Did the Quaternary climatic fluctuations really influence the tempo and mode of diversification in European rodents?

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1 **Abstract**

2   The objective of the present study is to establish if the Quaternary climatic fluctuations

3 influenced the tempo and mode of diversification in European rodents. Our case study is the

4 subgenus *Microtus* (*Terricola*) distributed from Western Europe to the Caucasus.

5 Mitochondrial cytochrome *b* gene sequences from several representatives of all the species

6 were used to generate maximum-likelihood and Bayesian phylogenetic trees, to estimate

7 divergence times, to identify biogeographic ancestral areas and to study the rate of

8 diversification. Results showed that phylogenetic tree topologies were similar to previous

9 published studies but with a better resolution at some nodes. The origin of *Microtus*

10 (*Terricola*) is dated back to approximately 4.05 Myr in the Early Pliocene, and molecular

11 dating for most *Terricola* species corresponds to several glacial periods of the Pleistocene.

12 Results of the biogeographic ancestral area reconstruction suggest that *Microtus* (*Terricola*)

13 diversified from the Caucasus/Turkey/Iran area through Western Europe. Several periods of

14 diversity variation were highlighted: two period of diversity increase, between 3 and 2 Myr,

15 and after 1 Myr; two periods of diversity decrease, before 3 Myr, and between 2 and 1 Myr.

16 The diversification rate of *Microtus* (*Terricola*) was 0.353 ± 0.004 event/Myr, a rate similar to

17 that of the Muridae family. To conclude, although the Pleistocene glacial conditions had an

18 impact on the speciation events, the Quaternary does not appear however as a period with an

19 exceptional rate of diversification for European rodents.

20

1 Introduction

Estimating diversification rates appears essential in ecology and evolutionary biology to understand how the biodiversity varies across space and time (Ricklefs 2007; Morlon 2014). Because speciation and extinction processes require thousands to millions of years to happen, diversification has long been studied from fossil data. Since the 1990's, a phylogenetic alternative is commonly used to estimate speciation, extinction, and thus diversification rates because phylogenies contain information about evolutionary relationships among species with a temporal dimension (Hey 1992; Nee et al. 1994; Sanderson and Donoghue 1996; Paradis 1997; Ricklefs 2007; Morlon 2014) and a characteristic signature left by extinction events (Nee 2001; Rabosky 2009). Molecular phylogenies were used to infer diversification rates, for instance, in plants (Magallón and Sanderson 2001; Hughes and Eastwood 2006), insects (Barraclough and Vogler 2002), amphibians (Kozak et al. 2006) and birds (Zink et al. 2004). In mammals, a fluctuating diversification rate since the Cretaceous/Tertiary boundary was recently highlighted (Stadler 2011). Several shifts in diversification rates were even identified in the most diversified mammalian clade, the Rodentia (Steppan et al. 2004; Fabre et al. 2012). Several hypotheses were proposed to explain these shifts as key innovations, biogeographic events, absence of competition or predation, chromosomal rearrangements as well as environmental changes (Rowe et al. 2011; Fabre et al. 2012).

Quaternary climatic fluctuations (during the last 2.6 Myr, Cohen and Gibbard 2012) are thought to have influenced plant and animal distribution through repeated range contraction (population isolation) and expansion (colonization by tracking favourable climatic space). For this reason, they are often considered as a major driving force of allopatric diversification. In particular, the role of Quaternary glacial cycles is often considered to explain the extraordinary diversification of the rodent genus *Microtus* Schrank, 1798 from the family Cricetidae (Chaline 1987; Chaline et al. 1999; Jaarola et al. 2004). The ancestor of the *Microtus* species is apparently within species of the genus *Allophaiomys* Kormos, 1930 (Chaline et al. 1999). Early radiation and diversification about 2.4 – 2 Myr ago (Early Pleistocene; Chaline and Graf 1988; Chaline et al. 1999; Zheng and Zhang 2000) would have generated *Microtus* subgenera that subsequently would have led to the appearance of the diverse extant species. One of the most studied radiations from systematic, paleontological, odontometric, cytogenetic, ethological, morphometric or genetic standpoints (*e.g.* Bastos-Silveira et al. 2012; Brunet-Lecomte 1988, 1989, 1990; Brunet-Lecomte and Chaline 1990, 1991, 1992; Castiglia et al. 2008; Chaline and Graf 1988; Chaline et al. 1988, 1999; Giannoni

et al. 1993; Haring et al. 2000; Jaarola et al. 2004; Kryštufek et al. 1996; Macholán et al. 2001; Martínková and Dudich 2003; Martínková and Moravec 2012; Martínková et al. 2004, 2007; Meylan 1972; Mitsainas et al. 2009; Rovastos and Giagia-Athanasopoulou 2012; Santos et al. 2009; Storch and Winking 1977; Thanou et al. 2005; Tougard et al. 2008a; Zagorodnyuk 1990) is the radiation of the subgenus *Terricola* Fatio, 1867 (European ground voles). The oldest fossil remains of *Microtus (Terricola)* are known from the end of Early Pleistocene (around 0.9 Myr) in Italy and they were attributed to *M.* (*T.*) *arvalidens* Kretzoi, 1958 (Masini and Sala 2007).

The subgenus *Microtus* (*Terricola*) is characterized by a "pitymyan rhombus" on the first lower molar, a primitive character found in evolved species of *Allophaiomys* (Chaline et al. 1999). First grouped with some Nearctic voles under the subgenus *M.* (*Pitymys*) McMurtrie, 1831, evolutionary studies and phylogenetic analyses underlined that the European ground voles are closer to European *M.* (*Microtus*) than to the American subgenera (Graf, 1982; Chaline et al. 1988; Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al. 2008). Indeed, although Palearctic and Nearctic ground voles seem to share the same *Allophaiomys* ancestor, they evolved independently leading to two monophyletic subgenera, respectively *M.* (*Terricola*) and *M.* (*Pitymys*) (Chaline et al. 1988, 1999). If the phylogenetic position of the European ground voles within the Arvicolinae is now clearly established (Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al. 2008), attempts to reconstruct phylogenetic relationships among *M.* (*Terricola*) from molecular markers have failed maybe because of the dataset incompleteness: mitochondrial cytochrome *b* gene (cytb) sequences of one or several individuals but not all species represented (Jaarola et al. 2004; Robovský et al. 2008); concatenated supermatrix (72.8% of missing data) of mitochondrial (cytb, control region, cytochrome *c* oxidase subunit I and NADH dehydrogenase subunit 4) and nuclear (interphotoreceptor retinoid-binding protein, growth hormone receptor, exon 10, sex-determining region Y and lecithin cholesterol acyl transferase, exons 2-5) markers for one individual of each species (Martínková and Moravec 2012).

