



Ancient origin of endemic Iberian earth-boring dung beetles (Geotrupidae)

Regina L. Cunha^{a,b}, José R. Verdú^c, Jorge M. Lobo^{a,*}, Rafael Zardoya^a

^aDepartamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, José Gutiérrez Abascal, 2, 28006 Madrid, Spain

^bCCMAR, Campus de Gambelas, Universidade do Algarve, 8005-139 Faro, Portugal

^cCentro Iberoamericano de la Biodiversidad (CIBIO), Universidad de Alicante, San Vicente del Raspeig, E-03080 Alicante, Spain

ARTICLE INFO

Article history:

Received 9 July 2010

Revised 25 February 2011

Accepted 29 March 2011

Available online 5 April 2011

Keywords:

Molecular phylogeny

Geotrupidae

Paleoendemism

Apterism

Molecular dating

Diversification rates

ABSTRACT

The earth-boring dung beetles belong to the family Geotrupidae that includes more than 350 species classified into three subfamilies Geotrupinae, Lethrinae, and Taurocerastinae, mainly distributed across temperate regions. Phylogenetic relationships within the family are based exclusively on morphology and remain controversial. In the Iberian Peninsula there are 33 species, 20 of them endemic, which suggests that these lineages might have experienced a radiation event. The evolution of morphological adaptations to the Iberian semi-arid environments such as the loss of wings (apterism) or the ability to exploit alternative food resources is thought to have promoted diversification. Here, we present a phylogenetic analysis of 31 species of Geotrupidae, 17 endemic to the Iberian Peninsula, and the remaining from southeastern Europe, Morocco, and Austral South America based on partial mitochondrial and nuclear gene sequence data. The reconstructed maximum likelihood and Bayesian inference phylogenies recovered Geotrupinae and Lethrinae as sister groups to the exclusion of Taurocerastinae. Monophyly of the analyzed geotrupid genera was supported but phylogenetic relationships among genera were poorly resolved. Ancestral character-state reconstruction of wing loss evolution, dating, and diversification tests altogether showed neither evidence of a burst of cladogenesis of the Iberian Peninsula group nor an association between apterism and higher diversification rates. Loss of flight did not accelerate speciation rates but it was likely responsible for the high levels of endemism of Iberian geotrupids by preventing their expansion to central Europe. These Iberian flightless beetle lineages are probably paleoendemics that have survived since the Tertiary in this refuge area during Plio-Pleistocene climatic fluctuations by evolving adaptations to arid and semi-arid environments.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

The earth-boring dung beetles belong to the highly diversified family Geotrupidae Latreille, 1802 that includes around 350 species (Schoolmeesters, 2010), currently classified into three subfamilies (Browne and Scholtz, 1999; Scholtz and Browne, 1996): Geotrupinae mainly distributed across the Holarctic region (≈ 234 spp.), Lethrinae present in eastern Europe and Asia (≈ 114 spp.), and Taurocerastinae with only three recognized species distributed in South America (but see Browne and Scholtz, 1995; Howden, 1982; Howden and Peck, 1987; Zunino, 1984, which included Taurocerastinae within Geotrupinae). Former classifications (Howden, 1982; Lawrence and Newton, 1995; Zunino, 1984) included the basically pantropical Bolboceratidae (≈ 561 spp.) as a subfamily of Geotrupidae, a proposal currently not supported by available phylogenies based on larval and morphological characters (Browne and Scholtz, 1999; Verdú et al.,

2004; but see Smith et al., 2006). Geotrupidae are thought to represent an early offshoot of the superfamily Scarabaeoidea that may have diverged from the remaining dung beetles at least during the Lower Cretaceous according to fossil data (Krell, 2007). Although earth-boring dung beetles are fairly represented in the fossil record (Krell, 2007) there is no information regarding lineage-splitting events within the family that lead to current species diversity.

The Iberian Peninsula hosts a remarkably diversified assemblage of earth-boring dung beetles (López-Colón, 2000). A total of 11 genera are present in the Iberian Peninsula representing about 65% and 23% of the total number of European and World Geotrupidae genera, respectively. These 11 Iberian genera include 33 species, which correspond to 62% and 9% of the European and World Geotrupidae species, respectively. Eighteen of the 20 Iberian endemic geotrupid species are apterous (i.e. have lost flight due to atrophy of hindwings and fusion of elytra); thus, their dispersal capacity is limited (Lobo et al., 2006). As in other beetles (e.g. Chown et al., 1998), the origin of apterism in Iberian Geotrupidae may be originally related to adaptation to

* Corresponding author. Fax: +34 91 5645078.

E-mail addresses: rcunha@ualg.pt (R.L. Cunha), jr.verdu@ua.es (J.R. Verdú), mcnj117@mnscn.csic.es (J.M. Lobo), rafaz@mnscn.csic.es (R. Zardoya).

arid or semi-arid conditions since the fusion of elytra diminishes loss of body water.

Despite the wealth of molecular studies centred on Scarabaeoidea (Cabrero-Sañudo and Zardoya, 2004; Forgie et al., 2006; Monaghan et al., 2007; Price, 2009; Smith et al., 2006; Sole and Scholtz, 2010; Villalba et al., 2002), few studies have focused on Geotrupidae, and phylogenetic relationships among earth-boring dung beetles remain poorly understood. Thus far, phylogenetic studies on Geotrupidae have been mostly based on morphological data, and rendered poorly resolved or even contradictory results (Palmer and Cambefort, 1997; Verdú et al., 2004; Zunino, 1984). The only attempt to examine the internal relationships within Geotrupidae based on molecular data focused on the biogeographical patterns of the genus *Trypocopris* (Carisio et al., 2004).

Here, we studied 31 species of earth-boring dung beetles inhabiting the Iberian Peninsula, southeastern Europe, Morocco, Mexico, and Austral South America. We conducted a phylogenetic analysis using both mitochondrial (mt) and nuclear sequence data. In particular, we assessed the phylogenetic placement of the subfamily Taurocerastinae, as well as provided a first phylogenetic hypothesis on the internal relationships within Geotrupinae. As Iberian Geotrupidae species are characterized by a high proportion of endemics we used the recovered phylogenies to test the hypothesis that adaptation to arid and semi-arid environments together with the associated loss of wings could be a major driving force for speciation in this group. First, we performed an ancestral character-state reconstruction to gain insights into the evolution of wing loss within this family. Subsequently, we dated lineage-splitting events within Geotrupidae to finally examine rates of lineage diversification through time in Iberian earth-boring dung beetles.

2. Materials and methods

2.1. DNA sources and extraction

A total of 31 specimens representing the same number of species, 13 genera and the three subfamilies of Geotrupidae were collected from the localities indicated in Fig. 1. All biological material used in this study is deposited in the DNA and tissues collection of the Museo Nacional de Ciencias Naturales of Madrid. Based on previous work (Cabrero-Sañudo and Zardoya, 2004; Villalba et al., 2002) species belonging to the other two lineages of dung beetles, Aphodiidae (*Aphodius conjugatus*) and Scarabaeidae (*Bubas bison*) were selected as outgroups. Total DNA was extracted from frozen tissue with a DNA Easy extraction kit (Qiagen).

