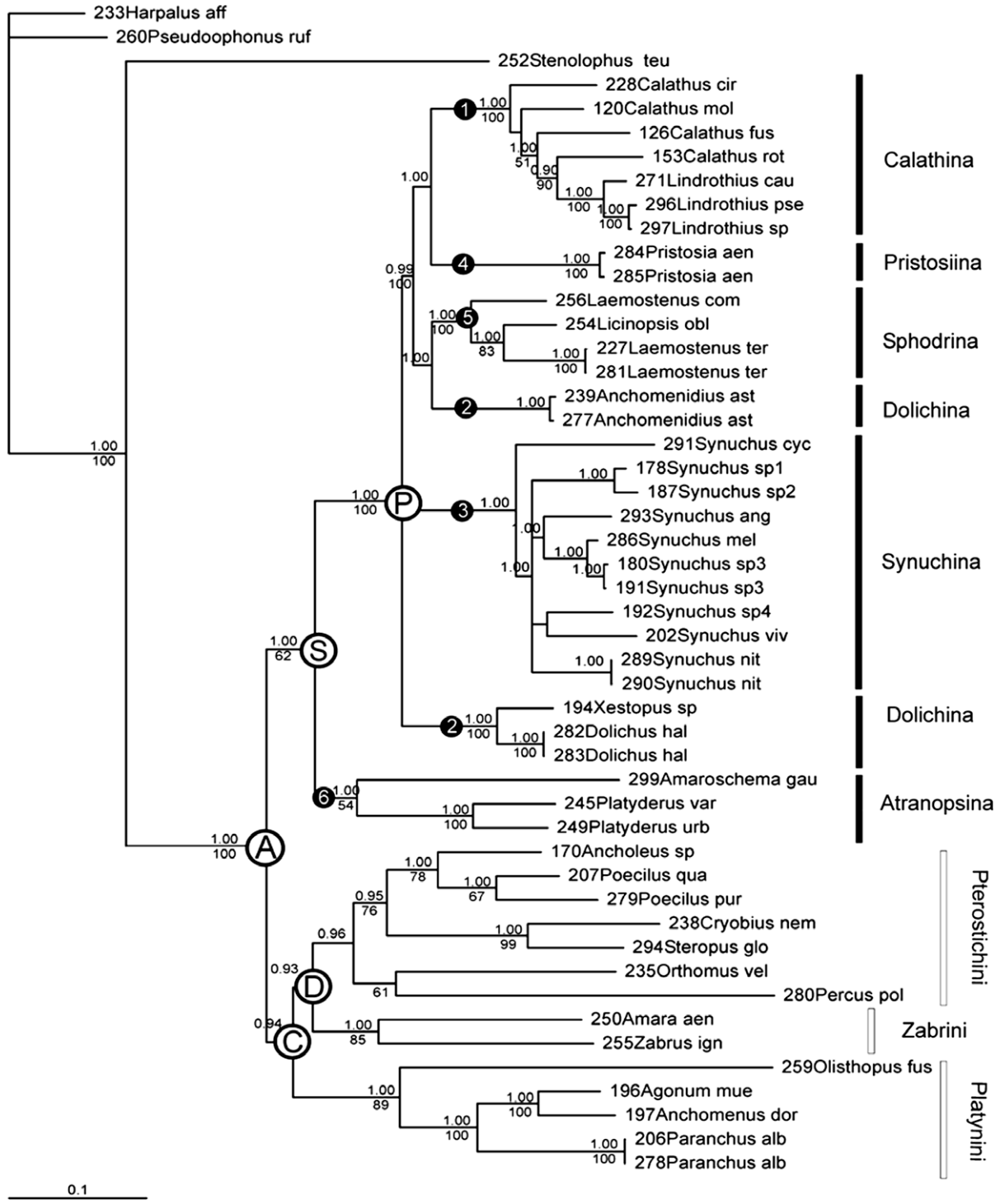

Fig. 4. The 50% majority consensus tree resulting from the BA of the combined data aligned with CW approach (GOP/GEP selected: default parameters- 18S: 15/6.6, 28S: 15/6.6; intron: 15/6.6 with both ends anchored). Number above node shows posterior probability ⩾0.90. Bootstrap support values are shown above the nodes. Lettering and bars as in Fig. 3.

evolution of sexual characters in this tribe. In Platynini and Atranopsina the male right paramere is not styloid but small, and there is a sensorial fovea in the apical segment of the gonostylus; hence, the right paramere seems to have become styloid or filamentous and the sensorial fovea has been lost in some of the other subtribes.

