ISSN 1062-3590, Biology Bulletin, 2019, Vol. 46, No. 4, pp. 353–360. © Pleiades Publishing, Inc., 2019.
Russian Text © The Author(s), 2019, published in Izvestiya Akademii Nauk, Seriya Biologicheskaya, 2019, No. 4, pp. 380–388.

BIOCHEMISTRY

Protein Polyacrylamide Gel Electrophoresis as a Common Vole Sibling Species Identification Method (*Microtus arvalis* PALLAS, 1779 and *M. rossiaemeridionalis* OGNEV, 1924 (Rodentia, Cricetidae))

I. A. Zhigarev[a, b, *], D. I. Zhigarev[a, c], V. V. Alpatov[a, b], V. V. Lapkovskii[b], V. M. Malygin[d], and S. V. Simak[e]

[a] *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, 119071 Russia*
[b] *Moscow State Pedagogical University, Institute of Biology and Chemistry, Moscow, 129164 Russia*
[c] *Pirogov Russian National Research Medical University, Moscow, 117997 Russia*
[d] *Moscow State University, Faculty of Biology, Moscow, 119234 Russia*
[e] *Nayanova Samara State Regional Academy, Samara, 443001 Russia*
**e-mail: i.zhigarev@gmail.com*

Received October 16, 2017; revised January 30, 2018; accepted March 22, 2018

Abstract—A new protocol for the identification of two sibling species of voles *Microtus arvalis* and *M. rossiaemeridionalis* was proposed by electrophoresis of blood proteins in polyacrylamide gel (PAGE). Animals captured in ten districts of Moscow, Kaluga, and Samara oblasts and Moscow natural territories (1030 individuals), as well as those taken from vivarium collections (five hybrids of the two species), were studied. A comparison of electrophoresis in PAGE, agarose gel, and cellulose acetate plates was carried out. The use of different organs and tissues for species identification was assessed. The relative electrophoretic mobility and the mass of a species-specific blood protein of the southern vole were determined.

DOI: 10.1134/S1062359019030154

INTRODUCTION

The existence of sympatric and, sometimes, symbiotopic, but genetically closed to one another population systems, the representatives of which appear to researchers to be extremely similar and, apparently, use the habitat in the same way, is of interest to both taxonomists and ecologists and evolutionists. Using this natural phenomenon, many problems can be solved. However, the identification of sibling species of the common vole *Microtus arvalis* s.l. is a difficult issue. Currently, in order to recognize two sympatric and symbiotopic closely related vole species, the common vole *M. arvalis* Pallas, 1779 and the southern vole *M. rossiaemeridionalis* Ognev, 1924 (= *M. levis* Miller, 1908; the priority of this name requires additional research to be justified), four groups of methods exist.

The cytogenetic method is reliable for determining these species (Meyer *et al.,* 1969; Malygin, 1970, 1983; Orlov and Malygin, 1974; Zagorodnyuk, 1991a; Baskevich *et al.,* 2016). Voles have different sets of chromosomes: their numbers and forms differ. In the karyotype of *M. rossiaemeridionalis* ($2n = 54$), acrocentric chromosomes prevail (the number of chromosome arms (NF) is 56), and in *M. arvalis* ($2n = 46$) submetacentric and metacentric (chromosomal forms "arvalis" (NF = 84) and "obscurus" (NF = 72)) predominate. There is evidence of relatively wide variability of NF in the karyoforms "arvalis" and "obscurus," which is due to the different number of observed acrocentrics in the chromosome set, which does not interfere with reliably distinguishing 54- and 46-chromosome species (Malygin, 1983; Zagorodnyuk, 1991a, etc.). The disadvantages of the method include its complexity, duration, and the need to have original living material for the use of bone marrow cells. In environmental studies, this method of identifying species can be used only when working with initially living objects (the method of tagging and recapture, laboratory experiments). The cytogenetic method does not allow identifying voles obtained by crushers and cylinders.

Until recently, the morphometric method was not considered reliable at all. No more or less distinct, discrete morphological features have been found yet that could be used to distinguish the southern and common voles (Malygin, 1978, 1983; Zagorodnyuk, 1991b, etc.). The use of mathematical methods, including the discriminant analysis of exterior, craniometric (Meier and Dityatev, 1989; Malygin and Panteleichuk Santos Luis, 1996), and odontological features (Markova *et al.,* 2003; Markova *et al.,* 2010), allows researchers to distinguish these species with some caution. How-

ever, the differences between the sibling species revealed in the above papers are more of a "tendency." The proportion of errors in determining the species is quite high (20–25%), which makes it possible to differentiate the species only with a certain probability. Thus, even a statistical analysis of a variety of morphological features should be recognized as an unreliable method.

