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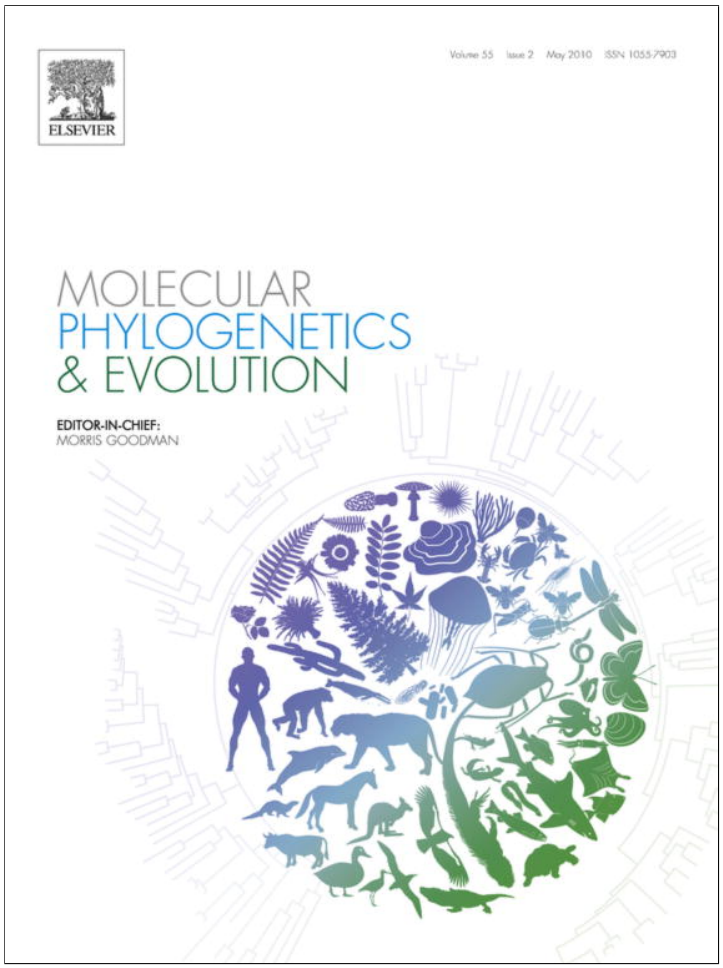

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Molecular systematics and evolutionary biogeography of the genus *Talpa* (Soricomorpha: Talpidae)

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ABSTRACT

The range of the genus *Talpa* covers almost all Europe up to Western Asia. This genus has never been the object of comprehensive systematic studies using molecular and genetic techniques, such that the evolutionary relationships among species remain unclear.

Talpa shows high levels of endemism, and the influence of past glaciation cycles on the distribution pattern of several species has been hypothesized.

In this work, we assessed the molecular systematics of the genus using the mitochondrial gene cytochrome *b* from eight of the nine extant species of *Talpa* moles. Furthermore, molecular clock estimations were used to hypothesize a biogeographic scenario in concordance with fossil data.

Results suggest a monophyletic origin of the genus and a common ancestor for the western European moles *T. europaea*, *T. caeca*, *T. romana* and *T. occidentalis*. The eastern species *T. altaica* and *T. caucasica* are basally divergent. The estimated ages of divergence among lineages are in accordance with a Miocene origin of the extant moles. The genus likely originated in Asia, spreading into Europe during the Pliocene. The evolution of moles appears to have been driven by changes in moisture levels that influenced extinction and speciation events during the Miocene and the Pliocene. Pleistocene climatic oscillations likely caused the range shrinkages and expansions that led to the current distribution pattern of most *Talpa* species.

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1. Introduction

Talpa species are widely distributed throughout the Western Palaearctic region, from the Iberian Peninsula to Siberia. All the extant, strictly subterranean Eurasian species belong to the genera *Mogera*, *Euroscaptor* and *Talpa* (subfamily Talpinae, tribe Talpini). The Talpinae subfamily belongs to the family Talpidae, which also includes the subfamilies Scalopinae and Uropsilinae (Hutterer, 2005). Yates and Moore (1990) suggested that this family originated in Europe during the Eocene and underwent explosive radiation in Eurasia and North America during the Miocene.

Despite the abundance of moles in their natural habitats, few data are available on the genus *Talpa* in recent scientific literature, and several aspects of its evolution, ecology and behavior have yet to be resolved. All species of this genus can usually be found in areas where the soil is deep and soft enough to dig tunnels, while they are absent from deserts and dry steppes (Nowak, 1999). Moles show a high tolerance to altitude and temperature and habitats range from sea level up to mountains where the ground is covered by snow for several months of the year (Nowak, 1999).

The distribution pattern of *Talpa* species is characteristic, showing high levels of endemism. In fact, only one species, *T. europaea*, is widespread throughout Eurasia from the Urals to the Pyrenees, while all other species are restricted to relatively small areas of Europe and Asia (Hutterer, 2005). Nine species were recognized in the last revision of the genus (Hutterer, 2005): the Altai mole *T. altaica* Nikolasky, 1883, the Caucasian mole *T. caucasica* Satunin, 1908, the Levant mole *T. levantis* (Thomas, 1906), the Père David's mole *T. davidiana* Milne-Edwards, 1884, the blind mole *T. caeca* Savi, 1882, the common mole *T. europaea* Linnaeus, 1758, the Iberian blind mole *T. occidentalis* Cabrera, 1907, the Roman mole *T. romana* Thomas, 1965 and the Balkan mole *T. stankovici* (Martino and Martino, 1931).

