

Phylogenetic relationships of Western Mediterranean subterranean Trechini groundbeetles (Coleoptera: Carabidae)

ARNAUD FAILLE*, ACHILLE CASALE & IGNACIO RIBERA

Phylogeny of Mediterranean hypogean Trechini
Faille *et al.*

Faille, A., Casale, A. & Ribera, I. (20xx) Phylogenetic relationships of Western Mediterranean subterranean Trechini groundbeetles (Coleoptera: Carabidae). *Zoologica Scripta*, 00, 000-000. Carabid beetles of tribe Trechini (Coleoptera) are one of the main groups of insects that colonized the subterranean environment. Many species of this group have developed similar morphological modifications related to the subterranean life, resulting in a characteristic *Aphaenops*-like phenotype that obscures their phylogenetic relationships (depigmented, blind, elongated body and appendages, narrow head and pronotum). We present here the result of a molecular study using a combination of nuclear (*SSU*, *LSU*) and mitochondrial (*cox1*, *cyb*, *rrnL*, *trnL*, *nad1*) genes in order to investigate the phylogenetic placement of the highly modified genera of subterranean Trechini from the west Mediterranean area (France, Spain, Morocco and Sardinia). Our results confirm the multiple independent origin of troglomorphy among these genera, and reveal a pattern largely determined by geographical proximity. We discuss the validity of some groups proposed on the base of morphological features, and provide estimates of divergence between subterranean genera and other groups of Trechini, including epigeal species of the same area. We compare the estimated age for the origin of the main groups resulting from two different calibrations, using one the standard mitochondrial mutation rate (2.3% divergence per MY) and the other the separation between Sardinia and mainland 33 My ago. Under the first scenario the main groups of genera would have a late Miocene origin, with a subsequent colonization of north Africa at the Pliocene-Pleistocene boundary. The assumption that the main groups originated through vicariance due to the separation of the Sardinian plate in the Oligocene results in a Messinian origin of the north African subterranean taxa, and a global mitochondrial rate reduced to 1% divergence per MY.

Corresponding author : A. Faille, Institut de Biologia Evolutiva (CSIC), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain and Muséum National d'Histoire Naturelle, C.P.50, UMR 7205 du CNRS / USM 601, Département Systématique et Evolution, Entomologie, 45 rue Buffon, F-75005 Paris, France. E-mail: faille@mnhn.fr

Achille Casale, Dipartimento di Zoologia e Genetica Evoluzionistica, Università di Sassari, Via Muroni 25, 07100 Sassari, Italy. E-mail: casale@uniss.it; a_casale@libero.it

Ignacio Ribera, Institut de Biologia Evolutiva (CSIC), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain. E-mail: ignacio.ribera@ibe.upf-csic.es

*Present address: Zoologische Staatssammlung München, Münchhausenstraße 21
81247 München (Germany)

Introduction

There are many groups of insects in which some isolated species have subterranean habits, but only some of them seem to have a marked tendency for repeated colonisations of the underground, with the possibility of extensive radiations in this environment. This is the case of two groups of Coleoptera, the Leiodidae Leptodirini and the Carabidae Trechini (Casale et al. 1998; Giachino et al. 1998). Subterranean species of Coleoptera usually show similar morphological and physiological modifications interpreted as convergent adaptations, which give them a typical facies: depigmented, blind, apterous, and either small size and short and robust appendages in the endogean species, or larger size, slender shape (in particular a narrow and elongate pronotum and head) and elongate appendages in species typical of caves or less open interstitial spaces (Racovitza 1907; Jeannel 1943; Barr & Holsinger 1985; Culver et al 1990). The later facies has often been defined as “aphaenopsian”, in reference to one of the earliest described genera of subterranean Coleoptera, *Aphaenops* Bonvouloir, 1861, which includes some species endemic to the Pyrenees sharing these modifications.

In the west Mediterranean area (south-western France, the Iberian peninsula, Morocco and Sardinia) there are several troglobitic genera of Trechini, of which the most prominent is the Pyrenean radiation of the genera *Aphaenops*, *Hydrapphaenops* Jeannel, 1926 and *Geotrechus* Jeannel, 1919 with about 80 species (Jeannel 1941; Lorenz 2005; Faille et al 2010a). A recent molecular phylogeny demonstrated that all the highly modified subterranean species of the Pyrenees share a single origin, but even at this reduced geographical scale convergent evolution is common, resulting in the polyphyly of the subterranean genera as established on morphological characters (Faille et al 2010a). In addition to this Pyrenean radiation, in the western Mediterranean there are several genera of subterranean Trechini with more or less highly modified, steno-endemic species that have often been hypothesized to be related to the Pyrenean radiation due to their “aphaenopsian” facies. They also share some internal morphological characters, such as the lateral position of the copulatory piece in their endophallus (“anisotopic” genitalia in the terminology of Jeannel 1943). These include *Speotrechus* Jeannel, 1922 in southern France, *Hydrotrechus* Carabajal, García & Rodríguez, 1999 and *Apoduvalius* Jeannel, 1953 in the Cantabrian mountains, *Sardapphaenops* Cerruti & Henrot, 1956 in Sardinia, *Parapphaenops* Jeannel, 1916 in the Mediterranean coast of Spain, and *Antoinella* Jeannel, 1937 and *Subilsia* Español, 1967 in Morocco. The aim of this work is to place these subterranean genera in a general phylogenetic framework including a

representative sample of the western Mediterranean Trechini, and provide a temporal framework for studying their origin and biogeography.

Material and Methods

Taxon sampling

We included in the study species of all subterranean genera of west Mediterranean Trechini with only two exceptions, *Subilsia* and *Hydrotrechus*. We also include some subterranean species of genera with more eastern distributions, included in the *Duvalius* phyletic lineage (Jeannel 1928a; Casale & Laneyrie 1982): *Duvalius* Delarouzée, 1859, *Agostinia* Jeannel, 1928 and *Trichaphaenops* Jeannel, 1916. Among the epigeal taxa we include a representation of the western European and Moroccan species of *Trechus* Clairville, 1806 plus some genera of Trechini of uncertain relationships: *Iberotrechus* Jeannel, 1920 (Ortuño & Toribio 2006) and *Doderotrechus* Vigna Taglianti 1968, plus representatives of other subtribes of Trechini: (*Perileptus*, *Aepopsis* and *Thalassophilus*, (Jeannel, 1926; Casale & Laneyrie 1982) (Table 2). As outgroups we used some species of the Trechinae tribes Bembidiini and Patrobini. Trees were rooted in *Penetretus* (Patrobini), likely to be the sister group of Trechini plus Bembidiini (e.g. Ribera et al. 2005; Maddison et al. 2009).

