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ORIGINAL ARTICLE

Late Miocene origin of an Ibero-Maghrebian clade of ground beetles with multiple colonizations of the subterranean environment

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ABSTRACT

Aim To test different biogeographical scenarios for the evolution of the ground beetles of the *Trechus fulvus* group, a lineage with many narrowly distributed flightless subterranean species, highly suitable for tracing their biogeographical history.

Location The Western Palaearctic, focusing on the Betic–Rifean area between south-east Iberia and north Morocco.

Methods We sequenced 3.3 kb of four mitochondrial and two nuclear genes of 30 individuals of 15 species of the *T. fulvus* group, plus 29 outgroups. We reconstructed their phylogeny and estimated divergence times using Bayesian probabilities and a priori evolutionary rates, and their ancestral distribution using maximum likelihood.

Results The phylogenetic reconstruction uncovered multiple independent colonizations of the subterranean environment within the *T. fulvus* group, a scenario also supported by variation in troglomorphic characters. Most of the Moroccan and south-east Iberian species form a clade with strong geographical structure, including the former genus *Antoinella*. The biogeographical model best fitting the current distribution and phylogeny of the group was a late Miocene palaeogeographical scenario with isolated populations on the Betic and Rifean areas and a south-eastern Iberian origin of the north Moroccan species. The widespread *T. fulvus* was sister to a central Moroccan species, suggesting a second Iberian–North African vicariance event within the group. One of the species, *T. lallemantii*, expanded its range to the east (Algeria and Tunisia) and the north (extreme south of the Iberian Peninsula) during the Pleistocene.

Main conclusions The *T. fulvus* group originated in the early Miocene, and the south-eastern Iberian lineage in the Tortonian. The lineage dispersed to Morocco during the Messinian, diversifying in rapid succession in the Atlas and the Rif and colonizing the subterranean environment multiple times. The geography of the Betic–Rifean region at the end of the Miocene can still be traced from the distribution of the extant species of the group.

Keywords

Atlas mountains, Betic, cave beetles, dispersal, diversification, Miocene, phylogenetic reconstruction, Rif, subterranean environment, *Trechus*.

INTRODUCTION

The relationship between the fauna and flora of the Iberian Betic cordilleras and that of the Moroccan Rif (the 'Gibraltar Arc') is one of the classic themes in biogeography (e.g. Bolívar, 1915; de Jong, 1998; Rodríguez-Sánchez *et al.*, 2008). This area had a very complex geological history during the Neogene (Alvinerie *et al.*, 1992; Martín *et al.*, 2009; Platt *et al.*, 2013), with multiple connections being formed between the African and Eurasian plates. Part of the present Betic–Rifean belt was integrated with the Iberian Peninsula until 30 Ma, close to present-day Catalonia, together with

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the Kabylies, Balearic Islands, Sardinia and Corsica (Rosenbaum *et al.*, 2002). During the Oligocene, these microplates started their independent rotation and migration, and whereas the Kabylies reached their present-day location in Northern Algeria during the Miocene, the Betic plates became fragmented and continued their migration towards the Gibraltar area, finally reaching their current location on both sides of the Alboran Sea after the Messinian (Rosenbaum *et al.*, 2002; Meulenkamp & Sissingh, 2003; Martín *et al.*, 2009).

Many groups of plants and animals have been hypothesized to have a Betic–Rifean origin (for examples in different animal groups, see Prüser & Mossakowski, 1998; Gantenbein & Largiadèr, 2003; Carranza *et al.*, 2004; Busack *et al.*, 2005; Pinho *et al.*, 2006), but, owing to the complex history of the area and the short distance between continental lands at the Strait of Gibraltar, it is very difficult to discern for most species whether the Betic–Rifean distribution pattern is related to the common geological history or originated by more recent (post-Messinian) dispersal, favoured by the proximity and current similar ecological conditions in southern Iberia and North Africa [as proposed for salamanders (Veith *et al.*, 2004), other reptiles and amphibians (Carranza *et al.*, 2004, 2006), and small mammals (Cosson *et al.*, 2005)]. Even when a pre-Messinian age can be established for a lineage, it is not usually possible to determine its precise geographical origin within the Iberian Peninsula because of subsequent range movements. Thus, the isolation of the Betic microplates during the Tortonian have been hypothesized to have promoted the diversification of some groups (e.g. Hydrochidae water beetles, Hidalgo-Galiana & Ribera, 2011; *Mesocarabus* ground beetles, Andújar *et al.*, 2012a), but the geographical signal of the ancestral distribution of the species is usually too weak to provide any detailed evidence.

In this work, we study the biogeography of a group of ground beetles with a mostly south Iberian–north Moroccan distribution, the *Trechus fulvus* group (Carabidae, Trechinae) (Jeannel, 1927). It includes many apterous taxa (or populations) with subterranean habits and distributions restricted to discrete geological units, and is thus suitable for tracing the history of subterranean taxa through the complex geological history of the area. With more than 800 species distributed mainly in the Holarctic, *Trechus* is the most species-rich genus in the tribe Trechini, and – as currently understood – is a vast paraphyletic complex with many derived genera nested within it (Casale *et al.*, 1998; Moravec *et al.*, 2003; Faille *et al.*, 2010, 2011, 2013). It includes species with disparate ecological traits, although most are highly hygrophilous and with very narrow geographical distributions. Among the species of *Trechus*, there are many instances of colonization of the subterranean environment, as is the case within the *T. fulvus* group (Jeannel, 1927; Faille *et al.*, 2013).

We specifically aim to reconstruct the biogeographical history of the *Trechus fulvus* group, and relate it to the palaeogeography of the Betic–Rifean area. For that purpose, we built a phylogeny using a combination of mitochondrial and nuclear sequences to estimate the relationships and age of divergence between species. More specifically, using a dispersal–extinction–cladogenesis model, we test whether the reconstructed phylogeny and the distribution of the species are better explained by the current geography or by the late Miocene palaeogeography of the Gibraltar area.

MATERIALS AND METHODS

Taxonomic background

The *Trechus fulvus* group, initially recognized by Jeannel (1927), currently includes 33 Western Palaearctic species, some of them regularly found in subterranean environments (Casale & Laneyrie, 1982; Ortuño & Toribio, 2005; Quéinnec & Ollivier, 2013) (see Appendix S1a in Supporting Information). Jeannel (1927) considered the group to have an Ibero-Mauritanian origin, with possible affinities with some Abyssinian species.

Trechus fulvus Dejean, 1831 was considered to be a relict of a Tertiary Lusitanian fauna by Jeannel (1920). It is widely distributed in western Europe and North Africa, with many isolated populations under different ecological conditions, although always requiring high humidity. It occurs from the northern Faroe Islands, through the British Isles, Scandinavia, northern Europe and coastal areas of France, to northern Morocco and the island of Madeira (Moravec *et al.*, 2003). North of the Pyrenees, *T. fulvus* is lapidicolous and coastal (Jeannel, 1920; Bruneau de Miré, 1961; Zorgati, 2009), whereas in the Iberian Peninsula, it tends to occur in subterranean environments (Jeannel, 1920, 1927). The existence of multiple isolated populations is likely to favour its morphological variability, with some populations having only apterous individuals with reduced eyes, whereas other populations also include macropterous specimens. In addition to the epigean and widespread *T. fulvus*, there is only one species north of the Pyrenees, *T. delhermi* Saulcy, 1906, known from a cave in the Lot department of southern France and from flood detritus up to several hundred kilometres downstream, reaching the Garonne estuary (Jeannel, 1927; Tempère, 1962).

In the Iberian Peninsula, there are two groups of species within the *T. fulvus* group found in subterranean environments, one restricted to the north-eastern Prebetic system and a second in Portugal. The first, the '*martinezi* lineage' *sensu* Ortuño & Arillo (2005), contains four species from the north-eastern Betic area (Appendix S1a). A fifth species, *T. breuili* Jeannel, described from a single cave in the Penibetic system (Málaga), was originally considered to be a subterranean form of *T. fulvus*, later considered by Jeannel (1927) to be close to *T. lallemantii* and finally included by Ortuño (2008) in the *T. martinezi* group alongside three Algerian species. Four Portuguese species, which are apparently close to *T. fulvus* and lack the morphological modifications associated to the subterranean life, occur in the Estremenho and Montejunto massifs (Appendix S1a) (Reboleira *et al.*, 2009, 2010).

We also included *Trechus lencinai* (Mateu & Ortuño, 2006), known from a cave in the Prebetic system (Sierra del Segura).

It was originally placed in a different genus, *Duvalius*, which is mostly diversified in central Europe, but was later transferred to *Trechus* based on its external morphology and distribution (Mateu & Ortuño, 2006; Ortuño & Barranco, 2013). *Trechus gloriensis* Jeanne, 1970, also an Iberian species, is usually considered a member of the *T. fulvus* group, but the structure of the male genitalia suggests that it may be related to other lineages within *Trechus* (Jeanne, 1970).

