

A genetic characterization of CITES-listed Iranian tortoises (*Testudo graeca*) through the sequencing of topotypic samples and a 19th century holotype

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The *Testudo graeca* complex is a CITES-listed group of taxa (species or subspecies) that is under threat from over-harvesting for the Western pet and Asian food trade. Taxonomically, *T. graeca* populations are in a state of flux. Middle Eastern *T. graeca* in particular are very poorly known because of a lack of adequate sampling, especially in Iran. Two recent studies generated mitochondrial sequence data from Iran using non-overlapping mitochondrial (mt) markers from samples from localities with largely non-overlapping geographical distributions. By generating new sequence data from key Iranian specimens, this study allows for a more comprehensive appraisal of mt phylogeography. Using historic DNA methods, we provide mtDNA sequence data from a 19th century holotype (*T. zarudnyi* Nikolsky, 1896) along with new data from two recently collected topotypes of other putatively valid taxa (*T. buxtoni* Boulenger, 1921; *Testudo perses* Perälä, 2002). The new data are used to assess the taxonomic assignments of previously published mtDNA sequences, reinforce the signal of considerable admixture among mt lineages in northwest Iran, establish a reference database for forensic studies of trade specimens and identify areas for future sampling.

Key words: Historic DNA, Iran, Testudinidae, tortoise, trade, *zarudnyi*

INTRODUCTION

In addition to providing evidence for taxonomic studies, understanding the distribution of mitochondrial (mt) DNA haplotypes can help reveal the taxonomic identity and provenance of trade specimens (Fong et al., 2007; Spinks & Shaffer, 2007; Baker, 2008). One highly traded species is the spur-thigh tortoise, *Testudo graeca* Linnaeus, 1758. The genus *Testudo* represents almost 80% of all recorded international tortoise trade (Theile, 2002) and the *T. graeca* complex (including Middle Eastern populations) comprises 37% of traded *Testudo* (Türkozan et al., 2008). All Middle Eastern populations of *T. graeca* are presently listed as CITES II, requiring permits for the cross-border transport of any material of this species. However, a large undocumented (illegal) international pet trade persists (Türkozan & Kiremit, 2007; Türkozan et al., 2008).

Given the highly fluctuating taxonomy and extreme morphological variation among tortoise populations in Western Asia (reviewed in Parham et al., 2006), the potential for laundering tortoise material under false species names is high, especially for countries that have poorly characterized tortoise faunas such as the Islamic

Republic of Iran. Some Iranian populations have either been described or elevated as full species, adding additional incentive for hobbyists to seek and illegally collect specimens as exotic rarities (Stuart et al., 2006). Whereas most scientists eschew these highly split taxonomic schemes (van der Kuyl et al., 2002, 2005; Parham et al., 2006; Türkozan et al., 2010), the prevalence of these taxa in the hobbyist literature (e.g., Vetter, 2002; Guyot, 2004) indicates a prospective threat. Furthermore, the demand for the Chinese food and medicine trade has already initiated the illegal harvest of tortoise populations in Pakistani Balochistan (Guriro, 2009). It is therefore likely that adjacent Iranian Balochistan tortoise populations are also being harvested. The ability to track the international trade of *T. graeca* depends largely on seizure of shipments and market surveys, but also the genetic characterization of multiple populations from within the range.

The purpose of this study is to provide new data from topotypic specimens (including one late 19th century holotype) in order to better characterize the distribution of mt haplotypes of a poorly studied and trade-threatened tortoise fauna. As more data on the *T. graeca* complex are collected, it should become simpler to assign trade samples to known localities, thereby allowing for more

detailed tracking of the trade (e.g., Fritz et al., 2009). In some parts of the world, especially eastern Asia, turtle populations are so depleted that the lack of known-locality specimens in phylogeographic studies greatly hinders scientists' ability to track the massive trade (van Dijk et al., 2000) and even identify species boundaries within heavily harvested countries (e.g., Parham et al., 2001; Fong et al., 2002; Stuart & Parham, 2004; Fong et al., 2007; Spinks et al., 2009). In the case of Iranian tortoises, the relative inaccessibility of populations to many herpetologists has led to a similar lack of reliable data.

