



Diversification of subgenus *Calathus* (Coleoptera: Carabidae) in the Mediterranean region – glacial refugia and taxon pulses

Carlos Ruiz^{1*}, Bjarte H. Jordal² and José Serrano¹

¹Departamento de Zoología y Antropología Física, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain, ²Museum of Natural History, University of Bergen, NO-5007 Bergen, Norway

ABSTRACT

Aim To investigate the effects of Pleistocene climatic variations on the diversification rate of the subgenus *Calathus* (Coleoptera: Carabidae), and to estimate the role of vicariance and dispersal for explaining current distributional patterns.

Location Western Palaearctic Region, particularly the Mediterranean Basin.

Methods Fragments of the mitochondrial *cox1-cox2* and the nuclear *28S* and *EF1 α* genes were analysed by Bayesian inference. Lineage divergence times were estimated using a Bayesian relaxed molecular clock. Three diversification rate analyses were conducted, namely gamma (γ)-statistic, birth–death likelihood (BDL) test and survival analyses, in order to test departures from a constant rate model of diversification. A Bayesian approach to dispersal–vicariance analysis was developed to reconstruct the most probable ancestral area of subgenus *Calathus* and subsequent events of dispersal and colonization.

Results A constant rate of speciation events from the late Miocene onwards was found for the subgenus *Calathus*, whereas recent Pleistocene climatic oscillations played an important role only in shaping intraspecific diversity. Overall diversification patterns for the subgenus are best explained by at least four westward dispersal events from the eastern Mediterranean Basin. Three distinct phylogroups were found for the widely distributed *Calathus fuscipes*. Incongruence between mitochondrial and nuclear loci was found for a number of species.

Main conclusions Diversification analyses suggest either a constant rate of diversification (BDL analysis) or a decrease in diversification rates for the subgenus (survival or γ -statistics analyses), but not an increase related to the effects of glaciation cycles. Diversification patterns in the subgenus *Calathus* agree with predictions of the taxon pulse model. From the middle Miocene onwards the Anatolian Peninsula was possibly the main centre of diversification, with successive dispersal events towards the western Mediterranean Basin. Range expansion and secondary contact zones are postulated between members of different phylogroups in *C. fuscipes*.

Keywords

BDL test, dispersal–vicariance analysis, divergence time, diversification analyses, Pleistocene climatic oscillations, subgenus *Calathus*, taxon pulse model, Western Palaearctic Region.

*Correspondence: Carlos Ruiz, Departamento de Zoología y Antropología Física, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, 30071 Murcia, Spain.
E-mail: caruiz@um.es

INTRODUCTION

The Mediterranean Basin is one of the most diverse biological hotspots in the world (Myers *et al.*, 2000). A rich biota has

developed there from the closing of the Tethys Sea in the Oligocene due to complex geological and climatic changes (Krijgsman, 2002; Mannion, 2008). Collisions and splits of tectonic plates have shaped the patterns of dispersal and

vicariance among populations, creating major barriers as well as new corridors in different geological periods. Thus, the Mediterranean biota is a complex mixture of species of different ages and diverse biogeographical origins, with some elements evolving *in situ* and others arriving from other regions (Weiss & Ferrand, 2006; Blondel *et al.*, 2010).

The Pleistocene glaciations are recent events that have deeply affected patterns of biodiversity in the Mediterranean region (Avice, 2000; Hewitt, 2000). Extreme climatic changes strongly affected the Mediterranean massifs (Hughes *et al.*, 2006), causing recurrent extinction and shifts in the elevational and latitudinal ranges of populations. The Mediterranean peninsulas are thought to have acted as major glacial refugia for the European flora and fauna during these climatic oscillations (Stewart *et al.*, 2010). However, the effect of these climatic changes in shaping the extant Mediterranean biota is a topic of much debate. According to the classic Pleistocene refugium hypothesis (Haffer, 1969), recurrent glacial cycles over the last 2.6 million years (Myr) have promoted speciation by isolating populations for extended periods in glacial refugia. Several molecular studies have supported this hypothesis in different taxa (Knowles, 2001; Lister, 2004; Ribera & Vogler, 2004).

A second hypothesis suggests a slowdown in diversification rates during the Pleistocene as a result of a decrease in speciation rates or an increase in extinction rates. According to some authors (Knowles, 2000; Coope, 2004), there was a decrease in speciation rates because glacial periods were not long enough to develop reproductive isolation, while extinction rates increased due to climate changes during Pleistocene glaciations (Zink & Slowinski, 1995). A slowdown in diversification rates has been postulated based on Pleistocene beetle fossils from northern areas of Europe and North America, which show a remarkable degree of morphological stasis, indicating stability with respect to ecological requirements during Ice Age climatic oscillations (Coope, 2004; Elias *et al.*, 2006). This hypothesis has been supported by molecular studies (Zink & Slowinski, 1995; Zink *et al.*, 2004; Burbrink & Pyron, 2010), with most speciation events pre-dating Pleistocene glacial cycles (Moritz *et al.*, 2000; Joger *et al.*, 2007).

A third hypothesis states that diversification has occurred more or less continuously with no significant difference in diversification rates between the Tertiary and Quaternary (Barnosky, 2005; Lovette, 2005; Zink & Klicka, 2006; Rull, 2008). This hypothesis is supported by recent studies showing that the tempo and mode of speciation have, rather, been influenced by: (1) biogeographical factors such as latitude (Weir & Schluter, 2007), elevation (Weir, 2006) and topography (Hewitt, 1999); (2) biological factors such as vagility (Jansson & Dynesius, 2002; Kodandaramaiah, 2009), geographical range (Jablonski & Roy, 2003), ecological tolerance (Knowles, 2000; Jansson & Dynesius, 2002) and generation time (Thomas *et al.*, 2010), and (3) the historical contingency of random processes (Blount *et al.*, 2008). Therefore, the evolutionary history of European biota cannot be described by a single pattern (Taberlet *et al.*, 1998; Stewart, 2009).

Here we investigate the tempo and mode of diversification of the subgenus *Calathus* Bonelli, 1810, one of the two principal subgenera of the homonymous genus (Coleoptera: Carabidae). This taxon is particularly diverse in the mountains of the Mediterranean peninsulas, and includes about 50 currently recognized species (Ruiz *et al.*, 2010). Most of these show restricted distributional ranges, with 12 of these species endemic to the Iberian Peninsula, 5 to Italy, 8 to the Balkan Mountains and 12 to Anatolia. These endemic taxa are flightless, show a preference for montane and forested habitats, and share similar latitudinal and elevational ranges. *Calathus fuscipes* constitutes an exception because it has a wide distribution across Europe and North Africa and is an eurytopic and wing-dimorphic taxon which has colonized a wide variety of habitats. Despite its dispersal capacity, *C. fuscipes* includes six subspecies, which indicates significant regional differentiation (Hovorka & Sciaky, 2003).

Based on the distribution of the subgenus *Calathus* in the forested Mediterranean massifs, we hypothesize that glaciation events caused shifts in the elevational and latitudinal ranges of most species, thus accelerating speciation rates during the Pleistocene. Their similar biotic and abiotic requirements (vagility, ecological preferences, generation time, distribution, latitudinal and elevational range) lead us to hypothesize that taxa surviving in Mediterranean ice-free refugia were affected by the same set of environmental factors, and probably responded to these factors in a similar way. This similarity reduces potential evolutionary 'noise' when attempting to determine changes in the diversification rate of a particular group.