According to Ricklefs (2007), using phylogenetic information to estimate diversification rates depends on three assumptions: the completeness of the phylogenetic data, a reliable time scale and the constancy of speciation and extinction rates through all clades. Because of their high specific diversity (14 extant and 11 extinct species; Brunet-Lecomte 1990; Brunet-Lecomte et al. 1992; Gil 1996; Kowalski 2001; Musser and Carleton 2005), their geographic distribution (from the Caucasus Mountains to the Iberian Peninsula; Musser

and Carleton 2005), the knowledge of their fossil record and their evolutionary history rooted in the Quaternary (Chaline and Graf 1988; Chaline et al. 1999; Zheng and Zhang 2000), the European ground voles are a species group particularly suitable to understand if and how the climatic fluctuations of the Quaternary could have promoted the diversification of rapid evolving small mammals such as rodents in temperate zones. In other words, does the level of diversification during this period exceed other mammal rates? In this context, the aim of the present study is as follows: to investigate the phylogenetic framework of the diversification from sequences of multiple representatives of all the *Microtus* (*Terricola*) species, and subspecies when sequences were available; to identify the origin of the diversification; to estimate the rate of diversification. For simplicity, the present study follows the systematic designation of Musser and Carleton (2005), and when dealing with the species, it refers to the genus (*Microtus* or *M.*) rather than the subgenus name (*Microtus* or *Terricola*), and to *Terricola* rather than *Microtus* (*Terricola*) when dealing with the subgenus.

Material and Methods

Data

Our dataset included original (15) and GenBank (85) DNA sequences for 100 specimens representing all the *Microtus* (*Terricola*) species (according to Musser and Carleton 2005) and some *Microtus* (*Microtus*) species, these latter being used as outgroup (Table 1). For original data, total DNA was extracted from 96% ethanol-preserved tissue pieces (skin or foot) following standard procedures (Sambrook et al. 1989). The complete cytochrome *b* gene (cytb) was PCR-amplified (Tm = 50°C) with universal primers located in the flanking tRNAs (L7 5'–ACCAATGACATGAAAAATCATCGTT–3' and H6 5'–TCTCCATTTCTGGTTTACAAGAC–3'). Direct sequencing was performed in both directions to confirm polymorphic sites by Macrogen Inc (Seoul, Korea). Original and GenBank sequences were aligned by hand using MEGA v5.2.2 (Tamura et al. 2011). Details about the sampling and sequences are in Fig. 1 and Table 1.

Phylogenetic inference

Phylogenetic trees were reconstructed using a maximum-likelihood approach (ML) and a Bayesian inference (BI) through the technical facilities of the Platform Montpellier Bioinformatics Biodiversity (MBB) of the "Institut des Sciences de l'Evolution de

Montpellier" (Centre Méditerranéen de l'Environnement et de la Biodiversité, Montpellier, France; ISEM and CeMEB). Best-fitting models of nucleotide substitution were determined using MrModeltest v2.3 (Nylander 2004). The optimal fitted model in both cases is identified by the minimum value of the Akaike Information Criterion.

ML reconstruction was conducted using PhyML v3.0 (Guindon et al. 2010) under the GTR model (Yang 1994) with a proportion of invariable sites (I) and a gamma distribution (G). Nodal robustness was estimated by bootstrap percentage values (BP) after 1000 pseudo-replicates. In BI, a mixed model analysis was applied according to the cytb codon positions: (1) and (2) HKY (Hasegawa et al. 1985) +I+G; (3) GTR+I+G. Five independent runs of five Markov chain Monte Carlo (MCMC) chains were simultaneously carried out for 5,000,000 generations using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of trees sampled every 100th generation after a burn-in stage of 25,000.

Alternative hypotheses of intra-*Terricola* relationships were compared with the test of Shimodaira and Hasegawa (1999) as implemented in PAUP*4.010b (Swofford 2002).

Intra- and intergroup genetic distances were estimated by the Kimura-2-parameter distance (K2P; Kimura 1980) with MEGA. An internal branch test was performed also with MEGA to determine whether short internal branches in the phylogeny are solved relationships or polytomies. A neighbour-joining distance-based method was used to build a tree under the K2P nucleotide substitution model.

Divergence time estimates

Time to the most recent common ancestor (TMRCA) was estimated for several clades, and especially each *Terricola* species, by a Bayesian coalescent analysis under the GTR+I+G model with BEAST, v2.3.1 (Bouckaert et al. 2014). Through the technical facilities of the Platform MBB of ISEM (CeMEB), divergence time estimates were achieved under the recently introduced "fossilized birth-death" (FBD) process (Heath et al. 2014). The FBD model considers the diversification of extant and fossil species are part of the same macro-evolutionary process. Four parameters (speciation, extinction and fossilization rates as well as proportion of sampled extant species) are taken into account to inform the amount of temporal uncertainty associated with speciation events on the tree (Heath et al. 2014). Three molecular clocks (strict, relaxed uncorrelated lognormal or relaxed uncorrelated exponential) were compared using Bayes factor values (BF) to test which of them best fit our data. BF significance was then determined from 2lnBF results (Brandley et al. 2005). BEAST analyses

consisted of five independent runs of 20 million generations with the first 10% discarded as burn-in. Results of these five independent runs were combined with LogCombiner v2.3.1 (Bouckaert et al. 2014) to estimate TMRCA and 95% confidence intervals. A consensus tree was generated using TreeAnnotator v2.3.1 (Bouckaert et al. 2014) with mean node heights as node heights option and maximum clade credibility as target tree type option.

The literature regarding the evolutionary history of the subgenus *Terricola* is rich but full of temporal inaccuracies (a time period such as, for instance, the Cromerian or the Middle Pleistocene in Brunet-Lecomte 1990, Kowalski 2001; a relative dating as, for instance, in Cuenca- Bescós et al. 2010, Masini et al. 2005, Masini and Sala 2007; rarely an absolute dating as in Bonfiglio et al. 2008) or contradictory relationships (notably for the phylogenetic position of *Allophaiomys* or for the relationships between extinct and extant species as in Chaline, 1987, Chaline et al., 1999). For these reasons, only the dates of 0.800 ± 0.100 Myr for the occurrence of the ancestor of *M. subterraneus*, *i.e*. *M. arvalidens* (Kowalski 2001; Masini et al. 2005; Masini and Sala 2007; Cuenca- Bescós et al. 2010), as well as the date of 0.475 ± 0.025 Myr for the origin of *M. arvalis* (Miesenheim I, Germany; Kowalski 2001) were used as fossil node and tip calibration points, respectively.

Identification of the ancestral area and diversification rate

Present-day distribution range (Fig. 1) of *Terricola* was divided into 11 biogeographic areas, based on the occurrence of one or more *Terricola* species (Austria, the Balkans, the Carpathians, the Caucasus/Turkey/Iran area, France, Germany/Bavaria, Italy, Portugal, Spain, Switzerland and eastern Europe). The possible ancestral areas of the *Terricola* main clades were then reconstructed using the Statistical Dispersal-Vicariance Analysis method (S-DIVA; Yu et al. 2010) implemented in RASP v2.1b (Yu et al. 2012) from 2,000 trees obtained from the five combined BEAST runs.

Diversification rates were estimated using BayesRate v1.63b (Silvestro et al. 2011) and BEAST. With BEAST, the analysis was performed simultaneously with the divergence time estimates as previously mentioned on a dataset including the outgroup. To evaluate the accumulation of lineages through time (LTT), a LTT plot was constructed with Tracer1.6 (Rambaut et al. 2014). With BayesRate, marginal likelihoods via the thermodynamic integration were calculated to select the best-fitting model of diversification between the pure-birth or birth-death processes, under the following parameters: 100,000 MCMC iterations per five chains for 1,000 randomly subsampled posterior BEAST trees excluding

1 the outgroup. Marginal likelihoods were then compared using Bayes factor tests as previously

2 mentioned. Speciation (λ), extinction (μ) and diversification ($\lambda - \mu$) rates through time were

3 finally estimated with the selected model and previously mentioned parameters. The results

4 were checked using Tracer.

