

Phylogenetic Relationships of Iberian Dung Beetles (Coleoptera: Scarabaeinae): Insights on the Evolution of Nesting Behavior

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Abstract. A phylogeny of the main lineages of dung beetles (Coleoptera: Scarabaeinae) from the Iberian Peninsula was based on partial nucleotide sequences (about 1221 bp) of the mitochondrial cytochrome oxidase I and II genes of 33 taxa. Our phylogenetic analyses confirmed the validity and composition of most of the recognized tribes within the subfamily. Interestingly, the Onitini showed an evolutionary rate significantly higher than that of the other tribes. The molecular phylogeny supports a sister-group relationship of the tribes Onitini and Oniticellini + Onthophagini. A close relationship of Scarabaeini, Gymnopleurini, and Sisyphini is also suggested but lacks bootstrap support. Surprisingly, the Coprini, which had always been related to the Oniticellini and Onthophagini, were placed closer to the Scarabaeini, Gymnopleurini, and Sisyphini. The inferred molecular phylogeny was used to assess the main evolutionary trends of nesting behavior. Our results suggest tentative single origins for both the tunneling and the rolling behaviors, and the possibility that the rolling behavior could have been lost secondarily in *Copris*.

Key words: Scarabaeinae — Nesting behavior — Iberian beetles — Onthophagini

Introduction

The Scarabaeinae (dung beetles) are a worldwide-distributed, highly successful subfamily of Coleoptera with nearly 5000 species grouped in 234 genera (Hanski and Cambefort 1991). Ever since Linnaeus' *Systema Naturae*, dung beetles have received wide attention from entomologists because of their singular adaptations in exploiting vertebrate dung pads (e.g., Fabre 1897, 1899; Heymons and von Lengerken 1922; Burmeister 1930; Heymons 1930; Prasse 1957; Rommel, 1961; Balthasar 1963; Halffter and Matthews 1966; Halffter and Edmonds 1982). Scarabaeids are one of the best-studied groups of beetles in terms of taxonomy (Janssens 1949; Balthasar 1963; Iablokov-Khznorian 1977; Zunino 1984; Browne and Scholtz 1998; for a review see Martín-Piera 2000), ecology (Hanski and Cambefort 1991), natural history (Halffter and Matthews 1966), and behavior (Halffter and Edmonds 1982).

Several recent and comprehensive studies have examined adult and larval morphological traits to infer phylogenetic relationships within the superfamily Scarabaeoidea (Howden 1982; Browne and Scholtz 1995, 1999) and within the family Scarabaeidae (Browne and Scholtz 1998). Twenty-three synapomorphic characters of the hindwing articulation and wing base defined the monophyly of the Scarabaeidae (Browne and Scholtz 1999). Within scarabaeids, there were 44 synapomorphies that closely related the Scarabaeinae to the Aphodiinae, *Aulonocnemis*, and the Aegialiinae (Browne and Scholtz 1998). The monophyly of the Scarabaeinae was supported by 12

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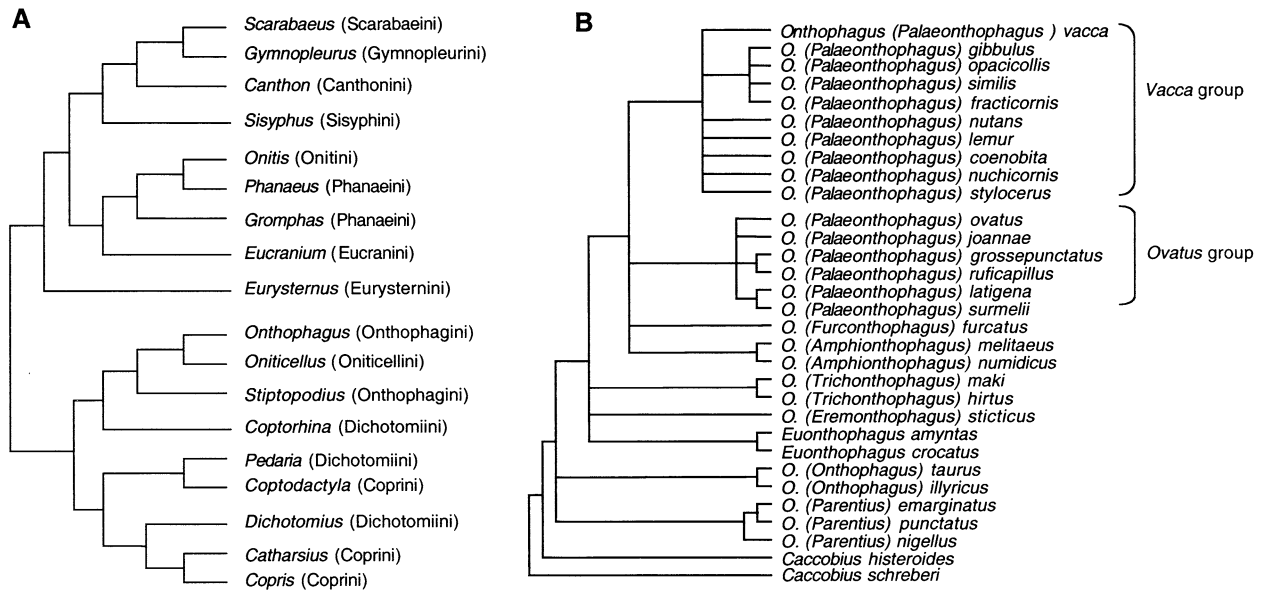


Fig. 1. Phylogenetic relationships (A) within the subfamily Scarabaeinae (Zunino 1984) and (B) within the tribe Onthophagini (Martín-Piera 2000) based on morphological data.

synapomorphies (Browne and Scholtz 1998). However, these studies did not analyze the phylogenetic relationships within the subfamily Scarabaeinae.

