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ABSTRACT

We examined the natural population structure of the Turkish hamster (*Mesocricetus brandti*) by analysing partial mitochondrial sequences of the control region and the cytochrome *b* gene. Evolutionary lineages were defined on haplotype clusters in genetic trees and a median-joining network. Most significant divergence events in *M. brandti* nested in the lower Pleistocene. Gene flow prevented spatial genetic differentiation among most populations contrasting previous ideas about potential subspeciation in Anatolia. None of the mitochondrial lineages showed significant signs of recent expansion indicating relatively stable ecological condition during recent population history. Furthermore, we discussed aspects of the evolution of *M. brandti* and the genus *Mesocricetus* in the context of available fossils.

Keywords

Turkish hamster, *Mesocricetus brandti*, mitochondrial DNA, genetic diversity, population structure, haplotype lineages, separation times

INTRODUCTION

The Eastern Mediterranean region harbours an exceptionally high number of mammalian species and subspecies (Kryštufek et al. 2009). Unravelling the origins of this diversity may therefore significantly contribute to a better understanding of the processes governing speciation and population differentiation within this animal class as a whole.

Several reasons were put forward to explain the species richness in this region. Moderate climatic oscillations during the Quaternary did not cause extensive species or population losses. Hence, long lasting persistence and relatively stable population sizes allowed the accumulation of many allelic variants as well as deep genetic differentiation within and among populations (Bilgin et al. 2008, Kryśtufek et al. 2009). Additionally, the area provided an important refuge for several mammalian groups, which led to the immigration of new elements during glacial heights (Michaux et al. 2004, Sert et al. 2005, Dubey et al. 2006, 2007). Furthermore, due to its geographic location, areas like Asia Minor function as a gateway for migration among neighbouring faunal complexes enabling a frequent species exchange between Europe, Arabia, northern Africa and Iran (Koufos et al. 2003, Ansell et al. 2011, Bilgin 2011).

The hamster genus *Mesocricetus* (order: Rodentia, subfamily: Cricetinae) comprises an autochthonous and characteristic element of the Eastern Mediterranean steppe fauna. It may therefore provide a useful model to study the mechanisms of population differentiation in the region, working over a relatively long period.

Mesocricetus originated ~ 8 - 11 million years (my) ago according to genetic data and forms a distinct phylogenetic lineage inside the subfamily Cricetinae (Neumann et al. 2006). To date, the oldest fossils identified as *M. primitivus* or *M. aff. primitivus* were excavated from the late Miocene and Pliocene layers in Greece (De Bruijn et. al. 1970, Vasileiadou et al. 2003, Vasileiadou et al. 2012), Turkey (Sen et al. 1998, Üney and Brujin 1998, Seyrek et al. 2008, Van den Hoek Ostende et al. 2015) and Israel (Tchernov 1986). Modern *Mesocricetus* replaced the species eventually during the upper Pliocene and early Pleistocene (Vereshchagin 1959, Storch 1975, Tchernov 1975, Gülec 1999). All four contemporary species (*M. auratus*, *M. raddei*, *M. brandti* and *M. newtoni*) are exclusively distributed in south-eastern Europe (Balkan), the Caucasus area and the Near East (Hamar and Shutowa 1966, Neumann et al. 2006). Although the historic species count and its distribution was slightly larger than today, it

appears that *Mesocricetus* evolved mainly in its current geographic area. There is no evidence from fossils that the genus experienced any dramatic range shifts during the past. Based on DNA sequences, recent *Mesocricetus* fall into two main lineages. One is formed by *M. auratus* and *M. raddei* and a second contains *M. brandti* and *M. newtoni*. Molecular clock calculations date their separation at about 2.5 - 2.7 my ago (Neumann et al. 2006).

The Turkish or Brandt's hamster (*M. brandti*) occupies by far the widest geographic range of all *Mesocricetus* species. Its distribution covers Turkish Anatolia, the Transcaucasus (Armenia, Georgia, Azerbaijan) as well as northwestern Iran (Doğramacı et al 1994, Yiğit et al. 2006). Noteworthy, an isolated Ciscaucasian population exists in Dagestan/Russia (Pavlinov et al. 2002). In this region, *M. brandti* meets the range of another the *Mesocricetus* species *M. raddei*. *Mesocricetus brandti* inhabits arid and semi-arid steppe habitats in lowlands and in mountainous areas up to 2500 metres. It also occurs in cultivated land but the species is much less dependent on agricultural fields than the golden hamster (*Mesocricetus auratus*). Information concerning the Turkish hamster's biology and population structure is limited (Lyman et al. 1981, Pohl 1985, Yiğit et al. 1997, Pavlinov et al. 2002). Yiğit et al. (2000, 2006) reported substantial morphological and chromosomal variation among populations in Turkey and Iran and suspected a high degree of subspeciation. They concluded that animals from eastern Turkey and Iran might represent an ancestral line. Chromosomal polymorphisms were documented from various populations. The diploid chromosome number of *M. brandti* totals 2n = 42 but there are reports of an additional rare *M. brandti* karyotype of 2n = 44 near Ankara in inner Turkey (Lyman and O'Brien 1977, Popescu and Di Paolo 1980). Differences in the fundamental number of arms of chromosomes (FN) were also specifically found in eastern animals (Todd et al. 1972, Yiğit et al. 2007, O'Brien et al. 2006).

Here we provide a first study on the genetic population structure of the Turkish hamster. Molecular and fossil data were aligned to reconstruct the species' population history. Since *Mesocricetus brandti* is a typical member of the widespread Anatolian steppe habitats, its genetic and spatial structure may provide a key model for diversification and speciation in many other Turkish rodents. Furthermore, our research will enhance the general knowledge of the various phylogeographic patterns existing in mammals from this still underexplored region (Michaux et al. 2004, 2005, Gündüz et al. 2007).