2.2. PCR amplification and sequencing

Primers COI-Sca-F, COI-Sca-R, COIIam-Sca, and COIIB-605-Sca (Villalba et al., 2002), were used to amplify by PCR two overlapping fragments that comprised the 3' end of the mt *cox1*, the adjacent complete *trnL* (*uur*), and the 5' end of the *cox2* genes of 31 specimens of earth-boring dung beetles, as well as of two outgroup species. Additionally, primers 8029fin-4F and 8029fin-7R (Regier et al., 2008) were used to amplify by PCR a fragment of 718 bp of the nuclear gene neurofibromin. Initially, only a few samples were amplified successfully. Therefore, two internal primers Sca nuc-F (CATATGATGTGGGGTGAYATCGC) and Sca-nuc-R (AAACGCTCGC-GATCTGGCGAAGC) were designed to amplify by PCR a fragment of 420 bp in a total of 23 specimens, including the outgroup *Bubas*

Table 1

List of subfamilies and species used in this study, geographic range, endemic species from the Iberian Peninsula (IP), and GenBank accession numbers for nuclear and mitochondrial gene fragments.

Subfamily	Species	Geographic range	Iberian Peninsul endemics	GeneBank accession number	
				Nuclear	<i>cox1</i> + <i>LEU</i> + <i>cox2</i>
Geotrupinae	<i>Ceratophyus hoffmannseggi</i>	Southwestern Europe, North Africa	No		GU984631
	<i>Ceratophyus martinezi</i>	North-Central IP	Yes		GU984624
	<i>Geotrupes stercorarius</i>	Western Europe	No	GU984581	GU984634
	<i>Geotrupes ibericus</i>	IP	Yes	GU984590	GU984608
	<i>Geotrupes mutator</i>	Western Europe	No	GU984579	GU984606
	<i>Geotrupes spiniger</i>	Europe-Western Asia	No		GU984614
	<i>Jekelius albarracinus</i>	Central-southeastern IP	Yes	GU984592	GU984604
	<i>Jekelius catalonicus</i>	Northeastern IP	Yes	GU984578	GU984629
	<i>Jekelius castillanus</i>	Central Iberian Peninsula	Yes	GU984597	GU984603
	<i>Jekelius hispanus</i>	Southwestern IP	Yes	GU984599	GU984628
	<i>Jekelius hernandezii</i>	Southeastern IP	Yes	GU984593	GU984627
	<i>Jekelius nitidus</i>	Northwestern IP	Yes	GU984583	GU984626
	<i>Jekelius punctatolineatus</i>	Southeastern IP	Yes	GU984582	GU984625
	<i>Jekelius balearicus</i>	Balearic islands	Yes	GU984580	HM625867
	<i>Silphotrupes orocantabricus</i>	Northwestern IP	Yes	GU984591	GU984623
	<i>Silphotrupes punctatissimus</i>	North-Central IP	Yes	GU984588	GU984622
	<i>Thorectes baraudi</i>	South-Central IP	Yes	GU984598	GU984636
	<i>Thorectes ferreri</i>	South IP	Yes	GU984585	GU984635
	<i>Thorectes lusitanicus</i>	South IP	Yes		GU984615
	<i>Thorectes valencianus</i>	Southeastern IP	Yes	GU984584	GU984632
	<i>Thorectes armifrons</i>	North Africa	No		GU984605
	<i>Trypocopris pyrenaicus</i>	Europe	No	GU984596	GU984621
	<i>Typhaeus momus</i>	South IP	Yes	GU984600	GU984620
	<i>Typhaeus typhoeus</i>	Europe-North Africa	No		GU984619
	<i>Haplogeotrupes guatemalensis</i>	Central America	No	GU984601	GU984617
	<i>Sericotrupes niger</i>	Southwestern Europe, North Africa	No	GU984589	GU984609
	<i>Onthotrupes nebularium</i>	Mexico	No	GU984594	GU984618
<i>Onthotrupes herbeus</i>	Mexico	No		GU984616	
Taurocerastinae	<i>Taurocerastes patagonicus</i>	Austral South America			GU984611
	<i>Frickius variolosus</i>	Austral South America		GU984595	GU984610
Lethrinae	<i>Lethrus raymondi</i>	Southeastern Europe, West Asia			GU984607
Aphodiinae	<i>Aphodius conjugatus</i>	–			AY223690
Scarabaeinae	<i>Bubas bison</i>	–		GU984602	AY039339

bison. A total of 35–40 PCR cycles (denaturing at 94 °C for 30 s, annealing at 42 °C for 30 s, and extending at 72 °C for 60 to 90 s) with an initial denaturing step at 94 °C for 5 min, and a final extending step at 72 °C for 5 min were performed to amplify both mt and nuclear fragments. All PCR amplifications were conducted in 25- μ l reactions containing 1 mM MgCl₂, a 0.4 mM of each dNTP, 0.4 μ M of each primer, template DNA (10 to 100 ng), and Taq DNA polymerase (1 unit, Biotools). After PCR purification using ethanol/sodium acetate precipitation, samples were directly sequenced using the corresponding PCR primers. Samples were sequenced in an automated DNA sequencer (ABI PRISM 3700) using the Big-Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems) following manufacturer's instructions. Sequences were deposited in GeneBank under the accession numbers given in Table 1.

2.3. Phylogenetic analyses

DNA sequences were aligned with CLUSTAL X v1.83 (Thompson et al., 1997) using the default options and further optimized by eye. The partial nucleotide sequences of the mt *cox1*, *cox2* genes, and the complete sequence of the mt *trnL* (*uur*) gene from 31 geotrupid species and two outgroups were concatenated with the partial sequences of the neurofibromin nuclear gene of 22 geotrupid species and *Bubas bison* into a single data set of 1508 positions (neurofibromin sequence data not available for 9 geotrupid species and one outgroup were treated as missing data). The combined data set included 775 constant, and 524 parsimony-informative positions.

The Akaike information criterion (Akaike, 1973) as implemented in MODELTEST v3.7 (Posada and Crandall, 1998) selected the GTR + I + Γ ($I = 0.35$; $\Gamma = 0.61$) as the evolutionary model that best fit the data set. The selected model and model parameters were used in the ML analysis performed with PHYML v.2.4.4 (Guindon and Gascuel, 2003). The robustness of the inferred trees was tested by nonparametric bootstrapping (BP) using 1000 pseudoreplicates. ML analysis was carried out at the Mobylye platform (<http://mobylye.pasteur.fr/cgi-bin/portal.py>).