There is still a lack of agreement regarding the taxonomic status of the Scarabaeinae. Janssens (1949) subdivided the group into six tribes: Coprini (including Dichotomiina, Phanaeina, and Ennearabdina), Eurysternini, Oniticellini, Onitini, Onthophagini, and Scarabaeini (including Eucraniina, Canthoniina, Gymnopleurina, Scarabaeina, and Sisyphina). However, Balthasar (1963) ranked the group as a family comprising two subfamilies: Coprinae and Scarabaeinae. The former subfamily included the tribes Coprini, Dichotomiini, Phanaeini, Oniticellini, Onitini, and Onthophagini, whereas the latter subfamily included the tribes Eucraniini, Eurysternini, Canthonini, Gymnopleurini, Scarabaeini, and Sisyphini. Both classifications have been followed by different authors until the present. For instance, the studies on the breeding and nesting behavior of dung beetles by Halffter and Edmonds (1982) used Janssens' taxonomy, whereas the comprehensive ecological study of dung beetles by Hanski and Cambefort (1991) followed Balthasar's views. In this study, we accept the subfamily status of Scarabaeinae (e.g., Brown and Scholtz 1998) and follow Balthasar's (1963) classification of tribes. Regardless of the taxonomic debate, it is generally assumed that the tribes reflect natural groups (based on the general evolutionary trends detected in the subfamily) that lack a formal systematic definition based on modern phylogenetic methods. Hence, despite the wealth of biological information accumulated, disagreement among workers remains, and a robust phylogeny of the subfamily awaits.

So far, the only study which has focused on the systematics of the subfamily Scarabaeinae was based on the comparative analysis of the male and female genitalia (Fig. 1A) (Zunino 1984). The recovered phylogeny did not support previous taxonomic classifications of the subfamily (Janssens 1949; Balthasar 1963). In that study, the monophyly of the tribes Onitini, Coprini, and Dichotomiini was questioned (Zunino 1984). Surprisingly, the tribes Onitini and Phanaeini were placed deep within the Scarabaeinae (*sensu* Balthasar 1963) (Fig. 1A). Within the Coprinae (*sensu* Balthasar 1963), a sister-group relationship of Oniticellini and Onthophagini was suggested (Zunino 1984) (Fig. 1A). Other studies focused on the phylogenetic relationships within some of the tribes further confirmed the nonmonophyly of Coprini and Dichotomiini (Montreuil 1998). Moreover, some studies based on larval and adult characters focused on the phylogenetic relationships within the most speciose tribe, the Onthophagini (Zunino 1979; Lumaret and Kim 1989; Palestrini 1985; Martín-Piera 1986, 2000; Martín-Piera and Zunino 1983, 1986) (Fig. 1B). The most recent and complete phylogeny of the Onthophagini was based on 12 external and 12 internal morphological traits (Martín-Piera 2000). In that study, a basal position of the genera *Caccobius* and *Euonthophagus*, as well as of some of the *Onthophagus* subgenera (*Onthophagus*, *Parentius*, *Eremonthophagus*, and *Trichonthophagus*) was suggested (Fig. 1B). In addition, two groups (*vacca* and *ovatus*) were proposed within the more derived *Onthophagus* subgenus *Palaeonthophagus* (Fig. 1B).

The ability of the Scarabaeinae to eat dung (coprophagia) from large herbivorous mammals is the key to their evolutionary success and involved nu-

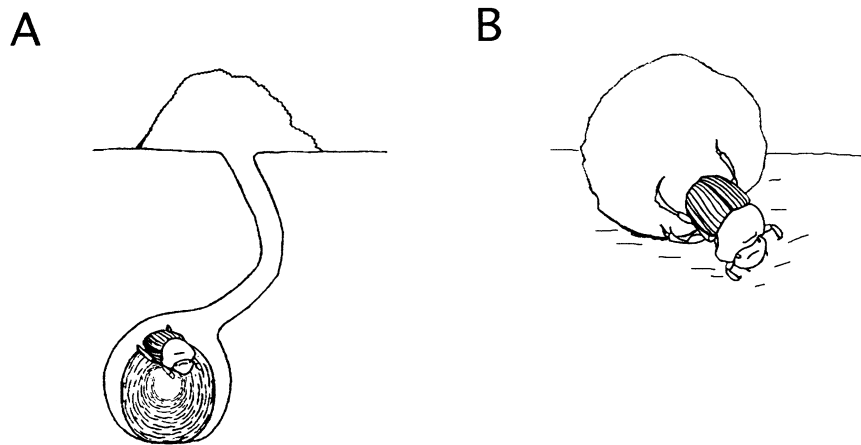


Fig. 2. Patterns of nesting behavior in dung beetles. **A Tunneling:** Members of the tribes Coprini, Dichotomini, Phanaeini, Oniticellini, Onitini, and Onthophagini dig nests in the soil that are filled with dung, out of which the brood balls are made. **B Rolling:** Members of the tribes Eucraniini, Eurysternini, Canthonini, Gymnopleurini, Scarabaeini, and Sisyphini prepare a ball of dung which is transported and buried away from the food source.

merous morphological and behavioral adaptations. This capacity to exploit excrements, which was already present in the Upper Cretaceous (Scholtz and Chown 1995; Chin and Gill 1996), is shared with the related scarabaeid subfamily Aphodiinae and the scarabaeid family Geotrupidae. However, the latter two groups exhibit relatively simple behaviors associated with the exploitation of dung compared with the Scarabaeinae. The larvae of the Aphodiinae eat and live generally inside dung directly beneath the surface of the soil (but see Barbero and Palestini 1995; Gittings and Giller 1997), whereas the Geotrupidae simply pack larval food into the end of a burrow. In contrast, the Scarabaeinae have evolved a wide array of feeding and nesting behaviors (Halffter and Matthews 1966; Halffter and Edmonds 1982; Cambefort and Hanski 1991). There are two essential ways of processing the excrement within the Scarabaeinae: tunneling and rolling behaviors (Fig. 2).