In order to develop a reference database for identifying Iranian tortoises, we summarize and integrate all existing geographically referenced mtDNA data from this region. We also present new genetic data for topotypic specimens of two Iranian tortoise taxa that have recently been considered valid (*T. buxtoni* Boulenger, 1921 and *T. perses* Perälä, 2002) as well as the methods used to obtain genetic data from the holotype of a third named taxon *T. zarudnyi* Nikolsky, 1896 (Fig. 1). The taxon *T. zarudnyi* has also recently been considered valid either as a species (Perälä, 2002), or more recently as a subspecies (Fritz et al., 2007). The new data presented here facilitate

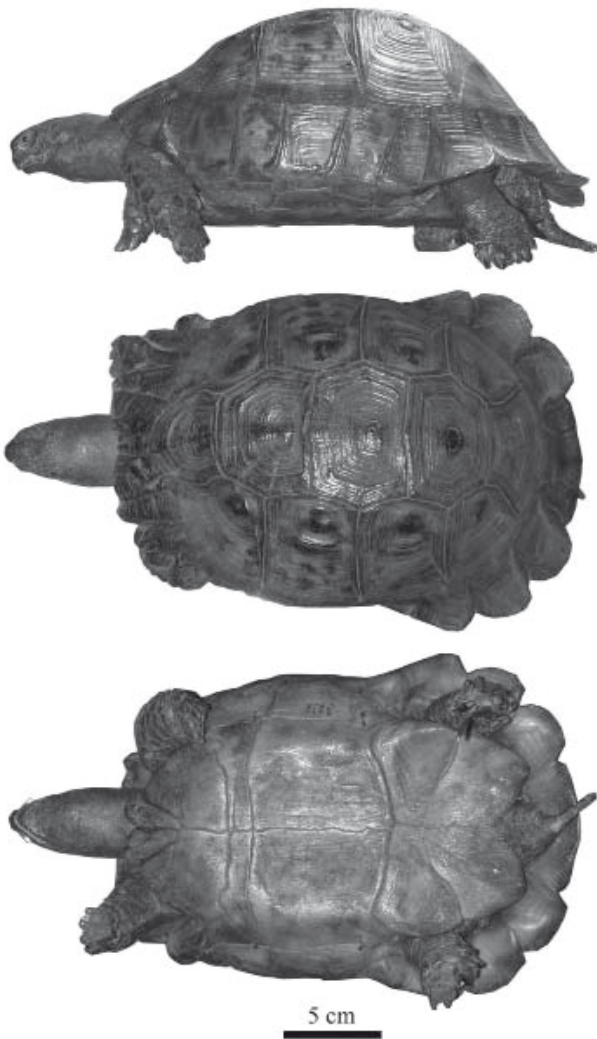


Fig. 1. ZIN 8738, the holotype of *T. zarudnyi*, a dried and stuffed specimen, in lateral, dorsal, and ventral views.

the integration of two recently published studies (Parham et al., 2006; Fritz et al., 2007) that report geographically referenced mtDNA sequences, thereby clarifying the distribution of haplotypes and haploclades (Fig. 2). In doing so, we provide a more comprehensive database for genetic identification of samples. Genetic data from topotypic and holotype specimens are important because they also assist the more detailed studies necessary to better define alpha taxonomic units among Iranian tortoise populations.

