Molecular data supports the inclusion of *Ildobates neboti* Español in Zuphiini (Coleoptera: Carabidae: Harpalinae)

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The phylogenetic relationships of *Ildobates neboti* Español (Coleoptera: Carabidae: Harpalinae) were investigated based on three nuclear genes (full 18S rRNA, and a fragment of each 28S rRNA and *wingless*). We compiled a data set using published sequences of 32 members of Harpalinae including one example each of Dryptini (genus *Desera*), Galeritini (*Galerita*) and Zuphiini (*Thalpius*), plus three Brachininae as outgroups. These three tribes form the "Dryptitae", within which various relationships of *Ildobates* had been proposed. The analyses of the data matrix using parsimony (with equally weighted and reweighted characters) and Bayesian posterior probabilities all support the monophyly of the three tribes in "Dryptitae", as well as a closest relationship of *Ildobates* with *Thalpius* to the exclusion of *Desera* plus *Galerita*. This confirms the previous inclusion of *Ildobates* among the Zuphiini, and corroborates current taxonomic classifications based on morphological criteria.

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1. Introduction

Among the diverse fauna of Coleoptera of the Iberian peninsula, *Ildobates neboti* Español stands out as one of the most emblematic taxa, both because of its morphology [with extreme modifications for cave life (Español 1966, Bellés 1987)] and its rarity, being known for only a handful of specimens from three caves in the Mediterranean coast of Spain, in the province of Castellón (Ortuño *et al.* 2004). Although in the original description the species was identified as a "Dryptidae" (Español 1966), its precise relationships were contentious, with characters relating it to several of the tribes within the group [see Ortuño *et al.* (2004) for a summary of the taxonomic history of the species]. The current consensus view seems to include *Ildobates* among

Table 1. Sequences used in the study. Taxonomic ordination follows Löbl & Smetana (2003).

No	Subfamily	Tribe	Species	28S rRNA	*wingless*	18S rRNA
1	BRACHININAE		*Brachinus hirsutus* Bates	AF398693	AF398572	AF012478
2			*Pheropsophus aequinoctialis* Linné	AF398678	AF398619	AF012477
3			*Aptinus displosor* Dufour	AF398638	AF398569	AF012480
4	HARPALINAE	Anthiini	*Cypholoba* sp.	AF398695	AF398584	
5		Calophaenini	*Calophaena* sp. n.	AF398666	AF398575	
6		Catapieseini	*Catapiesis brasiliensis* Gray	AF398645	AF398577	AF012476
7		Chlaeniini	*Chlaenius ruficauda* Chaudoir	AF398680	AF398578	AF002777
8		Cnemalobini	*Cnemalobus sulcifer* Philippi	AF398706	AF398580	AF012474
9		Ctenodactylini	*Leptotrachelus dorsalis* Fabricius	AF398646	AF398599	
10		Cyclosomini	*Tetragonoderus latipennis* LeConte	AF398653	AF398631	AF012471
11		Graphipterini	*Graphipterus cordiger* Dejean	AF398711	AF398593	
12		Harpalini	*Discoderus cordicollis* Horn	AF398652	AF398588	AF002776
13			*Pelmatellus* sp.	AF398690	AF398615	AF398720
14		Helluonini	*Omphra* sp.	AF398657	AF398610	
15		Lachnophorini	*Calybe laetula* LeConte	AF398705	AF398576	AF002772
16		Lebiini	*Cymindis puntigera* LeConte	AF398651	AF398583	AF002773
17		Licinini	*Dicaelus ambiguus* LaFerté-Sénectère	AF398655	AF398586	
18		Loxandrini	*Loxandrus* nr. *amplithorax* Straneo	AF398661	AF398600	AF002778
19		Metriini	*Metrius* sp.	AF398654	AF398604	AF012475
20		Morionini	*Morion aridus* Allen	AF398698	AF398606	AF002783
21			*Moriosomus seticollis* MacLeay	AF398701	AF398607	AF398721
22		Odocanthini	*Colliuris pennsylvanica* Linné	AF398712	AF398581	
23		Oodini	*Stenocrepis elegans* LeConte	AF398668	AF398627	
24		Orthogoniini	*Orthogonius* sp.	AF398709	AF398611	AF398719
25		Panagaeini	*Panagaeus sallei* Chaudoir	AF398691	AF398612	
26		Peleciini	*Pelecium* nr. *sulcipenne* Chaudoir	AF398672	AF398614	AF398715
27		Perigonini	*Perigona nigriceps* Dejean	AF398665	AF398617	
28		Platynini	*Agonum extensicolle* Say	AF398643	AF398564	AF002775
29		Pseudomorphini	*Pseudomorpha* nr. *angustata* Horn	AF398714	AF398622	AF002782
30			*Sphallomorpha* sp.	AF398679	AF398636	AF398717
31		Pterostichini	*Pterostichus melanarius* Illiger	AF398707	AF398623	AF002779
32		Zabrini	*Amara apricaria* Paykull	AF398694	AF398565	AF002774
33	"Dryptitae"	Galeritini	*Galerita lecontei lecontei* Dejean	AF398686	AF398590	AF002780
34		Dryptini	*Desera australis* Péringuey	AF398659	AF398585	per.com.
35		Zuphiini	*Thalpius* nr. *rufulus* LeConte	AF398697	AF398632	AF002781
36			*Ildobates neboti* Español	AM051084	AM051083	DQ130051