The tribes Coprini, Dichotomini, Phanaeini, Oniticellini, Onitini, and Onthophagini [i.e., the subfamily Coprinae (*sensu* Balthasar 1963)] exhibit tunneling behavior, in which a rather sophisticated nest is dug in advance, then filled with dung, out of which the brood balls are made. The tribes Eucraniini, Eurysternini, Canthonini, Gymnopleurini, Scarabaeini, and Sisyphini [i.e., the subfamily Scarabaeinae (*sensu* Balthasar 1963)] show a rolling behavior in which a ball of dung is prepared first, then transported by rolling, and finally, buried some distance away from the food source. This unique behavior apparently avoids ecological competition close to the food source and seems to have evolved once early on in the history of the subfamily (Halffter and Matthews 1966; Halffter and Edmonds 1982). Interestingly, however, some tropical Scarabaeini exhibit facultative ball rolling and coprine nesting and brooding behaviors (Sato and Imamori 1986; Sato 1998).

The Iberian Peninsula hosts one of the most diverse representation of dung beetles in the Palearctic region (Lumaret and Lobo 1996). Its historical role as a temperate refuge during the glaciations has con-

Table 1. World Scarabaeinae tribes and number of known genera (gen) and species (spe) in different regions

Tribe	World		Palearctic		Europe		Iberian Peninsula	
	Gen	Spe	Gen	Spe	Gen	Spe	Gen	Spea
Canthonini	93	771	1	3	0	0	0	0
Coprini	10	374	2	7	2	5	1	2 (2)
Dichotomiini	30	722	0	0	0	0	0	0
Eucraniini	4	19	0	0	0	0	0	0
Eurysternini	1	26	0	0	0	0	0	0
Gymnopleurini	4	104	1	6	1	5	1	3 (2)
Oniticellini	14	165	3	14	2	4	1	3 (2)
Onthophagini	35	2213	3	259	3	68	3	30 (19)
Onitini	18	195	3	21	3	14	3	6 (5)
Phanaeini	12	148	0	0	0	0	0	0
Scarabaeini	11	145	3	24	1	12	1	7 (2)
Sisyphini	3	58	1	1	1	1	1	1 (1)
Total	235	4940	17	335	13	109	11	52 (33)

^aThe number of Iberian species analyzed is given in parentheses. Data from Hanski and Cambefort (1991), Baraud (1992), and Martín-Piera (2000).

tributed decisively to the actual richness of Iberian fauna (Hewitt 1999). There is a total of 11 genera (65% of all palearctic genera) and 52 species (16% of all palearctic species) of Scarabaeinae in the Iberian Peninsula (Table 1). They represent 5 of the 6 tribes proposed by Janssens (1949) and 7 of the 12 admitted by Balthasar (1963). Hence, a phylogenetic study of the Iberian dung beetles can be viewed as a fair approximation to the systematics of the family.

In this study, we determined the DNA sequences of the mitochondrial cytochrome oxidase I and II genes in most of the species of dung beetles occurring in the Iberian Peninsula (Table 2). These genes were found to provide phylogenetic resolution among different groups of beetles, including scarabaeids (Emerson and Wallis 1995). The aim of this work is to clarify phylogenetic relationships within the Iberian species of the subfamily Scarabaeinae and to examine further the origin of the two main strategies of feeding and nesting behavior within the subfamily.

Table 2. Dung beetles included in the present study

Taxon	Locality	Altitude	Dung source
Scarabaeinae			
Onthophagini			
<i>Onthophagus punctatus</i>	Conil (Cádiz)	50	Cow
<i>Onthophagus maki</i>	Facinas (Cádiz)	25	Horse
<i>Onthophagus hirtus</i>	El Algar (Cádiz)	210	Cow
<i>Onthophagus nutans</i>	El Tiemblo (Avila)	1220	Cow
<i>Onthophagus grossepunctatus</i>	El Tiemblo (Avila)	1220	Cow
<i>Onthophagus ovatus</i>	Piasca (Santander)	700	Cow
<i>Onthophagus ruficapillus</i>	Vadillo Castril (Jaen)	1000	Horse
<i>Onthophagus melitaeus</i>	Los Hurones (Cádiz)	280	Cow
<i>Onthophagus lemur</i>	Huermece (Burgos)	880	Sheep
<i>Onthophagus latigena</i>	El Chorro (Jaen)	1200	Fallow deer
<i>Onthophagus coenobita</i>	El Tiemblo (Avila)	1220	Cow
<i>Onthophagus similis</i>	Pto. Somosierra (Madrid)	1420	Horse
<i>Onthophagus stylocerus</i>	Pto. Navafria (Madrid)	1700	Cow
<i>Onthophagus vacca</i>	Pto. Somosierra (Madrid)	1420	Horse
<i>Onthophagus merdarius</i>	Ontigola (Toledo)	600	Sheep
<i>Onthophagus furcatus</i>	Conil (Cádiz)	50	Cow
<i>Onthophagus taurus</i>	Ontigola (Toledo)	600	Sheep
<i>Euonthophagus amyntas</i>	El Tiemblo (Avila)	1220	Cow
<i>Caccobius schreberi</i>	Los Hurones (Cádiz)	280	Cow
Onitini			
<i>Cheironitis hungaricus</i>	Villasequilla de Yepes (Toledo)	480	Cow
<i>Onitis belial</i>	Conil (Cádiz)	50	Cow
<i>Onitis ion</i>	Pedro Martínez (Granada)	1150	Sheep
<i>Bubas bubalus</i>	El Bosque (Cádiz)	400	Cow
<i>Bubas bison</i>	El Bosque (Cádiz)	400	Cow
Oniticellini			
<i>Euoniticellus fulvus</i>	El Bosque (Cádiz)	400	Cow
<i>Euoniticellus pallipes</i>	El Algar (Cádiz)	210	Cow
Scarabaeini			
<i>Scarabaeus sacer</i>	Agua Amarga (Almeria)	10	Human
<i>Scarabaeus cicatricosus</i>	Cabo Roche (Cádiz)	30	Cow
Gymnopleurini			
<i>Gymnopleurus mopsus</i>	Quijorna (Madrid)	750	Cow
<i>Gymnopleurus flagellatus</i>	Vadillo Castril (Jaen)	1000	Horse
Sisyphini			
<i>Sisyphus schaefferi</i>	Vadillo Castril (Jaen)	1000	Horse
Coprini			
<i>Copris hispanicus</i>	El Bosque (Cádiz)	400	Cow
<i>Copris lunaris</i>	Rio Madarquillos (Madrid)	1020	Cow
Aphodiinae			
<i>Aphodius merdarius</i>	El Bosque (Cádiz)	400	Cow
<i>Aphodius quadrimaculatus</i>	El Chorro (Jaen)	1200	Fallow deer
<i>Aphodius satellitius</i>	Prádena (Madrid)		Cow
<i>Aphodius constans</i>	Pto. Navafria (Madrid)	1700	Cow
Geotrupidae			
<i>Thorectes lusitanicus</i>	Las Palomas (Cádiz)	1300	Sheep
<i>Typhaeus momus</i>	Cabo Roche (Cádiz)	30	Cow
<i>Geotrupes mutator</i>	Piasca (Santander)	700	Cow
<i>Geotrupes stercorarius</i>	Pto. Navafria (Madrid)	1700	Cow

