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GENERAL BIOLOGY

Taxonomic Interpretation of Allopatric Mammalian Forms on the Example of Two Karyoforms of *Microtus (Terricola) subterraneus* (Rodentia, Arvicolinae) from Eastern Europe

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Abstract—New European pine vole records from the Novgorod, Kaluga, Voronezh, and Belgorod oblasts were studied by sequencing of the mtDNA cytb gene (1143 bp) and by karyotyping (routine staining and G-banding techniques). The results enabled us to summarize chromosome variability of this species throughout Eastern Europe. In the sample studied, two geographically replacing chromosomal forms have been identified: northern, $2n = 54$ (Novgorod and Kaluga oblasts), and southern, $2n = 52$ (Voronezh and, presumably, Belgorod oblasts). Our data make the boundaries of these two karyoforms in Eastern Europe more precise and testify to intraspecific level of their taxonomic differentiation.

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To determine the taxonomic status of the allopatric mammalian forms (species or subspecies of a polytypic species), the species concept adopted by a researcher is of great importance. The most widely used in theriology is the biological concept suggesting that species are the groups of really or potentially inbred natural populations which are reproductively isolated from other similar groups [1]. In the case of the allopatric population distribution, the use of the biological species concept obviously depends on a subjective decision. In practice, this concept is often used when essential chromosomal differences characteristic of the "good" sympatric species of a given group are found in allopatric forms [2]. In recent years, the genetic species concept formulated in 1909 became widely spread in mammalian systematics. According to this concept, species identification depends on the genetic rather than reproductive isolation [3]. The genetic species concept development for almost a century has been described in [4], where, along with the problems of species and speciation, the practical use of genetic species concept is discussed. It is emphasized that the genetic concept is based on a search for correlations between genetic distances and the level of taxonomic differentiation in mammals. To clarify the taxonomic status of the allopatric populations using the genetic species concept, a test has been proposed which is based on analysis of the nucleotide sequence variability of the mtDNA cytochrome *b* gene (*cytb*) in several members of four mammalian orders. This test determines how genetic distances correspond to different levels of taxonomic differentiation, but the authors emphasize that differences of other genetic markers should be taken into account simultaneously. The use of the above-mentioned concepts for taxonomically complex and, in particular, highly genetically polymorphic species and species complexes is of unconditional interest.

The European pine vole *Microtus (Terricola) subterraneus* s. lato is a former polytypic species that inhabits a mosaic Eurasian area extending from Asia Minor and the Balkan Mountains to the Onega region and Estonia (south to north), and from countries of Southern and Southwestern Europe to Left Bank Ukraine, right bank of the Don, and the Middle and Upper Volga regions (west to east). Within this species, up to 25 subspecies were differentiated [5], many of which (*majori, daghestanicus, multiplex, likhtensteini,* et al.) have been subsequently recognized as independent karyologically discrete species (Niethammer and Krapp, 1982, cited from [12]). In addition, in the European pine vole proper, *M. (T.) subterraneus* s. str., the intraspecific (including geographic) karyotypic variability has been found by

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Table 1. Diploid chromosome sets of European pine voles from Eastern Europe

The sample number	Country, region	The place of catching	Coordinates		$2n$	Source
			N	E		
1	Russia, Leningrad oblast	In the vicinity of Boksitogorsck town	59.473	33.836	54	Sablina et al.., 1989, cited from [12]
2	Poland, northern-eastern part	Belavezha	52.698	23.846	54	Jordan et al., 1971, cited from [12]
3	Russia, Tver oblast	In the vicinity of the Seliger Lake	56.70	33.83	54	[13]
4	Tver oblast	In the vicinity of Bubonitsy village, Toropetski district	56.73	31.512	54	[12]
5	Bryansk oblast	In the vicinity of Savichki village, Novozybkovski district	52.43	31.86	54	[12]
6*	Novgorod oblast	Valdai National Park, in the vicinity of Lake Krenye	57.977	33.367	54	Our data
7*	Kaluga oblast	Kaluzhskie Zaseki, in the vicinity of Nagoe village	53.567	35.742	54	Our date
8	Ukraine, Kiev oblast	In the vicinity of Kiev city	50.39	30.39	52	[11]
9	Cherkassk oblast	Kanevsky Reserve	49.723	31.530	52	[11]
10	Zakarpatska oblast	Petros mountain, Eastern Carpathians	48.173	24.420	52	[11]
11	Zakarpatska oblast	Foot of the Pozhyzhevskaya mountain, Eastern Carpathians	48.14	24.49	52	[11]
12	Invano-Frankovsk oblast	Weather station at the mountain Pozhyzhevskaya, Eastern Carpathians	48.15	24.50	52	Baskevich, 1997, cited from [12]
13	Chernovitsa oblast	In the vicinity of Chernavka village	48.418	26.010	52	[12]
14	Poland, South-eastern part	Bieszczady Mountains, (Eastern Carpathians)	49.283	22.483	52	Jordan et al., 1971, cited from [12]
15	Russia, Voronezh oblast	Falenberg Field	51.878	39.664	52	[12] and our data
16*	Voronezh oblast	The right bank of the Usmanka River	51.937	39.684	52	[12] and our data
17	Ryazan oblast	Shatsk	53.83	41.5	52	[13]
18	Penza oblast	Zametchino	53.33	40.67	52	[13]