We also aim to test various hypotheses concerning the location and importance of geographical barriers, and to estimate the relative importance of vicariance and dispersal for explaining current distributional patterns of *Calathus*. The vicariance models tested here include: (1) a single vicariance model, in which a widely distributed common ancestor became isolated and diverged into separate species in a given peninsula (Fig. 1b), and (2) a multiple vicariance model, in which several ancestral taxa diversified in each peninsula (Fig. 1c). The dispersal models include: (3) an eastwards dispersal model, originating from the Iberian Peninsula (Fig. 1d), and (4) a westwards dispersal model originating from the Anatolian Peninsula (Fig. 1e). To test these hypotheses, we used mitochondrial and nuclear DNA in a comprehensive sampling of this subgenus.

MATERIALS AND METHODS

Taxa studied

Sampling covered most areas of the Mediterranean Basin together with adjacent areas (northern Europe and western Asia; see Table S1 in Appendix S1 in Supporting Information). Molecular data were obtained from 122 individuals in 36 species (out of the 50 described species) of the subgenus

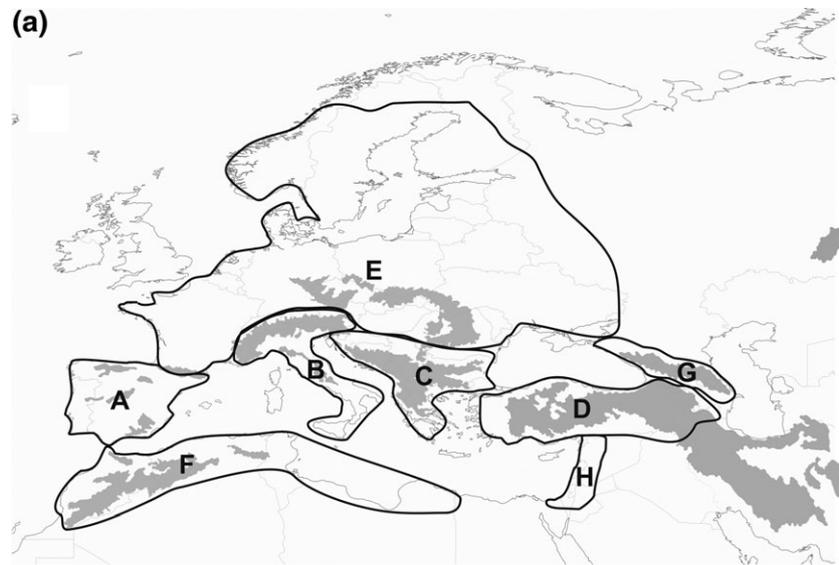
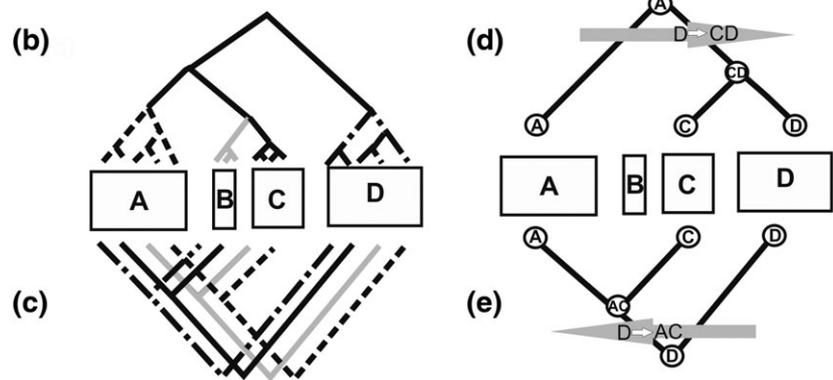


Figure 1 (a) Main areas of distribution of subgenus *Calathus*: A, Iberian Peninsula; B, Italian Peninsula; C, Balkan Peninsula; D, Anatolian Peninsula; E, North and Central Europe; F, North Africa; G, Caucasus; H, Levant region. (b–c) Vicariance models: (b) single vicariance event and posterior diversification in each peninsula; (c) multiple and independent vicariance events in each peninsula. (d–e) Dispersal models: (d) an eastwards dispersal from the Iberian Peninsula; (e) a westwards dispersal from the Anatolian Peninsula.



Calathus. Samples of the *Calathus fuscipes* group are shown in Fig. S1 (Appendix S2). Taxa related to the subgenus *Calathus*, according to previous molecular studies (clade 2 in Ruiz *et al.*, 2010), are included in this study, namely *C. metallicus*, the *C. granatensis* group and *Synuchidius*. Other more distantly related lineages (*Amphyginus*, *Iberocalathus*, *Lindrothius* and *Neocalathus*) are included as outgroups.

Molecular markers

DNA was extracted with the Qiagen DNeasy tissue kit (Qiagen, Hilden, Germany). A fragment of the mitochondrial cytochrome *c* oxidase subunits I and II genes (*cox1–cox2*; 1623 bp), which includes part of the *cox1* gene (860 bp), and the complete tRNA-Leu (62 bp) and *cox2* genes (701 bp), was sequenced. We also amplified two nuclear genes: a partial sequence of the 28S ribosomal RNA (D2–D4 region: about 858 bp) and the protein-coding elongation factor 1 α gene (*EF1 α* C0 copy; Jordal, 2002: 771; bp, no introns). Primers, polymerase chain reaction (PCR) conditions and sequencing procedures are described in Ruiz *et al.* (2009). Sequences are deposited in GenBank under the accession numbers provided in Table S1.

Sequence alignment

Sequences of *cox1–cox2* and *EF1 α* were unambiguously aligned; only tRNA-Leu showed length variations, ranging from 59 to 62 bp. 28S rDNA sequences had little length variation (from 841 to 844 bp). Two different alignment strategies were carried out, the first with MAFFT 6 software (<http://mafft.cbrc.jp/alignment/server/>) using the parameter G-INS-i and 200PAM/ $\kappa = 2$ (Katoh & Toh, 2008), and the second with CLUSTAL W 1.6 (Thompson *et al.*, 1994) in MEGA 4 (Tamura *et al.*, 2007) with default GOP/GEP parameters.

Phylogenetic analysis

Each data partition was analysed separately and in combination under maximum parsimony (MP) as implemented in TNT 1.1 (Goloboff *et al.*, 2008) and Bayesian analysis (BA) with MRBAYES 3.1 (Ronquist & Huelsenbeck, 2003). Details of parsimony and Bayesian analyses are given in Appendix S3. Genealogical relationships among populations of the *Calathus fuscipes* complex were also evaluated by the median-joining network approach (Bandelt *et al.*, 1999), as implemented in NETWORK 4.5.1 (<http://www.fluxus-engineering.com>), using

default settings. Mitochondrial DNA (mtDNA) haplotypes were collapsed using star contraction (Forster *et al.*, 2001) to summarize and contract any star-like phylogenetic cluster into one ancestral type.

Evolutionary rates and age estimation

We used mtDNA data to calibrate evolutionary rates following two independent strategies. Analysis A involved a multiple calibration using available external outgroups with suitable calibration points. For this purpose, 246 individuals belonging to the tribe Sphodrini were included with a major representation from the genus *Calathus* (Ruiz *et al.*, 2009, 2010). The fossil of *Calathus elpis* from the Eocene (Ortuño & Arillo, 2009) was used in two independent calibration analyses: (1) setting a minimum age of 34 Ma for the genus *Calathus* clade, and (2) incorporating the uncertainty in dating this fossil as a normal distribution ranging from 26 to 42 Ma (mean: 34 Ma; SD: 5.0; analysis A2). The origin of different Macaronesian islands was used as ‘maximum age’ for eight independent Macaronesian clades according to the geological data indicated by Guillou *et al.* (2004) (nodes b ≤ 10 Ma; c ≤ 1 Ma; d ≤ 11.6 Ma; e ≤ 11.6 Ma; f ≤ 15 Ma; g ≤ 11.6 Ma; h ≤ 1 Ma; i ≤ 14 Ma) using a uniform distribution as a prior. Five out of nine calibration points were placed in closely related species of the subgenus *Calathus* within the genus *Calathus*.