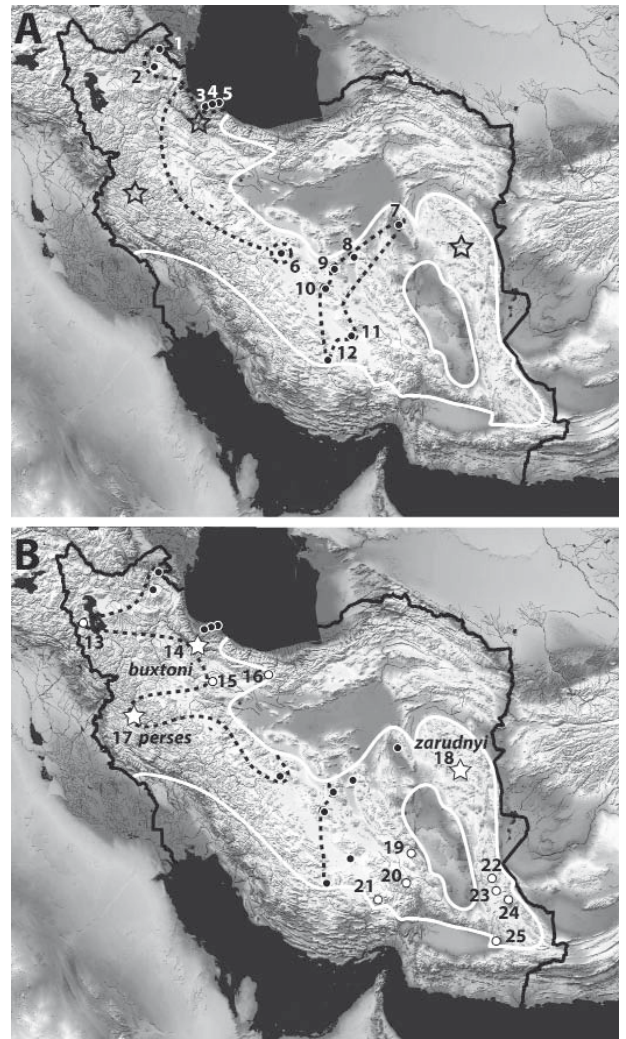


Fig. 2. Map of Iran showing distribution of localities and mtDNA haploclades. Numbers refer to Appendix 1. Solid black line is Iranian border. Solid white line is approximate range of *T. graeca* in Iran. Dashed black line is the estimated range of haploclades. Black circles are localities sampled by Fritz et al. (2007) and sequenced for *cob* by that study. White circles are localities sampled by Parham et al. (2006) and sequenced for *nad4* by that study. White stars (topotypes and holotype) are localities sequenced for *nad4* by Parham et al. (2006) and sequenced for *cob* in this study. A: Map showing the distribution of haploclades based on localities sampled by Fritz et al. (2007). B: Map showing distribution of haploclades based on a combined analysis of Parham et al. (2006) and Fritz et al. (2007).

Background

The tortoise fauna of Iran is poorly studied systematically and so its taxonomy remains in a state of flux (Rhodin et al., 2008). Different authors recognize anywhere between three species (Perälä, 2002 [*T. buxtoni*, *T. perses*, *T. zarudnyi*]), one species with two subspecies (Fritz et al., 2007 [*T. g. buxtoni*, *T. g. zarudnyi*]) or one provisional taxon pending further data (Parham et al., 2006 [*T. graeca*]). Fritz et al. (2007) recommended assigning subspecies names to mtDNA clades, but we follow Parham et al. (2006) and provisionally refer all populations to *T. graeca* pending studies that demonstrate a concordance of multiple lines of evidence (e.g., morphology, mtDNA and nuclear genome sequences). To date, the genetic characterization of this fauna is based largely on mtDNA (Parham et al., 2006; Fritz et al., 2007). These two studies appeared nearly simultaneously and presented geographically referenced, mtDNA data sets for 12 localities each. Each study used non-overlapping genetic markers and had complementary geographic coverage (Fig. 2). Whereas the overall results and conclusions of the two studies were largely in agreement, the potential to better characterize the geographic distribution of haplotype diversity through the combination of these data sets remained an open issue until now. In particular, the study of Fritz et al. (2007) lacked topotypic specimens, adding an additional assumption about the taxonomic assignment of haplotypes. For example, their samples assigned to the name *zarudnyi* are from outside the previously known range of tortoises in Iran (Anderson, 1979; Guyot, 2004). As such, these new localities were never assigned to a taxon by any previous author. By providing new sequence from topotypes and a holotype, we can combine the phylogeographic patterns from previously published data sets.