Bayesian inference (BI) analysis was performed with MRBAYES v.3.1.2 (Ronquist and Huelsenbeck, 2003), running for 1×10^7 generations (four simultaneous Markov chains; sample frequency, 100). Four independent runs were performed. Eight data partitions were analysed: mt *cox1* gene (first, second, and third codon positions), mt *cox2* gene (first, second, and third codon positions), mt *trnL* (*uur*) gene, and the nuclear neurofibromin gene. Model and model parameters were estimated independently for each of the respective data partitions using the unlink command in MRBAYES. We plotted the ln likelihood scores against generation number for each run in EXCEL to calculate the burn-in (samples obtained before the chain reached stationarity). Burn-in was set to the first 1,000,000 generations. The robustness of the inferred Bayesian trees was determined using Bayesian posterior probabilities (BPP; as obtained from majority-rule consensus trees of the post burn-in trees). Bayesian inference analysis was performed on the Computational Biology Service Unit at Cornell University (<http://cbsuapps.tc.cornell.edu/index.aspx>), and on Biportal at University of Oslo (<http://www.biportal.uio.no>).

Ancestral character-state reconstruction for the evolution of wings (character states: wings present or absent) under unweighted parsimony was performed in MACCLADE v4.08 (Maddison and Maddison, 2001) based upon the recovered ML topology. Both accelerated (ACCTRAN) and delayed (DELTRAN) transformations among character states were tested. In ACCTRAN, changes are assigned along branches of a phylogenetic tree as close to the root as possible. In DELTRAN, changes are assigned along branches as close to the tips as possible. We further used a randomization procedure also implemented in MACCLADE to test whether presence/ab-

sence of wings is significantly correlated with phylogeny. To obtain the null distribution of the number of transitions of the character, 1000 random trees were generated. Tree lengths were compared with the mean number of steps of the null distribution to test whether the observed number of transitions was significantly more correlated with phylogeny than expected at random.

In order to date lineage-splitting events within Geotrupidae we used a Bayesian relaxed molecular-clock approach as implemented in BEAST version 1.6.1 (Drummond and Rambaut, 2007). This methodology uses probabilistic calibration priors instead of point calibrations, allowing the incorporation of fossil uncertainties (Drummond et al., 2006). We chose the Yule speciation model that assumes a constant rate of speciation (Yule, 1924), which provides useful priors for species-level phylogenies (Drummond et al., 2006). This tree prior is most suitable for trees describing the relationships between individuals from different species (Drummond et al., 2006). The dating analysis was performed under the GTR + I + Γ substitution model, as selected by MODELTEST. *Cretogetrupes convexus* Nikolajev from the Lower Cretaceous was used as the minimum age for the splitting between Geotrupidae and its sister group (Krell, 2007), rejecting *Geotrupes lithographicus* (Deichmüller) from the Upper Jurassic because its doubtful status. The second calibration point was the fossil *Geotrupes atavus* (Oustalet) from the Oligocene (Krell, 2007) representing the minimum age for the appearance of the genus *Geotrupes*. The inclusion of *G. messelensis* Meunier dating from the Eocene was also rejected because it was also doubtful (Krell, 2007). Calibrations using the two fossils were modelled with a lognormal distribution, where 95% of the prior weight fell within the geological interval in which each fossil was discovered. For *Cretogetrupes convexus* [145.5–99.6 My] (Krell, 2007), the parameters of the lognormal calibration prior were: hard minimum bound 99.60, mean 3.13, and standard deviation 0.42. For *Geotrupes atavus* [33.9–23.03 My] (Krell, 2007), the parameters of the lognormal calibration prior were: hard minimum bound was 23.03, mean 1.69, and standard deviation 0.42. A lognormal distribution was used because evolutionary rates change along the branches instead of converging at the nodes as occurs in the exponential distribution (Drummond et al., 2006). MCMC were performed in BEAST with 5×10^7 , following a discarded burn-in of 5,000,000 steps. The convergence to the stationary distribution was confirmed by inspection of the MCMC samples using the program TRACER v1.5 (Rambaut and Drummond, 2007) that provides a measure of whether the chain has run for an adequate length. Tree and log output files from four independent runs were combined using LOGCOMBINER (Drummond and Rambaut, 2007). Bayesian dating was performed on the Computational Biology Service Unit at Cornell University (<http://cbsuapps.tc.cornell.edu/index.aspx>).

We investigated whether rates of cladogenesis have changed through time in the Iberian Geotrupinae using the constant-rate (CR) test with γ statistic (Pybus and Harvey, 2000; Pybus and Rambaut, 2002) as implemented in the R package LASER v.2.3 (Rabosky, 2006a,b) to determine whether the lineage through time (LTT) plots were consistent with a constant rate of diversification through time. Significant γ values indicate that rates are not constant. Positive γ values indicate an increase in the diversification rates towards the presents whereas negative values suggest a slowdown in the rate of diversification through time, when compared with expectations of a constant diversification model (Pybus and Harvey, 2000). We applied the Monte Carlo constant-rates (MCCR) test (Pybus, 2000) that takes incomplete taxon sampling into account also using the R package LASER. We further examined rates of diversification across the reconstructed Bayesian chronogram using the relative cladogenesis statistics implemented in ENDEPI v1.01 (Rambaut et al., 1997) that allows the identification of branches that have higher than expected rates of cladogenesis.

3. Results and discussion

3.1. Phylogenetic relationships of earth-boring dung beetles

Phylogenetic relationships within Geotrupidae have been a long-standing debate encouraged by the wealth of conflicting evolutionary proposals based on morphological studies (Lawrence and Newton, 1995; Scholtz and Browne, 1996; Smith et al., 2006; Verdú et al., 2004; Zunino, 1984). The subfamily Taurocerastinae has been alternatively considered as a separate clade (Browne and Scholtz, 1999) or included within the Geotrupinae (Howden, 1982; Howden and Peck, 1987). The reconstructed ML ($-\ln L = 13359.47$) and BI ($-\ln L = 12777.31$) phylogenies reject placement of Taurocerastinae within Geotrupinae, and instead strongly support its placement in a basal position with respect to all the remaining Geotrupidae, i.e. Lethrinae + Geotrupinae (Fig. 1). The basal position of Taurocerastinae is in agreement with the study of Zunino (1984) based on genitalic characters.

All analyzed genera within Geotrupinae except *Onthotrupes* are recovered as monophyletic with strong support (Fig. 1). The inclusion of *Haplogeotrupes* within *Onthotrupes* is not statistically supported, and may be a spurious result due to the rather incomplete taxon sampling of Nearctic earth-boring dung beetles. Former phylogenetic analyses and relatively recent classifications (e.g. Löbl and Smetana, 2006) included the genera *Jekelius* and *Silphotrupes* within *Thorectes* (see López-Colón, 1996). Our results suggest that each of the above-mentioned putative genera represents an independent lineage that appeared at a similar period of time of other well-established Geotrupidae genera (e.g., *Geotrupes* or *Typhaeus*). A sister group relationship of *Sericotrupes* and *Geotrupes* is strongly supported in all analyses, as already proposed by Zunino (1984) based on morphology. Although without support, a potential close relationship between *Ceratophyus*, *Typhaeus*, and *Onthotrupes* + *Haplogeotrupes* was found, as previously suggested based on adult characters and larval morphology (Howden, 1982; Howden and Peck, 1987).