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Due to their convergent adaptation to underground life, these species are very similar in their external morphology (Kryštufek and Vohralík, 2001), and species recognition is often difficult. The most recent and significant taxonomic progress has been obtained through morphometrics (Corti et al., 1985; Corti and Loy, 1987; Loy et al., 1993; Rohlf et al., 1996), and genetic analyses (Filippucci et al., 1987). Both these approaches have provided evidence for the specific status of *T. europaea*, *T. caeca*, *T. romana*, *T. occidentalis*, and *T. stankovici*, while the phylogenetic relationships among these taxa are still controversial. Moreover, no information is available on the relationships between western European taxa and the eastern *T. altaica*, *T. caucasica*, *T. levantis*, and *T. davidiana*.

Filippucci et al. (1987) found that *T. caeca* shares the highest number of alleles with all the other European moles, and suggested that this species has remained genetically more similar to the hypothetical common ancestor of the group. Furthermore, these authors found that *T. romana* and *T. stankovici* have a high degree of genetic difference, as do *T. occidentalis* and *T. caeca*. In contrast to these data, geometric morphometrics of skulls Loy et al. (1993) suggested closer phenetic similarities between *T. romana* and *T. stankovici*, and between *T. caeca* and *T. occidentalis*, while *T. europaea* appeared to be most different from these four species. Rohlf et al. (1996) confirmed the morphological similarity between *T. romana* and *T. stankovici*, and found that *T. europaea*, *T. occidentalis* and *T. caeca* are phenetically closely related to the Asian mole *Mogera latouchei* than to the other *Talpa* species.

With the exception of *T. caucasica*, the karyotype of the genus *Talpa* is very conservative. Comparison of G and C bands of *T. europaea* (Zima, 1983) and *T. occidentalis* (Jimenez et al., 1984) did not show any clear difference between these two species, and *in situ* hybridization highlighted only a few karyological differences in the distribution of 5S rRNA genes in *T. europaea* and *T. romana* (Gornung et al., 2008). Nonetheless, based on chromosome morphology and the presence of minute acrocentric chromosomes, Capanna (1981), suggested an evolutionary scenario in which three species, *T. romana*, *T. stankovici* and *T. caeca*, originated from an ancestral *T. europaea* stock.

Finally, the center of origin of the genus is controversial. The oldest fossil (20–22 Mya) was found in Germany (*T. tenuidentata*; Ziegler, 1990). Miocene fossils have also been located in Asia (Fortelius, 2008), where two other closely related genera of strictly subterranean moles, *Mogera* and *Euroscaptor*, are found.

Considering the contradiction in the available data, it is important to first establish the phylogeny of the European species and their relationships with eastern species. Secondly, a need exists for a comprehensive biogeographic hypothetical framework that takes into account the origin of the genus and the processes that led to the distribution patterns of the species today.

To address these issues, we adopted a molecular approach to investigate evolutionary relationships and biogeographic patterns within the genus *Talpa*. We used cytochrome *b* to build a molecular phylogeny and to calibrate a molecular clock in order to estimate the age of divergence among lineages. The comparison between molecular results with fossil data allowed us to construct a biogeographic scenario for the genus.

2. Materials and methods

2.1. Materials

Analyses were performed on 32 specimens belonging to *T. altaica*, *T. caucasica*, *T. stankovici*, *T. levantis*, *T. europaea*, *T. occidentalis*, *T. romana* and *T. caeca*. No tissues were available for the rare species *T. davidiana*. Collection localities are reported in Table 1. Tissues were stored in the collection of the Museo di Anatomia Comparata of the University of Rome "La Sapienza", in the Lomonosov Moscow State University, and in the Science and Research Centre of the University of Primorska.

Table 1
Species, ID, country, locality and EMBL database accession numbers.

Species	ID	Country	Locality	EMBL code
T. occidentalis	107[a]	Portugal	Montemor	FN640553
T. occidentalis	110[a]	Portugal	Montemor	FN640554
T. occidentalis	111[a]	Portugal	Montemor	FN640555
T. occidentalis	3009[a]	Spain	North-eastern Spain	FN640556
T. occidentalis	3013[a]	Spain	North-eastern Spain	FN640557
T. europaea	BV2[a]	Italy	Belvedere, Perugia	FN640551
T. europaea	MO4[a]	Italy	Modena	FN640549
T. europaea	PNA[a]	Italy	Prune Alto, Assisi	FN640552
T. europaea	FRA1[a]	France	Trieves	FN640550
T. caeca	TC03[a]	Italy	Monte Cucco, Umbria	FN640559
T. caeca	PRT6[a]	Italy	Prati di Tivo	FN640558
T. caeca	5689[*,c]	Montenegro	Niksic	FN640560
T. caeca	180/07[*,c]	Montenegro	Mt. Lovćen	FN640561
T. romana	FOR24[a]	Italy	Formello, Roma	FN640564
T. romana	CA2[a]	Italy	Decollatura, Catanzaro	FN640563
T. romana	CA6[a]	Italy	Decollatura, Catanzaro	FN640562
T. romana	TLP2[a]	Italy	Pizzone, Isernia	FN640565
T. stankovici	VIS18[*,a]	Greece	Western Macedonia	FN640566
T. stankovici	AXI1[a]	Greece	Axiopulus	FN640567
T. stankovici	VIT2[*,a]	Greece	Vitzi	FN640568
T. stankovici	34/06[c]	Macedonia	Mt. Galicica	FN640569
T. levantis	174/05[c]	Turkey	Cam Gecidi, Ardahan	FN640570
T. levantis	T10650[b]	Turkey	Ulu Dag	FN640571
T. levantis	T10299[b]	Turkey	Samsun, Kurtler	FN640572
T. levantis	ZMMU186085[b]	Kabardino-Balkaria	Northern Caucasus	FN640573
T. levantis	ZMMU186086[b]	Kabardino-Balkaria	Northern Caucasus	FN640574
T. caucasica	ZMMU186088[b]	Kabardino-Balkaria	Northern Caucasus	FN640575
T. caucasica	ZMMU186089[b]	Kabardino-Balkaria	Northern Caucasus	FN640576
T. caucasica	ZMMU186089[b]	Adygeya	Kisha River	FN640577
T. caucasica	TC4[b]	Russia	Krasnodar region	FN640578
T. altaica	TL1[b]	Russia	Teletskoe Lake, Altai region	FN640579
T. altaica	TL2[b]	Russia	Teletskoe Lake, Altai region	FN640580