Materials and Methods

DNA Sources and Extraction

A total of 33 Scarabaeinae species representing all the Iberian tribes and genera was used in the present analysis. Four species of Aphodiinae and four species of Geotrupidae were used as out-group. The complete list of taxa is presented in Table 2. Total DNA was extracted from one or two adult specimens per species. For each specimen, the head and the thorax were ground in liquid

nitrogen and resuspended in 500 µl of extraction buffer (Townner 1991). After homogenization, DNA was purified by a standard phenol/chloroform procedure followed by ethanol precipitation.

PCR Amplification, Cloning, and Sequencing

Initially, two universal insect primers—COIIam, 5'-AAT ATG GCA GAT TAG TGC A-3', and COIIB-605, 5'-GCT CCA CAA ATT TCT GAG CA-3' (Simon et al. 1994; Emerson and Wallis

Table 3. Parameters obtained in the phylogenetic analysis

Parameter	COI				COII			
	All	1st	2nd	3rd	All	1st	2nd	3rd
Parsimony info sites	249	65	22	162	278	84	33	161
Proportion of invariable sites	0.50	0.61	0.82	0.07	0.43	0.49	0.74	0.06
Ts/Tv ratio (<i>K</i>)	1.0 (2.5)	2.3 (5.0)	1.0 (1.7)	1.6 (8.5)	1.1 (2.9)	2.8 (6.0)	1.2 (2.4)	1.7 (10.3)
Gamma shape (α) ^a	0.30	0.21	0.12	2.02	0.39	0.37	0.18	1.68
A (%)	0.32	0.29	0.21	0.45	0.37	0.34	0.28	0.47
C (%)	0.16	0.16	0.24	0.08	0.15	0.17	0.21	0.08
G (%)	0.12	0.22	0.13	0.02	0.11	0.20	0.11	0.02
T (%)	0.40	0.33	0.42	0.45	0.37	0.29	0.40	0.43
Chi-square test ^b								
χ^2	90.14	35.39	6.51	281.21	81.20	34.74	8.85	255.84
df	120	120	120	120	120	120	120	120
<i>p</i>	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	<0.05
Saturation ^c	Yes (Ts)	No	No	Yes (Ts)	Yes (Ts)	No	No	Yes (Ts)
No MP trees	2	8	>1000	2	3	29	>1000	5

^a Calculated by the method of Yang and Kumar (1996).

^b Chi-square test of homogeneity of base frequencies across taxa as implemented in PAUP* (Swofford 1998).

^c Saturation was defined as a nonlinear relationship between uncorrected *p* distances and transitions or transversions (see Fig. 3).

1995)—were used to amplify the mitochondrial cytochrome oxidase II (COII) gene of dung beetles. Unfortunately, the amplifications worked in only a few samples, and four new primers (COIIam-Sca, 5'-GCA GAT TAG TGC AAT GAA TTT AA-3'; COIIB-605-Sea, 5'-GCT CCA CAA ATT TCT GAG CAT TG-3'; COI-Sca F, 5'-GGA ATT AAA ATT TTT AGA TGA TTA GC-3'; COI-Sca R, 5'-TGA TTA TGR AAA AAA TTT ART TGT TCT AT-3') were designed to amplify two overlapping fragments (1221 bp) that comprised the 3' end of the mitochondrial cytochrome oxidase I (COI) gene, the adjacent complete tRNA-Leu (UUR) gene, and the 5' end of the COII gene of Iberian dung beetles. Thirty-five PCR cycles (denaturing at 94°C for 60 s, annealing at 42°C for 60 s, and extending at 72°C for 90 s) were performed in 25- μ l reactions containing 67 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, a 0.4 mM concentration of each dNTP, a 2.5 μ M concentration of each primer, template DNA (10–100 ng), and Taq DNA polymerase (1 U; BioTools).

PCR products were ethanol precipitated and either directly sequenced using the corresponding PCR primers or cloned into the pGEM-T vector (Promega) and sequenced using the M13 universal (forward and reverse) sequencing primers. DNA sequences of both strands were obtained using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems Inc.) on an automated DNA sequencer (Applied Biosystems Prism 3700) following the manufacturer's instructions. The nucleotide sequence data determined for the present paper were deposited in GenBank under accession Nos. AY039337-AY039377.

