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ANIMAL GENETICS

# Molecular Differentiation and Taxonomy of the Sunwatcher Toad-Headed Agama Species Complex *Phrynocephalus* Superspecies *helioscopus* (Pallas 1771) (Reptilia: Agamidae)

**E. N. Solovyeva[a], N. A. Poyarkov[a], E. A. Dunaev[b], T. N. Duysebayeva[c], and A. A. Bannikova[a]**

[a] *Department of Vertebrate Zoology, Lomonosov Moscow State University, Moscow, 119991 Russia*
*e-mail: anolis@yandex.ru*
[b] *Zoological Museum, Lomonosov Moscow State University, Moscow, 125009 Russia*
[c] *Institute of Zoology, Almaty, 480060 Kazakhstan*

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**Abstract**—Lizards of the sunwatcher toad-headed agama species complex *Phrynocephalus* superspecies *helioscopus*, mostly distributed in Central Asia and Middle East, were examined using analysis of variation at the mitochondrial cytochrome oxidase c subunit I gene fragment and fingerprint analysis of nuclear DNA (inter-SINE PCR technique). A total of 86 individual tissue samples from 53 populations, to the full extent representing different parts of the species complex range, were subjected to molecular genetic examination, and surprisingly deep differentiation was revealed. The populations analyzed split into 12 isolated phylogroups, many of which were characterized by a narrow range and genetic isolation. Monophyly of sunwatcher (*Ph. helioscopus*) and Persian (*Ph. persicus*) toad-headed agamas was confirmed. However, both of these species probably represent the species complexes. Zoogeography of Central Asiais discussed.

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## INTRODUCTION

Sunwatcher toad-headed agama *Phrynocephalus* superspecies *helioscopus* is a wide-range species complex (hereafter designated as the *helioscopus–persicus* complex), which is found in CentralAsia, northwestern China, northern Iran, and Transcaucasia [1]. From the time of its first description (P. S. Pallas, 1771) till nowadays, no stable opinions on the species phylogeny have been formed. To solve this issue, mostly morphological characters, the indices of which strongly overlap, were used. Sunwatcher toad-headed agamas are characterized by polymorphism in the chromosome number and the ratio between macro- and microchromosomes [2–4]. According to V. K. Eremchenko and A.M. Panfilov [5], karyotype of 48 chromosomes (M/m = 24/24) is ancestral to *Ph. helioscopus*. Karyotype of Persian toad-headed agama (*Ph. persicus horvathi*) was found to be most different from ancestral variant. Populations of true sunwatcher toad-headed agama (*Ph. helioscopus*) are indistinguishable in the muscle tissue thermostability. However, *Ph. p. horvathi* from the south of Azerbaijan possess the same cytophysiological characteristics as *Ph. helioscopus* from Central Asia [6].

Development of molecular genetic approaches made it possible to put in order the concepts on the species complex *Ph. helioscopus–Ph. persicus*. However, genetic variation of this group was the subject of investigation in only few studies. In these studies, from three [5, 7] to 5–12 [8–14] phyletic clades are discussed. Thus, according to current ideas, within the species of *Phrynocephalus helioscopus* three subspecies, *Ph. helioscopus helioscopus*, *Ph. h. saidalievi*, and *Ph. h. varius* are distinguished. In turn, within *Phrynocephalus persicus* two subspecies, *Ph. p. persicus* and *Ph. p. horvathi*, are identified. In general, taxonomic relationships within the species complex of toad-headed agamas remain unresolved.

Since the range of sunwatcher and Persian toad-headed agamas covers nearly all Central Asiaand adjacent territories, it seems likely that analysis of phylogenetic relationships within this wide-range species complex will shed light on the events, which influenced the dispersal and speciation of many other organisms, inhabiting this region.

## MATERIALS AND METHODS

### *Sample Description*

Molecular genetic analysis was performed using tissue samples of 86 *Phrynocephalus helioscopus* lizards representing 53 populations covering the whole range (Fig. 1, Table 1). Tissue samples of *Ph. axillaris* (ZMMU R-12302), *Ph. mystaceus* (ZMMU R-12149), and *Ph. interscapularis* (ZMMU R-12260) were taken as outgroups. Most of the samples examined were held in herpetological collections of the Zoological Museum of Lomonosov Moscow State University (ZMMU, 70 samples) and the Institute of Zoology and Parasitology, Uzbek Academy of Sciences, Republic of

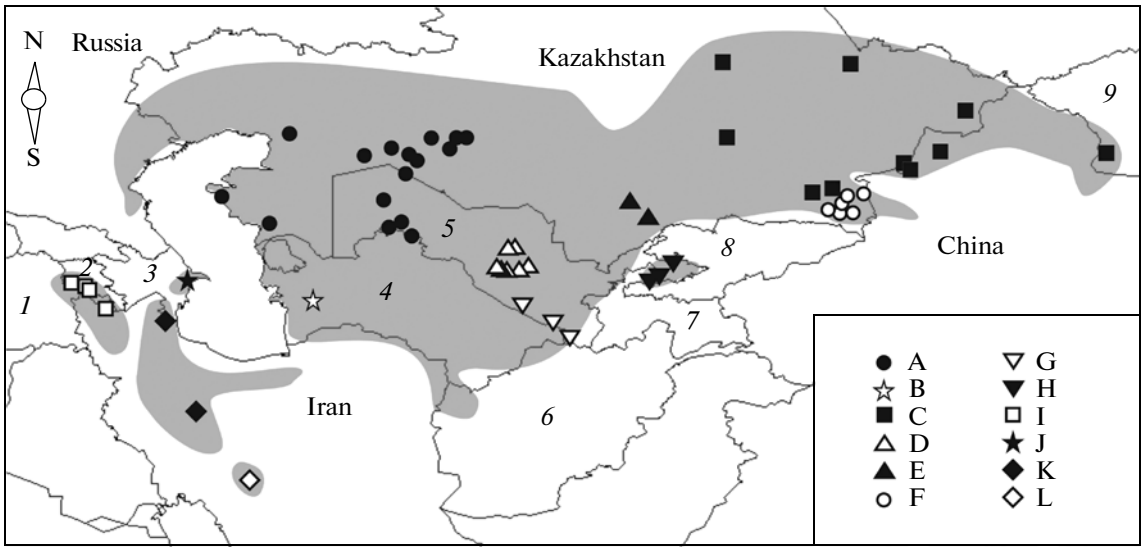


**Fig. 1.** Distribution of modern taxa of the *Ph. helioscopus* complex (symbols designate the sampling sites used in the molecular analysis; the letters correspond to the phylogroups identified (see Table 1)). Countries designated by figures are as follows: 1, Turkey; 2, Armenia; 3. Azerbaijan; 4, Turkmenistan; 5, Uzbekistan; 6, Afghanistan; 7, Tajikistan; 8, Kirghizia; 9, Mongolia.

Uzbekistan (IZIP, three samples). Some samples were kindly provided by our colleagues.

In molecular analysis, the gene fragments for the subunit I of the mitochondrial cytochrome oxidase c (*COI*) were compared, as well as nuclear DNA (nDNA) fingerprints obtained using the inter-SINE PCR technique. Inter-SINE PCR analysis with MIR-MIL primer system was carried out for 33 samples and the same analysis with the OMIR-OMIL primer system was carried out for 31 samples. The integrated matrix, which conbined the data for two systems of primers, contained the data for 25 samples representing 11 populations from the whole range of the *helioscopus–persicus* complex.

### *DNA Extraction*

DNA was isolated either from liver and muscles samples kept in 70 or 96% ethanol, or from dried skins. The extractions were performed using the DNA Prep 200 kit (Isogene). Alternatively, the samples were incubated in the presence of proteinase K and DNA was isolated using the standard phenol–chloroform method with subsequent sedimentation by isopropanol [15].