Congruence of these results was compared in a second calibration using various substitution rates (analysis B); one was the standard 2.3% pairwise divergence Myr^{-1} (Analysis B1), which is frequently used for arthropod mitochondrial genes (Brower, 1994). This calibration was set assuming uncertainty with a normal distribution of 1.15% and a standard deviation of 0.001 (95% range: 0.65–1.64%; pairwise: 1.3–3.28%). Other calibration rates correspond to recent studies in Coleoptera (*cox1*: 1.5–1.7% in Papadopoulou *et al.*, 2010; Ribera *et al.*, 2010; and 8.6% and 2.6% for *cox1* and *cox2*, respectively, in Pons *et al.*, 2010). Rate variation was codified (Analysis B2) with a uniform distribution (*cox1*: 1.5–8.6%; *cox2*: 2.6–3.14%).

A Bayesian relaxed molecular clock with Markov chain Monte Carlo (MCMC) was used (Drummond *et al.*, 2006), as implemented in BEAST 1.5.4 (Drummond & Rambaut, 2007) following standard procedures (see Ruiz *et al.*, 2009 and Appendix S3).

Timing and rates of diversification

In order to explore the effects of past climate changes on the diversification rates of *Calathus* species, diversification rate analyses were conducted on a species-level tree created by pruning all individuals but one per species from the ultrametric tree obtained by BEAST analysis. We plotted the log-transformed number of lineages accumulating through time (LTT; Nee *et al.*, 1994a) to visualize the changes in diversification rates. Three statistical analyses were carried out to test for significant departures from a constant speciation rate

model (CR): the gamma (γ)-statistic (Pybus & Harvey, 2000), the birth–death likelihood (BDL) test (Rabosky, 2006), and survival analyses (Paradis, 1997). The BDL test was carried out using Akaike’s information criterion (AIC). The difference (ΔAIC) between the AIC of rate-constancy models (AIC_{RC}) and of rate-variable models (AIC_{RV}) indicates which model fits the data better. Details of these analyses are given in Appendix S3. In order to detect any spurious decline in diversification rates over time (Pybus & Harvey, 2000), incomplete taxon sampling was taken into account by generating sets of 5000 trees under the null hypothesis of constant rate pure birth, and thereafter randomly pruning taxa from these trees by assuming that our sample ($n = 36$) represents a fraction f (from 0.1 to 0.5) of the true number of lineages.

Corrected pairwise sequence divergences were calculated using MEGA 4.0 for eight sister-species pairs selected from the phylogenetic analysis, as well as for the main clades 1–11, A–F. To test the effect of glacial cycles on speciation processes, we followed the criterion that the Pliocene–Pleistocene boundary occurred 2.6 Ma, according to Gibbard *et al.* (2010). The Pliocene–Miocene transition was set at 5.2 Ma (Berggren *et al.*, 1995).

Biogeographical inference

A Bayesian approach to dispersal–vicariance analysis (Ronquist, 1997) was implemented in statistical dispersal–vicariance analysis (S-DIVA 1.9; Yu *et al.*, 2010) to reconstruct the most probable ancestral area and the sequence of dispersal events. As there is no constraint to follow a hierarchical pattern of area relationships, the method is suitable for reconstructing reticulate biogeographical scenarios (Sanmartín, 2007). Ancestral reconstructions were calculated over a Bayesian sample of 5000 trees generated in BEAST from the combined analysis. Eight areas were considered for the analysis (Fig. 1). Given that most species of the subgenus *Calathus* are endemic to particular Mediterranean peninsulas, the parameter ‘maximum areas’ at ancestral nodes was constrained to three.

RESULTS

Phylogenetic analysis

The Bayesian analysis of the mtDNA data resulted in a well-supported lineage A that included the subgenus *Calathus*, as defined in Lorenz (2005), plus taxa assigned to other subgenera within the genus *Calathus* or to related genera [posterior probability (PP): 1.0; Fig. S2]. The deepest split in clade A corresponded to the Balkans *Synuchidius ganglbaueri* (clade 1) and a well-supported clade B that included the Anatolian *Calathus (Neocalathus) deplanatus* (clade 2), the Ibero-Maghrebian *C. granatensis* and *C. opacus* (clade 3), the Balkans *C. (Neocalathus) metallicus* (clade 4) and clade D. This last clade included all taxa currently contained within the