the Zuphiini, as reflected in the most recent taxonomic catalogues [both Iberian (Serrano 2003) and Palaearctic (Löbl & Smetana 2003)] and in morphological studies (Ortuño *et al.* 2004). At present, however, there are no phylogenetic studies (either morphological or molecular) including *Ildobates*, therefore all discussions on its relationships have been purely descriptive.

In this contribution we present the first formal phylogenetic study including *Ildobates neboti*, based on sequences of three nuclear gene fragments and including a good taxonomic coverage of Harpalinae (with one example each of the three tribes to which *Ildobates* has been related). Our aim is to provide for the first time a sound phylogenetic ground for the taxonomic placement of this emblematic species, as well as to make feasible future studies on its evolutionary and geographic origin by the identification of its potential closest living relatives.

2. Material and methods

2.1. DNA extraction and sequencing

Genomic DNA was obtained through a standard phenol-chloroform extraction using abdominal

tissue (voucher specimen kept in X. Bellés coll., DNA aliquots kept in the MNCN ref. 6409). Three nuclear fragments were amplified, those for which there were enough published sequences of related species: the full 18S rRNA, and a fragment of each *wingless* and 28S rRNA.

The 18S rRNA sequence was obtained by direct sequencing of four overlapping fragments amplified with internal primers (see Shull et al. 2001 for details of the primers and PCR conditions used). Both forward and reverse sequences were obtained for each fragment, which were contigged and edited using Sequencher 4.2 (GeneCodes Corporation). The *wingless* and 28S rRNA sequences were obtained using the primers and the PCR protocol described in Ober (2002). Accession numbers for the sequences are DQ130051 (18S rRNA), AM051083 (*wingless*) and AM051084 (28S rRNA) (Table 1).

2.2. Additional molecular data

We restricted our analyses to Harpalinae, as the inclusion of *Ildobates* within this clade is not contentious. We considered Brachininae as the outgroup, following Ober (2002) and Ribera *et al.* (2005). Sequences were compiled for all species of Harpalinae and Brachininae for which the three studied fragments were available (25 species), plus one example of each of the Harpalinae tribes for which no 18S rRNA was available (ten additional species; Table 1). Sequences were obtained from Maddison *et al.* (1998) (GenBank accession numbers AF002772–AF002783), Maddison *et al.* (1999) (7 sequences among AF012471–AF012480) and Ober (2002) (5 sequences among AF398715–AF398721) (Table 1). *Wingless* and 28S rRNA sequences were obtained from Ober (2002) (35 sequences of each among AF398569–AF398636 and AF398638–AF398564, respectively) (Table 1). The 18S rRNA sequence of *Desera australis* Peringuey (Harpalinae, Dryptini) was obtained from D. Maddison (personal communication; June 2005).