Four species from the Maghreb are generally considered to belong to the *T. fulvus* group (Appendix S1a). In addition to these four species, it has been proposed that the hypogean genus *Antoinella*, now considered a synonym of *Trechus* (Casale, 2011), is close to the *T. fulvus* group both based on morphology (especially the male genitalia; Jeannel, 1937; Casale, 1982, 1983) and molecular data (Faille *et al.*, 2011). It includes nine species that are only found in caves in the Moroccan Rif and the Atlas (Comas & Mateu, 2008; Casale, 2011). Mateu & Comas (2006) described the subgenus *Irinea* for the first anophthalmous species of North African *Trechus*, *T. (Irinea) aurouxi* (Mateu & Comas, 2006).

Taxon sampling and DNA sequencing

We studied representatives of epigean and subterranean species of the *T. fulvus* group from across its distribution area, with the exception of the eastern Maghreb (Algeria and Tunisia) (Fig. 1); the species in this area have been hypothesized to be closely related to *T. lallemantii* (Quéinnec & Ollivier, 2013). Three geological units were sampled in Morocco: Rif, Middle Atlas and High Atlas. In the Iberian Peninsula, specimens were mainly collected in caves from Cantabria and the Betic System (see Appendix S1b for details). Specimens were collected by hand and immediately killed in 96% ethanol, or by means of pitfall traps containing propylene glycol as the preserving agent. DNA was extracted non-destructively from whole specimens using commercial extraction kits (Qiagen, Hilden, Germany). Voucher specimens have been deposited in MZB (Barcelona), ZSM (Munich) and MNHN (Paris); DNA aliquots are kept in the DNA collections of ZSM (Munich) and IBE (Barcelona).

We used 17 species of *Trechus* of different species groups (Faille *et al.*, 2011, 2013) as outgroups, together with representatives of seven genera in the tribe Trechini (Appendix S1b) that were used to root the tree.

We sequenced two mitochondrial fragments, including four genes (3′ end of cytochrome *c* oxidase subunit I, *COI*; and a continuous fragment, *rrnL+trnL+nad1*, including the 3′ end of the gene for the large ribosomal RNA subunit, the leucine transfer RNA gene, and the 5′ end of the NADH dehydrogenase subunit 1 gene) and two nuclear genes (5′ end of the small ribosomal RNA subunit gene, *SSU*; and an internal fragment of the large ribosomal unit, *LSU*) (see Appendix S2 for the primers used and standard PCR conditions). Sequences were assembled and edited with Bioedit 7 (Hall, 1999) or Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Some sequences were obtained from Faille *et al.* (2010, 2011, 2012). New sequences have been deposited in EMBL (Appendix S1b).

Estimation of the phylogeny and ages of divergence

Protein-coding and ribosomal genes were aligned with the online version of mafft 6 (Katoh & Toh, 2008) using the EINS-i and QINS-i algorithms, respectively, with other parameters set to their defaults. The correct translation to amino acids was checked in mega 4 (Tamura *et al.*, 2007) for the protein-coding genes, which were subsequently divided into two codon partitions ('12p', pooling first and second codon

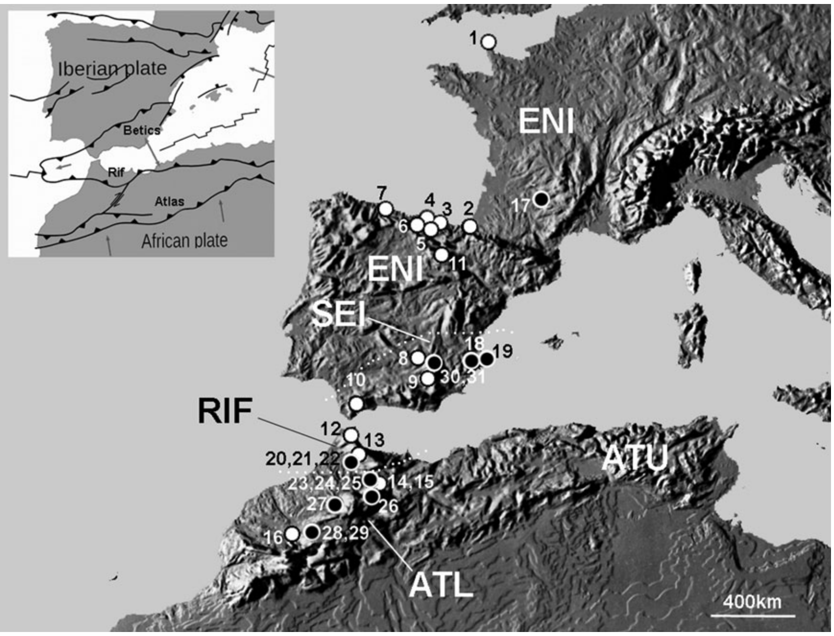

Figure 1 Map of the localities sampled for the studied species in the *Trechus fulvus* group, with the biogeographical areas used in Lagrange. White circles, epigean species; black circles, subterranean species. Geographical areas (separated by white dotted lines): ATL, Atlas; RIF, Rif; ATU, Algeria–Tunisia; SEI, South-East Iberia; ENI, rest of Europe + North Iberia. 1–7, *T. fulvus*; 8–10, *T. fulvus andalusiacus*; 11, *T. arribasi*; 12–15, *T. lallemantii*; 16, *T. zaerensis*; 17, *T. delhermi*; 18, *T. martinezi*; 19, *T. alicantinus*; 20–21, *T. sendrai*; 22, *T. djebalicus*; 23, *T. groubei*; 24–25, *T. gigoni*; 26, *T. iblanensis*; 27, *T. espanyoli*; 28–29, *T. (Irinea) aurouxi*; 30-31, *T. lencinai*. Inset, schematic map of the extent of the tectonic plates in the western Mediterranean, with the direction and sense of movement (arrows).

positions; and '3p', third codon positions). We used jModelTest 3.7 (Posada, 2008) to identify the model of nucleotide substitution best fitting each gene and codon partition, and ran analyses with the genes separately to check for potential incongruences in the resulting topologies.

beast 1.7 (Drummond *et al.*, 2012) was used to obtain a combined estimation of an ultrametric phylogenetic tree and ages of diversification. Analyses were conducted applying the best-fitting model of substitution to each gene or codon partition as above. A Yule speciation prior was applied and analyses were run for 50 million generations, sampling one tree every 2000 generations. In the molecular clock settings, only gene partitions were considered, and three independent analyses were conducted under three alternative molecular-clock hypotheses: (1) applying an uncorrelated lognormal clock (ULN) to all genes, (2) applying a strict clock (SC) to all genes, or (3) using ULN for nuclear and SC for mitochondrial genes. Alternative molecular-clock settings were compared using Bayes factors as estimated with the stepping-stone (SS) and the path-sampling (PS) algorithms in beast (Baele *et al.*, 2012), and with the harmonic mean estimator (HME) in Tracer 1.5 (Rambaut & Drummond, 2007); this last case required an improvement in marginal likelihood of 10 units per additional parameter for a more complex model to be accepted (Pagel & Meade, 2004; Miller *et al.*, 2009). We assumed one extra parameter when comparing ULN with SC (Drummond *et al.*, 2006). As calibration priors for estimating the diversification ages of nodes, we used the rates of molecular evolution obtained for the related genus *Carabus* (in the same family, Carabidae) and with the same analytical settings and DNA fragments studied here (Andújar *et al.*, 2012b). A uniform prior on the mean substitution rate was applied for each gene, with maximum and minimum bounds matching the 95% confidence interval of the rates obtained by Andújar *et al.* (2012b). For the *COI* fragment, the allowed rates ranged from 0.0100 to 0.0198 substitutions per site per million years per lineage, for *rrnL* from 0.0010 to 0.0022, and for *LSU* from 0.0007 to 0.0020. For *SSU*, which was not included in Andújar *et al.* (2012b), rates were not restricted. We used Tracer to determine convergence, measure the effective sample size of each parameter and calculate the mean and 95% highest posterior density interval for divergence times.

Consensus trees were estimated with TreeAnnotator (Drummond *et al.*, 2012) discarding a conservative 20% burn-in fraction, after checking the effective sample sizes of the likelihood, evolutionary rates and root age values, and ensuring that the tree likelihood values had reached a plateau. Posterior probabilities were considered as a measure of node support.

Ancestral area reconstruction

We used Lagrange C++ (Ree & Smith, 2008) to reconstruct the ancestral distribution area and to estimate the likelihoods of different geographical scenarios related to the biogeographical history of the species of the *T. fulvus* group. Lagrange implements likelihood models for geographical range evolution based on calibrated phylogenetic trees, the current distribution of the species and a predefined matrix containing a measure of the connectivity between areas. We defined five biogeographical areas: Atlas (ATL); Rif (RIF); Algeria–Tunisia (ATU); south-east Iberia (SEI); and the rest of Europe + North Iberia (ENI) (see Fig. 1 and Table 1 for the current distribution of the 13 species of the *T. fulvus* group included in this study). SEI covered all the Betic cordilleras and the Guadalquivir basin. We identified sea and land barriers between the areas using two geographical scenarios, the present and a reconstruction of the palaeogeography of the late Miocene. The geological history of the Gibraltar area during the late Palaeogene and Miocene was very complex, but the main trends were, in the north, the isolation of the Betic area from the Iberian platform first by sea corridors and then the Guadalquivir basin in the west, and, in the south, the isolation of the Rif area from the Atlas by the Rifean corridors (Sanz de Galdeano & Rodríguez-Fernández, 1996; Meulenkamp & Sissingh, 2003; Martín *et al.*, 2009; Platt *et al.*, 2013). Although these areas were not likely to be completely isolated during this period and there are many uncertainties in the exact reconstruction of the emerged coastline (e.g. Martín *et al.*, 2009), the main difference from the current geography is the recognition of three units (Iberia, Betic–Rifean area and Atlas) instead of the current two (Iberia and North Africa). We used the late Miocene geographical scenario in isolation and in combination with the present, applying the two matrices across different periods (with the transition at 5, 6 or 7 Ma) to account for uncertainties in the geological and phylogenetic reconstructions (Table 2a). For each geographical sce-

Table 1 Current distribution of the studied species in the *Trechus fulvus* group. Presence of the species in the area considered is indicated by (1), absence by (0).