MATERIALS AND METHODS

Note on the holotype status of ZIN 8738

In 1896, Nikolsky described *T. zarudnyi* (in Latin) without designating a type or providing any illustrations. In 1897, Nikolsky published an expanded diagnosis, gave measurements and indicated a single specimen from the Zoological Institute (ZIN), Russian Academy of Sciences, St. Petersburg, Russia (ZIN 8738) collected by N.A. Zarudny from the Birjand, Iran (see Nikolsky, 1897:309:

“A single specimen of this species was collected by N.A. Zarudny in mountains of the Persian Province Birdzhan”). The lack of a type designation in the original publication has led to some confusion. Perälä (2002) argued that any material used for the 1896 paper should be regarded as syntypes. As such, he considered ZIN 8738 and BMNH 1947.3.5.17 (formerly 99.7.25.1) as syntypes of *T. zarudnyi*. This assertion was supported by the fact that BMNH 1947.3.5.17 was received from the St. Petersburg Museum with a label indicating that it is the “Type of *Testudo zarudnyi*.” However, according to Nikolsky (1899), Zarudny did not collect specimens of *T. zarudnyi* from Zirkuch until 1898 (there are two specimens at ZIN). So it is clear that in 1896, Nikolsky could not have a type series of *zarudnyi*, but only one specimen (ZIN 8738) and so it should be considered the holotype (ICZN Art. 73.1.2). This is confirmed by direct words of Nikolsky (1897) and by data on Zarudny expeditions to Persia. Therefore, Perälä’s (2002) designation of a lectotype is not necessary. Finally, Danilov and Milto (2004) insisted that ZIN 8738 was a holotype, but the editors replaced “holotype” with “lectotype.”

Historic DNA methods

A piece of dried skin was excised from the holotype specimen of *T. zarudnyi* (ZIN 8738). DNA extraction and precautions against contamination followed the protocol of Kearney & Stuart (2004). The same 880 bp fragment used by Parham et al. (2006) for other Middle Eastern *T. graeca* (see below) was obtained by amplifying and sequencing four fragments of 158–476 bp using primers (Table 1) designed from a *T. zarudnyi* sequence obtained in a separate laboratory (Parham, unpublished data). To avoid generating chimeric sequences (Olson & Hassanin, 2003), the primers were designed so that the four DNA fragments overlapped by at least 49 bp after primer sequences were trimmed off (Table 2). Fragments were amplified by PCR (polymerase chain reaction; 94°C 45s, 48°C 30s, 72°C 50s) for 40 cycles using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences). The 25 µl PCR reactions contained 1.5 µl of each primer, 3.0 µl of DNA extraction and 19.0 µl of sterile water. PCR products were visualized, sequenced using the amplifying primers, edited and translated as described by Diesmos et al. (2005).

Table 1. Oligonucleotide primer sequences used to amplify and sequence mitochondrial DNA from the holotype specimen of *T. zarudnyi*. ‘L’ and ‘H’ refer to light and heavy strands, respectively. L-ND4 taken from Stuart & Parham (2004).

Primer	Sequence
L-ND4-TG	5'-GTAGAGGCCCAATTGCAG-3'
H-340ND4zar	5'-TGAGTGAGGAGTAATATTCG-3'
L-ND4	5'-GTAGAAGCCCAATCGCAG-3'
H-520ND4zar	5'-TATAAGTGGCGGTAATTAGTG-3'
L-465ND4zar	5'-ATAGGAGAGTTAACAATCATT-3'
H-630ND4zar	5'-GAATGTGTAGTGTATGAGG-3'
L-575ND4zar	5'-ATTATAACACAATGAGGAGA-3'
H-Leu-TG	5'-TGTACTTTTACTTGGGAATTGCACCA-3'

Good quality mitochondrial DNA sequences were obtained using all primer pairs listed in Table 2. Identical sequences were obtained in all overlapping fragments. To our knowledge, no testudinid turtle DNA had ever been extracted or sequenced in the laboratory where the holotype fragment was obtained. Consequently, we are confident that the sequence of the holotype of *T. zarudnyi* used in analyses here is authentic and does not represent a contaminant or chimeric sequence.