* Samples used for molecular genetic analysis.

karyotyping. For instance, in the European part of this species area, two chromosomal forms have been determined with $2n = 52$ (southern) and $2n = 54$ (northern); in Asia Minor, a special geographically isolated 54-chromosome form has been identified, which differed in some karyotype features from the 54-chromosome form of the Northern Europe [6]. The species form with 52 chromosomes, which inhabits broad-leaved forests of the south of Eastern Europe, has been recognized as a separate species *M. (T.) dacius* Miller, 1912, whereas, according to this view, the 54-chromosome form of the north Eastern Europe has remained in the species *M. (T.) subterraneus* s. str. [7].

The objective of this study was to clarify the distribution of the two chromosomal forms of the European pine vole *M. (T.) subterraneus* s. str. in Eastern Europe, as well as to assess the level of their karyological and molecular-genetic differentiation and determine their taxonomic status using both biological and genetic species concepts. In addition, we studied the molecular genetic difference between the Eastern Europe populations of this species on the one hand and between them and populations of Southwestern Europe and Asia Minor on the other hand.

Our own material of the European pine vole from Eastern Europe included 13 specimens from four localities of the Novgorod oblast (Valdai National Park, the vicinity of Lake Krenje; nos. 11-56, 11-57, 11-59, 13-70), Kaluga oblast (Kaluga Zaseki, a neighborhood of the Nagoe village; nos. 13-146, 13-179, 13-180, MS-1, MS-2), Voronezh oblast (the right bank of the Usmanka River; 03-67, 03-192), and Belgorod oblast (Belogor'e, Yamskaya Steppe; nos. 694, 696). In all specimens, the complete nucleotide sequences

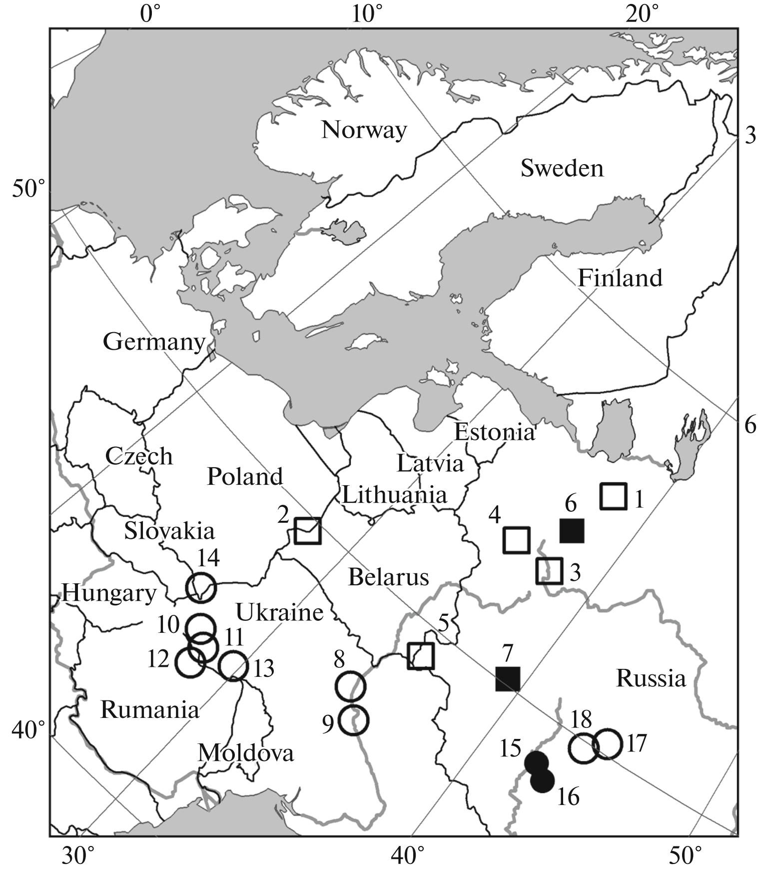

Fig. 1. Locations of two geographically replacing chromosomal forms of the European pine vole: with $2n = 54$ (square) and with $2n = 52$ (circle) in Eastern Europe. The figures marked near symbols using AreGis software correspond to numeration in the table. Our own records are shown in black.

of the mtDNA *cytb* gene were determined (1143 bp); karyotyping was made for 11 specimens (except for the voles from Belgorod oblast). Along with our own material, the following nucleotide sequences published earlier and those from the GenBank database [8] were included in the molecular genetic analysis: the *cytb* gene sequences of pine voles from Austria (AY513833, Glocknerhaus), Greece (AY513832, Seli) and Turkey (AY513834, AY513835, Çiğlikara). The *cytb* sequences of the pine voles *M. (T.) majori* (AY513814, Damar) and *M. (T.) daghestanicus* (AY513792, Handere) from Turkey served as an outgroup.