### *Amplification and Sequencing of Mitochondrial DNA*

Polymerase chain reaction (PCR) was performed using a dry reagent kit for DNA amplification (GenePak PCR CorE, Isogene). The MasterMix tube already containing lyophilized components (200 μM dNTP; *Taq* polymerase, inhibited until hot start; 2.5 mM $MgCl_2$; optimized buffer; and dye)was supplemented with 10 μl of the Diluent solvent; 5 μl of primer mix with the concentration of 1 AU/μl; and 5 μl of the DNA solution. Amplification was run using a Bio-Rad MyCycler thermal cycler. PCR was carried out using pair of primers standard for lower vertebrates: VF1d (5'-TTCTCAACCAACCACAA(RGA(Y)AT(Y)GG-3') and VR1 (5'-TAGACTTCTGGGTGGCC(R)AA(R)AA(Y)-3') [16]. The size of amplified DNA fragment was 680 bp.

The *COI* amplification protocol was as follows: initial denaturation for 1 min at 94°C; denaturation at 94°C for 30 s; and extension at 72°C for 1 min. The annealing conditions were variable. During the first five cycles, annealing was performed at 45°C for 40 s; in the following 30 to 35 cycles annealing conditions were 53°C for 40 s. Final extension was performed at 72°C for 10 min. Amplification was controlled by electrophoresis in 1% agarose gel with addition of ethidium bromide. The *COI* sequence in a number of samples (68 in total) was determined through direct sequencing performed at the Canadian Center of DNA Barcoding (Canada) using the method of N. V. Ivanova et al. [16]. A number of samples (37 in total) were sequenced in the laboratory of the Molecular Diagnostic Center, All-Russia Center for Control, Standardizations and Certification of Veterinary Preparations and Forage and in the Genome Center of Collective Use. Sequencing was performed using automated ABI 377 and ABI 3100-Avant sequencers, an ABI PRISM®Big-DyeTM Terminator, version 3.1, and a Big-Dye Ready-Reaction kit (United States). For sequencing, from 10 to 40 ng of purified PCR products were used and the same primers as for amplification.

### *Inter-SINE PCR Conditions*

Inter-SINE PCR method is based on polymorphism analysis of the DNA fragments located between the copies of short retroposons (SINEs). In this study, the SINE

**Table 1.** A list of samples of *Ph. helioscopus* and *Ph. persicus* used in molecular phylogenetic analysis

| No. | Locality | Accession numbers | *N* |
|---|---|---|---|
| | **A – Uzbekistan: Karakalpakia; Western Kazakhstan: Cisaralia, Ustyurt, Northwestern Kyzylkum (*N* = 30)** | | |
| 1 | Uzbekistan, Kungradskii raion, Outskirts of the settlement of Raushan; N42.933, E59.133 | IZIP 829(4401) R-12150 | 2 |
| 2 | Uzbekistan, Ustyurt, the settlement of Zhaslyk; 43.933, E57.498 | IZIP 003 | 1 |
| 3 | Uzbekistan, Kungradskii raion, southwest of the Kungrad Mountain; N42.683, E58.550 | R-12152 | 1 |
| 4 | Uzbekistan, Kungradskii raion, Ustyur; N43.991, E57.972 | R-12153 | 2 |
| 5 | Uzbekistan, Aral, Vozrozhdeniye Island; N45.167, E59.333 | R-12813 | 1 |
| 6 | Kazakhstan, Kyzylorda oblast, Aralsk–Kamyshlybash highway; N46.228, E61.367 | R-13241 | 1 |
| 7 | Kazakhstan, Kyzylorda oblast, outskirts of the city of Aralsk; N46.800, E61.667 | R-13242 | 5 |
| 8 | Kazakhstan, Kyzylorda oblast, outskirts of the city of Aralsk; N46.794, E62.133 | R-13240 | 3 |
| 9 | Kazakhstan, Mangistau oblast, the settlement of Ozektyk; N45.983, E57.467 | R-12151, R-12155 | 3 |
| 10 | Kazakhstan, Mangistau oblast, Mangyshlak Peninsula; N44.100, E50.950 | R-12157 | 1 |
| 11 | Kazakhstan, Kyzylorda oblast, Bol'shie Barsuki sandy desert; N46.283, E58.700 | R-12158 | 1 |
| 12 | Kazakhstan, Kyzylorda oblast, the settlement of Akespe; 46.783, 60.500 | R-12156, R-12160 | 3 |
| 13 | Kazakhstan, Kyzylorda oblast, Aral, Kulandy Peninsula; N46.017, E59.500 | R-12162 | 1 |
| 14 | Kazakhstan, Kyzylorda oblast, Barsa-Kelmes Island; N45.700, E59.867 | R-12519 | 1 |
| 15 | Kazakhstan, Mangistau raion, Ustyurt Nature Reserve; N42.883, E53.083 | R-12525 | 1 |
| 16 | Kazakhstan, Zhambyl oblast, outskirts of the settlement of Kyzyl-Kii; N41.650, E69.367 | R-12161 | 2 |
| 17 | Kazakhstan, Karaganda oblast, northward of the settlement of Zhankala; N50.217, E73.817 | R-12527 | 1 |
| | **B – Western Turkmenistan: outskirts of Nebit-Dag (*N* = 1)** | | |
| 18 | Turkmenistan, 70 km from the settlement of Nebit-Dag, to the city of Ashkhabad; N39.350, E55.200 | R-12789 | 1 |
| | **C – Eastern Kazakhstan, Chinese and Mongolian Dzungaria (*N* = 9)** | | |
| 19 | Kazakhstan, Eastern Kazakhstan oblast, Semei-Tau Mountains; N50.150, E79.667 | R-12163 | 2 |
| 20 | Kazakhstan, Eastern Kazakhstan oblast, surroundings of the Lake Zalanashkol; N45.571, E82.212 | R-12164, R-12524 | 2 |
| 21 | Kazakhstan, Alma-Ata oblast, northern part of Ili depression; N44.167, E78.783 | R-12810 | 2 |
| 22 | China, Xinjiang Uighur Autonomous Region, Toli county | R-13089 | 1 |
| 23 | Mongolia, Khovd, Ikcher-Toli, westward of Bulgan; N46.100, E91.350 | R-12903 | 2 |
| | **D – Northeastern Uzbekistan (*N* = 7)** | | |
| 24 | Uzbekistan, Bukhara oblast, surroundings of the Lake Ayakagytma; N40.667, E64.483 | R-12166 | 1 |
| 25 | Uzbekistan, Navoi oblast, Central Kyzylkum; N41.650, E64.300 | R-12167 | 1 |
| 26 | Uzbekistan, Navoi oblast, northwestward of the city of Zafarabad; N40.850, E64.933 | R-12259 | 1 |
| 27 | Uzbekistan, Navoi oblast, northeastward of the city of Zarafshan; N41.700, E64.033 | R-12264 | 1 |
| 28 | Uzbekistan, Navoi oblast, outskirts of the settlement of Shuruk; N40.683, E63.8 | R-12281 | 1 |
| 29 | Uzbekistan, Navoi oblast, near the settlement of Dzhangeldy; N40.800, E63.600 | R-12286 | 1 |
| 30 | Uzbekistan, Navoi oblast, Navoi oblast; N40.836, E63.483 | R-12310 | 1 |
| | **E – Northern Karatau (*N* = 2)** | | |
| 31 | Kazakhstan, Zhambyl oblast, outskirts of the settlement of Suzak; N44.133, E68.467 | R-12662 | 1 |
| 32 | Kazakhstan, Zhambyl oblast, surroundings of the Lake Kyzylkol; N43.784, E69.553 | R-12165 | 1 |
| | **F – Ili depression (*N* = 10)** | | |
| 33 | Kazakhstan, Alma-Ata oblast, road to the settlement of Kegen; N43.346, E79.22 | – | 2 |
| 34 | Kazakhstan, Alma-Ata oblast, Southern part of Ili depression | – | 1 |
| 35 | Kazakhstan, Alma-Ata oblast, Southern part of Ili depression; N43.600, E79.317 | R-12664 | 1 |