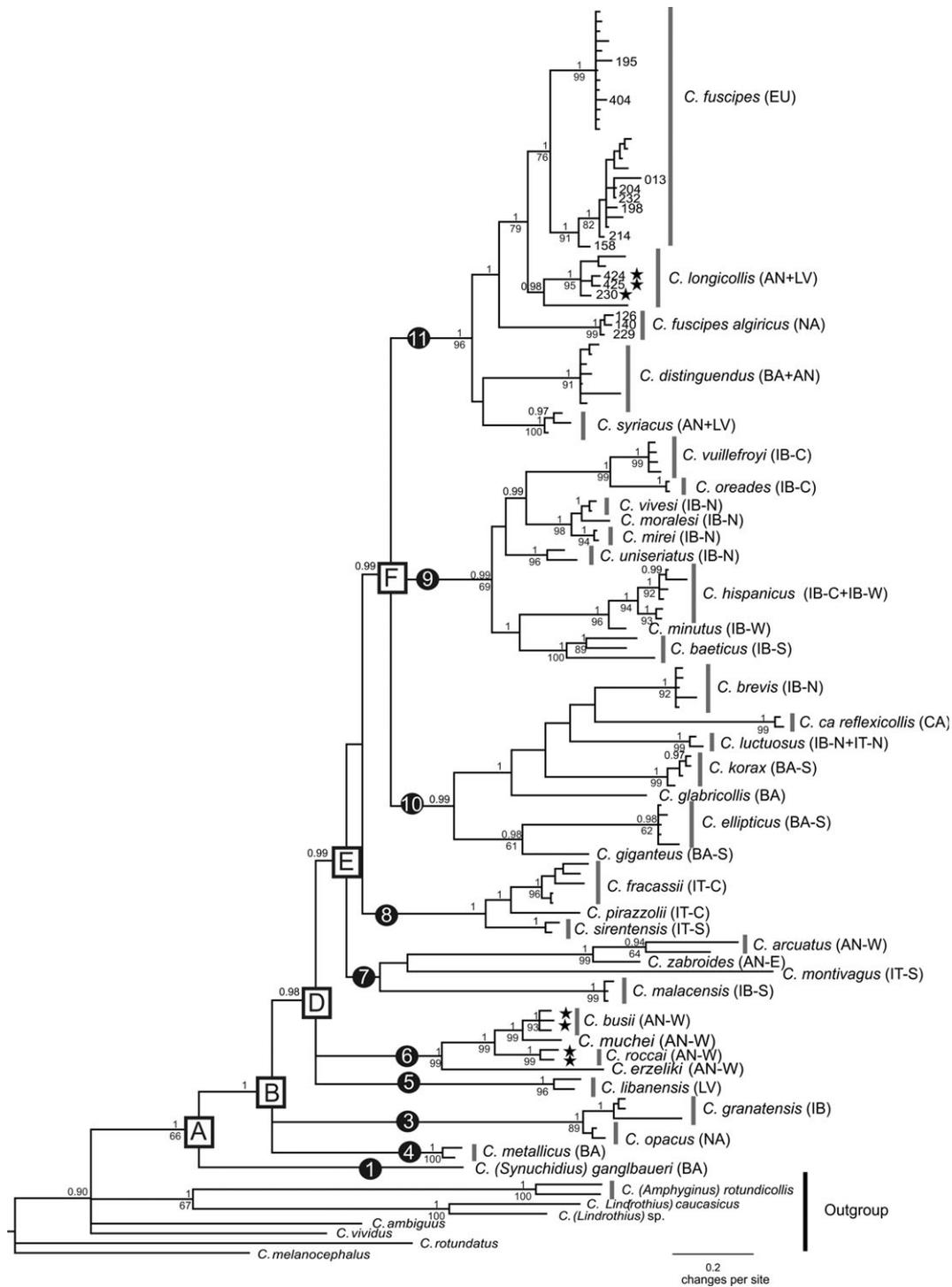


Figure 2 The 50% majority consensus tree of the subgenus *Calathus* resulting from the Bayesian analysis of the combined data (*cox1–cox2*, *EF1 α* and 28S aligned with MAFFT). Numbers above nodes show posterior probability ≥ 0.90 . Bootstrap support values are shown below nodes. Circled numbers and squared letters refer to particular clades which are discussed in the text. Stars refer to incongruence between nuclear and mitochondrial markers. Numbers in terminal clades refer to individuals cited in the text. NA, North Africa; IB, Iberian Peninsula; IT, Italian Peninsula; BA, Balkan Peninsula; AN, Anatolian Peninsula; CA, Caucasus, LV, Levant region; EU, Europe; -S, southern distribution; -C, central distribution; -N, northern distribution; -W, western distribution; -E, eastern distribution.

subgenus *Calathus*. In clade D, clade 7 is particularly interesting because it includes four species with a disjunct distribution: the south Iberian *C. malacensis*, central and south

Italian *C. montivagus*, and the north Anatolian *C. arcuatus* and *C. zabroides*. This distribution in different peninsulas was also found for clade 10. On the other hand clades 6, 8 and 9 were

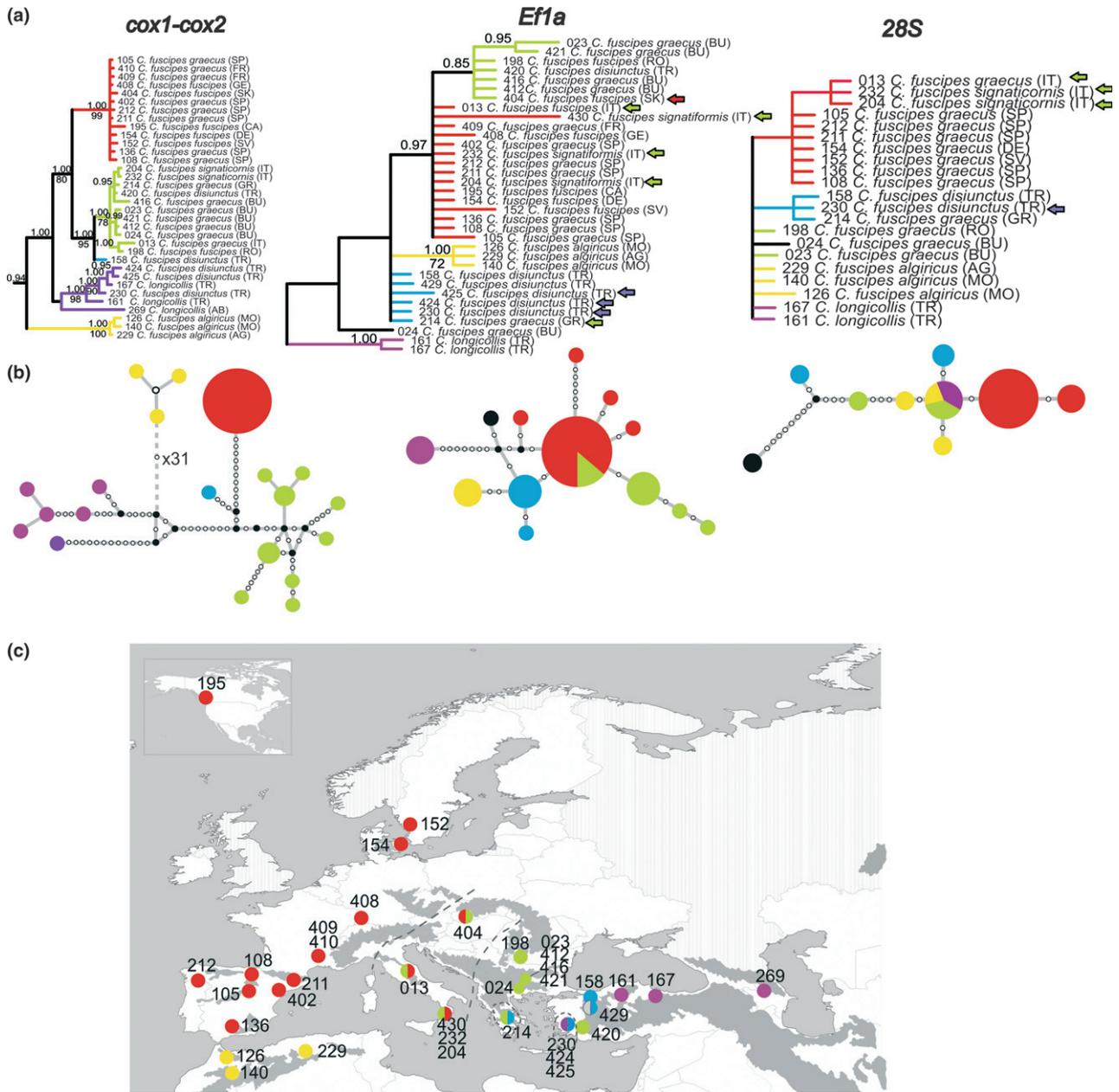


Figure 3 Reconstructed evolutionary relationships and geographical distribution of the morphologically related *Calathus fuscipes* and *C. longicollis*. (a) *cox1-cox2*, *EF1α* and *28S* Bayesian analyses. Arrows in colour denote incongruence between markers. (b) Median-joining network for each marker; circle size is proportional to haplotype frequency. (c) Geographical distribution. Pie charts indicate mitochondrial haplotype on the left side and nuclear haplotype on the right side.

each restricted to a single Mediterranean peninsula. Clade 11 was made up of the *fuscipes* group, as defined by Battoni & Vereschagina (1984), and includes *Calathus syriacus*, *C. distinguendus*, and the two morphologically similar species, *C. longicollis* and *C. fuscipes*.

Trees resulting from the analysis of the *EF1α* data generally agreed with the mtDNA topology, but the resolution and node support was low for the basal clades, with only mitochondrial clades 3, 4 and 8 fully recovered (Fig. S3). Clades 6, 9 and 11 in the mitochondrial topology were only partially recovered by the *EF1α* data. No differences in topology or support levels

were found when the protein-coding genes *cox1-cox2* and *EF1α* were partitioned by codon position.

