The LINNEAN SOCIETY of London ZOOLOGICAL Journal of the Linnean Society

*Zoological Journal of the Linnean Society*, 2015, **175**, 930–948. With 5 figures

# An underground burst of diversity – a new look at the phylogeny and taxonomy of the genus *Talpa* Linnaeus, 1758 (Mammalia: Talpidae) as revealed by nuclear and mitochondrial genes

ANNA A. BANNIKOVA[1]*, ELENA D. ZEMLEMEROVA[1], PAOLO COLANGELO[2], MUSTAFA SÖZEN[3], M. SEVINDIK[3], ARTEM A. KIDOV[4], RUSLAN I. DZUEV[5], BORIS KRYŠTUFEK[6] and VLADIMIR S. LEBEDEV[7]

[1]*Lomonosov Moscow State University, Vorobievy Gory, 1/12, Moscow, Russia*
[2]*Department of Biology and Biotechnology 'Charles Darwin', University of Rome 'La Sapienza', Roma, Italy*
[3]*Department of Biology, Faculty of Arts and Sciences, Bülent Ecevit University, 67100, Zonguldak, Turkey*
[4]*Depertment of Zoology, Russian State Agrarian University – Timiryazev Moscow Agricultural Academy, Timiryazevskaya, 49, Moscow, Russia*
[5]*Kabardino-Balkarian State University, 360004, Nalchik, Russia*
[6]*Slovenian Museum of Natural History, P.O. Box 290, 1001 Ljubljana, Slovenia*
[7]*Zoological Museum of Moscow State University, B. Nikitskaya 6, 125009, Moscow, Russia*

*Received 16 December 2014; revised 13 May 2015; accepted for publication 28 May 2015*

Using both nuclear and mitochondrial sequences, we demonstrate high genetic differentiation in the genus *Talpa* and confirm the existence of cryptic species in the Caucasus and Anatolia, namely, *T. talyschensis* Vereschagin, 1945, *T. ognevi* Stroganov, 1948, and *Talpa* ex gr. *levantis*. Our data support four clades in the genus *Talpa* that showed strong geographical associations. The 'europaea' group includes six species from the western portion of the genus' range (*T. europaea*, *T. occidentalis, T. romana*, *T. caeca*, *T. stankovici*, and *T. levantis* s.l.); another three groups are distributed further east: the 'caucasica' group (Caucasus), the 'davidiana' group (eastern Anatolia and Elburz) and *T. altaica* (Siberia). The phylogenetic position of *T. davidiana* was highlighted for the first time. The order of basal branching remains controversial, which can be attributed to rapid diversification events. The molecular time estimates based on nuclear concatenation estimated the basal divergence of the crown *Talpa* during the latest Miocene. A putative scenario of *Talpa* radiation and issues of species delimitation are discussed.


doi: 10.1111/zoj.12298

ADDITIONAL KEYWORDS: Caucasus – cryptic diversity – mole – molecular dating – nuclear genes – phylogenetics – species delimitation – Turkey.

## INTRODUCTION

The genus *Talpa* is one of the most successful lineages of the family Talpidae. Moles of the genus *Talpa* range from Europe to Central Siberia where they occupy broad-leaf and mixed forest zones, also reaching subalpine meadows and the boreal coniferous belt. Most extant species have relatively small and predominantly non-overlapping ranges (Loy *et al*., 2005).

The most recent taxonomic revision of the genus *Talpa* recognised nine species: the common mole *T. europaea*

*Corresponding author. E-mail: hylomys@mail.ru

Linnaeus, 1758, the blind mole *T. caeca* Savi, 1882, the Iberian blind mole *T. occidentalis* Cabrera, 1907, the Roman mole *T. romana* Thomas, 1902, the Balkan mole *T. stankovici* Martino and Martino, 1931, the Caucasian mole *T. caucasica* Satunin, 1908, the Levant mole *T. levantis* Thomas, 1906, Père David's mole *T. davidiana* Milne-Edwards, 1884, and the Altai mole *T. altaica* Nikolsky, 1883 (Hutterer, 2005). The uniformity of their external morphology due to their adaptation to the narrow fossorial niche renders the identification of these species difficult (Kryštufek & Vohralík, 2001). In particular, in the Balkans, which harbour the highest number of species, taxonomic identification based on morphology alone was substantially complicated by instability of meristic characters (Kryštufek, 1994). However, morphometric analysis was for a long time the most widely used method in species identification and taxonomic studies (Corti *et al*., 1985; Corti & Loy, 1987; Loy, Corti & Marcus, 1993; Kryštufek, 1994; Rohlf, Loy & Corti, 1996; Loy & Capanna, 1998; Kryštufek & Benda, 2002). This was partly explained by the fact that basic karyological data contributed little information to evolutionary and taxonomic studies in *Talpa* (but see Gornung *et al*., 2008). In contrast with the extreme chromosomal diversity of other subterranean mammals (Nevo, 1979; Steinberg & Patton, 2000), moles exhibit a striking karyotype stability, with most species of the genus *Talpa* having a diploid chromosome number ($2n$) of 34 and 62–64 autosomal arms (NFa) (Meylan, 1966; Dzuev, Tembotov & Ivanov, 1972; Todorović, Soldatović & Dunderski, 1972; Capanna, 1981; Jimenez, Burgos & De La Guardia, 1984; Sözen *et al*., 2012). Only *T. caeca* ($2n = 36$, NFa = 64) and *T. caucasica* ($2n = 38$, NFa = 62) differ in this respect (Meylan, 1966; Dzuev *et al*., 1972; Todorović *et al*., 1972).

Allozymic data analysis (Filippucci *et al*., 1987; Suchentrunk *et al*., 1995) yielded encouraging results for the delimitation of *Talpa* species while *cytb* sequence data were successfully used in phylogenetic reconstructions and molecular time estimations (Colangelo *et al*., 2010). However, in both studies, the taxonomic and geographic scope remained incomplete.

Moles demonstrate most of the major life history attributes characteristic of other subterranean mammals (Nevo, 1979). This includes a high degree of territoriality (Ognev, 1928; Stein, 1950; Godfrey, 1957; Loy *et al*., 1994), competitive exclusion, limited dispersal distance (Ellerman, 1956; Steinberg & Patton, 2000), and morphological specialization (Lessa, 1990). However, while the majority of fossorial mammals inhabits open and semi-open arid landscapes, moles are associated exclusively with humid habitats, which were heavily influenced by climatic changes during the Late Neogene and Pleistocene. Therefore, it seems plausible that moles persisted as isolated or semi-isolated local populations, which can result in restrictions to gene flow and phylogeographic discontinuities (Avise, 2000). Phylogeographic assessments performed to date on *T. romana* (Canestrelli *et al*., 2010), *T. stankovici* (Tryfonopoulos *et al*., 2009), and *T. europaea* (Feuda *et al*., 2015) suggest that a high level of geographic structuring is widespread in *Talpa*. Therefore, in a group with uniform external morphology and a stable karyotype, as is the case with *Talpa*, the genetic variability is crucial for assessing species boundaries.

In the present study, we sequenced fragments of four nuclear genes and the complete mitochondrial cytochrome *b* gene (*cytb*) in all recent species of the genus *Talpa* from throughout their ranges. Our aims were to estimate inter- and intra-specific genetic differentiation, evaluate the nuclear evolutionary history of this genus and compare it with the mitochondrial phylogeny.

## MATERIAL AND METHODS

### Taxon sampling

The original material consists of 83 specimens of all nine currently recognised species of the genus *Talpa* as well as five other genera of the family Talpidae which were used for rooting phylogenetic trees. Material was collected from 37 localities across the entire range of the genus (Fig. 1, Table 1). An additional 60 sequences of *Talpa* and 26 sequences of various species of the genera *Condylura*, *Desmana*, *Euroscaptor*, *Galemys*, *Mogera, Parascalops, Parascaptor*, and *Scaptochirus*, used as the outgroup were downloaded from GenBank (Supporting Information 1).

### Tissue collection, DNA extraction, PCR amplification and sequencing

A large part of the sample was obtained by small tissue biopsies of live-trapped animals. In other cases we used muscles preserved in ethanol or dried skins from museum vouchers deposited in different collections (Table 1).

Genomic DNA from ethanol-preserved tissues was extracted using a standard protocol of proteinase K digestion, phenol–chloroform deproteinization, and isopropanol precipitation (Sambrook, Fritsch & Maniatis, 1989). DNA was also extracted from dried skins of Zoological Museum of Moscow State University, Russia (ZMMU) collection specimens, including six *T. caucasica ognevi* (collected in 1963 and 1980 in Georgia, including the vicinity of the type locality) and eight *T. levantis minima* (collected from the type locality in 1957). The DNA was purified directly using the MiniElute PCR Purification Kit (QIAGEN) following the recommendations of Yang *et al*. (1998).

