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Running header: Karstificability and dispersal in a subterranean species

Substratum karstificability, dispersal and genetic structure in a strictly subterranean beetle

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ABSTRACT

Aim The deep subterranean environment is an ideal system to test the effect of physical constraints on the ecology and evolution of species, as it is very homogeneous and with simple communities. We studied the effect of substratum karstificability in the dispersal of the strictly subterranean *Trogloch*arinus *ferreri* (Reitter) (Coleoptera, Leiodidae) by comparing the genetic diversity and structure of populations in limestone (more soluble) and dolostone (less soluble) in the same karstic system.

Location *Trogloch*arinus *ferreri* is only known from ca.100 vertical shafts in an area of less than 500 km^2 SW of Barcelona (Spain).

Methods We sequenced mitochondrial and nuclear markers of a representative sample to identify main lineages within *T. ferreri* and estimate their temporal origin, and used mitochondrial data of 129 specimens from 41 caves to reconstruct their demographic history and estimate dispersal among caves.

Results *Trogloch*arinus *ferreri* diverged from its sister in the Early Pliocene, with an initial divergence of the sampled populations in the Early Pleistocene. The best demographic model was a constant population size with a fast population increase in the middle Pleistocene. The ancestral population was likely in limestone, with a probability of transition from limestone to dolostone triple to that from dolostone to limestone, suggesting a higher permeability of limestone to the transit of individuals. Populations in dolostone caves had lower gene flow between them and a stronger isolation by distance, although the low genetic variability for the studied markers and the lower abundance of dolostone caves decreased the statistical power of the analyses.

Main conclusions Our results point to the physical characteristic of the substratum as a determinant of dispersal and gene flow, potentially conditioning the long-term evolution of subterranean biodiversity.

Keywords

Gene flow, isolation, substratum permeability, subterranean environment, *Trogloch*arinus, troglomorphism

INTRODUCTION

It has long been recognised that some physical properties of the habitats may constraint the characteristics of the organisms living in them. Some of these constraints may affect dispersal and gene flow between populations, thus potentially also acting at longer, evolutionary time scales. This general principle has been articulated in different theoretical frameworks (see e.g. Grime, 1977 for plants, or the "habitat templet" of Southwood, 1977 for terrestrial invertebrates), but it has been difficult to precisely identify which habitat characteristics determine these constraints, especially those promoting dispersal, divergence and speciation. Some of the best studied examples include long-term habitat stability, especially in aquatic systems, both marine (mediated through the type of larval development, e.g. Emlet, 1995) and freshwater (Ribera & Vogler, 2000; see Ribera, 2008 for a review). In terrestrial systems, likely due to their higher complexity, it has been more difficult to identify habitat characteristics linking processes affecting individuals or populations to those affecting metapopulations or species, and ultimately whole lineages (but see Papadopoulou *et al*., 2009 for an example with Coleoptera).

A system that potentially may simplify this complexity is the deep subterranean environment. In the deep parts of caves and the associated network of voids and fissures the environmental conditions of the habitat are extremely constant and homogeneous, with a permanent darkness and nearly constant humidity and temperature through the year, which is approximately equal to the average annual temperature of the surface (Racovitza, 1907; Poulson & White, 1969; Culver & Pipan, 2009). Subterranean organisms have a very limited range of options to take advantage of local climatic heterogeneities, and their general lack of mobility reduces the possibility of migration when conditions become unfavourable. In addition, the general scarcity of resources in the deep subterranean environment imposes stringent requirements on the species, resulting in simple communities showing low local diversity (Poulson & Culver, 1969; Culver, 1976).

The subterranean environment is a discontinuous medium with a highly variable degree of connectivity, and populations of troglobitic species are in general more fragmented than populations of species living on the surface (Crouau-Roy, 1989; Culver & Pipan, 2009). Due to the difficulty to disperse, gene flow among geographically close populations can be very restricted, resulting in a strong micro-endemism (Juberthie *et al*., 1980; Faille *et al*., 2015a,b). These conditions offer a particularly favourable

92 situation for exploring the relationship between environmental factors and the genetic
93 structure of populations, as due to the geographical proximity and the generally
94 homogeneous environment the number of confounding variables should be greatly
95 reduced.
96 One of the main factors potentially constraining dispersal within the
97 subterranean environment is the karstificability of the substratum, which is highly
98 dependent on its lithology and geochemical composition. The role of geological barriers
99 (i.e. non karstificable strata) has been traditionally recognised as a factor shaping the
100 distribution and evolution of the subterranean fauna (e.g. Bellés, 1973; Bellés &
101 Martínez, 1980), and the degree of fragmentation of the karst has recently being related
102 with ongoing speciation processes in some Pyrenean subterranean species (Faille *et al*.,
103 2015a,b). These studies, however, did not establish a direct link between the geology of
104 the substratum and the genetic structure of the studied species, and the factors
105 potentially affecting dispersal could not be identified with precision. Ideally, the role of
106 karstificability in determining dispersal and genetic structure should be investigated
107 with populations of the same species in different geological substrata, but otherwise
108 with similar general characteristics and with the same biogeographic history.
109 Here we study a system that matches these conditions, the troglobitic species
110 *Troglocharinus ferreri* (Reitter) (Coleoptera, Leioididae, Leptodirini) in the
111 subterranean environment of the Garraf massif, in the vicinity of Barcelona (NE of the
112 Iberian peninsula). In a previous study we found that this strictly subterranean genus
113 expanded its range from the central Pyrenees to the coastal area of Catalonia in the early
114 Pliocene, where it subsequently diversified during the late Pliocene and the Pleistocene
115 (Rizzo *et al*., 2013). Within the coastal clade of the genus *Troglocharinus*, *T. ferreri* is
116 distributed in the Garraf massif, isolated by Pleistocene sedimentary basins of three
117 rivers (Llobregat in the north-east, Anoia in the north and Foix in the southwest, Fig. 1).
118 Preliminary results suggested a high level of genetic diversity within *T. ferreri*,
119 consistent with the complex geological structure of the plateau of the Garraf massif
120 (Rizzo *et al*., 2013), with one mitochondrial lineage mostly associated with a more
121 homogenous limestone of Cretaceous origin and its sister (including a recognised
122 subspecies, *T. ferreri pallaresi* Bellés) inhabiting also some areas with Jurassic and
123 Triassic dolostone. Dolomite ($CaMg(CO_3)_2$) has a similar structure to calcite but with
124 approximately half of its Ca replaced by Mg. The dissolution rate of dolomite is
125 considerably lower than that of calcite (Goldscheider & Drew, 2007), and in a recent

study Appelo & Postma (2005) estimated that under the same chemical and physical conditions of the water it takes more than 100 times longer to reach 95% saturation for dolomite than for calcite. In addition, erosion in dolomitic karsts results in fine sand that fills sinkholes and caves, partially blocking the ground water circulation and further reducing the development of a subterranean medium (Renault, 1970; Gilli, 2015). This lower karstificability should result in an environment less permeable for the subterranean fauna, with impeded dispersal and reduced contact between populations in different areas of the massif.