Convincing differences between sibling species and the evaluation of intraspecific polymorphism are demonstrated by molecular genetic methods. One of them is the evaluation of restriction fragment polymorphism (RFLP) of DNA (Kalendar' and Glazko, 2002; Juskeviciute and Paulauskas, 2003), based on processing the isolated DNA with various restrictases, with further separation of restriction fragments by electrophoresis and their hybridization with specific DNA probes. Another group of methods is the analysis of anonymous sequences using the polymerase chain reaction (RAPD, AFLP, and ISSR), where specific primers are used, and discrete DNA amplification products are obtained and analyzed. For the voles of the species complex *M. arvalis* s.l., in various studies, both nuclear and mitochondrial DNA (mtDNA) analysis was used (Potapov *et al.,* 1999; Fink *et al.,* 2004; Baskevich *et al.,* 2009, 2012; Bulatova *et al.,* 2010). Since 2004, the popularity of DNA barcoding has been growing, using short specific markers from the reference genome regions to determine the taxonomic position of the organism under study. For this purpose, a DNA library of reference genome regions is being created (Galan *et al.,* 2012).

The deciphered mtDNA sequences of these species in the GenBank database (NCBI) will make it possible to develop promising methods for differentiation of sibling vole species in the future.

Hemoglobin electrophoresis has become the most promising, moderately costly, and reliable method for differentiating closely related species of common voles (Dobrokhotov and Malygin, 1982). Species identification is based on the fact that the hemoglobin of the common vole has one broad (major) protein fraction migrating during electrophoresis, whereas the hemoglobin of the southern vole has two: one wide, similar to the previous species, and one minor, narrower, and more mobile (therefore, migrating slightly farther in electrophoresis).

The technique of horizontal cell electrophoresis on cellulose acetate plates (Dobrokhotov, 1985) has worked well and has been used in many studies (Zagorodnyuk and Teslenko, 1986; Baranovskii and Okhotskii, 1988; Zhigarev, 1993, 2004; Tikhonov *et al.,* 1998, 2010; Baskevich *et al.,* 2005; Tikhonova *et al.,* 2005; Mikhailova *et al.,* 2008; Oparin *et al.,* 2010, etc.). Recently, however, the facilities and equipment of biochemical laboratories have changed significantly. Modern electrophoresis of proteins is carried out mainly in agarose gel and PAGE. These analyses can be carried out in any biochemical laboratory.

The aim of this work is to create a new, modern protocol for electrophoresis of blood proteins in PAGE for the differentiation of two sibling species: *M. arvalis* and *M. rossiaemeridionalis*.

MATERIALS AND METHODS

Nine specimens of cytogenetically identified individuals of *M. arvalis* and *M. rossiaemeridionalis* caught in Kaluga and Moscow oblasts and in the natural territories of Moscow (serving as the control in electrophoresis), as well as their hybrids (five specimens, collection of the Department of Vertebrate Zoology, Moscow State University), were used. In addition, cytogenetically unidentified *Microtus* voles captured in Kaluga oblast (environs of the town of Balabanovo, Zhukovskii district: 21 specimens), in Moscow oblast (environs of the town of Chernogolovka, Noginskii district: five specimens), in Samara oblast (Samarskaya Luka National Park, Zhigulevskii Reserve, and the surroundings of the city of Tolyatti: 983 specimens), and in the forest–park districts of Moscow (the Setun' River valley: 12 specimens) were used.

Preparations for the determination of the chromosome set were made according to standard methods from bone marrow cells (Ford and Hamerton, 1956).

In order to identify the sibling species by hemoglobin electrophoresis, it is sufficient to take a few drops of blood from living specimens in field conditions or use the organs of captured animals containing blood. To practice various methods, we used fresh blood, blood clots, and lung, heart, spleen, liver, and skeletal muscles of the obtained animals. In living specimens, blood was taken from the hypoglossal vein. Both fresh and frozen organs were used in the analysis. The freezing was carried out in two modes: at −18°C (the temperature of standard household freezers) and at −80°C.

Electrophoresis of blood proteins (> 2600 analyses) was performed using three techniques: on cellulose acetate plates, in an agarose gel, and in PAGE.

The molecular mass of the species-specific protein (the minor band in a phoregram) was evaluated using denaturing electrophoresis in tricine buffer, which has a higher resolution than native electrophoresis, as described below. Denaturing electrophoresis requires large time and material costs.

The following equipment was used for electrophoresis in PAGE: Mini-PROTEAN Tetra Cell (Bio-Rad, United States, cat. no. 165-8000) vertical electrophoresis chamber (designed for 1–4 gels), pH meter (Akvilon pH-410, Russia), and an Elf-4 power supply (DNK-tekhnologiya, Russia, cat. no. PS-400). For electrophoresis in agarose gel and on cellulose acetate plates, a horizontal electrophoresis chamber SE-1 (Khelikon, article SE-1), an Elf-4 power supply, and Vladipor cellulose acetate plates 90 × 90 (MFFS-SCHS-1) were used.

