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Karyotype Evolution and Phylogenetic Relationships of *Cricetulus sokolovi* Orlov et Malygin 1988 (Cricetidae, Rodentia) Inferred from Chromosomal Painting and Molecular Data

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Abstract
Sokolov's dwarf hamster (*Cricetulus sokolovi*) is the least studied representative of the striped hamsters (*Cricetulus barabensis* species group), the taxonomy of which remains controversial. The species was described based on chromosome morphology, but neither the details of the karyotype nor the phylogenetic relationships with other *Cricetulus* are known. In the present study, the karyotype of *C. sokolovi* was examined using cross-species chromosome painting. Molecular and cytogenetic data were employed to determine the phylogenetic position of Sokolov's hamster and to analyze the potential pathways of chromosome evolution in *Cricetulus*. Both the chromosome and molecular data support the species status of Sokolov's hamster. Phylogenetic analysis of the *CYTB* data placed *C. sokolovi* as sister to all other striped hamsters (sequence divergence of 8.1%). FISH data revealed that the karyotype of *C. sokolovi* is highly rearranged, with the most parsimonious scenario of its origin implying at least 4 robertsonian events and a centromere shift. Comparative cytogenetic data on Cricetinae suggest that their evolutionary history includes both periods of chromosomal conservatism and episodes of rapid chromosomal change.

Cytogenetic studies have provided important insights into the taxonomy of Palearctic hamsters (subfamily Cricetinae, Cricetidae, Rodentia), as exemplified by the case of striped hamsters (*Cricetulus barabensis* group). Traditionally, this group was considered as a single polymorphic species inhabiting a vast range in steppe and semidesert zones of Siberia, the Russian Far East, Mongolia, and China [e.g., Argyropulo, 1933]. However, sub-

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sequently, it was found to comprise 4 allopatric chromosomal races [Malygin et al., 1992]. Three of them – "*barabensis*" (2n = 20), "*griseus*" (2n = 22), and "*pseudogriseus*" (2n = 24) – differ from each other by 1 or 2 robertsonian rearrangements [Kral et al., 1984; Romanenko et al., 2007a]. Although some authors have argued that they deserve species rank [e.g., Malygin et al., 1992], they are commonly treated as subspecies of *C. barabensis* sensu lato (s.l.) [Musser and Carleton, 2005]. By contrast, the fourth taxon, *Cricetulus sokolovi* Orlov et Malygin, 1988 (Sokolov's dwarf hamster, 2n = 20), which was described as a distinct species due to its specific chromosome morphology [Orlov et al., 1978; Orlov and Malygin, 1988], retains the status of a presumptive species. This taxonomic treatment was supported by a craniometric study that demonstrated a high level of differentiation between *C. sokolovi* and *C. barabensis* s.l. compared with that of the 3 other chromosomal races [Lebedev and Lisovsky, 2008]. However, there is still an obvious lack of information on Sokolov's hamster because it is rare throughout its range (Gobi desert) and is thus poorly represented in museum collections and genetic databases. The relationships of *C. sokolovi* with *C. barabensis* s.l. and the exact nature of the chromosomal rearrangements responsible for the formation of the *C. sokolovi* karyotype remain to be elucidated.

In the present study, a comprehensive analysis of the *C. sokolovi* karyotype was performed using cross-species chromosome painting. Based on a combination of cytogenetic and molecular data, we determined the phylogenetic position of *C. sokolovi* and examined potential scenarios of chromosome evolution in *Cricetulus*.

Materials and Methods

Sampling

Hamsters from 3 localities in Mongolia were examined: the eastern and south-western (type locality) banks of the Orog-Nuur lake (referred to as West population below in the text) and the northern part of Ongon Els sands (East population) (online suppl. Table 1; see www.karger.com/doi/10.1159/000477521 for all online suppl. material). The original sampling localities and previous collecting sites of *C. sokolovi* are illustrated in online supplementary Figure 1.