Using a comprehensive sample of *T. ferreri* across the different geologic layers found through its entire geographic range we investigate the relationship between the karstificability of the substratum and the genetic diversity and structure of the populations living on it, which may be an indication of differences in the rate of dispersal between them. Since we compare individuals in a reduced geographical space, originating from the same colonization event and with a shared evolutionary history and the same ecology, biology and physiology, it is expected that differences among them are mainly the result of the geology of the substratum in which they are found. Our specific objectives are (1) determine the phylogeographic structure and demographic history of *T. ferreri* within its entire distributional range; (2) reconstruct the direction and frequency of the transitions between different types of substratum; and (3) compare the genetic structure of populations within geological layers with different degrees of karstificability.

MATERIALS AND METHODS

Geological and taxonomic background

The Garraf Massif is a calcareous horst with an area of around 500 km^2 in the north-east of the Iberian Peninsula, 30 km south-west of the city of Barcelona. It is part of the Catalonian littoral cordillera, composed mainly of Jurassic and Cretaceous limestone and dolostone and isolated by Quaternary sedimentary layers from the rest of the coastal area (Daura *et al*., 2014; Figs 1,2). The central part of the massif is dominated by Cretaceous limestone rocks (Moreno, 2007), where most of the karst formations have developed, while the Jurassic dolomite strata in the high plains present less significant karst development and fewer dolines (Rubinat, 2004). Outcrops of Cretaceous marls and marly limestone are also found in a few areas (Moreno, 2007), while the Triassic carbonates comprise strata that only crop out in the north-east of the Garraf Massif rim

(Fig. 2). There are more than three hundred shafts documented in the central Garraf Massif, which have been explored since the late 19th century (Cardona i Oliván, 1990), in which the fauna is well documented due to the long biospeleological tradition of the city of Barcelona (e.g. Zariquieyi, 1917; Lagar, 1954; Español, 1961; Bellés, 1973).

Troglocharius ferreri currently includes three recognised subspecies, *T. ferreri ferreri*, *T. ferreri pallaresi* and *T. ferreri abadi* Lagar, all restricted to the Garraf Massif (Salgado *et al*., 2008; Fresneda & Salgado, 2017). Four other subspecies were described based mostly on small differences in the shape of the pronotum and the antennae, but are now considered to be synonyms of *T. ferreri ferreri* due to the presence of multiple populations with intermediate morphologies (Salgado *et al*., 2008; Appendix S1a). The two recognised subspecies were maintained in part due to the geologic isolation of their populations (Salgado *et al*., 2008). *Troglocharinus ferreri ferreri* is known from 123 cavities distributed through the Garraf Massif (Fresneda & Salgado, 2017; Appendix S2a), although this number may certainly increase with additional explorations. The other two subspecies have, however, very restricted distributions: *T. ferreri abadi* is only known from three cavities in close proximity situated in the northwest of the Massif, and *T. ferreri pallaresi* from four cavities in the north-east, also in close proximity (Fresneda & Salgado, 2017).

Taxon sampling and DNA sequencing

We sequenced a total of 126 specimens of *T. ferreri ferreri* from 40 shafts distributed through its entire known distribution area, and three *T. ferreri pallaresi* from the type locality (Avenc de Montmany) (Fig. 2; Appendix S1a). Caves were chosen with the aim to include the whole range of geographic, geologic and taxonomic diversity, including the type locality of the species (Avenc d'en Roca) and those of the synonymised subspecies, of which we obtained two (Appendix S1a). Despite several attempts we could not obtain specimens of *T. ferreri abadi* for study, and we are not aware of recent records (the last collected specimens to our knowledge being from Cova Miserachs in 1987, J. Comas personal communication, 2015). The three shafts in which the subspecies is known are very dry, and the specimens, always in reduced numbers, have only being found in the deepest, more humid areas (Salgado *et al*., 2008).

Specimens were collected in caves with the use of baits laid 24h in advance or through manual searches. We sequenced five specimens of each cave whenever available (Appendix S1a). Virtually all cavities in the Garraf Massif are vertical shafts

with no or very limited horizontal development, and in most cases beetles were collected at the base of the shaft, so the coordinates of the entrance are a good approximation to the actual location of the samples. To increase the statistical power of some analyses of molecular diversity we pooled specimens from caves known to be part of the same system, connected through the subterranean medium and without any apparent geological discontinuity between them (Fig. 2; Appendix S1a).

DNA extractions of single specimens were non-destructive, using commercial kits (mostly DNeasy Tissue Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. Vouchers and DNA samples are kept in Institute of Evolutionary Biology, Barcelona (IBE). We amplified fragments of five mitochondrial and four nuclear genes in eight amplification reactions: (1) 5' end (the "barcode", *cox1-5'*) and (2) 3' end of the cytochrome c oxidase subunit (*cox1-3'*); (3) an internal fragment of the cytochrome oxidase b (*cob*); (4) 5' end of the large ribosomal unit plus the Leucine transfer plus the 3' end of NADH dehydrogenase subunit 1 (*rrnL+trnL+nad1*); (5) 5' end of the small ribosomal unit, 18S rRNA (*SSU*); (6) an internal fragment of the large ribosomal unit, 28S rRNA (*LSU*); and one fragment each of (7) the histone H3 (*H3*) and (8) wingless (*Wg*) (see Appendix S2b for the primers used, and Appendix S1a for the specimens sequenced for each of the fragments). Owing to the generally low levels of variability found in preliminary analyses, only a limited sample of specimens was sequenced for the nuclear markers (Appendix S1a). New sequences (285) were deposited in the EMBL database under accession numbers LT797170- LT797446, LT799421- LT799428) (Appendix S1a).

Phylogenetic and phylogeographic analyses

We aligned the gene fragments with MAFFT 6 (http://mafft.cbrc.jp/alignment/ server). Protein-coding genes were aligned using the FFT-NS-1 algorithm, and ribosomal genes with the Q-INS-i algorithm, which considers the secondary structure (Katoh *et al.*, 2009).