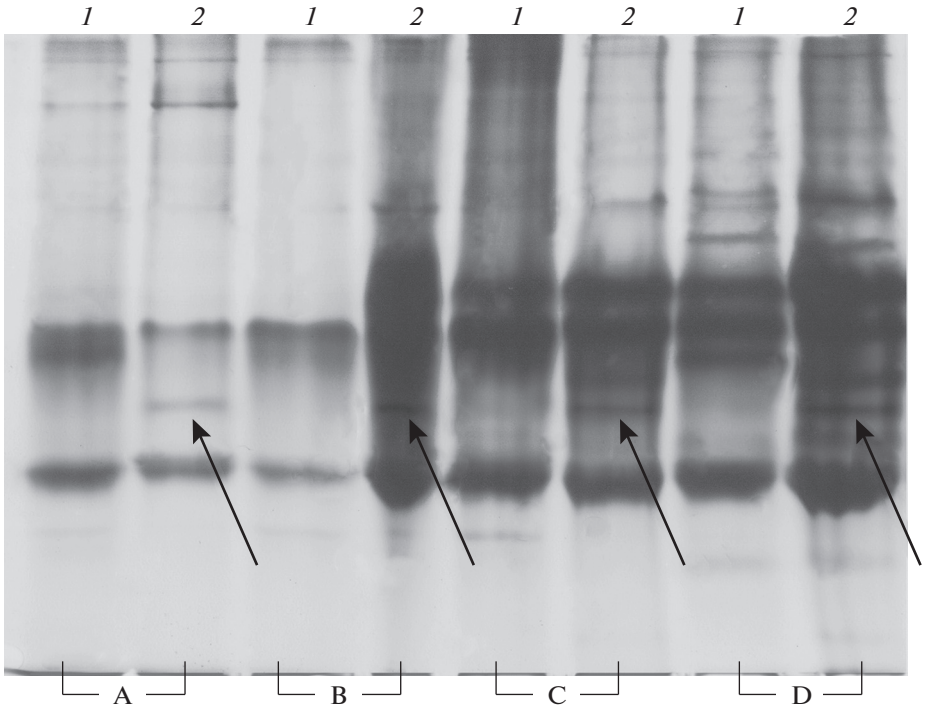

Fig. 1. Electrophoregram of the proteins of lysates of different organs of two sibling species (*1*) *Microtus arvalis* and (*2*) *M. rossiaemeridionalis* in PAGE. (A) Lung with blood clots, (B) spleen, (C) liver, and (D) heart. The arrows indicate the species-specific protein band for the southern part (Rf = 0.584); for Figs. 1 and 2.

RESULTS AND DISCUSSION

A comparative analysis of the methods of separation of proteins showed that, of the three existing types, electrophoresis in PAGE is the most informative in the differentiation of the sibling species of common voles, while that on cellulose acetate plates is the least informative. Electrophoresis of blood proteins in agarose gel (2.5%) is not applicable for the separation of sibling species, since diagnostic protein fractions are not separated due to the low resolving power of this gel.

The sterile interspecific hybrids of *M. arvalis* and *M. rossiaemeridionalis* determine the distribution of diagnostic zones, which is similar to that determined by the "pure" species *M. rossiaemeridionalis*, as well as by the interspecific hybrids of *M. rossiaemeridionalis* and *M. transcaspicus* (Zhigarev *et al.,* 2011). In this case, the second (minor) band is diagnosed.

The information content of electrophoregrams carried out in PAGE hardly depends on whether whole blood (plasma and formed elements) or washed erythrocytes are used in the analysis. Both variants give acceptable results of differentiation of the species studied. The use of whole blood somewhat overloads the electrophoregram with additional bands of plasma proteins, but this work requires less effort and time. In addition, whole blood is more informative, as it includes other proteins that can be used to differentiate interspecific hybrids (Zhigarev *et al.,* 2011).

Analysis of the results of electrophoresis of lysates (in EDTA) of various organs and tissues (blood, lungs, heart, spleen, liver, and skeletal muscles) showed the suitability of all samples for the identification of sibling species by electrophoresis in PAGE. A clearer distinction between sibling species was found in samples of fresh blood and clots of coagulated blood (including frozen). Good results are obtained when analyzing lysates of the spleen, lungs, and liver; much worse are the results of the analysis of muscle, heart, and lung lysates that do not have blood clots (Fig. 1). The presence of hemoglobin in the lysate is important. It should be borne in mind that, when using the liver to differentiate species, many proteins appear on the electrophoregram; therefore, chambers with a low mileage (having short gel plates) can produce difficult-to-distinguish bands, which are of little use for identification.