5

6 **Results**

7

8 **Sequence analysis and phylogenetic reconstructions**

9 The alignment of the complete cytb was 1143 nucleotides long with 348 phylogenetically

10 informative sites (368 with the outgroup). The new sequences were deposited in the EMBL

11 Nucleotide Sequence Database under the accession numbers LT222298-LT222312. Base

12 composition (A = 31%, C = 29%, G = 13%, T = 27%) was quite similar to that of other

13 rodents (Martin et al. 2000; Lecompte et al. 2002; Montgelard et al. 2002), and especially

14 *Microtus* rodents (Conroy and Cook 1999; Tougard et al. 2008). This indicated that no

15 artificial cluster occurred due to a misleading compositional signal in the dataset.

16   Phylogenetic reconstructions provided similar tree topologies in ML (Fig. 2 with BP

17 and PP robustness values) and BI (data not shown). The monophyly of the subgenus *Terricola*

18 is highly supported in both approaches (BP = 98%; PP = 1.00). Among *Terricola*, all the

19 species are also supported with high values (88% < BP < 100%; 0.96 < PP < 1.00). Four

20 internal nodes characterized by short branches remain unsolved as in previously published

21 studies (Jaarola et al. 2004; Martínková and Moravec 2012). The internal branch test indicates

22 that these branches are significantly different from zero with length confidence probabilities

23 higher than 95% (data not shown; Nei and Kumar 2000; Tamura et al. 2011). On the other

24 hand, some nodes, not supported in previously published studies (Jaarola et al. 2004;

25 Martínková and Moravec 2012; Robovský et al. 2008), are here moderately or highly

26 supported: the position of *M. majori* as the first offshoot of the *Terricola* species (BP = 98%;

27 PP = 1.00); the cluster of *M. gerbei* with *M. duodecimcostatus* and *M. lusitanicus* (BP = 60%;

28 PP = 0.87). As in Castiglia et al. (2008), *M. savii* appeared paraphyletic because of the

29 internal position of *M. brachycercus* in this group (BP = 100%; PP = 1.00). The present study

30 also confirmed that *M. tatricus* belongs to the subgenus *Terricola* as suggested by several

31 authors (Chaline et al. 1988; Jaarola et al. 2004; Martínková and Moravec 2012), and that *M.*

32 *schelkovnikovi* does not seem to be a *Terricola* species.

The intra-*Terricola* relationships from the present ML tree topology (Fig. 2) were compared with the intra-*Terricola* relationships presented in Martínková and Moravec (2012) with the test of Shimodaira and Hasegawa (1999). The best ML tree is the tree presented in Fig. 2. However, the tree topology from Martínková and Moravec (2012) is not significantly worse at the 5% confidence level ($P = 0.076$). These trees differ by the position of *M. majori* as the sister group of all the *Terricola* species (present study) or of the *daghestanicus* / *subterraneus* clade (Martínková and Moravec 2012), as well as the position of *M. thomasi* and *M. tatricus* as the sister group of either *M. felteni* or the *multiplex* complex.

Genetic distances were estimated within and between *Terricola* species or subspecies (Table S1). Intragroup distances are from $0.1 \pm 0.1\%$ for *M. thomasi atticus* to $2.8 \pm 0.4\%$ for *M. daghestanicus*, while intergroup distances are from $0.8 \pm 0.3\%$ (*M. thomasi atticus*/*M. thomasi evia*) to $12.6 \pm 1.5\%$ (*M. brachycercus*+*M. savii niethammericus*/*M. daghestanicus*).

Molecular dating, biogeographic history and diversification rates

Likelihood differences suggested that the relaxed uncorrelated lognormal clock is significantly more adapted to our dataset (2lnBF > 10). For this reason, divergence times for TMRCA in the subgenus *Terricola* were estimated under a lognormal molecular clock and the fossilized birth-death process. Convergence to stable values was checked with Tracer 1.6, obtaining an effective sample size (ESS) greater than 200 for all parameters. Divergence times are provided in Fig. 3. The origin of this subgenus is dated back to approximately 4.05 Myr in the Early Pliocene, and molecular dating for most *Terricola* species corresponds to several glacial periods of the Pleistocene: from 1.81 Myr for the *savii* complex to 0.08 Myr for *M. majori*. In this context, the substitution rate is estimated at 0.033 ± 0.004 substitution / site / Myr which seems lower than the rate (0.08) estimated for the genus *Microtus* (represented by *M. levis* and *M. kikuchii* Kuroda, 1920) but closer to that of others rodent genera such as *Mus* Linnaeus, 1758, *Rattus* Fisher, 1903, *Ctenomys* Blainville, 1826 and *Chaetopidus* Merriam, 1889 (Triant and DeWoody 2006).

Results of the ancestral area reconstructions with RASP are presented in Fig. 3. S-DIVA suggests that the subgenus *Terricola* most likely diversified early in the Caucasus/Turkey/Iran area (CTI in Fig. 3; frequency of occurrence = 100%). Subsequently, multiple dispersal (7) and vicariance (5) events occurred. Within the CTI area, two clades diverged giving rise to *M. majori* and the clade containing all the other *Terricola* species with an ancestor distribution including the CTI area and Southern Europe (100%). With an occurrence frequency of 100%, possible ancestor ranges should be: the CTI area for *M.*

1   *daghestanicus* and *M. subterraneus*; Italy for the *savii* complex; Spain + France for *M. gerbei*,

2   *M. lusitanicus* and *M. duodecimcostatus*; the Balkans for *M. felteni* and *M. thomasi*; the

3   Carpathians for *M. tatricus*. The biogeographic history of the *multiplex* complex (*M.*

4   *multiplex*, *M. liechtensteini* and *M. bavaricus*) is much more difficult to interpret with Italy,

5   the Balkans, France, Switzerland and/or Austria as possible ancestral area.

6   In diversification estimates, BF values provided a strong support (2lnBF > 10) for the

7   birth-death process. The diversification rate detected for this model was 0.353 ± 0.004 ($\lambda =$

8   5.767 ± 0.033 and $\mu = 5.414 \pm 0.031$) and 0.483 ± 0.051 event/Myr with BayesRate and

9   BEAST, respectively. The LTT plot (Fig. 4) reflects two periods of diversity increase

10   (between 3 and 2 Myr, and after 1 Myr) and decrease (before 3 Myr, and between 2 and 1

11   Myr).

12

13   **Discussion**

14

15   **Phylogenetic framework of the diversification**

16   From a molecular standpoint, the phylogeny of the subgenus *Terricola* was never studied for

17   itself but it was always studied in a larger context, *i.e.* included in the phylogeny of the

18   Arvicolinae or of the genus *Microtus* (Jaarola et al. 2004; Martínková and Moravec 2012;

19   Robovský et al. 2008). For this reason, its evolutionary history was never deeply investigated.

20   The use of only cytb sequences should be seen as a limitation of the study. Nevertheless, our

21   phylogeny based on a single mitochondrial marker but including several representatives of all

22   the *Terricola* species appears better resolved.

23   As in previously published studies, the present study confirms the monophyly of the

24   subgenus *Terricola*. Several clusters are not questionable, notably the *multiplex* complex

25   (Haring et al. 2000; Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al.