Table 2. Oligonucleotide primer pairs used to amplify and sequence mitochondrial DNA from the holotype specimen of *T. zarudnyi*. Amplicon size refers to the number of basepairs (bp) after trimming off the primer sequences.

Primer Pair	Amplicon Size (bp)
L-ND4-TG / H-340ND4zar	338
L-ND4 / H-520ND4zar	476
L-465ND4zar / H-630ND4zar	158

Other materials

In addition to the sequence from the holotype of *T. zarudnyi*, our study contributes new DNA sequences from four other key specimens of tortoises with whole body vouchers in museum collections. Two specimens were collected by the first author in northwestern Iran and are now housed in the Museum of Vertebrate Zoology (MVZ), Berkeley, California, USA (MVZ 245921, topotype of *T. buxtoni*; MVZ 245923, topotype of *T. perses*). These two samples were originally sequenced for NADH dehydrogenase 4 and adjacent tRNAs (*trn-H-S-L*) by Parham et al. (2006). For simplicity, we will refer to this section of the mt genome as “*nad4*” hereafter. In order to match haplotypes with the study of Fritz et al. (2007), we provide new sequences of cytochrome b and the adjacent tRNA (*trn-T*) (simply “*cob*” hereafter) for these specimens using the methods and primers of Diesmos et al. (2005). An additional specimen from eastern Iran, MVZ 234284, with a *nad4* haplotype matching that of the *zarudnyi* holotype (see below) was also sequenced for *cob*. We also sequenced *cob* from a topotypic sample of *Testudo graeca armeniaca* Chkhikvadze and Bakradze, 1991 (ZIN 23026). This specimen was sequenced for *nad4* in Parham et al. (2006), but was sequenced for *cob* here to be used as an outgroup.

Phylogenetic methods

A phylogenetic analysis was performed for the *cob* data set (the *nad4* data set for Iran included just three haplotypes, each corresponding to a type locality). All methods (e.g., Maximum Likelihood [ML], parsimony, neighbor joining) reveal the same topology so only the ML analysis is shown (Fig. 3). The best model of evolution (k81uf, “Kimura 1981 model” with unequal base frequencies) was determined using the Akaike criterion with Modeltest 3.7 (Posada & Crandall, 1998). Using this model and parameters given by Modeltest, the phylogeny was reconstructed using PAUP* 4.0b10 (Swofford, 2002). We include all sequences from the 12