Background on the taxonomy and the systematic placement of west Mediterranean subterranean Trechini

***Sardaphaenops* Cerruti & Henrot, 1956**

This genus includes three highly modified troglobitic taxa endemic to Sardinia, *S. supramontanus supramontanus* Cerruti & Henrot, 1956 and its markedly distinct subspecies *S. supramontanus graffittii* Casale and Giachino, 1988, and *S. adelphus* Casale, 2004. When this remarkable aphaenopsian genus was described, it was considered to be related to *Duvalius* (Cerruti & Henrot 1956, followed by Jeannel 1956 and Juberthie & Massoud 1980). The genus *Duvalius* is highly diversified from the Alps to China and includes hundreds species described so far, but is represented in the western Mediterranean region by only a few taxa: two species in Spain, two species in Mallorca (one of them attributed to the subgenus *Trechopsis*), one species in Sardinia, and four in Algeria (three of them attributed to the subgenus *Trechopsis*) (Moravec et al. 2003; Casale & Laneyrie 1982). Subsequently, Casale

& Laneyrie (1982) included *Sardaphaenops* in the *Aphaenops* phyletic lineage (see also Vigna Taglianti 1982, Casale & Giachino 1988, and Casale 2004). Although the detailed relationships of this genus seem difficult to be establish based on morphological characters – as it is the case for most of the species considered to be “palaeoendemic” (Vigna Taglianti 1982, Ortuño et al. 2004) –, Casale & Giachino (1988), Casale & Vigna Taglianti (1996), Casale (2004) and Casale & Marcia (2007) suggested that it could present affinities with the genus *Paraphaenops* from Catalonia (see below).

***Paraphaenops* Jeannel, 1916**

Paraphaenops breuilianus was described by Jeannel (1916) as a subgenus of the mostly epigean genus *Trechus*. This monospecific genus is known only from some caves in the north of the Iberian system in Tarragona, an area with several hypogean species of various zoological groups considered to be “palaeoendemic” (see Ortuño et al. 2004 for a review). It is geographically very isolated from all the other aphaenopsian species of Trechini. Jeannel (1928a) considered that it was closely related to the genus *Speotrechus* from the French Cevennes (see below), an opinion followed by Español (1950, 1965). Subsequent authors placed this enigmatic insect closer to *Sardaphaenops* and the Pyrenean troglobitic genera *Aphaenops*, *Geotrechus* and *Hydraphaenops* (Casale & Laneyrie 1982).

***Apoduvalius* Jeannel, 1953**

Apoduvalius are small blind and depigmented cave beetles known by 14 species located along the Cantabrian chain, in northwest Spain (Serrano 2003). The two first species, *Apoduvalius negrei* Jeannel, 1953 and *A. drescoi* Jeannel, 1953, were described by Jeannel (1953), who named the genus to underline the strong similarities with the *Duvalius* group of genera. This group is extremely diversified in the Alps and in central Europe, but represented in Spain by two species only, the first endemic to Catalonia, *Duvalius berthae* (Jeannel, 1910), the second only known from Sierra de Alcaraz, *Duvalius lencinai* Mateu & Ortuño, 2006. The *Duvalius* phyletic lineage (Jeannel 1928a) is morphologically well characterised by the dorsal (“isotopic”) position of the copulatory piece, a part of the endophallus that is usually well sclerified. The point of view of Jeannel (1953) was shared by subsequent authors (Español 1965, Casale et Laneyrie 1982), but in a revision of the genus *Apoduvalius* Vives i Noguera (1980) noted that all species of this genus have a lateral copulatory piece (“position anisotope”, Jeannel 1955) (see also Deuve 1991, Dupré 1995 and Salgado & Peláez 2004). Therefore, these authors concluded that the species of *Apoduvalius* were close to the endemic

Pyrenean genus *Geotrechus*, with 22 described species mainly located on the foothills of the French Pyrenees (Casale & Laneyrie 1982; Moravec et al. 2003). The presence of pubescent protibiae is another character shared by these two genera, although this is also shared with *Duvalius*.

***Antoinella* Jeannel, 1937**

The genus *Antoinella* was created by Jeannel for a single troglobitic species of Morocco, *Antoinella groubei* (Antoine, 1935), originally attributed to the genus *Duvalius* by Antoine (1935). From the features of male genitalia, Jeannel (1937) suggested that *Antoinella* is close to *Trechus*, more especially to the *Trechus fulvus* Dejean, 1831 species group, a point of view followed by Casale (1982) but not by Mateu & Escolà (2006), who assumed a relationship with *Duvalius*. It currently includes eight species, all endemic to Morocco (Comas & Mateu 2008).

***Speotrechus* Jeannel, 1922**

The monospecific genus *Speotrechus* was erected by Jeannel for a species of the French Alps and the Cevennes. It was attributed to the *Aphaenops* phyletic lineage, close to *Paraphaenops* (Jeannel 1928a).

The Pyrenean radiation: *Aphaenops* Bonvouloir, 1861, *Hydraphaenops* Jeannel, 1926 and *Geotrechus* Jeannel, 1919

All highly modified subterranean species of Trechini in the Pyrenean area were shown to form a monophyletic group by Faille et al. (2010a). The three genera were found to be para- or polyphyletic, showing extensive morphological convergence presumably as a result of similar ecological requirements (see Faille et al. 2010a for details).

***Hydrotrechus* Carabajal, García et Rodríguez, 1999 and *Subilsia* Español, 1967**

The genus *Hydrotrechus* was described for a species (*H. cantabricus* Carabajal et al. 1999) found in loose soil saturated with water in the Cantabrian mountains in north west Spain. According to the authors, it seems related to the genus *Trechus*, differing mainly by the presence of pubescence in the elytra and the absence of eyes (Carabajal et al. 1999). This genus should be considered as synonym of *Trechus* (Ortuño & Jimenez-Valverde in press).