26   2008). The cluster composed of *M. subterraneus* and *M. daghestanicus* is also always highly

27   supported, although Macholán et al. (2001) suggested the paraphyly of *M. subterraneus* with

28   *M. majori* and *M. daghestanicus* from karyotypic and allozymic data. Regarding the *savii*

29   complex, three clades (*M. s. savii*, *M. s. nebrodensis* and *M. brachycercus*+*M. s.*

30   *niethamericus*) were identified as in Castiglia et al. (2008). These latter authors suggested first

31   the paraphyly of *M. savii* because of the internal position of *M. brachycercus*, and they then

32   proposed a specific status for both species in considering *M. s. niethammericus* and *M.*

33   *brachycercus* as conspecific. As for *M. s. nebrodensis*, some authors supported the hypothesis

that it is an endemic species of Sicily because of its phylogenetic position in the *savii* complex and its high morphological, cytogenetic, mitochondrial and nuclear genetic divergence (Castiglia et al. 2008; Bezerra et al. 2015). The cluster of *M. gerbei* with the clade formed by *M. lusitanicus* and *M. duodecimcostatus* is also highly supported, as in Robovský et al. (2008), although Chaline et al. (1988, 1999) considered *M. gerbei* as a species of the *savii* complex from paleontological and morphological data. Lastly, *M. schelkovnikovi* does not seem to be a *Terricola* species as proposed by morphological, karyological and molecular studies (Nadachowski 2007; Martínková and Moravec 2012). Nadachowski (2007) even considered this species as the sole member of the subgenus *Microtus* (*Hyrcanicola*).

By contrast, the present study strongly supports the position of *M. majori* at the basis of the *Terricola* clade, what is in opposition with previous published works (Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al. 2008) where *M. majori* is clustered with *M. subterraneus* and *M. daghestanicus* but with low supports. Based on its karyotype, Zagorodnyuk (1990) considered besides *M. majori* as the sole member of its own species complex. An other point of divergence between the present and previously published studies (Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al. 2008) is the phylogenetic position of *M. thomasi* and *M. tatricus*. Regarding *M. thomasi*, three groups are identified in the present study and they are attributed to three (*thomasi*, *atticus* and *evia*) out of the five *M. thomasi* subspecies, as in Rovatsos and Giagia-Athanasopoulou (2012) but here with better supports. Based on reproductive, cytogenetic and cytb data, these latter authors proposed to consider *M. thomasi* and *M. atticus* (including the two "chromosome races" *atticus* and *evia*) as separate biological species. In the present study, *M. thomasi* would be the sister group of the *multiplex* complex, while *M. tatricus* would be the sister species of *M. felteni*. It is the opposite situation in previously published studies (Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al. 2008), but, in any case, relationships are poorly supported. No information in the literature can support a hypothesis rather than the other, except maybe the geographic proximity of *M. thomasi* and *M. felteni* in the Balkans (Mitchell-Jones et al. 1999; Musser and Carleton 2005; Shenbrot and Krasnov 2005).

Unfortunately, two internal clusters remain not supported among the *Terricola* species (Fig. 2) and are characterized by short branches. Several studies at generic level recovered polytomies among species, leading some authors to the conclusion of a burst of species diversification with no time left for the accumulation of synapomorphies in mitochondrial DNA (Conroy and Cook 1999; Galewski et al. 2006; Jaarola et al. 2004; Lessa and Cook

1998). In the present case, the internal branches not supported appear not significantly short that suggests a lack of phylogenetic resolution rather than fast lineage differentiations.

Origin of the diversification

From the fossil record, the evolutionary history of the genus *Microtus*, and thus of the subgenus *Terricola*, seems rooted in the Quaternary because the oldest fossil remains attributed to the ancestor of *Microtus* (*i.e. Allophaiomys*) are from the Early Pleistocene (2.4 - 2 Myr; Chaline and Graf 1988; Chaline et al. 1999; Zheng and Zhang 2000). However, our molecular dating and ancestral biogeographic reconstruction place the origin of *Terricola* at the end of the Early Pliocene (≈ 4 Myr) in a region including the Caucasus, Turkey and Iran (CTI; Fig. 3). Indeed, this subgenus shares a common ancestor with the subgenus *Microtus* (Jaarola et al. 2004; Martínková and Moravec 2012; Robovský et al. 2008) of which most species are distributed in Eastern Europe, Asia Minor and Western Central Asia, including the CTI area (Musser and Carleton 2005; Shenbrot and Krasnov 2005). In this region, the orogenic activity linked to the collision between the Arabian and Eurasian plates was at its acme during the late Miocene-Pliocene and it was accompagnied by a rapid mountain uplift that could potentially lead to the isolation of ancestral *Terricola* populations (Mosar et al. 2010; Ruban et al. 2007). A vicariant event (≈ 3.6 Myr) seems then at the origin of the split between two ancestral lineages: one including the species of the *subterraneus* complex and the other leading to all endemic European species. After 3 Myr, the progressive global cooling leading to a more suitable composition of the vegetation (from sub-tropical forested environments to temperate broad-leaved deciduous or coniferous forests and boreal vegetation; Fauquette and Bertini 2003; Pross and Klotz 2002; Thompson and Fleming, 1996; Willis et al. 1999) for *Terricola* species would have allowed the colonization of Europe westwards.

From the Pleistocene climatic records, glacial conditions dominated between the Praetiglian (2.6 – 2.4 Myr) to the Menapian (1.20 – 1.07 Myr) stages (Cohen and Gibbard 2012; De Jong 1988; Vandenberghe 2001). They should be at the origin of the differentiation of ancestral evolutionary lineages in Mediterranean peninsulas (Italy, the Balkans and the Iberian Peninsula; Fig. 3). Indeed, these Mediterranean areas are often considered as glacial refugia and sources of northwards postglacial colonization (Hewitt 1996, 2000, 2004; Taberlet et al. 1998) or as a hotspot of endemism for small mammals (Bilton et al. 1998). In both cases, geographic isolation of small populations in these peninsulas during Pleistocene glaciations could have served as "speciation traps", thus promoting allopatric speciation

events (Chaline 1987). This was notably exemplified by the *in situ* differentiation of the *savii* or *multiplex* complex ancestral lineages in Italy or in the Alps, respectively (Fig. 3). Even if Italy is recognized as a potential glacial refugium, few Italian populations would have participated to postglacial (re)colonization of northern Europe because of the Alps (Hewitt 2000; Taberlet et al. 1998), although the Alps are considered either as a barrier to postglacial expansion as for the *savii* complex species or as a glacial refugium (in southwestern, southeastern and/or northern marginal areas) as for the *multiplex* complex species (Haring et al. 2000; Hewitt 2000, 2004; Schmitt and Varga 2012).

From the present molecular dating, most extant *Terricola* species have their origin after the end of the Cromerian interglacial ($\approx$ 0.5 Myr; Fig. 3), *i. e.* during a period characterized by the development of regular and vast North European ice sheets and mountain caps (Böse et al. 2012). This results combined with the evolutionary hypotheses emitted on the ancestral lineages, the knowledge on the origin and present-day restricted geographic distribution (Fig. 1) of these *Terricola* species suggests that their speciation occurred after a long-term isolation in Mediterranean (Italy, the Balkans and the Iberian Peninsula including Southern France) and extra-Mediterranean (marginal areas of the Alps and the Carpathians, and the Caucasus) glacial refugia (Hewitt 1996, 2000, 2004; Schmitt and Varga 2012; Taberlet et al. 1998) according to an allopatric model. Only *M. subterraneus* and *M. daghestanicus* seem to have an older origin (respectively, 0.79 and 0.84 Myr). They probably evolved *in situ* as suggested by our reconstruction of the biogeographic ancestral areas (Fig. 3) and by Baskevich (1997) for *M. daghestanicus*. As mentioned previously, *M. arvalidens* is considered as being the ancestor of *M. subterraneus*. These two species, the former extinct and the latter extant, were and are the only widespread *Terricola* species in Europe with a nearly similar geographic distribution. Moreover, the distribution of *M. subterraneus* seems limited by the interspecific competition (Quéré and Le Louarn 2011), and, for this reason, it could have colonized Europe from the CTI area by replacing *M. arvalidens* by competitive exclusion (Krause 1986; Futuyma 1997).