We sequenced the complete mitochondrial cytochrome *b* (*cytb*) gene and fragments of four nuclear loci: exon

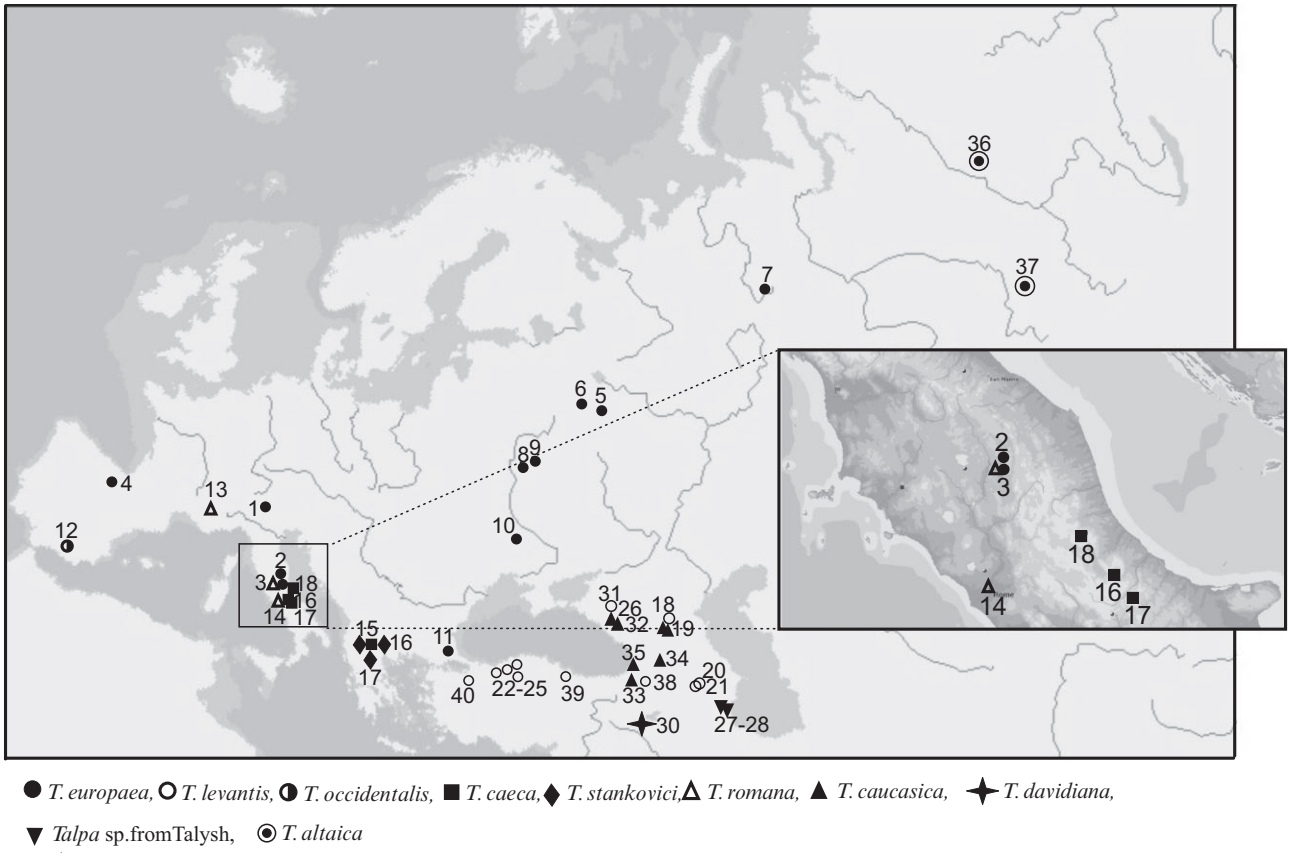


**Figure 1.** Map of sampling localities for specimens of the genus *Talpa* used in this study. Localities 1–37 are listed in Table 1 (original material), localities 38–57 correspond to the sequences retrieved from GenBank and are listed in Supporting Information 1.

11 of the breast cancer type 1 susceptibility protein (*BRCA1*), breast cancer type 2 susceptibility protein (*BRCA2*), apolipoprotein B (*ApoB*), and recombination activating gene 1 (*RAG1*). Nucleotide sequences of the original primers specially designed for amplification and sequencing are provided in the Supporting Information, Table S1. To amplify the *RAG1* fragments we also used the primers R2951 (Teeling *et al*., 2000), F2401 (Shinohara *et al*., 2004), F1851, and R2486 (Sato *et al*., 2004). Cytochrome *b* sequences (1140 bp) were obtained using the combination of primers L14734 (Ohdachi *et al*., 2001) and H15906 (Lebedev *et al*., 2007). The PCR protocol for all genes was initial denaturation at 94 °C for 3 min, then 30 cycles of 94 °C for 30 s, 52–65 °C (depending on the primer pair) for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 6 min.

DNA extracted from the old museum specimens was highly degraded thus, only short fragments were obtained using the combination of internal primers L393/H525 (Table S1). To ensure the authenticity of the *cytb* amplification products, four additional internal primers were designed to obtain fragments of approximately 260–400 bp in the following combinations: L148/H510, L148/H330a, and L50/H330a (Table S1). To avoid contamination, the extraction and amplification of the DNA from the museum specimens was carried out in a laboratory, where no previous work on mole tissues had been performed (Laboratory of Evolution of Eukaryotic Genomes, Engelhardt Institute of Molecular Biology).

PCR products were visualised on 1.5% agarose gel and then purified using ammonium-ethanol precipitation. Approximately 10–30 ng of the purified PCR product was used for sequencing with each primer by the autosequencing system ABI 3100-Avant using the ABI PRISM®BigDyeTM Terminator v. 3.1 (Applied Biosystems, Foster City, CA, USA). The sequences obtained in this study can be accessed via GenBank (accession numbers: KP717098–KP717379, Table S2).

## Phylogenetic analysis

### *Alignment, partitioning and base composition*

All sequences were aligned by eye using BioEdit version 7.0.9.0 (Hall, 1999). Heterozygous positions (at which

**Table 1.** Characterization of the original material: species, ID of specimens, collection and geographic location. Samples were collected from different collections: ZMMU – Zoological Museum of Moscow State University, Russia; ZIN – Zoological Institute of St.-Peterburg; PMS – Slovenian Museum of Natural History, Ljubljana, Slovenia; MAC – Museo di Anatomia Comparata, University of Rome 'La Sapienza'; ZMNK – Zoological Museum of National Museum of Natural History, Kiev, Ukraine; PISR – Pechora-Ilych State Reserve, Russia; MS – personal collection of M. Sozen

| Species | Specimen code in Figures 2–3, S1, S2 | Museum catalog number or tissue collection code (ID) | Collecting locality (region, closest city and Figure 1 ref. in the brackets) |
|---|---|---|---|
| *T. europaea* | Te VAL1 | aa1496, MAC | Italy, Valtellina, Sondalo (1) |
| | Te VAL3 | aa1498, MAC | Italy, Valtellina, Sondalo (1) |
| | Te MV17 | aa1409, MAC | Italy, Valfabbrica, Monte Villano, PG (2) |
| | Te CCS3 | aa1360, MAC | Italy, Assisi, Casa Casella, PG (3) |
| | Te 3109 | 3109, MAC | Spain, Haro (4) |
| | Te Moscow 09 | Moscow 09 | Russia, Moscow (5) |
| | Te 2001-10 | 2001-10 | Russia, Tver Region, vill. Krutitcy (6) |
| | Te 2001-3 | 2001-3 | Russia, Tver Region, vill. Krutitcy (6) |
| | Te PI 08–19-4 | PI 08–19-4, PISR | Russia, Northern Urals, Pechora-Ilych NR (7) |
| | Te Kiev 08-1 | Kiev 08-1, ZMNK | Ukraine, Kiev (8) |
| | Te Brovary 09-2 | Brovary 09-2, ZMNK | Ukraine, Kiev region, Brovary (9) |
| | Te Cherk | Cherk, ZMNK | Ukraine, Cherkassk region, Kanevskiy NR (10) |
| | Te 6341 | MS 6341 | Turkey, Kuleli Köyü – Babaeski, Kirklareli (11) |
| *T. occidentalis* | Toc 2 | *presented by A.B. Sánchez | Spain, Granada (12) |
| | Toc 24 | *presented by A.B. Sánchez | Spain, Granada (12) |
| | Toc 30 | *presented by A.B. Sánchez | Spain, Granada (12) |
| | Toc 31 | *presented by A.B. Sánchez | Spain, Granada (12) |
| *T. romana* | Trom CDM5 | aa1369, MAC | Italy, Assisi, Croce di Mora, PG (3) |
| | Trom FRA1 | FRA1, MAC | France, Trieves (13) |
| | Trom LAU | aa1398, MAC | Italy, Laurentina, Roma (14) |
| *T. caeca* | Tc 184-08 BK | PMS 16823 | Macedonia, Pelister (15) |
| | Tc TLP10 | TLP10, MAC | Italy, Capracotta (17) |
| | Tc MAJ | MAJ, MAC | Italy, Majelletta (16) |
| | Tc PRT3 | aa1455, MAC | Italy, Prati di Tivo, Gran Sasso (18) |
| | Tc TLP11 | TLP11, MAC | Italy, Monti Simbruini |
| *T. stankovici* | Tst 153-09 BK | PMS 16820 | Macedonia, Pelister (15) |
| | Tst 181-08 BK | PMS 16821 | Macedonia, Bistra (16) |
| | Tst VIS18 | VIS18, MAC | Greece, western Macedonia (17) |
| *T. levantis transcaucasica* | Tl KB 10-1 | ZMMU S-192847 | Russia, Nalchik vicinity (18) |
| | Tl KB 10-3 | ZMMU S-192848 | Russia, Nalchik vicinity (18) |
| | Tl KB 2000-3 | ZMMU S-186085 | Russia, Nalchik region, Khasanya (19) |
| | Tl KB 2000-4 | ZMMU S-186086 | Russia, Nalchik region, Khasanya (19) |
| | Tl Arm11-1 | ZMMU S-192844 | Armenia, Margahovit (20) |
| | Tl Arm11-2 | ZMMU S-192845 | Armenia, Fioletovo (21) |
| | Tl Arm11-3 | ZMMU S-192846 | Armenia, Fioletovo (21) |
| *T. levantis* ssp. | Tl 5185 | MS 5185 | Turkey, Uzungüney Köyü, Zonguldak (22) |
| | Tl 5628 | MS 5628 | Turkey, Çaycuma, Zonguldak (23) |
| | Tl 5923 | MS 5923 | Turkey, Sefercik – Filyos, Zonguldak (24) |
| | Tl 5931 | MS 5931 | Turkey, Sefercik – Filyos, Zonguldak (24) |
| | Tl 5925 | MS 5925 | Turkey, Alaplı, Zonguldak (25) |
| *T. l. minima* | | ZMMU S-61975 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| | | ZMMU S-61976 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| | | ZMMU S-61977 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| | | ZMMU S-61978 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| | | ZMMU S-61979 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| | | ZMMU S-61981 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| | | ZMMU S-61982 | Russia, Adygea, Upper Belaya R., Khamyshki (26) |
| *Talpa* sp. *(T. l. talyschensis* according to MSW3) | Talysh 1 | ZMMU S-182088 | Azerbaijan, Astara, Sim village (27) |
| | Talysh 3 | ZMMU S-188886 | Azerbaijan, Astara, Vazezamin (28) |
| | Talysh 2 | ZMMU S-188890 | Azerbaijan, Astara, Vazezamin (28) |
| *T. davidiana* | Td | PMS 21503 | Turkey, Tatvan (29) |
| | Td 6226 | MS 6226 | Turkey, Çataklı Köyü – Tatvan, Bitlis (30) |
| | Td 6233 | MS 6233 | Turkey, Çataklı Köyü – Tatvan, Bitlis (30) |
| | Td 6240 | MS 6240 | Turkey, Çataklı Köyü – Tatvan, Bitlis (30) |
| | Td 6241 | MS 6241 | Turkey, Çataklı Köyü – Tatvan, Bitlis (30) |
| | Td 6242 | MS 6242 | Turkey, Çataklı Köyü – Tatvan, Bitlis (30) |