Alignments of the *28S* data made by Clustal (CW) and MAFFT showed similar results although the posterior probability was higher when analysing with MAFFT. Trees resulting from the Bayesian analysis showed almost no resolution for deep nodes, with only clades 3 and 6 fully resolved and supported (Fig. S4), and clades 9 and 10 partially resolved.

Combined analysis of the protein-coding gene sequences (mtDNA + *EF1α*) revealed no substantial differences in topol-

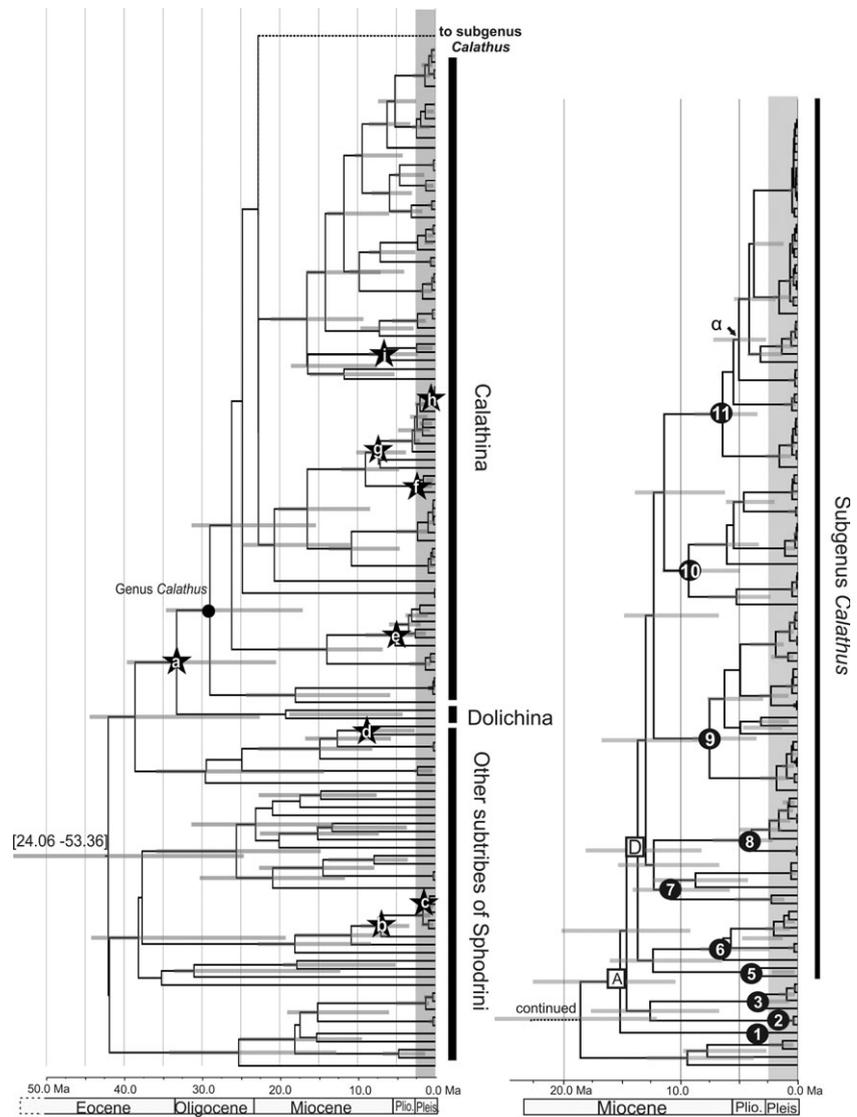


Figure 4 Divergence times for the subgenus *Calathus* and outgroups estimated from *cox1-cox2* sequences in BEAST. Nine points of calibration (a–i) were used to assess maximum or minimum ages (analysis A2, see text for details). Grey bars at each node show 95% highest posterior density intervals for the main nodes. Circled numbers and bars are as in Fig. 3. Stars represent calibration points (nodes a \geq 34 Ma, b \leq 10 Ma; c \leq 1 Ma; d \leq 11.6 Ma; e \leq 11.6 Ma; f \leq 15 Ma; g \leq 11.6 Ma; h \leq 1 Ma; i \leq 14 Ma; Guillou *et al.*, 2004). The shaded area represents Pleistocene glacial cycles.

ogy or support compared with the mtDNA analysis. The same applies to the analysis in which data were partitioned by codon. Combined analysis of all nuclear DNA (*EF1 α* + 28S) also resulted in an overall similar topology to the mtDNA topology, although some basal nodes were only weakly supported. Combined analysis of the three markers (Fig. 2) showed the same main clades found in the separate analysis of the mtDNA data. The positive value of Bayes factor (BF = 553.419) indicates that partitioning by gene and codon (1 + 2, 3) was favoured over partitioning by gene.

Phylogeographical analysis of *Calathus fuscipes*

A median-joining network based on the mitochondrial data (Fig. 3) was obtained for the *C. fuscipes* complex. The genealogical structure was highly congruent with the results from the Bayesian analyses of these data. Five main clades were separated by several mutational steps: a western European clade, a Balkans clade, a mainly Anatolian clade, a North

African clade and the *longicollis* clade divided into one Caucasian and one Anatolian subclade. The *C. fuscipes* sample from western Canada (no. 195) grouped with western European samples, suggesting a recent introduction.

Incongruence between mitochondrial and nuclear markers

Mitochondrial results were not always congruent with those derived from nuclear data. (1) All Italian individuals of *C. fuscipes* (samples 013, 204, 232, 430) were found nested within the western European clade based on mtDNA data, but were placed closer to the Balkans population based on the nuclear data. The inverse pattern was observed for the Slovakian sample (404) (Fig. 3). (2) Three Anatolian individuals of *C. fuscipes disiunctus* (samples 230, 424 and 425) were related to *C. longicollis* according to the mtDNA data (1.00/98) but were related to other *C. fuscipes* samples according to *EF1 α* and 28S data, although with much lower support (Fig. 3). (3)

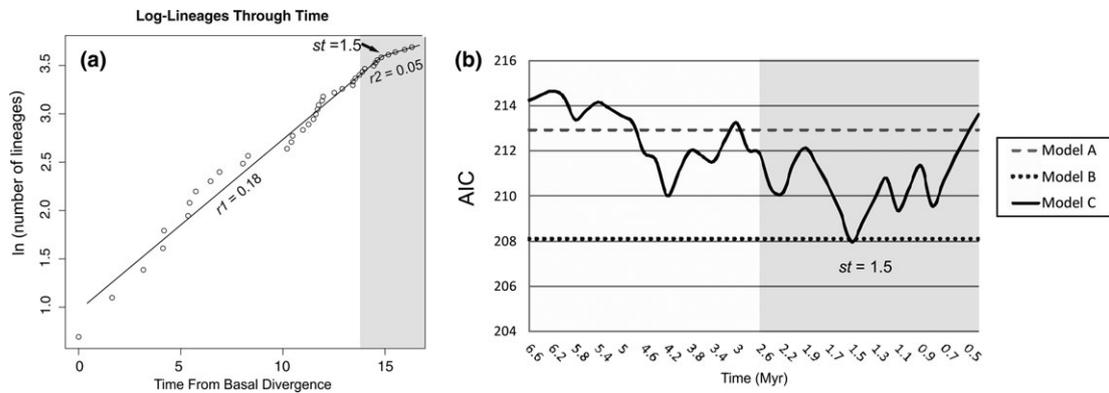


Figure 5 (a) Lineage-through-time plots derived from Bayesian relaxed clock estimates of divergence dates for the subgenus *Calathus*. r_1 and r_2 are diversification rates per million years and st is the time of rate shift (Myr). The shaded area represents Pleistocene glacial cycles. (b) Survival analysis: Akaike information criterion (AIC) values for diversification models A, B and C. Model A implies a constant rate of diversification, model B a gradually changing rate, and model C a pattern with an abrupt change of diversification. The lowest AIC value identifies the best model.