Within Atranopsina, *Amaroschema gaudini* represent the sister taxon of the remaining Atranopsina, indicating an ancient colonization event of Tenerife in the Canary archipelago (Fig. 3, node 6). A second and more recent colonization event in the Canary Islands possibly occurred in the related taxa *Gomerina* and *Paraeutrichopus*.

Within the subtribe Calathina, *Lindrothius* is clearly nested within the genus *Calathus* and closely related to *Calathus rotundicollis*. This suggests that *Lindrothius* should be treated as a subgenus of *Calathus* (Lindroth, 1956; Lorenz, 2005), instead of a separate genus (Hovorka and Sciaky, 2003).

In the subtribe Sphodrina, the Canarian taxa *Licinopsis obliterata* and *Calathidius accuminatus* are closely related (Fig. 3, node 5) as

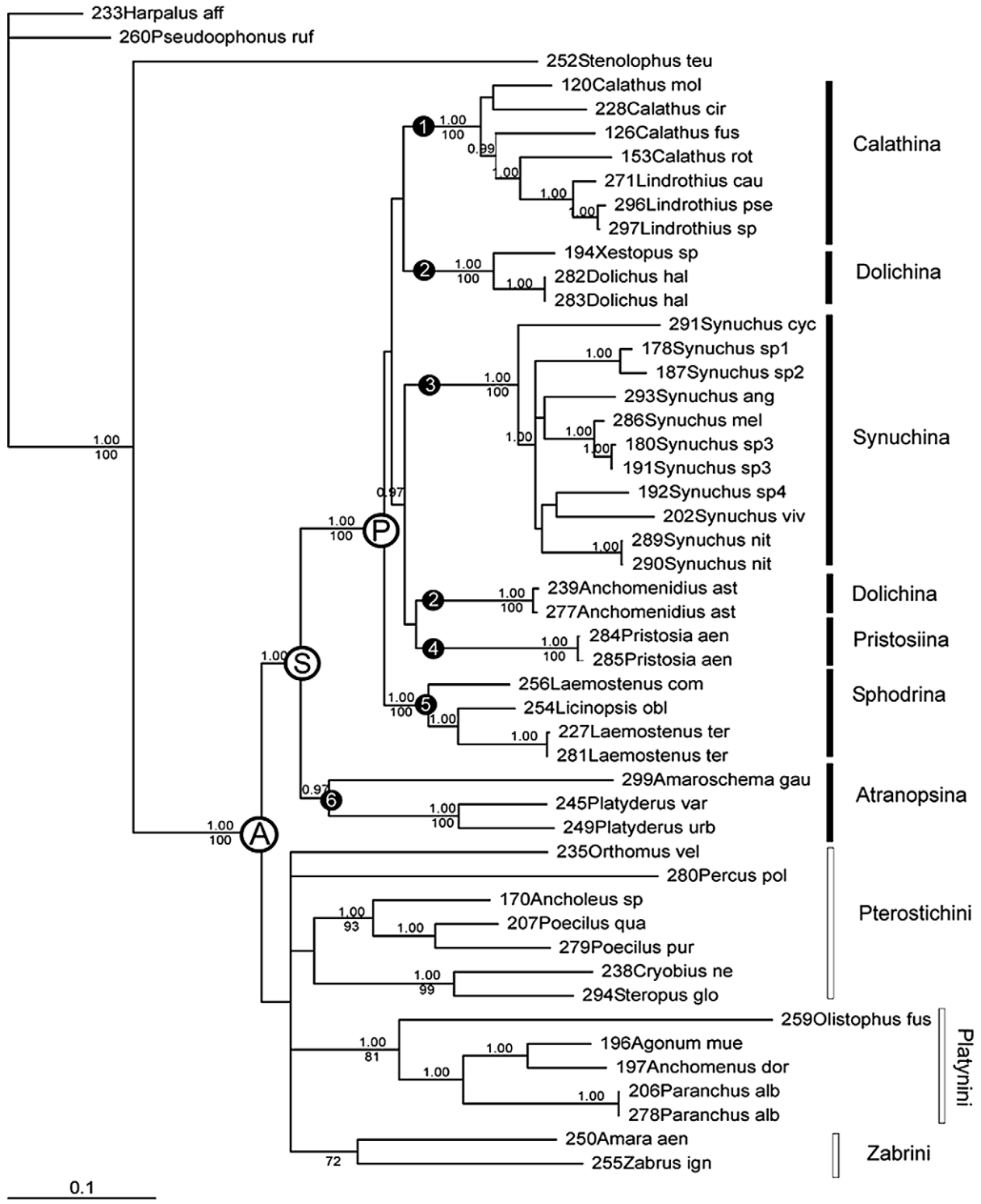

Fig. 5. The 50% majority consensus tree resulting from the BA of the combined data aligned with MX approach (GOP/GEP selected 18S: 4/0.5, 28S: 8/1; intron: 15/6.6 with both ends anchored). Number above node shows posterior probability ⩾0.90. Bootstrap support values are shown above the nodes. Circle letters are discussed in the text. Lettering and bars as in Fig. 3.

suggested by Casale (1988) and Machado (1992), and they are included with high support within the large genus *Laemostenus*. Further studies are needed to assess the relationships of these taxa.

Within the genus *Synuchus* (subtribe Synuchina), *Synuchus cycloderus* (subgenus *Synuchus*) from Japan was always found as a sister clade of the remaining *Synuchus*. A species in a different subgenus, *Synuchus (Diplosaccus) angusticeps*, was nested within taxa of the nominal subgenus, what suggests that a taxonomic revision is needed for this genus.

4.1.3. Resolution of molecular data

In spite of the analysis of 6467 bp that contributed 1438 phylogenetically informative sites from different molecular markers, several nodes were poorly resolved and received low support.