Two specimens of *C. sokolovi* from the Orog-Nuur and 5 specimens from Ongon Els were karyotyped using the standard method [Ford and Hamerton, 1956] and routine staining. It should be noted that the Ongon Els site has not been studied cytogenetically previously. Chromosomal painting and C-banding were performed for 4 males from the Orog-Nuur (West) samples (CSOK1m, CSOK2m, CSOK3m, CSOK4m).

Complete *CYTB* sequences were obtained for 27 specimens of *C. sokolovi*. Additionally, 15 specimens of other species of hamsters were included in the phylogenetic reconstructions (our data and sequences from GenBank, see online suppl. Table 1). In this study, we treated *C. longicaudatus*, *C. sokolovi*, and *C. barabensis* s.l. as *Cricetulus* sensu stricto (s.s.). *Allocricetulus*, *Cricetus*, and *Cricetulus migratorius* were used as the outgroup for *Cricetulus* s.s. following previous molecular reconstructions [Neumann et al., 2006].

Cytogenetic Analysis

Primary fibroblast cell lines were established in the Laboratory of Animal Cytogenetics, the Institute of Molecular and Cellular Biology, Russia, using enzymatic treatment of tissues as described previously [Stanyon and Galleni, 1991; Romanenko et al., 2015]. The fibroblast cell lines were derived from lung and breastbone (CSOK1m) and tail biopsies (CSOK1m, CSOK2m, CSOK3m, and CSOK4m). All cell lines were deposited in the IMCB SB RAS cell bank ("The general collection of cell cultures," No. 0310-2016-0002). Metaphase chromosome spreads were prepared from chromosome suspensions obtained from early passages of primary fibroblast cultures as described previously [Yang et al., 1999; Graphodatsky et al., 2000, 2001]. C-banding was done as described by Gladkikh et al. [2016]. GTG-banding was performed on chromosomes of all 4 animals prior to FISH using the standard trypsin/Giemsa treatment procedure [Seabright, 1971]. Additionally, short-term cultures were established from bone marrow for CSOK1m [Graphodatsky and Radjabli, 1988].

The set of chromosome-specific and microdissected painting probes of the golden hamster *Mesocricetus auratus* (2n = 44) used here was described in Romanenko et al. [2006]. FISH was performed following previously published protocols [Yang et al., 1999; Graphodatsky et al., 2000]. Images were captured using VideoTest-FISH software (Imicrotec) with a JenOptic CCD camera mounted on an Olympus BX53 microscope. Hybridization signals were assigned to specific chromosome regions defined by G-banding patterns previously photographed and captured by the CCD camera. All images were processed using Paint Shop Photo Pro X3 (Corel Corporation).

DNA Analysis

DNA was isolated from ethanol-fixed tissues using the standard phenol-chloroform method and proteinase K [Sambrook et al., 1989]. Complete sequencing of the *CYTB* gene (1,140 bp) was conducted using a combination of primers and PCR conditions employed in a previous study on striped hamsters [Poplavskaya et al., 2012].

Phylogenetic Analysis

The ML tree for Cricetinae was reconstructed using TREEFINDER [Jobb, 2008]. The alignment was partitioned into codon positions, and separate substitution models were selected for each subset in TREEFINDER using the BIC criterion. Bootstrap support was estimated based on 1,000 pseudoreplicates with a substitution model and rate parameters fixed at the ML values.

The relationships among the haplotypes of *C. sokolovi* were examined using a median-joining network constructed in Network 4.6.1.1 using the Median Joining tool [Bandelt et al., 1999] with the default options.