MATERIAL AND METHODS

Animal sampling

Altogether, we sampled tissues of 47 *M. brandti* at different localities in Turkey, western Iran, Armenia and Dagestan/Russia. Table 1 summarizes the information about sampling localities and the corresponding numbers of collected animals. Figure 1 provides geographic details about the species' current distribution range and sampling sites.

DNA extraction and processing

Genomic DNA isolation from fresh or ethanol fixed materials such as ear, liver and muscle followed a protocol supplied with the E.Z.N.A. Tissue DNA Kit II (PEQLAB Biotechnologie).

For DNA analyses, we used sequence information of two partial mitochondrial segments, the control region (*ctr*; 380bp) and the cytochrome b gene (*cytb*; 925bp). PCR-amplification, fragment purification and sequencing followed largely as described in Neumann *et al*. (2004, 2005).

DNA sequence statistics

Sequences were aligned and edited in PROSEQ 3.5 (Filatov 2002). Haplotype diversities (*Hd*) with corresponding standard deviations (*SD*) of single and concatenated sequences as well as nucleotide diversity values (π) were calculated in DNASP 5 (Rozas and Rozas 1999). Coalescent simulations (10 000 replicates) implemented in the same program were used to define the 95% confidence limits of π. Population statistics as well as phylogenetic analyses were exclusively performed on concatenated DNA sequences to enhance statistical power. DNA sequences of *M. newtoni* (KY404082, AJ97338), *M. auratus* (AM904616, EU660218) and two subspecies of *M. raddei* (*M. r. avaricus*: AJ973383, KX023777; *M. r. nigriculus*: AJ973382, KX024778) served as outgroups.

Haplotype group structure was deduced from phylogenies obtained with gene trees and a sequence network. Tree building was carried out by distance using the maximum likelihood (ML) and neighbour joining (NJ) methods, as implemented in MEGA 6.06

(Tamura et al. 2013). For the construction of the ML tree we used the HKY+Γ+I algorithm which proved among the most appropriate substitution models. Model tests were carried out with MEGA 6.06 using the AIC criteria (Tamura et al. 2013) and FINDMODEL (Weighbor method based on Jukes-Cantor distances). The web application FINDMODEL (http://hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was developed from MODELTEST (Posada and Crandall, 1998). A NJ tree was built using the popular Kimura-2 parameter (K2P) distance. Robustness of nodes was confirmed by bootstrapping (1000 replicates). Additionally, a Bayesian tree was constructed in MrBayes v. 3.2 (Ronquist et al. 2012) with the HKY+Γ+I model. Sequence data were used in a single partition. A Markov chain was run for one million generations with sampling every 500 generations until convergence was achieved (standard deviation close to 0.01). A median-joining network based on concatenated sequences was computed in NETWORK 4.1.0.1 (Röhl 2000). The network associates haplotypes according to the number of dividing mutational steps creating a 1-step haplotype topology. The method produces rather robust networks from non-recombinant sequences (Bandelt et al. 1999, Wooley et al. 2008). An analysis of molecular variance (AMOVA, Excoffier et al. 1992) was conducted to verify haplotype structuring. The test was performed in ARLEQUIN version 3.10 (Excoffier et al. 2005).

A global clock test (Hasegawa et al. 1985) was applied to detect potential rate variation between phylogenetic groups (MEGA 6.06). The method compares the ML values of a given tree topology under the presumptions of a strict (SC) and a relaxed clock (RC). Divergence dates were estimated using Bayesian inferences implemented in BEAST 1.8.2 (Drummond et al. 2012). We used two calibration constraints based on relaxed clock data of our recent hamster phylogeny (Neumann et al 2006). The first one corresponds to the divergence time between *M. brandti*+*M. newtoni*/*M. auratus*+*M. raddei* at 2.7 ± 0.8 my. The second calibration was the split between *M. newtoni*/*M. brandti*, which is estimated at 1.7 ± 0.6 my. One *M. auratus* and one *M. newtoni* sequences were added to our dataset to calibrate the tree. We applied an exponential prior on the tmrca (time of the most recent ancestor) of all taxa, which required specification of only the offset and mean. The model of nucleotide substitution that best fitted the dataset was estimated with FINDMODEL. Analyses were performed under the GTR+G+I, an uncorrelated lognormal molecular clock, and a Bayesian skyline coalescent tree model. These priors were selected because they better fitted the data than any other molecular clock and population models according to the Bayes factor

calculated to compare the models. Two independent runs with MCMC length of 50.10^6 were performed with sampling every 5000 generations. Convergence of the chains to the stationary distribution was checked using TRACER 1.5 (Rambaut et al. 2009). Additionally, we estimated mean genetic differences among haplotype groups assuming a strict clock using the Kimura-2 parameter (K2P)-model. Calculations were carried out using MEGA 6.06. Corresponding variance was estimated by bootstrapping (1000 replicates). Separation time estimates were based on strict clock data (Neumann et al. 2006). The following references were used: *M. brandti*+*M. newtoni* - *M. auratus*+*M. raddei* = 2.5 - 2.7 ± 0.2 my, *M. newtoni*/*M. brandti* = 1.7 - 1.8 ± 0.1 my. R2-statistics was calculated to detect potential signs of recent population expansion in haplotype groups because it proved well suited for small sample sizes (Ramos-Onsins and Rozas 2002). The test was run in DNASP 5 and *p*-values were obtained by coalescence simulations over 1000 replicates as implemented in the same software.