The morphologically peculiar genus *Subilsia* was originally described from a female, and considered to be close to *Duvalius* (Español, 1967). The subsequent discovery of the male

(Español 1970) showed that the copulatory piece of the endophallus was of “anisotropic” type; therefore, this peculiar genus from the Middle Atlas in Morocco seems to be more likely related to *Trechus* and *Antoinella* (Casale & Laneyrie 1982). We have not obtained fresh specimens for a molecular study of these two species.

DNA extraction, PCR amplification and sequencing

Specimens were collected alive in the field and directly killed and preserved in 96% ethanol. DNA was extracted from whole specimens by a standard non-destructive extraction (Rowley et al. 2007) using commercial extraction kits. Voucher specimens are kept in the MNHN (Paris), DNA aliquots are kept in the tissue collections of the MNHN and IBE (CSIC-UPF, Barcelona).

We sequenced three mitochondrial genome fragments including five genes (3' end of cytochrome c oxidase subunit 1, *cox1*; an internal fragment of the cytochrome b, *cyb*; and a continuous fragment including the 3' end of the large ribosomal unit plus the Leucine transfer plus the 5' end of NADH dehydrogenase subunit 1, *rrnL+trnL+nad1*) and two nuclear genes (5' end of the small ribosomal unit, SSU and an internal fragment of the large ribosomal unit, LSU) (see Table 1 for the primers used). Sequences were assembled and edited with Bioedit v. 7.00 (Hall, 1999) or Sequencher 4.6 (Gene Codes, Inc., Ann Arbor, MI). New sequences have been deposited in GenBank with Acc. Nos. XXXXXX (Table 2). Protein coding genes were not length variable, and the ribosomal genes were aligned with the online version of MAFFT v.6 using the GINS-i algorithm and default parameters (Katoh et al., 2002; Katoh and Toh, 2008).

Part of the sequences were taken from Faille et al. (2010a) and Faille et al. (2010b). We added 14 new specimens and a total of 230 new sequences to the dataset (Table 2), including 151 sequences for the specimens already in Faille et al. (2010a), thus avoiding the use of chimerical sequences for eight of the taxa.

Phylogenetic analyses

Bayesian analyses were conducted on a combined data matrix with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), using six partitions corresponding to the six sequenced genes (the small *trnL* was included in the same partition as the *rrnL* gene). Evolutionary models were estimated prior to the analysis with Model-Test 3.7 (Posada and Crandall, 1998).

MrBayes ran for 10^7 generations using default values, saving trees each 1000. “Burn-in” values were established after visual examination of a plot of the standard deviation of the split frequencies between two simultaneous runs. We also used Maximum Likelihood as implemented in the on-line version of RAxML (which includes an estimation of bootstrap node support, Stamatakis et al., 2008), using GTR+G as the evolutionary model and the same five gene partitions used in MrBayes.

Estimation of divergence times

To estimate the relative age of divergence of the lineages we used the Bayesian relaxed phylogenetic approach implemented in BEAST v1.4.7 (Drummond and Rambaut, 2007), which allows variation in substitution rates among branches (Drummond et al., 2006). We implemented a GTR+I+G model of DNA substitution with four rate categories using the mitochondrial data set only and pruning specimens with missing gene fragments, with an uncorrelated lognormal relaxed molecular clock model to estimate substitution rates and the Yule process of speciation as the tree prior. The main nodes of the topology were constrained to match that of the tree obtained with the whole dataset (mitochondrial plus nuclear) in MrBayes. We ran two independent analyses for each group sampling each 1000 generations, and used TRACER version 1.4 to determine convergence, measure the effective sample size of each parameter, and calculate the mean and 95% highest posterior density interval (HPD) for divergence times. Results of the two runs were combined with LogCombiner v1.4.7 and the consensus tree compiled with TreeAnnotator v1.4.7 (Drummond and Rambaut, 2007). The analyses were run for 20×10^6 generations, with the initial 10% discarded as burn-in. We calibrated the trees in two different forms:

- 1- It has been hypothesized that the genus *Sardaphaenops* originated through vicariance with SW mainland Europe due to the tectonic separation of Sardinia during the Oligocene and Early Miocene (Casale & Vigna Taglianti 1996; Casale 2004). This calibration point has been used for the estimation of divergence rates among west-Palaeartic lineages of Leptodirini subterranean beetles (Caccone & Sbordoni 2001; Ribera et al. 2010). The prior of the age of the node separating *Sardaphaenops* from its sister group was defined as a normal distribution with average 33 MY (following Schettino & Turco 2006, see Ribera et al. 2010 for a detailed justification) and a standard deviation of 2.0 MY.

- 2- We used a standard mitochondrial mutation rate for insects of 2.3% divergence per MY, equivalent to a per-branch rate of 0.0115 substitutions/site/MY (Brower, 1994). Despite the obvious limitations of the use of an a-priori universal standard rate, recent works have shown that for Coleoptera the rate of a combination of ribosomal and protein coding mitochondrial genes is remarkably consistent and close to this figure (e.g. Papadopoulou et al. 2010; Ribera et al. 2010). We used as prior for the rate a normal distribution with average 0.0115 substitutions/site/MY and a standard deviation of 0.0001.

Results

The two runs of the Bayesian analyses converged at approximately 6.5×10^6 generations (taken as burn-in), with a standard deviation of the split frequencies lower than 0.01. The two runs were combined to produce a single consensus phylogram based on 7,000 trees. The convergence of the parameters was good, as measured both with the estimated effective population size and the convergence diagnostic parameter in MrBayes (Huesenbelck & Ronquist 2001). The topology obtained with Maximum Likelihood in RAxML was very similar to that obtained with MrBayes, with only some poorly supported nodes showing incongruence (Fig. 1).

The monophyly of Trechini was strongly supported (Bayesian posterior probability, Bpp = 1; ML bootstrap, BT= 100, Fig.1), but Bembidiini appeared as paraphyletic, with one well supported node excluding *Philochthus* from the rest of Bembidiini plus Trechini. Within Trechini the two genera *Perileptus* and *Thalassophilus* (subtribes Perileptina and Trechodina respectively) were sister to a large clade including subtribes Trechina and Aepyna in a series of well supported lineages with poorly resolved relationships among them (Fig. 1).