After conducting field studies, it is not always possible to analyze the data obtained immediately. The material has to be stored in the freezer. We checked the effect of storage of biological material in a refrigerator on the quality of information received. It turned out that biological material (dead bodies of voles or frozen blood and organs, including EDTA solutions) stored in a freezer (−18°C) for two to three years gives an acceptable result for identification and hardly changes the nature of protein distribution in electrophoresis. Older samples are also suitable for identification. Over

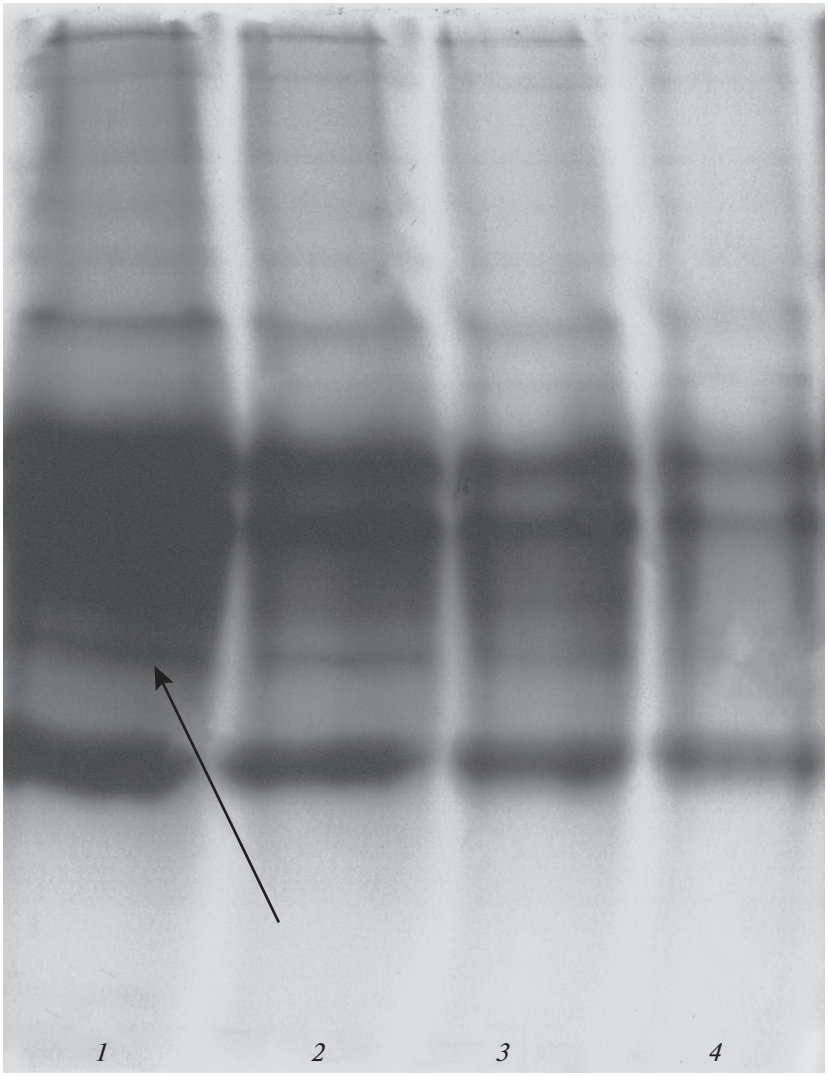

Fig. 2. Electrophoregram of *Microtus rossiaemeridionalis* liver lysate proteins in PAGE upon dilution with EDTA. (*1*) One-fold (reddish lysate), (*2*) two-fold (light pink lysate), (*3*) four-fold, and (*4*) eight-fold dilution.

time, proteins are destroyed and the electrophoregram gives a wide band that does not allow us to separate the species. Repeated freezing and thawing of the biomaterial leads to the same result. Storage at –80°C can significantly extend (at least up to 6–8 years) the quality of the material.

Electrophoregrams in PAGE can vary significantly in informativeness depending on the concentrations of the substances and methods used. Attention should be paid to the degree of dilution of samples with an EDTA solution before loading into the gel. When using concentrated samples, blurred bands are obtained; excessive dilution leads to the impossibility of distinguishing the species-specific bands on the electrophoregram (Fig. 2). To obtain good results, it is necessary to dilute the biomaterial with a solution of EDTA to a light pink color.

Different concentrations of PAGE (6.25–12.5%) were used, and it was revealed experimentally that a 10.4% gel was the most informative for identifying sibling species.

As a result of studies with the Totallab TL120 computer program (info@totallab.com), the molecular mass of a species-specific protein was estimated (a minor band in a phoregram, which allows us to identify *M. rossiaemeridionalis*). The masses of marker proteins are 12.8–13 kDa (Fig. 3). The relative electrophoretic mobility (Rf) of the minor band protein in native electrophoresis (10.4% gel) was 0.58–0.59. The cytogenetic and electrophoretic analyses of animals (1030 specimens) captured in ten geographical areas of Moscow, Kaluga, and Samara oblasts and natural areas of Moscow showed the numerical dominance of *M. arvalis*. Only in Moscow did the southern vole prevail (Table 1).

The protocol for a detailed description of electrophoresis of blood proteins in PAGE is designed for a biochemist–researcher of any qualification, therefore some descriptions will seem superfluous for highly qualified specialists. Electrophoresis requires small time and material costs.

PROTOCOL OF ELECTROPHORESIS OF BLOOD PROTEINS OF SIBLING SPECIES *M. arvalis* AND *M. rossiaemeridionalis* IN 10.4% PAGE: PREPARATION OF SOLUTIONS

Tris-HCl buffers. For electrophoresis, two solutions with pH 8.8 and 6.8 are necessary. In both cases, tris (tris(hydroxymethyl)aminomethane (Panreac, Spain, cat. No. 141940.1209)) is used as the acid–base buffer, a one-molar solution of which has an alkaline pH. To bring the solutions to the required acidity value, 1 M of chemically pure hydrochloric acid is used (OAO Kaustik, Sterlitamak, Russia).

