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GENERAL GENETICS

Polymorphism of the 12S rRNA Gene and Phylogeography of the Central Asian Tortoises *Agrionemys horsfieldii* Gray, 1844

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Abstract—Based on intraspecific polymorphism of 12S rRNA gene, genetic variation of isolated populations of the Central Asian tortoise, *Agrionemys horsfieldii*, was for the first time investigated on a large part of the species distribution range, encompassing Uzbekistan, southern Kazakhstan, and northern and eastern Iran. In 59 tortoises, four haplotypes were discovered, including two (AH1 and AH2), described earlier. Haplotype AH1 was detected in 52 tortoises, inhabiting southern Kazakhstan and Uzbekistan. Haplotype AH2 was found in four tortoises from the border territory between Uzbekistan, Tajikistan, and Afghanistan. Two novel haplotypes, AH3 and AH4, were detected in the three tortoises from Iran. Based on nucleotide substitutions in the 12S rDNA sequence, the possible divergence time between the tortoises from different parts of the range was estimated. Possible pathways of the formation of modern intraspecific groups of *A. horsfieldii* are discussed.

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INTRODUCTION

The range of Central Asian tortoise, *Agrionemys horsfieldii*, Gray, 1884, occupies a large territory, encompassing the plains and low mountains of Uzbekistan, Turkmenistan, Tajikistan, Kirgizia, southern Kazakhstan, Afghanistan, northeastern Iran, northwestern China, and northwestern Pakistan. The central Asian tortoise is treated as a separate genus *Agrionemys*, where it is represented by a single species [1]. However, some authors still assign *A. horsfieldii* to the genus *Testudo*, or to its subgenus [2, 3].

Populations of *A. horsfieldii* differ by a number of diagnostic characters (coloration, and the shape of the carapace and the frontal edge of the plastron), based on which three subspecies are recognized [4]. Some authors made a rather groundless suggestion that these subspecies should be elevated to the species level [5]. Nominative subspecies *A. horsfieldii* Gray, 1984, inhabits Afghanistan, eastern Turkmenistan, and Tajikistan; *A. h. rustamovi* Ckhikvadze, Amiranshvili et Ataev, 1990, is found in southwestern Turkmenistan; and *A. h. kazakhstanica* Ckhikvadze, occurs in the northern part of the species range [1, 4].

Earlier, analysis of mitochondrial DNA polymorphism, specifically, that of 12S rRNA gene, in different terrestrial tortoises of the genus *Testudo* revealed high haplotype diversity [6–8]. On the other hand, in *A. horsfieldii* individuals from Kazakhstan and Uzbekistan only two hapolotypes, differing from one another by a single transition, were identified [7, 9]. However, phylogeography, i.e., territorial geographic distribution of intraspecific gene flows in natural populations [10] of this species was not examined. Comparative analysis of nucleotide substitutions in mtDNA validated the recognition of the Central Asian tortoise as a separate genus [7]. This finding was later confirmed by a comparison of RAPD variation patterns in Mediterranean and Central Asian tortoises [11]. Genetic variation in the populations of Central Asian tortoise from different parts of the species range still remains poorly studied. To investigate intraspecific differentiation of *A. horsfieldii* based on the 12S rRNA intraspecific polymorphism, in this study, for the first time, an analysis of isolated populations from the substantial part of the species range, encompassing Uzbekistan, southern Kazakhstan, and Northern and Eastern Iran, was carried out.

MATERIALS AND METHODS

Polymorphism of the mitochondrial 12S rRNA gene fragment was examined using DNA samples of 59 tortoises (29 males and 30 females). Blood samples were taken from the cervical vein of the adult animals from natural populations of Uzbekistan ($n = 45$), Kazakhstan ($n = 11$), and Iran ($n = 3$) in 2004 through 2006 (Fig. 1).

Fresh blood samples were preserved in 0.05 M EDTA (pH 8.0) or in 96% ethanol, and stored at 4°C.