These well supported lineages included three highly diversified groups: 1) all the Pyrenean subterranean taxa, i.e. the *Aphaenops* group of genera, *Aphaenops*, *Hydraphaenops* and *Geotrechus* (in agreement with Faille et al. 2010a); 2) the *Duvalius* group of genera, including *Duvalius*, *Agostinia* and *Trichaphaenops* in a well supported clade, plus the genera *Doderotrechus*, *Aepopsis* (subtribe Aepyna) and *Iberotrechus* with poorly resolved relationships within this group; and 3) the *Trechus* group of genera, a clade including all epigeal species of *Trechus* with the only exception of two Iberian species (*T. comasi* and *T. schaufussi* complex) plus the subterranean genera *Apoduvalius*, *Antoinella* and *Speotrechus*.

In addition to these three major lineages there were a number of less diversified genera with poorly supported relationships: *Sardaphaenops*, *Paraphaenops* and the two Iberian species of *Trechus* not included with the rest of the genus. In both the Bayesian and the Maximum likelihood trees the *Aphaenops* and the *Duvalius* lineages were sisters, and the *Trechus* group was included in a clade with *Sardaphaenops*, *Paraphaenops* and the Iberian *Trechus*, although with low support in both cases (Bpp = 0.76, BT= 62 and 74 respectively; Fig. 1). The genera *Sardaphaenops* and *Paraphaenops* never appeared directly related to the Pyrenean *Aphaenops* group.

Of the three main lineages within Trechini, only the *Aphaenops* group in the Pyrenees included exclusively subterranean species. The internal phylogeny of this group was in agreement with the results of Faille et al. (2010a), with the effect of the new sequences being mostly reflected in the increase of support of some nodes. The hypogean genus *Geotrechus* formed a paraphyletic series at the base of the lineage, within which there were two independent evolutions of first an intermediate *Hydraphaenops* morphology and then the *Aphaenops* morphology, forming two radiations in the East and West of the chain (Fig. 1; see Faille et al. 2010a).

The west Mediterranean species of *Duvalius* included in the analyses (*D. berthae* Jeannel, from Catalonia) formed a well supported clade with the Alpine species of the genus, with two subterranean genera with an “aphaenopsian” facies (*Agostinia* and *Trichaphaenops*) nested within it.

The single species of *Antoinella* included in the analyses formed a well supported clade with two epigeal species of *Trechus*, *T. martinezi* from the south east of the Iberian peninsula and *T. fulvus*, widely spread in the western Palearctic region. The Cantabrian genus *Apoduvalius* appears as polyphyletic and again nested within a lineage of epigeal *Trechus* (Fig. 1), with the species *A. alberichae* (included in the subgenus *Trichapoduvalius*) related to some epigeal *Trechus* from the same geographical area, and the species of *Apoduvalius* sensu stricto sister to them. The genus *Speotrechus* from the Alps appears as the sister to the complex formed by the epigeal *Trechus* and the subterranean genera nested within it, i.e. *Antoinella* and *Apoduvalius*.

Estimation of divergence dates

The two independent runs of Beast for each of the analyses reached convergence in less than the 10% of the generations used as burnin by default, as measured with the effective sample

size estimated in Tracer. For each analysis the two independent runs were combined to produce a single ultrametric tree. The relationship between the estimated age of the nodes when using an a-priori standard rate of 2.3% divergence/MY and when the age of the node including the *Trechus* group of genera, *Sardaphaenops*, *Paraphaenops* and *T. aff. schaufussi* plus *T. comasi* was set to 33 MY followed a perfect linear relationship, with slope 2.33 and an intercept of 0.065 (Table 3). When the tectonic separation of Sardinia was used as a calibration point, the estimated average rate for the combined mitochondrial sequence decreased by a factor of 2.3, to a value of 0.005 substitutions/site/MY (Table 3). Under this scenario, the origin of Trechina dated back to mid Eocene, and that of the main lineages to late Oligocene or early Miocene (Fig. 2A). The separation between the Moroccan *Antoinella* and the SE Iberian *Trechus martinezi* was 5.66 MY, in good agreement with a vicariance produced by the end of the Messinian salinity crisis at 5.3 MY (Chalouan et al. 2008).

On the contrary, using a standard rate of 2.3% divergence per MY the origin of Trechina was estimated to be in early Miocene, and the origin of the main lineages late Miocene (Fig. 2B). The separation between *Sardaphaenops*, *Paraphaenops* and the species of *Trechus* was estimated to have occurred in middle Miocene, during the Tortonian, and the separation between *Antoinella* and *T. martinezi* in the Pliocene-Pleistocene transition.

Discussion

Phylogeny of Trechini

We found a strong support for the monophyly of the subtribe Trechina, one of the most diverse lineages of ground beetles (Lorenz 2005), but only with the inclusion of *Aepopsis*, a genus including one only intertidal species of the Atlantic coast from Britain to Morocco. This result is in accordance with recent work based on larval morphology (Grebennikov & Maddison 2005). This genus has been grouped with other intertidal species from both Europe (*Aepus*) and sub-Antarctic territories (Chile, Patagonia, Falkland islands and New Zealand) in the tribe Aepini (Jeannel 1926, Casale & Laneyrie 1982). Our results suggest that the morphology of *Aepopsis* could be a highly specialised, derived combination of characters within the subtribe Trechina, likely to be close to the European *Aepus*, but of uncertain affinities with the southern taxa.

Within Trechina, the genera included in our study grouped largely according to their geographical distribution, with three strongly supported clades including the Pyrenean subterranean taxa (*Aphaenops* group), some genera mostly distributed in the Alps and central Europe (*Duvalius* group), and a series of species of *Trechus* with a western Mediterranean distribution (*Trechus* group). These three groups largely agree with the traditional phyletic lineages of *Aphaenops*, *Duvalius* and (part of) *Trechus* (Jeannel 1927, 1928a, Casale & Laneyrie 1982, Casale et al. 1998), although their composition is not exactly the same (in particular for the troglobitic genera studied here) and the morphological characters used to define them are highly homoplastic (pubescence of the protibiae and presence of eyes). Preliminary results suggest that the widespread genus *Duvalius* should be polyphyletic, as observed for *Trechus*.