Preliminary searches were conducted with the 28S rRNA and wingless genes alone to test for the possibility that *Ildobates* could be related to any of the tribes with missing 18S rRNA sequences. As this was not the case, a reduced dataset was constructed with only the species for which all genes were available (26 including *Ildobates*), and all subsequent analyses were conducted with this dataset.

2.3. Phylogenetic analyses

Three hyper-variable regions of the 18S rRNA gene and six of the 28S rRNA gene were excluded from the analyses, and the remaining sequence was aligned by hand. To align the *wingless* gene we translated the nucleotide sequence with McClade 4.0 (Maddison & Maddison 2000), and aligned the aminoacid sequence manually. The nucleotide sequence was thus aligned using the aminoacid sequence as a template. An additional dataset was constructed with the only four species of "Dryptitae" included in the analyses (Table 1), with the full sequences (including hypervariable regions), which were aligned by hand. This was aimed to increase the support for the internal nodes within the group.

Parsimony analysis was conducted in PAUP* version 4.0b10 (Swofford 2002) using TBR heuristic searches with 1,000 random sequence addition replicates. In all searches gaps were coded as a missing character state. To increase resolution, characters were re-weighted according to the rescaled consistency index (Farris 1969), and new heuristic searches conducted starting with the trees obtained with equally weighted characters. Node support was measured with non-parametric bootstrapping with 1,000 pseudo-replicates of 100 random sequence additions each.

Previous analyses including the sequences used in this study (Maddison *et al.* 1998, 1999, Ober 2002, Ribera *et al.* 2005) showed the likely presence of artefacts due to the distorting effects of long branches (Felsenstein 1978). Therefore, we conducted additional analyses using model-based phylogenetic methods, which should in principle be less sensitive than parsimony to the biases introduced by highly saturated or homoplasious data (e.g. Swofford *et al.* 1996, Felsenstein 2004).

The optimal model of nucleotide substitution was determined with Modeltest 3.7 (Posada & Crandall 1998), for the three genes separately. In all cases a generalised time reversible (GTR)
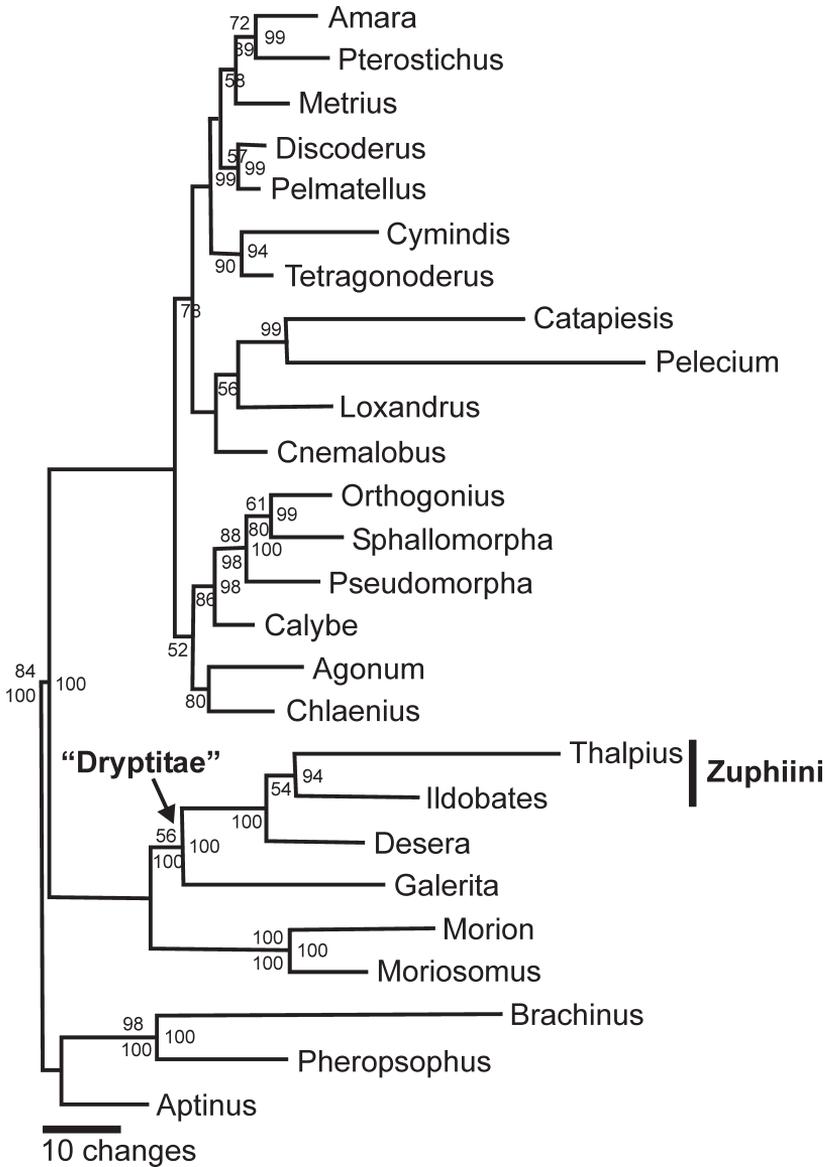
Fig. 1. Phylogram of the single most parsimonious tree resulting from the heuristic search on the re-weighted combined dataset. Numbers above branches, bootstrap support values on the equally weighted dataset; below branches, bootstrap support values on the re-weighted dataset; inside nodes, posterior Bayesian probabilities of the search in MrBayes (×100) (see Methods for details).