Phylogenetic Analyses

DNA sequences were aligned using CLUSTALX (Thompson et al. 1997). No ambiguous alignments were found within the ORFs but a gap was postulated between the 3' end of the COII gene and the beginning of the tRNA-Leu (UUR) due to an insertion of 26 bp in the sequence of *Copris lunaris*. Maximum-parsimony (MP) analyses [PAUP* version 4.0b4a (Swofford 1998)] were performed using heuristic searches (TBR branch swapping; MulTrees option in effect) with 10 random stepwise additions of taxa. A 2:1 transversions (Tv):transitions (Ts) weighing scheme was used based on empirical evidence (Table 3). Minimum-evolution (ME) (Rzhetsky and Nei 1992) analyses were performed with PAUP* using general

time-reversible (GTR) distances (Rodríguez et al. 1990). Maximum-likelihood (ML) analyses using the GTR model were performed in PAUP*. Robustness of the inferred MP and ME trees was tested by bootstrapping (Felsenstein 1985) (as implemented in PAUP*, with 500 pseudoreplications each), and that of ML trees by quartet puzzling (10,000 puzzling steps) (Strimmer and von Haeseler 1996). A rate constancy test [two-cluster test (Takezaki et al. 1995)] was performed with PHYLTEST version 2.0 (Kumar 1996) using the HKY85 distance to detect those taxa that showed significantly different substitution rates (Z statistic, at the 5% level). Additionally, a likelihood-ratio test based on the HKY85 model with and without the molecular clock enforced was performed in PUZZLE version 4.0 (Strimmer and von Haeseler 1996).

Results

Sequence Variation

Mitochondrial COI, tRNA-Leu (UUR), and COII gene sequences were combined into a single data set that produced an alignment of 1250 positions. Of these, 37 positions were excluded from the analyses because of ambiguity in the homology assignment, 585 were constant (48.2%), and 539 were parsimony-informative. In the protein-coding genes, most phylogenetically informative sites were found at third codon positions (Table 3). The mean base composition for COI was 32% adenine, 16% cytosine, 12% guanine, and 40% thymine. The mean base composition for COII was 37% adenine, 15% cytosine, 11% guanine, and 37% thymine. First and second positions showed considerable anticytosine and antiguanine biases, respectively (Table 3). Third codon positions showed a strong adenosine + thymine bias (Table 3). Homogeneity of base frequencies among taxa was rejected in both genes at third codon positions (Table 3) by a chi-square test