The position of *Sardaphaenops*, *Paraphaenops* and two Iberian species of *Trechus* was not well supported, although all analyses grouped them with the *Trechus* group, in agreement with their geographic distribution and with morphology, especially the structure of the endophallus (anisotropic position of copulatory piece). In any case, both *Paraphaenops* and *Sardaphaenops* (and the two species of *Trechus*) are isolated lineages within the western Mediterranean Trechini, not closely related to the Pyrenean radiation. These two Iberian species of *Trechus*, *T. comasi* and a species of the *T. schaufussi* complex, were not grouped with the rest of the species of the genus. *Trechus comasi* has been said to be close to *Trechus brucki* Fairmaire, 1862 (Hernando 2001), a member of the “*Trechus uhagoni* group” (Jeannel 1941). However, *T. uhagoni* Crotch, 1869 was included in the analyses and unambiguously placed inside the *Trechus* clade. The specimen considered as *T. schaufussi* in Faille et al (2010a), from Ciudad Real, should better be considered as a member of a complex of species under the name *T. schaufussi*, which has seven recognised subspecies (Serrano 2003) and is in need of a taxonomic revision.

Within the exclusively subterranean Pyrenean *Aphaenops* group our results basically agree with Faille et al. (2010a), although the addition of new species increased the evidence of the para- or polyphyly of the recognised genera. This implies the transition from an “anophthalmous” habit (*Geotrechus*) to a subterranean life strongly linked to the presence of high humidity and a film of water (*Hydraphaenops*) and finally, twice independently, to the typical *Aphaenops* morphology. The appearance of the *Aphaenops* morphology in the Pyrenean clade was apparently accompanied by an increase in the diversification rate and a range expansion, once in the eastern and a second time in the western part of the Pyrenees (Faille et al. 2010a). The same “Aphaenopsian” morphological type developed independently

in other lineages of Trechini, but in these cases without diversification or range expansions (*Paraphaenops*, *Sardaphaenops*), even if, according to our estimations, these taxa are of more ancient origin (see below). A possible reason could be the lack of suitable habitat for their geographical expansion, either for being confined to an island (*Sardaphaenops*) or for the higher aridity of the area (*Paraphaenops*).

The inclusion of *Iberotrechus* in the *Duvalius* group is surprising, and had never been hypothesized before. Originally described within *Trechus*, Jeannel (1920) finally erected a new genus and subsequently postulated its affinities with the American Homaloderini (Jeannel 1927). However Moore (1972), followed by Casale & Laneyrie (1982), synonymised Homaloderini with Trechini, but the affinities of the highly distinctive genus *Iberotrechus* remained obscure (e.g. Ortuño & Toribio, 2006). *Iberotrechus bolivari* (Jeannel, 1913) was considered to be exclusive of a single cave in Cantabria, but has recently been found to be a typical inhabitant of shaded and very moist deciduous forest, usually close to streams or springs (Ortuño & Toribio 2006).

The *Trechus* group of genera as considered here includes two subterranean genera nested within it, one of them (*Apoduvalius*) polyphyletic, and a third subterranean genus (*Speotrechus*) as sister to the remaining species. The most incomplete sampling of the large and widespread genus *Trechus* prevents the introduction of any formal taxonomic change, but from our results it is clear that a deep taxonomic rearrangement will be necessary, with the separation of what is currently known as *Trechus* in several genera. The inclusion of *Antoinella* within the *T. fulvus* group is in agreement with morphological expectations (Jeannel 1937; Casale 1982), in particular its proximity to species distributed in the Baetic cordilleras (*T. martinezi*). Hypogean lifestyles are common in this group of species, and several of them are subterranean in Spain and North Africa (Jeannel 1927; Antoine 1955).

The inclusion of *Apoduvalius* as part of a group of NW Iberian species of *Trechus*, although coherent from a geographical perspective, was more unexpected. The subgenus *Trichapoduvalius* seems to be distinct from the rest of the species, and of more recent origin. The relationships of *Apoduvalius* with *Trechus* but not the Pyrenean *Aphaenops* group (and in particular *Geotrechus*) is supported by two additional morphological characters: the disposition of the humeral setae of the elytra, aggregated on the external edge of elytra (not aligned in any species of the *Aphaenops* group, Jeannel 1926), and the presence of complete frontal furrows (incomplete in the *Aphaenops* group, Jeannel 1926).

Estimations of divergence times within Trechini

The two alternative calibrations, assuming a vicariant origin of *Sardaphaenops* due to the tectonic isolation of Sardinia or assuming a standard mitochondrial rate, resulted in two very different scenarios for the origin and evolution of the subterranean west Mediterranean Trechini. Under the classic hypothesis of a tectonic origin for *Sardaphaenops*, its origin – and thus the origin of the *Trechus* group plus *Paraphaenops* and the two Iberian *Trechus* – was set to be early Oligocene. The divergence between the Moroccan *Antoinella* and its Iberian relatives was accordingly estimated to have occurred at the end of the Messinian period, in agreement with many examples of vicariance produced by the last opening of the Strait of Gibraltar 5.2 MY ago (e.g. Martínez-Solano et al. 2004; Carranza & Amat 2005; Agustí et al., 2006). The origin time of the *Aphaenops* group would be early Miocene, a period of global warming that could be associated with subterranean colonization of hygrophilous species (Uriarte 2003, Zachos et al. 2001). With this calibration the Trechini radiation would still be younger than the origin of the Pyrenean radiation of the other main lineage of subterranean beetles (Leptodirini), as estimated using the same vicariant event (Ribera et al. 2010).

The estimated average rate when the tree is calibrated using the tectonic separation of the Sardinian plate (0.005 substitutions/site/MY) (Fig. 2A) was ca. 2.5 times lower than the standard mitochondrial rate of 2.3% of divergence per MY (Fig. 2B) (Brower 1994). The standard rate has shown to be surprisingly accurate for different groups of beetles when using a mixture of protein coding and ribosomal mitochondrial genes, as it was the case here. Thus, as noted above, using Leptodirini beetles and the same calibration point the estimation of Ribera et al. (2010) for a very similar set of genes was 2%; and Papadopoulo et al. (2010) using Tenebrionidae and the age of the Aegean trench obtained an estimate of 2.7%. Using only protein-coding genes gives higher estimates, e.g. 3% for *cox1-cox2* for the Canarian *Trechus* (Contreras-Díaz et al. 2007), or 2.6% for the complete combined protein coding mitochondrial set and for deep divergences (which tend to underestimate the rates) (Pons et al. 2010). In the later work it is shown that the branches for the protein coding genes of suborders Polyphaga and Myxophaga were about double the length of those of Archostemata and Adephaga, with the rate for the later estimated to be ca. 2%. However, even assuming a slower rate for Adephaga, the estimated rate of 0.005 substitutions/position/MY for the combination of protein coding and ribosomal mitochondrial genes seems to be exceedingly low in comparison to published results (see above). There does not seem to be any obvious difference in the estimated branch lengths of the subterranean vs. the epigeal species of

Trechini that could justify a lower rate as a consequence of the subterranean adaptations (longer life cycles, cold adaptation, e.g. Culver 1982).