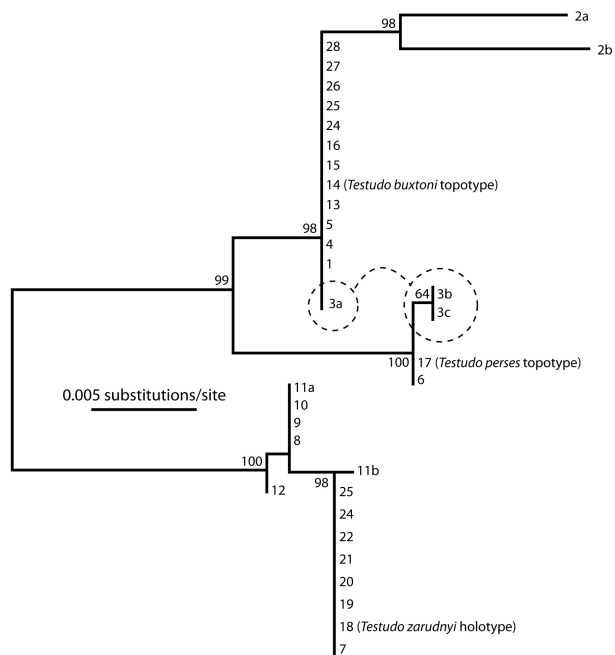


Fig. 3. ML phylogenetic analysis of geographically referenced Iranian *T. graeca* haplotypes. Numbers on terminal branches refer to localities listed in Fig. 2 and Appendix. Numbers above nodes refer to ML bootstraps (1000 replicates). Dashed lines illustrate the presence of haplotypes matching *buxtoni* and *perses* haplotypes at a single locality (3).

Iranian localities sampled by Fritz et al. (2007) along with three new Iranian sequences (the two topotypes and MVZ 234284). MVZ 234284 is from eastern Iran and has a *nad4* haplotype matching the *T. zarudnyi* holotype. Other samples sequenced for Parham et al. (2006) were confidently placed on the *cob* tree based on presence of identical *nad4* haplotypes to those from the topotypes and MVZ 234284.

RESULTS

Our analysis reveals two major haploclades within Iran, corresponding to the northwestern and southeastern regions of the country. These haploclades differ by ~4.1% uncorrected pairwise sequence divergence. MVZ 235824 (locality 24; Fig. 2B) shares an identical *cob* haplotype with MTD T 2273 (locality 7; Fig. 2A) and the *zarudnyi* holotype (locality 18; Fig. 2B), thereby showing that clade F of Fritz et al. (2007) is indeed equivalent to clade 3 (“*zarudnyi*”) of Parham et al. (2006). The overall pairwise sequence divergence within this clade is very low (~0.4%). The *cob* haplotype recovered for the *buxtoni* topotype (Fig. 3) show that clade E of Fritz et al. (2007) is equivalent to clade 4 (“*buxtoni*”) of Parham et al. (2006). We refer to these haploclades as the *zarudnyi* and *buxtoni* haploclades, but do not endorse the formal recognition of these haploclades as mito-subspecies (e.g., Fritz et al. 2007). Within the *buxtoni* haploclade there are two subclades, separated by 2.9% uncorrected pairwise sequence divergence. The distribution of these haplotype subclades does not correspond well to geography or

recently recognized taxa. Most notably, locality 3 (“S Resht”) very near the type locality of *buxtoni* has tortoises with haplotypes that match both the *buxtoni* and *perses* topotypes. We cannot explain the long branches subtending samples from locality 2 (“6 km NE Meshgin Shahr”, Fig. 3) based on sequences provided by Fritz et al. (2007). These long branches are also present in the previous study (Fritz et al., 2007; Fig. 2).

DISCUSSION

By combining two geographically referenced mtDNA data sets, the distribution of *T. graeca* complex haplotypes in Iran becomes clearer. In northwestern Iran, the presence of haplotypes close to both topotypic *buxtoni* and *perses* at a single locality (3), well within the proposed range of “*T. buxtoni*” or “*T. g. buxtoni*,” indicates significant admixture of these mt subclades. This pattern further argues against the recognition of *perses* as a taxon separate from the much older-named and morphologically similar *buxtoni*. In contrast to the apparent geographic discordance within the *buxtoni* subclades, the provenance of *zarudnyi* haplotypes are easier to understand. The relatively westernmost localities 8–12 are distinct from those in Balochistan (localities 22–25) and the type region (localities 7, 18).

At present, both *zarudnyi* and *buxtoni* haploclades are only reported from Iran. However, the possibility that the *buxtoni* clade exists in adjacent countries such as Turkey or Azerbaijan cannot be discounted. For example, locality 13 (Fig. 2B) is very near the Turkish border and Türkozan et al. (2004) records the presence of “*Testudo perses*” (AKA *T. g. buxtoni*) from easternmost Turkey based on morphological characters. In general, the distributional limit of the *buxtoni* haploclade is still emerging, but appears to be centered on the northern Zagros Mountains.