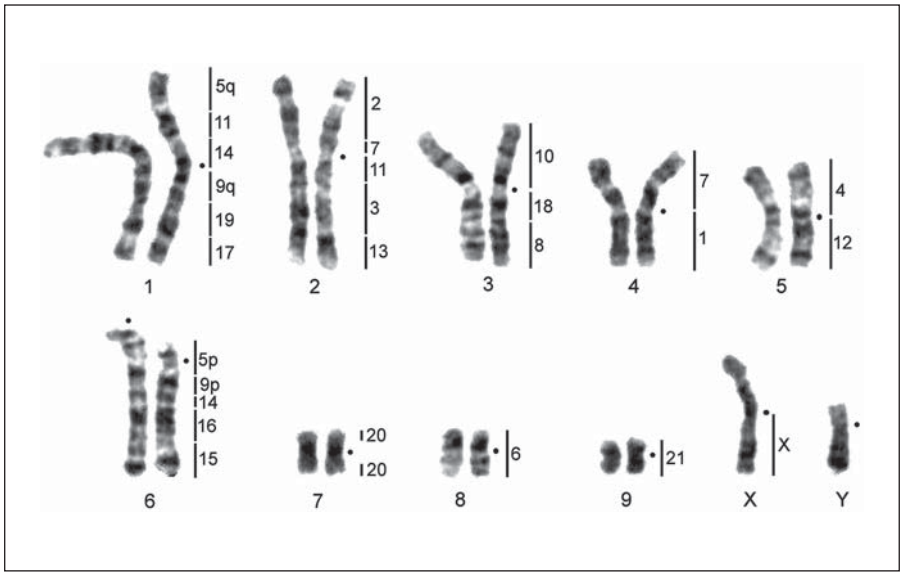

Fig. 1. Localization of the chromosomal segments of *Mesocricetus auratus* (marked by vertical bars) in the karyotype of *Cricetulus sokolovi*. The black dots mark the positions of centromeres. The nomenclature of the chromosomes follows the Atlas of Mammalian Chromosomes [Radjabli et al., 2006].

The number of haplotypes, haplotype diversity, nucleotide diversity, Tajima's *D*, and Fu's *Fs* neutrality tests for the West and East populations were calculated using ARLEQUIN, version 3.5 [Schneider et al., 2000]. The significance of the corrected average pairwise difference between the 2 populations was estimated based on 1,000 replicates.

To reconstruct the demographic history of Sokolov's hamster, a Bayesian skyline analysis was conducted in BEAST ver. 1.8.1 software [Drummond et al., 2012] using 2 runs with a chain length of 100 million steps. Convergence diagnostics and skyline plots were generated in Tracer v1.6 [Rambaut et al., 2014].

Ancestral Karyotype Reconstruction

Based on the FISH results, we reconstructed ancestral karyotypes of the *Cricetulus* s.s. clade (AKC) and those of the *C. barabensis* group (= *C. barabensis* s.l. + *C. sokolovi*) (AKCbs) under the maximum parsimony criterion. For reconstruction, we accepted the pattern of phylogenetic relationships inferred in molecular studies [Neumann et al., 2006, original results]. *Cricetus*, *Allocricetulus*, and *C. migratorius* were used as outgroups. Other genera of Palearctic hamsters were excluded from consideration due to their large phylogenetic distance from *Cricetulus* (*Mesocricetus* and *Phodopus*) or to an extremely high level of reorganization of their karyotype (*Tscherskia*). To obtain the most parsimonious reconstructions of ancestral karyotypes, we considered all plausible karyotypes for internal nodes and estimated the minimum number of rearrangements (centric fusions, fissions, and whole arm reciprocal translocations [WARTs]) required to explain the observed pattern. Optimal scenarios of chromosome evolution under different weighting schemes (fission/fusion weight ratios) were compared. Hemiplasy was not considered. We did not reconstruct the phylogenetic tree based on cytogenetic data because, if structural transformations are used as characters following the suggestion by Dobigny et al. [2004], the character state matrix in our case cannot be constructed unambiguously.

Results

Cytogenetic Analysis

The karyotypes of all *C. sokolovi* specimens examined using routine staining generally corresponded to the original description [Orlov and Malygin, 1988; Radjabli et al., 2006 as *C. obscurus*]. The karyotypes consisted of 2 pairs of large metacentric chromosomes, 1 pair of large subtelocentrics (or submetacentrics, pair 6 in Radjabli et al. [2006]), 3 pairs of medium-sized metacentrics, and 3 pairs of small metacentrics (Fig. 1). Three specimens had a heteromorphic pair 6 represented by 1 large acrocentric and 1 large submetacentric; in the fourth animal (CSOK4m), it consisted of 2 large submetacentrics. Additionally, it is necessary to note that in the Atlas of Mammalian Chromosomes, the 2nd pair of autosomes was shown in the wrong orientation – the q arm above the p arm [Radjabli et al., 2006]. The X chromosome was submetacentric and similar in size to the autosomal pair 4. The submetacentric Y chromosome was smaller than the autosomal pair 5. One of the examined specimens (CSOK3m) had an additional Y chromosome in all metaphases studied (online suppl. Fig. 2).