First, prepare a 1 M solution of tris in dH_2O. For 100 mL of the final solution, 12.1 g of crystalline tris are taken. It is dissolved in half of the final volume of water. After complete dissolution, water is added to 100 mL. The solution is poured into two containers (20 and 80 mL, by volume 1 : 4). The larger volume is adjusted to pH 8.8, carefully adding HCl, while tracking the change in pH on the potentiometer. The smaller volume is similarly adjusted to pH 6.8. The prepared solutions are stored in the refrigerator (+4°C). This volume is sufficient for analysis of 600–800 samples.

Loading buffer (4X-solution). One hundred mL of glycerol (Labtekh, Russia) and 50 mL of Tris-HCl buffer (pH 6.8) are mixed until a homogeneous thick dark blue liquid is obtained and bromophenol blue is added (Panreac, Spain, cat. No. 131165.1604) at the tip of the spatula. The stock buffer is stored in the refrigerator for an unlimited time. This volume is sufficient to work with 15000 samples.

Acrylamide solution. The solution of acrylamide (MP biomedicals, United States, cat. no. 814326) and N,N'-methylene bisacrylamide (MP biomedicals, United States, cat. no. 800173) in water is the main gel-forming component of PAGE. To prepare 100 mL of the solution, first, 0.9 g of bisacrylamide (crosslinking component) is completely dissolved in 50 mL of dH_2O, 24.1 g of acrylamide are added, and the volume is brought to 100 mL with distilled water. The prepared 25% acrylamide solution is stored in a refrigerator in a dark container. This volume is sufficient to analyze several hundred samples, depending on the size of the electrophoresis chamber.

Tris-glycine buffer (5X-solution). Tris-glycine buffer (TGB) is the main electrode buffer in both chambers of the electrophoretic system. To prepare a five-fold tris-glycine buffer, 15.1 g of tris (Panreac, Spain, cat. no. 141940.1209) and 94 g of glycine (Panreac, Spain, cat. no. 141340.1209) are required, which must be diluted with distilled water to 1 liter. Stock TGB is stored in the refrigerator. Immediately before use, it must be diluted with distilled water five times.

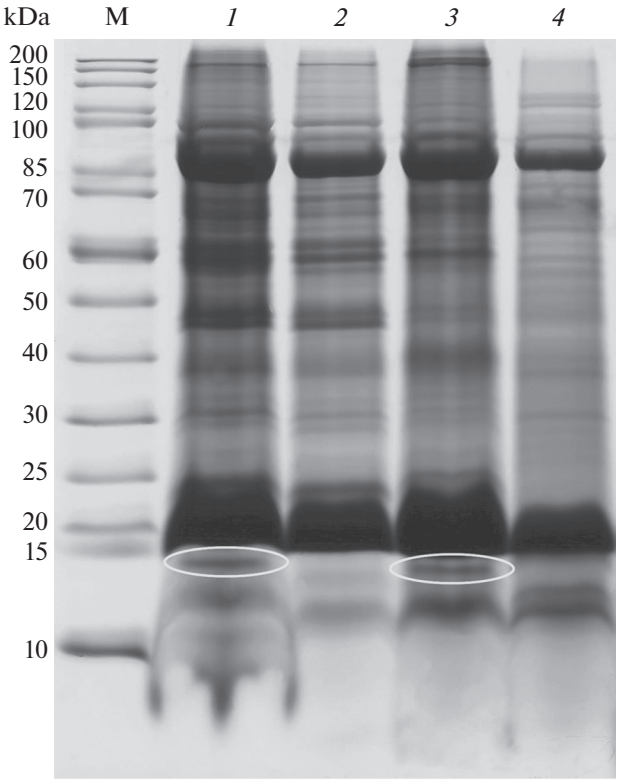

Fig. 3. Evaluation of the molecular weight of species-specific proteins of the southern vole. (*1*) *Microtus rossiaemeridionalis* (blood clot), (*2*) *M. arvalis* (blood clot), (*3*) *M. rossiaemeridionalis* (spleen), (*4*) *M. arvalis* (spleen), (M) set of protein markers. The ovals are protein bands that are species-specific for the southern vole.

Ammonium persulfate solution, 10%. Crystalline ammonium persulfate (APS) (Sigma-Aldrich, United States, cat. no. A3678) is a polymerization activator that must be dissolved in distilled water, bringing the concentration to 10%. It should be kept in the refrigerator for no more than two weeks.

Coomassie solution (brilliant blue G-250). To prepare 100 mL of the solution, 0.2 g of brilliant blue G-250 (Panreac, Spain, cat. no. 254933.1606), 45 mL of ethyl alcohol, 10 mL of glacial acetic acid of "kh.ch." (chemically pure) grade (Kupavnareaktiv, Russia), and 45 mL of distilled water are required. The components should be mixed until a homogenous dark blue liquid is obtained. The resulting solution is stored at room temperature.