model (Tavaré 1986) with gamma distributed among site rate variation and estimating the proportion of invariable sites (Yang 1993) was selected as the best fit to the data. To analyse the data we used Bayesian probabilities (Rannala & Yang 1996) as implemented in the computer program MrBayes 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003), as it allows the estimation of different evolutionary models for the user-defined data partitions. The parameters of the three partitions (i.e., genes) were estimated independently. Two independent

searches were conducted using the default priors (uniform probabilities) starting with random trees, with three heated and one cold Markov chains for 2,000,000 generations, sampled at intervals of 100 generations. To determine the point at which the Markov chains reached stationarity, the log-likelihood scores were plotted against generation time, and visually determined when the log-likelihood values reached a stable equilibrium. The parameter estimations (including tree topologies) obtained before reaching the stationarity are discarded as a "burnin", and only

the trees sampled after that point are considered (Huelsenbeck & Ronquist 2001).

Posterior probabilities were used to assess node stability. Although generally higher than bootstrap support values, posterior probabilities above the standard 95% threshold can be taken as indicative of strong node stability (Suzuki *et al.* 2002, Alfaro *et al.* 2003, Douady *et al.* 2003, Simmons *et al.* 2004, Huelsenbeck & Rannala 2004).

3. Results

The matrix with the 36 species with 28S rRNA and *wingless* sequences (Table 1) had 1,187 characters, of which 359 were parsimony informative. A heuristic search in PAUP resulted in two equally shortest trees of 2,669 steps, in which *Ildobates* was included in a clade with *Desera*, *Thalpius* and *Catapiesis* with 69% bootstrap support, none of them with 18S rRNA missing (Table 1). The more inclusive clades containing *Ildobates* had bootstrap support values lower than 50%.