**Table 1.** (Contd.)

| No. | Locality | Accession numbers | *N* |
|---|---|---|---|
| 36 | Kazakhstan, Alma-Ata oblast, outskirts of the settlement of Khorgos; N44.217, E80.250 | R-12665 | 2 |
| 37 | Kazakhstan, Alma-Ata oblast, outskirts of the settlement of Tashkarasu; N43.733, E79.467 | R-12668 | 1 |
| 38 | Kazakhstan, Alma-Ata oblast, north of the Syugatinskaya valley; N43.450, E78.900 | R-12669 | 2 |
| 39 | Kazakhstan, Alma-Ata oblast, eastward of the Aktau hills; N44.083, E79.467 | R-12812 | 1 |
| | **G – Northeastern Turkmenistan, Southeastern Uzbekistan (*N* = 5)** | | |
| 40 | Uzbekistan, Surkhandarya oblast, Pashkhurd steppe; N37.583, E66.667 | R-12801 | 2 |
| 41 | Uzbekistan, Bukhara oblast, surrounding of the Bukhara nursery "Dzheiran"; N39.567, E64.767 | R-12803 | 1 |
| 42 | Uzbekistan, border between the Bukhara and Kashkadarya oblast, surroundings of the Lake Devkhon; N39.200, E64.650 | R-12804 | 2 |
| | **H – Fergana valley (*N* = 4)** | | |
| 43 | Uzbekistan, Fergana oblast, 25 km northward of the city of Kokand; N40.767, E70.967 | R-12678 | 1 |
| 44 | Uzbekistan, Fergana oblast, outskirts of the city of Kokand; N40.517, E70.933 | R-12802 | 3 |
| | **I – Transcaucasia (*N* = 9)** | | |
| 45 | Armenia, Armavir Marz, the settlement of Gorovan; N39.917, E44.733 | R-13243 | 4 |
| 46 | Armenia, Armavir Marz, outskirts of the city of Armavir; N40.067, E44.050 | – | 2 |
| 47 | Armenia, Armavir Marz, outskirts of the settlement of Oktemberyan; N40.150, E44.033 | R-12322 | 1 |
| 48 | Armenia, Armavir Marz, outskirts of the settlement of Vedi; N39.933, E44.717 | R-3843 | 1 |
| 49 | Azerbaijan, outskirts of the city of Nakhichevan; N39.217, E45.417 | R-12466 | 1 |
| | **J – Nakhichevan (*N* = 1)** | | |
| 50 | Azerbaijan, Nakhichevan, outskirts of the settlement of Sangachaly; N40.167, E49.450 | R-12786 | 1 |
| | **K – Northern and Central Iran (*N* = 2)** | | |
| 51 | Iran, province of Markazi, outskirts of the city of Arak; N34.240, E49.750 | – | 1 |
| 52 | Iran, province of Eastern Azerbaijan, outskirts of the city of Ardebil; N38.393, E48.368 | – | 1 |
| | **L – outskirts of the city of Abadeh (*N* = 2)** | | |
| 53 | Iran, province of Fars, outskirts of the city of Abadeh; N31.129, E52.203 | – | 2 |

Note: *N*, number of individuals. The collection accession numbers shown are: R, herpetological collection of ZMMU, Moscow; IZIP, collection of the Institute of Zoology and Parazitology, Tashkent, Uzbekistan.

family studied was earlier successfully used in mammal phylogenetic studies. The family is designated as MIR (mammalian interspersed repeats), and it is thought to be present in all vertebrates [17, 18]. Amplification was performed using primers complementary to most conserved central core sequence of MIR element [18]: O-MIR (5'-ACCTTGAGCAAGTCACT-3') and O-MIL (5'-CGGAGTCAAAGGAGTAG-3'), as well as primers MIR (5'-AGTGACTTGCTCAAGGT-3') and MIL (5'-GCCTCAGTTTCCTCATC-3'). The inter-SINE PCR conditions and analysis of the data obtained are described by Bannikova et al. [17].

### *Phylogenetic Analysis of the COI Sequencing Data*

In all, 680-bp sequences of the *COI* gene fragment were obtained for 77 samples (GenBank NCBI nos. JF756679–JF756689, JF769363–JF769421). Sequences were aligned by hand using the BioEdit Sequence Alignment Editor 5.0.9 software program [19]. Final alignment used in phylogenetic analysis was 662 bp in size.

Phylogenetic analysis was carried out in the PAUP version 4.0b4a [20] and MEGA 4.0 [21] software programs. Dendrograms were constructed using the neighbor joining method (NJ dendrograms) and based on the maximum composite likelihood model distances, taking into consideration transitions, transversions, and all coding positions. Cladograms were constructed using Wagner maximum parsimony algorithm (MP cladograms).

### *Phylogenetic Analysis of Inter-SINE PCR Data*

Fingerprints obtained in MIR- and MIL-inter-SINE PCR reactions were compiled into binary matrices (where 1 or 0 were the band presence or absence, respectively). The matrices were analyzed within the PAUP version 4.0b4a software package [20]

**Table 2.** Genetic diversity (p, nucleotide; *H*, haplotype) diversity and substitutions (Ti, transitions; Tv, transversions) in some phylogroups (1, lineages F, C, A, D, H, and I) and geographic populations (2, shown are the population numbers from Table 1 and the numbers of individuals)

| (1) | A ($N=30$) | C ($N=9$) | D ($N=7$) | F ($N=9$) | H ($N=4$) | I ($N=9$) |
|---|---|---|---|---|---|---|
| π | 0.38 | 0.84 | 0.16 | 1.29 | 0.20 | 1.21 |
| *H* | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| (2) | Armenia (45–48; $N=8$) | Eastern Kazakhstan and Dzungaria (19–23; $N=9$) | Cisaralia (6–8, 11–14; $N=15$) | Ustyurt (1–4, 9–10, 15; $N=11$) | Left bank of Ili River (33–35, 37, 38; $N=7$) | Uzbekistan (24–30; $N=7$) |
| π | 0.00 | 0.92 | 0.28 | 0.35 | 0.69 | 0.17 |
| *H* | 0.71 | 0.96 | 0.95 | 0.91 | 1.00 | 0.81 |
| Ti | 0 | 18 | 6 | 6 | 7 | 3 |
| Tv | 0 | 5 | 2 | 3 | 9 | 0 |

using the neighbor joining and Wagner maximum parsimony methods. Statistical significance of the groupings obtained was tested by means of bootstrap analysis (BS) with 1000 replicates. A total of three NJ dendrograms were generated. One of these trees was constructed using the first system of primers, the second one, with the second primer system, and the third tree summarized the data of the first two (the total number of characters in summarized matrix was 332). Statistical significance of the groupings generated as a result of the inter-SINE PCR profile analysis was evaluated with the help of multidimensional scaling based on Nei and Li genetic distances ($D_{NL}$) [22], as implemented in the STATISTICA software package [23].

### *Genetic Diversity Estimates*

To evaluate the contribution of the within- and between-group variation to total variation, analysis of molecular variance (AMOVA) was performed [24]. Frequency distribution of pairwise nucleotide differences (mismatch distribution), as well as the indices of nucleotide (π) and haplotype (*H*) diversity were computed using the Arlequin ver. 3.5.1.2 software program [25]. Nucleotide diversity (π) was calculated according to the formula: $\pi = (\theta_\pi/N_{nu}) \times 100\%$, where $\theta_\pi$, population parameter θ calculated based on the number of pairwise differences [26], and $N_{nu}$, the number of nucleotides ($N_{nu} = 656$). In addition, in all sequences the number of polymorphic loci was determined.

To calculate some of population genetic indices, the total sample represented by 53 populations of the *helioscopus–persicus* complex was subdivided into individual groups, roughly corresponding to geographic populations of toad-headed agamas. In this case, the following perceptions were observed: the groups were composed of the population samples belonging to one cluster of haplotypes from geographically close populations not divided by the boarders, known to be the barriers to the dispersal of toad-headed agamas (like mountain ridges, beds of the large rivers, etc.). A total of six groups were distinguished (Table 2), which contained the populations from the valley of Arax River in Armenia (four populations, lineage I), Eastern Kazakhstan and the adjacent regions of Xinjiang Uighur Autonomous Region of China (five populations, lineage C), Cis-Aralia (six populations, lineage A), Ustyurt (seven populations, lineage A), Central Uzbekistan (seven populations, lineage D), and the populations from the left bank region of the Ili River, Ili depression (five populations, lineage F). The sizes of the groupings analyzed were only slightly variable (from seven to 16 individuals), providing comparison of the calculations made for individual groups.