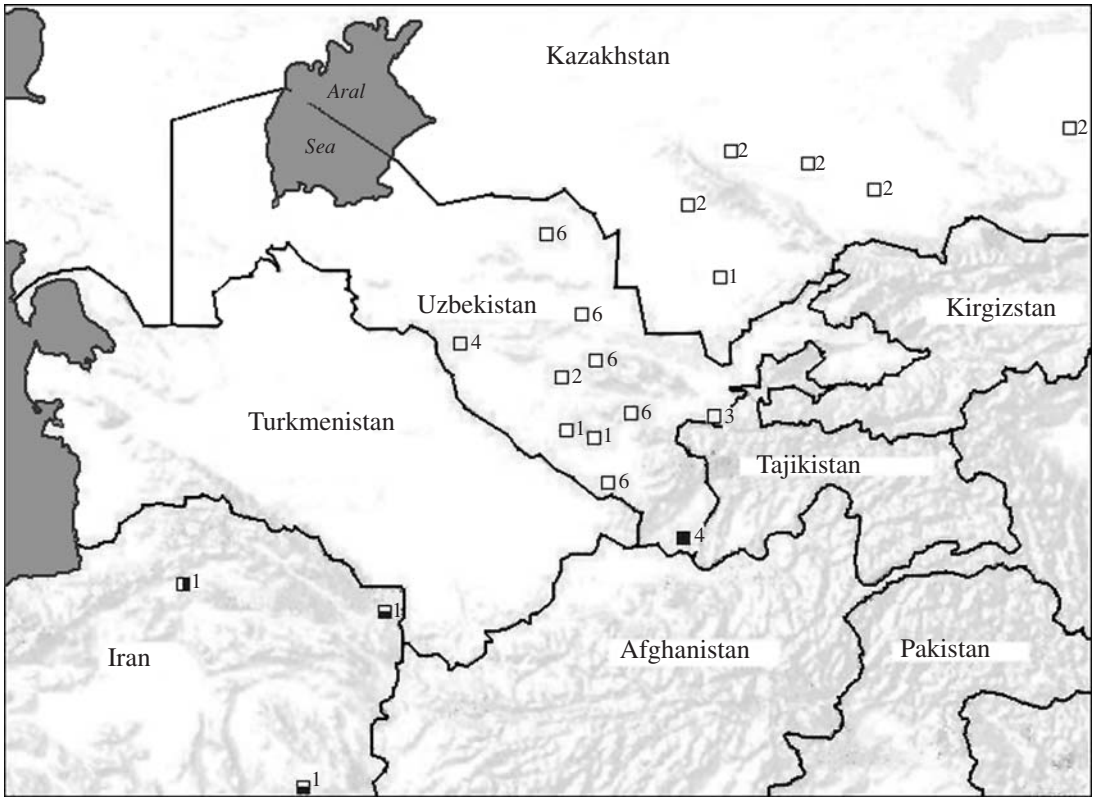

Fig. 1. Blood sampling sites and distribution of *A. horsfieldii* Mt haplotypes on the territory of Central Asia. Haplotype designations: □ AH1; ■ AH2; ⬓ AH3; ◨ AH4; numbers indicate the number of animals examined.

Total DNA was extracted using the standard phenol–chloroform method [12].

Amplification of mitochondrial 12S rRNA gene fragment was performed using universal 12S primers, L 01091 (forward) and H 01478 (reverse), providing synthesis of the fragments of 400 bp in size [6–8,13]. PCR amplification was done using the following protocol: 94°C for 2 min; 93°C for 1 min; 50°C for 1 min; 72°C for 2 min (35 cycles); and 72°C for 10 min. The 25 μl-reactions contained 60 mM Tris–HCl; 10 mM $(NH_4)_2SO_4$; 0.1% Tween-20; 100 μM of each dNTP; 4 μM $MgCl_2$; 1.5 pmol of each primer; 0.6 to 0.7 units of *Taq* polymerase (Dialat, Moscow); and 25 to 100 ng of total DNA template. Sequencing of amplificants was performed in both directions using the ABI PRISM^R BigDye^{TM} Terminator v. 3.1 reagent kit with further analysis of the reaction products on an ABI PRISM^R 3100-Avant automated DNA sequencer. Obtained sequences were aligned manually based on the 12S rRNA sequence of *T. horsfieldii* from the GenBank (AF175328, AB090020). Reconstruction of phylogenetic relationships and estimation of divergence times between the haplotypes was performed using the MEGA 3 software package [14]. Dendrograms were constructed using Kimura's two-parameter model and the NJ algorithm [15, 16].