Table 1 Testing models of diversification in the subgenus *Calathus* using birth–death likelihood.

Model	Pure Birth	Birth–Death	DDL	DDX	yule2rate	SPVAR	EXVAR	BOTHVAR
parameters	$r_1 = 0.1537$	$r_1 = 0.1537$ $a = 0$	$r_1 = 0.2686$ $k = 55.0561$	$r_1 = 0.4677$ $x = 0.3849$	$r_1 = 0.1842$ $r_2 = 0.0535$	$\lambda = 0.302$ $k = 0.060$	$\lambda = 0.154$ $z = 1.002$	$\lambda = 0.300$ $k = 0.060$ $z = 0.001$
					$st = 1.492$	$\mu = 0.001$	$\mu = 0.001$	$\mu = 0.001$
Ln(L)	−3.3009	−3.3009	−1.2494	−1.7807	−0.3247	−2.23006	−3.318	−2.226563
AIC	8.6018	10.6018	6.6494	7.5614	6.4988	10.4601	12.63615	12.4513

DDL and DDX, logistic and exponential density-dependent speciation models, respectively; yule2rate, multi-rate variant of the Yule model; SPVAR, time-varying speciation only, with a constant extinction rate; EXVAR, time-varying extinction only, with a constant speciation rate; BOTHVAR, both speciation and extinction vary over time; r_1 – r_2 , net diversification rate (speciation event per million years); a , extinction fraction; st , time of rate shift (Myr); k , parameter in the logistic density dependent model; x , parameter in the density-dependent exponential model; λ , initial speciation rate; k , parameter of the exponential change in speciation rate; z , parameter of the exponential change in extinction rate; μ , final extinction rate; Ln(L), log-likelihood; AIC, Akaike information criterion; Δ AIC, difference in AIC scores between each model and the overall best-fit model.

North African *C. fuscipes algiricus* (samples 126, 140, 229) did not appear as monophyletic with the remaining *C. fuscipes* samples based on the mtDNA data, but was monophyletic according to the *EF1 α* data (without high support); its relationships were unresolved by 28S data (Fig. 3). (4) The Greek *C. fuscipes* sample 214 showed mtDNA typical for the Balkans population, but was related to the Anatolian clade according to nuclear DNA (Fig. 3). (5) The Iberian *C. oreades* and *C. mirei* appeared as sister species according to the nuclear data (PP: 0.99–1.00) but were separate and included in distinct clades by the mtDNA data (PP: 1.00) (Fig. S5). (6) Likewise, *C. hispanicus* and *C. baeticus* appeared as sister taxa in the 28S topology (PP: 0.94), but as separate clades in the *cox1*–*cox2* analysis (Fig. S5).

Tempo and mode of diversification of the subgenus *Calathus*

The results based on multiple calibration points (analyses A1 and A2) yielded broad ranges for the most basal nodes (Fig. 4 and Table S2 in Appendix S1). Both analyses resulted in

similar estimates for pairwise substitution rates, 1.52% using uniform distribution of the root age (analysis A1) and 1.92% when root age had a normal distribution (analysis A2). Applying the fixed 2.3% rate younger estimates of age were obtained but these estimates overlapped with those resulting from analysis A1 and A2. Analysis using recently reported rates (B2) resulted in even younger estimations (Fig. S6, Table S2).

Calibration analyses A1 and A2 placed the origin of the most common recent ancestor of subgenus *Calathus* between 16 and 20 Ma [confidence interval (CI) = 9.89–26.70 Ma] (Fig. 4, Table S2) and the origin of main lineages of subgenus *Calathus* (Fig. 4: clades 6–11) in the late Miocene 5–12 Ma (CI 2.10–16.05 Ma). Most pairs of sister species probably originated during the last 5 Myr, apparently at a near constant rate (Fig. S6), whereas divergence occurring during the Pleistocene mainly affected intraspecific lineages. Calibration analyses using higher substitution rates (Table S2) placed the origin of the main lineages from 3 to 7 Ma (B1), or during the early Pleistocene (B2).

Lineage-through-time (LTT) plots (Fig. 5a) showed that the diversification rates have slowed down for the last 1.5 Myr.

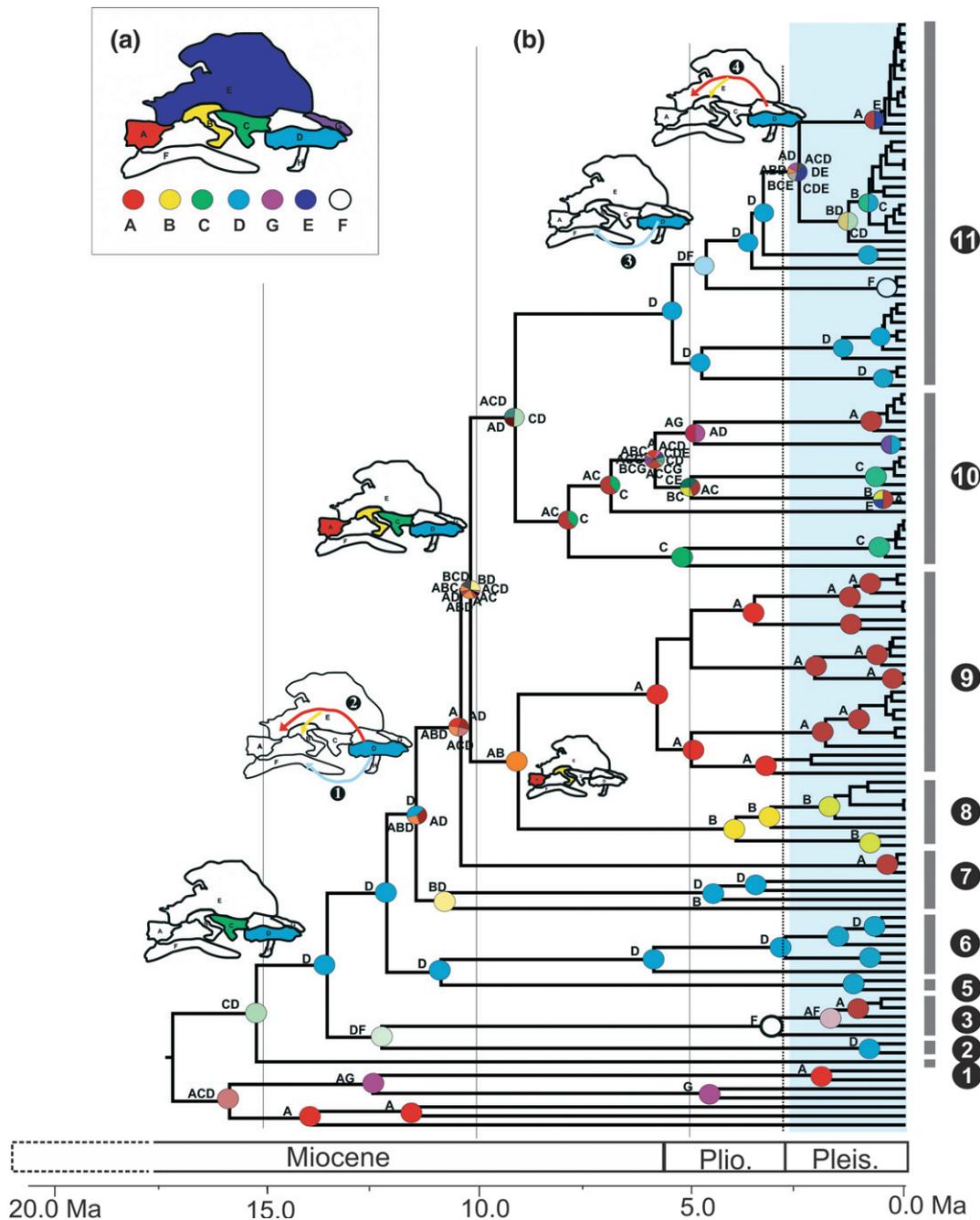


Figure 6 (a) Analysed biogeographical areas (A to H). Single areas are represented by solid colours. (b) Combined chronogram (analysis A2) and biogeographical analysis of subgenus *Calathus*. Pie charts represent the relative probabilities of alternative ancestral distributions obtained by integrating dispersal–vicariance analysis (S-DIVA) optimizations over the 5000 Bayesian trees from the combined analysis.