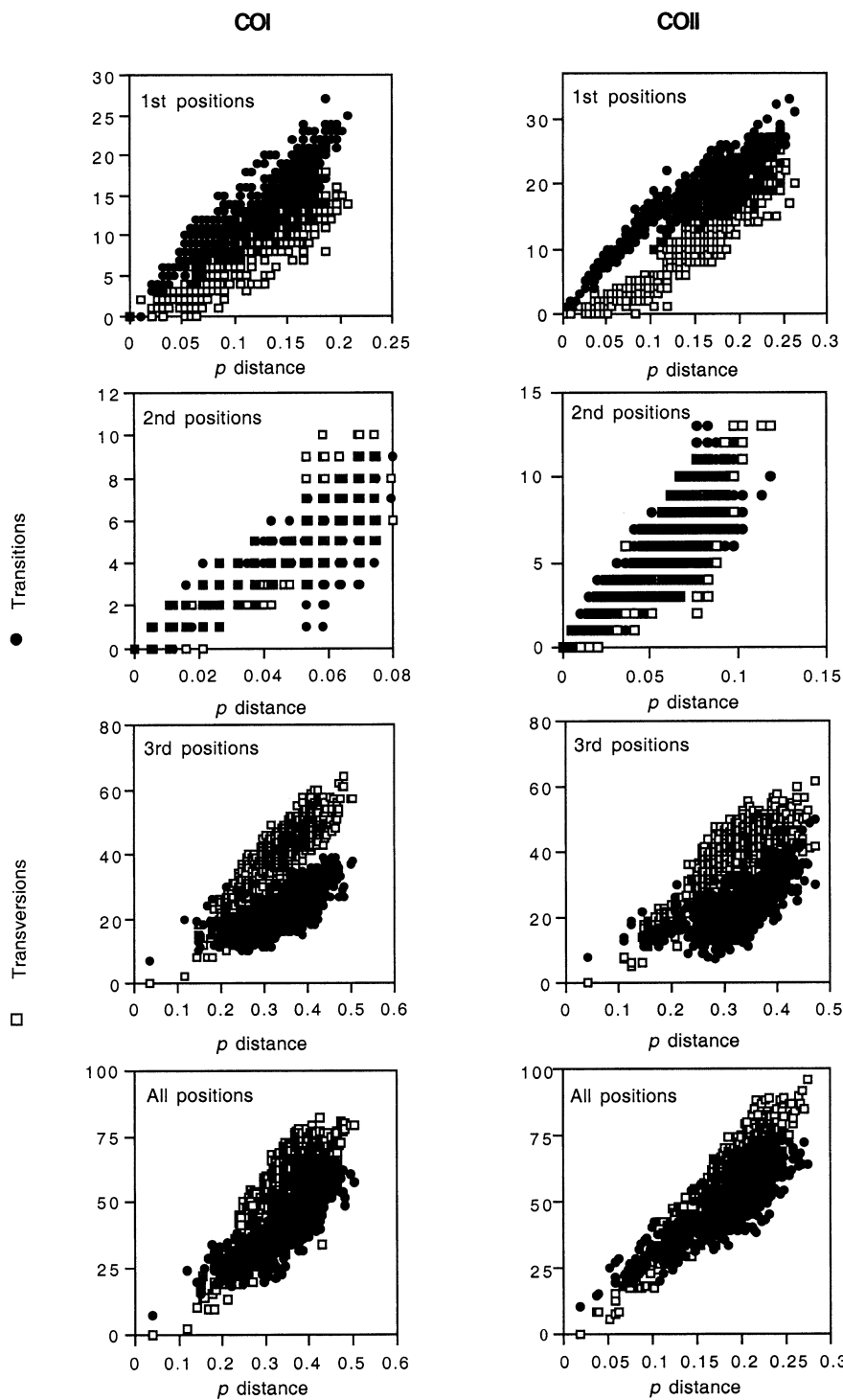


Fig. 3. Substitution pattern of cytochrome oxidase I and II gene sequences. Transitions (*filled circles*) and transversions (*open squares*) in all, first, second, and third codon positions of the scarabaeid data set were plotted against total sequence divergences (unconnected p distances) for all pairwise comparisons.

comparing the nucleotide composition of each sequence to the frequency distribution assumed in the GTR model.

The maximum pairwise uncorrected p distance found among all ingroup taxa was 0.225 between *Onitis belial* and *Sisyphus schaefferi*, and the minimum was 0.014 between *Onthophagus maki* and *O. hirtus*. The average pairwise divergence value within tribes is 0.11, whereas the average pairwise distance

among tribes is 0.18. However, preliminary data showed that almost no sequence variation was found when four or five individuals of geographically separated populations of the same species were analyzed (not shown). Overall, in both genes transitions and transversions increased linearly with genetic distance (Fig. 3). However, some levels of transition saturation were found in third codon positions for distantly related taxa (above 0.2 se-

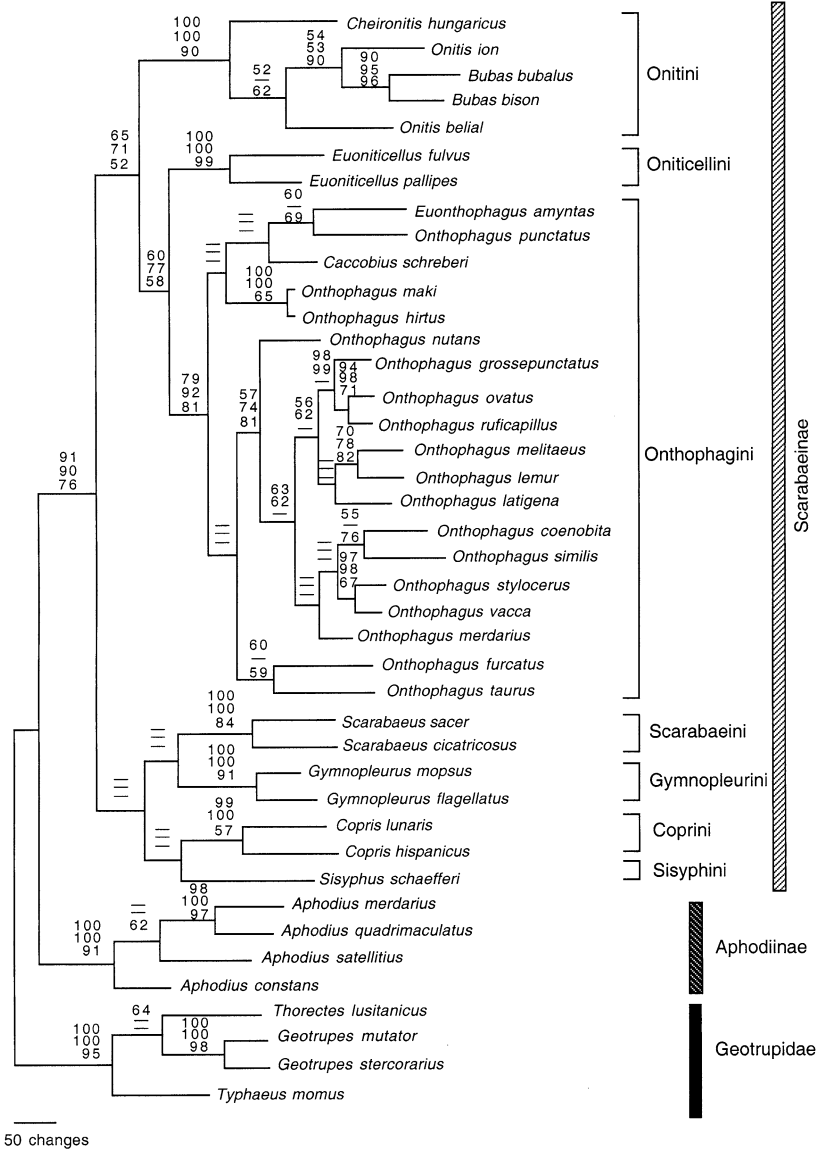


Fig. 4. Single MP tree (based on a Tv:Ts weighting of 2:1) depicting phylogenetic relationships of Iberian Scarabaeinae. The numbers above branches are bootstrap values corresponding to the MP (upper value of each triplet of numbers), ME (middle value of each triplet of numbers), and ML (lowest value of each triplet of numbers) phylogenetic analyses. Only values above 50% are shown.

quence divergence) (Fig. 3). Furthermore, empirical Ts/Tv ratios were found to vary between 1 and 2, also indicating some general effect of saturation (Table 3 and Fig. 3).

Interestingly, Onitini, Onthophagini, and Scarabaeini taxa have an extra amino acid (serine) at the carboxyl end of the COI deduced protein with respect to Oniticellini, Gymnopleurini, Sisyphini, Coprini, Aphodiinae, and Geotrupidae taxa. Moreover, Oniticellini, Gymnopleurini, Coprini, and Aphodiinae taxa have, in addition, intergenic nucleotides separating the end of the COI gene and the beginning of the tRNA-Leu (UUR) gene.