## RESULTS

### *Characterization of the MtDNA Sequences*

The guanine nucleotide content in the *COI* gene fragment examined was decreased, which was typical of mtDNA: T, 28.10%; A, 30.84%; G, 13.45%; and C, 27.60%. Among the 656 *COI* fragments, 249 were found to be variable with 181 of these, which were parsimoniously informative. The total number of substitutions was 293, of which 98 were transversions and 195, transitions. In most of the populations examined, the number of transitions was higher than that of transversions (Table 2).

### *Phylogenetic Analysis of MtDNA*

Our studies revealed considerable structuring of the mitochondrial gene pool of toad-headed lizards from the *helioscopus–persicus* complex. On topologically identical MP and NJ dendrograms, constructed based on phylogenetic analysis of the *COI* gene fragment (Fig. 2), at least 15 monophyletic clusters of haplotypes were identified by high or medium bootstrap support levels (90 to 99% for NJ-, and 75 to 100% for MP analysis). The mean uncorrected *p*-distances between the lineages identified are demonstrated in Table 3. The levels of cluster genetic isolation were different. All together, the clusters could be

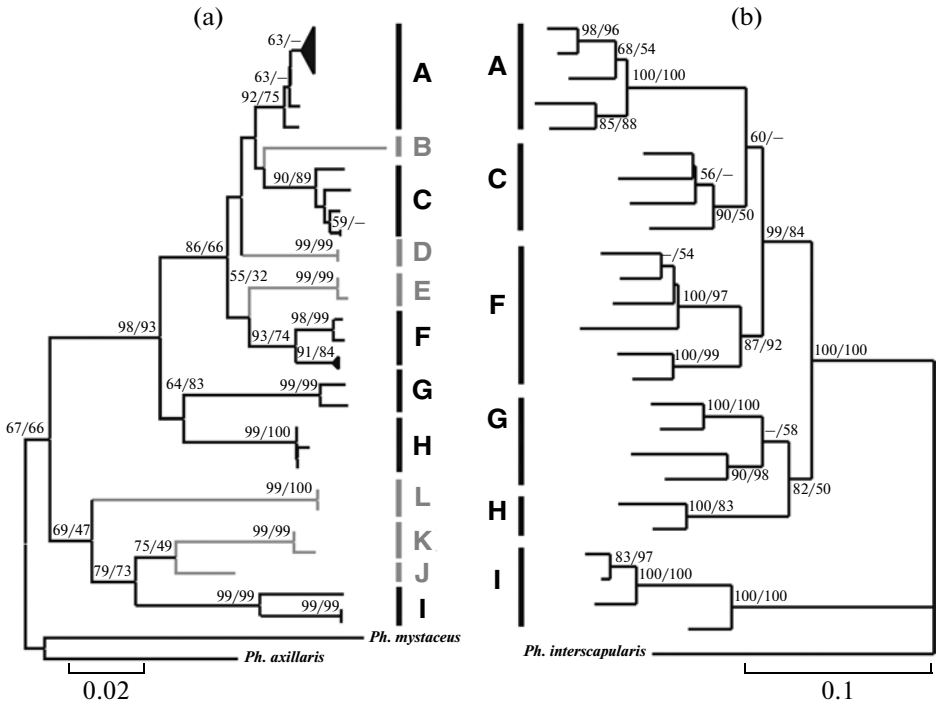


**Fig. 2.** Schemes of molecular genetic relationships between the representatives of the *Ph. helioscopus* complex inferred from the data of mtDNA and nDNA marker analysis. a, NJ dendrogram constructed based on the *COI* fragment sequence data; b, integrated NJ dendrogram based on the Nei and Li genetic distances inferred from the Inter-SINE PCR data. For the clusters, statistical significance of which is higher than 50%, bootstrap support indices are demonstrated (% from 1000 replicates): before the slash, in neighbor-joining (NJ) analysis; after the slash, in parsimonious analysis (MP). Letter symbols of the groups correspond to those in Table 1: A, Northwestern Uzbekistan; Western Kazakhstan: Cisaralia, Ustyurt, Northwestern Kyzylkum; B, Western Turkmenistan: Nebit-Dag; C, Eastern Kazakhstan, Chinese and Mongolian Dzungaria; D, Northeastern Uzbekistan; E, Kazakhstan: Northern Karatau; F, Eastern Kazakhstan: Ili depression; G, Southeastern Uzbekistan: Surkhandarya and south of the Bukhara oblast; H, Uzbekistan: Fergana valley; I, Armenia; Azerbaijan, Nakhichevan; J, Azerbaijan: Apsheron Peninsula; K, Northern and Central Iran: Ardebil and Arak; L, Southern Iran: Abadeh.

grouped into 12 monophyletic lineages (designated as A to L), the mean uncorrected *p*-distances between which exceeded the conditional level of $p = 4.0\%$ (Table 3). Furthermore, maximum level of the within-group variation did not exceed $p = 1.4$ (for lineage G). Lineage F from Ili depression deserves special interest. This lineage is represented by two deeply isolated statistically significantly monophyletic subclades $F_1$ and $F_2$ ($p = 3.0\%$, Table 3), divided by the bed of the Ili River. In general, in the complex of sunwatcher toad-headed agamas, the within-group variation at the mtDNA marker examined is substantially lower than for the between-group comparisons, and do not overlap with the latter.

Within the sunwatcher toad-headed agama complex, two statistically significantly monophyletic clusters can be distinguished. These clusters correspond to two currently recognized species of the complex, *Ph. helioscopus* from the territory of Middle and Central Asia and Ciscaucasia, and *Ph. persicus* from Iranian and plateaus. Genetic isolation of these clusters is remarkably high ($p = 14.8\%$). Moreover, the mean uncorrected *p*-distances between the lineages of sunwatcher toad-headed agama complex and out groups (other representatives of the genus *Phrynocephalus*) vary from 12.1 to 19.3%. The interspecific distances between different groups of true sunwatcher toad-headed agamas and between different groups of Persian toad-headed agamas vary from 5% (between phylogroups E and K) to 16.1% (between C and I). The overlap of the genetic distance values between the species of the *helioscopus–persicus* complex and the other species of the genus *Phrynocephalus* is probably associated with saturation of relatively rapidly evolving *COI* gene, which however, is not of much significance for analysis of phylogenetic relationships within the complex.

Thus, within *Ph. helioscopus* and *Ph. persicus*, a total of 12 mitochondrial lineages are distinguished. The distribution of these lineages is described in Discussion. The wide-range species *Ph. heliscopus* is represented by clusters grouping eight haplotype lineages. Six of these lineages (A to F; Figs. 1, 2a) inhabit northern and central parts of the species range, and another two lineages (G and H) are found in the southern part of the range. Although the bootstrap support for monophyly of these clusters is low, we hereafter designate them as the populations of "northern" and "southern" sunwatcher toad-headed agamas, respectively. In true sunwatcher toad-headed agamas, the lowest mean *p*-distance constituted 3.0% (between the

**Table 3.** Mean uncorrected *p*-distances (in %) inferred from the *COI* gene fragment sequence data: (1) *p*-distances between the lineages of *Ph. helioscopus–Ph. persicus* complex (below the diagonal); (2) *p*-distances between some species of the genus *Phrynocephalus* (below the diagonal). Above the diagonal, standard deviation; on the diagonal, mean uncorrected within-group *p*-distances (in semi-bold type)