[*] Tissues obtained from museum specimens (dry skins or alcool preserved).
[a] Museo di Anatomia Comparata, University of Rome "La Sapienza".
[b] Zoological *Museum*, Lomonosov Moscow State University.
[c] Science and Research Centre, University of Primorska.

2.2. DNA extraction, amplification and sequencing

DNA was extracted from ethanol-preserved tissues or from museum skins following the salting out procedure described by Aljanabi and Martinez (1997).

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The entire cytochrome *b* mitochondrial gene was amplified by polymerase chain reaction (PCR) using a MJ Research PTC-150HB MiniCycler. A 1140 bp fragment of cytochrome *b* was amplified by combining primers L14723 (5′-ACCAATGACATGAAAAATCATCGTT-3′) and H15915 (5′-TCTCCATTTCTGGTTTACAAGAC-3′) as follows: a first cycle of initial denaturation at 94 °C for 1 min, then 35 cycles with denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min. These steps were followed by a 10 min extension at 72 °C. To amplify the complete cytochrome *b* we also used internal primers L15408 (5′-ATAGACAAATCCCATTCCA-3′) and H15553 (5′-TAGGCAAATAGGA AATATCATTCTGGT-3′). DNA extracted from old museum specimens was highly degraded. To ensure the complete amplification of cytochrome *b*, four new primers were designed to obtain fragments of 300–400 bp: TLP-H1 (5′-TGTAATTACCGTTGCACCTCA-3′), TLP-H2 (5′-GCATTGGCTGATAGGTCGAA-3′), TLP-L1 (5′-GCATTCATAGGG TACGTTTTACC-3′), TLP-L2 (5′-CGCTATCCTACGATCAATTCCT-3′). Primers were combined as follows: L14723 + TLP-H1, TLP-L1 + H15553, L15408 + TLP-H2, TLP-L2 + H15915.

PCR reactions were carried out in 25 μL reaction volume including 200 ng of each primer, 2.5 μL of 10× Tris buffer, 2.5 μL $MgCl_2$ 50 mM, 0.2 mM dNTP, 2 U *Taq* polymerase (BioLine), and 50–500 ng of template DNA. Double stranded PCR products were purified using Sure Clean (*BioLine*) and prepared for automated sequencing using the same primers as used for the amplification.

2.3. Sequences analysis and genetic distances

The number of parsimoniously informative sites, variable sites and the nucleotide composition (Irwin et al., 1991) at all the three codon positions were calculated. Possible saturation for substitutions at the three codon positions was evaluated using a graphical survey, by plotting the number of transitions and transversions versus uncorrected genetic distances (p distances) calculated using the MEGA4 program (Tamura et al., 2007).

Average interspecific genetic divergence was calculated with the Kimura 2-parameter model (Kimura, 1980) using the MEGA4 program.

2.4. Phylogenetic analyses

Cytochrome *b* sequences were used to obtain phylogenies using maximum parsimony (MP), maximum likelihood (ML), neighbor-joining (NJ) and Bayesian inference (BA).

Cytochrome *b* sequences of Asian moles of the genus *Euroscaptor mizura* and *Mogera wogura* (Accession Nos. AB076828 and AB033612, respectively) were also included in the analysis. Sequences of *Galemys pyrenaicus* and *Desmana moschata* (Accession Nos. AY833419 and AB076836, respectively) were used as an outgroup in MP and NJ analyses, while only *G. pyrenaicus* was used in ML analysis.

The appropriate model of evolution for the whole dataset was chosen using the software Modelgenerator (Keane et al., 2006) which implements likelihood ratio tests (LRT), two different kinds of Akaike information criteria (AIC1 and AIC2), and the Bayesian information criterion (BIC) to find the best model. Likelihood ratio test found that the best fitting model for our dataset was the general time-reversible model (GTR) with gamma-distributed rate variation across sites (Γ) and a proportion of invariable sites (I), while AIC1 chose TrN+Γ+I, a simpler model of the same family of GTR. Both AIC2 and BIC chose HKY+Γ+I. Posada and Buckley (2004) suggested that the Bayesian and AIC approaches present several advantages compared to the LRT. Furthermore, Posada and Crandall (2001) suggested that the "empirically tuned" AIC2 could perform better compared to AIC1. As AIC2 and BIC converge on the same model, we chose the HKY+Γ+I model for the maximum likelihood and Bayesian analyses. The choice of the simplest model can also avoid the negative effects of over-parameterization, which can introduce biases in branch length estimates due to the reduction of degrees of freedom (Sullivan and Joyce, 2005).

Three different MP searches were performed using the Paup4.0b10 program (Swofford, 1998). The first MP was carried out without *a priori* weighting of characters, and the second and third by applying different weighting schemes (ts:tv = 1:5, ts:tv = 1:10), to correct for the possible effects of saturation of transition at the 3rd codon position, and using a heuristic search with tree-bisection-reconnection (TBR). A consensus tree with support for each clade was obtained after 1000 bootstrap replications for both weighted and unweighted searches.