RESULTS AND DISCUSSION

Analysis of the mitochondrial 12S rRNA gene polymorphism in 59 representatives of Central Asian tortoise resulted in identification of four haplotypes. Two of these haplotypes differed by a single transition (T–C) in nucleotide position (np) 140. These haplotypes were described earlier (AF175328 and AB090020) for the tortoises from Central Asia (geographic regions are unknown) [7, 9]. In this study, haplotype AH2 (AB090020) was found in four tortoises inhabiting the region of the Babatag mountain ridge (the region of Uzbekistan bordering Tajikistan and Afghanistan). In the remaining 52 animals the second known haplotype, AH1 (AF175328), was identified (Fig. 1). In three tortoises from Iran, two novel haplotypes, AH3 and AH4, differing from the earlier described sequences by a single transition (T–C) in np 303, were identified. In turn, haplotypes AH3 and AH4 differed from one another by a single nucleotide substitution (C–T) in position 359 (table).

Geographic distribution of haplotypes AH1 and AH2 partly coincides with the ranges of *A. h. horsfieldii* and *A. h. kazakhstanica*, while the range borders of these subspecies are still disputable. It seems likely, that haplotype AH1 is typical of *A. h. kazakhstanica*, inhabiting the territory of Kazakhstan and the most part of Uzbekistan.

Distribution and characteristics of the 12S rRNA haplotypes in the studied populations of two putative subspecies of Central Asian tortoise *A. horsfieldii*

Haplotype		Haplotype distribution	No. of individuals examined	Nucleotide substitutions		
Definition	GeneBank			140 np	303 np	359 np
AH 1	AF175328	Southern Kazakhstan and Uzbekistan *A. h. kazakhstanica*	52	T	T	C
AH 2	AB090020	Uzbekistan, the region of Babatag Mountain Range *A. h. horsfieldii*	4	C	T	C
AH 3		Eastern Iran, Mozdevand; Northeastern Iran, 45 km northwest of the city of Nehbandan *A. h. horsfieldii*	2	T	C	C
AH 4		Northern Iran, valley between the Alborz and Jaghatai Mountain Ranges *A. h. horsfieldii*	1	T	C	T

Haplotype AH2, found in the tortoises living at the border between Uzbekistan and Tajikistan, and probably, in Tajikistan, is distributed throughout the northeastern part of the range of nominative subspecies *A. h. horsfieldii*. However, according to V. M. Chkhikvadze [4], the nominative subspecies inhabits not only Tajikistan, but also Afghanistan, where it was described, as well as southern Turkmenistan (Murgab River valley, northern Kopet Dag, Badhis). In this respect, the discovery of the third haplotype (AH3), differing from haplotype AH2, in eastern and northeastern Iran deserves special interest. The sites where this haplotype was detected are located at the border of Iran with Turkmenistan and Afghanistan. Similarly to haplotype AH2, location of the haplotype of interest also coincides with the range of nominative subspecies. Haplotype AH3 was identified in the tortoises from two populations, located 495 km apart from one another along the Afghani border. It is thus evident that the distribution area of the latter haplotype is much wider, encompassing the territory of Afghanistan and southern Turkmenistan.

The fourth haplotype (AH4) was detected in a single tortoise from the north of Iran (a valley between the Alborz and Jaghatai Mountain Ranges), which made impossible to evaluate the haplotype frequency and prevalence in the populations. No data on the 12S rRNA gene polymorphism in the third subspecies *A. h. rustamovi* described from southwestern Turkmenistan are currently available [17]. If haplotype AH4 is found not only in Iran but also in Turkmenistan and if it is typical of *A. h. rustamovi*, then the range of this subspecies can be substantially extended southwards. On the other hand, based on the subspecies description, *A. h. rustamovi* individuals are small (carapace length less than 17 cm). However, the *A. h. rustamovi* female found on the territory of Iran in the course of the present study, was big (carapace length, 19 cm). Hence, it is more probable that the northern part of Iran is inhabited by *A. h. horsfieldii* with haplotype AH4 than that this hapotype is typical of *A. h. rustamovi*.