| (1) | Lineage | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Ph. mystaceus* | – | 2.4 | 2.8 | 2.8 | 2.5 | 2.5 | 2.8 | 2.8 | 2.4 | 3.0 | 2.8 | 2.8 | 2.6 | 2.6 | 2.6 |
| 2 | *Ph. axillaris* | 13.7 | – | 2.3 | 2.1 | 2.2 | 2.2 | 2.1 | 2.1 | 2.3 | 2.4 | 2.3 | 2.5 | 2.4 | 2.4 | 2.2 |
| 3 | K | 17.9 | 14.0 | **0.6** | 2.1 | 1.2 | 1.7 | 2.4 | 2.3 | 2.2 | 2.3 | 2.3 | 2.2 | 2.4 | 2.1 | 2.3 |
| 4 | L | 17.7 | 12.1 | 12.0 | **0.0** | 1.9 | 2.0 | 2.4 | 2.2 | 2.3 | 2.4 | 2.3 | 2.3 | 2.5 | 2.5 | 2.4 |
| 5 | J | 14.6 | 13.7 | 14.0 | 13.8 | – | 1.5 | 2.2 | 2.1 | 1.9 | 2.0 | 1.9 | 2.0 | 2.2 | 2.0 | 2.2 |
| 6 | I | 15.5 | 14.5 | 9.0 | 12.6 | 8.3 | **1.0** | 2.3 | 2.2 | 2.1 | 2.3 | 2.4 | 2.2 | 2.4 | 2.4 | 2.4 |
| 7 | G | 17.1 | 12.4 | 15.0 | 15.7 | 13.4 | 13.7 | **1.4** | 1.5 | 2.0 | 1.8 | 1.9 | 1.9 | 2.0 | 1.7 | 1.7 |
| 8 | H | 17.5 | 13.1 | 15.0 | 13.9 | 14.0 | 14.8 | 7.5 | **0.2** | 1.8 | 1.6 | 1.6 | 1.8 | 1.8 | 1.7 | 1.6 |
| 9 | E | 14.8 | 12.4 | 5.0 | 9.7 | 11.2 | 8.4 | 14.0 | 13.4 | **0.3** | 1.3 | 1.1 | 1.3 | 1.4 | 1.3 | 1.2 |
| 10 | $F_1$ | 19.3 | 14.9 | 14.0 | 14.7 | 11.0 | 15.2 | 10.1 | 7.7 | 11.0 | **0.6** | 0.8 | 1.3 | 1.4 | 1.3 | 1.3 |
| 11 | $F_2$ | 17.5 | 14.2 | 14.0 | 14.0 | 11.1 | 15.6 | 10.8 | 8.1 | 11.1 | 3.0 | **0.2** | 1.1 | 1.3 | 1.2 | 1.2 |
| 12 | D | 18.3 | 15.3 | 14.0 | 14.4 | 11.7 | 14.0 | 10.6 | 9.0 | 11.8 | 5.0 | 5.0 | **0.0** | 1.4 | 1.3 | 1.2 |
| 13 | B | 16.6 | 14.7 | 15.0 | 15.5 | 12.5 | 15.3 | 11.1 | 10.1 | 12.5 | 7.0 | 6.0 | 6.0 | – | 1.1 | 1.2 |
| 14 | C | 16.4 | 14.7 | 14.0 | 15.8 | 11.8 | 16.1 | 10.0 | 9.1 | 11.7 | 6.0 | 6.0 | 6.0 | 5.0 | **0.8** | 1.0 |
| 15 | A | 16.9 | 13.1 | 14.0 | 14.5 | 12.9 | 15.5 | 8.8 | 7.9 | 12.9 | 6.0 | 5.0 | 5.0 | 5.0 | 4.0 | **0.5** |

| (2) | Species | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| 1 | *Ph. helioscopus* | **4.3** | 2.0 | 2.0 | 2.5 |
| 2 | *Ph. persicus* | 14.8 | **6.3** | 2.0 | 2.3 |
| 3 | *Ph. mystaceus* | 17.1 | 16.1 | – | 2.2 |
| 4 | *Ph. axillaris* | 13.8 | 13.9 | 13.7 | – |

Note: In the analysis, clade F from the Ili River valley was subdivided into two subclades, $F_1$ (population from the right bank of the Ili River) and $F_2$ (population from the left bank of the Ili River).

populations from the right ($F_1$) and left ($F_2$) banks of the Ili River), and the maximum such distance constituted 12.5% (between the samples from Nebit Dag (B) and the populations from North Karatau (E)). The *Ph. persicus* is characterized by the range consisting of isolated populations. In our study, this species is represented by four haplotype lineages (I to L; Figs. 1, 2a). Genetic isolation of these lineages is considerable (*p*-distances between the lineages are high and vary from 9 to 14%; the mean uncorrected within-group *p*-distance for the species constitutes 6.3%; Table 3), which is noticeably higher than that in *Ph. helioscopus*.

### *Genetic Diversity of the Identified Lineages*

For the whole complex of sunwatcher toad-headed agamas, the mean uncorrected *p*-distance inferred from the *COI* gene fragment data constituted 7.6%. According to the AMOVA results, the contribution of the between-group variation to the total genetic diversity (92.7%) was many times higher than the contribution of the within-group variation (1.9% between the populations within the groups, and 5.4% in the populations within groups).

Nucleotide diversity ($\pi$) was the highest in the populations from East Kazakhstan and Dzungaria (C) (0.92). On the remaining territory, nucleotide diversity was rather low, varying from 0.17 to 0.69 (Table 2, (1)). The haplotype diversity (*H*) varied from 0.71 (in group I) to 1 (in group F). In other words, most of the lizards examined had unique haplotypes. Among all phylogroups, the highest $\pi$ value was observed for phylogroups F (1.29) and I (1.21).

### *Frequency Distribution of Pairwise Nucleotide Differences*

The mismatch distribution pattern in three haplotype clusters was clearly unimodal (Fig. 3). These were the populations from the Northeast Uzbekistan (lineage D) and the populations from Ustyurt and Cisaralia (Figs. 3c, 3d). This finding suggests recent demographic expansion, or a series of expansions, which were accompanied by exponential growth of the population number and high migration rate between neighboring populations

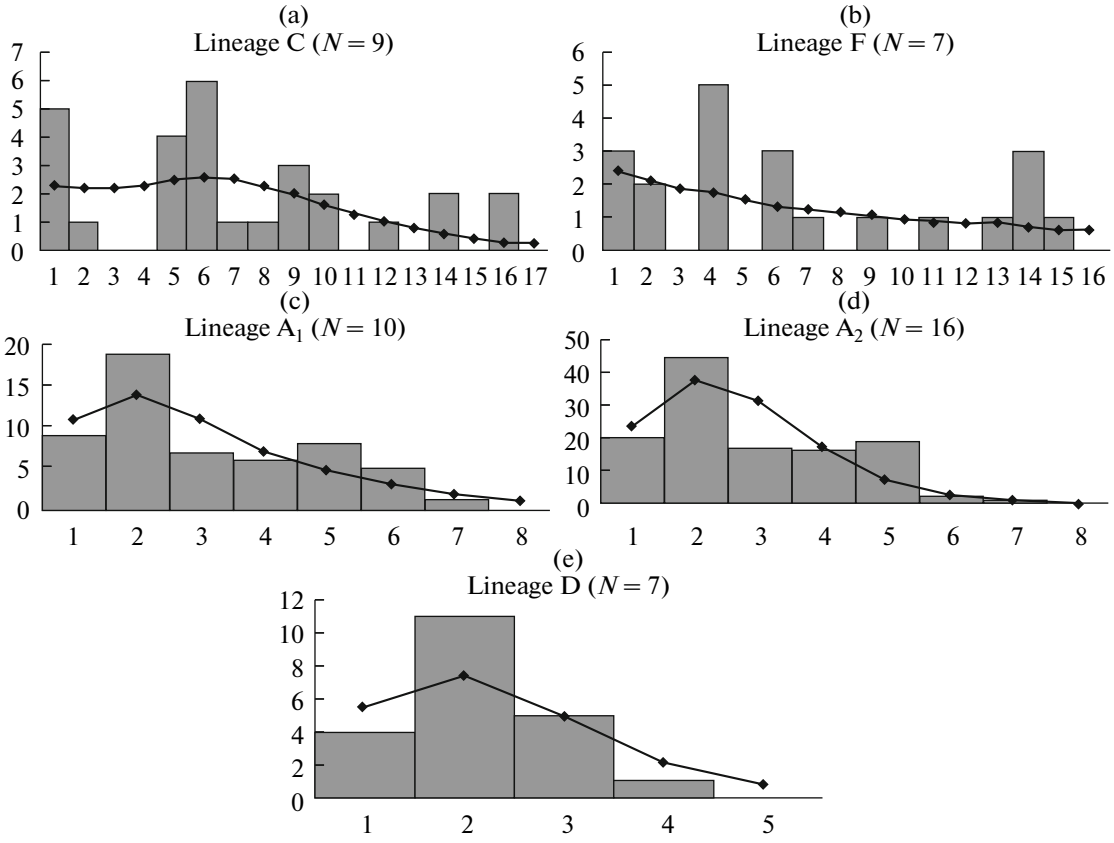


**Fig. 3.** Analysis of the mismatch distribution (histograms, observed distribution; curve, expected distribution; above the plots, the number of individuals included into the analysis): a, Eastern Kazakhstan and Dzungaria (C); b, left bank of Ili River (F); c, Ustyurt($A_1$); d, Cisaralia ($A_2$); e, Northeastern Uzbekistan (D). On abscissa, the number of pairwise differences; on, ordinate, frequency.