Aside from providing a forensic tool and highlighting the need for range-wide geographic sampling, this study should guide future taxonomic studies in the following ways. First, by clarifying the distribution of haploclades (subspecies sensu Fritz et al., 2007) we draw attention to areas that require additional genetic sampling. After all, the patterns of genetic and morphological variation among populations along the border of parapatric taxa should drive alpha taxonomy (Türkozan et al., 2010). Therefore, closing the distributional gaps among haploclades in Iran and other countries should be a priority. Second, future studies that combine multiple molecular markers and morphology may ultimately define one or more robustly diagnosable species within Iranian *T. graeca*. If so, DNA data from holotype and topotypic specimens should play a crucial role in assigning the appropriate names.

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APPENDIX

Numbers refer to localities shown in map Fig. 2 and tree Fig. 3. Localities with multiple specimens listed once after #a. GenBank numbers for new sequences are provided in brackets. Specimens from ZIN and MVZ include whole body vouchers. Despite inquiry, the status of MTD specimens (localities 1–12) remains unclear and so some or all may be blood samples from pet trade specimens. Outgroup) ZIN 23026: Meghri, Armenia [*cob*, GQ855759]; 1) MTD T 2286: Between Germi and Razay-Amir Abad, Amir Kandi, Ardabil Province; 2a) MTD T 1427: 6 km northeast of Meshgin Shahr, Ardabil Province; 2b) MTD T 1428; 3a) MTD T 2265: South of Resht, between Saravan and Rostamabad, Gilan Province; 3b) MTD T 2267; 3c) MTD T 2268; 4) MTD T 1999: Sefid Rud, Gilan Province; 5) MTD T 2260: Nowshar near Manjil, Gilan Province; 6) MTD T 2269: East of Esfahan, Khūhpāyeh, Esfahan Province; 7) MTD T 2273: Tabas, southern border of Kavir Desert, South Khorasan Province; 8) MTD T 2280: Sāghand, southern border of Kavir Desert, South Khorasan Province; 9) MTD T 2283: Anjir Avand, Yazd Province; 10) MTD T 2272: Nir, Yazd Province; 11a) MTD T 2284: Shahr-e Babak, Maymand, Kerman Province; 11b) MTD T 2285; 12) Neyriz, Fars Province; 13) MVZ 236881: 15 km south (by road to Dizaj) at junction with the Orūmiyeh to Turkey border highway, East Azerbaijan Province; 14) MVZ 245921, topotype of *Testudo buxtoni*: Harzevil, Manjil, Gilan Province [*cob*, GQ855760]; 15) MVZ 236882: 3 km south of Buin, which is 55 km south of Qazvin, Qazvin Province; 16) MVZ 234290: Lar Dam, Tehran Province; 17) MVZ 245923, topotype of *Testudo perses*: 5 km, by air, west of Lalabad Village which is 40 km, by air, northwest of Kermānshāh, Kermānshāh Province [*cob*, GQ855761]; 18) ZIN 8738, holotype of *Testudo zarudnyi*: Birjand, South Khorasan Province [*nad4*, GQ855763]; 19) MVZ 234423: West side of Sirch Tunnel, on the road from the Kermān-Māhān road to Shahdād, Kermān Province; 20) MVZ 243879: 36.25 miles south-southwest, by air, of Qariat al Arab, Kermān Province; 21a) MVZ 234291: Desert between Khabar Mountain and Mazar Mountain, 30 km west of Khabar, Kermān Province; 21b) MVZ 234292; 22) MVZ 234285: Ziārat, 30 km west, by road, of Zāhedān, Sistan and Baluchestan Province; 23) MVZ 234282: 93 km north-northwest of Khash (by road to Zāhedān), Sistan and Baluchestan Province; 24) MVZ 234284: Jam Chin Valley, Kuh-e Taftan, 25 km north, by air, of Khash, Sistan and Baluchestan Province [*cob*, GQ855762]; 25) MVZ 234509: Garm Beyt, Sistan and Baluchestan Province.