The assumption of a standard rate of 2.3% divergence per MY resulted in a linear increase of all estimated dates (Fig. 2B), with the origin of *Sardaphaenops*, *Paraphaenops*, the Iberian *Trechus* and the *Trechus* group of genera in the Tortonian. During this period the SW Mediterranean experienced a complex geological setting, with multiple events of isolation of terrains in the Baetic ranges and the Rif Area (Braga et al., 2003; Jolivet et al., 2006), in agreement with an almost simultaneous split of taxa in mainland Iberia and Europe (*Trechus* group), the Baetic ranges (the two isolated Iberian *Trechus*), some Mediterranean coastal ranges (*Paraphaenops*) and Sardinia (*Sardaphaenops*). This would require the subsequent expansion of the distribution of some species of the *Trechus* group, including the colonization of north Africa by *Antoinella* at the Pliocene-Pleistocene boundary, and the recent expansion of *T. fulvus* and other species of the genus (i.e. *T. obtusus*, see Jeannel 1927, 1928b).

The study of another genus of Trechini in the same area, e.g. *Duvalius*, with the troglobitic species *Duvalius sardous* (Doderò, 1917) in Sardinia, considered to be close to the Catalan *D. berthae* (Vigna Taglianti 1982), or the Mallorcan *D. balearicus* Henrot, 1964, apparently close to the Algerian *D. jurjurae* Peyerimhoff, 1909 could shed further light on the diversification of the west Mediterranean subterranean Trechini and the origin of some of the most emblematic subterranean species of this area.

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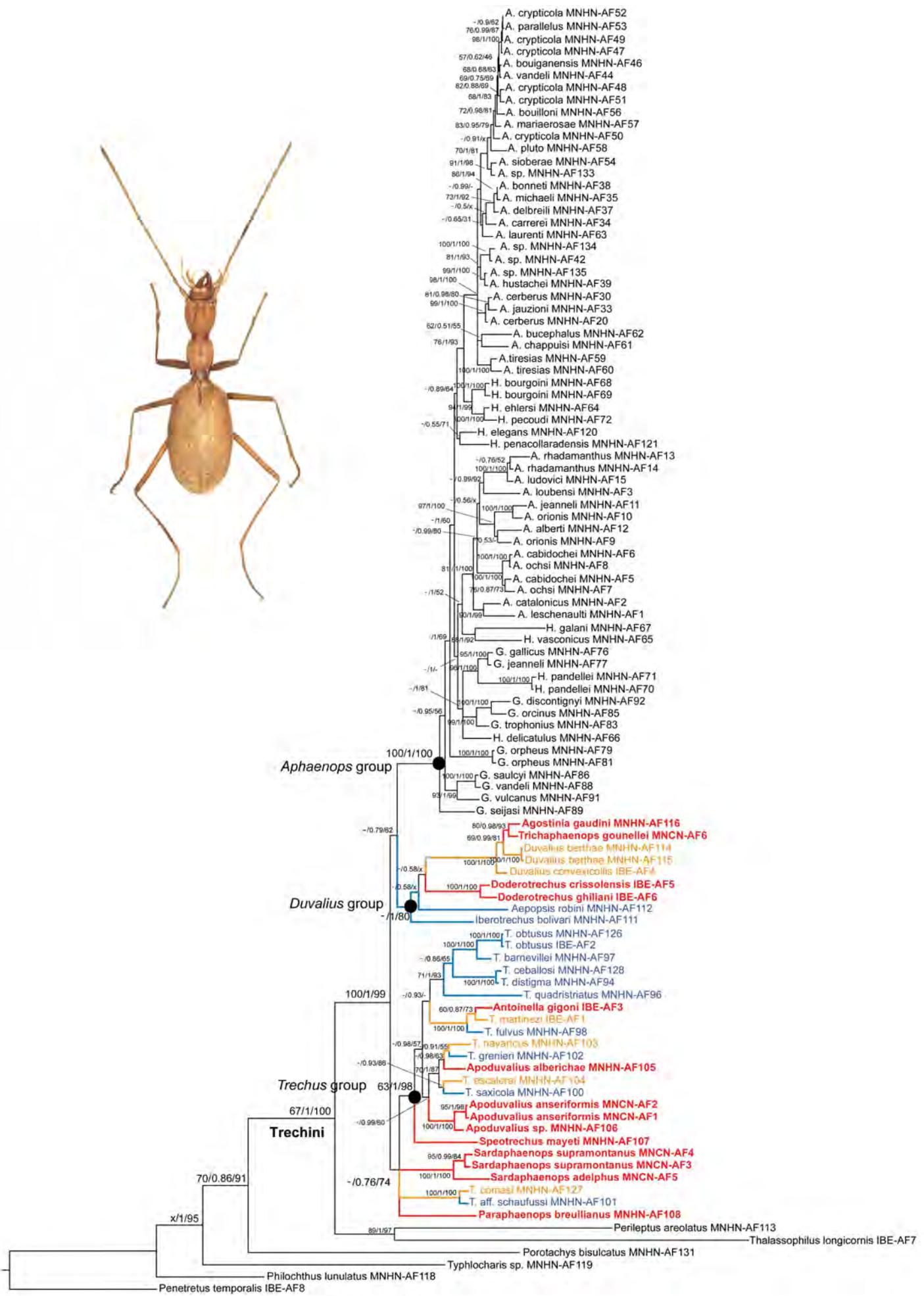
Fig. 1. Phylogram of Western Mediterranean subterranean Trechini obtained with maximum likelihood in Garli, using the combined data matrix. Number in nodes, Bayesian posterior probability, obtained in MrBayes / ML bootstrap (see Material & Methods for details). In red, troglobitic genera; in orange, hypogean depigmented and eye-regressed species of the two genera *Trechus* and *Duvalius*; in blue, epigeal species (including intertidal (*Aepopsis*) or cryophilic (*T. saxicola*) species and species with some populations located in caves (*Iberotrechus*, *T. uhagoni*, *T. barnevillei*) (see Table 2). -: node present but not supported, x : node lacking in ML (For interpretation of colours mentioned in this figure the reader is referred to the web version of the article.)