[27–29]. At the same time, isolated populations from the Ili River valley (lineage F), enclosed by Dzungarian Alatau from the north, by the Boro-Khoro Mountain Range from the east, and by Zailisky Alatau and Ketmen ranges, from the south, are characterized by multimodal mismatch distribution pattern (Fig. 3b). This pattern implies the existence of demographic equilibrium, i.e., of prolonged population stability along with the absence of the exponential growth of the population number. Multimodal mismatch distribution pattern was also identified for the populations from lineage C (*Ph. h. varius*), which unlike the populations from lineage F, are spread over rather vast territory from Cisbalkhashia till Mongolian Dzungaria. However, the histogram of mismatch distributions for the haplotypes of this lineage (Fig. 3a) showed the presence of a peak in the range of values from five to six (number of differences), which does not exclude a recent population expansion.

### *Phylogenetic Analysis of Inter-SINE PCR Data*

In the profile of PCR products obtained with MIR–MIL primer system a total of 185 fragments were identified. Primers OMIR–OMIL produced 147 fragments. Distance and parsimonious dendrograms constructed based on Inter-MIR- and inter-OMIR PCR data similarly group the samples examined into six statistically significant (BS > 75%) groups (representatives of lineages A, C, F, G, H, and I participated in the analysis. The two primer systems used similarly subdivided the sample examined into two main groups. These groups united the samples of *Ph. helioscopus* (lineages A to H) and *Ph. persicus* (lineage I), respectively. According to the Inter-MIR and Inter-OMIR PCR data, molecular genetic relationships between individual lineages within *Ph. helioscopus* (lineages A to H) in a number of nodes are resolved in different ways. Since bootstrap support values of these groups were not high (and noticeably lower than the support values obtained as a result of the analysis of integrated matrix), we thought it possible to consider the difference between Inter-MIR and Inter-OMIR PCR data as nonsignificant, combine the binary matrices, and discuss the integrated Inter-SINE PCR matrix.

Table 4 presents the values of Nei and Li genetic distances ($D_{NL}$) between the lineages of the *helioscopus–persicus* complex and the outgroup (*Ph. interscapularis*). These distances were based on the data of Inter-SINE PCR analysis with MIR–MIL/OMIR–

**Table 4.** Mean Nei and Li genetic distances ($D_{NL}$) between the lineages of *Ph. helioscopus–Ph. persicus* complex inferred from the data of Inter-SINE PCR analysis (integrated matrix for MIR–MIL and OMIR–OMIL primer systems). Below the diagonal, mean between-group genetic distances; on the diagonal, mean within-group genetic distances (in semi-bold type).

| | *Ph.int.* | F | C | A | H | G | I |
|---|---|---|---|---|---|---|---|
| *Ph.int.* | – | – | – | – | – | – | – |
| F | 1.65 | **0.30** | – | – | – | – | – |
| C | 1.89 | 0.44 | **0.21** | – | – | – | – |
| A | 1.76 | 0.59 | 0.46 | **0.16** | – | – | – |
| H | 1.52 | 0.61 | 0.61 | 0.68 | **0.13** | – | – |
| G | 1.57 | 0.70 | 0.63 | 0.68 | 0.49 | **0.25** | – |
| I | 1.24 | 1.18 | 1.22 | 1.26 | 1.20 | 1.25 | **0.11** |

OMIL primer systems. The within-group genetic distances at all six groups identified varied from 0.1 to 0.30 (the mean value of $D_{NL} = 0.19 \pm 0.07$). At the same time, genetic distances between individual lineages of *Ph. helioscopus* varied from 0.44 (between lineages F and C) to 0.70 (between lineages F and G) (the mean value of $D_{NL} = 0.59 \pm 0.09$). In Inter-SINE PCR analysis, Persian toad-headed agamas were represented by a single population. Because of this, estimation of intraspecific variation of *Ph. persicus* is impossible. The Nei and Li genetic distance between *Ph. helioscopus* and *Ph. persicus* constituted $D_{NL} = 1.22 \pm 0.03$), and between the representatives of the *helioscopus–persicus* complex and *Ph. interscapularis* it was somewhat higher, ranging from 1.24 to 1.89 (the mean value of $D_{NL} = 1.60 \pm 0.22$). Thus, according to Inter-SINE PCR analysis, ranges of the Nei and Li genetic distance values did not overlap.

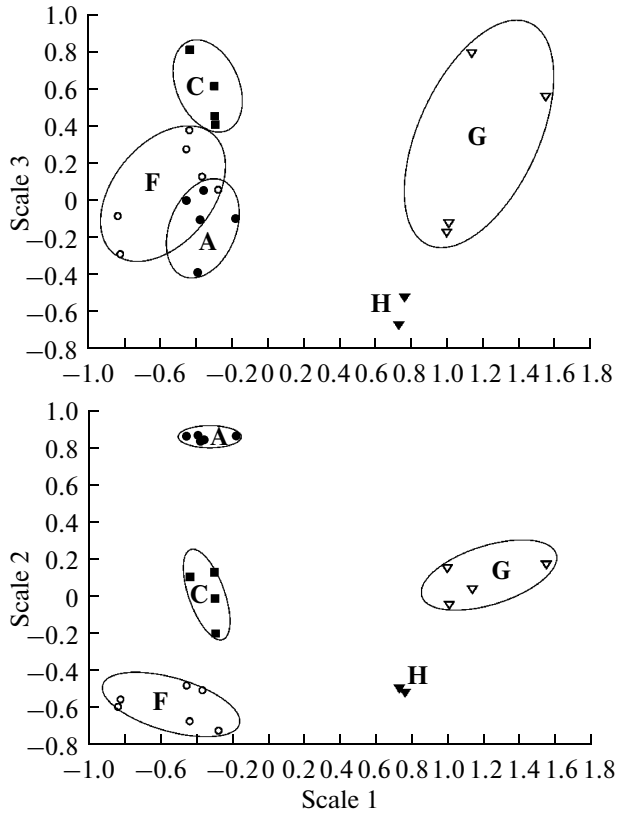


**Fig. 4.** Multidimensional scaling of the genetic variation of intraspecific phylogenetic lineages of *Ph. helioscopus* based on the analysis of 332 characters of integrated Inter-SINE PCR matrix (MIR–MIL and OMIR–OMIL primer systems) in the space of three coordinates. The considerably distant from *Ph. helioscopus* outgroup (*Ph. interscapularis*), as well as the lineages included into *Ph. persicus* were excluded from the analysis. Symbols and letters designating phylogenetic lineages of *Ph. helioscopus* correspond to those in Figs. 1 and 2.

Molecular genetic relationships are graphically represented in Fig. 4. This figure demonstrates the results of multidimensional scaling of genetic variation based on the analysis of integrated Inter-SINE PCR matrix. Due to considerable genetic isolation of certain toad-headed agama species, and to avoid false interpretation of the results, multidimensional scaling was performed only for the populations of true *Ph. helioscopus* with exclusion from the analysis of outgroup and *Ph. persicus*. As seen in Fig. 4, comparison of the first and second variation scales showed that all five lineages of sunwatcher toad-headed agama, included into the analysis, were clearly distinguishable without overlapping. These lineages formed compact grouping, which noticeably stood apart from one another. Small overlapping was observed between relatively more close lineages A and F, only upon the comparison of the first and third variation scales.