In all chromosomes, blocks of constitutive heterochromatin were found in the pericentromeric regions (online suppl. Fig. 2). Chromosome 6 had a small C-block in the q arm. Chromosomes 7 and 9 had large C-blocks in the pericentromeric regions of both arms. The entire p arm of the X chromosome was heterochromatic, and the Y chromosome was almost completely heterochromatic.

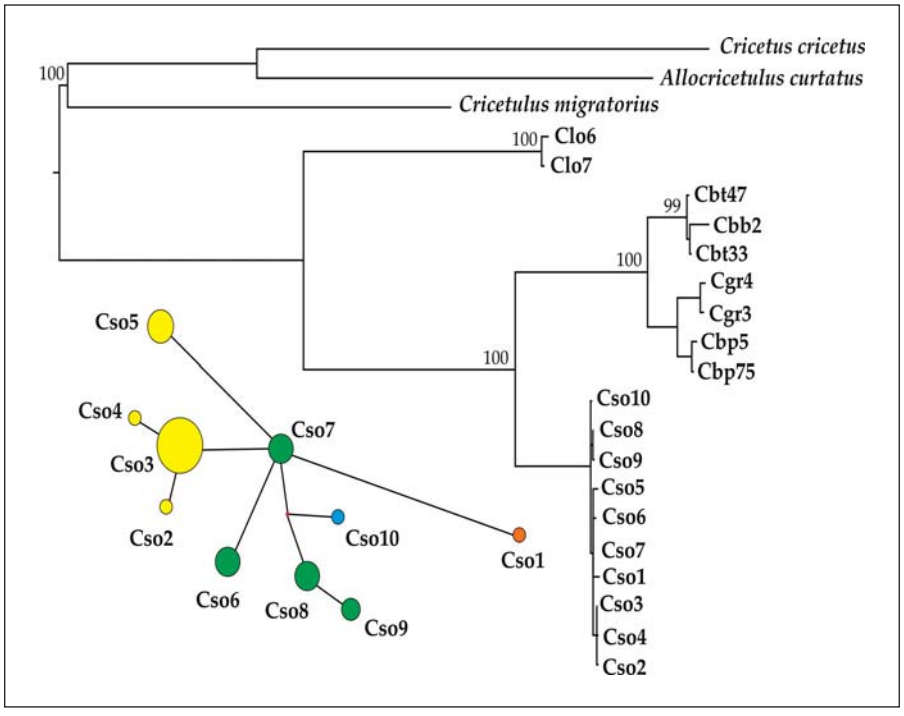

Fig. 2. ML-tree of the phylogenetic relationships (numbers on the tree correspond to bootstrap values) and a median-joining network of haplotypes of *Cricetulus sokolovi* based on complete *CYTB* sequence data. The colors on the network correspond to those in the map in online supplementary Figure 1.

Fluorescence in situ Hybridization

The following associations were revealed in the genome of *C. sokolovi* with the set of *M. auratus* (MAU) autosome-specific flow-sorted probes: MAU1/7a, 2/7b/11a/3/13, 4/12, 5p/9p/14a/16/15, 5q/11b/14b/9q/19/17, and 8/18/10 (Fig. 1). Examples of FISH are presented in online supplementary Figure 3.

Molecular Analysis

In total, 10 haplotypes of *C. sokolovi* were found (online suppl. Table 1). In the ML-tree (Fig. 2), all of them were grouped together in a highly supported cluster that is placed as sister to *C. barabensis* s.l. The mean nucleotide diversity between *C. sokolovi* and *C. barabensis* s.l. was equal to 8.1%, whereas that between *C. sokolovi* and *C. longicaudatus* was close to 13%. The inferred relationships between *Cricetulus* and other genera are consistent with those of previous results [Neumann et al., 2006].