RESULTS

Sequence diversity in M. brandti

Ctr sequences showed 37 mutations (15 singletons, 7 transversions) at 35 variable sites resulting in 31 haplotypes. *Hd* measured 0.963 ± 0.017 with π = 0.016 ± 0.001. Accession numbers were AM904643 - AM904663, KF149996 - KF150003, and KX023779 - KX0237784.

Thirty-seven *cytb* haplotypes (AM904620 - AM904642, KF149989 - KF149995, KX023785 - KX023791) were detected comprising 155 mutations at 149 sites (12 singletons, 11 transversions). Fifteen substitutions led to amino acid changes. Haplotype diversity (*Hd*) measured 0.979 ± 0.012 and nucleotide diversity (π) equaled 0.046 ± 0.005.

Our data set revealed a slightly lower variation in *ctr* than in *cytb*. Differences cannot be explained by high levels of saturation because of the low numbers of transversions (n = 3) and the lack of sites with multiple substitutions observed in the control region. However, similar reduced rates of sequence evolution in the *ctr* region were already reported in other species (Koh et al. 2000, Ingman et al. 2000).

Concatenation of *mt* sequences assigned to 40 different haplotypes with *Hd* = 0.984 ± 0.012 and π = 0.037 ± 0.003. All diversity values are summarized in Table 2.

Population structure and divergence times

ML-, NJ- and Bayesian trees (Figure 2) performed on the concatenated dataset exhibited almost identical topologies with four main haplotype lineages (LI-LIV) in *M. brandti*. Lineage LI forms a mix of individuals from south and southeast of the Konya basin (Konya, Yesilköy, Nigde, Meydan) in Central Anatolia as well as hamsters from Eastern Anatolia (Ardahan, Eleşkirt-Ağrı, Erzurum-Çat Yolu, Muş-Malazgirt, Erzurum-Horasan) in Turkey. A second main lineage LII is formed by animals from Central Anatolia north/northwest of the Konya basin (Çorum, Kırşehir, Kayseri), Eastern Anatolia (Van, Ardahan, Erzurum-Çat Yolu, Erzurum, Erzurum-Horasan) and Armenia. Lineages LIII and LIV contain animals from Iran and Dagestan/Russia, respectively. Lineages LI and LII displayed also further subdivision.

The haplotype network of the Turkish hamsters clearly defined four different clades divided by 36 to 74 mutational steps. In this respect, network as well as gene trees gave consistent results. However, the positions of lineages LIII and LIV were even more distinguished in the network than in trees.

Altogether, all applied clustering methods revealed deep genetic structuring and defined haplotypes to at least four main monophyletic lineages. Their distinctiveness in trees was supported by high bootstrap values (70 - 100%). Haplotype structuring is also proven by AMOVA results (based on subdivision in four lineages, *Fst* = 0.789, p < 0.001). About 79% of all mtDNA variation was found between lineages and only 21% within.

A global clock test revealed that not all concatenated sequences mutated in a clockwise manner (p<0.05). Therefore, we calculated divergence times among lineages according to a strict clock (K2P distance) and a relaxed clock model. All distance and divergence time values were listed in Table 3. K2P distances measured from 0.016 (±0.003) - 0.081 (±0.008) and the corresponding divergence times were as follows: LI ~1.2 - 2.6 my, LII ~0.6 - 1.3 my, LIII and LIV split ~0.6 - 1.2 my. Absolute values of an uncorrelated lognormal clock based splits appeared slightly younger ~0.2 - 2.3 my. Unfortunately, the use of two rather short mitochondrial fragments and only two time calibration points lead to very large confidence intervals and hence not very reliable time estimates with the BEAST program. However, all estimates suggested that divergence in *M. brandti* largely occurred in the middle and older parts of the Pleistocene. Most recent glacial and interglacial events did not significantly influence the main genetic structure of the Turkish hamster.

Except for the four main genetic groups, further diversification was particularly observed in LII, which harbors the largest number of individuals and sites. Noteworthy was the separation of an eastern subgroup formed by individuals from Eastern Anatolia and Armenia from Central Anatolian hamsters. Animals from the Lake Van form also a single subclade. The potential divergence times of the two subclades inside LII were placed in the middle Pleistocene (SC: 0.3 - 0.5 my).

Demographic parameter R2

The analyses of concatenated sequences delivered no significant signs of expansion in any of the *M. brandti* lineages. Only LI edged significance (*R2* = 0.166, p = 0.053). The remaining values were as follows: LII (*R2* = 0.120, p = 0.209), LIII (*R2* = 0.241, p = 0.720) and LIV (*R2* = 0.245, p = 0.313). However, in particular the results of LIII and LIV must be considered with care because the low number of individuals (n = 5) surely inflates the power of the statistics. Further analysis on a much larger sample is required in the future.

DISCUSSION

Genetic diversity and haplotype structure

Although the overall sample size in this study is relatively small, the data set still covers most of the species known range (except Georgia and Azerbaijan) and may therefore provide a representative overview with regards to the genetic and spatial diversification in Turkish hamsters. Mitochondrial haplotype and sequence diversities proved very high in contemporary *M. brandti* and in this respect corroborate previous reports on significant protein polymorphisms seen in hamsters from Turkey and Iran (Yiğit et al. 2007). The high number of observed haplotypes is associated by deep genetic divergence, where *K2P*-distances of combined haplotypes measured up to 9.3% (Eleşkirt-Ağrı/Van). Such distance values are higher than the inter-species divergence found in some other steppe rodents (*Spermophilus, Microtus*) described from the region but match well with mitochondrial divergence data in cytotypes of Anatolian mole rats (Jaarola et al. 2004, Gündüz et al. 2007, Arslan et al. 2010, Kankılıç and Gürpınar 2014). However, high genetic diversity in *M. brandti* is not surprising since the species not only evolved but also persisted in its current distribution range without suffering dramatic population declines, a phenomenon so typical for many central and northern European species. Based on this relatively stable population system, *M. brandti* developed a highly complex and differentiated population structure over the last 2.5 my.