3.2. Dates of major lineage-splitting events within earth-boring dung beetles

It has been hypothesized that the relict distribution of Taurocerastinae in the southern tip of South America could result from a vicariant event related with the break up of Gondwana and the separation of South America (Zunino, 1973, 1984) during the Lower Cretaceous (Storey, 1995). Our Bayesian dating analysis indicated that Taurocerastinae branched off around 130 [110–161] My (Fig. 3) supporting such hypothesis (Zunino, 1973, 1984). The divergence between Lethrinae and Geotrupinae occurred around 108 [83–136] My at the end of the Lower Cretaceous coinciding with the orogeny of Eastern Alps (Handy et al., 2010).

It is widely accepted that genera *Thorectes*, *Jekelius*, and *Silphotrupes* originated in the western Mediterranean region (Palmer and Cambefort, 2000). However, authors differ on the age of the ancestral lineage that originated this clade: either at the beginning (Krieken, 1981) or at the end (Palmer and Cambefort, 1997; Zunino, 1984) of the Tertiary. According to our results, the estimated age of the divergence of the stem lineage leading to the three above mentioned genera is 65 [50–82] My (Fig. 3) around the Cretaceous-Tertiary boundary. During this period, only the western part of the present Iberian Peninsula was emerged, the Central Iberian Chain emerges, and first signs of the Pyrenean collision appear (Andeweg, 2002).

Pleistocene climatic fluctuations had unquestionably a considerable effect on species distributions (Hewitt, 1996, 1999). However, there is a long-standing debate on whether species

dispersal in response to Pleistocene climate change promoted speciation (Cooper, 2004). Establishing comparisons between rates of diversification during glacial and interglacial periods may be used to investigate the role of glaciations in speciation (Knowles, 2001). In the present study, all clades have a pre-Pleistocene origin, which precludes any comparison of divergence rates between glacial and interglacial periods. Moreover, according to our Bayesian dating analysis, the early diversifications of most of the Geotrupinae genera with Iberian endemic species date back to the Paleogene, when the Iberian plate was amalgamated with the Eurasian plate (Grimaud et al., 1982), moving back in time previous estimates and modifying proposed evolutionary scenarios (Palmer and Cambefort, 2000). Therefore, cladogenesis in this region could hardly be supported by the refuge theory (Hewitt, 2000) during the Pleistocene glaciations, since species divergence started prior to this epoch by far. For instance, the current range of *T. valencianus* at the border of the Northeast and Southeast Iberia biogeographic areas was explained by the existence of marine saline lakes in this region at about 16 My ago (Palmer and Cambefort, 2000). Instead, our Bayesian dating analyses indicate an earlier Oligocene origin for this species at 28 [18–39] My (Fig. 3) not supporting this scenario. An alternative explanation for *T. valencianus* diversification could be related to the extension of plate boundary to the Provençal-Ligurian Basin until the Valencia Trough (Andeweg, 2002).

3.3. Drivers of diversification and endemism in the Iberian Geotrupinae

The Iberian Peninsula harbours a relatively high number of earth-boring dung beetle species; 19 of the 39 world species belonging to *Jekelius*, *Silphotrupes*, and *Thorectes* genera occur in the Iberian Peninsula (Schoolmeesters, 2010), a territory with the greatest proportion of geotrupid endemic species in the Palaearctic Region. It has been hypothesized that diversification of Iberian geotrupids could be related with early adaptations to arid or semi-arid environments (Zunino, 1984; Verdú and Galante, 2002), the most striking one being the fusion of elytra (modified forewing), which diminishes loss of body water. According to this hypothesis, apterism would prevent dispersal and thus, promote genetic isolation leading to diversification and endemism. In this study, we tested whether the large number of endemic species of Iberian Geotrupinae could be the result of a radiation event in the region provoked by the apterism of this lineage. The obtained negative value of $\gamma = -0.11$ suggests an early burst of cladogenesis (Pybus and Harvey, 2000). However, results of the MCCR/CR tests (species sampled = 17; estimated total number of species = 20; critical value one tailed test = -1.57 , $P = 0.45$) showed that overall diversification rates through time did not differ significantly from a constant pure-birth model, and thus the hypothesis of an early radiation was not supported. Moreover, we mapped the presence/absence of wings onto the reconstructed ML phylogeny (Fig. 2) in order to evaluate whether a higher diversification rate could be associated with the hypothetical key innovation (apterism) (Ree, 2005). Despite low resolution of internal nodes of the ML tree, the unweighted parsimony reconstruction of wing evolution in Geotrupidae (both using either ACCTRAN or DELTRAN algorithms) confidently showed that apterism (wing loss) arose recurrently throughout earth-boring dung beetle evolution. The tracing of the two-character states “present” and “absent” wings in Geotrupidae resulted in a 5-step tree, which was not in the 95% confidence interval of the null distribution (Fig. 2). This result indicates that apterism arose at least five times in the Geotrupinae and that presence/absence of the wings is significantly correlated with the phylogeny. However, with the present analysis, it is not possible to resolve whether the presence of wings was the plesiomorphic condition in the group, although it is likely since wing loss