Discrepancies exist between molecules and fossils on the origin of the subgenus *Terricola* and *Terricola* species. Indeed, the fossil record documents at best the first appearance of a morphologically recognizable group (*Allophaiomys* or *Terricola* morphotypes) and not the time when species became genetically isolated (Douzery et al. 2004; Yang 2014). In a way, this situation is comparable with what it is observed with the cryptic species: they are morphologically similar but genetically distinct, and for this reason,

they can have diverged thousand to millions of years ago (for instance, Hulva et al. 2004; Irwin et al. 2001; Šlapeta et al. 2006; Tougard et al. 2013).

Rate of diversification

The Quaternary climatic fluctuations, and especially the glacial periods, are thought to have an impact on species diversity and distribution of plants and animals. In the present context, most extinct *Terricola* species originated from the Cromerian interglacial or other later and shorter interglacials but it seems, from the fossil record, they did not survived these periods (Brunet-Lecomte 1989; Cuenca- Bescós et al. 2010; Gil 1996; Kowalski 2001). According to several authors (Dalén et al. 2007; Hewitt 1996; Provan and Bennett 2008; Stewart et al. 2010), populations, with a dispersal ability that does not allow to track retreating habitats when climatic conditions became harsher, went extinct. There is no evidence (no fossil remain) allowing to say that the extinct *Terricola* species, mostly endemic to some restricted continental or insular localities, migrated southwards when colder climatic conditions occurred nor if there is an ancestor-descendant relation between some of these extinct and extant *Terricola* species, except for *M. arvalidens* and *M. subterraneus*. On the other hand, most extant *Terricola* species found their origin during glacial periods because of a long-term divergence of ancestral lineages in Mediterranean and extra-Mediterranean glacial refugia thus seen as allopatric speciation traps (Chaline 1987; Haring et al. 2011; Hewitt 2004; Martínková and Moravec 2012). All the extant *Terricola* species have a relatively recent origin (between 0.84 and 0.08 Myr; Fig. 3), while the "lifespan" for some extinct *Terricola* species is estimated between 0.2 and 0.4 Myr (Brunet-Lecomte 1988). According to Avise et al. (1998), speciation in mammals requires a time frame of 2.2 ± 1.0 Myr, and thus the fossil record and our molecular dating could suggest that the time frame may be too short in voles to achieve complete speciation. Chaline et al. (1999) considered however that the speciation duration in vole species is relatively short, from 0.3 to less than 1.5 Myr, compared to other mammal species.

From the present study, it seems that the Pleistocene glacial conditions had an impact on the speciation and extinction events, but does that mean that the rate of diversification during the Quaternary was heightened? The LTT plot (Fig. 4) underlined several shifts in the *Terricola* diversification rate with two phases of increase (between 3 and 2 Myr and after 1 Myr), more or less corresponding to periods dominated by cool or cold climate conditions. The estimated rate of diversification for the *Terricola* species was 0.353 ± 0.004 events / Myr. Diversification rates were also estimated for numerous plant and animal groups but rarely at

the subgenus level. In rodents, several shifts in diversification rates were identified (Steppan et al. 2004; Fabre et al. 2012). Seven of these shifts were notably underlined in the Cricetidae (Fabre et al. 2012). Unfortunately, no study focused specifically on diversification rates of this rodent family. With 1.36 events / Myr, the cricetid *Akodon* of the subfamily Sigmodontinae presents a higher diversification rate than the subgenus *Terricola* (Leite et al. 2014), whereas *Akodon* originated at the beginning of the Pleistocene (between 2.65 and 2 Myr; Smith and Patton 2007). It thus presents a diversification rate among the highest reported for vertebrates, that is to say four times higher than that of the subgenus *Terricola*. This rate for *Terricola* is rather comparable to the average diversification rate of another rodent family, the Muridae (around 0.36 events / Myr; Stanley 1998). Consequently, the Quaternary does not appear as a period with an exceptional rate of diversification compared to other animal groups, although some periods in the Quaternary appear more favourable for the diversification of this subgenus.

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Figure Legends

1   Figure 1. Map showing the geographic distribution (1) of the *Microtus* (*Terricola*) species

2   according to the IUCN Red List, and the sampling localities (2) for the specimens used in the

3   present study. Details about the localities and/or the samples are in Table 1.

4

5   Figure 2. Maximum-likelihood tree topology reconstructed from cytochrome *b* gene

6   sequences of *Microtus* (*Terricola*) species as well as *M*. (*Microtus*) *arvalis* and *M*. (*M*.) *levis*

7   used as outgroup. Numbers at node are for maximum-likelihood bootstrap values (BP ≥ 50%)

8   and Bayesian posterior probabilities (PP ≥ 0.85). Black crosses indicate nodes with BP =

9   100% and PP = 1.00, while grey crosses are for nodes with BP < 50% and PP = < 0.85. The

10   species names are indicated on the right and are followed by the symbols used in Fig. 1.

11   Details about specimen numbers are given in Table 1.

12

13   Figure 3. Chronogram showing the divergence time estimates and the reconstruction of the

14   biogeographic ancestral areas. Values in bold and under species names (at right) reflect the

15   time to the most recent common ancestor and, in brackets, the 95% confidence interval, while

16   letters (biogeographic areas) and values (occurrence frequencies) in regular are related to the

17   biogeographic ancestral area analysis.

18

19   Figure 4. Lineages through time plot of all the *Microtus* species (in- and outgroup included)

20   taken into account in the present study.

21

22   **List of supporting information**

23

24   Table S1. Genetic distance within and between *Microtus* (*Terricola*) species and the

25   outgroup.

26

Tables

Table 1. List of taxa used in the present analyses. Systematic arrangement is from Musser and Carleton (2005). Sample localities, GenBank accession numbers and author references are indicated for each cytochrome *b* gene sequence. Locality numbers are for sample numbers of the Fig. 2. Letters in regular are for published data (see the footnote below the table for details), whereas letters in bold are the initials of the tissue providers (see details in acknowledgements).