**Table 1.** *Continued*

| Species | Specimen code in Figures 2–3, S1, S2 | Museum catalog number or tissue collection code (ID) | Collecting locality (region, closest city and Figure 1 ref. in the brackets) |
|---|---|---|---|
| *T. caucasica caucasica* | Tcau KB 10-2 | ZMMU S-192842 | Russia, Nalchik vicinity (18) |
| | Tcau KB 10-4 | ZMMU S-192843 | Russia, Nalchik vicinity (18) |
| | Tcau KB 2000-1 | ZMMU S-186088 | Russia, Nalchik region, Khasanya (19) |
| | Tcau KB 2000-2 | ZMMU S-186089 | Russia, Nalchik region, Khasanya (19) |
| *T. c. orientalis* | Tcau Kisha 1997-1 | ZMMU S-186090 | Russia, Adygea, Kisha river (31) |
| | Tcau Ad 10-5 | ZMMU S-192841 | Russia, Adygea, Kisha river (31) |
| | Tcau Zin 01 | ZIN 85582 | Russia, Krasnodar Krai, Krasnaya Polyana (32) |
| *T. c. ognevi* ? | Tcau 6205 | MS 6205 | Turkey, Cumhuriyet Mahallesi – Hopa, Artvin (33) |
| | Tcau 6477 | MS 6477 | Turkey, Cumhuriyet Mahallesi – Hopa, Artvin (33) |
| | Tcau 6478 | MS 6478 | Turkey, Cumhuriyet Mahallesi – Hopa, Artvin (33) |
| *T. c. ognevi* | | ZMMU S-118038 | Georgia, Bakurniani, Borzhomi (34) |
| | | ZMMU S-118039 | Georgia, Bakurniani, Borzhomi (34) |
| | | ZMMU S-118041 | Georgia, Bakurniani, Borzhomi (34) |
| | | ZMMU S-136234 | Georgia, Bakurniani, Batumi (35) |
| | | ZMMU S-136235 | Georgia, Bakurniani, Batumi (35) |
| | | ZMMU S-136237 | Georgia, Bakurniani, Batumi (35) |
| *T. altaica* | Talt 08–1015 | ZMMU S-184036 | Russia, Krasnoyarsk region, Middle Yenisei (36) |
| | Talt 08–1185 | ZMMU S-184043 | Russia, Krasnoyarsk region, Middle Yenisei (36) |
| | Talt 08–204 | tissue alt_08–204 | Russia, Krasnoyarsk region, Middle Yenisei (36) |
| | Talt 08–1079 | ZMMU S-184039 | Russia, Krasnoyarsk region, Middle Yenisei (36) |
| | Talt 08–1113 | ZMMU S-184040 | Russia, Krasnoyarsk region, Middle Yenisei (36) |
| | Talt 08–53 | ZMMU S-185683 | Russia, Krasnoyarsk region, Middle Yenisei (36) |
| | Talt 03-1 | *Tl1, presented by Yu.Litvinov | Russia, Altai, Teletskoye Lake (37) |
| | Talt 03-2 | *Tl12, presented by Yu.Litvinov | Russia, Altai, Teletskoye Lake (37) |
| *Mogera robusta* | Mrob 1990-9 | ZMMU S-176638 | Russia, Ussuri Nature Reserve |
| | Mrob 1990-16 | ZMMU S-176639 | Russia, Ussuri Nature Reserve |
| | Mrob 1990-17 | ZMMU S-176640 | Russia, Ussuri Nature Reserve |
| *Mogera latouchei* | Mlat AVA 13–114 | Zool. Inst. of Hanoi | Northern Vietnam, Son La Province, NW of Phu Yen |
| *Desmana moschata* | Desm1 | ZMMU S-181101 | Russia, Vladimir region |
| *Euroscaptor parvidens* | Eu par 15 | ZIN 98917 | Vietnam, province Lamdong, Bidup |
| *Euroscaptor longirostris* | Eu long 167 | ZIN 97789 | Vietnam, province Laokai, Sa Pa |
| *Condylura cristata* | NA1 | *presented by K.Campbell | USA |
| *Parascalops breweri* | NA5 | *presented by K.Campbell | USA |
| | Pbrew PP2002 | ZMMU S-175514 | USA, Pennsylvania Powdermill |

two peaks of approximately equal intensity are observed) were coded using the IUB ambiguity codes. In all analyses sequences were used as unphased genotypes.

Phylogenetic reconstructions were performed with the following data sets: (1) each nuclear gene separately; (2) all nuclear genes combined; (3) an extended sample of taxa for *cytb*; and (4) nuclear and mitochondrial *cytb* sequences combined in a species tree estimation.

The program PartitionFinder (Lanfear *et al*., 2012) was used to determine the best partitioning strategy for nuclear concatenation among five a priori candidate schemes: (1) partitioning by gene; (2) partitioning by codon position; (3) partitioning by gene and codon position (three subsets per gene); (4) as in variant 3 but with the 1st and 2nd codon positions combined (2 subsets per gene); and (5) no partitioning. The *cytb* data set was always partitioned into three codon positions.

Tests for base composition homogeneity in the 3rd codon positions and estimation of K2P distances (Kimura, 1980) were conducted in MEGA5 (Tamura *et al*., 2007).

*Phylogenetic tree reconstruction*

Phylogenetic trees were generated by maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). An unweighted parsimony analysis was performed in PAUP* 4.0b10 (Swofford, 2003) with the following options: random addition sequence with 20 replicates, no limit for the number of optimal trees and TBR-branch swapping. Clade stability was assessed based on 1000 bootstrap replicates obtained with the same tree search parameters. An incongruence length difference (ILD) test (Farris *et al*., 1995) was implemented to check for significant discordances among nuclear genes (1000 replicates).

ML analysis was performed in Treefinder (October 2008 version) (Jobb, 2008). The appropriate models of

sequence evolution were selected for each partition, employing the routine implemented in Treefinder and using BIC as the criterion. A tree search was conducted with the following options: parameter optimisation simultaneous with tree search, optimised partition rates, proportional branch lengths for all partitions, and maximum search depth. Bootstrap support (1000 pseudoreplicates) was estimated using model parameters and rate values optimised for the ML topology. Alternative topological hypotheses were tested using the AU test (Shimodaira, 2002) as implemented in Treefinder.

A Bayesian tree reconstruction was performed by MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Models with either two or six rate matrix parameters were selected for each partition based on the results of the model selection in Treefinder. Each analysis included two independent runs of four chains (one cold plus three heated following the default settings). The chain length was set at 20 million generations with sampling every 5000 generations. With these settings, the effective sample size exceeded 200 for all estimated parameters. Tracer 1.5 software (Rambaut & Drummond, 2005) was used to check for convergence and determine the necessary burn-in fraction, which was set to 2 million generations in all cases.

To estimate the species tree from data on five potentially discordant independent loci (including mtDNA), we employed a Bayesian coalescent framework as implemented in *BEAST (Heled & Drummond, 2010). The units of the analyses correspond to species or well-supported intraspecific groups as inferred in *cytb* analysis. Following the results of the molecular clock tests (detailed below), we used separate strict clock models for nuclear genes and an uncorrelated lognormal relaxed clock model for the mtDNA. No calibration information was utilised, and the mean rate for *BRCA1* was set to one. We used the same partitioning scheme and models as in the ML analysis. Yule prior for the species tree shape and the piecewise constant population size model were assumed. Default priors were used for all other parameters. In total, we conducted three runs of 500 million generations each in BEAST version 1.8.0 (Drummond *et al*., 2012). Parameter convergence was assessed in Tracer 1.5.