To reconstruct the general phylogenetic relationships and time of divergence among the main lineages within *T. ferreri* we built a data matrix with all mitochondrial and nuclear markers, using as outgroups a selection of 26 specimens from both the coastal and Pyrenean clades of *Troglocharinus* following the topology obtained in Rizzo *et al*. (2013) (Appendix S1a). We reconstructed phylogenetic relationships with RAxML 7 (Stamatakis *et al*., 2008) using GTR+I+G as an evolutionary model and a

partition by gene, separating the two *cox1* fragments (due to the unequal sampling) and pooling the two mitochondrial ribosomal genes (*rrnL+trnL*). The optimum topology was that of the best likelihood amongst 100 replicates, and node support was estimated with 500 bootstrap replicates using the CAT approximation (Stamatakis *et al*., 2008). We analysed the nuclear sequence data separately using the same methods.

To obtain an estimation of the divergence time we analysed the matrix in BEAST 1.8 (Drummond *et al*., 2012), using as priors the rates obtained by Cieslak *et al*. (2014) for the same group of organisms and genes, calibrated using the tectonic separation of Sardinia from mainland Europe ca. 33 Ma (Schettino & Turco, 2006). We used an uncorrelated relaxed clock with normally distributed prior average rates of 0.015 substitutions/site/MY for mitochondrial protein coding genes (*cox1-5*, *cox1-3*, *cob*, *nad1*), 0.004 for nuclear ribosomal (*LSU*, *SSU*), and 0.006 for mitochondrial ribosomal genes (*rrnL+trnL*), all of them with a standard deviation of 0.001. For the nuclear protein coding genes (*H3*, *Wg*) we used default flat priors. We constrained the monophyly of the coastal and Pyrenean clades respectively, following the topology obtained in Rizzo *et al*. (2013) and ran two independent analyses for 200 million (M) generations, logged every 5000, with 20 M (10%) as burn-in fractions. Preliminary analyses with the complete matrix using either a Yule speciation (YL) or a Birth-Death with incomplete sampling model (BD, Stadler, 2009) failed to converge, so we used a sample of 27 specimens of *T. ferreri* with complete sequence data, including all main lineages as identified in the RAxML analysis and a wide representation of different caves and types of substratum. We used a GTR+I+G and HYK evolutionary models for each of the two mitochondrial and nuclear partitions respectively, and TRACER 1.6 (Drummond *et al*., 2012) to assess convergence, measure the effective sample size of each parameter and check that the used burn-in fraction was sufficient. We run two different sets of analyses using YL or BD models, comparing their likelihoods using 100 replicas of the Akaike's Information Criterion for MCMC samples (AICM) statistic (known to perform better than harmonic mean estimators, Baele *et al*., 2013) in TRACER 1.6.

Demographic history

To estimate the demographic history of *T. ferreri* we used the mitochondrial *cox1-5* fragment only, for which the sampling was most complete and presented sufficient variation, with a GTR+I+G evolutionary model and an a-priori mean rate of 0.015

substitutions/site/MY with a standard deviation of 0.001. We run two analyses in BEAST 1.8, one with a strict clock and a second with a relaxed lognormal, and comparing their likelihoods using 100 replicas of the AICM statistic. Analyses were run for 100 M generations and convergence and burn-in fraction were assessed as in previous BEAST analyses.

We compared four demographic models implemented in BEAST 1.8: constant population size (CT), population expansion (ES), logistic growth (LG) and exponential growth (EL). We also constructed a Bayesian skyline plot (BSP, Drummond *et al*., 2005) with the results of a separate analysis. The likelihood of the trees built with the different models were compared with 100 replicas of the AICM statistic.

Estimation of transition rates between different geological substrata

To estimate the transition rates between different geological substrates we assigned each of the caves to different categories according to the dissolvability of the substratum in which they are found, as estimated with detailed lithological maps (ICC, 2010; Appendix S1a). We divided the caves in two categories: (1) Cretaceous limestone and (2) Jurassic and Triassic dolostone. Due to the lower number of dolostone caves we pooled all dolostone caves in a single category. We did an ancestral trait reconstruction in BEAST 1.8, with the geological type of the cave as a discrete character and a reduced *cox1-5* matrix randomly selecting one specimen per cave when multiple haplotypes in the same cave were monophyletic (Appendix S1a). We used a single partition with a simple HKY model with estimated nucleotide frequencies, as preliminary analyses showed convergence problems when using more complex models. We set up an asymmetric discrete phylogeographic (CTMC) model (Lemey *et al*., 2009), using as priors a uniform distribution between 0 and 1 for the frequencies, and a Gamma with shape 1 and scale 1 for the rates. We also implemented the best clock and demographic model as estimated above and uniform root frequencies for the trait. The analyses were run for 100 M generations, and convergence was assessed as in the previous analyses.

Genetic versus geologic or geographic distances

To estimate the role of the lithology in the genetic isolation among *T. ferreri* populations we compared the relationship between phylogenetic and geographical distances in populations in dolostone and limestone. Given the heterogeneity of the spatial distribution of dolostone and limestone in the Garraf massif, in addition to the

simple geographic distance between caves we also used a weighted measure taking into account the permeability to the subterranean fauna of the different geological substrata in between them.

Phylogenetic distances were obtained in MESQUITE 3.8 (http://mesquiteproject.org) from the ultrametric tree obtained with the *cox1-5* sequence, using the best demographic and clock models as estimated previously. Geographic distances were measured from the cave entrance and specimens from the same cave were considered to have a geographic distance of zero. These distances were expressed in decimal degrees and calculated in ARCGIS 9.3 (ESRI, Redlands, CA, USA). For the geological data we used the "Mapa de grups litològics de Catalunya LITO250M_v1 (2010)" downloaded from the Institut Cartogràfic i Geològic de Catalunya (www.igc.cat), converted into a raster file at a resolution of 0.001º (c. 90 m). We pooled all dolostone caves (Jurassic and Triassic) in a single category, and added two non-karstificable layers (with no known caves), recognising a total of four layers: (1) Cretaceous limestone; (2) Jurassic and Triassic dolostone; (3) sedimentary rocks with no or few carbonated constituents (mainly marly limestone); (4) metamorphic or sedimentary siliceous rocks (marls, slate) (Fig. 2; Appendixes S1a,S2c). We assigned a value of geological resistance to each one of these categories as a proxy of the suitability for the dispersal of the subterranean fauna, with lower values for cells with higher geologic permeability (i.e. low resistance). We tested the sensitivity of our results to different values of resistance by trying five different combinations, always maintaining the ranking of substratum resistance (1<2<3<4) (Appendix S2c).