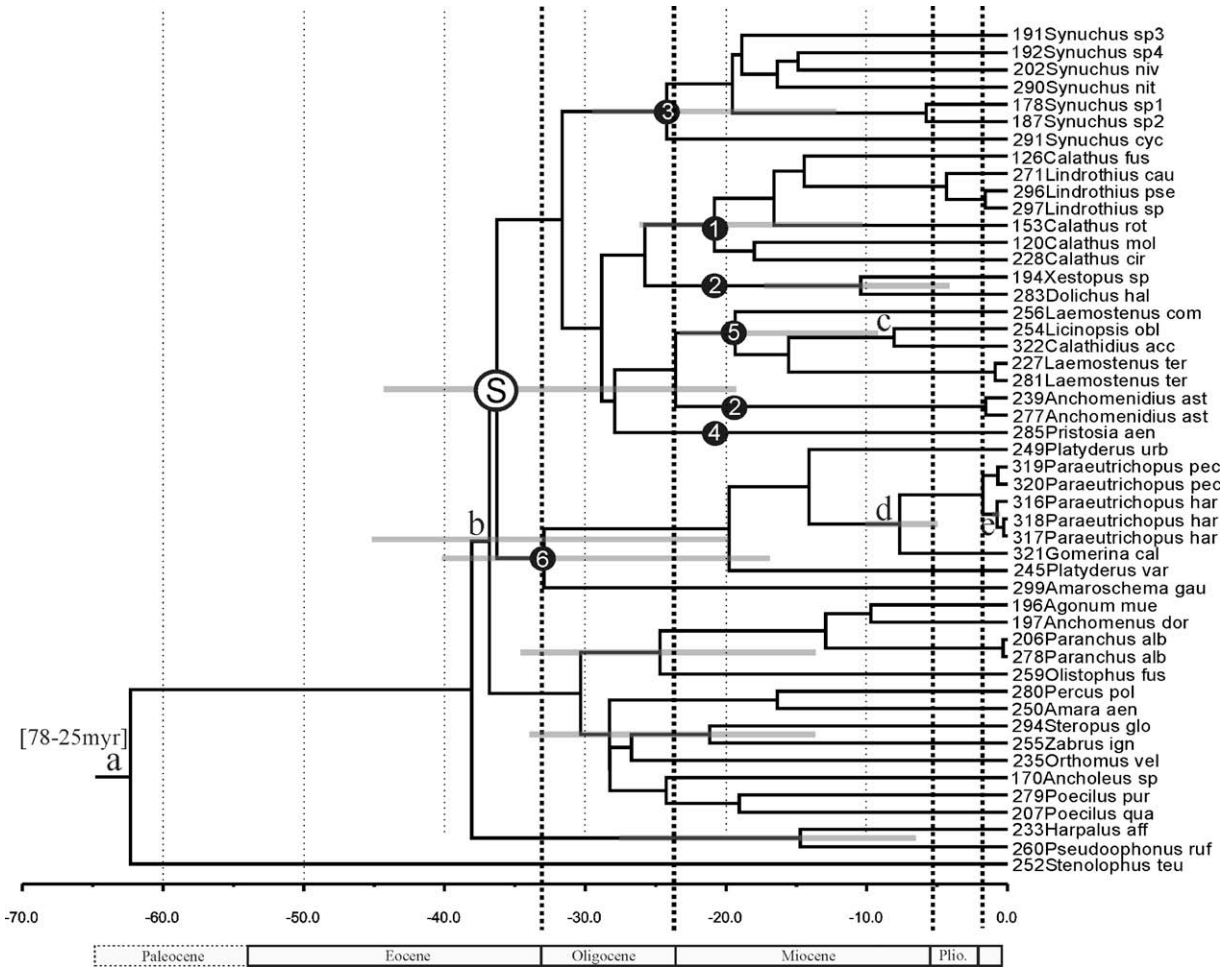

Fig. 6. Phylogeny of Tribe Sphodrini with divergences times estimates based on cox1-cox2 sequences. Lower case letters (a–e) represent the nodes used to calibrate the tree (a: normal 100 Myr, Stdev:20; b: normal 35 Myr Stdev:10; c ⩽ 11.6 Myr; d ⩽ 10 Myr; e ⩽ 1 Myr). Grey bars at each node show 95% highest posterior density interval for the main nodes. Root 95% highest posterior density intervals in square brackets. Lettering and bars as in Fig. 3.

The occurrence of deep nodes with short internal branches is characteristic for 'soft' polytomies, a frequent problem in phylogenetic analysis (Whitfield and Lockhart, 2007; Whitfield and Kjer, 2008). Lack of resolution is usually due either to non-optimal substitution rates, inappropriate phylogenetic methods or substitution models, or insufficient or conflicting phylogenetic signal. Alternatively, it might be that an explosive radiation of a clade has resulted in the polytomy, a phenomenon defined by some authors as a 'hard' polytomy (Maddison, 1989). Our results showed that the first three alternatives can be discarded. Molecular data taken from different unlinked loci showed very different substitution rates and should therefore cancel out any bias with respect to saturation. We furthermore applied a range of phylogenetic methods that take different substitution rates into account. Finally, the ILD tests and PBS analyses also showed that polytomies were not due to incongruence between different gene trees, and the PBS analyses revealed an overall low support for particular nodes (Fig. 1, indicated with arrows). Only the problem of insufficient data remains to be explored in depth.

Poorly resolved nodes were found in the relationship between the four tribes (clade B, C, D and E, Figs. 3–5, and Fig. 11, Supplementary material). As noted above, Platynini and Sphodrini were not clearly recovered as sister taxa. Likewise, the relationships between subtribes of Sphodrini (clade P) were poorly resolved and sensitive to alignment strategy. The hypothesis outlined by Habu (1978) and Casale (1988) suggesting a close relationship between Synuchina and Dolichina (based on the absence of sensorial fovea in the apical segment of the gonostylus) was not ruled out by a combined analysis with a CW alignment under default parameters (Fig. 4), but it was not supported with other alignment strategies (Figs. 3 and 5, and Fig. 11, Supplementary material) or in separate analyses of cox1-cox2 and EF-1α C0 (Fig. 2). These results suggest that the right styloid paramere (that relates Calathina, Sphodrina and Dolichina, and perhaps Pristosiina) is homoplastic, and the same can be said about the lack of sensorial fovea that potentially relates Dolichina and Synuchina.