Fig. 2.A-B. Ultrametric tree of the Phylogeny of Western Mediterranean subterranean Trechini obtained with Beast, using A) the separation of the Sardinian species with a prior age of 33MY; B) a standard mitochondrial rate (0.0115 substitutions/site/MY) (see Material and Methods for details). Number in nodes, estimated age (in MY); grey band, 95% confidence intervals

Table 1. Primers used in the study.

Table 2. Suppl. Table 1. Sequenced specimens, with locality, collectors, sequence accession numbers and ecology (T: troglobitic, E: endogean, Ep: Epigeal).

Table 3. Estimated parameters in the Beast runs for the two hypothesis of calibration:

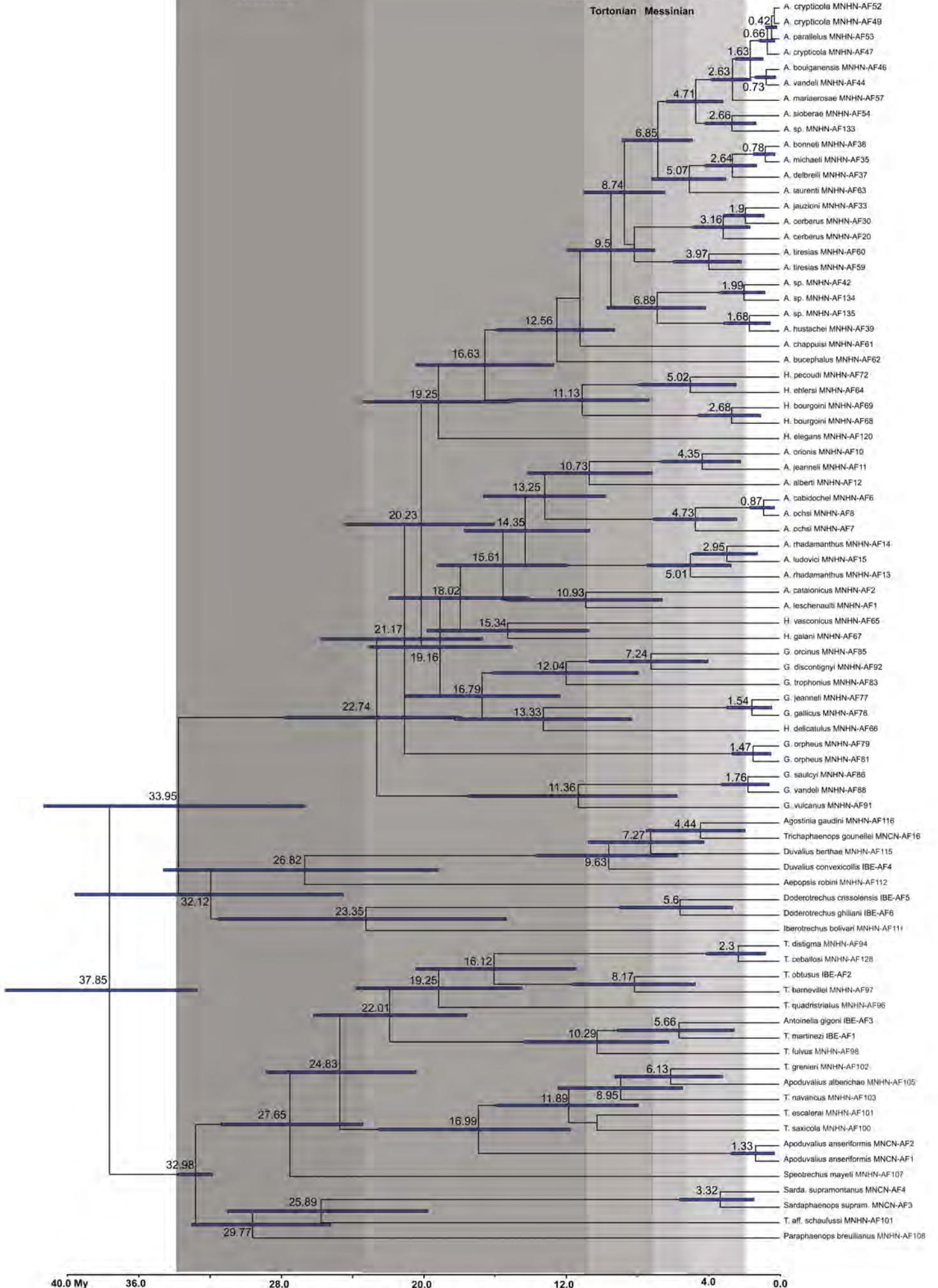


OLIGOCENE

MIOCENE

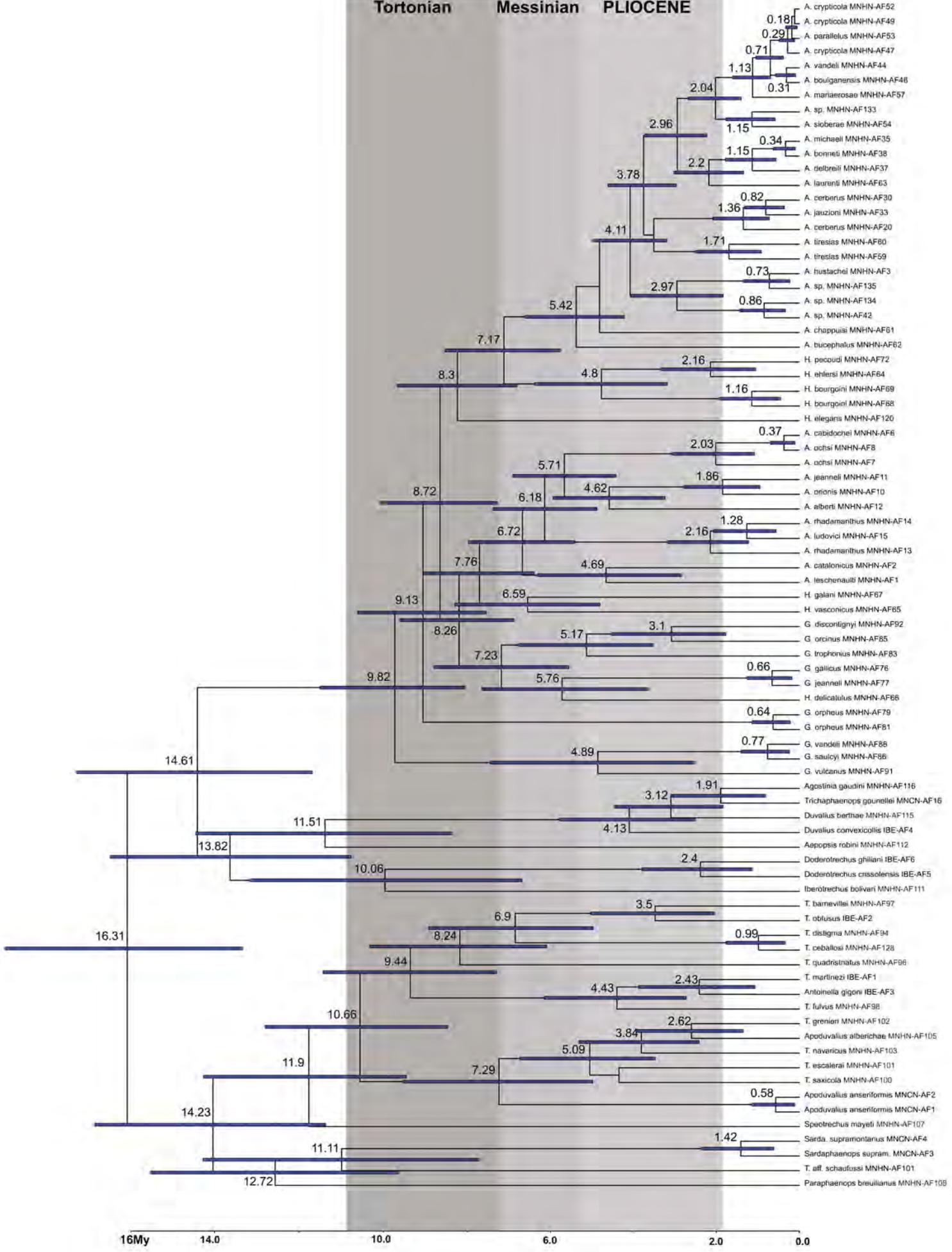
PLIOCENE

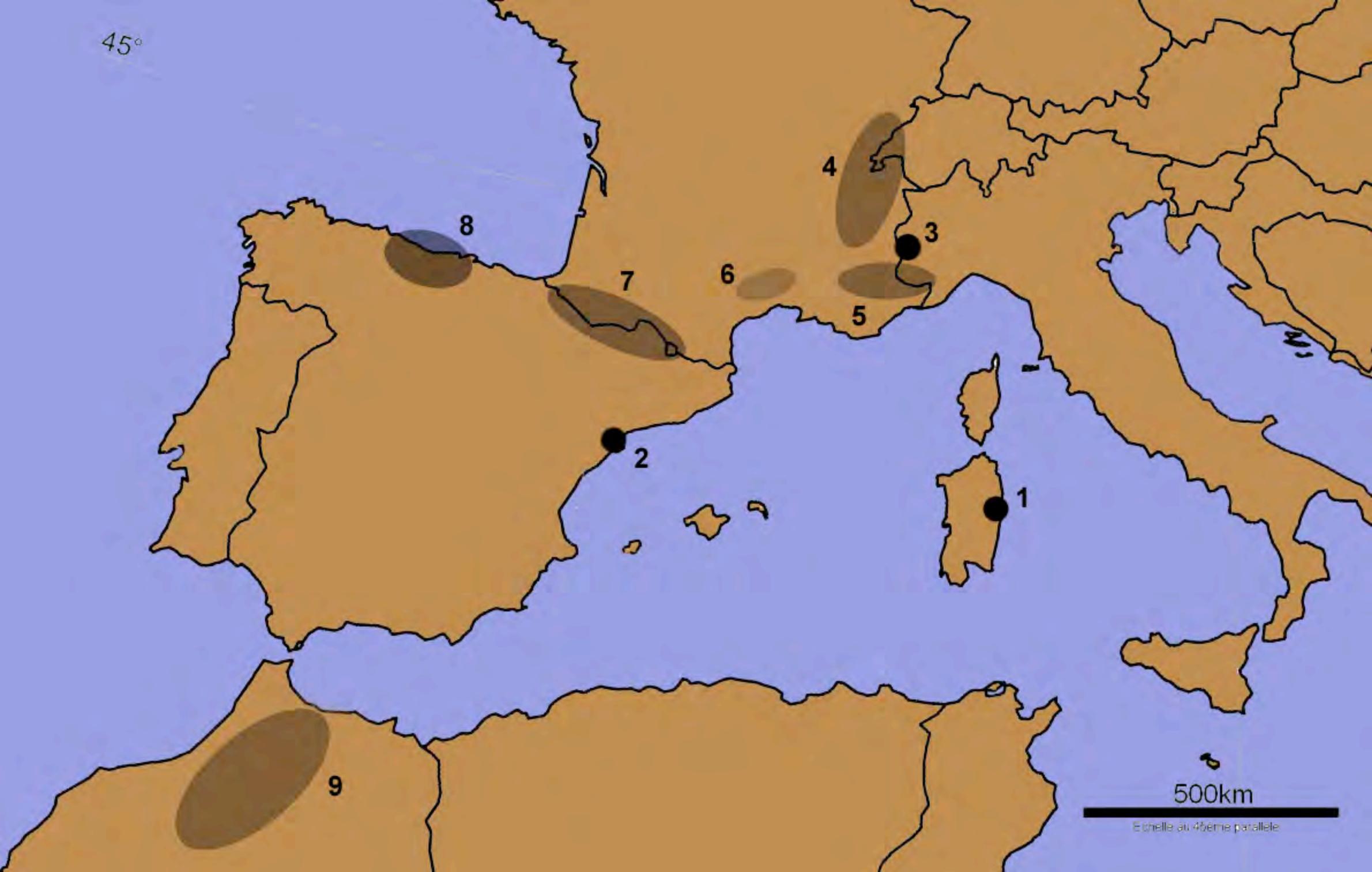
Tortonian Messinian



40.0 My 36.0 28.0 20.0 12.0 4.0 0.0

Tortonian Messinian Pliocene





45°

8

7

6

4

3

5

2

1

9

500km

Echelle au 45ème parallèle