On the western bank of the Orog-Nuur Lake, 4 haplotypes were found (Cso2, Cso3, Cso4, and Cso5), among which Cso3 was the most common. At the same time, the specimen from the eastern bank of the Orog Nuur Lake had a unique Cso1 haplotype, which was rather distant from other haplotypes (Fig. 2). It should be noted that a distance of only 30 km separates the 2 localities. Four other haplotypes (Cso6, Cso7, Cso8, and Cso9) were detected in the sample from Ongon-Els sands. No shared haplotypes between the West and East populations were found.

Among the *CYTB* sequences available from the GenBank and identified as "*C. griseus*," one sequence (AB033693) was grouped with *C. sokolovi* (haplotype Cso10, online suppl. Table 1). No individuals with this haplotype were found in the other samples under study. Unfortunately, the geographical origin of this sequence is unknown.

Haplotype (h) and nucleotide (p) diversity in the populations of *C. sokolovi* were as follows: West, $h = 0.65 \pm 0.1$, $p = 0.005914 \pm 0.0033$; East, $h = 0.82 \pm 0.062$, $p = 0.002 \pm 0.001344$.

The West and East populations are separated by the average inter-haplotype distance of 0.53%, which corresponds to a net-distance of 0.137%, $p < 0.0001$.

Tajima's and Fu's tests did not reject the hypothesis of population stability for both samples. Demographic analysis based on skyline plots also revealed no large changes in the effective population number over time.

Ancestral Karyotype Reconstruction

The reconstruction of the ancestral karyotypes was based on the following logical steps:

1. According to the molecular data, the sister group to *Cricetulus* s.s. is the clade comprising *Cricetus*, *C. mi-*

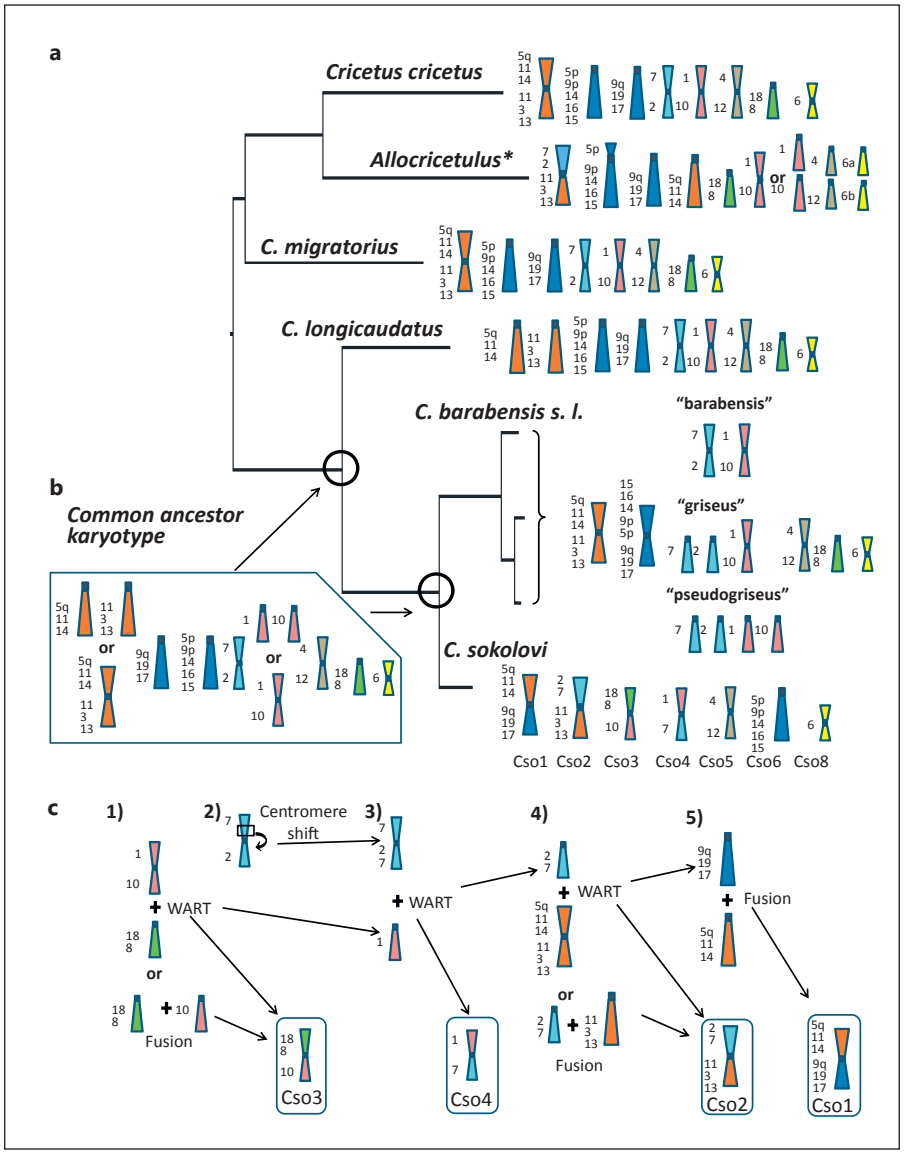

Fig. 3. a Phylogenetic relationships of *Cricetulus sokolovi* and other taxa of Cricetinae (based on mitochondrial data) and their karyotypes with marked elements of the *Mesocricetus auratus* genome (on the left) according to Romanenko et al. [2007a]. * Ancestral karyotype of *Allocricetulus* [according to Romanenko et al., 2013]. The nomenclature of the *C. sokolovi* chromosomes (Cso1–Cso8) follows the Atlas of Mammalian Chromosomes [Radjabli et al., 2006]. **b** AKC (equal to AKCbs). **c** Possible paths of *C. sokolovi* karyotype formation from AKC. 1–5, sequence of rearrangements.

gratorius, and *Allocricetulus*. The karyotypes of the former two consist of the same set of associations, whereas that of *Allocricetulus* is significantly rearranged [Romanenko et al., 2013]. The most parsimonious scenario implies that the ancestral karyotype for this clade (AKACCm) is essentially equivalent to those of *Cricetus* and *C. migratorius*.

2. The comparison of karyotypes of *Cricetulus* s.s. species and AKACCm demonstrated that chromosomal evolution in this group was predominantly robertsonian, with nearly all chromosomal arms retaining their integrity. The only exception was the element MAU7 in the *C. sokolovi* karyotype (see below).
3. Several autosome pairs are invariably present in the karyotypes of all *Cricetulus* s.s. and AKACCm and are thus considered part of AKCbs and AKC. These include MAU4/12, MAU6, MAU20, and MAU21.
4. The association between the synteny blocks MAU9q/19/17 and 15/16/14/9p/5p is a synapomorphy of *C. barabensis* s.l. The most parsimonious scenario suggests that the 2 blocks are present as separate chromosomes in both AKCbs and AKC.
5. The element MAU8/18 does not form any association in all taxa other than *C. sokolovi*; therefore, it should be included as a separate chromosome in both AKCbs and AKC.