### Divergence-time estimation

Divergence times in Talpini were estimated based on the concatenation of the four nuclear genes. The molecular clock assumption was tested separately for each gene using likelihood ratio tests with likelihood values calculated in PAML 4.7 (Yang, 2007). The analysis was performed in BEAST version 1.8.0 (Drummond *et al*., 2012), assuming separate clock rates for each gene, a Yule prior for the tree shape and optimum models (as in the ML analysis) for each partition. Two runs of 100 million generations were conducted.

Calibration information combined evidence of two types. First, we used two fossil-based calibration points that were previously employed by Colangelo *et al*. (2010): the appearance of Talpini in MP21 (Ziegler, 2012) and the earliest representative of *levantis*-related moles in the Late Pliocene (Popov, 2004). Both dates were used as lower boundaries for the separation of corresponding clades. Phylogenetic affinities of fossil moles can not be established with adequate precision (van Cleef-Roders & van den Hoek Ostende, 2001; Popov, 2004; van den Hoek Ostende & Fejfa, 2006), thus, it is difficult to define objectively fossil-based upper bounds or probability distribution on split times within Talpini. Therefore, we had to employ external secondary calibration points. Although this approach is sometimes criticised for a high risk of bias (e.g. Hipsley & Müller, 2014), here we attempted to minimise the error by constructing priors that incorporate all evident sources of uncertainty associated with secondary calibrations. Secondary calibrations were obtained in the course of an additional molecular clock analysis, which was performed in MCMCTREE (part of PAML 4.7) and BEAST using an extended set of 25 nuclear genes retrieved from GenBank and employing published calibration data for additional mammalian outgroups (for details see Supporting Information 2 and Tables S3, S4). The posterior densities for five nodes (Talpini/Condylurini, Talpini/Scalopini, Talpini/Desmanini, *Talpa*/*Mogera*, and *T. altaica*/*T. europaea*) inferred in this primary analysis were used to construct lognormal priors for subsequent BI of divergence times within Talpini conducted in BEAST (Tables 2, S5).

### Species delimitation and validation analyses

As mentioned above, *Talpa* species are characterized by a high level of allopatry and relatively rare cases of parapatry. As such they present little evidence of absence or presence of gene flow in contact zones of different species. In cases like this, species boundaries can be identified with the help of operational criteria employed by the Genetic Species Concept sensu Bradley & Baker (2001) and Baker & Bradley (2006), i.e., by comparison of the levels of divergence between lineages of uncertain taxonomic status with those between indisputable species within the clade of interest.

The closest indisputable sister species in our analysis were *T. europaea* and *T. occidentalis*. Therefore, we used the value of the genetic distance between these two species or the age of their last common ancestor as a crude proxy for a threshold between inter- and intra-specific divergence. As a second approach, we defined the limit between intra- and inter-species variation based on the distribution of times of the most recent common ancestors (*tmrca*) within *Talpa*. Among

**Table 2.** Clade age estimates based on concatenation of four nuclear genes (Fig. 2)

| | Concatenation | |
|---|---|---|
| Clades | Mean (Mya) | 95% HPD lower/upper |
| Talpini+Scalopini+Desmanini+Condylurini | 36.02 | 33.74–38.36 |
| Desmanini+Talpini | 34.85 | 32.69–36.84 |
| *Desmana+Galemys* | 9.51 | 6.84–12.22 |
| Talpini | 12.24 | 10.57–13.81 |
| *Mogera+Euroscaptor+Parascaptor+Scaptochirus* | 8.22 | 6.95–9.53 |
| *Talpa* | 6.56 | 5.49–7.64 |
| (*T. davidiana+T. talyschensis*) clade including stem | 6.15 | 5.12–7.22 |
| *T. altaica* clade including stem | 5.48 | 4.55–6.44 |
| *Talpa*_Western clade* | 3.84 | 3.09–4.65 |
| (*T.e. + T.oc. + T.rom. + T.cae. + T. stank.)* | 3.15 | 2.46–3.83 |
| *T. caucasica* clade | 2.75 | 1.90–3.66 |
| *T. levantis* clade | 2.68 | 1.92–3.51 |
| (*T.e. + T.oc. + T.rom. + T.cae.*) | 2.68 | 2.09–3.27 |
| *T. davidiana+ T. talyschensis* | 2.53 | 1.75–3.34 |
| *T. romana+T. caeca* | 2.48 | 1.84–3.17 |
| *T. europaea+T. occidentalis* | 1.88 | 1.36–2.42 |
| *T. europaea* (all lineages) | 1.31 | 0.91–1.75 |
| *T. caucasica*_northern subclade | 1.07 | 0.58–1.63 |
| *T. europaea*_without_Italy and Spain | 1.00 | 0.59–1.46 |
| *T. caeca* | 0.98 | 0.42–1.57 |
| *T. europaea*_Italy | 0.65 | 0.34–1.03 |
| *T. levantis*_eastern subclade | 0.53 | 0.19–0.95 |
| *T. talyschensis* | 0.53 | 0.11–1.03 |
| *T. altaica* | 0.50 | 0.18–0.86 |
| *T. levantis*_western subclade | 0.43 | 0.13–0.76 |
| *T. stankovici* | 0.34 | 0.01–0.83 |
| *T. occidentalis* | 0.23 | 0.03–0.50 |
| *T. davidiana* | 0.23 | 0.04–0.46 |
| *T. romana* | 0.17 | 0.01–0.40 |
| *T. caucasica*_southern subclade | 0.15 | 0.00–0.36 |

**Talpa*_Western clade – *T. europaea+T. occidentalis+T. romana+T. caeca+T. stankovici+T. levantis*.

the methods that are functional in the absence of reliable *a priori* taxonomic information, we applied to our *cytb* data set the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al*., 2012) and the General Mixed Yule-coalescent model (GMYC) (Pons *et al*., 2006; Reid & Carstens, 2012). The automatic identification of the 'barcode gap' was performed using the ABGD application available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html under the following parameters: Pmin (prior minimal distance) = 0.01, Pmax (prior maximal distance) = 0.1, X (relative gap width) = 1.0. A matrix of pairwise K2P distances was taken as input.

We used the Bayesian implementation of the GMYC that can take into account phylogenetic uncertainty (bGMYC software available at http://sites.google.com/site/noahmreid/home/software). The input sample consisted of one hundred ultrametric trees extracted from the posterior tree sample produced by BEAST version 1.8.0. The preliminary analysis was performed as recommended in Reid & Carstens (2012).

Finally, to objectively elucidate species boundaries among cryptic lineages in our phylogenetic trees, we analysed several potential taxa using a multilocus species validation method, implemented in the Bayesian posterior probabilities (BPP) 2.1 software (Rannala & Yang, 2003; Yang & Rannala, 2010). This method tests the hypothesis that the observed pattern of variation is better explained by the existence of two independent lineages than by a single panmictic population. The Bayesian algorithm used in BPP computes posterior probabilities for the nodes of a predefined species tree using a reversible jump Markov chain Monte Carlo (rjMCMC) approach. The method is widely used and was shown to be one of the most accurate techniques of species delimitation (Camargo *et al*., 2012).

Separate analyses were run for the samples of *T. levantis*, *T. caucasica*, *T. europaea*, and *T. altaica* using either nuclear loci or a combination of nuclear and mitochondrial loci. The guide species trees contained only one or two nodes (e.g., *T. caucasica*_Caucasus + *T. caucasica*_Turkey). We used Gamma (2100) as a prior distribution for both θ and τ. The relative rates of the loci were fixed at values inferred by BEAST. In each case, we performed several runs using different rjMCMC algorithms as described in Yang & Rannala (2010). The MCMC parameters were as follows: chain length of five million generations, burn-in period of ten thousand generations, and sampling frequency set at 1/1000.

## RESULTS

### Alignment, partitioning and base composition

In the combined analyses of four nuclear genes, the final alignment consisted of 4362 nucleotide positions including 1263 bp of *BRCA1*, 975 bp of *ApoB*, 1113 bp of *BRCA2*, and 1011 bp of *RAG1*. In total, the data set contained 60 specimens including 12 outgroups. The final alignment of *cytb* included 1140 bp for 114 specimens of *Talpa* and 27 outgroups. The optimum partitioning scheme for the nuclear genes identified by PartitionFinder under the BIC criterion corresponded to variant 4 (partitioning by gene and codon position but with the 1st and 2nd positions combined).The best-fit substitution models employed for each of the subsets are given in Table S6.

Composition homogeneity tests revealed no highly significant difference ($P < 0.01$) between any pair of sequences within *Talpa* for any of the nuclear genes. In *cytb*, the variation in base composition is more pronounced; however, neither test was significant at the $P < 0.002$ level. Thus, in general, the composition heterogeneity was regarded as low and was therefore neglected.

### Analysis of the nuclear genes and a combined nuclear tree

No contradicting nodes with high support were found among gene genealogies inferred from separate nuclear genes (Fig. S1, Table S7). The ILD test did not reject the H0 of partition homogeneity ($P = 0.105$). The results of AU tests on individual gene topologies suggested that the best topology inferred from the concatenation was not rejected by *BRCA1*, *BRCA2* or *RAG1* but is significantly worse for *ApoB* ($P < 0.01$). Given that the gene tree of *ApoB* is less resolved, we do not regard this outcome as an unambiguous indication of a true conflict among genes.

The concatenated ML and Bayesian analyses recovered identical topologies, thus, only the Bayesian tree is shown (Fig. 2). At a shallow phylogenetic level the tree showed significant support for 10 clades. Nine correspond to the currently recognised species, and one includes individuals from the Talysh Mts (denoted subsequently *Talpa* sp. Talysh; localities 27–28 in Fig. 1).