4.1.4. Alignment consideration

Length variable regions of ribosomal DNA differ considerably from conserved regions in their substitution rates and the probability of nucleotide insertion and deletion. Therefore, the use of average gap cost, as implemented in a standard Clustal (CW) approach, does not seem to be an optimal solution (Kjer, 1995). Secondary structure information can be used to better determine the boundaries between conserved and ambiguous alignment regions and to enable the assessment of a proper gap cost. For this reason it is expected that the mixed strategy (MX), in which conserved regions are delimited with secondary structure, will produce a better primary assessment about homology than CW. Further, Clustal tends to place gaps at the end of the variable region during alignment with increasing gap cost, which may lead to incongruent topologies. In the analysis of the intron the ends were anchored,

which resulted in an increased congruence as shown by the sensitivity analysis (Fig. 7, Supplementary material).

Despite the many potential problems associated with ambiguous alignment regions, these regions contain considerable phylogenetic information as indicated by the increased overall PBS scores when such regions were included in the analyses (Fig. 10, Supplementary material). These results agree well with some previous studies that highlighted the importance of including such ambiguous regions (Lee, 2001; Jordal et al., 2008; Lindgren and Daly, 2007). Care must nevertheless be taken as certain nodes can be very sensitive to the alignment strategy (e.g., clades C and D, in Fig. 4 and clade E in Fig. 11, Supplementary material); (Giribet and Wheeler, 1999; Maddison et al., 1999; Ogden and Whiting, 2003). This problem is particularly relevant to clades defined by short internal branches or polytomies which leaves few traces of taxon relationships. In these sections of a phylogeny can relatively few alignment errors have a large effect on phylogenetic inference (Kjer, 1995; Kumar and Filipski, 2007). For example, the subtribes Calathina and Pristosiina made up a clade with high support when the 28S data were aligned with default parameters in Clustal in both the separate (Fig. 2) and combined analyses (Fig. 4), a relationship not found for other markers or parameters. Removal of alignment ambiguous regions is a more conservative and less controversial approach for data inclusion although a common tradeoff is reduced tree resolution (Gatesy et al., 1993; Wheeler et al., 1995). In our case exclusion of ambiguous alignment regions also resulted in lower resolution in some of the analyses, but this effect was mainly observed in the separate analyses of ribosomal RNAs. The exclusion of such regions in the combined analyses did not change the topology nor the support of the main nodes in either of the parsimony and Bayesian analyses. Thus, in combined analysis it may be safer to exclude ambiguous alignment regions.

4.2. Timing and radiation of ancestral Sphodrini

Ranges of estimated dates on the origin and radiation of the tribe Sphodrini are broad (around ±20 myr) and reflect the uncertainty in fossil identification and calibration. The origin of Sphodrini is here dated from late Eocene to early Miocene (95% confidence interval of 45.2 to 19.5 mya), later than the late Cretaceous epoch suggested by Casale (1988). This calculation is based on a pairwise substitution rate of 0.92%, twofold times lower than that commonly reported for insects of 2.3% (Brower, 1994). Similar low substitution rates have been reported in other Coleopteran groups, e.g. 0.76% in Chrysomelidae (Gómez-Zurita et al., 2000) and 0.39–0.98% in Carabidae (Prüser and Mossakowski, 1998).

The estimated age of Sphodrini coincides with the onset of geological events in the late Eocene that gave rise to the present configuration of the Mediterranean basin. It may thus be hypothesized that sphodrines originated from Platynini-like ancestors (as suggested by Casale, 1988) inhabiting central Asia (Angaria) in that period and then radiated towards both sides of the Palaearctic region. The basal subtribe Atranopsina (that includes the genera *Platyderus, Amaroschema* and *Gomerina*, among others) has more extant representatives in the western Palaearctic that concentrate in the Canary Islands, and particularly in the remnants of the laurisilva forests that formerly occupied the Mediterranean basin (Mai, 1989). There have been at least two colonizations of the Macaronesian archipelagos by continental members of this subtribe. *Amaroschema* started to diverge from its sister lineage 32 mya (95% HPD: 17–40), much earlier than the origin of Tenerife (11.6 Myr). This could be explained by incomplete sampling of extant taxa or lineage extinction on the continent. Analysis of continental *Amphimasoreus* (Near East) and *Broter* (India) may greatly help in assessing these hypotheses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.09.023.

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