Washing solution. To prepare 100 mL of the washing solution, it is necessary to mix 20 mL of ethyl alcohol, 4 mL of glacial acetic acid of "kh.ch." (chemically pure) grade, and 76 mL of distilled water. The solution is stored at room temperature.

Table 1. Places of capture and number of determined (cytogenetically and (or) by blood protein electrophoresis) sibling species *Microtus arvalis* and *M. rossiaemeridionalis*

Place of capture	Number of individuals		
	total	*M. arv.*	*M. rossi.*
Moscow, Setun' River valley, intersection of Minskaya ul. (N 55.721037, E 37.501080), Ryabinovaya ul. (N 55.701107, E 37.412872) and Amin'evskoe sh. (N 55.710323, E 37.460100)	14	0	14
Moscow oblast, Noginskii district, vicinity of Chernogolovka (N 56.037164, E 38.423409; N 56.022408, E 38.415616)	7	6	1
Kaluga oblast, Zhukovskii district, vicinity of Balabanovo (N 55.196806, E 36.717885; N 55.188731, E 36.705424)	26	26	0
Samara oblast, Samarskaya Luka National Park, environs of Brusyany (N 53.229453, E 49.374495)	297	287	10
Samara oblast, Samarskaya Luka National Park, environs of Malaya Ryazan' (N 53.229107, E 49.302547)	217	213	4
Samara oblast, Samarskaya Luka National Park, environs of Kol'tsovo (N 53.189586, E 49.419946)	206	202	4
Samara oblast, Zhigulevskii State Nature Biosphere Reserve, environs of Bakhilova Polyana (N 53.428487, E 49.669588)	91	88	3
Samara oblast, environs of Tolyatti, in the vicinity of the TogliattiAzot plant (N 53.532389, E 49.632381)	35	34	1
Samara oblast, environs of Tolyatti, in the vicinity of Vasil'evskie sewage treatment facilities (N 53.539804, E 49.567104)	59	57	2
Samara oblast, Tolyatti environs, in the vicinity of the former AO Fosfor (N 53.537076, E 49.508280)	78	77	1
Total	1030	990	40

ELECTROPHORESIS PROCEDURE

Sample preparation. A small amount of fresh tissue or organs should be placed in an Eppendorf tube and 0.5% solution of ethylenediaminetetraacetic acid (EDTA-disodium salt AppliChem A1103.0500) poured over the top of it. This solution can be stored for a long time in a freezer or 1–2 days at room temperature (in this case, before loading the sample onto the gel, it is advisable to freeze–thaw it for effective destruction of the erythrocyte membranes). If the organs were previously frozen, they are thawed and filled with EDTA solution. The solution should have a light pink color; it must be pipetted using a 1 mL pipette and added to the loading buffer. In order to do this, 10 μl of loading buffer should be poured into clean Eppendorf tubes and 30 μl of the investigated lysate should be added and mixed well.

Loading the gels and conducting electrophoresis. In the method proposed, the gel in which the separation of proteins occurs is two-component. It consists of resolving (lower) and stacking (upper) gels. Both monomer solutions are prepared separately.

To prepare 12 mL (for four gels, i.e., 3 mL for each) of the resolving gel, the following protocol is used: in a clean glass container, the previously prepared 25% acrylamide solution (5 mL), dH_2O (2.4 mL), tris-HCl, pH 8.8 (4.5 mL), 10% ammonium persulfate solution in water (0.13 mL), and TEMED (0.01 mL) are mixed.

To initiate the polymerization reaction, TEMED must be present in the solution. Therefore, before adding it, the electrophoretic device glasses should be correctly installed (according to the device instructions) and all other components of the solution are added. After adding TEMED and persulfate, the solution of monomers of the resolving gel is poured into the space between the glass plates as soon as possible using a 1 mL pipette. To even the top surface of the gel, 300 mL of butanol are layered on top (can be replaced with ethanol). Full polymerization occurs 30 minutes after the addition of TEMED and persulfate. Upon completion of the polymerization, the butanol layer must be removed using filter paper. The resulting gel resolving, its concentration is 10.4%.

To prepare 8 mL (for four gels, 2 mL per each) of the stacking gel, it is necessary to mix the previously prepared 25% acrylamide solution (1.34 mL), dH_2O (5.5 mL), tris-HCl, pH 6.8 (1 mL), 10% water solution of APS (0.08 mL), and TEMED (0.008 mL).

As with the preparation of the resolving gel, TEMED is added prior to casting the gel. The stacking gel is layered over the already frozen resolving gel to the upper border of the glasses. To create loading pockets, a Teflon comb is inserted until it stops in the space between the panes. The top gel hardens completely in 15 minutes.