Current mitochondrial haplotype structure

Our data revealed that modern *M. brandti* populations fall into four deeply diverged mitochondrial lineages, which probably arose from different diversification events. Two haplotype lineages LI and LII are characteristic of Turkey and Transcaucasia, the main distribution area of the species, but they form no obvious spatial pattern. Especially Eastern Anatolia represents a true genetic mix where several localities (e.g. Ardahan, Erzurum region) contain both LI and LII haplotypes.

The absence of a clear haplotype separation between Eastern and Central Anatolian hamsters was unexpected. Previous morphological studies predicted high levels of subspeciation in *M. brandti* across Anatolia and discussed in particular differences between eastern and western populations (Yiğit et al. 2000, 2006). Yiğit et al. (2006, personal communication) found that animals from Eastern Turkey exhibit more locally

distributed color morphs and chromosomal variation than Central Anatolian hamsters. However, pelage color variation proved already a poor indicator of genetic differentiation in other Turkish mammals e.g. the Anatolian brown hare (Sert et al. 2005). The expression of different pelage morphs in Eastern Anatolia and Transcaucasia potentially reflects an environment, which differs from the Central Anatolian plains. Altogether, today's LI and LII pattern in *M. brandti* underlines the only temporarily effectiveness of geographical and other migration barriers such as the eastern "Anatolian Diagonal" mountains (Dubey et al. 2006, Ansell et al. 2011).

The deep haplotype divergence distinguishes *M. brandti* from another widely distributed Turkish steppe rodent, the Anatolian ground squirrel (*Spermophilus xanthoprymnus*). Ground squirrels fall in several mitochondrial lineages from west to east but exhibit a much shallower genetic divergence (Gündüz et al. 2007).

However, we found also some evidence for substructuring of Central and Eastern Anatolian populations inside lineage LII. This could be due to limited sampling but may equally imply that ecological and geographic conditions led to moderate population fragmentation. There is also evidence for further structuring in Eastern Anatolia as indicated by the distinctive position of animals from the Lake Van area in gene trees. However, a pronounced genetic fine structure is expected in a rodent with a great population dynamics such as *M. brandti* and should be further explored using other genetic markers.

The situation in Central Anatolia appears slightly different from Eastern Turkey. Haplotypes of lineage LI were found specifically in south and west of the Konya basin whereas lineage LII is present more easterly of the basin. A similar horizontal structure was also observed in other Turkish rodents e.g. the Anatolian ground squirrel (*Spermophilus xanthoprymnus*) which shows two horizontally located and partially overlapping lineages in Central Turkey (Gündüz et al. 2007). The lack of shared haplotypes in our samples could be related to small sample size (n= 4 from the southern part of Central Anatolia). However, another example of horizontal population zonation in this part of Turkey represents the broad-toothed field mouse (*Apodemus mystacinus*) (Michaux et al. 2005). An enzyme study on the grey hamster (*Cricetulus migratorius*) populations in Turkey identified animals from the southern Konya-region as the most diverged group according to an UPGMA-tree (İbiş et al. 2011). Despite limited sampling, the genetic pattern observed in Central Anatolian *M. brandti* and probably on other Turkish steppe animals is very likely associated with the historic

formation, current geography and climate peculiarities of the large Konya basin, a drainage basin covering a total of 55 000 km^2 (Erol 1978, Kuzucuoğlu et al. 1999). Today, the Konya basin represents a mix of highly xeric steppe and marshy areas. Its demanding environmental conditions may still impede extensive gene flow among hamster populations in Central Anatolia.

Animals from Zanjan in Iran and Dagestan/Russia form two other highly distinguished haplotype lineages in Turkish hamsters. Although both groups appear to be well differentiated, they belong to a superior haplotype cluster. It is well possible that they split during a major species expansion event. At this time, one group could have moved northwards, bypassing or crossing the Caucasus Mountains and the other westwards, settling in the western Iranian plains. Today, the Caucasus population from Dagestan represents the most geographically isolated *M. brandti* population so far. It may even be considered to form a distinct subspecies. This could also apply to the Turkish hamsters in northwestern Iran at the eastern distribution edge (Karami et al. 2008). Our data point towards a geographic and genetic distinctiveness of Iranian hamsters. However, a more extensive sampling is required to investigate a potential genetic overlap between Anatolian and Iranian haplotypes in eastern Turkey.

Timing of lineage separation and the evolution of *M. brandti*

Estimating separation times of haplotype groups in *M. brandti* appeared difficult due to some uncertainties concerning the variances in the evolutionary mode, crude mutation rates and the lack of fossil based calibration points. However, since we consider differences on an intraspecific level we still think that a strict clock is acceptable for divergence time calculations. This view is supported by the fact that most absolute relaxed clock estimates are not too far outside the time range of the strict clock based values in our study. All calculations congruently point out that the four main haplotype lineages in *M. brandti* formed around 0.3 - 2.6 my ago during the early and middle Pleistocene. None of the major separation events occurred during the last period of glaciation.