Negative values of the γ -statistic ($\gamma = -1.913787$) were significant ($P = 0.0278$) and indicated a decrease in net diversification rates rather than a constant rate model. The γ -statistic was sensitive to incomplete sampling and failed to reject ($P > 0.05$) a constant rate model when the number of missing *Calathus* taxa (f) exceeded 30% of the true clade diversity.

BDL analyses resulted in positive values of ΔAIC (2.10304) for the subgenus *Calathus*, thus showing that rate-variable models fit the data better than the rate-constant model. The

best fitting model was a two-parameter rate-variable model that estimated a diversification rate of $0.18 \text{ species Myr}^{-1}$ from 23 to 1.5 Ma, and a threefold slowdown in the speciation rate (0.05) during the last 1.5 Myr (Fig. 5a, Table 1). However, the observed $\Delta\text{AIC}_{\text{RC}}$ statistic indicated no significant departure from the null hypothesis of rate constancy ($P = 0.12$), therefore the rate-variable model was not fully supported. Simulations based on incomplete taxon sampling ($f = 0.1\text{--}0.5$) also failed to reject the null hypothesis ($P = 0.07\text{--}0.19$). A

constant extinction rate model (SPVAR) provided a better fit to the data compared to the variable extinction rates models (EXVAR and BOTHVAR, Table 1).

Survival analysis indicated that model B (gradual change in diversification) was always better supported than model A (constant diversification) by both AIC (Fig. 5b) and likelihood ratio test (LRT; $\chi^2 = 6.813$, $P = 0.009$). The β -value of model B was higher than 1 ($\beta = 1.436$), suggesting a decreasing diversification rate. The AIC score for model C depends on the hypothesized breakpoint in diversification rate. When the value of the break-point obtained for the BDL analysis (1.5 Ma) was used, the likelihood improved and model C was the best model both according to AIC score and LRT ($\chi^2 = 6.97$, d.f. = 1, $P = 0.0083$). This result indicates an abrupt decrease ($\delta_1: 0.055 < \delta_2: 0.206$) in the diversification rate at 1.5 Ma.

Biogeographical analysis

S-DIVA analyses indicated an eastern Mediterranean distribution for the ancestor of the subgenus *Calathus* (Fig. 6b). Four successive westward dispersal movements through both sides of the Mediterranean Basin were inferred during the Miocene and later (Fig. 6b).

DISCUSSION

Diversification of the subgenus *Calathus*

Most taxa of the subgenus *Calathus* are found south of a line connecting Bordeaux, Lyon, the Southern Alps, the Carpathians and the Black Sea, the so-called Holdhaus line in Europe (Holdhaus, 1954). Moreover, most species of this subgenus are restricted to forested areas of the mountain ranges around the Mediterranean peninsulas. Both facts suggest that the distribution and speciation patterns of the subgenus *Calathus* were notably influenced by the climatic oscillations during the Pleistocene, as found for many other taxa (Hewitt, 1999; Schmitt, 2009). However, this hypothesis does not fit well with the divergence times reconstructed for this group, which are based on a substitution rate from 1.52 to 1.92% or with the diversification analyses (Fig. 5, Table 1 and Table S2). On the contrary, data support a relatively constant rate of speciation possibly beginning in the late Miocene (Figs 4 & 5a and Fig. S6). Most of the species splits occurred during the Pliocene (5–2.6 Ma) according to analysis A (Fig. S6), while the Pleistocene seems to have been a period mainly characterized by intraspecific divergence, a pattern also seen for other animal groups (Avice *et al.*, 1998; Joger *et al.*, 2007).

The scarcity of *Calathus* fossils does not allow for a calibration of divergence dates as robust as one may wish. Therefore, our analyses resulted in relatively broad ranges of estimated divergence times, in particular for the most basal nodes. The estimated rates in analysis A (1.52% to 1.92%) are congruent with others reported for various Coleopteran groups, with pairwise substitution rates ranging from 0.7% to

2% (Adephaga: Prüser & Mossakowski, 1998; Polyphaga: Gómez-Zurita *et al.*, 2000; Farrell, 2001; Sota & Hayashi, 2007; Ruiz *et al.*, 2009; Faille *et al.*, 2010). These estimates are slightly lower than the standard 2.3% rate assumed in numerous studies of insect taxa and lower than others recently reported in Coleoptera: *cox1*, 3–17.6% and *cox2*, 5.2% (Papadopoulou *et al.*, 2010; Pons *et al.*, 2010; Ribera *et al.*, 2010). Broad rate variations have been found across genes and lineages in many animal studies (Kumar, 2005). In Coleoptera, Pons *et al.* (2010) found a large rate variation, depending on the lineage (almost twofold: Adephaga, 1.94% vs. Elateriformia, 3.56%) and gene (15-fold: *nad4*, 1.11% vs. *cox1*, 17.2%). Moreover this rate variation may have been due to methodological problems (Papadopoulou *et al.*, 2010). We have therefore taken into account various methodological issues to minimize potential artefacts, including partitioning by codon (Pons *et al.*, 2010), using rate heterogeneity among sites (Papadopoulou *et al.*, 2010) and using multiple calibration constraints on independent nodes (Ho & Phillips, 2009). The broad analytical approach used here enabled us to document how some published rates are too high and thus not suitable for *Calathus* time estimation. This was particularly evident for analysis B2, which estimated a very late origin of the genus (in the late Miocene, 5.84–9.26 Ma), an age highly incongruent with the Eocene age of the *Calathus elpis* fossil (Ortuño & Arillo, 2009).

Diversification analyses showed moderate rates from 0.05 to 0.18 species Myr^{-1} , which are not far from those reported for other carabids [*Cicindela*: 0.22–0.29 species Myr^{-1} (Barracough & Vogler, 2002); Harpalinae: 0.07–0.10 species Myr^{-1} (Ober & Heider, 2010)], and are distinctly lower than those reported for rapid radiations [from 0.56 to 0.8 species Myr^{-1} (Baldwin & Sanderson, 1998; Kozak *et al.*, 2006; Ricklefs *et al.*, 2007)]. Our analyses indicate that there has been either a constant rate of diversification (BDL analysis) or a decrease in diversification rates for the subgenus (survival or γ -statistics analyses), but certainly no increase during the Pleistocene, falsifying our initial hypothesis of increased diversification during this period. Wider sampling is needed to test the first two hypotheses, because a decrease in the diversification rate could be the result of incomplete sampling. It might also be argued that Pleistocene glaciations caused an increase of extinctions that led to a decrease of diversification rates, but this hypothesis is not supported by likelihood models, which point to a roughly constant rate of extinction.