The frozen gels are removed from the casting trays and gates and placed into the electrophoretic device. The chambers of the device are filled with TGB (5X-solution) diluted five times with distilled water. In this study, 10-well Teflon combs were used (ten samples, respectively, were accelerated on one gel). The prepared samples, mixed with the loading buffer, are loaded into the wells, 15 μL per well. Further, the device should be connected to a power source (setting the output voltage of 150 V and the output power of 60 W). Electrophoresis is carried out in two stages, in which the values of the amperage differ. The first stage lasts 35 minutes, and the fixed current rate is 50 mA. After this, the front should shift to the boundary of the stacking and resolving gels. The current is then increased to 80 mA. The second stage lasts 60–80 minutes. Electrophoresis is considered complete when the front approaches the bottom edge of the gel. The gels are removed and dyed with a pre-prepared solution of diamond blue G-250 for several hours (left overnight). After dyeing, it is necessary to wash the excess dye from the gels. To do this, the washing solution is used, in which the gels are soaked until distinct blue protein bands appear against the background of the transparent gel. Then, the samples can be photographed and analyzed. For this, it is necessary to calculate *Rf* on the electrophoregram. The presence of a band in the *Rf* region = 0.58–0.59 diagnoses the southern vole.

ACKNOWLEDGMENTS

The authors are grateful to E.V. Cherepanova (Institute of the Problems of Ecology and Evolution, Russian Academy of Sciences) for help in conducting a cytogenetic analysis.

FUNDING

This study was supported by the Russian Science Foundation (project no. 16-14-10269).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

REFERENCES

Baranovskii, P.M. and Okhotskii, Yu.V., Territory use, diurnal activity, and mobility of sibling species *Microtus arvalis* and *Microtus rossiaemeridionalis* (Rodentia, Microtinae), *Zool. Zh.*, 1988, vol. 67, no. 7, pp. 1090–1094.

Baskevich, M.I., Okulova, N.M., Sapel'nikov, S.F., Balakirev, A.E., Ryabina, S.B., and Malygin, V.M., Cytogenetic and electrophoretic differentiation of sibling species *Microtus arvalis* s.l. (Rodentia, Arvicolidae) in the Voronezh Reserve, *Zool. Zh.*, 2005, vol. 84, no. 10, pp. 1298–1309.

Baskevich, M.I., Potapov, S.G., Okulova, N.M., Capel'nikov, S.F., Vlasov, A.A., Oparin, M.L., Mironova, T.A., and Avilova, E.A., The distribution and variability of sibling species *Microtus arvalis* sensu lato (Rodentia, Arvicolinae) in the Central Black Earth Region according to chromosomal and molecular genetic data, *Zool. Zh.*, 2009, vol. 88, no. 4, pp. 473–483.

Baskevich, M.I., Okulova, N.M., Potapov, S.G., Mironova, T.A., Sapel'nikov, S.F., Sapel'nikova, I.I., Egorov, S.V., and Vlasov, A.A., New data on the distribution of sibling species and hybridization of 46 chromosome forms of M*icrotus arvalis* sensu lato (Rodentia, Arvicolinae) in the Central Black Earth region, *Zool. Zh.*, 2012, vol. 91, no. 8, pp. 994–1005.

Baskevich, M.I., Mironova, T.A., Cherepanova, E.V., and Krivonogov, D.M., New data on chromosomal variability, distribution of sibling species, and hybridization of 46-chromosomal forms of *Microtus arvalis* sensu lato (Rodentia, Arvicolinae) in the Upper Volga basin, *Biol. Bull. (Moscow)*, 2016, vol. 43, no. 9, pp. 1281–1291.

Bulatova, N.Sh., Potapov, S.G., and Lavrenchenko, L.A., Genomic versus chromosomal polytypy in studies of mitochondrial and nuclear DNA markers in the *Microtus arvalis* group, *Russ. J. Genet.*, 2010, vol. 46, no. 5, pp. 586–594.

Dobrokhotov, B.P. and Malygin, V.M., The use of hemoglobin electrophoresis for identification of voles (*Microtus*) of *arvalis* group (Rodentia, Cricetidae), *Zool. Zh.*, 1982, vol. 61, no. 3, pp. 436–439.

Dobrokhotov, B.P., Technique of hemoglobin electrophoresis for identification of some difficult-to-distinguish species of small mammals in the field, *Zool. Zh.*, 1985, vol. 64, no. 10, pp. 1569–1575.

Fink, S., Excoffier, L., and Heckel, G., Mitochondrial gene diversity in the common vole *Microtus arvalis* shaped by historical divergence and local adaptations, *Mol. Ecol.*, 2004, vol. 13, no. 11, pp. 3501–3514.

Ford, C.E. and Hamerton J.L., A colchicine hypotonic citrate, squash sequence for mammalian chromosomes, *Stain Technol.*, 1956, vol. 31, pp. 247–251.

Galan, M., Pages, M., and Cosson, J.-F., Next-generation sequencing for rodent barcoding: species identification from fresh, degraded and environmental samples, *PLoS One*, 2012, vol. 7, no. 11. https://doi.org/. Updated December 1, 2018.
https://doi.org/10.1371/journal.pone.0048374

Juskeviciute, E. and Paulauskas, A., Application of DNA fragment length polymorphism analysis for identification of small rodents, *Ecologija*, 2003, no. 1, pp. 21–23.