As there was no evidence of a close relationship of *Ildobates* with any of the species with a missing 18S rRNA sequence (Table 1), a new matrix was build with the 26 species with full data, with 2,959 characters of which 422 were parsimony informative. The heuristic parsimony search resulted in 11 shortest trees of 2,385 steps (CI: 0.382, RI: 0.312), with "Dryptitae" monophyletic (with only 56% bootstrap support) and *Galerita* sister to the rest, which formed a polytomy (not shown). After re-weighting the characters, the heuristic search on the 11 original trees resulted in a single tree, in which *Thalpius* was sister to *Ildobates* (i.e., a monophyletic Zuphiini), also with very low support (bootstrap 54%, Fig. 1). The bootstrap support for "Dryptitae" using re-weighted characters was very high (100%, Fig. 1). The tree obtained with Bayesian posterior probabilities had a large polytomy at the base of Harpalinae, although the nodes that were resolved were identical to those of the re-weighted parsimony tree (Fig. 1). The nodes defining the relationship of *Ildobates* had in general higher support, with a posterior probability of 1.0 for "Dryptitae" and 0.94 for the sister relationship
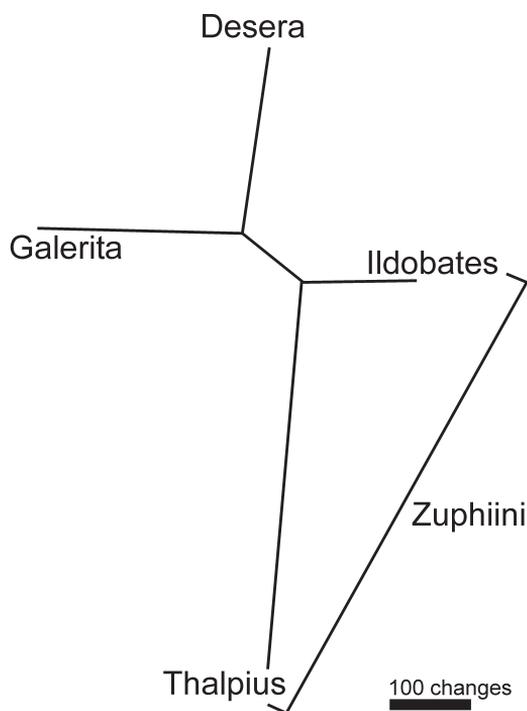

Fig. 2. Un-rooted tree of the exhaustive search on the combined dataset (*wingless*, 18S rRNA, 28S rRNA) of the four species of "Dryptitae", including hyper-variable regions of the ribosomal genes.

of *Ildobates* and *Thalpius* (Fig. 1).

The data matrix of the full sequences of the four species of "Dryptitae" (*Galerita*, *Desera*, *Thalpius* and *Ildobates*) had 3,846 characters, of which 114 were parsimony informative. In the shortest of the three possible unrooted trees, with 772 steps, the closest relative of *Ildobates* was *Thalpius*, with exclusion of *Galerita + Desera* (i.e., a monophyletic Zuphiini). The same relationship was found with a Bayesian probability search, with a posterior probability of 1.0 (Fig. 2).

4. Discussion

Despite the limited taxon sampling (with only one example of each of the potentially relevant groups), all our analyses recovered the monophyly of tribes Zuphiini, Galeritini and Dryptini in what could be called the supertribe "Dryptitae" [i.e., the subfamily Dryptinae of e.g. Serrano (2003)]. This was one of the few nodes with high support among the Harpalinae, both with parsi-

mony and Bayesian posterior probabilities. The resolution within "Dryptitae" is however low, although all analyses point to a sister relationship of the only included Zuphiini (i.e. *Thalpius*) and *Ildobates*, confirming the inclusion of *Ildobates* among the Zuphiini, as considered in Ortuño *et al.* (2004) based on a morphological analysis, and in agreement with the traditional taxonomic classification (e.g., Löbl & Smetana 2003, Serrano 2003).

Zuphiini is a tribe with an almost cosmopolitan distribution, with numerous species occupying the subterranean medium (both endogean and troglobitic) (Moore 1995, Casale *et al.* 1998). It is remarkable that some of these troglobitic species in very distant areas have a strikingly similar overall appearance, such as e.g. *Speozuphium* and *Speothalpius* in Australia (Moore 1995, Casale *et al.* 1998). Our results demonstrate the inclusion of *Ildobates* among Zuphiini, but are silent about any further detail on relationships within the tribe. It would be most interesting to know if *Ildobates* is most closely related to similar troglobitic forms in distant geographical areas – in which case it could be appropriately called a "relict" species, as it is usually considered (Casale *et al.* 1998, Ortuño *et al.* 2004). Alternatively, it could be most closely related with some of the European species, in which case the similarities in external morphology with distant cave species would be an interesting case of parallel or convergent evolution.

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