The oldest phylogenetic split inside *M. brandti* dates to ~1.2 - 2.6 my (SC) before present (BP) and coincides with the dissociation of *M. brandti* and *M. newtoni* ~1.7 - 1.8 my ago (Neumann et al. 2006). It seems that the lower Pleistocene marks some of the most fundamental population reorganization events inside the *M. brandti* complex. The timing includes the so-called Early Pleistocene Migration period (1.8 - 2.0 my BP),

a process of extensive species renewal and exchanges in the Eastern Mediterranean region (Koufos et al. 2005). It is possible, that during this diversification of *M. brandti*-type populations one of the most western Anatolian populations eventually moved to the Balkans and after physical isolation, evolved in today's *M. newtoni*. In contrast, frequent gene flow prevented from further speciation inside Anatolia. This scenario does not necessarily support the idea of a sole evolutionary centre in eastern Anatolia as postulated by Yiğit (2006). It could equally be that at least some diversification in *M. brandti* occurred in western Turkey. There is more evidence in favour of such a hypothesis. A survey on protein polymorphisms identified Turkish hamsters from central Anatolia as the most basal group compared to eastern Anatolian and Iranian specimen (Yiğit et al. 2007). Studies on morphological and karyological characteristics revealed no striking variation in central Anatolia compared to eastern Anatolia (Yiğit et al. 2000, Yiğit et al. 2006) but there are reports of a single rare karyotype variant (2n = 44) near Ankara (Lyman and O'Brian 1977, Popescu and Di Paolo 1980).

Although, the fossil record is far from conclusive; there is additional support for a hot spot of Mesocricetus/*M. brandti* evolution in a Western/Central Anatolian centre. At first, *M. primitivus*, the ancestor of modern *Mesocricetus,* existed during the Pliocene in Greece (De Bruijn et al. 1970) and is known as *M. cf. primitivus* from the Pliocene and Early Pleistocene layers at several fossil sites in Anatolia including western Turkey (Sen et al. 1998, Ünay and de Bruijn H. 1998, Suata-Alpaslan 2009, van den Hoek Ostende et al. 2015). It is well plausible, that *M. brandt*/*newtoni* descended from a western and/or central Anatolian population whereas a more eastern *M. primitivus* population was the ancestor of the *M. auratus/raddei* lineage. Unfortunately, reports on Pleistocene *M. brandti* fossils from its current range are scanty and faithful discrimination from *M. auratus* on the bases of bone fragments and teeth is often impossible (Yiğit et al. 2003, 2006). Despite these confinements, hamsters of the *auratus*/*brandti*-type occurred throughout the Quaternary in Anatolia (Storch et al. 1988, Sen et al. 1991, Güleç et al. 1999, Suata-Alpaslan 2011a, 2011b). An early record stems from the lower Pleistocene at Dursunlu in the Konya region/Central Anatolia (Güleç et al. 1999). There is no fossil record of *M. brandti* in eastern Anatolia dating from a comparable period. Another argument in favour of a western/central Anatolian evolutionary centre comes *from M. newtoni*. Fossils catalogued as *M. newtoni* and *M. cf. newtoni* were excavated in the Balkan area and Turkish Thrace (Santel and Königswald 1998, Munteanu et al. 2008). Oldest fossils date to the middle

Pleistocene implying that genetic differentiation between *M. brandti* and *M. newtoni* very likely occurred in western Turkey. The process of divergence itself could be associated with a fundamentally changing environment following the desiccation of the extensive Pliocene lake system in central Anatolia. Alternating periods of dryness and spreading lakes with marshy steppe caused dramatic oscillations in living conditions in inner and western Anatolia during the Pleistocene including the Konya basin (Erol 1978, Kuzucuoğlu et al. 1999, Özsayin et al. 2013). Spreading steppe corridors could also have allowed frequent emigration to eastern Anatolia. Differentiation between Central and typical Eastern Anatolian haplotypes as well substructuring inside Eastern Anatolia happened *ca*. 0.3 - 0.5 my (RC: ~0.2 my) ago. This middle Pleistocene period included several switches between warmer and colder periods, which may have had some significant effects on hamsters in Eastern Anatolia, and Transcaucasia (Cukur et al. 2014). The fossil record from the Caucasus is too limited to allow a reliable reconstruction of its colonization history by *M. brandti*. Specimens of *M. brandti*/*auratus* type were probably present in Transcaucasia since the Palaeolithic (Vereshchagin 1959, Pinhasi et al. 2011). *Mesocricetus* fossils were already found in the Galerian layers (>0.5 my BP) in the Transcaucasus but they mainly assemble *M. raddei* (Baryshnikov 2002). According to that, hamsters from the Transcaucasus may have experienced repeated species replacement and most likely retreated frequently from the mountains during very cold climate spells. They recolonized them from eastern Anatolian refugia. Fossils representing *M. brandti* in Armenia are not older than 50 000 years BP (Pinhasi et al. 2011, Kandel et al. 2011).

We estimated the split of the most eastern populations from Iran and Dagestan/Russia from Anatolian populations to approximately ~0.6 – 1.3 my ago (RC: ~0.6 my). Hamsters from Iran and Dagestan probably split soon afterwards ~ 0.5 – 1.2 my ago, (RC: ~0.3 my) perhaps in the course of further range extensions. The published fossil record does not allow any validation of the dating. The few *Mesocricetus* fossils from western Iran date to younger epochs of the Pleistocene e.g. the younger Mousterian period (Turnbull 1974, 1975) and the late Pleistocene (Hashemi et al. 2006). However, it is very likely that the Turkish hamster reached that area much earlier. *M. brandti* from Dagestan emerged from a northern advance probably along the shores of the Caspian Sea passing the Caucasus during a major drop of the sea level. Several significant sea level fluctuations of the Caspian Sea (e.g. the Bakunian Transgression ~ 0.85 – 0.88 my BP) occurred during the Pleistocene caused by plate tectonics and climate change

(Avdeev and Niemi 2011, Badertscher et al. 2011, Van Baak et al. 2013). During these periods land passages with xeric steppe conditions opened and allowed an exchange of terrestrial animals between the Iranian plateau and eastern Ciscaucasia (Vereshchagin 1959, Koufus et al. 2005). A water level rise may have finally sealed the isolation of the Dagestan population. Alternatively, migration across the high mountains during suitable climatic conditions appears also possible and may have happened alongside dry riverbeds. Although fossils provide proof of the presence of *Mesocricetus* in the Caucasus during the Pleistocene, findings around the Caspian Sea e.g. the Apsheron peninsula belong to younger Pleistocene layers (Vereshagin 1959).