NJ analysis was performed using the maximum composite likelihood method (Tamura et al., 2007) as implemented in the software MEGA4. This method calculates a distance matrix using a Tamura–Nei model (Tamura and Nei, 1993), maximizing the composite likelihood, calculated as the sum of the log-likelihood of all pairwise distances, to better estimate substitution parameters. The robustness of the tree was tested with 1000 bootstrap replications.

The ML tree was calculated using the PhyML program (Guindon and Gascuel, 2003). Robustness of the reconstruction was assessed through 500 bootstrap replicates.

Bayesian analysis was carried out using the Beast v1.4.7 program (Drummond and Rambaut, 2007). The search was performed with the model chosen using Modelgenerator (HKY) but allowing the software to estimate separate parameters for the first and second positions and the third position (HKY_{112}+CP_{112}+Γ_{112}). This model, which allows different rates of substitution for different codon positions, incorporates information from the genetic code, and this has been suggested to optimize performance of this model for coding sequences (Shapiro et al., 2006). The Markov chain Monte Carlo was run for 20,000,000 million generations, sampling trees every 1000 generations. The first 10% of trees (2000) were discarded and the remaining trees were used to build a maximum *a posteriori* tree that was visualized using the program Figtree v 1.1.2.

2.5. Molecular clock

Calibration of a molecular clock is often a critical issue due to both the heterogeneity of substitution rates among lineages (Britten, 1986) and the uncertainty of the fossil data used as calibration points (Wray, 2001; Benton and Ayala, 2003).

To address these problems we used a relaxed molecular clock (Drummond et al., 2006) as implemented in the Beast 1.4.7 package (Drummond and Rambaut, 2007). This program uses a Bayesian Markov chain Monte Carlo (MCMC) that allows co-estimation of phylogeny and divergence time. We assumed that rates among lineages were uncorrelated with the rate in each branch, independently drawn from a lognormal distribution (UCLN model, Drummond et al., 2006). With Beast 1.4.7, it is possible to use probabilistic calibration priors instead of point calibrations incorporating the uncertainness of calibrations due to the possible lack of knowledge of fossils (Drummond et al., 2006).

Five different calibration points were defined based on available fossil data, following McKenna and Bell (1997) and the NOW (Neogene of the Old World) database (Fortelius, 2008). Given the uncertainties in fossil data we assumed that the range for the age of the split between two sister clades can be roughly approximated by the time range of the earliest fossil definitely belonging to one of the clades. The priors for each calibration point have been hypothesized assuming a lognormal or a normal probability distribution. In the first case, the lower age range of the most ancient fossil was used to define a lower hard bound, and the age range to define the median and 95% high probability density (HPD). If a normal

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Table 2
Mean genetic distance between species calculated using the Kimura 2-parameter model. Species names are abbreviated as follows: *T. occidentalis* (occ), *T. europaea* (eur), *T. caeca* (cae), *T. romana* (rom), *T. stankovici* (sta), *T. levantis* (lev), *T. caucasica* (cau) and *T. altaica* (alt).

	occ	eur	cae	rom	sta	lev	cau
occ							
eur	0.086						
cae	0.094	0.100					
rom	0.111	0.106	0.095				
sta	0.101	0.106	0.097	0.119			
lev	0.104	0.108	0.094	0.115	0.092		
cau	0.149	0.145	0.138	0.147	0.142	0.142	
alt	0.145	0.148	0.153	0.156	0.142	0.137	0.149

distribution was used, fossil age ranges were used to define the mean and 95% HPD probability distributions, with both higher and lower soft bounds.

Firstly, we defined two calibration points external to the genus *Talpa*. The first appearance of Desmaninae (*Mygatalpa*, 34–24 Mya) and Talpinae (*Myxomygale*, 37–34 Mya) subfamilies was used to calibrate the root of the tree. The oldest record of the genus *Desmana* (9–11.2 Mya) was used to calibrate the split between the genera *Desmana* and *Galemys* not later than 9 Mya.

Successively, we defined three calibration points related to the genus *Talpa*. The oldest fossil of *Talpa* is dated to between 20 and 22 Mya (Ziegler, 1990), and by 16–20 Mya the genus had already settled all the Western Palearctic. Consequently, we calibrated the split between the genus *Talpa* and the other Asian moles of the genera *Euroscaptor* and *Mogera* considering that these lineages did not separate later than 16 Mya. In Western Europe, the two fossil species closely related to the extant European moles, i.e., *T. fossilis* and *T. minor* (Cleef-Roders and Hoek Ostende, 2001), co-existed between 4.2 and 5.3 Mya (Fortelius, 2008). This range was used to calibrate the split between *T. caucasica* and the other western European moles. Finally, the oldest fossil of *T. levantis* (1.95–2.6 Mya) was used to define the split between *T. stankovici* and *T. levantis*. Details for each calibration point are reported in Table 3.

3. Results

A significant deviation of the four bases from equal frequency was found in cytochrome *b* sequences at the second and third codon position. In particular, guanine at the second position appeared to be highly underrepresented, only 5.6% of the total, while adenine accounted for almost half of the nucleotides (47%) at this position. At the second codon position, thymine was the most highly represented (42%) with lower levels of guanine (14.2%) and adenine (19.7%). This pattern appeared similar to those found in several mammals (Irwin et al., 1991).

The graphical survey suggested there was a substantial saturation of nucleotide substitutions observed at the third codon position, due to an excess of transitions.

Of the 1140 bp used in the analysis, 379 were found to be parsimonious informative. As expected, the bulk of nucleotide variability was found at the third codon position, with 292 parsimoniously informative sites.

Almost all the obtained trees showed the same topology irrespectively of the method used (Figs. 1 and 2).