The relationships between species were not always resolved with high support, however, all analyses (BI, ML, and MP) robustly support a clade comprising six western species (*T. europaea*, *T. occidentalis*, *T. romana*, *T. caeca*, *T. stankovici*, and *T. levantis*) to the exclusion of four eastern species (*T. caucasica, T. altaica, T. davidiana*, *Talpa* sp. Talysh). Within this western ('europaea') clade, *T. levantis* is consistently placed as the most basal lineage, with *T. stankovici* branching next.

*T. occidentalis* and *T. europaea* formed a well-supported cluster; its relationships with *T. caeca* and *T. romana*, however, remained unresolved. A sister-relationship of *T. altaica* to the western clade was supported in all analyses (0.95/65/86 in BI, Ml and MP, respectively) but their common stem is very short. At the same time, the basal position of *T. caucasica* in the genus received less support (0.95/70/0). Moreover, the AU test did not reject the hypothesis postulating the basal position of *T. altaica* ($P < 0.23$). *T. davidiana*, and *Talpa* sp. Talysh constituted a separate lineage that is highly divergent from the other taxa.

The majority of species were further subdivided, and the sublineages showed strong geographical associations. Within both *T. caucasica* and *T. levantis*, two highly supported subclades were identified. The two subclades of *T. caucasica* are represented by samples from the North Caucasus (localities 18, 19, 31, 32 in Fig. 1) and northeastern Turkey (loc. 33 in Fig. 1), respectively. The main dichotomy within *T. levantis* was between northern Turkey (loc. 22–25, 39, 40 in Fig. 1) and the remaining samples including the North Caucasus (loc.18–21), Transcaucasia (loc. 26), and northeastern Turkey (Ardahan, loc. 38). *T. caeca* segregated into the Italian and the Balkan lineages. Within *T. europaea*, there are also two well-supported subclades, though these were significantly less divergent. This subdivision corresponds to geographical differentiation into the Italian (loc. 1–3, 52, 53) and the Central-East European (loc. 5–11, 13, 54, 55) lineages.

### Analysis of the mitochondrial cytb gene

#### *Mitochondrial phylogenetic tree*

The mitochondrial trees are mutually congruent regardless of the method employed (BI, ML, MP) and agree with the results of Colangelo *et al*. (2010). However, with a wider geographical and taxonomic sampling in this study, a more detailed pattern of relationships was retrieved. As evident from the nucleotide and protein *cytb* trees (Figs 3, S2), all sampled species of *Talpa* are strongly supported as monophyletic groups,

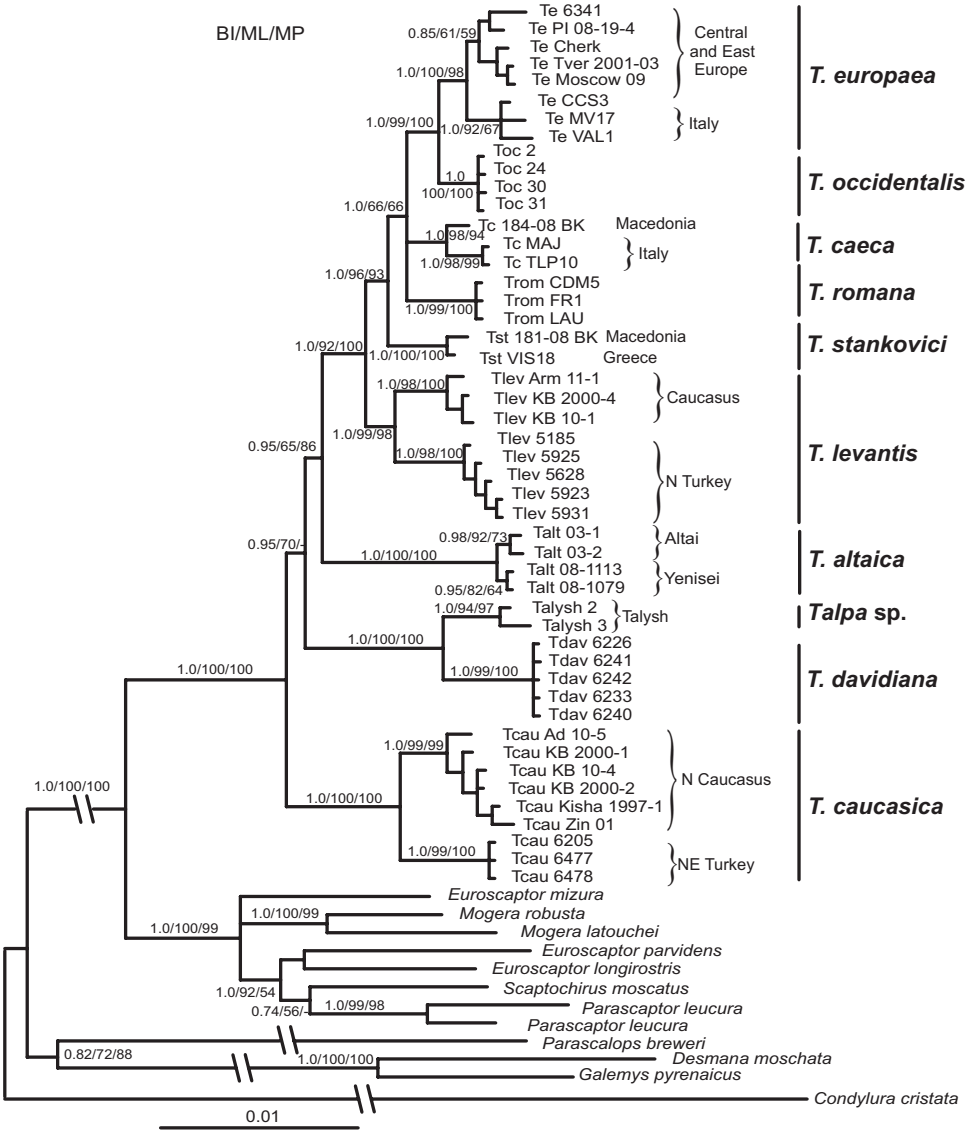


**Figure 2.** The Bayesian phylogeny of the genus *Talpa* as inferred from a concatenated alignment of four nuclear genes. Values above the branches correspond to Bayesian posterior probabilities (BPP) in MrBayes and bootstrap support (1000 pseudoreplicates) in ML and MP analyses, correspondingly. Representatives of the genera *Euroscaptor*, *Mogera*, *Parascaptor*, *Scaptochirus* and tribes Desmanini, Scalopini and Condylurini are used as outgroups.

except for *T. europaea*, which emerged paraphyletic with respect to *T. occidentalis*. Distances (K2P) between recognised species of *Talpa* vary from 9 to 15.6%. *Talpa* sp. Talysh forms a monophyletic group with *T. davidiana* (~13%). Further sublineages within *T. caucasica* and *T. levantis* are separated by high genetic distances: 10% in *T. caucasica* and 7% in *T. levantis*. The grouping of *T. stankovici* and *T. levantis* was reproduced in the protein sequence tree (Fig. S2) but not in the DNA tree. *T. altaica* appears basal to all other extant *Talpa* species although the internodes separating the basal branches of the tree are relatively short. The AU test rejected the hypothesis of the internal position of *T. altaica* in the mitochondrial tree ($P < 0.033$).

*The position of T. caucasica ognevi and T. levantis minima based on cytb data*

Samples of *T. caucasica* from Georgia (*T. caucasica ognevi*) and *T. levantis minima* from Adygea were not included in the phylogenetic analysis because of the short length of the sequenced fragments. In total, only 380 bp for *T. c. ognevi* and 253 bp for *T. l. minima* were sequenced with different combinations of primers (KP717344-46, KP717357-61).