Conclusions

The Turkish hamster displays considerable mitochondrial haplotype divergence in its contemporary geographic range. All main lineages probably evolved before the late Pleistocene and originated from major events of the species' evolutionary process. In Turkey and Transcaucasia, we found two main maternal lineages without a significant spatial structure. A finding, which contradicts morphological studies, predicted a high degree of differentiation between central and eastern Anatolian hamsters. The finding proves that mountain barriers in Turkey were no insurmountable obstacles for this particular steppe rodent and gene flow was frequent over time and across Anatolia. However, there is evidence that the complex basin structure in central Anatolia played an important role for population fragmentation and population diversification. Hamsters from Iran and Dagestan form another two main genetic lineages, which originated from east and northwards population expansions. Altogether, we postulate that complex climatic and tectonic events formed the phylogenetic shape of the Turkish hamster. There is also evidence that western/central Anatolia provided a major evolutionary hotspot for *M. brandti*. The analysis of more samples and the use of additional markers like nuclear microsatellites in the future could provide further clarification of the phylogenetic history of this interesting rodent.

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Tables

Table 1 Geographic origins and numbers of the *M. brandti* specimens sampled for mtDNA analyses.

Country	Geographic location	No of animals tested
Turkey	Konya (Central Turkey)	1
	Nigde (Central Turkey)	1
	Yesilköy (Central Turkey)	1
	Meydan (Central Turkey)	1
	Kırşehir Central Turkey)	4
	Kayseri (Central Turkey)	4
	Çorum (Central Turkey)	1
	Erzurum (Eastern Turkey)	1
	Erzurum-Çat Yolu (Eastern Turkey)	2
	Erzurum-Horasan (Eastern Turkey)	3
	Ardahan (Eastern Turkey)	10
	Van (Eastern Turkey)	4
	Muş-Malazgirt (Eastern Turkey)	1
	Eleşkirt-Ağrı (Eastern Turkey)	1
Iran	Zanjan	5
Armenia	Gyumri (Transcaucasus)	2
Russia	Levashi (Dagestan-Ciscaucasus)	5
	All	47

Table 2 Diversity measures of mitochondrial sequences (haplotype number N_H, nucleotide diversity π, haplotype diversity *Hd*) obtained from the entire *M. brandti* sample.

Mitochondrial sequences	N_H (*n*)	π ± SD	*Hd* ± SD
ctr	31 (47)	0.016 ± 0.001	0.963 ± 0.017
cyt b	37 (47)	0.046 ± 0.005	0.979 ± 0.012
ctr + cytb	40 (47)	0.037 ± 0.003	0.984 ± 0.012

Table 3 Genetic distance measures and divergence time estimates of mitochondrial haplotype lineages in *M. brandti* based on strict (SC) and relaxed clock (RC) calculations

Separation events between and	**Mean *K2P* distance/ Time of divergence in my (SC)**	**Bayes Test/ Time of divergence in my (RC)**
LI - LII, LIII, LIV	0.081 (0.008) ~1.4 - 2.6[1] ~1.2 - 2.1[2]	~2.3[1,2]
LII - LIII, LIV	0.040 (0.005) ~0.7 - 1.3[1] ~0.6 - 1.1[2]	~0.6[1,2]
LIII -LIV	0.034 (0.005) ~0.6 - 1.2[1] ~0.5 - 0.9[2]	~0.3[1,2]
LII (Central Anatolia - Eastern Anatolia)	0.017 (0.002) ~0.3 - 0.5[1] ~0.3 - 0.4[2]	~0.2[1,2]
LII (Ardahan, Erzurum, Armenia - Van)	0.016 (0.003) ~0.3 - 0.5[1] ~0.3 - 0.4[2]	~0.18[1,2]

SC and RC divergence time estimates are based on the splits of *M. auratus*+*M. raddei*/*M. brandti*+*M. newtoni* [1)] and *M. brandti*/*M. newtoni* [2)] (Neumann et al. 2006). Variances for K2P distances were calculated by bootstrapping (1000 replicates). Confidence values for RC estimates were too high and therefore not presented.

Figure captures

Figure 1 Distribution area of *Mesocricetus brandti* (grey with solid line) with sampling sites. White circles correspond to main haplotype lineage LI and black circles to LII. Circles in white and black indicate locations harbouring both lineages (LI and LII). The light grey triangle marks LIII and the dark grey square symbolizes LIV. Areas in light grey framed by a dotted line show distribution areas of the other three *Mesocricetus* species.

Figure 2 Topology of a ML-tree based on *M. brandti* haplotypes (*ctr*+*cytb*). Numbers on branches are bootstrap values (NJ-K2P/ML-HKY+Γ+I/Bayesian-HKY+Γ+I) based on 1000 replicates. LI-LIV mark main haplotype lineages.