6. The element MAU2/7 was found in *C. sokolovi* in association with MAU11/3/13. However, a part of MAU7 is dissociated from the former and joins MAU1. A plausible explanation of this pattern implies the presence of MAU2/7 as a separate metacentric in AKCbs (and AKC) with a subsequent centromere shift (repositioning or pericentric inversion) in the *C. sokolovi* lineage followed by 2 robertsonian events.
7. In *Cricetulus* karyotypes other than *C. sokolovi*, the synteny blocks MAU1, MAU10, MAU5q/11/14, and MAU11/3/13 are present as either metacentrics MAU1/10 and MAU5q/11/14/11/3/13 or as acrocentrics. Maximum parsimony reconstructions for the states of these elements in AKCbs and AKC are ambiguous depending on the weight of fissions relative to fusions, which are hard to determine objectively.
8. Regardless of the state of MAU1, MAU10, MAU5q/11/14, and MAU11/3/13 in AKCbs, the transition from AKCbs to the karyotype of *C. sokolovi* requires 4 robertsonian changes (and a centromere shift/inversion). If the above elements are represented by 2 metacentrics, the scenario includes 3 WARTS and 1 fusion. If these elements are present as acrocentrics, 1 WART and 3 fusion events are postulated. Thus, if the weights of WARTs and fusions are equal, the reconstruction of the optimum AKCbs depends only on the changes assumed for other branches. A potential sequence of rearrangements is illustrated in Figure 3.
9. Concerning the scenario for the entire tree, if the ratio of fission weight to fusion weight is less than 2 (e.g., if they are weighted equally), both AKCbs and AKC contain MAU1/10 and 5q/11/14/11/3/13 as metacentrics and are thus equivalent to AKACCm.

In summary, according to the results of our reconstructions, the ancestral karyotype of *Cricetulus* s.s. contains the following associations: MAU5q/11/14/11/3/13 (or separate 5q/11/14 and 11/3/13), 9q/19/17, 5p/9p/14/16/15, 2/7, 1/10 (or separate 1 and 10), 4/12, 8/18, 6, 21, and 20 (Fig. 3).

Discussion

The previous cladistic analysis of chromosomal rearrangements performed for a wider sample of hamsters did not resolve unambiguously the relationships between species of the *C. barabensis* species group [Romanenko et al., 2007a]. This could be accounted for by a relatively low number of chromosomal changes among these species and by the types of the changes detected. Following Dobigny et al. [2004], chromosomal rearrangements as robertsonian translocations and WARTs could be uninformative for reconstruction of chromosomal phylogeny. In particular, if both robertsonian translocations and WARTs could occur during chromosomal evolution, it may be impossible to choose which transformation should be used as a cladistic character [Dobigny et al., 2004]. This is precisely the situation which was found in Palearctic hamsters [Romanenko et al., 2007a] and, particularly, *C. sokolovi*.

Previously, *C. sokolovi* was included in comparisons using G-banding data only [Romanenko et al., 2007a]. It was shown that *C. sokolovi* was the sister group to the genus *Allocricetulus*, whereas other striped hamsters were closer to *Cricetus cricetus* and *C. longicaudatus*. The *Allocricetulus* + *C. sokolovi* clade was supported by 2 putative synapomorphies: the presence of a unique association MAU2/7/11/3/13 and fission of MAU6. However, the subsequent analysis of chromosomal painting data for *Allocricetulus* [Romanenko et al., 2013] and *C. sokolovi* (this study) demonstrated that the association of MAU2/7/11/3/13 is not completely identical between the 2 lineages. In contrast to *Allocricetulus*, the karyotype of *C. sokolovi* contains 2 fragments of MAU7, one of which is part of the MAU2/7/11/3/13 chromosome, while the other forms an association with MAU1. Apparently, the syntenies MAU2/7/11/3/13 in *Allocricetulus* and MAU2/7b/11/3/13 in *C. sokolovi* emerged independently. In addition, now it is clearly shown that the *C. sokolovi* karyotype includes a single element homologous to MAU6 and does not contain the associations MAU1/18/8, 10/20, contrasting what was reported previously [Romanenko et al., 2007a]. The fragmentation of MAU7 and the presence of associations MAU1/7a and 8/18/10 are unique features of *C. sokolovi* among Cricetinae. Another interesting result revealed by ZOO-FISH analysis was the presence of the synteny MAU5q/11/14 + 9q/19/17 in the karyotypes of *C. sokolovi* and *Allocricetulus curtatus*. As this synteny was not found in another *Allocricetulus*, one can suggest that the observed pattern can be explained by convergent evolution rather than shared ancestry. This finding highlights the importance of parallelisms in chromosomal evolution.