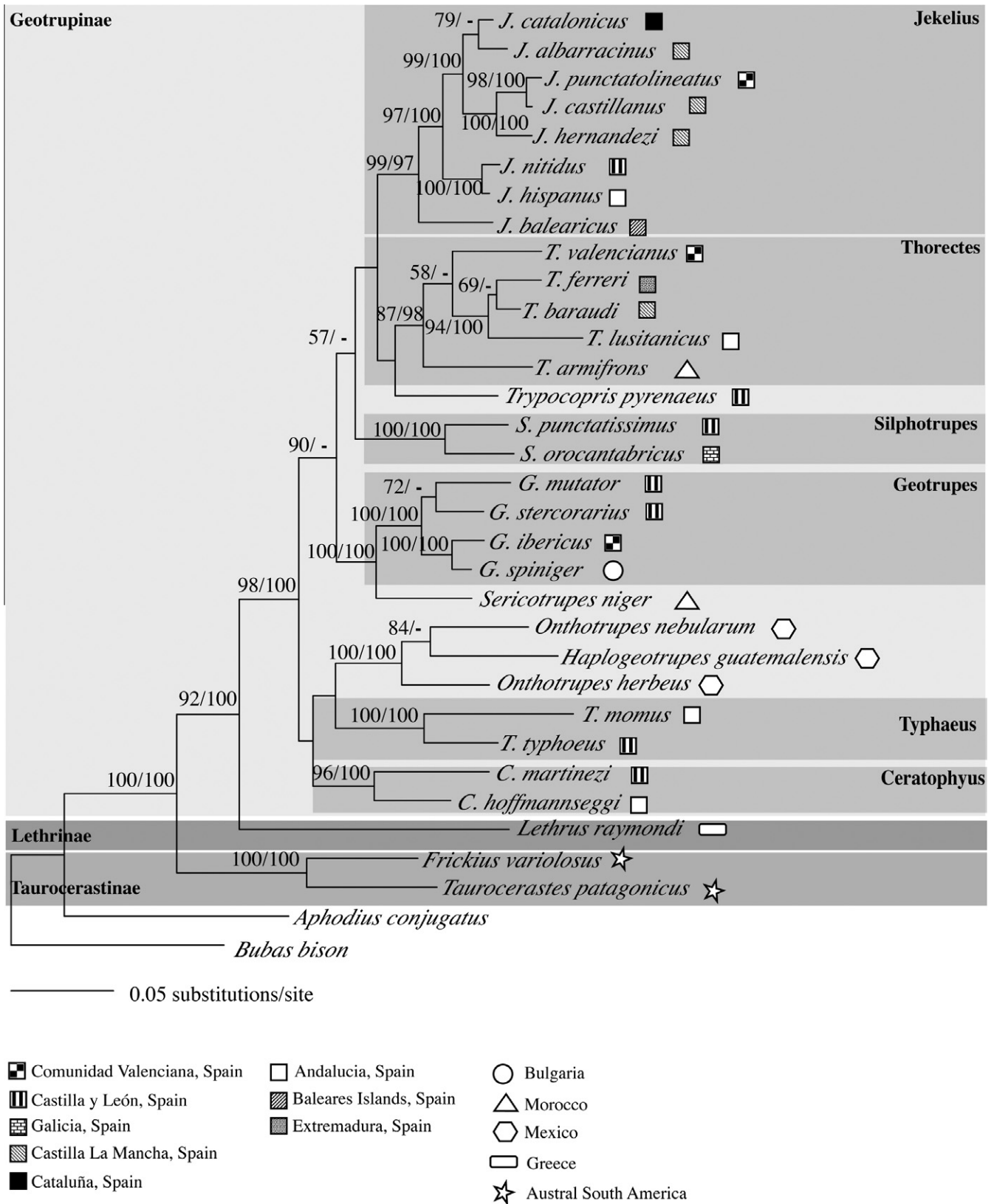


Fig. 1. Phylogenetic relationships of Geotrupidae based on a maximum likelihood (ML) analysis of a combined (mt and nuclear) data set using the GTR + I + Γ evolutionary model. This fragment includes the partial nucleotide sequences of the mt genes *cox1*, *cox2*, and the complete sequence of the *trnL-ur* gene as well as the partial sequence of the nuclear gene neurofibromin. Numbers in the nodes correspond to maximum likelihood bootstrap proportions – BP (first number) and Bayesian posterior probabilities – BPP (second number). Only BP and BPP values above 50% and 90%, respectively, are represented. Geographical origin of the samples is indicated.

is a much easier and likely evolutionary process that wing acquisition (see Whiting et al., 2003; Trueman et al., 2004).

The partitions of the phylogeny showing significantly higher rates of diversification identified by the relative cladogenesis test

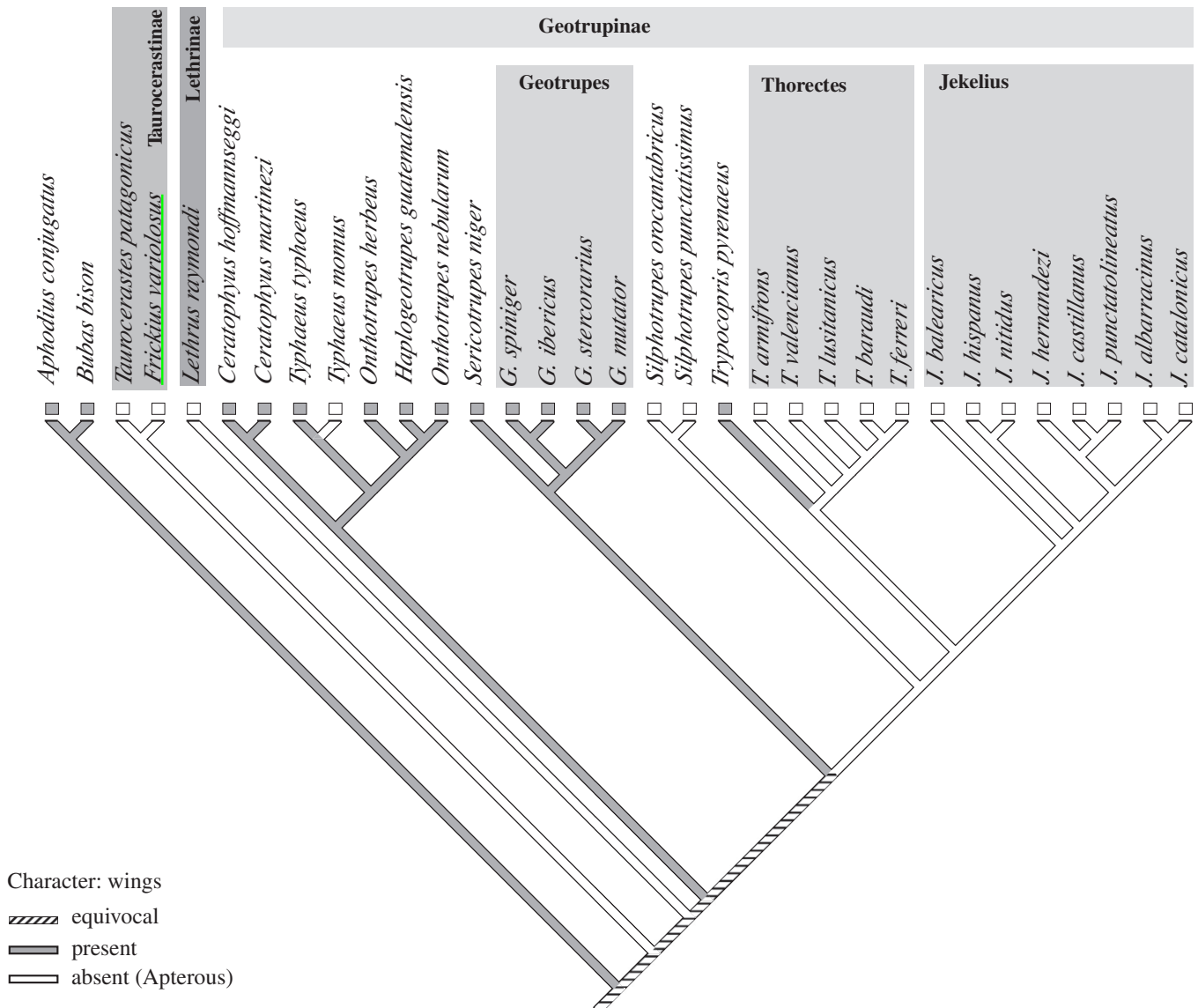


Fig. 2. Ancestral character-state reconstruction for the evolution of wings in the Geotrupidae based on unweighted parsimony under DELTRAN algorithm (ACCTRAN produced identical results) was mapped onto the ML phylogeny. Character states wings present and absent are represented in grey and white, respectively. When tracing the character is equivocal branches are represented by black stripes.