Topological differences were found in the unweighted MP and weighted MP with ts:tv = 1:10 (not shown). It is likely that the different result shown by the unweighted MP is due to artifacts introduced by transition saturation at the third codon position. Weighted parsimony with transitions at the third codon position down-weighted 1:10 differed in the branching order of *T. stankovici*, which is basal to *T. levantis*. This topological difference was not supported by bootstrap (<50) and was only found using this weighting scheme; thus, it is probably due to an excessive weight of transversions at the third codon position. Consequently, only weighted MP with ts:tv = 1:5 at third codon position, showing high bootstrap values and topological concordance with other methods, was retained and discussed.

MP (Fig. 1), NJ and BA (Fig. 2) analyses showed a high support for most of the nodes, while ML (Fig. 1) showed lower bootstrap values for some nodes.

In all the trees, the species of the genus *Talpa* form a monophyletic assemblage that is related to the eastern Asian moles of the genus *Mogera* and *Euroscaptor*. According to previous findings (Motokawa, 2004; Shinohara et al., 2005), the genus *Euroscaptor* is paraphyletic compared to the genus *Mogera*.

Within the genus *Talpa* it is possible to distinguish the two most divergent and basal species (Fig. 1 and Table 2), i.e., *T. altaica* and *T. caucasica*. The other six species are genetically more closely related (Table 2). The most basal species of the genus is *T. altaica* and its phylogenetic position is strongly supported by all the analyses (Figs. 1 and 2). The next species to diverge was *T. caucasica*, a species endemic to the Caucasian region. The remaining species can be divided into two main lineages. The first lineage includes *T. stankovici* and *T. levantis*, both present in the southern Balkan Peninsula and absent from Western Europe. The second lineage includes *T. europaea* and the three western European species, *T. occidentalis*, *T. caeca* and *T. romana*. Within this clade, the topology is fully re-

Table 3
Divergence time estimates and priors for calibration points. Mean, median and 95% HPD (high probability density) are expressed in millions of years.

Split	Priors for calibration points				Divergence estimates	
	Distribution	Mean	Median	95% HPD	Mean	95% HPD
Desmanini/Talpini	Normal	35		31.7–38.3	32.09	28.03–36.08
Galemys/*Desmana*	Lognormal		10	9.4–11.3	10.05	9.31–11.51
Mogera + *Euroscaptor*/*Talpa*	Lognormal		17	16.2–21.2	16.62	16.05–18.24
Euroscaptor/*Mogera*					10.97	6.86–14.99
T. altaica/other moles					10.64	6.56–15.53
T. caucasica/European moles	Normal	4.75		4.2–5.3	5.06	4.48–5.68
T. levantis + *T. stankovici*/other European moles					4.67	3.88–5.43
T. levantis/*T. stankovici*	Lognormal		2.3	2.1–2.8	3.00	2.14–4.20
T. levantis basal split					2.36	1.54–3.54
T. romana + *T. caeca*/*T. europaea* + *T. occidentalis*					4.3	3.52–5.14
T. europaea/*T. occidentalis*					3.32	2.32–4.44
T. romana/*T. caeca*					3.75	2.78–4.66
T. caeca basal split					2.33	1.4–3.23
T. occidentalis basal split					2.4	1.48–3.47
T. romana basal split					1.16	0.52–1.96
T. europaea basal split					0.7	0.33–1.38

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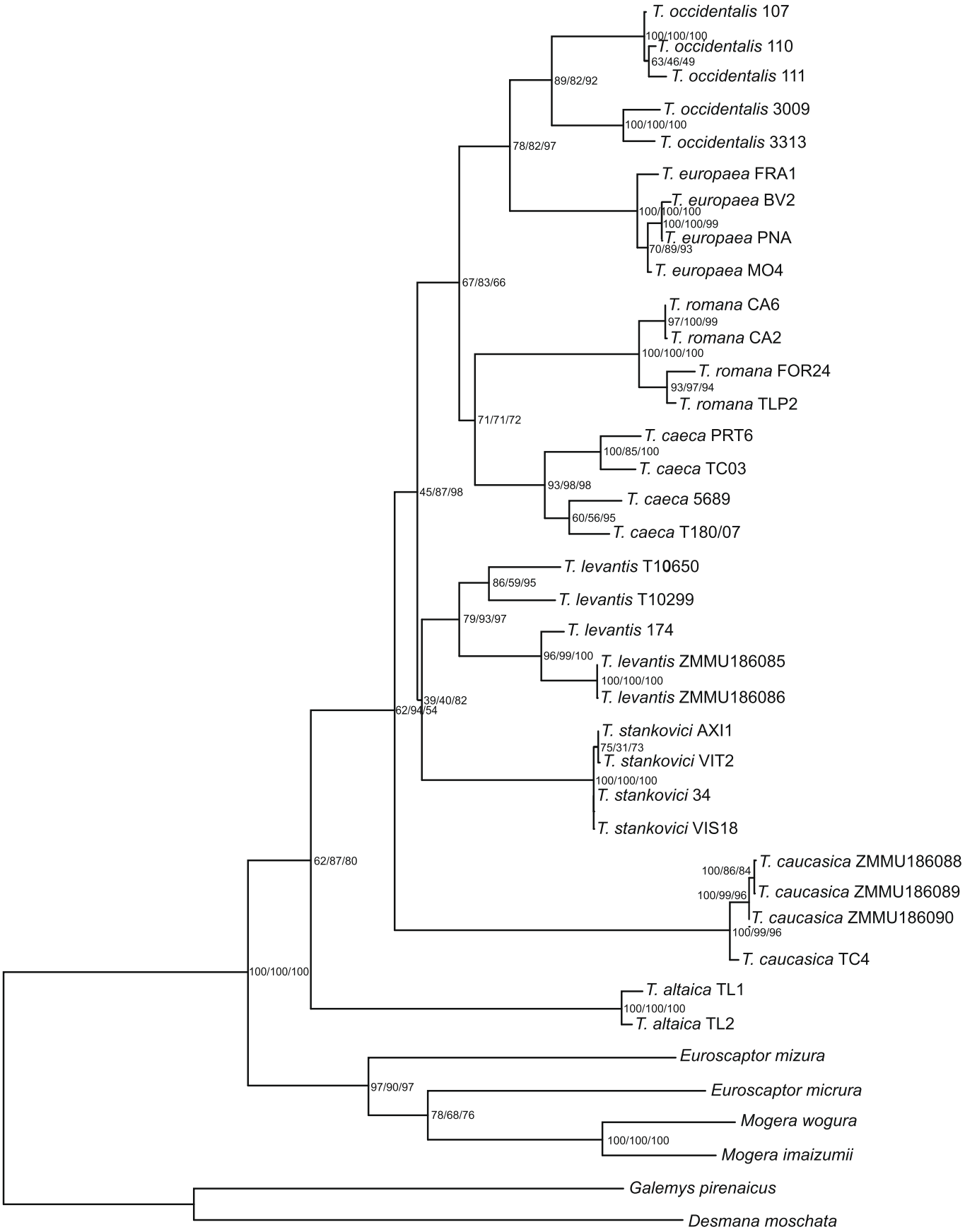