Air-dried slides of chromosomes from bone marrow cells were obtained using the standard technique. Analysis of the chromosome G-banding pattern was performed by Seabright's method [9]. DNA isolation and amplification, as well as the *cytb* gene sequencing, have been described earlier [8]. The molecular genetic data were processed statistically using the Mega 6.06 software [10]. Selection of the nucleotide substitution model was performed according to Bayesian Information Criterion (BIC). Dendrograms constructed by the maximum likelihood (ML) method and genetic distances (D) were calculated after the Tamura–Nei model with γ-distributed (TN93+G). Bootstrap values were determined from 1000 replications.

As determined by karyotyping of specimens from the Novgorod and Kaluga oblasts and those from Voronezh oblast, they belonged to the 54-chromosome and 52-chromosome forms, respectively. Our own and published data [11–13] on cytogenetic diagnosis of the two Eastern European chromosomal forms of European pine vole can be seen in Table 1 and Fig. 1. The results obtained provide additional information about their allopatric habitats and outline more exactly the southern boundary of the northern and northern boundary of the southern forms ($2n = 54$ and $2n = 52$, respectively). In general, our study confirmed the presumable scheme of the chromosomal forms isolation in Eastern Europe [11]. The species status of each form of European pine vole in Eastern Europe was postulated by Zagorodnyuk [7] who suggested that differences in ecology (they populated different stations and differed in abundance, fertility, and other demographic parameters) testified to their species independence. At the same time, we have found in this study that cytogenetic differences between two