Our results of the molecular phylogenetic analysis demonstrated that *C. sokolovi* is the sister group of *C. barabensis* s.l. The level of differentiation between these lineages is substantially higher than that among the chromosomal races "*barabensis*," "*pseudogriseus*," and "*griseus*" but lower than that between the *C. longicaudatus* and *C. barabensis* groups. The mean genetic distance be-

tween the *CYTB* haplotypes of *C. sokolovi* and *C. barabensis* s.l. (8%) falls within the range observed for congeneric sister species in Rodentia [Bradley and Baker, 2001]. The inferred phylogenetic position of *C. sokolovi* is in good agreement with the results of the craniometric study [Lebedev and Lisovsky, 2008], which produced an identical pattern of similarity/dissimilarity among *C. sokolovi, C. longicaudatus,* and subtaxa within *C. barabensis* s.l.

By contrast, the comparative cytogenetic data obtained here with FISH highlight the outlying position of *C. sokolovi* within *Cricetulus*. Although the karyotype of *C. sokolovi* was found to contain the associations MAU11a/3/13, 4/12, 5p/9p/14a/16/15, 5q/11b/14b, 9q/19/17, and 8/18 detected previously in the karyotype of all forms of *C. barabensis* s.l. and some *Allocricetulus,* we have not revealed associations specific exactly for *C. sokolovi, C. barabensis* s.l., and *C. longicaudatus*. Regardless of the ambiguities in ancestral karyotype reconstruction, the most parsimonious scenario suggested that the karyotype of *C. sokolovi* was highly rearranged, implying 4 robertsonian events (WARTS and fusions: MAU1 + 7, 2/7 + 11a/3/13, 5q/11b/14b + 9q/19/17, and 8/18/10) and a pericentric inversion/centromeric shift (in MAU2/7) as the cause of the difference between the ancestral karyotype of the *C. barabensis* group (AKCbs) and *C. sokolovi*. This result provides decisive support for the species status of Sokolov's hamster. The number of rearrangements inferred for other branches of the *Cricetulus* tree was relatively low; thus, the ancestral karyotype of *Cricetulus* (AKC) is identical to AKCbs and, moreover, may be identical to the ancestral karyotype of its sister clade (*Cricetus* + *Allocricetulus* + *C. migratorius*).

Comparative cytogenetic data on Palearctic hamsters highlight that periods of rather slow chromosomal evolution (or even stasis) characteristic for some segments of the Cricetinae tree alternate with periods of rapid chromosomal change in other lineages. Rapid chromosome evolution is exemplified by the highly rearranged karyotypes of *Tscherskia triton* [Romanenko et al., 2007a], the genus *Allocricetulus* [Romanenko et al., 2013], and *C. sokolovi* (this study). Moreover, the data on *C. sokolovi* indicate that even within a compact genus, such as *Cricetulus*, variation in the rates of karyotype evolution can be substantial. Similar cases are represented by the arvicoline genera *Ellobius* [Romanenko et al., 2007b], *Microtus* (subgenus *Alexandromys*) [Lemskaya et al., 2010], and *Lasiopodomys* [Gladkikh et al., 2016].

One may hypothesize that life history traits of *C. sokolovi* are at least partly responsible for the rapid chromosomal evolution in this species. In contrast to predominantly steppe-dwelling representatives of *C. barabensis* s.l., the range of *C. sokolovi* is restricted to the arid semidesert zone of Mongolia. However, a high population density was observed only in oases, which are often separated by long (~100 km) distances. A patchy habitat promotes isolation between demes that can be an important factor for the quick fixation of chromosomal rearrangements in small local populations [Bush et al., 1977; Britton-Davidian et al., 2000; Dobigny et al., 2002; Kawada et al., 2008]. Consequently, chromosomal divergence could contribute to the emergence of an effective reproductive barrier between *C. sokolovi* and other striped hamsters.

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Statement of Ethics

All experiments were performed in accordance with the European Community Council Directive of September 22, 2010 (2010/63/EU) and were approved by the Committee on the Ethics of Animal Experiments of the Institute of Molecular and Cellular Biology (IMCB) SB RAS, Russia.

Disclosure Statement

The authors declare that they have no competing interests.

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