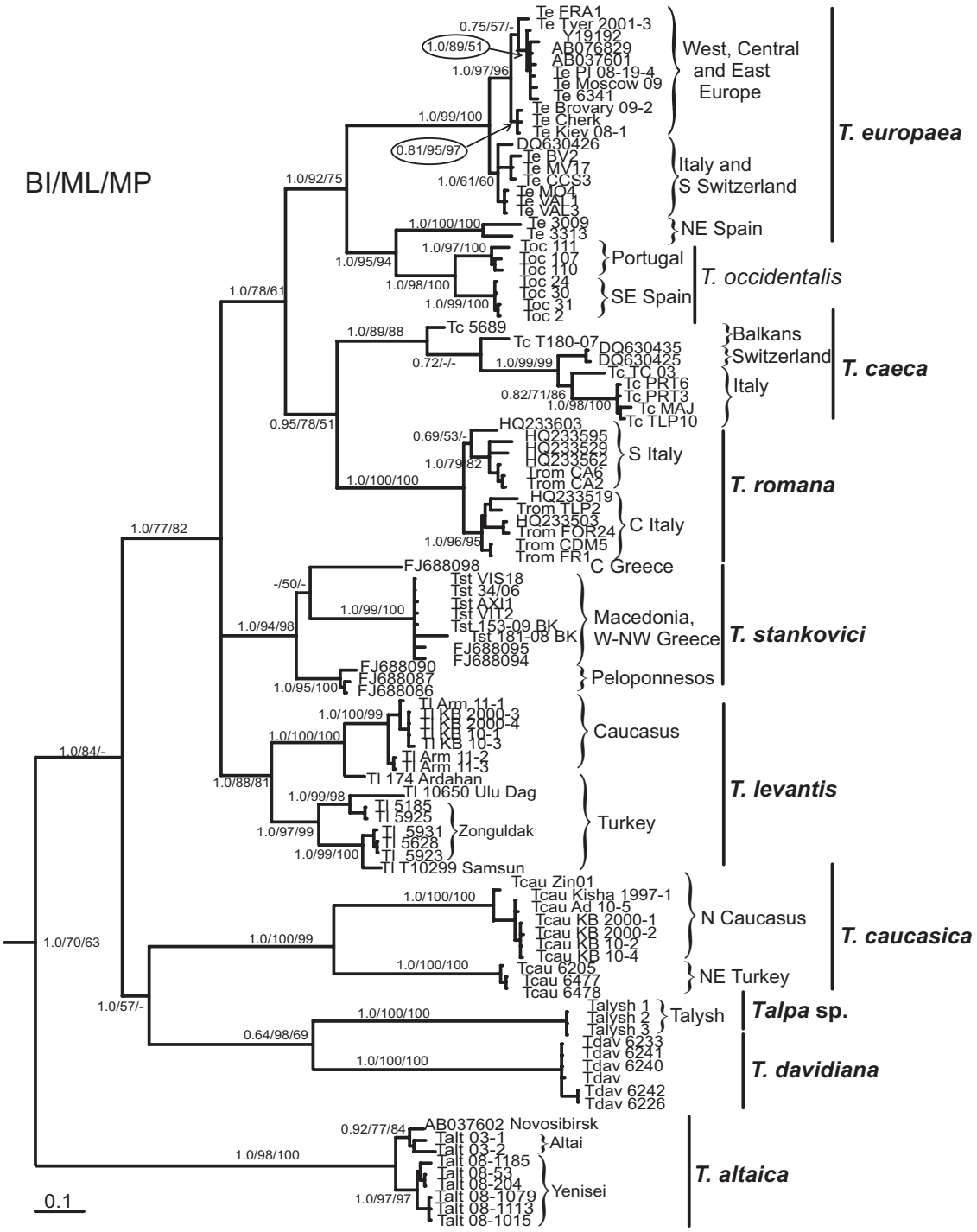


**Figure 3.** The Bayesian phylogeny of the genus *Talpa* as inferred from the complete *cytb* gene sequence. The designations are as in Figure 3. The outgroup (representatives of the genera *Euroscaptor*, *Mogera*, *Parascaptor*, *Scaptochirus* and tribes Desmanini, Scalopini and Condylurini) is not shown.

In the case of *T. c. ognevi* the obtained fragments were similar (p-distance of approximately 1.5%) to the corresponding parts of the complete sequence of *T. caucasica* from Hopa (Cumhuriyet Mahallesi, northeastern Turkey), comprising all diagnostic substitutions that distinguish the Turkish population from the North Caucasus one (Fig. S3A).

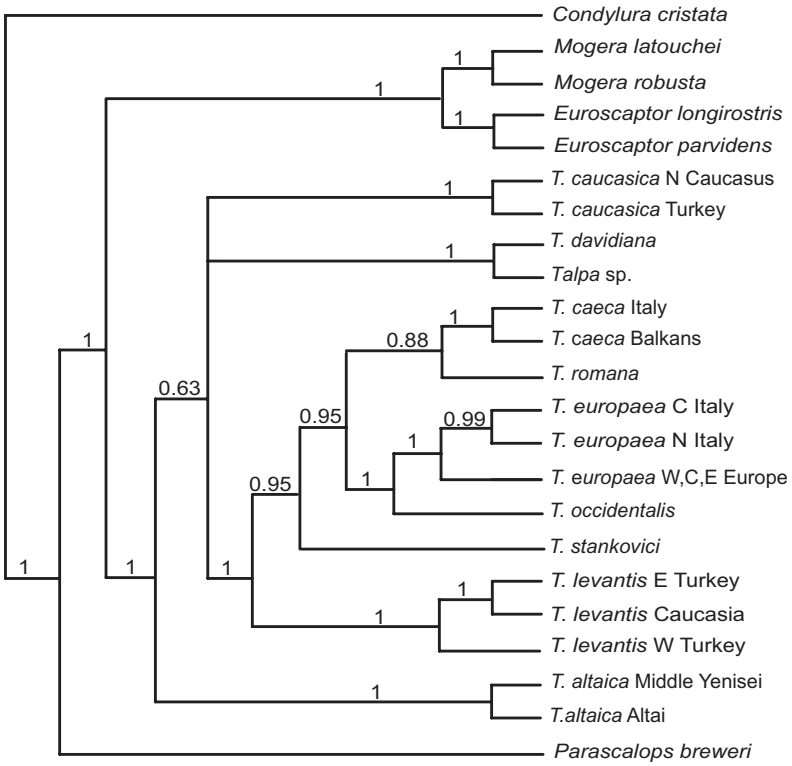


**Figure 4.** Species tree of *Talpa produced by the* *BEAST algorithm using the Bayesian multispecies coalescent approach. Values above the branches correspond to Bayesian posterior probabilities.

Concerning *T. l. minima*, the divergence between the sequences of the specimens from the terra typica (Adygea) and corresponding fragments of the sequences of the Levant moles from Kabardino-Balkaria, Armenia or northeastern Turkey (Ardahan) is approximately 3% (Fig. S3B).

### Comparison of mitochondrial and nuclear phylogeny and a species tree estimation

The topologies of the mitochondrial, nuclear and *BEAST species trees are essentially similar. Three points of disagreement should be mentioned. The first concerns the relationships among the four major clades. While in the mtDNA tree *T. altaica* is recovered as the basal-most branch, in the nuclear tree it is placed sister to the western clade. In the species tree, the basal branching pattern is poorly resolved (Fig. 4); *T. altaica* tends to be the earliest offshoot but with low support. Next, the grouping of *T. caeca* + *T. romana* is supported only by the mtDNA data, whereas the position of *T. levantis* as sister to the rest of the western clade is supported only by the nuclear concatenation. Both of these relationships are supported in the species tree.

### Molecular time estimates

Our analysis based on concatenated nuclear genes estimated the time of the most recent common ancestor (*tmrca*) of the genus *Talpa* at ~6.56 Myr (5.49–7.64 Myr) which corresponds to the Late Miocene (Messinian – latest Tortonian) (Fig. 5, Table 2). However, short interior branches of the phylogenetic tree and very close time estimates for the divergence of branches of *T. caucasica*, *T. altaica*, and *T. davidiana* + *Talpa* sp. Talysh (5.48–6.56 Myr) suggest successive divergences in a relatively narrow time span. Radiation of the western group of species was estimated to have occurred between 4 and 2 Myr, with the most common recent ancestor at approximately 3.8 Myr (3.09–4.65 Myr). Intraspecific divergence events were estimated to date back to the Early-Middle Pleistocene (from 1.4 to 0.17 Myr), but *T. levantis* and *T. caucasica* each split into two sublineages as early as the Late–Middle Pliocene, approximately 2.68 Myr (1.92–3.51 Myr) and 2.75 Myr (1.90–3.66 Myr), respectively. Linear regression of concatenation-based *tmrca* against relative times inferred by *BEAST indicates that the former are on average 480 Ky younger (SE = 140; $P = 0.003$; $R^2 = 0.970$).

### Species delimitation and validation

#### *Determination of species boundaries using molecular dating and genetic distances*

The analysis of the distribution of *tmrca* within the genus *Talpa* estimated from the concatenation of nuclear