Phylogenetic Relationships of Iberian Dung Beetles

Maximum-parsimony analysis of the mitochondrial sequence data arrived at one most-parsimo-

nious tree of 5330 steps when a 2:1 Tv:Ts weighting was assumed and Geotrupidae taxa were used as outgroup (CI = 0.27, RI = 0.49) (Fig. 4). Alternative weighting schemes (1:1, 3:1) recovered similar topologies. The ME (score = 6.45) and ML ($-\ln L = 15,669.6$) analyses using the GTR model arrived at similar and largely congruent trees with a consistent pattern of bootstrap support (Fig. 4).

The monophyly of each of the scarabaeid tribes was well supported with high bootstrap values. Within the Onitini, the monophyly of the genus *Onitis* was questioned because at least one Mediterranean species—*O. ion*—appeared to be more related to the genus *Bubas*. The Onthophagini were resolved into five distinct clades: *Euonthophagus amyntas* + *Onthophagus punctatus*, *Caccobius schreberi*, *Onthophagus maki* + *O. hirtus* (this clade is clustered with *C. schreberi* in the ME and ML

Table 4. Z statistics of the relative rate tests performed among the main tribes of Scarabaeidae

	Onitini	Oniticellini	Onthophagini	Scarabaeini	Gymnopleurini	Coprini	Sisyphini
Onitini	—						
Oniticellini	1.75	—					
Onthophagini	3.69 ^a	1.98 ^a	—				
Scarabaeini	2.42 ^a	0.76	0.79	—			
Gymnopleurini	2.80 ^a	1.14	0.46	0.32	—		
Coprini	2.94 ^a	1.27	0.34	0.48	0.13	—	
Sisyphini	2.29 ^a	0.77	0.68	0.06	0.22	0.35	—

^a Rate constancy was rejected at the 5% significance level.

analyses with 57 and 70% bootstrap values, respectively), *O. furcatus* + *O. taurus*, and a clade containing more derived *Onthophagus*. Within the latter clade, *O. nutans* showed a basal position with respect to two more derived groupings, the *vacca* and *ovatus* lineages (Fig. 4). The latter two groups showed evident internal incongruences in composition with respect to their former definitions (Martin-Piera 2000) (Fig. 1B). Phylogenetic relationships among Onthophagini clades could not be confidently resolved. A close phylogenetic relationship of Oniticellini and Onthophagini (58–77% bootstrap support) and of both tribes with Onitini (52–71% bootstrap support) was suggested. Hence, all tunneler taxa with the exception of *Coprins* are grouped together.

In the recovered MP and ML phylogenies, rollers are grouped together in a clade that is weakly supported (bootstrap value below 50%). Interestingly, the Coprini, which exhibit a tunneler behavior, are placed within the rollers (Fig. 4). In the ME phylogeny, a clade including Scarabaeini, Gymnopleurini, and Coprini was also recovered, albeit with no bootstrap support. *Sisyphus* is placed basal to the Onitini + Oniticellini + Onthophagini clade with no bootstrap support.

Rates of Evolution

A likelihood-ratio test with ($-\ln L = 19,248.41$) and without ($-\ln L = 19,164.99$) the molecular clock enforced rejected overall rate constancy ($\delta = 166.85$, $df = 39$). To understand further, why rate constancy among scarabaeid taxa was rejected, a two-cluster test (Takezaki et al. 1995) was conducted across tribes. Onitini taxa showed significantly higher rates of evolution with respect to the rest of the taxa (Table 4). Within the Onthophagini, two species (*Onthophagus furcatus* and *O. taurus*) showed significantly higher rates of sequence divergence with respect to other members of the tribe (data not shown).

Discussion

Systematics

The molecular phylogeny of Iberian dung beetles inferred from mitochondrial cytochrome oxidase gene sequences provides an independent and robust framework in which to evaluate previous taxonomic and phylogenetic hypotheses on the subfamily Scarabaeinae that were based on the analyses of morphological and behavioral characters. The main incongruences between the molecular phylogeny presented here and a previous phylogeny inferred from characters of the genitalia (Zunino 1984) refer to the relative position of the tribes Onitini and Coprini. Mitochondrial evidence places the Onitini as the sister group of Oniticellini + Onthophagini (Fig. 4). This branching pattern frontally contradicts Zunino's (1984, 1985) phylogenetic hypothesis, which placed Onitini (including Phanaeini) within the Scarabaeinae subfamily of Balthasar (1963) (see Fig. 1). Instead, it is congruent with Balthasar's (1963) taxonomy that grouped Onitini, Oniticellini, and Onthophagini together within the subfamily Coprinae. It is interesting to note that the Onitini showed a significantly higher rate of divergence with respect to other Scarabaeinae. The possibility that the Onitini sequences correspond to a transposed nuclear copy can be discarded since COI and COII fragments were independently amplified by PCR, the ORFs of both genes are not interrupted by indels or stop codons, and the base composition pattern of these sequences conforms with the consensus mitochondrial codon usage. A high rate of sequence divergence in Onitini might prompt the search in this tribe for higher metabolic or reproductive rates, which are the factors that are generally involved in the generation of higher substitution rates (Martin and Palumbi 1993). Both molecular and morphological data agree on the sister-group relationship of Oniticellini and Onthophagini and their derived position within the subfamily.