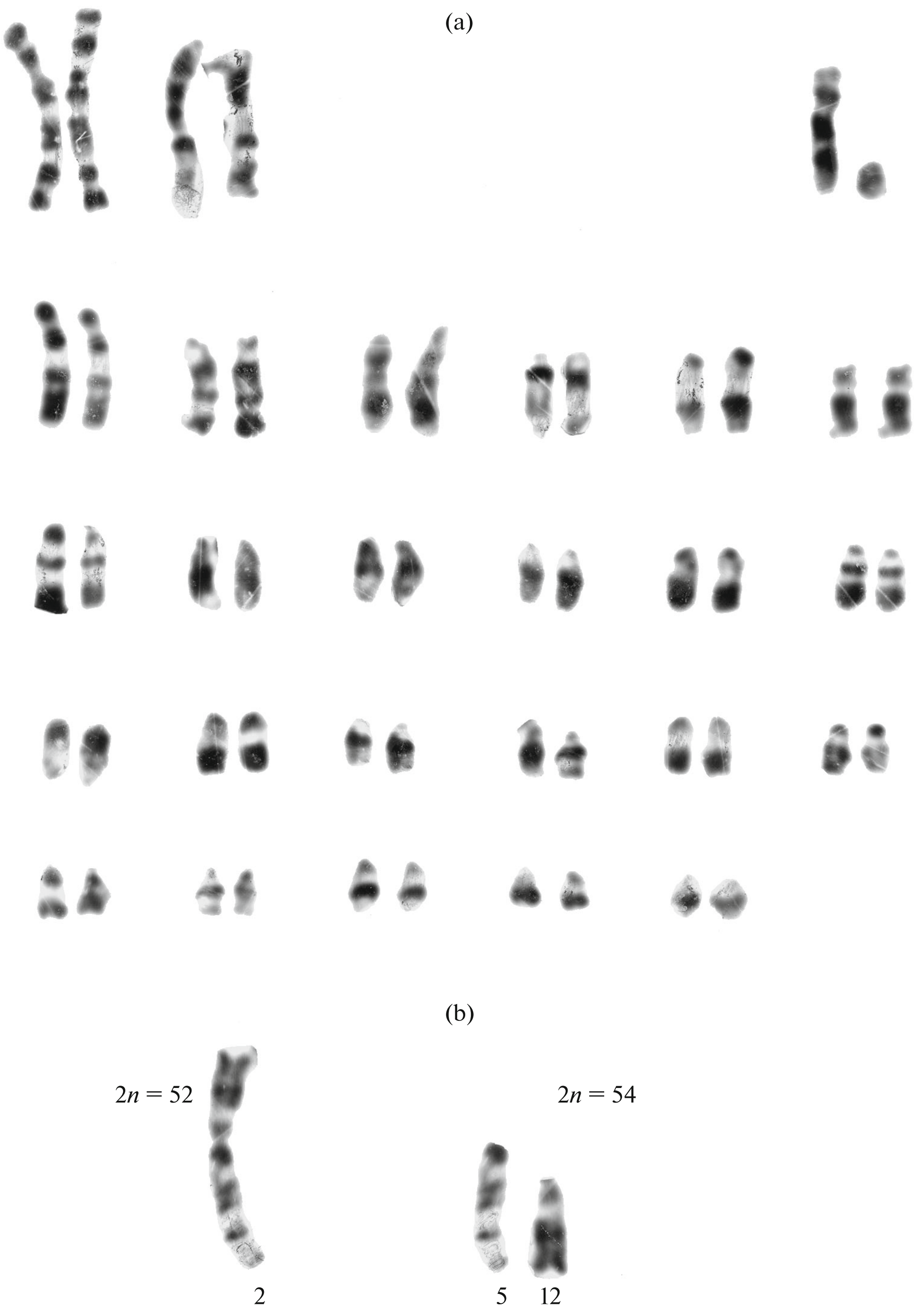

Fig. 2. (a) G-banding of the 52-chromosome karyotype of a male from the right bank of the Usmanka River, Voronezh oblast; (b) Robertsonian translocation that differentiates 52-chromosome and 54-chromosome forms (on the left and right, respectively) of the European pine vole from Eastern Europe.

karyoforms of the European pine vole from Eastern Europe depend on a single chromosomal rearrangement, Robertsonian translocation (Fig. 2), which is insufficient for their reproductive isolation. This is also confirmed by the results of experimental hybridization [14] and by data on Robertsonian polymorphism (Robertsonian fan) of a close species, Daghestan vole, *M.* (*T.*) *daghestanicus* [15].

In the framework of the genetic concept, the taxonomic status of the two allopatric chromosomal forms of the European pine vole from Eastern Europe was assessed by comparing the nucleotide sequences of

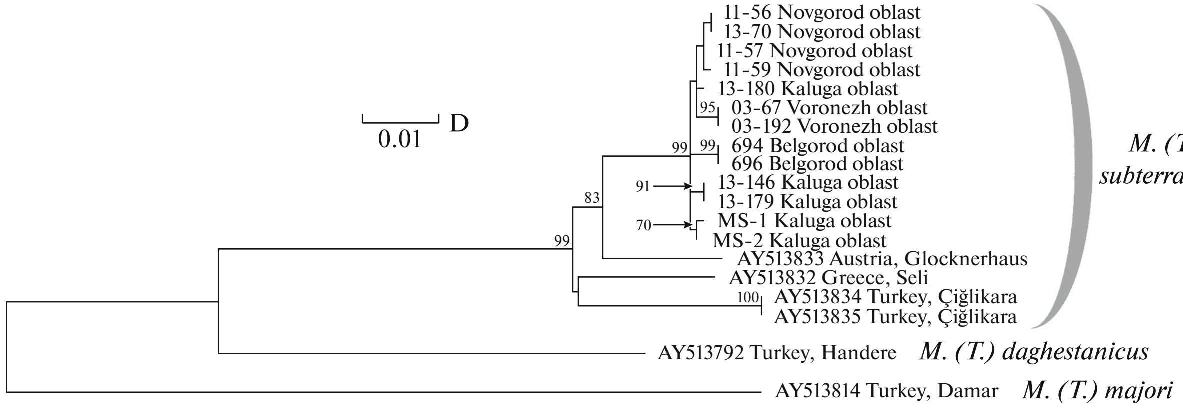

Fig. 3. ML dendrogram constructed by comparing the complete nucleotide sequences of *cytb* gene (1143 bp) of *M. (T.) subterraneus* from Eastern Europe, Southwestern Europe and Anatolia, as well as by comparing *M.* (*T.*) *daghestanicus* and *M.* (*T.*) *majori*. The bootstrap values exceeding 70% are given in the nodes of dendrogram branching.

their *cytb* genes (Fig. 3). The molecular genetic data confirmed the results of chromosomal analysis and showed that the level of differentiation between the two European pine vole karyoforms corresponded to the intraspecific level ($D = 0.005$) without their clear-cut separation on a dendrogram. At the same time, the individuals of a single haplogroup including both chromosomal forms of European pine vole from Eastern Europe differed markedly from individuals of the same species from Anatolia ($D = 0.037$), Austria ($D = 0.026$), and Greece ($D = 0.030$).

Thus, our analysis of two genetic traits of the European pine vole allopatric forms from Eastern Europe have shown that they are intraspecific forms. The use of neither biological nor genetic concepts has confirmed the species level of these two forms hypothesized earlier [7].

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