Figure 3 Median-joining network reconstructed from 47 *cytb* haplotypes obtained from different Turkish hamster populations. Squares (solid lines) refer two main haplotype lineages and circles (dotted lines) indicate potential subdivision. Numbers on links signify mutational steps (more than one) dividing haplotypes. Sizes of circles correlate with the frequency of haplotypes. LI-LIV mark main haplotype lineages. Abbreviations are as follows: CT= Central Turkey, ET= Eastern Turkey.

Figure 1

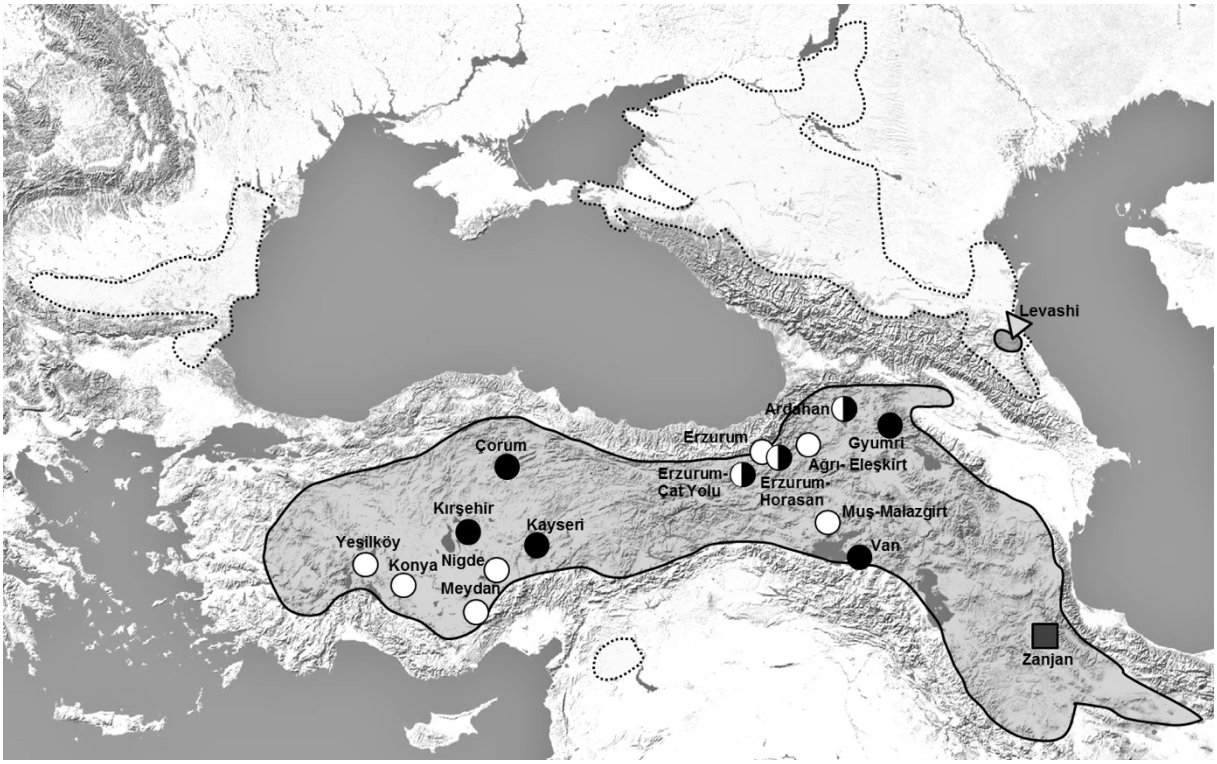

Levashi
Ardahan
Erzurum
Gyumri
Çorum
Ağrı- Eleşkirt
Erzurum-
Çat Yolu
Erzurum-
Horasan
Muş-Malazgirt
Kırşehir
Kayseri
Van
Yeşilköy
Konya
Nigde
Meydan
Zanjan