We used the software CIRCUITSCAPE 2.2 (McRae, 2006; McRae *et al.*, 2008) to estimate the pairwise connectivity (or current flow) between the cells with observed presences (i.e. caves with fauna) according to the five resistance maps (Appendixes S2c,d). CIRCUITSCAPE uses circuit theory to model connectivity in heterogeneous landscapes. Landscapes are represented as conductive surfaces, with low resistances assigned to landscape features types that are most permeable to movement or best promote gene flow, and high resistances assigned to movement barriers. We selected the pairwise option so that connectivity, or current flow, was calculated between all pairs of observed presence cells, and these pairwise current maps were then overlapped (and averaged) to obtain a single cumulative current (or connectivity) map (Appendix S2d).

The values of these five cumulative connectivity maps were then correlated (Pearson's correlation coefficient) with genetic distances using a Mantel test with 1000 randomizations, to estimate whether the phylogenetic distance between populations in caves in different substratum was best explained by geographic distance alone or by a compound measure of geologic isolation and geographic distance.

Genetic diversity and population structure

We compared population genetic statistics between caves from different geological substrata, to test if their different karstificability resulted in significant differences in dispersal among populations that could be reflected in their current genetic characteristics. Caves were classified as being on limestone or dolostone as described above (Appendix S1a). To increase the number of specimens per population, when caves were in the same substratum, in close proximity and within the same geological unit (i.e. without any geological or lithological discontinuity) we pooled individuals within the same clade following the topology of the previous phylogeographic analyses (Appendix S1a). We collapsed the *cox1-5* haplotypes of each population in Mesquite v3 and estimated *Fst* values between caves in ARLEQUIN v3.5 (Excoffier *et al*., 2005). We then compared the slope of the regression between geographic distance and *Fst*. If due to the lower permeability of the substratum nearby caves in dolostone are more isolated between them than caves in limestone, the *Fst* value should increase at a higher rate with geographic distance, resulting in a stepper slope. For each cave we identified all caves within a radius of 5 km using ARCGIS 9.3 and plotted the geographical distance (km) against the *Fst*. We separated three types of caves pairs considering the type of substratum, limestone-limestone, limestone-dolostone and dolostone-dolostone, and estimated the slope of the linear regression for each of them.

RESULTS

Phylogenetic and phylogeographic analyses

The complete final data set (Appendix S1a) included 5173 aligned nucleotides, with no length variation except for some regions in the *LSU* gene and a variation of one or two Asparagine residues in a repetitive region in the gene *Wg*. The topology of the coastal clade of *Troglocharinus* was very similar to that obtained in Rizzo *et al*. (2013), with *T. ferreri* monophyletic and sister to the rest of the species with very good support. Within *T. ferreri* two well-supported clades were recovered (clades 1 and 2), although with

only moderate support for most of the internal nodes in each of them (Appendix S3a). The nuclear sequence only recovered a monophyletic *T. ferreri*, although with low support, and with no support for internal relationships (Appendix S3b).

In the calibrated analysis in BEAST with a representative sample of *T. ferreri* the best tree model was BD (AICM YL= 28674.8, BD= 28625.4), although with a poorer convergence. The topology and age estimations of both analyses were, however, almost identical, with changes in some poorly supported terminal nodes (Appendix S3c,d). The separation between the coastal and Pyrenean clades was estimated to have occurred at the end of the Miocene-early Pliocene (5 +/- 1 Ma), with the separation between *T. ferreri* and the rest of species in close proximity (4.5 +/-1 Ma). The sampled *T. ferreri* had a last common ancestor in the transition Pliocene-Pleistocene (2.6 +/- 0.6 Ma), and most of the variation within the different lineages of *T. ferreri* had a Middle to Late Pleistocene origin (Appendix S3c).

Demographic history

The analysis of the *cox1-5* data (129 specimens, Appendix S1a) assuming a strict clock had better AICM than when assuming a lognormal clock (AICM strict= 4072.6; relaxed= 4084.2). In all subsequent analyses using the *cox1-5* data we thus implemented a strict clock.

The best demographic model was constant population size (CT) (Table 1). The Bayesian skyline plot (Fig. 3) suggested no substantial changes for most of the history of the species, with a rapid population growth starting at ca. 250-300 Kya and a pronounced shift at <100 Kya. In all subsequent analyses we implemented a CT model.

The Bayesian tree obtained with a strict clock and a CT coalescent model had good support for the two main clades within *T. ferreri* (Fig. 4), but only moderate or poor support for most of the lineages. Most haplotypes found in single caves were either exclusive or only found in other caves in close geographical proximity. Only one cave had individuals with mitochondrial haplotypes in the two main clades, the Avenc San Cristofol, and only three had individuals with haplotypes in different well supported lineages within each of the clades (Cuneta, Sogre and Bufi) (Figs 2,4). All four caves with individuals of mixed origins were in limestone (Appendix S1a). Of the 13 caves in dolostone, none had individuals with haplotypes in different main lineages, although due to the higher number of limestone caves (28) differences were not significant as measured with a Fisher's exact test in a 2x2 contingency table.

Estimation of transition rates between geological substrata

The reduced matrix selecting one specimen per cave when all sampled specimens were monophyletic had 82 haplotypes (Appendix S1a). The most likely lithology of the ancestral population was reconstructed as limestone, with a probability of 0.74 over 0.24 dolostone (Fig. 4). Similar probabilities were reconstructed for clade 2 (0.6 versus 0.4), while clade 1 was clearly reconstructed as having originated from limestone caves (0.98, Fig. 4). Within clade 1 there were some transitions to Jurassic or Triassic dolostones, but all very recent and with only one potential reversal to limestone. This reversal affected two caves (Roca and Rigol) at ca. 1.3 km from each other but in different geological substratum (limestone and Jurassic dolostone respectively), which populations had specimens with mixed haplotypes (Fig. 4). Within clade 2 there was one genetically and geologically isolated lineage sister to the rest, *T. ferreri pallaresi* in Montmany cave, in Triassic dolostones (Fig. 4). The remaining specimens were grouped in two poorly supported clades, one entirely on limestone caves (clade 2A) and the other reconstructed as having originated in Jurassic dolostone with only 56% probability (clade 2B). Within the later there was only one reversal to limestone, affecting two caves (Cristofol and Bufi) also in close proximity (ca. 0.6 Km) and surrounded by dolostone. In both caves there were also specimens with haplotypes belonging to other clades in limestone caves (Fig. 4), suggesting that they have a fauna of mixed origin.