Figure 2b shows a summarized NJ dendrogram constructed based on the analysis of two Inter-SINE PCR primer systems. The topology of summarized parsimonious (MP, 217 of parsimoniously-informative out of total number of 332) and distance (NJ) dendrograms was found to be similar. This topology generally coincided with that obtained based on the data of the mtDNA phylogenetic analysis (except the lineages not included in Inter-SINE PCR analysis: clades B, D, E, L, K, and J, designated by gray color in Fig. 2a). For example, a clear tendency to the subdivision of the *Ph. helioscopus* cluster into two popula-

tion groups, "northern" (lineages A, C, and F) and "southern" (lineages G and H), could be followed.

## DISCUSSION

In our study, Inter-SINE PCR technique was first applied for analysis of the phylogeny and intraspecific differentiation of agama lizards. Earlier, this method was successfully used in analysis of phylogeny of other reptiles [30–32] and mammals [33, 34]. The Inter-SINE PCR data pointed to considerable molecular structuring of nuclear gene pool of the *helioscopus–persicus* complex, largely coinciding with the geographical structure of mitochondrial gene pool.

### *Geographic Differentiation of Sunewatcher Toad-Headed Agamas and Distribution of the Lineages Identified*

The investigations performed revealed deep divergence of the *helioscopus–persicus* complex. According to our preliminary data, most of 12 phylogenetic lineages identified are characterized by either allopatric or parapatric distribution patterns. At present, the contact zones between the lineages identified remain scarcely investigated. The possibility of sympatry between the lineages D and G in Central Uzbekistan cannot be excluded. The contacts between lineages A, C, and E on the territory of Central Kazakhstan seem to be less probable.

Two phylogroups (A and C) spread over the whole northern and eastern parts of the range. Populations of the first phylogroup are found in North Uzbekistan (Karakalpakia) and Western Kazakhstan (Cisaralia, Ustyurt, and Northwestern Kyzylkum). Populations of the second lineage inhabit the eastern part of Kazakhstan, including the northern part of Ili depression, as well as Chinese and Mongolian Dzungaria. The southern part and the east of the northern part of Ili depression are inhabited by the representative of phylogroup F. Toad-headed agamas from the outskirts of North Karatau carry a number of specific features (clade E). Populations from Uzbekistan were found to be extremely diverse and belonged to four phylogroups. At the north of the country (Karakalpakia), phylogroup A is found. Populations of *Ph. h. saidalievi* (Fergana Valley) are represented by phylogroup H, while populations from Northwest Uzbekistan (Bukhara, Dzhambul, and Navoi oblasts) belong to phylogroup D, and populations from Southeastern Uzbekistan (Sukhan-Darya oblast and the north of Bukhara oblast) belong to phylogroup G. At the same time, a sample from the outskirts of Nebit-Dag in West Turkmenia represent an independent lineage (B), relative to "northern" phylogroups A to F. Persian toad-headed agama *Ph. p. persicus* includes four phylogroups, represented by phylogroup I from Transcaucasia (Armenia, Nakhichevan, and Armavir marz), phylogroup J from Apsheron Peninsula, Azerbaijan, phylogroup K, from Northwestern and Northern Iran, and phylogroup L, from Southern Iran (outskirts of Abadeh).

### *Tentative Taxonomy of the helioscopus–persicus Complex*

Monophyly of the *helioscopus–persicus* complex is beyond question [7, 35, 36]. Phylogenetic analysis of both mtDNA and nDNA markers confirms subdivision of the complex members into two deeply isolated groups, which correspond to two independent species, *Ph. helioscopus* and *Ph. persicus*. High diversity level (specified by a great number of groups along with the high genetic distances between them [37, 38]) in the southeastern part of the range suggests that initial differentiation of the *Ph. helioscopus–Ph. persicus* complex probably started in the region of Iranian Plateau [39]. Increased diversity in the southeastern part of the range implies the existence of the second, more recent center of differentiation already within the lineage of true sunwatcher toad-headed agamas (*Ph. helioscopus*). It seems likely that evolutionary history of Persian toad-headed agamas was longer than that of *Ph. helioscopus*. This suggestion is supported by higher values of the within-group mean uncorrected *p*-distance obtained for Persian toad-headed agamas.

### *Persian Toad-Headed Agamas (Ph. persicus)*

The populations from Iran and Transcaucasia studied clustered together into monophyletic group, within which four phylogroups can be distinguished (Fig. 2). These phylogroups are represented by nominative subspecies (K); toad-headed agamas from Abadeh (L); agamas from Transcaucasia, conditionally attributed to *Ph. h. horvathi* (I); and Apsheron population (J).

Toad-headed agamas from the outskirts of the city of Abadeh inhabit the branches of the Kushkazar range in the province of Fars and represent the southernmost population of the complex. This is allopatric population of *Ph. persicus*, which is considerably distant from the nominative subspecies range, and is blocked by the mountains. It seems likely that this isolation was sufficient for the formation of an independent form, which we preliminary treat as *Ph. persicus* ssp. According to our data, Transcaucasian toad-headed agamas (*Ph. persicus horvathi*) unambiguously group together with Persian agamas (Fig. 3). Thus, attribution of these lizards to *Ph. helioscopus* in the rank of the subspecies [40] is illegal. As suggested earlier [41], the population from Apsheron peninsula (J) formed an independent lineage, which was closer to nominative (K) Persian and Transcaucasian toad-headed agamas, then to nominative *Ph. helioscopus*. Independence of Apsheron toad-headed agamas agrees with preliminary conclusion of Golubev and Mezhzherin [42], made based on allozyme analysis.

*Sunwatcher Toad-Headed Agamas (Ph. helioscopus)*

Populations earlier included into the nominative subspecies *Ph. h. helioscopus*, according to the mitochondrial and nDNA data, form polyphyletic group (A, B, D, E, and G) (Fig. 2).

*"Southern" sunwatcher toad-headed agamas.* MtDNA analysis showed that populations from the south of Central Asiaand Turan formed the following isolated groups; Fergana toad-headed agamas *Ph. h. saidalievi* (H); populations of South Uzbekistan–East Turkmenistan (G); and populations from West Turkmenistan (B). Furthermore, analysis of both molecular markers showed that populations from southeastern part of the range (phylogroup H from Fergana valley and phylogroup G from Surkhandarya oblast) form monophyletic group, definitely isolated from all the other phylogroups of *Ph. helioscopus* ("northern" populations and phylogroup B). Phylogroup from West Turkmenistan (B) is not relative to toad-headed agamas from South Uzbekistan (G) and represents an independent lineage. Population of sunwatcher toad-headed agama from Northeast Iran, as may be supposed, belongs to phylogroup of West Turkmenistan.

*"Northern" sunwatcher toad-headed agamas.* Until recently it was suggested [9, 43] that northern part of the sunwatcher toad-headed agama range was occupied by nominative subspecies *Ph. helioscopus*. Populations inhabiting the territories from the Lake Balkhash to Mongolian and Chinese Dzungaria in the east, including Alakol' depression, Zaisan depression, and (as it was supposed earlier) Ili depression, were thought to belong to *Ph. h. varius*. Molecular analysis demonstrated polyphyly of this subspecies. Specifically, populations from Ili depression were shown to be deeply isolated and not closely relative to typical *Ph. h. varius*. It seems likely, that typical *Ph. h. varius* penetrates into the valley northward of the Ili River bed, till the mountain chain of Sholak–Katutau–Altyn-Emel–Koyandytau. Northern bank populations, eastwards from these mountains (surroundings of the Akutau Mountains, population no. 39; the city of Zharkent, population no. 34) together with the right bank populations (nos. 33, 35, 37, and 38, Syugatinsk valley, Kegen, Tashkarasu) form a separate lineage. Using the name suggested by Nikol'skii for sunwatcher toad-headed agamas from Kuldja (Xinjiang, China) [44], we preliminary designated this lineage as *Ph. h. cameranoi*. Similar pattern is observed in Ili depression for another pair of closely related taxa of toad-headed agamas, *Ph. guttatus kuschakewitschi* (*Ph. kuschakewitschi*) and *Ph. g. alpherakii* (*Ph. alpherakii*) ([45], Fig. 2).