		Atlas	Rif	Algeria–Tunisia	SE Iberia	Europe+ North Iberia
	Species	ATL	RIF	ATU	SEI	ENI
1	*T. alicantinus*	0	0	0	1	0
2	*T. delhermi*	0	0	0	0	1
3	*T. djebalicus*	0	1	0	0	0
4	*T. espanyoli*	1	0	0	0	0
5	*T. fulvus*	0	1	0	1	1
6	*T. gigoni*	1	0	0	0	0
7	*T. groubei*	1	0	0	0	0
8	*T. iblanensis*	1	0	0	0	0
9	*T. lallemantii*	1	1	1	1	0
10	*T. lencinai*	0	0	0	1	0
11	*T. martinezi*	0	0	0	1	0
12	*T. sendrai*	0	1	0	0	0
13	*T. zaerensis*	1	0	0	0	0

Table 2 Results of ancestral area reconstruction with Lagrange of the *Trechus fulvus* group using the BEAST consensus tree. (a) Different geographical scenarios tested, with the barriers between areas according to the present geography (ACT) and the late Miocene palaeogeography (MIO): S, sea barrier of less than 100 km; L, land barrier (i.e. with one area in between); > 2, more than two barriers; 1, contiguous areas. Geographical areas: ATL, Atlas; RI, Rif; ATU, Algeria–Tunisia; SEI, south-east Iberia; ENI, the rest of Europe + North Iberia. (b) Penalization values (*a–c*) used for the different types of barrier. The final value of the pairwise connectivity for each of the geographical scenarios is the default probability (1) minus the penalization value. (c) Results of the analyses of the consensus tree obtained in BEAST. When two different geographical matrices were used, the temporal range for each one is given. In this case, the penalization values with the best likelihood were used for each of the matrices. ACT, present geography; MIO, late Miocene palaeogeography (see text for details).

(a)

ACT/MIO	ATL	RIF	ATU	SEI	ENI
ATL	1				
RIF	1/S	1			
ATU	L/L	1/S	1		
SEI	L+S/S	S/1	L+S/S	1	
ENI	> 2	> 2/S	> 2	1/S	1

(b)

Barrier	Default	*a*	*b*	*c*
L	0	0.2	0.5	0.2
S	0	0.2	0.5	0.7
> 2	0	0.2	0	0

(c)

Penalization	Geography	−ln(likelihood)
Default	Default	26.5
a	ACT	25.5
a	MIO	25.5
b	ACT	25.9
b	MIO	24.8
c	ACT	26.4
c	MIO	24.7
a/*c*	ACT 0-5/MIO 5–20 Ma	26.1
a/*c*	ACT 0-6/MIO 6–20 Ma	25.8
a/*c*	ACT 0-7/MIO 7–20 Ma	25.7

nario, we estimated the likelihood of the Lagrange reconstruction using three combinations of values for the probability of dispersal through the barriers between the defined areas (Table 2b). For comparison, we used a default matrix with all probabilities of dispersal between areas equal to one.

For the selection of the best geographical model and dispersal parameters in Lagrange, we used as a unique topology the consensus tree obtained in BEAST, pruning all duplicated specimens of the same species. To account for topological uncertainty, we applied the optimal geographical model and parameter values to a selection of 1000 trees from the stationary (i.e. post-burn-in) set of BEAST trees using LogCombiner (Drummond *et al.*, 2012). We pruned the trees, ran Lagrange and parsed and edited the output with a custom script (available on request) and a spreadsheet.

RESULTS

Sequences for the protein-coding mitochondrial genes *COI* and *nad1* showed no length variation and were correctly translated to amino acids. The best substitution model for *COI* was HKY+I+G, whereas the 12p partition was best fitted by GTR+I and 3p by GTR+G. The mitochondrial *rrnL–trnL–nad1* fragment ranged from 725 to 746 bp and the best fit was a GTR+G model of evolution. The nuclear ribosomal *SSU* gene fragment ranged from 612 to 616 bp, best fitting a GTR+G model, and the *LSU* fragment ranged from 876 to 909 bp, again with a GTR+G model selected as optimal. The comparison of Bayes factors favoured the analyses with a strict clock (SC) for mitochondrial genes and an uncorrelated lognormal clock (ULN) for nuclear genes for both PS and SS, whereas the HME favoured ULN for all genes (Appendix S3a).

Phylogeny of the *Trechus fulvus* group

The monophyly of a clade including most of the species of the *T. fulvus* group was strongly supported, and its temporal origin was estimated to be the early Miocene (node A in Fig. 2). All the species currently considered to belong to the species group were included in this clade with the exception of *T. aurouxi* and *T. arribasi* Jeanne, 1988. *Trechus lencinai*, which is considered to be of uncertain affinities, was found to belong to the *T. fulvus* group with strong support. The *T. fulvus* group was sister to a group including *T. aurouxi* and some Iberian species, including subterranean species currently included in the genus *Apoduvalius* (Fig. 2).

Within the *T. fulvus* group, *T. delhermi* from the French Massif Central was sister to the remaining species, which were divided into two main lineages that are estimated to have separated during the Tortonian (clades C and D in Fig. 2). Clade D included the hypogean endemics from the Prebetic area (*T. alicantinus* and *T. martinezi* on the basal-most branch, and *T. lencinai* on the next lowest), and most of the North African species (clade G), which were estimated to have diversified during the late Miocene (Messinian) and the early Pliocene. The early diversification of clade F was estimated to have occurred in a narrow temporal window during the late Tortonian–early Messinian, with low support for the internodes (Fig. 2, Appendix S3b). The second main lineage (clade C) included the central Moroccan *T. iblanensis* (Mateu & Escolà, 2006) as the sister of *T. fulvus*, with an estimated divergence in the early Messinian.

Within the North African clade G, three lineages were identified with strong support: (1) the two epigean species (clade K); (2) a group of species from the Atlas (clade J); and (3) the species from the Rif (clade H). The last two nodes include some of the species formerly included in the genus *Antoinella*,