There were large differences in the geographic setting of the different clades. Clade 1 (limestone, with only some caves in Jurassic dolostone) occupies a large geographic area within the Garraf massif (mean geographic distance between caves of 7.0 km); while the two lineages within clade 2 (2A in limestone and 2B in Jurassic dolostone) both occupy a restricted area with a mean geographic distance between caves in each of them of 1.2 km (Fig. 2).

The estimated transition rate from limestone to dolostone was ca. three times that of dolostone to limestone (1.46 vs. 0.51), although with largely overlapping marginal probability distributions (Fig. 4).

Genetic versus geologic or geographic distances

The correlation between geographic and phylogenetic distance was strongly significant both for the whole *T. ferreri* and for the two clades separately, with or without the

inclusion of *T. ferreri pallaresi* (Montmany cave) (Table 2). None of the schemes with different values for the geological resistance could improve the correlation obtained with the simple geographical distance (Table 2), although the best (also with highly significant correlations) was the one with the highest resistance values for sedimentary and metamorphic rocks, which do not have void interstices (Table 2, Appendix S2c). The phylogenetic distances within the two main clades were very similar but, as noted above, clade 1 covers a much wider area (Fig. 2), which resulted in significantly different slopes of the regression lines between genetic and geographic distances (Table 2). Thus, in clade 2, with both dolostone and limestone caves, the same phylogenetic distances were attained at much shorter distances than in clade 1, mostly with limestone caves.

Genetic structure in populations in limestone vs dolostone

The relationship between geographical distance and *Fst* for caves closer than 5 km was non significant for pairs of caves in limestone (N= 188, y = 0.38+0.04x; p = 0.37; r^2 = 0.02). This relationship was significant for pairs of caves in different substratum (limestone-dolostone; N= 146, y = 0.39+0.07x; p = <0.01; r^2 = 0.06), and especially for pairs of caves in dolostone, with a higher slope (N= 22, y = 0.28+0.14x; p<0.001; r^2 = 0.50).

DISCUSSION

Despite the reduced extension of its geographic range, *T. ferreri* displays a strong phylogeographic structure, as could be expected from a strictly subterranean species with very limited dispersal capabilities (Kane *et al*., 1992). This phylogeographic variation, together with the general homogeneity of the subterranean environment and the lack of climatic differences due to its limited geographic range, render subterranean species ideal systems to study the effect of physical constraints imposed by the habitat (see e.g. Faille *et al*., 2015a,b for examples with a different group of Coleoptera).

According to our reconstruction, and in agreement with Rizzo *et al*. (2013), the ancestor of the coastal clade of the genus *Troglocharinus* colonized the area at the end of the Pliocene-early Pleistocene, likely in a window of favourable climatic conditions before the onset of the Mediterranean climate ca. 3.2 Ma (Suc, 1984). This range expansion from the ancestral area in the Pyrenees to the coast may have involved surface displacements, as there is no continuity in the subterranean medium between the

Pyrenean and coastal areas (Rizzo *et al*., 2013). The resistance of the species of *Troglocharinus* to relatively high temperatures (up to 20-23ºC, Rizzo *et al*., 2015) would allow surface displacements in periods with low seasonality and high precipitation, such as the Pliocene-Pleistocene transition (Suc & Cravatte, 1982; Jiménez-Moreno *et al*., 2010). The subsequent formation of sedimentary basins in the rivers surrounding the Garraf massif likely contributed to the isolation of the populations that would become the current *T. ferreri* (Rizzo *et al*., 2013).

The demographic reconstruction suggested a constant population size for most of the history of the species but with a fast population expansion starting at ca. 0.3 Ma. This expansion is in agreement with the available data of the development of the subterranean environment in the Garraf massif (Daura *et al*., 2014). According to these authors, the caves in the Garraf plateau have an endogenous origin, and the subterranean network of cavities and fissures did not developed completely, opening to the surface, before the middle-upper Pleistocene. The accessibility to an extensive subterranean system in the massif could have allowed the population expansion of *T. ferreri*. It must be noted that the ancestor of the coastal clade of *Troglocharinus* (and thus of *T. ferreri*) must have had all the morphological and physiological modifications currently associated with the subterranean life (Rizzo *et al*., 2013; Cieslak *et al*., 2014), so it could be expected that the subterranean environment was occupied whenever it become accessible.

We found a highly significant linear correlation between geographic and phylogenetic distance in all main lineages within *T. ferreri,* despite the reduced geographic area. This correlation is habitually interpreted as isolation by distance (Slatkin, 1993), without dispersal barriers or corridors that could respectively increase or decrease isolation with independence of geographic distance. Within the Garraf Massif the subterranean environment is not continuous, as there are geological discontinuities and non-calcareous layers which in principle should act as barriers to dispersal through the underground. This was apparent in the case of the subspecies *T. ferreri pallaresi*, isolated from the rest of the populations by a layer of non karstificable Triassic Keuper marl (ICC, 2010). However, and contrary to our expectations, the attempt to weight geographic distances with a-priori values for the permeability to dispersal of the different substrata did not result in any improvement in the correlation with phylogenetic distance, although the best result was obtained with the highest resistance values for non-karstificable materials (sedimentary and metamorphic rocks).

It must be considered that, although highly significant, this correlation was relatively small, with low slopes and a high dispersion except when the geographically and genetically very isolated population of *T. ferreri pallaresi* was included in the analyses. The low variance explained by distance alone suggests a more complex scenario, with possibly a number of uncontrolled factors determining dispersal acting at small scales, although it may just be that our exploration of the parametric space was insufficient.

Other than the possible existence of what could be considered strong barriers, preventing all movement through the subterranean habitat, the different composition of the substratum did also influence the dispersal capabilities and in consequence the genetic structure of the studied populations. The Garraf Massif is broadly divided in two bands of different composition perpendicular to the coast, limestone in the south and dolostone in the north (Fig. 2). Our results suggest that the initial colonization was in limestone, more soluble and thus likely to have developed a subterranean network of fissures earlier than the dolostone. The initial colonization of limestone may have biased the estimation of the transition probabilities (Davis *et al*., 2013), which had also wide marginal distributions likely due to the low number of dolostone caves. But in any case, our results indicate a much higher rate of transitions from limestone to dolostone than vice-versa, suggesting that limestone is more permeable to the movements of the subterranean fauna. This higher permeability agrees with the fact that in most dolostone caves only closely related specimens were found, in many cases forming a monophyletic lineage exclusive of a cave or a group of caves in close geographic proximity, although differences with limestone caves were not statistically significant. It is interesting to note that the limestone caves with fauna of mixed origin were all in the same area, and in close proximity of the caves in dolostone inhabited by populations of clade 2 (see the location of the caves in Fig. 2). There is the possibility that other factors favoured the transit of the subterranean fauna in this area, including some early transitions from limestone to dolostone in this clade. The high number of tectonic faults (ICC, 2010) may be one of these factors.