The 12S rRNA polymorphism data were used to reconstruct possible phylogenetic relationships and to estimate the divergence times for the subspecies (forms) of Central Asian tortoise (Fig. 2). The divergence time was estimated at substitution rate of 0.3% per 1 Myr, suggested earlier for comparison of mtDNA samples of the tortoises from different families [6, 7]. It is suggested that AH2 sequence found in the tortoises from Babatag can be considered as the most ancient haplotype. This sequence is typical of the representatives of the modern subspecies *A. h. horsfieldii*, radiation of which from the common ancestor could occur in lower Pleistocene, about 0.8 Myr ago. Somewhat later, during middle Pleistocene (about 0.6 Myr ago), radiation of *A. h. kazakhstanica*, carrying the corresponding haplotype AH1, took place. This finding agrees with the suggestion based on the analysis of fossil material [18]. Isolation of the group of Iranian tortoises with the corresponding haplotypes AH3 and AH4, occurred later, approximately during the middle Pleistocene.

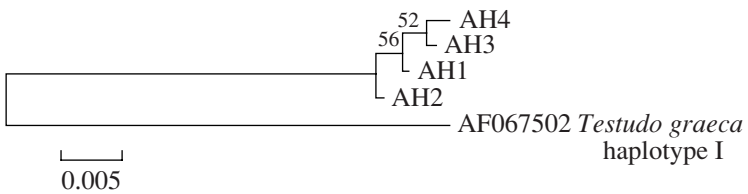

Fig. 2. NJ tree of phylogenetic relatiobnships of the 12S rRNA haplotypes found in the populations of the Central Asian tortoise, *A. horsfieldii* investigated. A fragment of the 12S rRNA gene sequence from Mediterranean tortoise (GenBank, AF067502 *Testudo graeca* haplotype I) was used as the outgroup. Numbers indicate bootstrap confidence levels (1000 iterations).

Thus, based on the analysis of the mitochondrial 12S rRNA gene, in the present study, intraspecific differentiation of the Central Asian tortoise, *A. horsfieldii*, was first demonstrated. Among the 59 tortoise individuals, four haplotypes, differing by one to three transitions, were discovered. Moreover, the range of putative nominative subspecies *A. h. horsfieldii* is inhabited by the animals having two haplotypes, differing by two transitions. Similar intraspecific genetic heterogeneity was described earlier in the populations of Mediterranean tortoise, *Testudo graeca*. It was shown that modern subspecies of *T. graeca* were characterized by the presence of a number of haplotypes, differing by one to seven nucleotide substitutions [6–8]. For instance, among the *T. g. graeca* populations from Africa and Spain, a total of six haplotypes, differing by one to five nucleotides, were described. In *T. g. whitei* individuals from Eastern Morocco four haplotypes, differing by one to three transitions were observed, while in the tortoises inhabiting a vast territory, encompassing Lebanon, Israel, Turkey, Sardinia, and Bulgaria, two haplotypes, differing by a single transition were observed. At the same time, the representatives of some territorially isolated and well morphologically distinguishable subspecies had identical haplotypes. For example, haplotype III was identified in *T. g. graeca* from Western Marocco, as well as in *T. g. whitei* from Eastern Marocco, while some *T. g. ibera* from Turkey were found to be similar to *T. g. nikolskii* from Krasnodar krai (Russia) [8]. Thus, there was no clear correlation between the number and quality (transition, transversion, and deletion) of the nucleotide substitutions and the isolation of subspecies groups in the *T. g. graeca* tortoises.

At present, the only suggestion that can be made based on the data obtained is that *A. h. kazakhstanica* has haplotype AH1, while *A. h. horsfieldii* carryhaplotypes AH2, AH3, and AH4. Small genetic differences between the populations examined suggest that they showid be assigned to one species. Clarification of phylogenetic relationships requires extension of the tortoise distribution region examined in Afghanistan and Turkmenistan along with the use of additional molecular genetic markers.

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