were indistinctly related with both winged and apterous species (Fig. 3). Therefore, apterism could not be related with higher rates of diversification in the Iberian Geotrupinae. Although wing loss did not enhance diversification, this adaptation might have promoted endemism in this ancient phylogenetic group. We suggest that these apterous species have remained isolated in the Iberian Peninsula since the Lower Tertiary probably due to the uplift of the Pyrenees (Le Pichon and Sibuet, 1971) and their low dispersal capabilities. While other species that used the Iberian Peninsula as glacial refuge were able to recolonize central Europe during Pleistocene interglacial periods, many Iberian geotrupids would have maintained their restricted distributions promoting current endemism. A similar pattern is observed with Taurocerastinae in the southern tip of South America where the loss of flight capacity may have enhanced the long term persistence of endemic species but not facilitate the occurrence of diversification processes (Domínguez et al., 2007). Wing loss is a common feature in species inhabiting isolated habitats such as oceanic islands. For instance, 18 of

the 20 endemic species of beetles on the island of Tristan da Cunha have lost or reduced their dispersal mechanisms (Cox and Moore, 2005). Long-distance dispersal abilities are not an advantage for terrestrial species that occur in habitats surrounded by vast stretches of open sea. The loss of wings was also observed in some island birds such as the kiwi from New Zealand (Cox and Moore, 2005). The lack of predators is thought to be the main reason for this adaptation because escaping is no longer required to maximize survival. Flightlessness frequently occurs in insects from semi-arid habitats and deserts. In some South African desert scarab beetles, the loss of flight seems to be related with water conservation capabilities (Scholtz, 2000; Capinera, 2008). It is also important to consider that together with wing loss, Iberian earth-boring dung beetle also evolved other adaptations to arid habitats such as e.g. the acquisition of strongly developed mandibular denticles, which allowed them to exploit dry pellets of wild small herbivores (mainly rabbits) and other unusual resources such as leaf litter, carrion, acorns, fresh fungi and fruits. These feeding adaptations

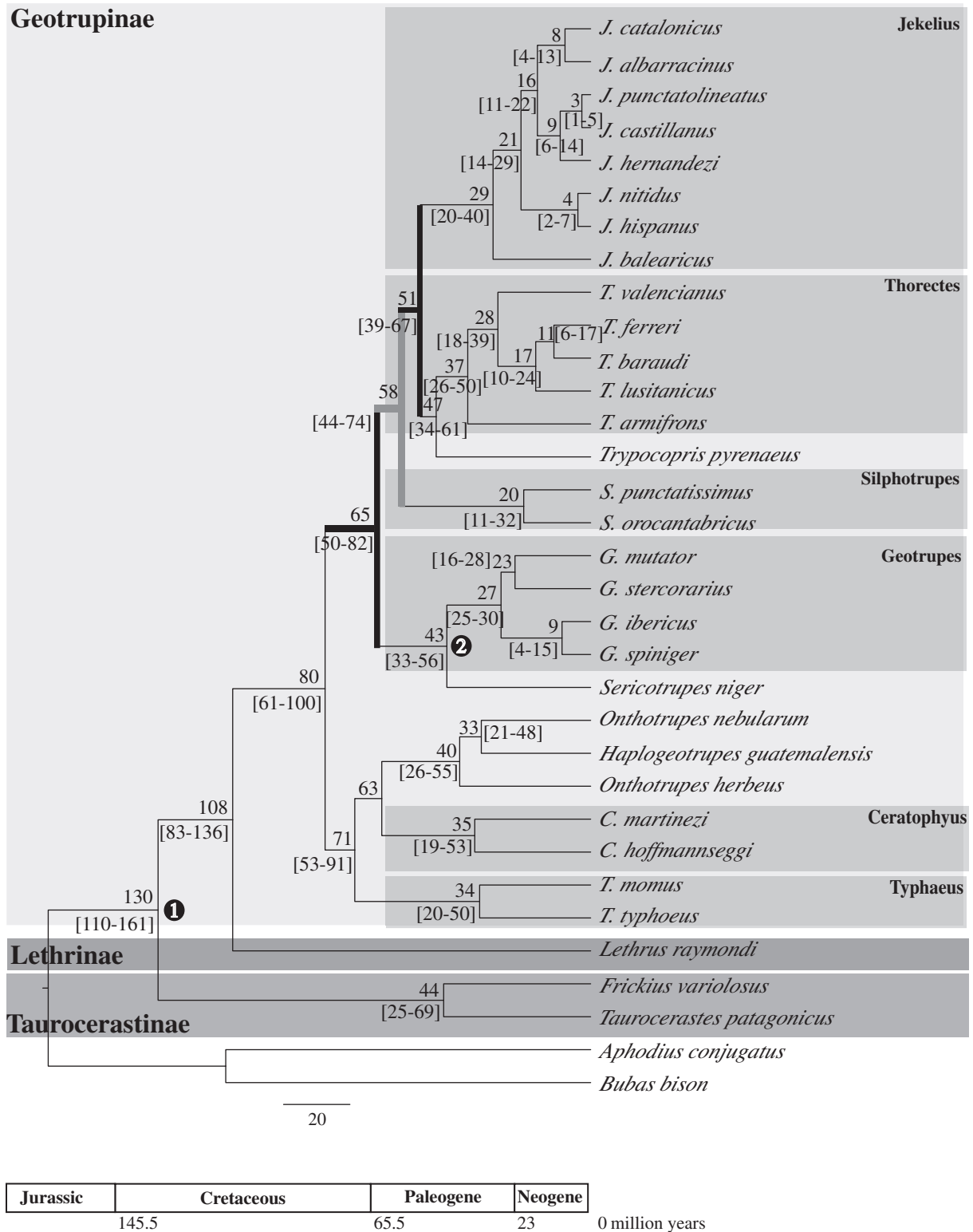


Fig. 3. Beast maximum clade credibility chronogram. Age estimates in million years and corresponding 95% highest posterior density intervals (values in square brackets) are depicted. Branches with higher rates of diversification identified by the relative cladogenesis test are represented in colour ($P < 0.05$, branches in bold; $P < 0.01$, branches in grey). Numbers 1 and 2 in the nodes indicate fossil constraints.

could further explain the long-term maintenance of these geotrupid endemics under the general Mediterranean conditions of the Iberian Peninsula (Verdú and Galante, 2004; Verdú et al., 2010).

4. Conclusions

Overall, our results indicate that current diversity of Iberian earth-boring dung beetles is the result of a constant accumulation

of species through time since the Lower Tertiary. Although wingless evolution did not trigger any burst of speciation, it is rather plausible that it may be underlying the evolution of the observed high proportion of endemics. Iberian geotrupids are paleoendemics that evolved early adaptations to hot and cold arid environments, among which the most remarkable ones were elytra fusion, wing loss and polyphagous habits. These apterous species lost dispersal capabilities and thus remained isolated in the Iberian Peninsula since the Lower Tertiary due to the uplift of the Pyrenees. While other species that used the Iberian Peninsula as glacial refuge were able to recolonize central Europe during Pleistocene interglacial periods, Iberian geotrupids maintained their restricted distributions promoting endemism.