Figure 2

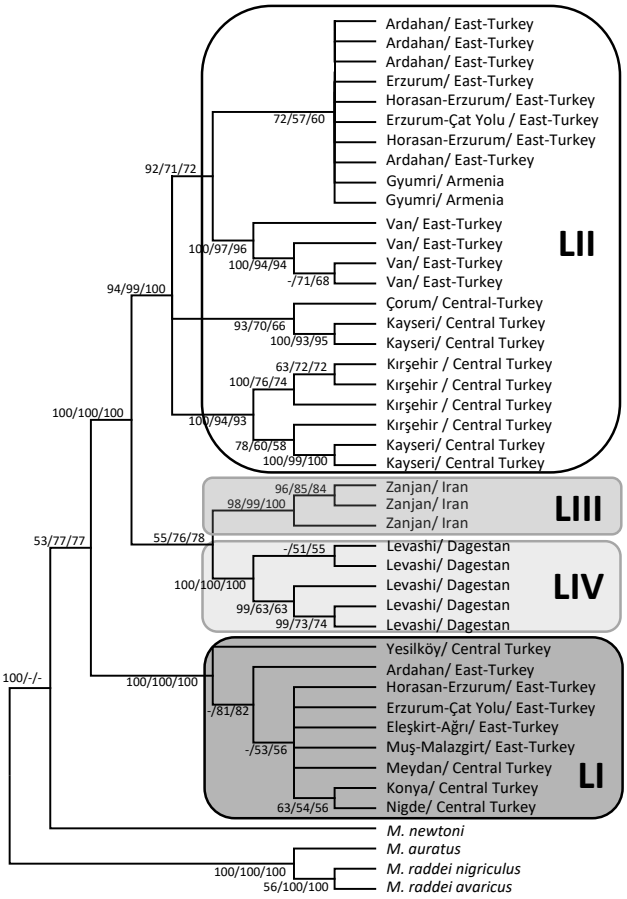
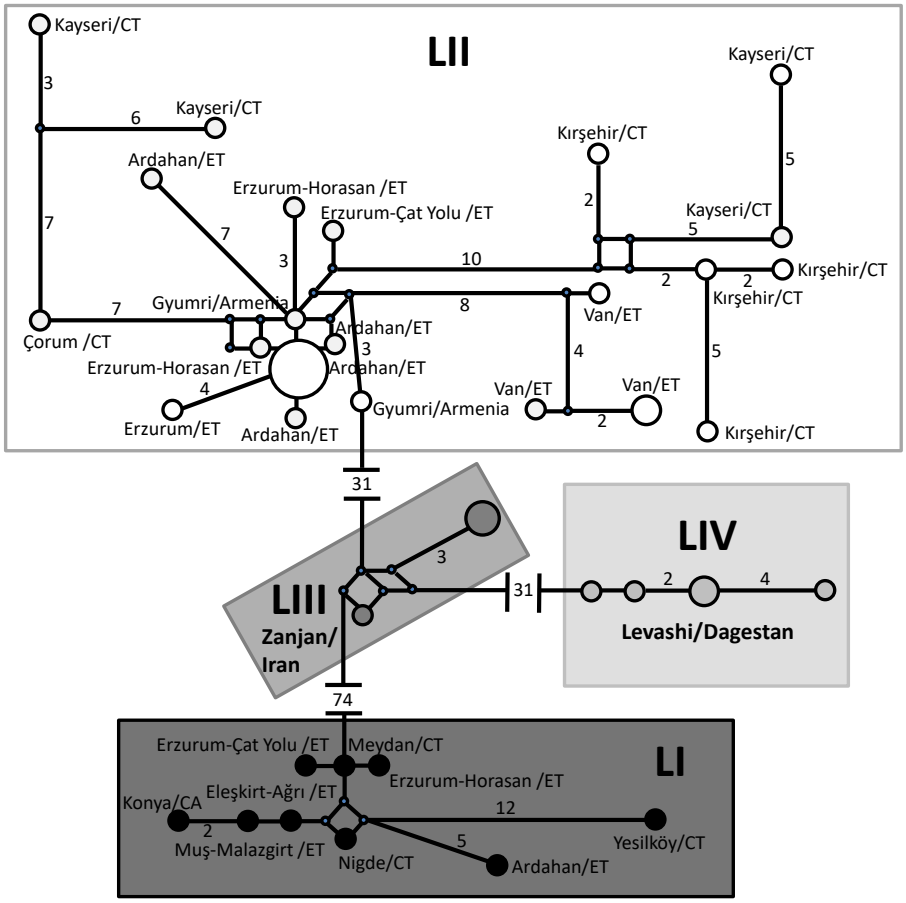

Ardahan/ East-Turkey
Ardahan/ East-Turkey
Ardahan/ East-Turkey
Erzurum/ East-Turkey
Horasan-Erzurum/ East-Turkey
Erzurum-Çat Yolu / East-Turkey
Horasan-Erzurum/ East-Turkey
Ardahan/ East-Turkey
Gyumri/ Armenia
Gyumri/ Armenia
Van/ East-Turkey
Van/ East-Turkey
Van/ East-Turkey
Van/ East-Turkey
Çorum/ Central-Turkey
Kayseri/ Central Turkey
Kayseri/ Central Turkey
Kırşehir / Central Turkey
Kırşehir / Central Turkey
Kırşehir / Central Turkey
Kırşehir / Central Turkey
Kayseri/ Central Turkey
Kayseri/ Central Turkey
LII
Zanjan/ Iran
Zanjan/ Iran
Zanjan/ Iran
LIII
Levashi/ Dagestan
Levashi/ Dagestan
Levashi/ Dagestan
Levashi/ Dagestan
Levashi/ Dagestan
LIV
Yesilköy/ Central Turkey
Ardahan/ East-Turkey
Horasan-Erzurum/ East-Turkey
Erzurum-Çat Yolu/ East-Turkey
Eleşkirt-Ağrı/ East-Turkey
Muş-Malazgirt/ East-Turkey
Meydan/ Central Turkey
Konya/ Central Turkey
Nigde/ Central Turkey
LI
M. newtoni
M. auratus
M. raddei nigriculus
M. raddei avaricus
72/57/60
92/71/72
100/97/96
100/94/94
-/71/68
94/99/100
93/70/66
100/93/95
63/72/72
100/76/74
100/94/93
78/60/58
100/99/100
100/100/100
96/85/84
98/99/100
53/77/77
55/76/78
-/51/55
100/100/100
99/63/63
99/73/74
100/-/-
100/100/100
-/81/82
-/53/56
63/54/56
100/100/100
56/100/100

Figure 3


LII
Kayseri/CT
Kayseri/CT
Kayseri/CT
Kırşehir/CT
Ardahan/ET
Erzurum-Horasan /ET
Erzurum-Çat Yolu /ET
Kayseri/CT
Kırşehir/CT
Kırşehir/CT
Gyumri/Armenia
Van/ET
Çorum /CT
Ardahan/ET
Erzurum-Horasan /ET
Ardahan/ET
Van/ET
Van/ET
Erzurum/ET
Ardahan/ET
Gyumri/Armenia
Kırşehir/CT
31
LIII
Zanjan/
Iran
LIV
Levashi/Dagestan
74
LI
Erzurum-Çat Yolu /ET
Meydan/CT
Erzurum-Horasan /ET
Konya/CA
Eleşkirt-Ağrı /ET
Muş-Malazgirt /ET
Nigde/CT
Yesilköy/CT
Ardahan/ET