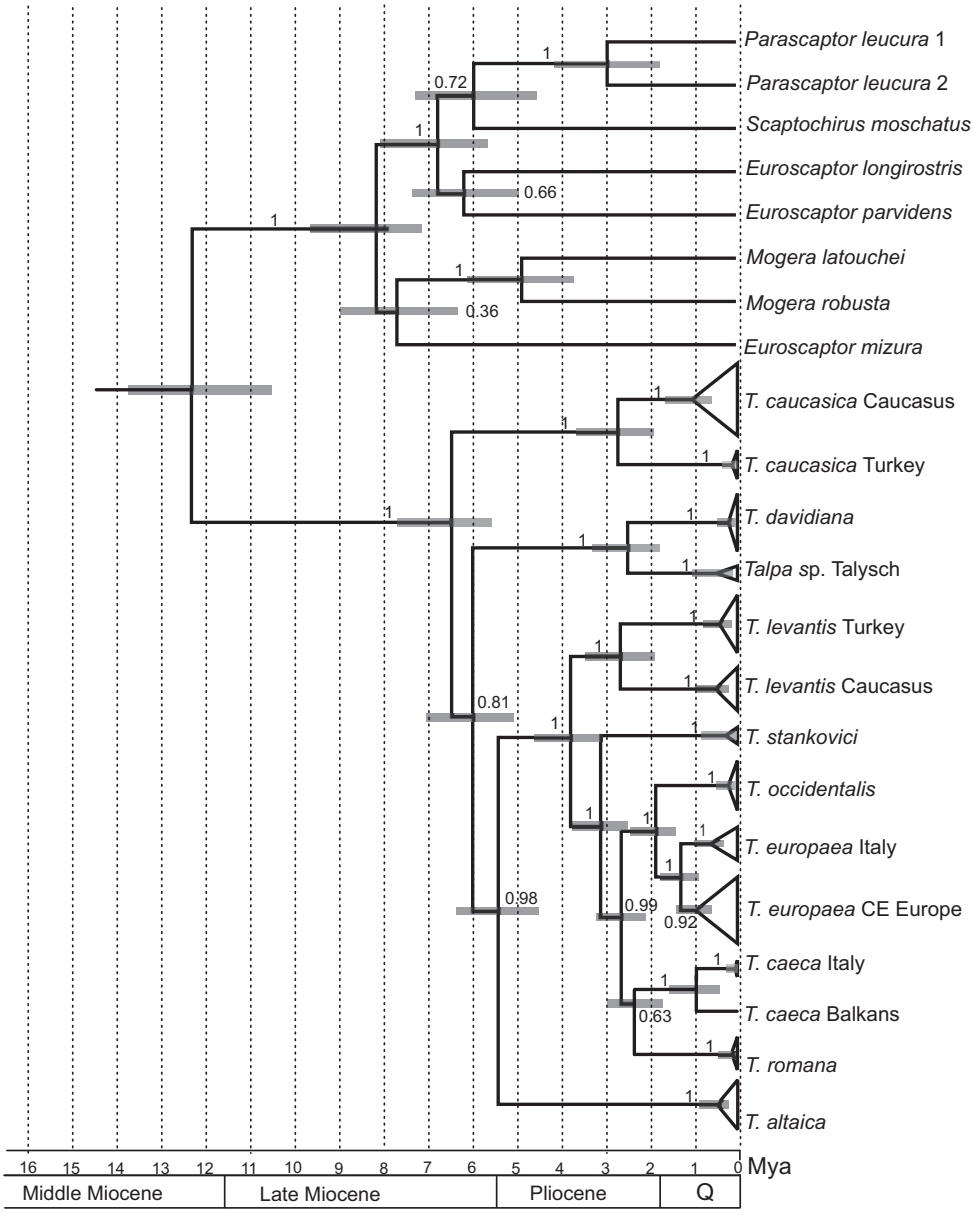


**Figure 5.** Timescale of major divergence events among *Talpa* based on nuclear concatenation (BEAST). The divergence times correspond to the mean posterior estimate of their age in Myr. The grey bars represent the 95% HPD interval. Numbers above the branches correspond to posterior probabilities for each node.

genes (Table 2) revealed three levels of divergence: (1) within species (*tmrca* < 1.5 Myr); (2) among species within the main clades (2–4 Myr); and (3) among the main clades (6–7 Myr) (Fig. S4). The *tmrca* of *T. caucasica* s.l. and *T. levantis* s.l. fall into the second group and correspond to the indisputable species level based on the minimum age of interspecific divergence as marked by the *tmrca* of the *T. europaea*/*T. occidentalis* clade (~2 Myr). The time of divergence of the two main lineages within *T. caeca* (~1 Myr) appeared close to the maximum limit of intraspecific divergence.

The frequency distribution of K2P values between *cytb* haplotypes has three peaks that roughly correspond to the *tmrca* clusters. The most evident gap was between 3.5 and 5% (Fig. S5). Based on this distribution, ABGD retrieved 16 groups for primary partitions and from 16 to 18 groups for recursive partitions. The 16 group solution corresponds to the following partition: *T. europaea s.s.*, *Talpa* aff. *europaea* from Spain, *T. romana*, *T. occidentalis*, *T. altaica*, *T. davidiana*, *Talpa* sp. Talysh, two groups within *T. caucasica*, two groups within *T. levantis*, two groups within *T. caeca*, and three groups within *T. stankovici* (Fig. S6).

The bGMYC analysis resulted in 19 putative species (Fig. S6) including all groups revealed by the ABGD method along with additional lineages within *T. altaica*, *T. romana*, and *T. europaea s.s.*

*Species delimitation using multilocus genetic sequence data*

Due to limited sampling of nuclear loci, the results of the BPP species delimitation analyses should be regarded as preliminary. The inspection of the output showed proper convergence and mixing regardless of the rjMC algorithm used. The analyses provided strong support for the recognition of *T. levantis* from the Caucasus region with the adjacent parts of northeastern Turkey on the one hand and the sample of Levant moles from northern Turkey on the other as separate lineages with 100% support based on the data set including only nuclear genes. Similar results were obtained for the two lineages of *T. caucasica*. The fully resolved tree had a posterior probability of 1.0 in each case (Table S8).

In a species delimitation analyses, *T. europaea* samples from North Italy and Central Italy were treated a priori either as separate taxa or as a single taxon. In both cases the delimitation results supported the Italian population as being a separate taxon with respect to all other populations of *T. europaea*. The separation of North Italy versus Central Italy, however, should be considered ambiguous, being supported in only ~90% cases. Moreover, the results of the delimitation contrasting Italian and non-Italian populations was mtDNA dependent. Namely, when the latter marker was excluded from the analysis, samples of *T. europaea* from the entire range emerged as a single species with 62% posterior probability. The results obtained for *T. altaica* subdivisions did not pass the test for presence of independent lineages even with mitochondrial data included ($P < 0.001$).

## DISCUSSION

### The problem of species delimitation in moles

Our study revealed high genetic diversity of the genus *Talpa* and the presence of strong phylogeographic structure within most of the currently recognised species. However, with numerous cryptic forms revealed, it is hard to draw the line between intra- and interspecific diversity. Different methods of species delimitation produced different groupings of potential species status; this phenomenon, however, is not unique for moles (e.g., Miralles & Vences, 2013; Salter, Carstens & Hedin, 2013). Nevertheless, we believe that species delimitation in *Talpa* may present an especially complex task due to specific features of evolution and life history of moles.

The fossorial ecological niche requires morphological specialisations and, at the same time, provides little space for resource partitioning (Nevo, 1979). Therefore, speciation events are usually not accompanied by pronounced morphological changes other than moderate size shifts. Correspondingly, due to competitive exclusion, species of moles may achieve sympatry only after an extended period of evolution in allopatry when a high level of divergence is reached. This hypothesis agrees with our finding that all sympatric species of moles diverged in pre-Pleistocene times. This also offers an explanation for the lack of gene flow in zones of sympatry and parapatry. Notably, ancient interspecies introgression between species of *Talpa*, has hitherto been confirmed only for *T. europaea* and *T. romana* in the parapatric contact zone in Central Italy, based on allozyme data (Loy *et al*., 2001). No case of mtDNA introgression has been documented so far. According to the Genetic Species Concept the species threshold is determined based on the level of differentiation between known genetically isolated lineages that maintain their genetic integrity in sympatry or parapatry (gene exchange is absent or negligible). If it is true that only highly divergent species of *Talpa* may co-occur, then a straightforward application of the Genetic Species Concept will lead to overestimation of the threshold between inter- and intraspecific variation.

A potential problem with the methods based on multispecies coalescent (GMYC, BPP) is that they assume panmixia within populations (Camargo & Sites, 2013). However, this is not the case for *Talpa* species, which typically demonstrate strong phylogeographic structure, evidently as a result of the reduced mobility and limited dispersal ability (Ognev, 1928; Stein, 1950; Gorman & Stone, 1990). Under such circumstances, species delimitation methods may be prone to split single species into multiple allopatric lineages and the bias tends to be stronger for methods based on a single marker such as GMYC (Lohse, 2009; Carstens *et al*., 2013). At the same time, a higher level of spatial structuring typical for subterranean mammals may lead to elevated diversification rates (Reig *et al*., 1990), hence, increasing the number of lineages of ambiguous taxonomic status. Taking into account all of these considerations, we regard our delimitation results as preliminary.

However, even if we accept a conservative approach to species delimitation, there are ample reasons to elevate three lineages to the species rank in addition to the nine currently recognised. High levels of genetic divergence and delimitation results corroborate the full species status of *Talpa* sp. from the Talysh Mts and indicate the existence of cryptic species within *T. caucasica* and *T. levantis*.

## Implications for the taxonomy of the genus *Talpa*

### *T. talyschenses Vereschagin, 1945*

Our molecular data provide unequivocal genetic evidence for a separate status of the moles from the Talysh Mts., which are deeply divergent from both the Caucasian mole and the Levant mole, being the sister branch to *T. davidiana*. According to our time estimates based on the nuclear data set, the age of the Talysh lineage is not less than 2 My. The moles from the Talysh Mts were previously treated as a subspecies of *T. levantis* (Sokolov & Tembotov, 1989; Hutterer, 2005), although Zaitsev (1999) after examination of type material expressed doubt whether *talyschensis* is conspecific with *T. levantis*.

We conclude that moles occupying an area along the southwestern banks of the Caspian Sea from southernmost Azerbaijan to the central Elburz Mts in Iran, actually belongs to *T. talyschensis* and not to *T. caucasica* or *T. levantis*, as previously proposed (Kryštufek, 2001; Kryštufek & Benda, 2002). This finding adds one more species to the list of the Hyrcanian endemic mammals, which includes *Crocidura caspica* (Zaitsev, 1991), *Apodemus hyrcanicus* (Vorontsov *et al*., 1992), *Microtus shelkovnikovi* (Nadachowski, 2007), and the Iranian lineage of *Glis glis* (Naderi *et al*., 2013).