Acknowledgements

We thank Federico Ocampo for the loan of the South American specimens. We are grateful to Rita Castilho for conceiving and drawing Fig. 1. Two anonymous reviewers provided insightful comments on an earlier version of the manuscript. Research was supported by a project of the Ministerio de Ciencia e Innovación of Spain (CGL2008-03878) to J. R. Verdú.

References

- Akaike, H., 1973. Information theory as an extension of the maximum likelihood principle. In: Csaki, B.N.P.A.F. (Ed.), 2nd International Symposium on Information Theory. Akademiai Kiado, Budapest, Hungary, pp. 267–281.
- Andeweg, B., 2002. Cenozoic tectonic evolution of the Iberian Peninsula: causes and effects of changing stress fields. PhD thesis, Vrije Universiteit Amsterdam.
- Browne, D.J., Scholtz, C.H., 1995. Phylogeny of the families of the Scarabaeoidea (Coleoptera) based on characters of the hindwing articulation, hindwing base and wing venation. *Systematic Entomology* 20, 145–173.
- Browne, J., Scholtz, C.H., 1999. A phylogeny of Scarabaeoidea (Coleoptera). *Systematic Entomology* 24, 51–84.
- Cabrero-Sañudo, F.J., Zardoya, R., 2004. Phylogenetic relationships of Iberian Aphodiini (Coleoptera: Scarabaeidae) based on morphological and molecular data. *Molecular Phylogenetics and Evolution* (Print) 31, 1084–1100.
- Capinera, J.L., 2008. *Encyclopedia of Entomology*. Springer, Heidelberg, Germany.
- Carisio, L., Cervalla, P., Palestrini, C., DelPiero, M., Rolando, A., 2004. Biogeographical patterns of genetic differentiation in dung beetles of the genus *Trypocopris* (Coleoptera, Geotrupidae) inferred from mtDNA and AFLP analysis. *Journal of Heredity* 31, 1149–1162.
- Chown, S.L., Pistorius, P., Scholtz, C.H., 1998. Morphological correlates of flightlessness in southern African Scarabaeinae (Coleoptera: Scarabaeidae): testing a condition of the water conservation hypothesis. *Canadian Journal of Zoology* 76, 1123–1133.
- Coope, C.R., 2004. Several million years of stability among insect species because of, or in spite of, ice age climatic instability? *Philosophical Transactions of the Royal Society* 359, 209–214.
- Cox, C.B., Moore, P.D., 2005. Life, death, and evolution on islands. In: Cox, C.B., Moore, P.D. (Eds.), *Biogeography: An Ecological and Evolutionary Approach*. Blackwell Publishing, Oxford, UK, pp. 165–197.
- Domínguez, M.C., Roig-Juñent, S., Tassin, J.J., Ocampo, F.C., Flores, G.E., 2007. Areas of endemism of the Patagonian steppe: an approach based on insect distributional patterns using endemism analysis. *Journal of Biogeography* 33, 1527–1537.
- Drummond, A.J., Rambaut, A., 2007. BEAST: bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7, 214.
- Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. *PLoS Biology* 4, e88.
- Forgie, S.A., Kryger, U., Bloomer, P., Scholtz, C.H., 2006. Evolutionary relationships among the Scarabaeini (Coleoptera: Scarabaeidae) based on combined molecular and morphological data. *Molecular Phylogenetics and Evolution* 40, 662–678.
- Grimaud, S., Boillot, G., Collette, B.J., Mauffret, A., Miles, P.R., Roberts, D.B., 1982. Western extension of the Iberian–European plate-boundary during early Cenozoic (Pyrenean) convergence. A new model. *Marine Geology* 45, 63–77.
- Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52, 696–704.
- Handy, M.R., Schmid, S.M., Bousquet, R., Kissling, E., Bernoulli, D., 2010. Reconciling plate-tectonic reconstructions of Alpine Tethys with the geological–geophysical record of spreading and subduction in the Alps. *Earth-Science Reviews* 102, 121–158.
- Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal Linnean Society* 58, 247–276.
- Hewitt, G.M., 1999. Postglacial re-colonisation of European biota. *Biological Journal of the Linnean Society* 68, 87–112.
- Hewitt, G.M., 2000. The genetic legacy of the Quaternary ages. *Nature* 405, 907–913.
- Howden, H.F., 1982. Larval and adult characters of *Frickius* Germain, its relationship to the Geotrupinae, and a phylogeny of some major taxa in the Scarabaeoidea (Insecta: Coleoptera). *Canadian Journal of Zoology* 60, 2713–2724.
- Howden, H.F., Peck, S.B., 1987. Adult habits, larval morphology, and phylogenetic placement of *Taurocerastes patagonicus* Philippi (Scarabaeidae: Geotrupinae). *Canadian Journal of Zoology* 65, 329–332.
- Knowles, L.L., 2001. Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Molecular Ecology* 10, 691–701.
- Krell, F.T., 2007. Catalogue of fossil Scarabaeoidea (Coleoptera: Polyphaga) of the Mesozoic and Tertiary, Version 2007. Denver Museum of Nature and Science Technical Report 2007–2008, pp. 1–79.
- Krieken, J., 1981. Geotrupidae from the Nepal Himalayas. New flightless species of *Geotrupes* Latreille, 1796, with a biogeographical discussion (Insecta: Coleoptera). *Senckenbergiana Biologica* 61, 369–381.
- Lawrence, J.F., Newton, A.F., 1995. Families and subfamilies of Coleoptera (with selected genera, notes, references and data on family-group names). In: Pakaluk, J., Slipinski, S.A. (Eds.), *Biology, Phylogeny and Classification of Coleoptera: Papers Celebrating the 80th birthday of Roy A. Crowson*. Muzeum i Instytut Zoologii PAN, Warsaw, pp. 779–1006.
- Le Pichon, X., Sibuet, J.C., 1971. Western extension of boundary between European and Iberian plates during the Pyrenean orogeny*. *Earth and Planetary Science Letters* 12, 83–88.
- Löbl, I., Smetana, A., 2006. Catalogue of Palaearctic Coleoptera, vol. 3: Scarabaeoidea, Scirtoidea, Dascilloidea, Buprestoidea, Byrrhoidea. Apollo Books, Stenstrup, Denmark.
- Lobo, J.M., Verdú, J.R., Numa, C., 2006. Environmental and geographical factors affecting the Iberian distribution of flightless *Jekelius* species (Coleoptera: Geotrupidae). *Diversity & Distributions* 12, 179–188.
- López-Colón, J.I., 1996. El género *Thorectes* Mulsant, 1842 (Coleoptera, Scarabaeoidea, Geotrupidae) en la fauna europea. *Giornale italiano di Entomologia* 7, 355–388.
- López-Colón, J.I., 2000. Familia Geotrupidae. Fauna Iberica. In: Ramos, M.A.e.a., Martín-Piera, F., López-Colón, J.I. (Eds.), *Coleoptera Scarabaeoidea I*. Museo Nacional de Ciencias Naturales, CSIC, Madrid, pp. 107–183.
- Maddison, W.P., Maddison, D.R., 2001. *MacClade 4 Version 4.03PPC*.
- Monaghan, M.T., Inward, D.J.G., Hunt, T., Vogler, A.P., 2007. A molecular phylogenetic analysis of the Scarabaeinae (dung beetles). *Molecular Phylogenetics and Evolution* 45, 674–692.
- Palmer, M., Cambefort, Y., 1997. Aptérisme et diversité dans le genre *Thorectes* Mulsant, 1842 (Coleoptera: Geotrupidae): Une étude phylogénétique et biogéographique des espèces méditerranéennes. *Annales de la Société Entomologique de France* 33, 3–18.
- Palmer, M., Cambefort, Y., 2000. Evidence for reticulate palaeogeography: beetle diversity linked to connection – disjunction cycles of the Gibraltar strait. *Journal of Biogeography* 27, 403–416.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Price, D.L., 2009. Phylogeny and biogeography of the dung beetle genus *Phanaeus* (Coleoptera: Scarabaeidae). *Systematic Entomology* 34, 137–150.
- Pybus, O.G., 2000. MCCRT. Department of Zoology, Univ. of Oxford, Oxford, U.K.
- Pybus, O.G., Harvey, P.H., 2000. Testing macro-evolutionary models using incomplete molecular phylogenies. *Proceedings of Royal Society London B* 267, 2267–2272.
- Pybus, O.G., Rambaut, A., 2002. GENIE: estimating demographic history from molecular phylogenies. *Bioinformatics* 18, 1404–1405.
- Rabosky, D.L., 2006a. LASER: a maximum likelihood toolkit for inferring temporal shifts in diversification rates. *Evolutionary Bioinformatics Online* 2, 257–260.
- Rabosky, D.L., 2006b. Likelihood methods for detecting temporal shifts in diversification rates. *Evolution* 1152, 1164.
- Rambaut, A., Drummond, A.J., 2007. *Tracer Version 1.4*.
- Rambaut, A., Harvey, P.H., Nee, S., 1997. End-Epi: an application for reconstructing phylogenetic and population processes from molecular sequences. *Computer Applications in the Biosciences* 13, 303–306.
- Ree, R.H., 2005. Detecting the historical signature of key innovations using stochastic models of character evolution and cladogenesis. *Evolution* 59, 257–265.
- Regier, J.C., Shultz, J.W., Ganley, A.R.D., Hussey, A., Shi, D., Ball, B., Zwick, A., Stajich, J.E., Cummings, M.P., Martin, J.W., 2008. Resolving arthropod phylogeny: exploring phylogenetic signal within 41 kb of protein-coding nuclear gene sequence. *Systematic Biology* 57, 920.
- Ronquist, F., Huelsenbeck, J.P., 2003. Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.
- Scholtz, C.H., 2000. Evolution of flightlessness in Scarabaeoidea. *Mitteilungen Museum für Naturkunde Berlin. Deutsche Entomologische Zeitschrift* 47, 5–28.
- Scholtz, C.H., Browne, D.J., 1996. Polyphyly in the Geotrupidae (Coleoptera: Scarabaeoidea): a case for a new family. *Journal of Natural History* 30, 597–614.
- Schoolmeesters, P., 2010. World Scarabaeidae database. In: Bisby, F.A., Ruggiero, M.A., Roskov, Y.R., Cachuela-Palacio, M., Kimani, S.W., Kirk, P.M., Soulier-Perkins, A., Hertum, J.v. (Eds.), *Species 2000 & ITIS Catalogue of Life: 2006 Annual Checklist*. Species 2000, Reading, UK.
- Smith, A.B.T., Hawks, D.C., Heraty, J.M., 2006. An overview of the classification and evolution of the major scarab beetle clades (Coleoptera: Scarabaeoidea) based on preliminary molecular analyses. *The Coleopterists Bulletin* 60, 35–46.