The general relationship between phylogenetic and geographic distances was also indicative of differences between the permeability of dolostone and limestone to the movement of the subterranean fauna. Thus, the clade with the highest proportion of dolostone caves (clade 2) had a steeper slope than the clade mostly on limestone caves (clade 1), suggesting a lower permeability to the displacement of the fauna. A similar result was obtained for the degree of genetic isolation (as measured with the *Fst*)

between the population of a cave and that of its closest neighbours. For caves in dolostone *Fst* values increased much faster with distance than when caves were in limestone, clearly indicating a stronger isolation likely due to the increased difficulty of displacement through the substratum.

There are few systems in which it is possible to trace the effect of a physical constraint from the individual to the macroevolution of the lineage. In this sense, the highly fragmented subterranean habitat offers an excellent opportunity, with the additional advantage of its abiotic and biotic simplicity and stability. Knowing the factors leading to the origin of the current diversity can greatly help to inform management and conservation decisions, but the lack of data of both genetic diversity and population viability in this unique system hampers our understanding of the potential effect of environmental changes (Rizzo *et al*., 2015; Sánchez-Fernández *et al*., 2016).

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686
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688

SUPPORTING INFORMATION

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

Appendix S1 Additional materials:

(a) List of the studied material.

Appendix S2 Additional methods:

(a) Location of caves in the Garraf.

(b) Primers used in the study.

(c) Categorisation of geological substrata and resistance.

(d) Resistance maps.

Appendix S3 Additional results:

(a) Best tree with mitochondrial and nuclear markers.

(b) Best tree with only nuclear markers.

(c) Tree obtained with a Yule speciation.

(d) Tree obtained with a birth death with incomplete sampling.

BIOSKETCH

Valeria Rizzo is a PhD student in the Institute of Evolutionary Biology in Barcelona. This paper is part of his thesis dissertation, focussed on the evolution of a lineage of subterranean beetles (genus *Troglocharinus*).

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Author Contributions: V.R. and I.R. conceived the work; V.R., J.P. and I.R. led the specimen and data collection; V.R. and R.A. obtained the molecular data; V.R., D.S-F. and I.R. analysed the data; V.R. and I.R. led the writing and all authors contributed to the discussion of results and writing.

TABLES

Table 1 Value of Akaike's information criterion for MCMC samples (AICM) of the four tested demographic models. CT, constant population size; ES, population expansion; LG, logistic growth; EL, exponential growth. Lower values of AICM indicate a better adjustment to the model.

model	AICM	stdv.	ΔAICM
CT	4008.9	0.08	-
ES	4013.3	0.14	-4.4
LG	4046.7	0.21	-37.8
EL	4012.6	0.10	-3.7

Table 2 Linear regression between phylogenetic distances, Euclidean geographic distances (in decimal degrees) and distances weighted by resistance schemes A, B & E (values for C&D were intermediate between B&E, data not shown). See Appendixes S2c,d for the resistance values applied to the different geologic substrata; and Appendix S1a and Fig. 4 for the composition of the clades (clade "2(A+B)" refers to Clade 2 with the exclusion of *T. ferreri pallaresi*).

distance	clade	intercept	SE (i)	slope	SE (s)	r2	p
geographic	1	0.22	0.004	1.78	0.051	0.27	<0.01
	2	0.20	0.003	3.57	0.059	0.77	<0.01
	2(A+B)	0.18	0.004	4.91	0.233	0.32	<0.01
A	1	0.32	0.003	0.001	0.001	0.00	n.s.
	2	0.17	0.005	0.02	0.001	0.58	n.s.
	2(A+B)	0.29	0.005	0.01	0.001	0.11	n.s.
B	1	0.29	0.004	0.02	0.002	0.02	n.s.
	2	0.14	0.005	0.08	0.002	0.60	n.s.
	2(A+B)	0.20	0.005	0.04	0.003	0.16	n.s.
E	1	0.21	0.006	0.11	0.005	0.11	<0.01
	2	0.07	0.006	0.24	0.005	0.65	<0.01
	2(A+B)	0.15	0.006	0.13	0.007	0.27	<0.01

740 **FIGURE LEGENDS**

741

742 **Figure 1** Distribution of the coastal clade of the genus *Troglocharinus*, with the main
743 tectonic and geological substrata (modified from Rizzo *et al*., 2013). Black squares, *T.*
744 *ferreri*; circles, other species in the coastal clade. Colour codes: yellow, Tertiary and
745 Quaternary sedimentary basins; green, folded Mesozoic cover; pink and purple:
746 Hercynian substratum; grey: Quaternary filling of Miocene and Quaternary fractures.
747 Lines represent tectonic features in standard geological notation. Note the isolation of
748 the distribution area of *T. ferreri* by Quaternary sediments.

749

750 **Figure 2** Simplified geological map with the location of the studied caves (circles).
751 Blue, caves in clade 1; yellow, caves in clade 2A (limestone lineage); red, caves in
752 clade 2B (dolostone lineage); black, *T. ferreri pallaresi* (upper right corner). Caves with
753 mixed colours had specimens of different clades. Geological substratum: yellow,
754 dolostone; green, limestone; grey and pink, different non-karstificable materials
755 (modified from ICC, 2010).

756

757 **Figure 3** Bayesian skyline plot of the analyses of the *cox1-5* matrix of 129 specimens of
758 *T. ferreri*, assuming a strict clock with a rate of 0.015 substitutions/site/MY. Thin lines,
759 95% confidence intervals; horizontal axis, time (MY); vertical axis, effective population
760 size (N_eT).