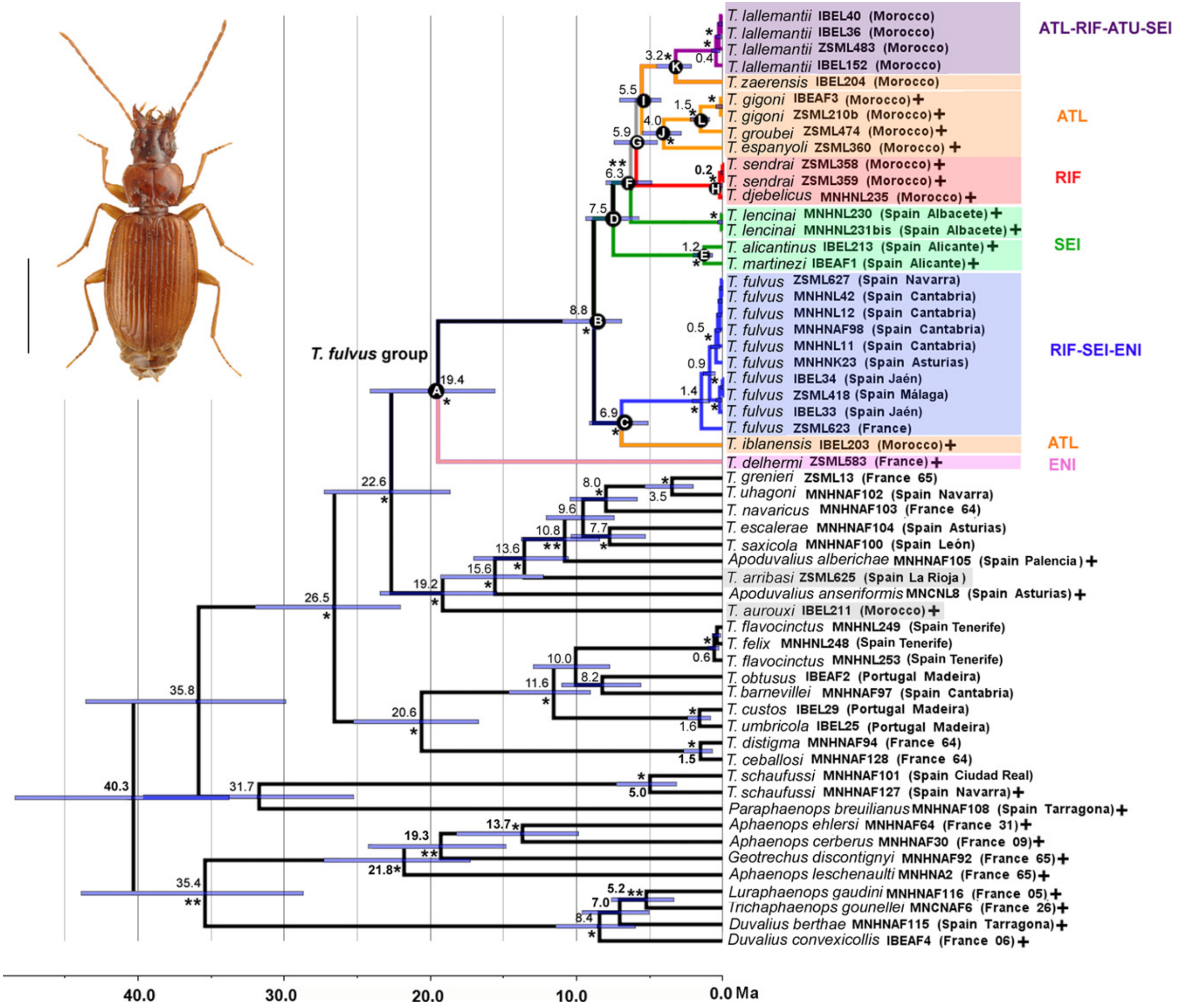

Figure 2 Ultrametric time-calibrated tree obtained with BEAST for the combined dataset. Blue bars, 95% confidence intervals of the estimated ages for the nodes; with *, nodes with posterior probability (PP) > 0.95; **, nodes with 0.95 > PP ≥ 0.90 (see Appendix S3b for detailed node support values); with +, subterranean species; in grey, species previously considered to belong to the *Trechus fulvus* group. See Table 1 for the codes of the distribution of the species, and Appendix S1b for detailed locality data of the specimens. Habitus photograph, *T. fulvus* (photo: A. Faille).

with some troglomorphic modifications and only found in caves. The sampled specimens of the widespread *T. lallemantii* and *T. fulvus* had some geographical structure, which originated in the Pleistocene. Specimens of *T. fulvus* from the French coast diverged *c.* 1.5 Ma from the Iberian populations, which in turn were divided into northern and southern populations (the latter recognized as *T. fulvus andalusiacus*) (Fig. 2, Appendix S3b).

Ancestral area reconstruction

The best likelihood of the ancestral area reconstruction in the BEAST consensus tree was that of the model based on the late Miocene palaeogeography, penalizing sea barriers more than land barriers (penalization probabilities of 0.7 and 0.2, respectively), and not allowing contact through two contiguous barriers (probability 0) (penalization *c* in Table 2c). This was only marginally better than a model with equal penalization for sea and land barriers (*b* in Table 2c). The three models using the late Miocene palaeogeography were equal (*a* in Table 2c) or better (*b* and *c* in Table 2c) than the best model using the present geographical scenario, which had a low penalization for sea or land barriers, allowing dispersal between non-contiguous areas. The model using the default matrix with all measures of connectivity between areas equal to one had the worst likelihood of all the tested models (Table 2c).

The use of different matrices with two time windows in the tree did not improve the likelihood of the model, irrespective of their age, although the best likelihood was obtained when the division between the current and the late Miocene scenarios was set at 7 Ma (early Messinian; Table 2c). We used the best model (i.e. the matrix based on the late Miocene palaeogeography and the penalization values as in *c* in Table 2c) to reconstruct the ancestral areas on the 1000 trees selected from the stationary phase in BEAST. The ancestral area reconstruction was very consistent, with most nodes having high frequencies of the preferred areas (Table 3). Eight of the 12 nodes present in the consensus tree (D–F and H–L) had a single preferred reconstructed area with a frequency over 95%, with the second best below 75% in all of them (Fig. 3, Table 3). In the other four nodes, there was more than one reconstructed area with a probability over 80%, with the three basal nodes with four areas over 85%.

The origin of the clade including most of the south-east Iberian and north Moroccan species (node D, with the stem between 8.8 and 7.5 Ma) was reconstructed as south-east Iberian (SEI) in all trees in which this node was present (82%; Table 3), with only *c.* 10% of the cases reconstructed as having an Atlas or Rifean origin. In the remaining 18% of trees, all alternative nodes also included SEI as an ancestral area with only one exception (i.e. a probability of 0.001) (Appendix S3c), which were reconstructed as originating in the Atlas and Rif areas. From south-east Iberia, LAGRANGE reconstructed an expansion to north Morocco during the late

Table 3 Results of the analyses of the 1000 post-burn-in trees in LAGRANGE of the *Trechus fulvus* group. No. trees are the number of the 1000 post-burn-in trees in which the node was present. ATL to ENI, frequency of reconstruction of each area as ancestral to the node in the 1000 post-burn-in BEAST trees (in brackets, percentage over the number of trees in which the node is present). Nodes as in Fig. 2. Geographical areas: ATL, Atlas; RIF, Rif; ATU, Algeria–Tunisia; SEI, south-east Iberia; ENI, rest of Europe + North Iberia.

Node	No. trees	ATL	RIF	ATU	SEI	ENI
A	1000	946(95)	886(89)	0	999(99.9)	1000(100)
B	1000	1000(100)	1000(100)	9(1)	1000(100)	979(98)
C	984	980(99.6)	955(97)	0	984(100)	955(97)
D	827	79(10)	88(11)	1(0.1)	826(99.9)	0
E	1000	66(7)	35(4)	1(0.1)	998(99.8)	0
F	889	643(72)	572(64)	10(1)	862(97)	0
G	323	296(92)	271(84)	3(1)	190(59)	0
H	1000	0	1000(100)	0	0	0
I	325	325(100)	122(38)	6(2)	170(52)	0
J	996	996(100)	0	0	0	0
K	1000	1000(100)	591(59)	533(53)	625(63)	0
L	1000	1000(100)	0	0	0	0

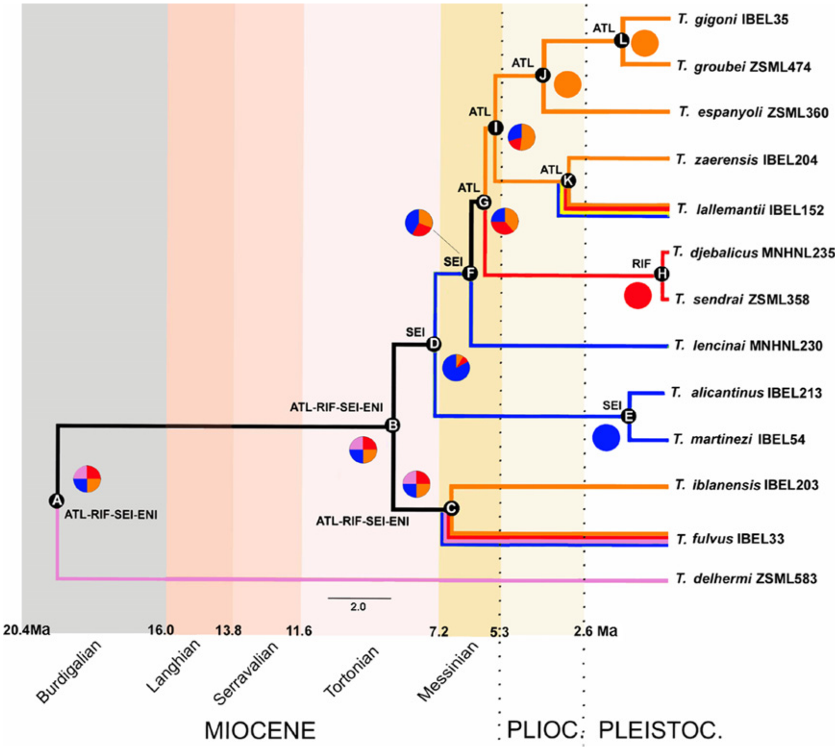

Figure 3 Pruned consensus tree of the *Trechus fulvus* group showing the result of the LAGRANGE analyses of the 1000 post-burn-in trees. Branch colour indicates current distribution and most likely reconstruction. Above nodes, area with the highest probability. Circles in nodes, approximate proportion of the reconstructed trees with each of the ancestral areas (see Table 3 for detailed results). Geographical areas: ATL, Atlas; RIF, Rif; SEI, south-east Iberia; ENI, rest of Europe + North Iberia.

Messinian (6.3–5.9 Ma; nodes F and G in Fig. 3). Both the Rif (node H) and the Atlas (node I) were occupied in a short temporal succession, with a subsequent Pleistocene expansion of *T. lallemantii* to the east and the extreme south of the Iberian Peninsula (Fig. 3).

DISCUSSION

Biogeographical history of the *T. fulvus* group

According to our results, the palaeogeography of the late Miocene, with an isolated Betic–Rifean area, fits the phylogenetic and geographical data of the species of the *Trechus fulvus* group better than the current geography. This was irrespective of the degree of isolation used for the different areas in the analyses of Lagrange, or the use of different time-frames within the late Miocene. Although the age estimates were based on rates of molecular evolution obtained for a related genus of ground beetles using a combination of fossil and biogeographical events (Andújar *et al.*, 2012b), they were in accordance with those obtained by previous studies of western Mediterranean Trechini (Faille *et al.*, 2011, 2013) that used the vicariant separation of the genus *Sardaphaenops* due to the tectonic drift of the Sardinian plate from the continent *c.* 33 Ma as a calibration point.