Toad-headed agamas of nominative (*Ph. h. helioscopus*) subspecies (A) are monomorphic across the whole territory occupied. The exclusion is the population from the Vozrozhdeniye Peninsula (Island) (Aral Sea) (no. 50 and the population from Ustyurt-Mangyshlak (nos. 10, 15). These poulations are most isolated. Low value of nucleotide diversity, along with unimodal mismatch distribution pattern, characteristic of the populations from phylogroup A (Table 2; Figs. 3c, 3d), points to recent expansion of the sunwatcher toad-headed agama nominative subspecies range.

Populations from the northern branches of the Karatu Mountains (south of Central Kazakhstan, phylogroup E) form an independent lineage, which is genetically isolated from eastern subspecies *Ph. h. varius* (phylogroup C), as well as from western nominative subspecies (phylogroup A). However, due to low bootstrap support values, phylogenetic position of this lineage is not determined so far.

High values of the genetic diversity measures, typical to the populations of "northern" sunwatcher toad-headed agamas from the east and south of Kazakhstan, and the number of mitochondrial lineages, identified on this territory, suggest that populations of this part of the range are relatively more ancient.

*Genetic Differentiation and Taxonomic Status of the Lineages Identified*

Mean uncorrected *p*-distances between sunwatctcher toad-headed and agamids from other genera (*Laudakia* and *Trapelus*), inferred from the *COI* fragment data, vary in the range from 19.3 to 28.6. Interspecific comparisons within the genus *Phrynocephalus* showed that minimum *p*-distance values were typical of closely related forms of taxonomically composite species complexes. For instance, between *Ph. raddei* and *Ph. strauchi* these distances are not higher than 6.5; between *Ph. raddei* and *Ph. reticulatus* they reach the value of 6.6; and between *Ph. strauchi* and *Ph. guttatus*, 8.9 (our unpublished data). The mean uncorrected *p*-distances between phylogroups of the *helioscopus–persicus* complex are often higher than the interspecies distances mentioned above (Table 3). Thus, judging by the level of differentiation mitochondrial lineages of the sunwatcher toad-headed agama complex are well comparable with the independent species of the genus *Phrynocephalus*.

The issue on taxonomic status of *Ph. h. saidalieri* deserves special attention. This form was earlier treated as an independent species [5, 46]. According to our data, the level of genetic isolation of the Fergana population from the other toad-headed agama lineages is comparable with the isolation of a number of other phylogroups of *Ph. helioscopus* (G and E). At the same time, this level is lower than for the phylogroups of *Ph. persicus* (I to L). In addition, since phylogroup H in both dendrograms presented was sister to phylogrop G, attribution of a species status the Fergana population automatically requires the same action relative to the phylogroup from Southeastern Uzbekistan. For these reasons, we consider the attribution of a species status to *Ph. h. saidalievi* as premature.

However, the mtDNA data alone are insufficient to serve as a basis for the species status. For this purpose, recruitment of the data on nDNA markers, larger sample size, and morphological data are required [47]. Short dis-

persed repeats (SINEs) are spread over the whole nuclear genome, and are the genetic markers independent from mtDNA. Fingerprint analysis performed with the help of Inter-SINE PCR technique supported monophyly of all mitochondrial groups tested (Fig. 2b). In addition, Inter-SINE PCR data definitely evidence in favor of monopyly of *Ph. helioscopus* and its isolation from *Ph. persicus*, which is considerably higher than the level of differences between the sunwatcher toad-headed agama lineages (Table 4).

In our study, the data on both mtDNA and nDNA point to the existence of the same lineages. Hence, the gene pool diversity of sunwatcher toad-headed agamas revealed cannot be explained only by linear inheritance of mtDNA. In addition, the lineages identified represent the evolutionary gropupings, i.e., the phylogroups. It should be noted that phylogenetic positions of some groups can be currently evaluated only based on mtDNA data. Nevertheless, taking into consideration the concordance of the patterns of geographic variation and not overlapping of the within- and between-group variation values for both types of molecular markers used, it can be preliminary concluded that the mitochondrial lineages identified correspond to the evolutionary isolated phyletic lineages. Independent taxonomic status of at least some of these phylogroups cannot be excluded. Clarification of this issue requires additional investigations. For thiese reasons, we preliminary recognize the existence of two polytypic species within the sunwatcher toad-headed agama complex. These species are represented by *Ph. persicus* (the species includes *Ph. p. persicus*, *Ph. p. horvathi*, and undescribed taxon from Southern Iran; taxonomic status of Apsheron population needs to be clarified) and *Ph. helioscopus* (includes *Ph. h. helioscopus*, *Ph. h. varius*, *Ph. h. cameranoi*, *Ph. h. saidalievi*, and a set of undescribed taxa from Southern Kazakhstan, Uzbekistan, and Turkmenistan). It cannot be excluded that in the light of new data, taxonomic status of the phylogroups described can be revised.

### *Intraspecific Differentiation of the Sunwatcher Toad-Headed Agamas and Biogeography of Middle Asia*

Surprisingly high number of deeply isolated phyletic lineages identified within the wide-range *helioscopus–persicus* complex is of considerable interest for the understanding of the biogeography of the region examined. Many of these lineages are characterize by narrow ranges (for example, phylogroup L from the outskirts of Abadeh, Southern Iran; phylogroup E from the surroundings of Northern Karatau; and populations of Fergana (H) and Ili (F) valleys). The distribution limits of a number of phylogroups can be predicted with certainty (as in the case of Persian and Fergana toad-headed agamas). In some cases these limits still remain unclear, and their clarification requires collection of additional material. First, the border between the phylogroups from Southeastern (G) and Northeastern Uzbekistan (D) is not established for certain. Relatively small distance between the uttermost populations examined along with the absence of visible geographic barriers between them enables suggestion on the existence of a contact zone between phylogroups G and D. Second, the border between *Ph. helioscopus* and *Ph. h. varius* remains undetermined. According to the opinion of some authors, there is wide intedrgradation zone between these species [9]. Due to scarce data from Central Kazakhstan, this proposal can be neither rejected, nor supported. Third, since only two populations of phylogroup E from Northern Karatau were available for the analysis, the limits of this phylogroup distribution can be hardly appraised.

The stretching back into antiquity age of arid biocoenoses of Central Asiaalong with far distant time of the *helioscopus–persicus* complex appearance [7, 48, 49] hamper the search for the factors determining such deep differentiation. The only currently made suggestion [7] refers to possible Middle and Late Pleistocene glaciation in Kazakhstan. Our molecular genetic data indirectly evidence in favor of more long, than it was earlier expected, evolutionary history of the *Ph. helioscopus* species complex. It is suggested that gradual intensification of orogenetic processes and caused by them progressive aridization in the Middle East and Central Asiaserved as one of the factors determining differentiation of the species complex of interest [50]. These factors could lead to the initial stages of the complex differentiation. Dispersal of sunwatcher toad-headed agamas over the vast territory of Middle Asia, as well as their intergroup differentiation could be considerably influenced by the geography of the beds of ancient rivers and their dynamics. It is suggested that that these factors played similar role in the history of dispersal and speciation of other reptile groups of Middle Asia, including *Ph. guttatus* [39].

Phylogenetic pattern presented in this study largely coincide with differentiation pattern of other reptile groups of Middle Asia, including racerunners (*Eremias*, Lacertidae), gekkonid lizards (*Cyrtopodion*, Gekkonidae), and other toad-headed agama species [39, 49, and our unpublished data]. In our study, mtDNA data were supported by the data on nDNA markers, pointing to high diversity and ancient differentiation of the wide-range lizard species investigated. It seems likely that similar phylogenetic patterns can be discovered in other reptile groups of this region.

The data obtained provided identification of isolated phylogenetic lineages within the *Ph. helioscopus–Ph. persicus* complex. The existence of most of these lineages was supported by the data of both mtDNA and nDNA markers. Four of these lineages belong to Persian toad-headed agama, and the remaining eight, to sunwatcher toad-headed agama. It is suggested, that on the territory of Turkmenistan two different forms can be found, eastern and western, while the territory of Uzbekistan is inhabited by four

independent phylogroups. Populations from the Ili River valley and the surroundings of Northern Karatau occupy isolated position in the eastern part of the range. Further development of the phylogeographic scenario for the sunwatcher toad-headed agama complex requires wider application of nuclear markers and performing of molecular timing in combination of detailed paleogeographic reconstruction of the history of Middle and Central Asia in Cenozoic.

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