The placement of the tribe Coprini as sister group of the Sisyphini, and within a clade that also included

Scarabaeini and Gymnopleurini (Fig. 4), was an unexpected result since previous phylogenetic and taxonomic hypotheses had always placed Coprini closer to Oniticellini and Onthophagini (Janssens 1949; Balthasar 1963; Zunino 1984). However, it is important to note that these phylogenetic relationships were supported by very low bootstrap values and, hence, need to be treated as tentative. In this regard, it is surprising that the traditionally well-established (Janssens 1949; Balthasar 1963; Zunino 1984) close relationships of Sisyphini, Gymnopleurini, and Scarabaeini cannot be recovered with statistical significance in our analyses. To investigate further the phylogenetic validity of this grouping, it will be important to include representatives of the tribes Canthonini, Eurysternini, and Eucranini (not present in the Iberian Peninsula) into future molecular analyses. Moreover, in agreement with former studies (Zunino 1984; Montreuil 1998), molecular evidence highlights the need to evaluate further both the internal and the external phylogenetic relationships of the tribe Coprini (and the related tribe Dichotomiini).

The molecular phylogeny also rendered some interesting insights on the relationships within the tribes Onitini and Onthophagini. The basal position of the genus *Cheironitis* within the tribe Onitini supported previous morphological evidence (Cambefort 1995). Of special interest is the nonmonophyly of the rather speciose genus *Onitis* as evidenced by the two Iberian representatives included in the phylogenetic analysis. In this regard, the grouping of *O. ion* and the genus *Bubas* needs to be treated as tentative because of its low bootstrap support.

Several studies have tried a natural reordering of the speciose and cosmopolitan tribe Onthophagini emphasizing its phylogenetic complexity (see, e.g., Zunino 1979; Palestini 1985; Martín-Piera 2000). The monophyletic condition of the *Onthophagus* subgenus *Trichonthophagus* (Zunino 1979; Palestini 1985) and probably *Palaeonthophagus* (Zunino 1979) was confirmed by the results (Fig. 4). The molecular phylogeny partly agrees with previous morphological evidence (Martín-Piera 2000) in placing the genera *Caccobius* and *Euonthophagus* and several *Onthophagus* subgenera (*Parentius*, *Trichonthophagus*, and *Onthophagus sensu stricto*) at the base of the Onthophagini, although they disagree in their relative position (see Figs. 1B and 4).

Within the derived subgenus *Palaeonthophagus*, the molecular phylogeny supports the validity of the *vacca* and *ovatus* groups but not their respective compositions. In particular, *O. nutans*, which was formerly included in the *vacca* group; appeared to be basal to both groupings. Other significant discrepancies are the relative positions of *O. lemur* and *O. melitaeus*. The proposed phylogenetic position of the latter species questions the validity of the subgenus

Amphionthophagus. A comprehensive morphological analysis including more characters may help to resolve the discrepancy between the molecular and the morphological phylogenetic hypotheses.

Evolution of the Tunneling and Rolling Nesting Behaviors

The traditional classification of the Scarabaeinae (Janssens 1949; Balthasar 1963) reflects to some extent the major evolutionary trends of the nesting behavior. Dung beetles are divided into those that exhibit a rolling behavior and those that show a tunneling behavior. It has always been assumed that because of the complexity of both alternative nesting systems, each one must have evolved only once (Halffter and Matthews 1966; Halffter and Edmonds 1982). The phylogenetic hypothesis of Zunino (1984) already questioned this idea by placing two tunneler tribes (Onitini and Phanaeini) deep within the roller tribes (Fig. 2A). The molecular phylogeny presented here (Fig. 4) contradicts Zunino's (1984) hypothesis and groups the Onitini together with other tunnelers with a moderately high bootstrap support.

It is more difficult to ascertain the monophyly of rollers from the MP topology (Fig. 4). The three rolling tribes analyzed (Sisyphini, Scarabaeini, and Gymnopleurini) are grouped together in a clade that is weakly supported (bootstrap value below 50%). Moreover, this clade also includes the *Coprini* genus, which exhibits a tunneling behavior. The lack of resolution at this level may indicate that the origin of the rolling behavior was coupled with a rapid adaptive radiation. If the MP topology is correct, it is plausible that *Coprini* derived from an ancestral rolling lineage that secondarily lost the rolling relocation behavior. Interestingly, some tropical species of Scarabaeini (Sato and Imamori 1986; Sato 1998), Canthonini and Eurysternini (Halffter and Matthews 1966) exhibit facultative coprine nesting and breeding behaviors. The molecular hypothesis (Fig. 4), if true, would emphasize the significant differences between the tunneling behavior of Coprini and that of other tunnelers. Alternatively, Coprini may be placed basal to the tunnelers (Onitini, Oniticellini, and Onthophagini) or to the rollers (Sisyphini, Scarabaeini, and Gymnopleurini). In both cases, the rolling behavior still would have evolved only once. Nevertheless, recurrent independent origins of the rolling behavior cannot be discarded based on our results, particularly, taking into account that the Coprini are not a monophyletic assemblage (Montreuil 1998) and that members of the Dichotomiini, Phanaeini, Eurysternini, Eucranini, and Canthonini need to be included in the analysis.

In conclusion, we present the first molecular hypothesis on the phylogenetic relationships of dung

beetles based on the sequence and phylogenetic analyses of mitochondrial cytochrome oxidase genes and using Iberian species as representatives of the main lineages within the subfamily Scarabaeinae. Our results support the close relationships of the Onitini, Oniticellini, and Onthophagini. Behavioral and morphological evidence strongly supports the monophyly of the rollers (Halffter and Edmonds 1982; Zunino 1984). However, our results are not as conclusive since we recover a clade that includes Sisyphini, Scarabaeini, and Gymnopleurini, but without bootstrap support. This lack of resolution may indicate a rapid adaptive radiation in the origin of the roller taxa. Hence, the molecular phylogeny does not contradict the traditional rolling–tunneling dichotomy within the subfamily and suggests that the elaborated rolling behavior may have been secondarily lost in *Copris*. Because some tribes of Scarabaeinae are not present or have few representatives on the Iberian Peninsula, our analysis leaves open the possibility that the rolling behavior might have evolved several times independently.

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