The geographical origin of the *T. fulvus* group could not be reconstructed without ambiguity, although the distribution of the sister group (mostly Iberian Peninsula and southern France) and of *T. delhermi* (Massif Central) suggest a western European origin, in agreement with the 'Lusitanian' origin originally proposed by Jeannel (1920). There are, however, still many uncertainties due to the incomplete sampling of the wider genus *Trechus* and of some species of the *T. fulvus* group that cannot be readily placed in our phylogeny based on morphological characters alone (e.g. *T. idriss* or *T. breuili*; see below).

Most species of the *T. fulvus* group were included in a clade reconstructed with high probability as having a south-eastern Iberian origin (node D). These results were not affected by the topological uncertainty of the phylogenetic tree, as shown with the use of 1000 trees of the post-burn-in fraction in beast, with 99.9% of the trees including south-east Iberia as an area of origin for the species in this clade but only *c.* 10% including the Atlas or the Rif. According to our reconstruction, the group colonized northern Morocco from the Betic region during the late Messinian, probably after the closure of the Rifean corridors (the Guadalhorce gateway; Martín *et al.*, 2009), but there is no evidence that they ever moved northwards from the Prebetic area. The late Miocene tectonic history of the Prebetic area, where the species of the *T. martinezi* group currently occur, is uncertain. It is formed of limestone deposits that transgressed over the Iberian platform with the thrust produced by the movement of the Alborán plate during the Miocene (Platt *et al.*, 2013). The south-eastern part of the Prebetic area was still mostly submerged during the early Tortonian (Sanz de Galdeano & Rodríguez-Fernández, 1996), and there could only have been an emerged substratum with subterranean environment to be colonized during the late Tortonian, in agreement with our estimated dates for the origin of the *T. martinezi* group and, later, *T. lencinai* (Fig. 4).

The subsequent diversification of the Betic lineage within Morocco occurred in a short period during the late Messinian, resulting in an unresolved topology with two clades in the Rif and the Atlas, respectively. Unexpectedly for a group of species that usually have very restricted distributions, one of them – *T. lallemantii* – expanded its range to the east (Algeria and Tunisia) and the north, reaching the southern tip of the Iberian Peninsula (Serrano, 2003). Although there are no molecular data on these Iberian populations, they are likely to have dispersed after the opening of the Strait of Gibraltar at the end of the Messinian salinity crisis 5.3 Ma, because the separation between *T. lallemantii* and its sister *T. zaerensis* (from the Moroccan Atlas) was estimated at 3.2 Ma. There are also no data on the ecological tolerance of *T. lallemantii*, but the only other species with a widespread distribution in the group, *T. fulvus*, is known to tolerate sea water, and is limited to coastal habitats throughout its range north of the Pyrenees (Jeannel, 1927; Bruneau de Miré, 1961). This northern form, originally described as

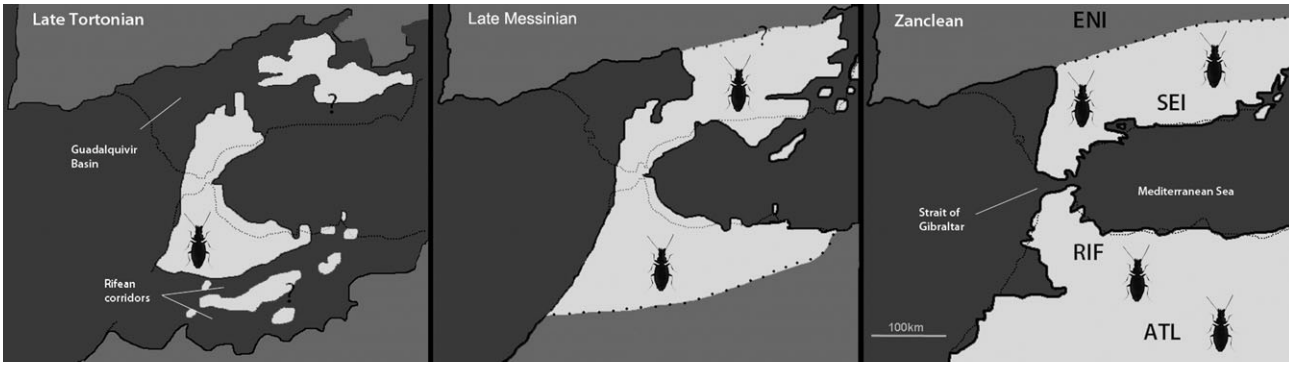

Figure 4 Reconstructed palaeogeographical scenario of the colonization of the Betic–Rifean area by the *Trechus fulvus* group from the late Tortonian to the Zanclean (early Pliocene), according to the results of Lagrange (modified from Martín *et al.*, 2009). Geographical areas: ATL, Atlas; RIF, Rif; SEI, South-East Iberia; ENI, rest of Europe + North Iberia.

T. lapidosus Dawson, 1849 but synonymized with *T. fulvus* by Jeannel (1920), was estimated to have diverged in the early Pleistocene from the Iberian populations, but more data on northern *T. fulvus* are needed before it can be raised to species status. *Trechus fulvus* is also present on the island of Madeira, and is assumed to have arrived there through long-distance dispersal (Serrano & Borges, 1995).

The Algerian and Tunisian species of the *T. fulvus* group not included in our study have been hypothesized to derive from isolated populations of *T. lallemantii* (Jeannel, 1920; Quéinnec & Ollivier, 2013), although other authors have suggested a relationship with the southern Iberian species of the group (Ortuño, 2008). The estimated age of the *T. fulvus* group (*c.* 20 Ma), however, excludes the possibility that the eastern Maghrebian species originated by the tectonic drift of the Kabylia microplate from the Iberian Peninsula, which started *c.* 35–30 Ma (Rosenbaum *et al.*, 2002; Schettino & Turco, 2006). The lack of species of the *T. fulvus* group in the Mediterranean Islands (Corsica, Sardinia and the Balearic Islands) supports the African origin of *T. lallemantii*, in agreement with our results and contrary to other groups with probable tectonic origins and which are present in other microplates derived from the fragmentation of the eastern Iberian Peninsula, such as Oligochaeta (Omodeo & Rota, 2008), spiders (Bidegaray-Batista & Arnedo, 2011), Leptodirini subterranean beetles (Ribera *et al.*, 2010), or some groups of Trechini ground beetles within the genus *Duvalius* (Jeannel, 1928; Faille *et al.*, 2013).

The phylogenetic position of *T. iblanensis* from Morocco as sister to the widespread *T. fulvus* is surprising, because some morphological features, especially the male genitalia, link it with the Rifean hypogean species. The species differs, however, in some characters, such as its larger size and the presence of pubescence on the elytra. With our data, it was not possible to discriminate between different alternatives for the origin of this lineage, but it seems likely that this represents another independent instance of North African colonization through a Tortonian corridor within the *T. fulvus* group.

The strong palaeogeographical signal in the distribution of the species of the *T. fulvus* group is likely to be related to their ecological habits, with an abundance of species occupying the subterranean environment. According to our results (and assuming that there is no possibility of reversal), there are likely to have been multiple independent colonizations, with each lineage having different degrees of morphological adaptation to the subterranean environment. The strongest factor driving the subterranean colonizations may have been the aridification of the climate since the late Miocene (Krijgsman *et al.*, 2000; Micheels *et al.*, 2009), as even the epigean populations of these insects are always hygrophilous.

The multiple origins of the subterranean populations or species within the *T. fulvus* group – supported both by the phylogenetic results and the variation in the degree of eye regression and other troglomorphic characters – is in contrast to hypotheses surrounding other radiations of subterranean beetles, especially in the Pyrenees (Faille *et al.*, 2010, 2013; Ribera *et al.*, 2010; Rizzo *et al.*, 2013; Cieslak *et al.*, 2014), where whole lineages of species are found exclusively within the deep subterranean environment with no known epigean relatives and without morphological variation in some troglomorphic characters (Jeannel, 1924, 1928; Salgado *et al.*, 2008). Some of the closely related species within the *T. fulvus* group may, however, have speciated within the subterranean environment. *Trechus groubei* and *T. gigoni* (found in the northern part of the Middle Atlas; Comas & Mateu, 2008) are sister to *T. espanyoli* (southern Middle Atlas); all three species (the *groubei* group *sensu* Comas & Mateu, 2008) share a similar morphology and degree of troglomorphism, and there is no evidence to suggest independent colonization of the subterranean environment. A similar situation is that of the species pair *T. sendrai* (Comas & Mateu, 2008) and *T. djebalicus* (Comas & Mateu, 2008) from the Rif, to which the morphologically similar and geographically close *T. messoulii* Casale, 2011, not included in our study, should probably be added. All these species were included in the genus *Antoinella* based on their subterranean habits and troglomorphic characters, but were recently transferred to *Trechus* by Casale (2011), who proposed close relationships with the *T. fulvus* group, as already suggested by Jeannel (1937).

We have shown that the distribution and phylogeny of the species of the *Trechus fulvus* group in the Betic–Rifean region is best explained by the palaeogeography of the late Miocene, providing a prime example of the role of the complex history of the area in the generation of Mediterranean biodiversity. This is likely to have been due to the unique combination of widespread epigean species with multiple colonizations of the subterranean environment, giving rise to species with very restricted geographical ranges that have persisted unaltered over long evolutionary periods.