Species (Common Name)	Sample Locality	Accession Number	Source
Order Rodentia			
Family Cricetidae			
Subfamily Arvicolinae			
Genus *Microtus*			
Subgenus *Microtus*			
M. arvalis	1	AM991045	I[a]
(Common vole)	2	AM991075	I
	3	AY220766	D
	4	AY220788	D
M. levis	1	DQ015676	J
(East European vole)	2	AY513820	E
M. tatricus	1. Tretie Rohácske pleso Lake, Western Tatra Mts, Slovakia	LT222298	**NM**
(Tatra vole)	2. Tretie Rohácske pleso Lake, Western Tatra Mts, Slovakia	AY513837	E
	3. Smutná dolina Valley, Western Tatra Mts, Slovakia	AY513838	E
	4. Velka studena dolina Valley, Slovakia	AY513839	E
	5. Rakytovská dolina Valley, Veľká Fatra Mts, Slovakia	DQ841699	F
	6. Dolný Harmanec, Veľká Fatra Mts, Slovakia	DQ841700	F
Subgenus *Terricola*			
M. (*T.*) *bavaricus*	1. Steinberg am Rofan, Northern Tyrol, Austria	LT222299	**NM**
(Bavarian pine vole)	2. Steinberg am Rofan, Northern Tyrol, Austria	DQ841693	F
	3. Garmisch-Partenkirchen, Bavaria, Germany	DQ841694	F
	4. Papuk Mountain, Croatia	GQ243218	K
M. (*T.*) *brachycercus*	1. Fiumefreddo, Sicily, Italy	AY513827	E
(Calabria pine vole)	2. Lago di Cecita, Calabria, Italy 3. Muro Lucano, Basilicata, Italy	EU158784	B
		EU158785	B
	4. Pollinello, Calabria, Italy	EU158787	B
	5. Muro Lucano, Basilicata, Italy	EU158776	B
M. (*T.*) *daghestanicus*	1. Cew Valley, Central Caucasus, Russia	LT222300	**FC**
(Caucasus pine vole)		AY513790	E
	2. Beniani, Georgia	AY513791	E

	3. Bagdasan, Turkey	AY513792	E
	4. Handere, Turkey		
M. (*T.*) *duodecimcostatus* (Mediterranean pine vole)	1. Jardin des Gardes, Hautes-Alpes, France	LT222301 AJ717744	**JPQ** I
	2. Montferrier, Hérault, France	AY513796	E
	3. Setubal, Portugal	AY513797	E
	4. Algarve, Portugal	JX424211	A
	5. Segovia, Spain		
M. (*T.*) *felteni* (Balkan pine vole)	1. Mt Pelister, Begova Cesma, Macedonia	AY513798	E
	2	DQ663661	C
M. (*T.*) *gerbei* (Pyrenean pine vole)	1. Vall d'Aran, Catalogna, Spain	AJ717748	I
	2. Armendarits, Pays Basque, France	LT222302	**JPQ**
	3. Vall d'Aran, Catalogna, Spain	AY513799	E
	4. Riba, Spain	AY513800	E
	5. Hecho, Spain	AY513801	E
M. (*T.*) *liechtensteini* (Liechtenstein's pine vole)	1. Brikini, Slovenia	LT222303	**FS**
	2. Campo di Mezzo, Pian del Cansiglio, Veneto, Italy	LT222304	**LN**
	3. Anhovo, Slovenia	AY513811	E
	4. Croatia	EF379100	F
M. (*T.*) *lusitanicus* (Lusitanian pine vole)	1. Saint-Pée-sur-Nivelle, Pays Basque, France	AJ717746 LT222305	I **JPQ**
	2. Saint-Pée-sur-Nivelle, Pays Basque, France	AY513812 AY513813	E E
	3. Burgos, Spain	JX424207	A
	4. Melgar de Fernamental, Spain	JX424208	A
	5. Rebordelo, Portugal		
	6. Grijo da Para, Portugal		
M. (*T.*) *majori* (Major's pine vole)	1. Damar, Turkey	AY513814	E
	2. Damar, Turkey	DQ841703	F
	3. Hopa, Turkey	DQ841704	F
M. (*T.*) *multiplex* (Alpine pine vole)	1. Saint Jean de Vaulx, Isère, France	LT222306	**PBL**
	2. Carmagnola, Piedmont, Italy	LT222307	**GB**
	3. Saint Martin de la Cluze, Isère, France	AJ717747 AY513817	I E
	4. Lillaz, Italy	AY513818	E
	5. Méribel, France		
M. (*T.*) *savii* (Savi's pine vole)			
M. (*T.*) *s. savii*	6. Palumbara, Sabina, Latium, Italy	LT222308	**NM**
	7. Viterbo, Lazio, Italy	AY513824	E
	8. Torino, Piedmont, Italy	AY513825	E
	9. Cerano, Piedmont, Italy	AY513826	E
	10. Velletri, Italy	EU158777	B
	11. Circeo, Italy	EU158778	B
	12. Capracotta, Molise, Italy	EU158780	B
M. (*T.*) *s. niethammericus*	13. Capracotta, Molise, Italy	EU158781	B
	14. Capracotta, Molise, Italy	EU158782	B
	15. Farindola, Italy	EU158790	B
	16. Farindola, Italy	EU158791	B

M. (T.) s. nebrodensis	17. Ficuzza, Sicily, Italy	EU158802	B
	18. Ficuzza, Sicily, Italy	EU158804	B
	19. Ficuzza, Sicily, Italy	EU158803	B
	20. Ficuzza, Sicily, Italy	EU158779	B
M. (T.) schelkovnikovi (Schelkovnikov's pine vole)	Talysh, Hyrkanian reserve, Azerbaidjan	LT222309	**FC**
M. (T.) subterraneus (Common pine vole)	1. Val Piora Ticino, Switzerland	AJ717745	I
	2. Úzka dolina Valley, Western Tatra Mts., Slovakia	LT222310	**NM**
	3. Tourch, Finistère, France	LT222311	**JPQ**
	4. Seli, Greece	AY513832	E
	5. Glocknerhaus, Austria	AY513833	E
	6. Ciglikara, Turkey	AY513834	E
	7. Nova Kapela, Croatia	FR869858	G
	8. Brussels, Waterloo, Belgium	FR869862	G
	9. Kasperske hory Mts, Czech Republic	FR869878	G
	10. Bialowieza, Poland	FR869884	G
M. (T.) thomasi (Thomas's pine vole)	1. Trebinje, Bosnia and Hezergovina	LT222312	**NM**
M. (T.) t. thomasi	2. Kalavryta, Greece	JN019756	H
	3. Kyparissia, Greece	AY513842	E
	4. Trebinje, Bosnia and Hezergovina	AY513844	E
	5. Ano Kastritsi, Greece	JN019765	H
	6. Ano Kastritsi, Greece	JN019766	H
	7. Vrodamas, Greece	JN019773	H
	8. Strofylia, Greece	JN019775	H
	9. Peleta, Greece	JN019778	H
	10. Strofylia, Greece	JN019780	H
	11. Aigies, Greece	JN019762	H
	12. Voutianoi, Greece	JN019763	H
	13. Agios Stefanos, Greece	AY513840	E
	14. Afidnes, Greece	JN019760	H
M. (T.) t. atticus	15. Afidnes, Greece	JN019761	H
	16. Agios Stefanos, Greece	JN019767	H
	17. Eretria, Greece	JN019764	H
	18. Vasiliko, Greece	JN019768	H
M. (T.) t. evia	19. Kimassi, Greece	JN019771	H