Fig. 1. Maximum likelihood tree. Numbers are bootstrap values of ML, MP and NJ, respectively.

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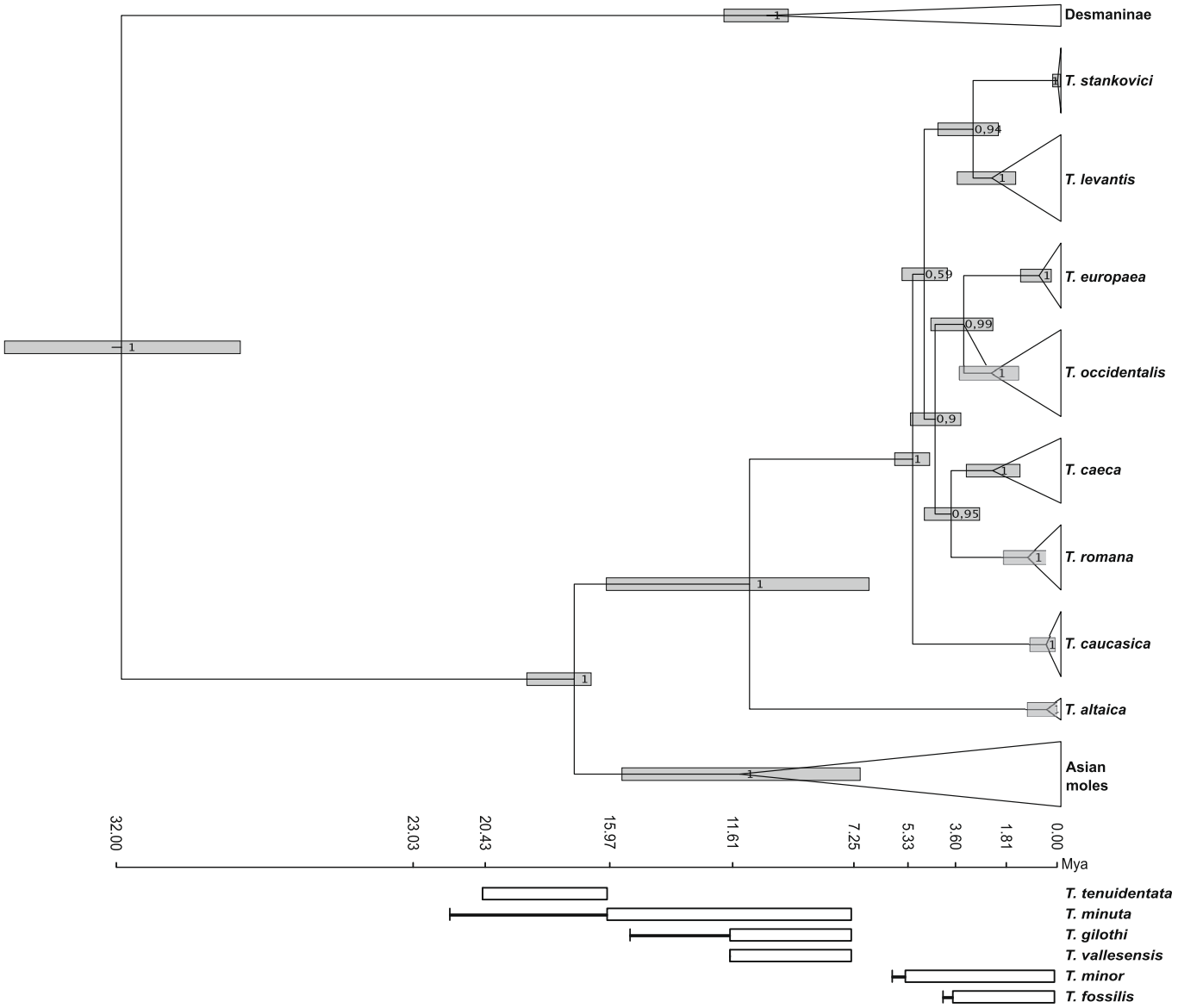

Fig. 2. Bayesian molecular clock estimations. Numbers are posterior probabilities for each node. Grey bars are 95% HPD of the node ages estimation (see Table 3 for divergence time estimations). Below the tree is reported the known range of six fossil species with the 95% confidence interval associated to the first occurrence (Marshall, 1990; Strauss and Sadler, 1989). The bars represent the known range of fossils (data downloaded from the Paleodatabase [http://paleodb.org/] on 1 December, 2008).

solved with good bootstrap values (Fig. 1) and high posterior probabilities (Fig. 2), clearly showing that *T. occidentalis* is the sister species of *T. europaea*, while *T. romana* is the sister species of *T. caeca*. Furthermore, *T. europaea* and *T. occidentalis* show the lowest interspecific genetic divergences within the genus, while *T. romana* and *T. caeca* show a slightly higher divergence (Table 2).