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SUPPORTING INFORMATION

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

Appendix S1 Additional materials. (a) List of the species of the *Trechus fulvus* group, including distribution and previous hypotheses of relationships. According to the results of this study the species *T. arribasi*, *T. aurouxi* and *T. gloriensis* are excluded from the group. (b) Additional data on the studied specimens: locality data, collector and sequence accession numbers.

Appendix S2 Additional methods: list of primers used for sequencing and standard PCR conditions.

Appendix S3 Additional phylogenetic results. (a) Bayes factor comparison of BEAST phylogenetic analyses conducted under alternative clock models. (b) Ultrametric time-calibrated tree obtained with BEAST for the combined dataset with detailed node support values. (c) Ancestral area reconstruction for alternative topologies of node D in Fig. 2.

BIOSKETCH

Arnaud Faille is a postdoctoral researcher at the Zoologische Staatssammlung, Munich. The main focus of his research is an understanding of the origin and evolution of subterranean biodiversity and its description, with Coleoptera as the target group. During the last few years he has studied phylogenetic relationships and speciation among some of the most species-rich radiations of hypogean ground beetles of the Western Palaearctic region.

Author contributions: A.F. and I.R. conceived the study; A.F., C.A., F.F. and I.R. led the specimen's collection; A.F. obtained the molecular data; A.F., C.A. and I.R. performed the analyses and led the writing; all authors discussed the results and commented on the manuscript.

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SUPPORTING INFORMATION

Late Miocene origin of an Ibero-Maghrebian clade of ground beetles with multiple colonizations of the subterranean environment

Arnaud Faille, Carmelo Andújar, Floren Fadrique and Ignacio Ribera

Appendix S1 Additional materials.

(a) List of the species of the *Trechus fulvus* group, including distribution and previous hypotheses of relationships. The species *T. arribasi*, *T. aurouxi* and *T. gloriensis*, previously considered to be part of the *T. fulvus* group, are excluded according to our results. Geographical areas: ATL, Atlas; RIF, Rif; ATU, Algeria–Tunisia; SEI, south-east Iberia; ENI, rest of Europe + North Iberia. Bold, species included in the study.

Species	Authority	Distribution	Previous hypotheses of relationships
T. fulvus	Dejean, 1831	ENI, Madeira, Morocco	
T. martinezi	Jeannel, 1927	SEI (Alicante)	*T. lallemantii* (Jeannel, 1927); *T. martinezi* lineage (Ortuño & Arrillo, 2005)
T. alicantinus	Español, 1971	SEI (Alicante)	*T. martinezi* (Español, 1971); *T. martinezi* lineage (Ortuño & Arrillo, 2005)
T. beltrani	Toribio, 1990	SEI (Alicante)	*T. martinezi* lineage (Ortuño & Arrillo, 2005)
T. torressalai	Ortuño & Arrillo, 2005	SEI (Dénia)	*T. martinezi* lineage (Ortuño & Arrillo, 2005)
T. breuili	Jeannel, 1913	SEI (Malaga)	*T. fulvus* (Jeannel, 1920); *T. lallemantii* (Jeannel, 1927); *T. martinezi* group (Ortuño, 2008)
T. lallemantii	Fairmaire, 1858	RIF, ATL, ATU, SEI	*T. fulvus* (Jeannel, 1920, 1927)
T. djbel-gloubensis	Queinnec & Ollivier, 2013	ATU (Tunisia)	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. zorgatii	Queinnec & Ollivier, 2013	ATU (Tunisia)	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. machadoi	Jeannel, 1941	ENI (Portugal)	*T. fulvus* (Jeannel, 1941)
T. lunai	Reboleira & Serrano, 2009	ENI (Portugal)	*T. fulvus* (Reboleira *et al.,* 2010)
T. gamae	Reboleira & Serrano, 2009	ENI (Portugal)	*T. fulvus* (Reboleira *et al.,* 2010)
T. tatai	Reboleira & Ortuño, 2010	ENI (Portugal)	*T. fulvus* (Reboleira *et al.,* 2010)
T. idriss	Peyerimhoff, 1924	ATL	*T. fulvus* group (Jeannel, 1927)
T. zaerensis	Antoine, 1928	ATL	*T. lallemantii* (Antoine, 1928)
T. rotroui	Antoine, 1934	ATL	*T. lallemantii* (Antoine, 1934)
T. groubei	Antoine, 1935	ATL	*Duvalius* (Antoine, 1935); *T. fulvus* (Jeannel, 1937; Casale, 1982); *T. groubei* group (Comas & Mateu, 2008)

Species	Authority	Distribution	Previous hypotheses of relationships
T. gigoni	Casale, 1982	ATL	*T. fulvus* (Casale, 1982); *T. groubei* group (Comas & Mateu, 2008)
T. iblanensis	Mateu & Escolà, 2006	ATL	*T. iblanensis* group (Comas & Mateu, 2008)
T. espanyoli	Mateu & Escolà, 2006	ATL	*T. groubei* group (Comas & Mateu, 2008)
T. fadriquei	Mateu & Escolà, 2006	ATL	*T. iblanensis* group (Comas & Mateu, 2008)
T. sendrai	Comas & Mateu, 2008	RIF	*T. iblanensis* group (Comas & Mateu, 2008)
T. djebalicus	Comas & Mateu, 2008	RIF	*T. iblanensis* group (Comas & Mateu, 2008)
T. messoulii	Casale, 2011	RIF	Moroccan species of the *T. fulvus* group ('*Antoinella*') (Casale, 2011)
T. oligops	Bedel, 1895	ATU	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. bedeli	Jeannel, 1922	ATU	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. atlasicus	Moravec & Lompe, 2003	ATU	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. kabylicus	Casale, 1983	ATU	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. incola	Peyerimhoff, 1909	ATU	*T. lallemantii* (Queinnec & Ollivier, 2013)
T. delhermi	Saulcy, 1880	ENI	
T. lencinai	Mateu & Ortuño, 2006	SEI	*Duvalius* (Mateu & Ortuño, 2006); *Trechus* (Ortuño & Barranco, 2013)

(b) Additional data on the studied specimens: locality data, collector and sequence accession numbers.

No.	Species	Code	Locality	Collector	*SSU*	*LSU*	*COI*	*rrnL+trnL+nad1*
Trechus fulvus group								
1	*T. fulvus*	ZSM-L623	Vauville (France: 50)	C. Vanderbergh		HG813170	HG813201	HG813225
2	*T. fulvus*	ZSM-L627	Endara (Spain: Navarra)	J. Fresneda		HG813169	HG813200	HG813224
3	*T. fulvus*	MNHN-L11	Cueva de la Lastrilla, Sámano (Spain: Cantabria)	A. Cieslak, A. Faille, J. Fresneda, I. Ribera, J.M. Salgado		HG813173	HG813204	HG813228
4	*T. fulvus*	MNHN-L42	Cueva de Los Moros, San Salvador – Medio Cudeyo (Spain: Cantabria)	C.G. Luque, J.M.Salgado		HG813171	HG813202	HG813226
5	*T. fulvus*	MNHN-L12	Mina Constante, Rasines (Spain: Cantabria)	A. Cieslak, A. Faille, J. Fresneda, I. Ribera, J.M. Salgado		HG813172	HG813203	HG813227
6	*T. fulvus*	MNHN-AF98	Cueva del Pis – Penilla-Santiurde de Toranzo (Spain: Cantabria)	C. Bourdeau, P. Déliot, A. Faille	FR733972	GQ293613	FR733909	GQ293729
7	*T. fulvus*	MNHN-K23	Cueva del Sidrón, Villobal-Borines (Spain: Asturias)	J.M. Salgado		HG813174	HG813205	HG932325
8	*T. fulvus andalusiacus* Jeannel, 1927	IBE-L34	Sierra del Relumbrar, carretera de Génave a Albaladejo (Spain: Jaén)	C. Andújar	HG813144	HG813168	HG813199	HG813223
9	*T. fulvus andalusiacus* Jeannel, 1927	IBE-L33	Cueva Secreta del Poyo Manquillo, Sierra de Cazorla (Spain: Jaén)	Grupo de Espeleología de Villacarrillo (GEV)	HG813147	HG813177	HG813208	HG813231
10	*T. fulvus andalusiacus* Jeannel, 1927	ZSM-L418	Cueva de Hundidero Montejaque, (Spain: Málaga)	F. Fadrique	HG813146	HG813176	HG813207	HG813230
11	*T. arribasi* Jeanne, 1988	ZSM-L625	Valdezcaray, Ezcaray, Sierra de la Demanda (Spain: La Rioja)	C. Bourdeau		HG813162	HG813193	HG813220