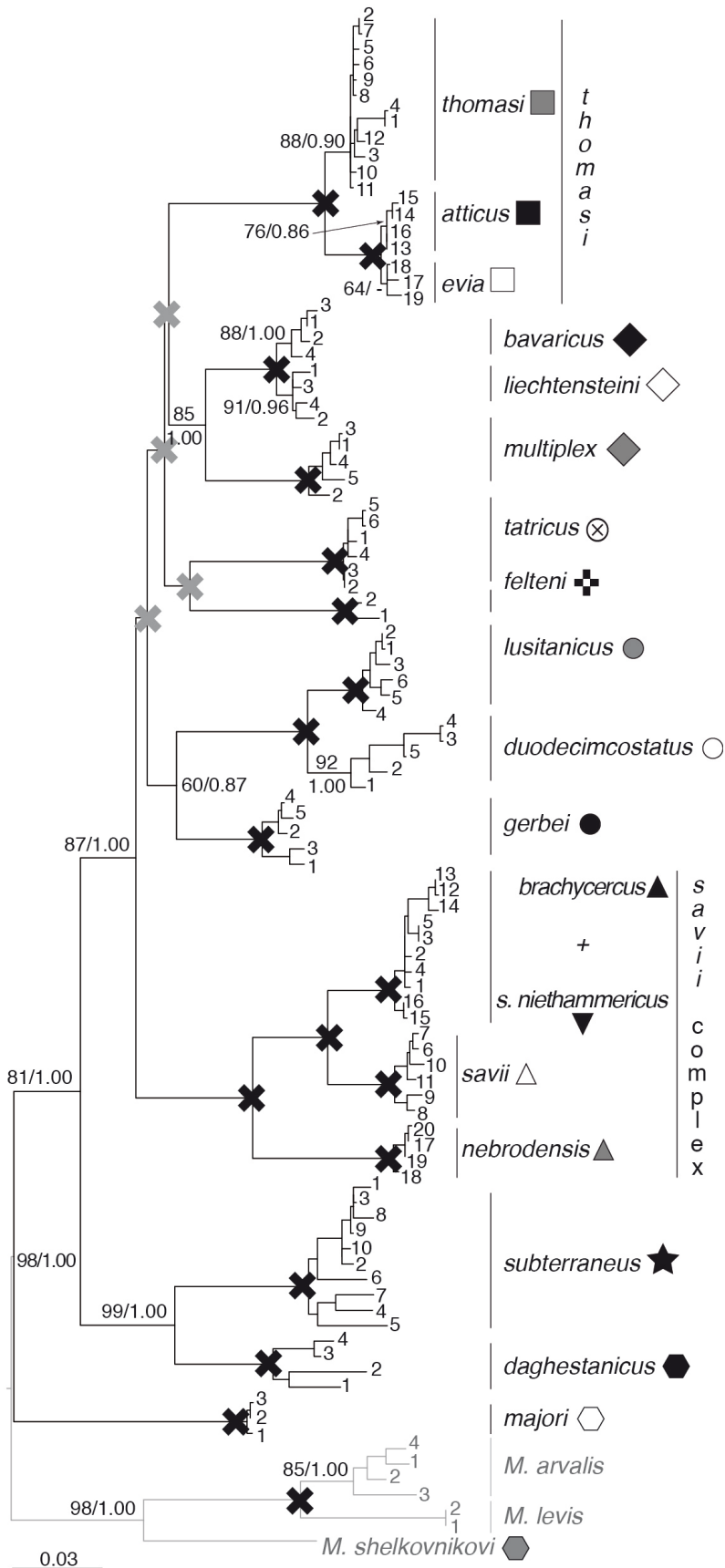1 [a] A: Bastos-Silveira et al. (2012); B: Castiglia et al. (2008); C: Fink et al. (2006); D: Haynes et
2 al. (2003); E: Jaarola et al. (2004); F: Martínková et al. (2007); G: Martínková et al. (unpubl.);
3 H: Rovastos and Giagia-Athanasopoulou (2012); I: Tougard et al. (2008b); J: Triant and
4 DeWoody (2006); K: Tvrtkovic et al. (2010).
5

1   Fig. 1

Legend:

1	2			1	2			1	2			1	2			1	2	
	○	M. duodecimcostatus			◇	M. liechtensteini			✛	M. felteni			□	M. thomasi evia			⬡	M. majori
	●	M. lusitanicus			◆	M. multiplex			⊗	M. tatricus			■	M. thomasi thomasi			⬣	M. daghestanicus
	●	M. gerbei			◆	M. bavaricus			★	M. subterraneus			■	M. thomasi atticus			⬣	M. shelkovnikovi

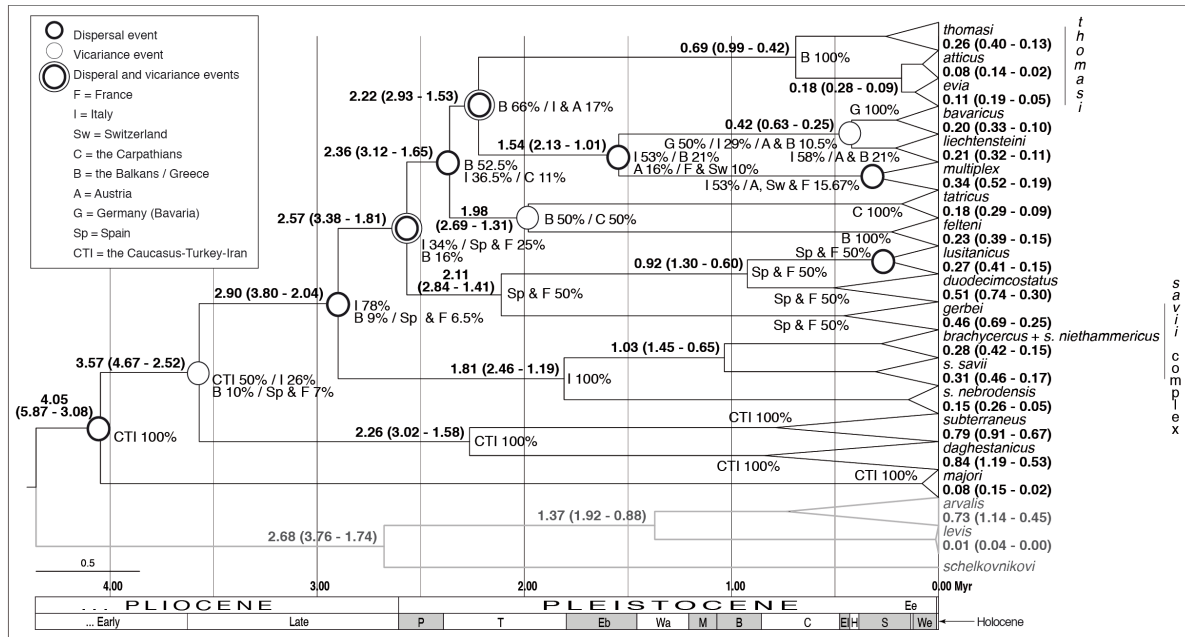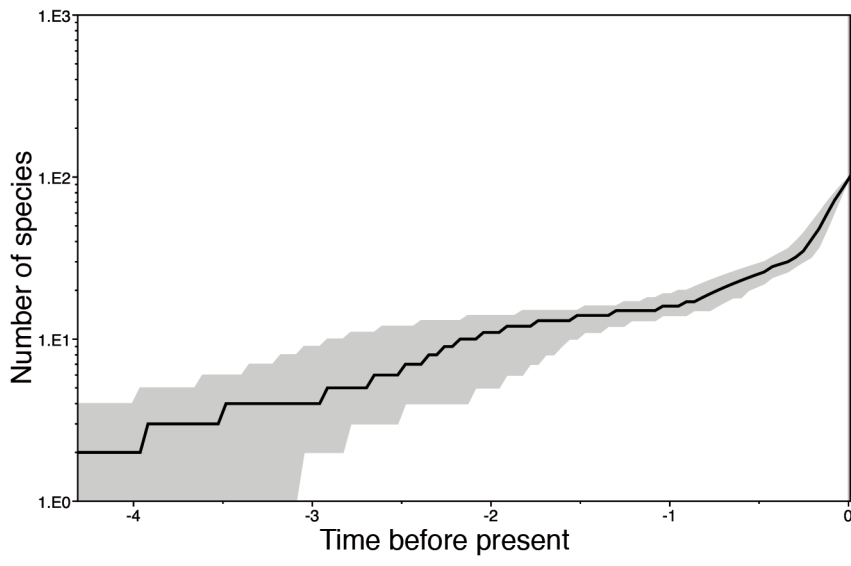| 1	2			1	2	
	△	M. savii savii			▼	M. savii niethammericus
	△	M. savii nebrodensis			▲	M. brachycercus
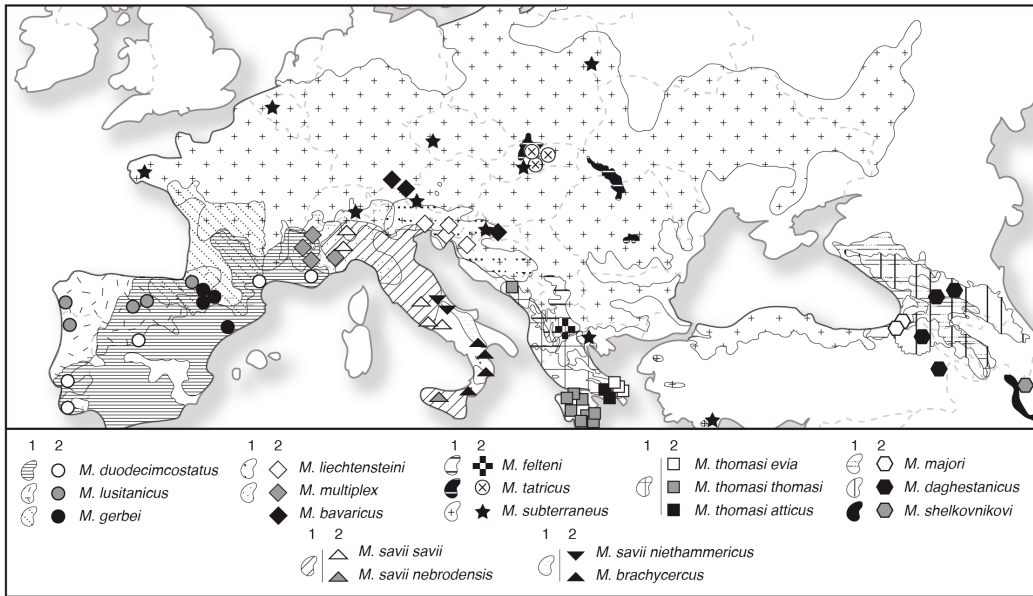
2
3