Results of molecular clock analysis revealed a mean clock rate of 0.01407 (standard deviation 0.00003168) substitutions/site/Myr and a coefficient of variation of substitution rate over the tree with a mean of 0.554 (standard deviation 0.004046), which suggests a departure from a strict molecular clock.

The split of the genus *Talpa* from the closest Asian genera can be placed at around 16–17 Mya during the early-middle Miocene (Fig. 2; Table 3). The origin of this genus was followed by first split of *T. altaica* during the middle-late Miocene. Successive divergence events seem to be more recent. First, *T. caucasica* splits from the rest of the species in the early Pliocene, while all successive divergence events can be roughly placed during the middle-late Pliocene (Fig. 2, Table 3). In particular, according to our estimations, the cladogenetic events that produced the four species currently present in Western Europe, i.e., *T. romana*, *T. caeca*, *T. occidentalis* and *T. europaea*, can be placed in a relatively narrow window of time during the late Pliocene. Finally, our molecular clock estimations suggest a basal split for the species *T. occidentalis*, *T. caeca*, and *T. levantis* that can be dated back to the Plio-Pleistocene boundary, while the basal split for both *T. romana* and *T. europaea* appears to be more recent and compatible with a full Pleistocene intraspecific divergence (Table 3).

4. Discussion

The genus *Talpa* comprises two easternmost basal species, *T. altaica* and *T. caucasica*, and two main European lineages. An eastern clade includes *T. stankovici* and *T. levantis* and a western clade includes *T. europaea*, *T. romana*, *T. caeca* and *T. occidentalis*. Within the latter, we found closer affinities between *T. romana* and *T. caeca*, and between *T. europaea* and *T. occidentalis*. These results partially disagree with previous findings.

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Our results reject the closer affinity between *T. romana* and *T. stankovici* and between *T. caeca* and *T. occidentalis* suggested by Martino and Martino (1931), Niethammer and Krapp (1990) and Loy et al. (1993) on the basis of morphological traits. On the contrary, they are in accordance with the pelvis morphology of these species. In fact, *T. europaea* and *T. occidentalis* share a europoid pelvis (with the 4th *foramen sacrale*, which is closed by a bone bridge connecting the ischium and the sacrum (Grulich, 1971)), while *T. romana* and *T. caeca* share a caecoid pelvis (4th *foramen sacrale* is posteriorly open). A caecoid pelvis is also shared by all the other species of the genus *Talpa* (Kryštufek and Vohralík, 2001), suggesting that the europoid pelvis is an apomorphic character of *T. europaea* and *T. occidentalis*.

According to our molecular phylogeny *T. stankovici* and *T. romana* do not share a recent common evolutionary history (Fig. 1), and our estimate suggests an early Pliocene divergence of these two lineages (Fig. 2). In particular, *T. stankovici* appears to be more closely related to *T. levantis*.

Our molecular clock agrees with fossil data in suggesting an origin of the genus *Talpa* during the early Miocene, as the oldest known *Talpa* fossil (*T. tenuidentata*) is dated between 20 and 22 Mya (Ziegler, 1990). Furthermore, our results support an Asian origin of the genus. This evidence is not fully supported by fossil record, as the oldest fossil was found in southern Germany (Ziegler, 1990). Nevertheless, other fossils of *Talpa* species from Asia, dated between 20 and 16 Mya, have been found in the Irkusk region, Russia (Fortelius, 2008).

Molecular clock estimation suggested an origin for the extant European species *T. europaea*, *T. occidentalis*, *T. romana*, *T. caeca*, *T. levantis* and *T. stankovici* during the early Pliocene (Fig. 2 and Table 3). The origin of these species should mark a second radiation event consequent to an extinction crisis at the end of the Miocene. The fossil record support the presence of three different extinct *Talpa* species in Europe since the early Miocene (Fig. 2): *T. minuta* (early Miocene to the first part of late Miocene), *T. gilothi* (middle Miocene to the first part of late Miocene) and *T. vallesensis* (middle Miocene). These species disappeared from the fossil record at the end of the Miocene (Fortelius, 2008), and there is a gap in the mole fossil record in Europe during the Messinian (7.2–5.3 Mya). This gap is followed by the appearance of two new species similar to *T. europaea* and *T. caeca*, i.e., *T. fossilis* and *T. minor*, in the early Pliocene in Europe, without any apparent continuity with the species from the Miocene (Fig. 2).

It is well known that the early and middle Miocene of Europe was humid and warm (Fortelius et al., 2002, 2003). This could have favoured a first mole radiation that gave rise to the family diversification. During the late Miocene, the climate of Europe became more xeric (Fortelius et al., 2002, 2006), which might have caused a drastic reduction of Talpinae diversity in Europe.

An increase in humidity occurring after the Messinian in Europe (Fortelius et al., 2002, 2006) may have triggered new mole diversification during the Pliocene, giving rise to all the extant European species.