No.	Species	Code	Locality	Collector	*SSU*	*LSU*	*COI*	*rrnL+trnL+nad1*
12	*T. lallemantii*	IBE-L152	Tetuan rd N Souk-Khemis-des-Anjra (Morocco)	A. Cieslak, I. Ribera	HG813145	HG813175	HG813206	
13	*T. lallemantii*	IBE-L40	17 km E of Chefchauen (Morocco)	Dpt Biologia Animal Univ. Murcia	HG813148	HG813178	HG813209	HG813232
14	*T. lallemantii*	IBE-L36	Rivière de Chara – Tabehirt-Semia (Tahla) (Morocco)	F. Alfambra, J. Comas, F. Fadrique, H. Mansouri	HG813150	HG813180	HG813211	HG813234
15	*T. lallemantii*	ZSM-L483	Aven Bab Bou Idir, Taza (Morocco)	F. Fadrique	HG813149	HG813179	HG813210	HG813233
16	*T. zaerensis*	IBE-L204	Ifri N'Taouya, Aït M'Hammed (Morocco)	F. Alfambra, F. Fadrique, A. Faille	HG813152	HG813182	HG813213	HG813236
17	*T. delhermi*	ZSM-L583	Grotte du Robinet, Marcilhac-sur-Célé (France: 46)	C. Bourdeau, A. Faille		HG813164	HG813195	HG813222
18	*T. martinezi*	IBE-AF1	Cova de les Meravelles, Cocentaina (Spain: Alicante)	C. Andújar, P. Arribas, A. Faille	FR733973	FR733996	HE817939	FR729576
19	*T. alicantinus*	IBE-L213	Cova del Somo – Castell de Castells (Spain: Alicante)	F. Fadrique		HG813161	HG813192	HG813219
20	*T. sendrai*	ZSM-L358	Foukmaggo, Maggou (Morocco)	F. Fadrique	HG813139	HG813157	HG813187	HG932320
21	*T. sendrai*	ZSM-L359	Foukmaggo, Maggou (Morocco)	F. Fadrique		HG813158	HG813188	HG932321
22	*T. djebalicus*	MNHN-L235	Kef Lahmar, Maggou (Morocco)	F. Fadrique		HG813153	HG813183	HG813214
23	*T. groubei*	ZSM-L474	Ghar Bied, Ain el Ouda, Taza (Morocco)	F. Fadrique	HG813138	HG813156	HG813186	HG813215
24	*T. gigoni*	IBE-AF3	Trou de la piste, Tabehirt-Semia, Tahla (Morocco)	F. Alfambra, J. Comas, F. Fadrique, H. Mansouri	FR733980	FR734000	FR733917	FR729583
25	*T. gigoni*	ZSM-L210b	Trou de la piste, Tabehirt-Semia, Tahla (Morocco)	F. Alfambra, F. Fadrique, A. Faille			HG813185	HG932319
26	*T. iblanensis*	IBE-L203	Tlat Izra, Bou Iblane (Morocco)	F. Fadrique, A. Faille	HG813136	HG813154		HG813237

No.	Species	Code	Locality	Collector	*SSU*	*LSU*	*COI*	*rrnL+trnL+nad1*
27	*T. espanyoli*	ZSM-L360	Ifri Berrit, Ain Khala, Ain Leuh (Morocco)	F. Fadrique	HG813137	HG813155	HG813184	HG932318
28	*T. (Irinea) aurouxi* Mateu & Comas, 2006	IBE-L211	Iri Nousgour (=Ousgre), Taguelft, Beni Mellal (Morocco)	F. Alfambra, F. Fadrique, A. Faille	HG813141	HG813160	HG813191	HG813218
29	*T. lencinai*	MNHN-L230	Cueva del Farallón, Riopar (Spain: Albacete)	C. Andújar, A. Faille	HG813140		HG813189	HG813216
30	*T. lencinai*	MNHN-L231bis	Cueva del Farallón, Riopar (Spain: Albacete)	C. Andújar, A. Faille		HG813159	HG813190	HG813217
Outgroups								
Out-1	*T. distigma* Kiesenwetter, 1851	MNHN-AF94	Aven de Nabails, Arthez d'Asson (France: 64)	C. Bourdeau, P. Déliot, A. Faille	FR733971	GQ293611	GQ293678	FR729575
Out-2	*T. barnevillei* Pandellé, 1867	MNHN-AF97	Cueva del Pis, Penilla, Santiurde de Toranzo (Spain: Cantabria)	C. Bourdeau, P. Déliot, A. Faille	GQ293533	GQ293607	GQ293680	GQ293727, GQ293783, GQ293848
Out-3	*T. saxicola* Putzeys, 1870	MNHN-AF100	Braña Caballo, Piedrafita (Spain: León)	C. Bourdeau, P. Déliot, A. Faille	FR733974	GQ293614	GQ293682	FR729577
Out-4	*T. aff. schaufussi* Putzeys, 1870	MNHN-AF101	El Boquerón, Navas de Estena (Spain: Ciudad Real)	A. Faille	GQ293532	GQ293620	FR733910	GQ293737, GQ293788, GQ293820
Out-5	*T. uhagoni uhagoni* Crotch, 1869	MNHN-AF102	Cueva de Orobe, Alsasua (Spain: Navarra)	C. Bourdeau	GQ293540	GQ293616	FR733911	GQ293730
Out-6	*T. escalerae* Abeille de Perrin, 1903	MNHN-AF104	Cueva de Porro Covañona, Covadonga (Spain: Asturias)	J.M. Salgado	GQ293538	GQ293612	FR733912	GQ293731, GQ293793, GQ293839
Out-7	*T. grenieri* Pandellé, 1867	ZSM-L13	Résurgence de la Hèche, Fréchet-Aure (France: 65)	J.P. Besson, C. Bourdeau, A. Faille	HG514715	HE817904	HE817920	HE817887
Out-8	T. *navaricus* (Vuillefroy, 1869)	MNHN-AF103	Grotte de Sare, Sare (France: 64)	C. Bourdeau	GQ293539	GQ293603	GQ293687	FR729578

No.	Species	Code	Locality	Collector	*SSU*	*LSU*	*COI*	*rrnL+trnL+nad1*
Out-9	*T. custos* Wollaston, 1854	IBE-L29	Ribeiro Frio, Madeira (Portugal)	A. Arraiol	HG813142	HG813163	HG813194	HG813221
Out-10	T. *umbricola* Wollaston, 1854	IBE-L25	Ribeiro Frio, Madeira (Portugal)	A. Arraiol	HG813151	HG813181	HG813212	HG813235
Out-11	*T. flavocinctus* Jeannel, 1922	MNHN-L249	Barranco de Hijuana, Anaga, Canarias (Spain: Tenerife)	A. Faille		HG813166	HG813197	HG932323
Out-12	*T. flavocinctus* Jeannel, 1922	MNHN-L253	Las Portelas, Monte del Agua, Canarias (Spain: Tenerife)	A. Faille		HG813165	HG813196	HG932322
Out-13	*T. felix* Wollaston, 1864	MNHN-L248	Barranco de Hijuana, Anaga, Canarias (Spain: Tenerife)	A. Faille	HG813143	HG813167	HG813198	HG932324
Out-14	*T. ceballosi* Mateu, 1953	MNHN-AF128	Aven de Licie Etsaut, Lanne-en-Barétous (France: 64)	C. Bourdeau, A. Faille	FR733978	GQ293610	FR733914	GQ293728, GQ293791, GQ293850
Out-15	*T. obtusus* Erichson, 1837	IBE–AF2	Estrada de Nicho, Madeira (Portugal)	A. Arraiol	FR733976	FR733997	FR733913	FR729579
Out-16	*T. schaufussi* ssp *comasi* Hernando, 2002	MNHN-AF127	Cueva Basaula, Barindano (Spain: Navarra)	J. Fresneda	FR733977	GQ293617	HE817940	FR729580
Out-17	*Apoduvalius anseriformis* Salgado & Peláez, 2004	MNCN-AF2	Cueva del Perro, Ludeña (Infiesto) (Spain: Asturias)	A. Cieslak, A. Faille, J. Fresneda, I. Ribera, J.M. Salgado	FR733979	FR733999	FR733916	FR729582
Out-18	*Apoduvalius alberichae* Español, 1971	MNHN-AF105	Cueva de Agudir, Cardaño de Abajo (Spain: Palencia)	J.M. Salgado	GQ293536	GQ293618	GQ293632	GQ293732, GQ293794, GQ293840
Out-19	*Duvalius convexicollis* Peyerimhoff, 1904	IBE-AF4	Faille du Pensier, Saint-Auban (France: 06)	J.M. Lemaire	HG514718	FR734005	FR733923	FR729589
Out-20	*Duvalius berthae* (Jeannel, 1910)	MNHN-AF115	Cova d'en Xoles, Pratdip (Spain: Tarragona)	C. Bourdeau, P. Déliot, F. Fadrique, A. Faille		GQ293606	GQ293626	FR729588
Out-21	*Paraphaenops breuilianus* (Jeannel, 1916)	MNHN-AF108	Cova Cambra, Tortosa (Spain: Tarragona)	C. Bourdeau, P. Déliot, A. Faille	GQ293541	GQ293551	GQ293685	FR729587

No.	Species	Code	Locality	Collector	*SSU*	*LSU*	*COI*	*rrnL+trnL+nad1*
Out-22	*Agostinia gaudini* (Jeannel, 1952)	MNHN-AF116	Puits des Bauges, Dévoluy (France: 05)	J.-Y. Bigot	GQ293543	GQ293604	FR733925	GQ293692, GQ293787, GQ293838
Out-23	*Trichaphaenops gounellei* (Bedel, 1879)	MNCN-AF6	Baume Cervière, Vassieux-en-Vercors (France: 26)	A. Faille	FR733985	FR734006	FR733924	FR729590
Out-24	*Aphaenops leschenaulti* Bonvouloir, 1861	MNHN-AF1	Grotte de Castelmouly, Bagnères-de-Bigorre (France: 65)	C. Bourdeau, P. Déliot, A. Faille	FR733945	GQ293593	GQ293629	GQ293739, GQ293757, GQ293822
Out-25	*A. (Cerbaphaenops) cerberus* (Dieck, 1869)	MNHN-AF30	Grotte du Sendé, Moulis (France: 09)	P. Déliot, A. Faille	FR733948	GQ293589	GQ293646	GQ293718, GQ293779, GQ293835
Out-26	*A. (Hydraphaenops) ehlersi* (Abeille de Perrin, 1872)	MNHN-AF64	Goueil-di-Her, Arbas (France: 31)	C. Bourdeau, P. Déliot, A. Faille	FR733957	GQ293565	GQ293683	FR729571
Out-27	*Geotrechus discontignyi* (Fairmaire, 1863)	MNHN-AF92	Grotte du Tuco, Bagnères-de-Bigorre (France: 65)	C. Bourdeau, P. Déliot, A. Faille	FR733966	GQ293560	FR733901	FR729572

Appendix S2 Additional methods: list of primers used for amplification and sequencing, and standard PCR conditions.