1   Fig. 3

2
3
4

1   Fig. 4

2

Tableau S1 of C. TOUGARD, Did the Quaternary climatic fluctuations really influence the tempo and mode of diversification in European rodents?

Genetic distance within and between *Microtus* (*Terricola*) species and the outgroup

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *majori*	**0.002 (0.001)**[a]	0,015[c]	0,015	0,016	0,017	0,016	0,014	0,013	0,013	0,013	0,014	0,015	0,016	0,016	0,014	0,013	0,013	0,012
2. *daghestanicus*	0,103[b]	**0.028 (0.004)**	0,010	0,014	0,015	0,015	0,012	0,014	0,014	0,012	0,012	0,013	0,013	0,013	0,012	0,012	0,012	0,013
3. *subterraneus*	0,106	0,080	**0.022 (0.003)**	0,014	0,015	0,014	0,013	0,014	0,014	0,013	0,013	0,013	0,013	0,013	0,010	0,012	0,011	0,013
4. *s. savii*	0,113	0,118	0,115	**0.011 (0.002)**	0,007	0,012	0,013	0,012	0,011	0,012	0,013	0,013	0,014	0,015	0,013	0,010	0,012	0,015
5. *brachycercus + s. niethamericus*	0,125	0,126	0,120	0,046	**0.011 (0.002)**	0,011	0,013	0,013	0,013	0,013	0,013	0,013	0,015	0,015	0,014	0,012	0,013	0,016
6. *nebrodensis*	0,121	0,124	0,112	0,071	0,077	**0.005 (0.002)**	0,015	0,014	0,013	0,014	0,012	0,013	0,014	0,014	0,013	0,013	0,014	0,015
7. *gerbei*	0,082	0,093	0,094	0,090	0,095	0,106	**0.013 (0.003)**	0,011	0,012	0,011	0,012	0,012	0,012	0,012	0,011	0,008	0,009	0,012
8. *duodecimcostatus*	0,088	0,113	0,112	0,095	0,101	0,109	0,080	**0.025 (0.005)**	0,007	0,012	0,012	0,013	0,014	0,014	0,011	0,012	0,012	0,014
9. *lusitanicus*	0,084	0,112	0,105	0,087	0,102	0,102	0,079	0,049	**0.009 (0.002)**	0,012	0,012	0,012	0,013	0,014	0,011	0,011	0,012	0,014
10. *felteni*	0,095	0,094	0,094	0,096	0,097	0,109	0,071	0,084	0,079	**0.010 (0.004)**	0,012	0,011	0,011	0,012	0,011	0,010	0,009	0,013
11. *tatricus*	0,097	0,104	0,104	0,099	0,106	0,101	0,075	0,097	0,092	0,079	**0.005 (0.002)**	0,013	0,013	0,014	0,011	0,010	0,011	0,013
12. *thomasi*	0,106	0,105	0,099	0,090	0,094	0,094	0,076	0,101	0,089	0,077	0,090	**0.008 (0.002)**	0,007	0,007	0,012	0,011	0,011	0,012
13. *atticus*	0,100	0,098	0,096	0,090	0,093	0,094	0,073	0,091	0,090	0,071	0,080	0,030	**0.001 (0.001)**	0,003	0,012	0,013	0,013	0,014
14. *evia*	0,099	0,095	0,094	0,097	0,100	0,102	0,071	0,094	0,094	0,074	0,088	0,034	0,008	**0.005 (0.002)**	0,012	0,013	0,013	0,013
15. *multiplex*	0,085	0,090	0,086	0,091	0,099	0,102	0,071	0,079	0,074	0,071	0,075	0,078	0,078	0,080	**0.009 (0.002)**	0,009	0,010	0,013
16. *liechtensteini*	0,081	0,097	0,087	0,082	0,093	0,094	0,057	0,073	0,072	0,072	0,076	0,073	0,078	0,079	0,056	**0.009 (0.003)**	0,004	0,013
17. *bavaricus*	0,086	0,089	0,080	0,088	0,096	0,099	0,059	0,077	0,075	0,065	0,079	0,072	0,082	0,083	0,057	0,021	**0.008 (0.002)**	0,013
18. outgroup	0,101	0,115	0,116	0,138	0,147	0,146	0,102	0,119	0,115	0,112	0,107	0,109	0,112	0,113	0,097	0,108	0,101	**0.047 (0.006)**

[a] Genetic distance within species (standard error)
[b] Genetic distance between lineages (below the diagonal)
[c] Standard error (above the diagonal)

savii group: rows 4, 5, 6

thomasi: rows 12, 13, 14

1

33