- Sole, C.L., Scholtz, C.H., 2010. Did dung beetles arise in Africa? A phylogenetic hypothesis based on five gene regions. *Molecular Phylogenetics and Evolution*. doi:10.1016/j.ympev.2010.04.023.
- Storey, B.C., 1995. The role of mantle plumes in continental break-up: case histories from Gondwanaland. *Nature* 301, 308.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, J., Higgins, D.G., 1997. The Clustal X windows interface. Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.
- Trueman, J.W.H., Pfeil, B.E., Kelchner, S.A., Yeates, D.K., 2004. Did stick insects really regain their wings? *Systematic Entomology* 29, 138–139.
- Verdú, J.R., Galante, E., 2002. Climatic stress, food availability and human activity as determinants of endemism patterns in the Mediterranean region: the case of dung beetles (Coleoptera, Scarabaeoidea) in the Iberian Peninsula. *Diversity and Distributions* 8, 259–274.
- Verdú, J.R., Galante, E., 2004. Behavioural and morphological adaptations for a low-quality resource in semi-arid environments: dung beetles Coleoptera, Scarabaeoidea associated with the European rabbit *Oryctolagus cuniculus* L. *Journal of Natural History* 38, 705–715.
- Verdú, J.R., Galante, E., Lumaret, J.P., Sañudo, F.J., 2004. Phylogenetic analysis of Geotrupidae (Coleoptera, Scarabaeoidea) based on larvae. *Systematic Entomology* 29, 509–523.
- Verdú, J.R., Casas, J.L., Lobo, J.M., Numa, C., 2010. Dung beetles eat acorns to increase their ovarian development and thermal tolerance. *PLoS ONE* 5, e10114.
- Villalba, S., Lobo, J.M., Martín-Piera, F., Zardoya, R., 2002. Phylogenetic relationships of Iberian dung beetles (Coleoptera: Scarabaeinae): insights on the evolution of nesting behavior. *Journal of molecular evolution* 55, 116–126.
- Whiting, M.F., Bradler, S., Maxwell, T., 2003. Loss and recovery of wings in stick insects. *Nature* 421, 264–267.
- Yule, G.U., 1924. A mathematical theory of evolution, based on the conclusions of Dr. J.C. Willis. *Philosophical Transactions of the Royal Society London Series B* 213, 21–87.
- Zunino, M., 1973. Il genere *Ceratophyus* Fish. (Coleoptera, Scarabaeoidea). *Bolletino del Museo di zoologia dell'Università di Torino* 2, 9–40.
- Zunino, M., 1984. Sistematica generica dei Geotrupinae (Coleoptera, Scarabaeoidea: Geotrupidae), filogenesi della sottofamiglia e considerazioni biogeografiche. *Bolletino del Museo Regionale di Scienze Naturali, Torino* 2, 9–162.