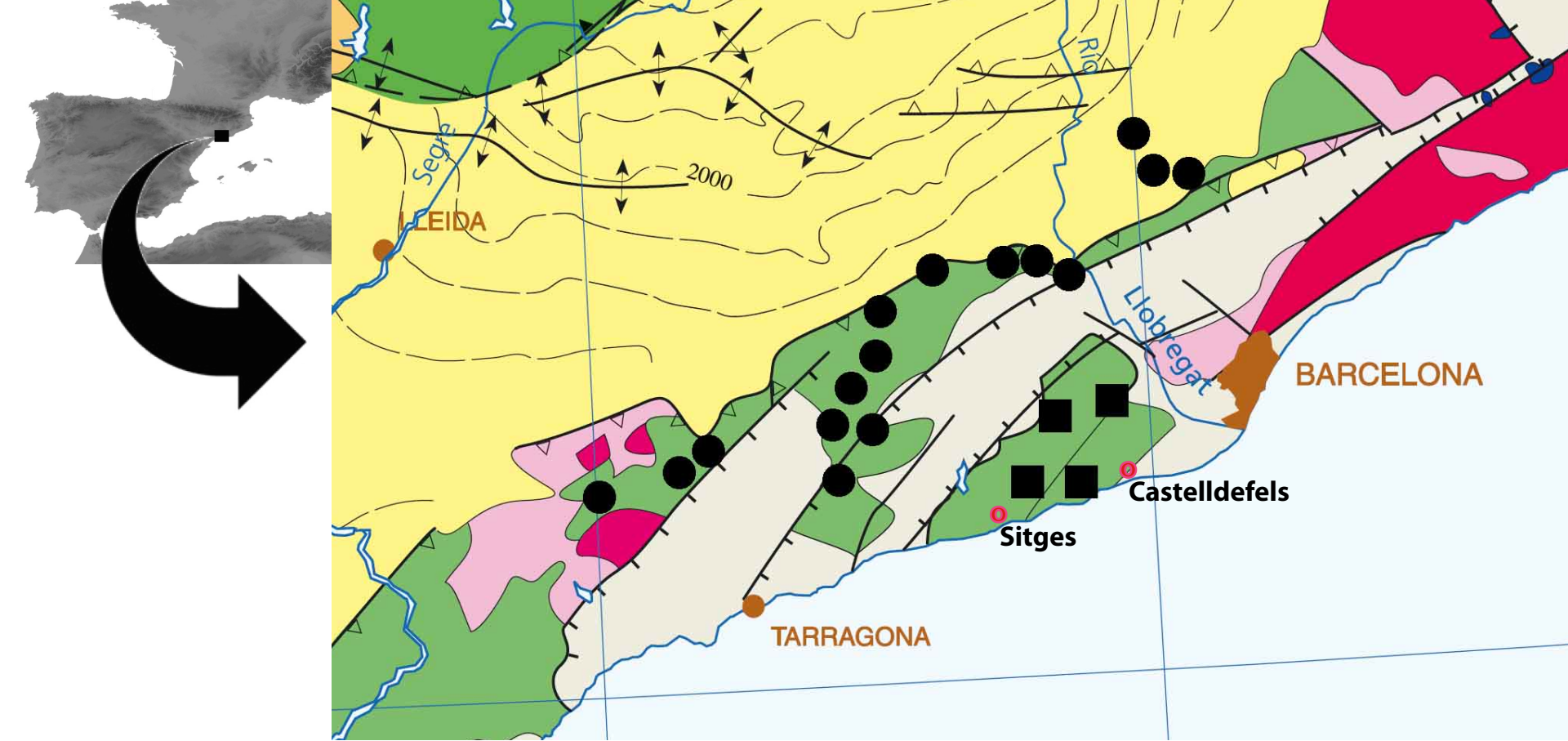
761

762 **Figure 4** Reconstruction of the ancestral lithology of the substratum in BEAST, using a
763 simplified *cox1-5* matrix and a strict clock with a rate of 0.015 substitutions/site/MY
764 and a constant population size. Numbers in nodes, probabilities of respectively
765 dolostone (red) and limestone (blue) (only when >0). With stars, specimens from caves
766 with fauna of mixed origin (two stars, different main clades; one, different subclades).
767 See Fig. 2 for the location of these caves. Insert: Marginal probability distribution of the
768 reconstructed transition rates from limestone to dolostone (grey) and dolostone to
769 limestone (blue). Habitus photograph by A. Messeger.