Two main lineages split during the early Pliocene (Fig. 2 and Table 3): a western lineage, including the *europaea–occidentalis* and the *caeca–romana* clades, and an eastern lineage, including the *stankovici–levantis* lineage. The successive cladogenetic events within each clade occurred almost contemporaneously during the middle-late Pliocene (Fig. 2 and Table 3).

The geographic pattern of this evolutionary framework involves both Asia and Europe. The fact that *T. altaica* and *T. caucasica* are basal to all the extant European species (Figs. 1 and 2) would suggest that the colonization of Europe started from Asia. Moreover, as *T. caucasica* is the sister species of the European moles, it is likely that the two eastern and western lineages covered a route from the Caucasus and the Black Sea, as a consequence of the increase in humidity and the spread of grasslands at the Miocene–Pliocene boundary (Cerling et al., 1997).

According to our molecular clock estimations *T. levantis* and *T. stankovici* diverged approximately during the middle-late Pliocene and it is possible that the split between these two species was likely a consequence of the allopatric isolation caused by a Pliocene connection between the Mediterranean and the Black Sea (Marinescu, 1992; Clauzon et al., 2005). *Talpa levantis*, that is mainly distributed across Turkey up to the Caucasus, is also present in a restricted area of Europe (Kryštufek and Vohralík, 2001). This would suggest that in some period, successive to the split from *T. stankovici*, *T. levantis* was able to cross the Bosphorus strait. Several mammalian species are known to have migrated from Asia to Europe during the early Pliocene following a route from Anatolia to the southern Balkans (Koufos et al., 2005). The same route could have been used by *T. levantis* and possibly by the ancestor of the *levantis–stankovici* lineage. However, the oldest known fossil of *T. levantis* was found in Bulgaria (late Pliocene, Popov, 2004) and the oldest *T. levantis* from Turkey is from the early Pleistocene (Fortelius, 2008). This fact would suggest a colonization route from Europe to Anatolia and not vice versa. However, because the absence of earlier fossils from Turkey could be due to a lack knowledge of fossil records, further molecular analyses, including new samples of *T. levantis* from Bulgaria, as well as new data from paleontologists, will be required to elucidate colonization routes in this area.

Concerning the four western European species, *T. europaea*, *T. occidentalis*, *T. romana* and *T. caeca*, our data reveal the existence of two distinct lineages: a southern European lineage including *T. romana* and *T. caeca*, and a northern lineage including *T. europaea* and *T. occidentalis*. According to our estimate, the split of the two lineages occurred during the early-middle Pliocene (Table 3). Although our data do not allow the depiction of the evolutionary processes and colonization routes that led to the present distribution of these taxa, some inferences can be made on the role of climate in shaping their actual distribution ranges.

According to Fanfani (2000) and Kotsakis et al. (2003), *Talpa* cf. *romana*, a talpid very similar to living endemic Italian species, appears for the first time in the Pleistocene (Late Biharian, 1.0–0.8 Mya) in Southern Italy (Sant'Arcangelo, Lucania). The species range shrank and expanded following the Pleistocene glaciation cycles. During warmer periods, *T. romana* fossils dominate the faunal associations in southern Italy, occurs as far north as the Friuli region near the Alps. During the coldest periods they are totally absent from northern Italian fossil associations (Kotsakis et al., 2003). These findings suggest that the current distribution patterns of *T. romana* and probably its closely related species, *T. europaea*, *T. caeca* and *T. occidentalis*, have been influenced by Pleistocene glaciations. In particular, *T. romana*, *T. caeca* and *T. occidentalis* were likely confined in the three southern European peninsulas that acted as Pleistocene glacial refugia for several other species of mammals (Randi, 2007). These three species were probably unable to later recolonize their previous ranges. This concurs with the view of Bilton et al. (1998), who suggested that Mediterranean refugia are hotspots of endemism but not the main source of postglacial recolonization of central and northern Europe.

On the contrary, *T. europaea* probably found multiple refuge sites, both in the European peninsulas and in eastern Europe, similar to many other small mammals (Jaarola and Searle, 2002; Koltlík et al., 2006), and was thus able to recolonize all those areas of Europe that were covered by permafrost during the last glacial maximum. In fact, evidence of secondary contact zones within *T. europaea*, likely related to the Pleistocene postglacial recolonization phases, have been highlighted by Loy and Corti (1996).

In conclusion, the cytochrome *b* phylogeny depicts a new phylogeny for the species of the genus *Talpa* and suggests the need for

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a critical review of the morphological traits traditionally used for phylogenetic analysis. The most widely used characters on which species affinity has been based are craniometric. However, it is likely that these characters, despite their usefulness in distinguishing species, do not accurately reflect evolutionary relationships. Other morphological characters of the post-cranial skeleton, such as the humerus, have been used to reconstruct the phylogeny of the family Talpidae, suggesting the presence of a good phylogenetic signal, congruent with characters from different sources (Sánchez-Villagra et al., 2004). It will be worth using these characters to test the phylogenetic and biogeographic hypotheses that have emerged from the present survey as they also allow the inclusion of fossil data.

The evolutionary scenario that emerges from our analyses proposes that the response to aridification levels of the Mio-Pliocene was one of the major forces driving extinction, diversification, and migration of the genus *Talpa*. Additionally, the Pleistocene glaciations seem to have affected the phylogeographic pattern of the extant species by reducing and fragmenting their ranges, resulting in the high degree of endemism and producing secondary contact zones in the widespread species *T. europaea*. Our results agree with the hypothesis proposed by Zink et al. (2004) who postulated the protraction of speciation events over the past 5 Myr, contradicting a view that the entire Pleistocene, including the last two glacial cycles, was important for speciation (Johnson and Cicero, 2004; Lister, 2004).

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