Gene	Name	Sense	Sequence	Reference
COI	Jerry (M202)	F	CAA CAT TTA TTT TGA TTT TTT GG	Simon *et al*. (1994)
	Pat (M70)	R	TCC A(A)T GCA CTA ATC TGC CAT ATT A	Simon *et al.* (1994)
	Chy	F	TWG TAG CCC AYT TTC ATT AYG T	Ribera *et al.* (2010)
	Tom	R	ACR TAA TGA AAR TGG GCT ACW A	Ribera *et al.* (2010)
rrnL–nad1	16saR (M14)	F	CGC CTG TTT AWC AAA AAC AT	Simon *et al.* (1994)
	ND1A (M223)	R	GGT CCC TTA CGA ATT TGA ATA TAT CCT	Simon *et al.* (1994)
SSU	5′	F	GAC AAC CTG GTT GAT CCT GCC AGT	Shull *et al.* (2001)
	b5.0	R	TAA CCG CAA CAA CTT TAA T	Shull *et al.* (2001)
LSU	D1	F	GGG AGG AAA AGA AAC TAA C	Ober (2002)
	D3	R	GCA TAG TTC ACC ATC TTT C	Ober (2002)

Step	*CO1*	*LSU*, *rrnL–nad1*	*SSU*
1	96 °C – 3 min	96 °C – 3 min	96 °C – 3 min
2	94 °C – 30 s	94 °C – 30 s	94 °C – 30 s
3	47 °C – 30 s	47 °C – 1 min	50 °C – 30 s
4	72 °C – 1 min	72 °C – 1 min	72 °C – 1 min
5	Go to 2, 39×	Go to 2, 34×	Go to 2, 39×
6	72 °C – 10 min	72 °C – 10 min	72 °C – 10 min

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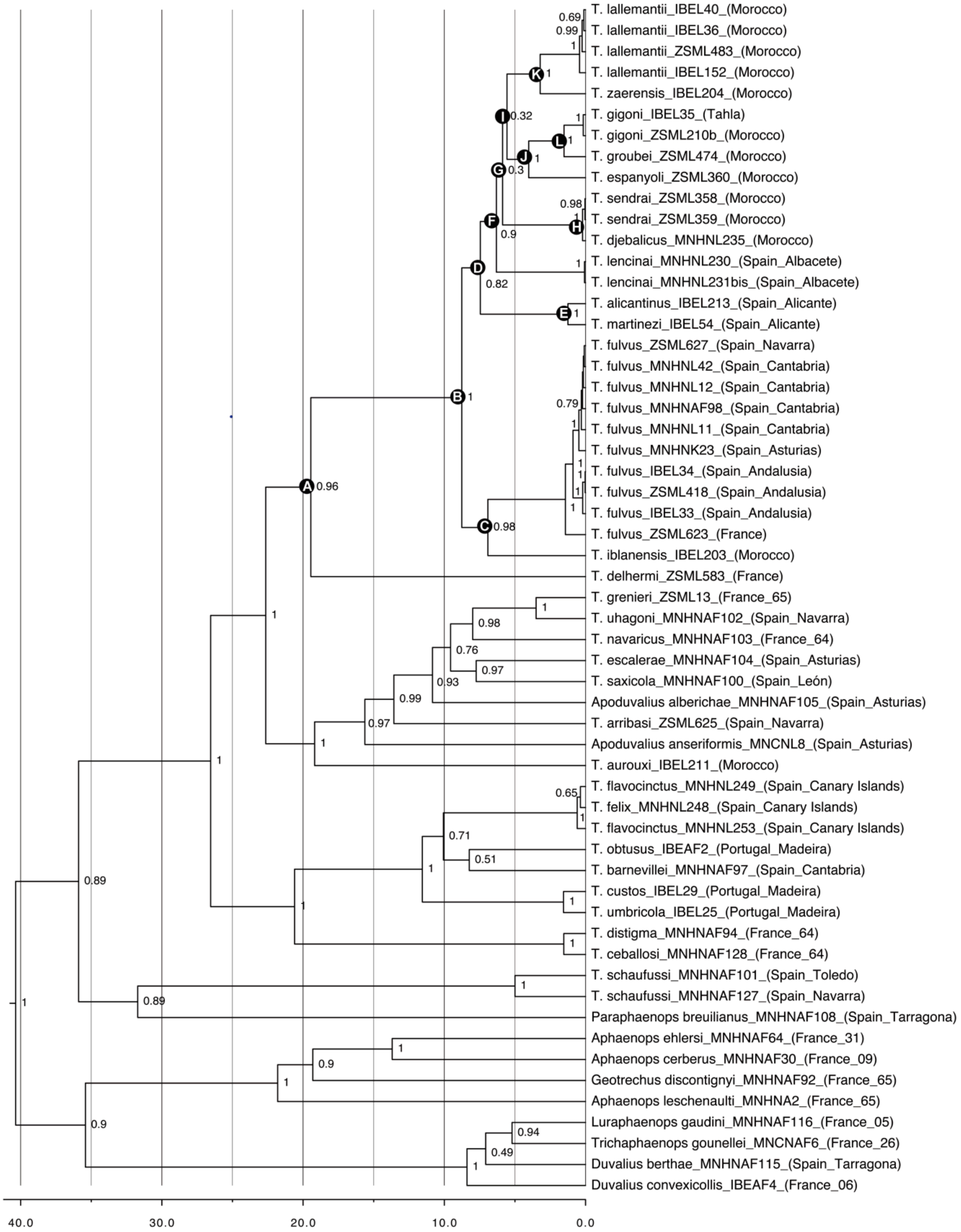
Appendix S3 Additional phylogenetic results.

(a) Bayes-factor comparison of the BEAST phylogenetic analyses conducted under alternative clock models: *, best model; PS, path-sampling method; SS, stepping-stone sampling method; HME, harmonic mean estimator; Par, relative number of parameters in the model; HME cor, harmonic-mean estimator penalized with ten units per additional parameter in the model; SC, strict clock applied to every gene fragment; SC-ULN, strict clock applied to mitochondrial and uncorrelated lognormal clock to nuclear genes; ULN, uncorrelated lognormal clock applied to every gene fragment.

	PS	SS	HME	Par	HME cor
SC	−14061.7	−14064.6	−13676.3	*x*	−13656.3
SC-ULN	**−14013.4***	**−14016.1***	−13598.6	*x* + 2	**−13598.6***
ULN	−14025.1	−14029.3	**−13578.6***	*x* + 4	−13598.6

(b) Ultrametric time-calibrated tree obtained with BEAST for the combined dataset with detailed node support values.



(c) Ancestral area reconstruction for alternative topologies of node D, the south-eastern Iberican–north Moroccan clade, in Fig. 2. Numbers refer to the frequency of reconstruction of each area as ancestral to the node in the 1000 post-burn-in trees of BEAST. Node, species included in the alternative topology. No. trees, number of the 1000 post-burn-in trees in which the node was present. See Table 1 for the areas.

Node	No. trees	ATL	RIF	ATU	SEI	ENI
T. djebalicus, T. espanyoli, T. gigoni, T. groubei, T. lencinai, T. sendrai	165	162	140	0	162	0
T. djebalicus, T. espanyoli, T. gigoni, T. groubei, T. sendrai	152	152	131	0	0	0
T. djebalicus, T. lallemanti, T. lencinai, T. sendrai, T. zaerensis	95	95	46	2	88	0
T. djebalicus, T. lallemanti, T. sendrai, T. zaerensis	188	128	188	3	128	0
T. djebalicus, T. lencinai, T. sendrai	263	0	221	0	262	0
T. espanyoli, T. gigoni, T. groubei, T. lallemanti, T. lencinai, T. zaerensis	192	178	15	1	188	0
T. espanyoli, T. gigoni, T. groubei, T. lencinai	182	182	0	0	178	0
T. lallemanti, T. lencinai, T. zaerensis	73	54	28	0	73	0