770

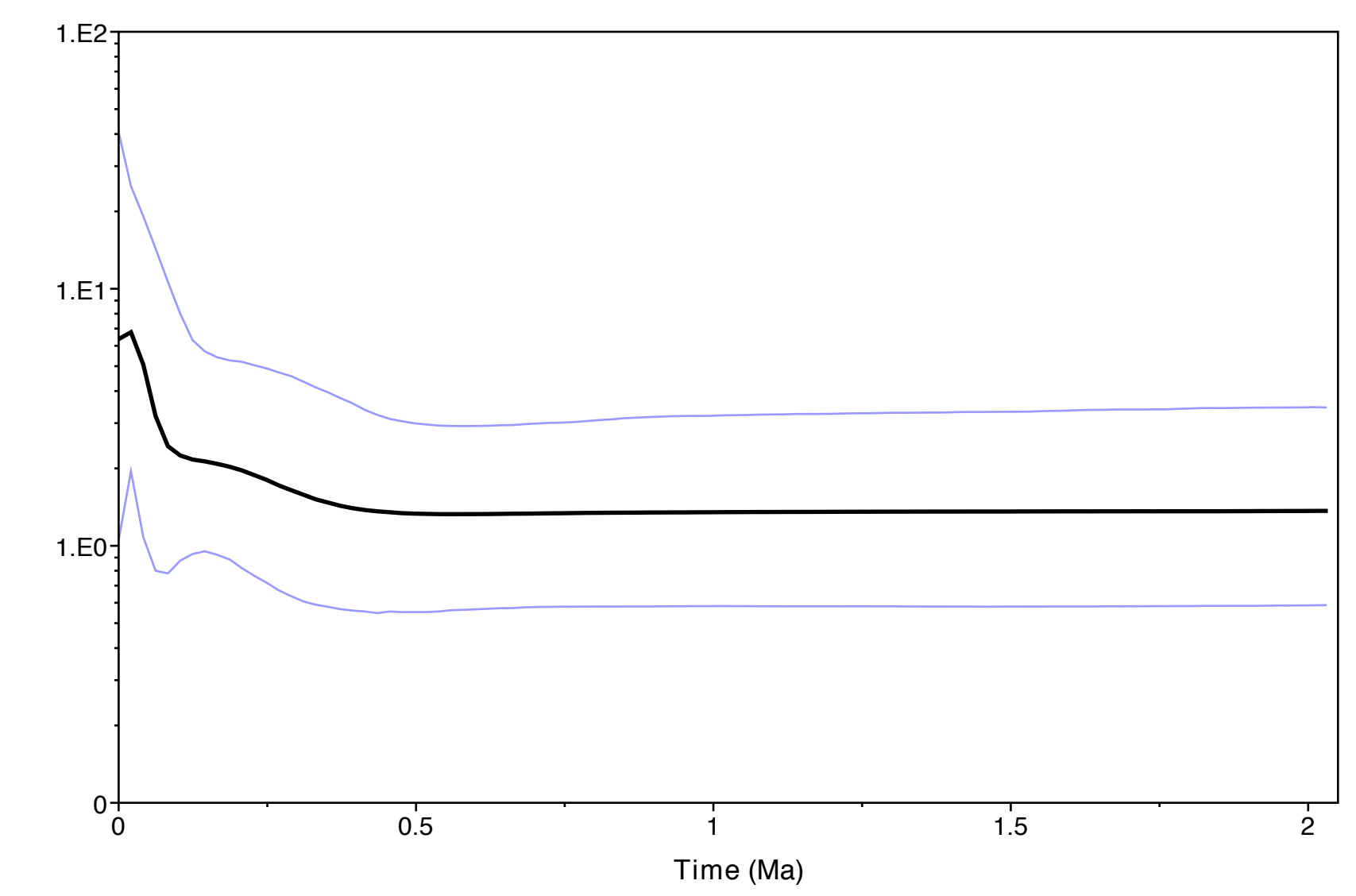
771

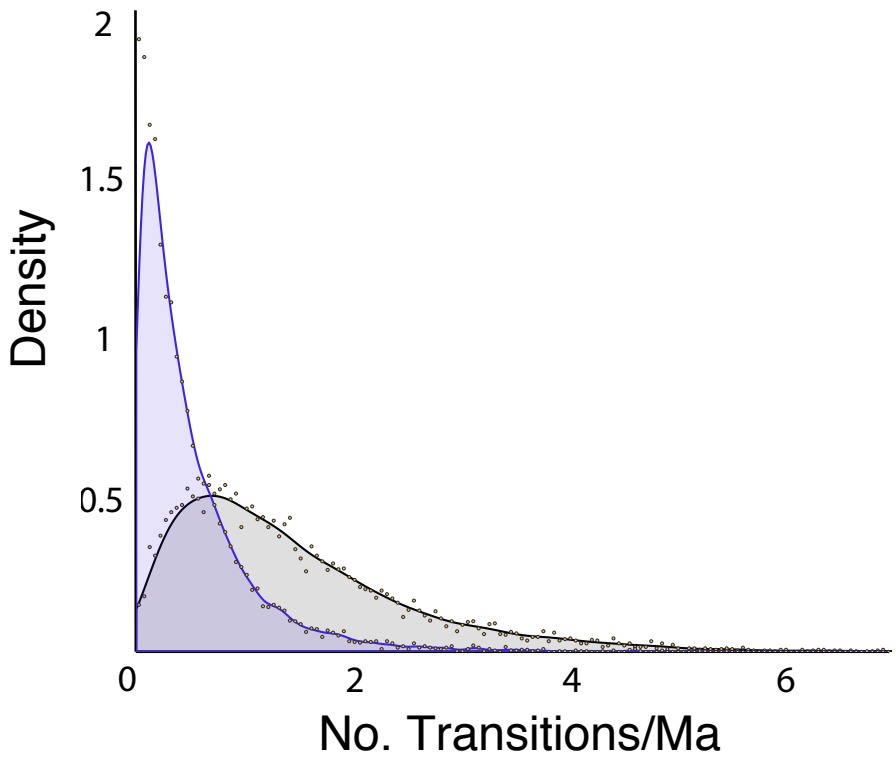
772


2000
Segre
LLEIDA
Llobregat
BARCELONA
Castelldefels
Sitges
TARRAGONA

Casteldefells

Sitges


1.E2
1.E1
1.E0
0
0
0.5
1
1.5
2
Time (Ma)



Clade 1

Clade 2

Clade 2A

Clade 2B

Troglocharinus ferreri

[0.26,0.74]

[0.43,0.57]

[0.36,0.64]

[0.59,0.41]

[0.56,0.44]

[0.53,0.47]

[0.97,0.03]

[0.04,0.96]

[0.02,0.98]

[0.01,0.99]

[0.03,0.97]

[0.06,0.94]

[0.23,0.77]

[0.12,0.88]

[0.11,0.89]

[0.07,0.93]

[0.92,0.08]

[0.01,0.99]

[0,1]

[1,0]

VR69Verdiell

VR68 Verdiell

AF172 Bardissa

VR70 Verdiell

VR44 Llinia

VR47 Llinia

VR118 Comes

VR62 Cuneta *

PB21 Pompeu Fabra

VR33 Verdaguer

RA1093 Rigol

PB22 Roca

RA1097 Rigol 2

RA1092 Roca

RA1098 Rigol 2

VR45 Llinia

VR46 Llinia

VR117 Esteles

RA1106 Geolegs

RA1107 Geolegs

VR104 Gori

RA1194 Gori

PB15 Cristofol **

RA1160 Marçal

VR119 Comes

PB1 Sogre *

VR105 Gori

RA1156 Pere

PB3 Pere

PB2 Pere

RA1108 Sadurnì

VR58 Marcel

VR81 Infern

RA1105 Pompeu Fabra

VR76 Vermell_1.939_41.347

RA1109 Sadurni

VR59 Marcel_1.938_41.351

RA1104 Pompeu Fabra

VR73 Vermell

RA1163 Marçal

RA748 Alzines Gran

VR108 Esquerra

VR111 Esquerra

VR112 Banderes

VR115 Banderes

PB11 Sogre *

VR60 Cuneta *

VR26 Corral Nou

VR48 Morgan i Comas

VR83 Pleta

VR27 Corral Nou

PB5 Serrano

VR128 Svabroc

VR49 Benjamin Digò

VR53 Benjamin Digò

RA1111 Trons

PB7 Trons

VR126 Svabroc

PB8 Trons

VR52 Benjamin Digò

RA1192 Pasant

VR82 Infern

RA1193 Pasant

VR90 Bufi *

VR80 Infern

PB6 Trons

VR50 Benjamin Digò

VR91 Bufi *

VR130 Troneda

VR41 Puigmolto

AI1065 Roc

VR55 Cabeza

PB14 Cristofol **

PB12 Cristofol **

VR92 Bufi *

VR42 Puigmolto

RA1100 Roc

RA1099 Roc

VR133 Troneda

RA1159 Pepi

VR31 Guerrillers

VR3 Montmany