According to the above mentioned conclusions, lineage diversification in the subgenus *Calathus* is to a great extent independent of Pleistocene climatic oscillations, and has, instead, been driven by fine-scale variables and species-specific life-history traits, among other factors. Similar conclusions have been reported for other taxa in studies based on fossils and molecular data (Taberlet *et al.*, 1998; Stewart, 2009; Stewart *et al.*, 2010). Moreover, studies based on ecological modelling have shown that topographical, climatic and geological variables explain a low proportion (on average 20%) of the variation in richness in Iberian *Calathus* (Gañán

et al., 2008) and in British Carabidae (Eyre *et al.*, 2005). Eyre *et al.* (2005) concluded that the key drivers in shaping distribution patterns are probably linked to fine-scale variation in habitat features.

Biogeography of the subgenus *Calathus*

The biogeographical analysis has shown that the diversification of *Calathus* did not follow a simplistic model of vicariance or dispersal. Instead it was found that at least four westward dispersal events occurred in the subgenus *Calathus*, from the east to the west Mediterranean region (Fig. 6b). The Anatolian and Balkan peninsulas are therefore the most likely ancestral areas of this group (Fig. 6b), an inference that is congruent with previous analyses that indicated a sister relationship between the subgenus and taxa from the Caucasus (clade 7 in Ruiz *et al.*, 2010).

Dispersal events were followed by isolation and differentiation on particular massifs in the Mediterranean peninsulas. The age of most of these events roughly coincides with the appearance of new dispersal routes during the Tertiary. Two westward dispersal events probably occurred during the middle Miocene. One occurred towards western Europe when landmass connections permitted dispersal across the Mediterranean Basin, resulting in trans-Mediterranean lineages (Oosterbroek & Arntzen, 1992). Another event seems to have occurred through North Africa, giving rise to the ancestor of clade 3. A third dispersal event was inferred through the North African corridor, possibly in the late Miocene, which allowed for the widespread distribution of the ancestor of the *Calathus fuscipes* species complex. This hypothesis is supported by the *EF1 α* network analysis and age estimates, and agrees with previous biogeographical studies of circum-Mediterranean Coleoptera (Sanmartín, 2003; Micó *et al.*, 2009). A fourth westward dispersal event during the early Pleistocene is thought to have given rise to the ancestor of the European *Calathus fuscipes* phylogroups.

The role of the Eastern Mediterranean region as a centre of origin and area of diversification with recurrent biotic western expansions has been postulated for many circum-Mediterranean lineages (e.g. Mansion *et al.*, 2008; Micó *et al.*, 2009). These biogeographical events agree with the taxon pulse model (Erwin, 1985), which is characterized by biotic expansion from a centre of origin with subsequent peripheral isolation that leads to vicariant speciation in successive pulses. Predictions of this model are congruent with the fact that basal lineages (clades 1, 2, 3, 4, 5 and 7) include few extant taxa with disjunct distributions, in contrast to younger, more species-rich, lineages that have radiated in particular geographical areas (clades 6, 8, 9, 10 and 11). These results are explained by constant rates of random extinction that tend to prune older widespread lineages (Nee *et al.*, 1994b), with few lineages surviving in disjunct areas (e.g. Iberian *C. malacensis*, Italian *C. montivagus*, and Anatolian *C. zabroides* and *C. arcuatus*), where they coexist with taxa of younger pulses.

Pleistocene divergence and introgression in *Calathus*

Calathus fuscipes is the only widely distributed species that extends its distribution north of the Holdhaus line. It is a generalist species with a moderate dispersal capacity, features that were perhaps selected in ancestral populations occupying northern latitudes (Jansson & Dynesius, 2002). In fact, there are fossil records of this species from England prior to the Last Glacial Maximum (LGM), documenting a long-term broad distribution (Buckland & Buckland, 2006). However, extant populations in western Europe revealed very low sequence variation, indicating potential bottlenecks and more recent dispersal from a single southern refugium. In addition to a distinct western European population, our molecular data indicated three additional clades for this species, corresponding to the Balkans, Anatolian and North African regions. The range of the three European lineages closely corresponds to the three main Pleistocene glacial refugia (Stewart *et al.*, 2010).

Node ages based on the mtDNA data indicate fragmentation of populations during the Pleistocene. Subsequent range expansion has led to secondary contact zones between west European and Balkans clades in Italy and Slovakia, and between Balkans and Anatolian clades in Greece (no. 214, Fig. 3c) and west Turkey (no. 420). These contact zones are characterized by the asymmetric introgression of genes, indicating that reproductive isolation has not been fully attained in these populations.

The existence of a distinct Anatolian clade suggests that the Anatolian Peninsula acted as a major refugium during the Pleistocene, giving rise to the subspecies *C. fuscipes disiunctus*. In this region hybridization probably occurred between *C. fuscipes disiunctus* and the morphologically related *C. longicollis*, especially in western Turkey (individuals 230, 424, 425). The fact that the mtDNA of *C. longicollis* is nested within *C. fuscipes* clades (Fig. 3a) indicates complete replacement of its original mtDNA by that of *C. fuscipes*. However, both taxa make up distinct clades according to *EF1 α* data, reflecting their morphological distinctiveness. The African populations (*C. fuscipes algiricus*) are similarly related to the Anatolian population as shown by the biogeographical analysis (Fig. 6), and by the proximity of North African and Anatolian clades in the *EF1 α* median-joining network (Fig. 3b). This close relationship should be corroborated using data from the Libyan *C. fuscipes kochi*.

The Iberian clade 9 includes nine closely related endemic species. Most of them are allopatric (Fig. S5) and, where two of them overlap, there is usually an elevational segregation (Nègre, 1969). The incongruence found between data sets in *C. oreades* and *C. vuillefroyi*, and between *C. mirei* and both *C. vivesi* and *C. moralesi*, are also possible instances of introgression events (Fig. S5). Finally, we note that introgression is not uncommon among carabid beetles (Düring *et al.*, 2006; Zhang & Sota, 2007), which highlights the need to investigate multiple and independent markers when analysing closely related species of Carabidae.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sampling localities of taxa of subgenus *Calathus* and related taxa included in the study, sequenced genes and accession numbers of sequences in GenBank (Table S1) and divergence time estimates in million years for the main nodes of the phylogenetic tree of subgenus *Calathus* in the four calibration analyses (Table S2).

Appendix S2 Supplementary figures on subgenus *Calathus* phylogeny (Figs S1–S6).

Appendix S3 Details of phylogenetic, calibration and diversification analyses.

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BIOSKETCHES

Carlos Ruiz is a postdoctoral researcher at University of Murcia. His research focuses on understanding the tempo and mode of diversification of carabid beetles. He has also worked on the phylogeny and phylogeography of Mesoamerican bees of the tribe Meliponini.

Bjarte H. Jordal is Associate Professor in Systematic Entomology at University of Bergen. His research focuses on the phylogenetics and taxonomy of beetles, in particular wood-boring weevils, with a strong emphasis on phylogenetic methods and their implementation in comparative analyses of key evolutionary transitions.

José Serrano is Professor of Zoology, University of Murcia, specializing in the systematics and evolution of ground beetles, and the phylogeny of the genus *Apis*. He is head of the research group ‘Filogenia y Evolución Animal’ of the University of Murcia. Together with papers dealing with phylogeny he has published regional faunas, monographs and catalogues on Iberian carabids.

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