### *T. caucasica Satunin, 1908 and T. ognevi Stroganov, 1948*

Our results suggest a species status for the two allopatric lineages of the Caucasian mole. The northern lineage is found in the North Caucasus and presumably corresponds to two recognised subspecies: *T. c. caucasica* Satunin, 1908 from the Central North Caucasus (Kabardino-Balkaria) and *T. c. orientalis* Ognev, 1926 from the Northwest Caucasus (Adygea). The southern lineage is represented by a sample from northeastern Turkey (Hopa, Cumhuriyet Mahallesi, Artvin), and is evidently very close to *T. c. ognevi* Stroganov, 1948 (type locality is Bakuriani, Georgia), which is reported for adjacent western Transcaucasia (Sokolov & Tembotov, 1989). Our additional analysis of *cytb* (Fig. S3) showed that sequences of Turkish specimens are very similar to the partial fragments obtained from archived museum specimens of *T. c. ognevi* originating from Batumi and Borzhomi (South Georgia); the latter is only ~20 km away from the type locality of *ognevi*. According to our results the time of the split between *T. caucasica* and *T. ognevi* dates back to the second half of the Pliocene (2.5–3 Myr). *T. ognevi* from Georgia and Turkey is morphologically distinct from true *T. caucasica* of the North Caucasus being characterized by larger size and more robust dentition (Sokolov & Tembotov, 1989; Kryštufek & Vohralík, 2001). These differences induced Stroganov (1948) to attribute *ognevi* to *T. romana* (as a subspecies *T. r. ognevi*). The details of the distributions of *T. caucasica* and *T. ognevi* remain to be clarified.

### *T. levantis group*

The most complex genetic structure was found in *T. levantis*. According to our nuclear data, the two major lineages within the Levant mole may correspond to distinct species that separated more than 2 Myr. The eastern lineage is distributed in the Caucasus, Transcaucasia, and the adjacent parts of northeastern Turkey (Cam Gecidi, Ardahan), while the western lineage occupies the major part of the Turkish range, stretching along the Black Sea shore westwards to the Marmara region. The nomenclatural treatment of these two lineages remains unclear because of the lack of genetic material from the type locality of *T. levantis* (Alindere, south of Trabzon), which is located between the sampled ranges of the and the eastern clades. If typical *T. levantis* belongs to the western clade, the senior synonym applicable for the eastern clade is *T. transcaucasica* Dahl, 1944 (type locality is Voskresenovka, Armenia). However, if the opposite is true, then no name is available for the western clade.

The Asian part of the range is believed to be disjointed (Kryštufek, 2001) with the gap between North–Northwest Turkey (Paphlagonia, i.e., between Zonguldak and Sinop). However, genetic data show no correspondence with this subdivision because the western clade is found both eastwards (Samsung) and westwards (Uludag) from the presumed gap.

Within the western and eastern clades, two additional subclades emerge from the mtDNA evidence, with the distance between them being 3–3.5%. Within the last one the substantial difference between morphologically specific lineage from the northwestern Caucasus (Adygea) and Kabardino-Balkarian sample give grounds to treat these subdivisions as subspecies and support the recognition of the Levant moles from northwestern Caucasus as *T. l. minima* Deparma, 1959.

### *Genetic differentiation in other species of Talpa*

Due to a limited genetic data we could not validate the species status of the major phylogroups within *T. caeca* and *T. stankovici* which are characterized by deep phylogeographic breaks in each of these species.

The populations of blind moles from Apennines, Swiss Alps, and Balkans are treated as different subspecies: *T. c. caeca* Savi, 1822 (Central Italy), *T. c. augustana* Capolongo and Panasci, 1978 (Switzerland and North Italy), *T. c. steini* Grulich, 1971 (Montenegro), and *T. c. hercegovinensis* Bolkay, 1925 (Herzegowina) (Niethammer, 1990). The mtDNA differentiation observed for the Apennines and the Swiss Alps (western clade) versus the Balkans (eastern clade)

is also supported by the divergence at conservative nuclear loci. According to our data the split of these lineages dates back to the Early Pleistocene (approximately 1.1 Myr). Notably, the karyotypes of the Blind mole in the Swiss Alps (Switzerland, Ticina) and Balkans differ in the chromosome fundamental number (Niethammer, 1990).

As demonstrated by Tryfonopoulos *et al*. (2009), the sample of *T. stankovici* from Greece contains three mitochondrial lineages (central Greece, Pelopónnēsos, and northwestern Greece). The K2P distances among them vary from 5% to 7%. Based on the available mitochondrial data the *tmrca* can be tentatively estimated at no less than 1 Myr.

Thus, it is probable that the accumulation of the nuclear data on geographically representative samples of *T. caeca* and *T. stankovici* will lead to the revision of the taxonomic status of certain phylogroups.

Finally, we cannot exclude that the Spanish lineage of *T. europaea* is also a new cryptic species rather than a result of past mtDNA introgression from *T. occidentalis* (Feuda *et al*., 2015). The available nuclear data on this population is restricted to a single sequence of *ApoB*, which appeared to be a distinct haplotype. Mitochondrial data placed the split of these lineages at approximately 1 Myr. In the case of *T. altaica* and *T. europaea s.s.*, the main mitochondrial phylogroups are also recovered with nuclear data. According to the mtDNA data (Feuda *et al*., 2015), the split between the Italian and European lineages of *T. europaea* occurred ~700–800 Kya. Thus, it could not be the result of population fragmentation during the last glacial maximum, but most likely reflects a more ancient divergence during some previous interglacial periods. Moreover, Loy & Corti (1996) found a sudden change in mandible morphology over a short geographic range, highlighting a significant morphological difference of both the Spanish and Italian *T. europaea* versus other populations from western and central Europe, respectively. A morphological gap was also shown between Spain and western France and there is one abrupt change from the Italian populations to geographically adjacent populations in Switzerland, Germany, and Austria (Loy & Corti, 1996).

*Subgeneric taxonomy*

As shown above, 12 species of the genus *Talpa* may be clearly divided into four divergent lineages. One of them (*T. altaica*) was previously attributed to a monotypic subgenus, *Asioscalops* Stroganov, 1941, which was established on specific pelvic and dental morphology. The remaining species were included into *Talpa s.s*. In some cases, *Asioscalops* was regarded even as a separate genus (e.g., Yudin, 1989). Its isolated position was moderately supported by mitochondrial data (Colangelo *et al*., 2010; our data) and cytogenetic evidence (Kratochvíl & Král, 1972; Kawada *et al*., 2002). However, the nuclear results provide no support for placement of *Asioscalops* as sister to *Talpa s.s*. and, hence, for its recognition as a distinct taxon of equivalent rank to the rest of *Talpa*. Based on our data, we find no justification for splitting extant *Talpa* into several genera. If the subgeneric rank of the *T. altaica* lineage is maintained, then one should also reconsider the taxonomic status of two other lineages (i.e., the 'davidiana' and 'caucasica' groups).

## Molecular time estimates and putative scenario of *Talpa* radiation

According to our data the basal part of the *Talpa* tree appears as a polytomy, which can be attributed to rapid diversification events. Molecular clock results suggest that the onset of this radiation dates back to the latest Miocene (Table 2) in agreement with previous mitochondrial data (Colangelo *et al*., 2010). The fact that three of the four basal lineages are distributed in Asia supports the hypothesis on re-colonisation of Europe from Asia advanced in the above study and indicates a key role of the Near East and Paratethys regions in the evolution of this genus. Whereas the Late Miocene Messinian climatic fluctuations could have led to a nearly complete extinction of Talpini in the western Europe (Fortelius, 2008), the mountainous areas of the Caucasus, Anatolia, and western Iran retained a sufficient level of humidity in the Late Miocene and Pliocene as follows from the available data on vegetation (Kovar-Eder, 2003; Kovar-Eder *et al*., 2006) and thus may have harboured isolated populations of several *Talpa* lineages ('caucasica' and 'davidiana' groups). One can hypothesise that the third branch ('europaea') also originated from this region. This proposition is consistent with the fact that the only Asian member of the clade, *T. levantis*, is the sister to the subclade containing all European species. Finally, the fourth major lineage (*T. altaica*) is a geographical outlier, being distributed in Siberia. The place of origin and range history of the Siberian lineage remains unclear.

In general, the evolutionary history of the genus *Talpa* is characterized by several rounds of radiation and extinction. Although the genus has a long evolutionary history with the first appearance recorded in the Early Miocene of Europe (Ziegler, 1990) it is evident from the molecular phylogenetic analysis that the modern diversity was formed de novo from a single Late Miocene lineage. It should be emphasised that the genus *Talpa* (including the Early Miocene taxa) is expected to be paraphyletic relative to the other extant genera of Talpini (i.e., the East Asian clade, including *Mogera*, *Euroscaptor*, *Parascaptor*, *Scaptochirus*) because the oldest fossil of *Talpa* is dated to 20–22 Myr (Ziegler, 1990) whereas the molecular data suggest a Middle

Miocene age for the most recent common ancestor of the crown Talpini (He *et al*., 2014; our data). The fact that both Early–Middle Miocene and extant species are attributed to the same genus is explained by morphological conservatism owing to the uniformity of fossorial adaptations (Barrow & Macleod, 2008). At the other extreme, slow morphological evolution in *Talpa* manifests as high cryptic diversity at the level of closely related species.

Further studies based on a more extensive sampling of genes and taxa should elucidate the true magnitude of cryptic speciation in fossorial moles and develop a more detailed evolutionary scenario for *Talpa*.

## ACKNOWLEDGEMENTS

The authors are grateful to V. Burskya, B. Sheftel and Yu. Litvinov for the *T. altaica* samples, A. Sánchez for the specimens of *T. occidentalis* and K. Campbell for American representatives of Talpidae used as outgroups. The work was supported by the Russian Foundation for Basic Research, project 14-04-00034a and Russian Science Foundation, project 14-50-00029 (the analysis of archive DNA from museum specimens). Turkish specimens were collected with the support of Bulent Ecevit University, project 2009-13-06-02.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Supporting Information 1.** The list of sequences retrieved from GenBank and Tables S1-S2.
**Supporting Information 2.** Additional molecular dating and Tables S3-S8.
**Supporting Information 3.** Figures S1–S6.
**Supporting Information 4.** Supplementary references.