Kalendar', R.N. and Glazko, V.I., Types of molecular genetic markers and their application, *Fiziol. Biokhim. Kult. Rast.*, 2002, vol. 34, no. 4, pp. 279–296.

Malygin, V.M., Systematics of the common vole superspecies, *Vestn. Mosk. Univ., Ser. Biol. Pochv.*, 1970 no. 5, pp. 89–91.

Malygin, V.M., Comparative morphological analysis of the species of voles from the group *Microtus arvalis* (Rodentia, Cricetidae), *Zool. Zh.*, 1978, vol. 57, no. 7, pp. 1062–1073.

Malygin, V.M., *Sistematika obyknovennykh polevok* (Systematics of Common Voles), Moscow: Nauka, 1983.

Malygin, V.M. and Panteleichuk Santush Luish T.M., Morphological criteria for determining taxon holotypes of common vole species (Microtus, Rodentia, Mammalia, *Dokl. Akad. Nauk,* 1996, vol. 349, no. 2, pp. 282–286.

Markova, E.A., Borodin, A.V., and Gileva, E.A., Odontological traits of the common vole (*Microtus arvalis* Pallas, 1779) and southern vole (*M. rossiaemeridionalis* Ognev, 1924) in the Urals region and their diagnostic value, *Zool. Zh.*, 2003, vol. 82, no. 9, pp. 1086–1094.

Markova, E., Malygin, V., Montuire, S., Nadochowski, A., and Quere, J.P., Dental variation in sibling species *Microtus arvalis* and *M. rossiaemeridionalis* (Arvicolinae, Rodentia): Between-species Comparisons and geography of morphotype dental patterns, *J. Mamm. Evol.*, 2010, vol. 17, no. 2, pp. 121–139.

Meier, M.N. and Dityatev, A.E., The use of linear discriminant analysis in the diagnosis of sibling species of the common vole (Rodentia, Microtus), *Zool. Zh.*, 1989, vol. 68, no. 7, pp. 119–129.

Meyer, M.N., Orlov, V.N., and Skhol', E.D., The use of karyological, physiological, and cytological analysis data for distinguishing a new species of rodents (Rodentia, Mammalia), *Dokl. Akad. Nauk SSSR*, 1969, vol. 188, no. 6, pp. 1411–1414.

Mikhailova, T.V., Bernshtein, A.D., Balakirev, A.E., Apekina, N.S., Al'bov, S.A., Novokhatka, A.D., and Dorofeev, E.M., Some features of biology of voles *Microtus arvalis* and *Microtus rossiaemeridionalis* (Rodentia, Cricetidae) and their relationship with the Tula hantavirus, *Zool. Zh.*, 2008, vol. 87, no. 2, pp. 239–247.

Oparin, M.L., Oparina, O.S., Matrosov, A.N., and Kuznetsov, A.A., Dynamics of mammalian fauna of the Volga–Urals interfluve steppes in the last century, *Povolzh. Ekol. Zh.*, 2010, no. 1, pp. 71–85.

Orlov, V.N. and Malygin, V.M., Kinship of species of the superspecies *Microtus arvalis* according to karyological data, *Byul. Mosk. O-va Ispyt. Prir., Otd. Biol.*, 1974, vol. 79, no. 2, pp. 23–28.

Potapov, S.G., Orlov, V.N., Koval'skaya, Yu.M., Malygin, V.M., and Ryskov, A.P., Genetic differentiation in the voles of the tribe Arvicolini (Cricetidae, Rodentia) using DNA taxonprint and RAPD–PCR, *Russ. J. Genet.*, 1999, vol. 35, no. 4, pp. 403–410.

Tikhonov, I.A., Tikhonova, G.N., and Polyakova, L.V., Sibling species *Microtus arvalis* and *M. rossiaemeridionalis* (Rodentia, Cricetidae) in the north-east of Moscow oblast, *Zool. Zh.*, 1998, vol. 77, no. 1, pp. 95–100.

Tikhonov, I.A., Muntyanu, A.I., Uspenskaya, I.G., Konovalov, Yu.N., Burlaku, V.I., Karaman, N.K., Nistreanu, V.B., Tikhonova, G.N., and Kotenkova, E.V., Biotopic distribution, population structure, and some features of reproduction of small mammals in Chisinau, *Povolzh. Ekol. Zh.*, 2010, no. 4, pp. 404–414.

Tikhonova, G.N., Tikhonov, I.A., Surov, A.V., Oparin, M.L., Bogomolov, P.L., and Koval'skaya, Yu.M., Ecological characteristics of common species of rodents of the steppes in the lower reaches of the Volga and Don rives, *Povolzh. Ekol. Zh.*, 2005, no. 3, pp. 281–291.

Zagorodnyuk, I.V. and Teslenko, S.V., Sibling species of the superspecies *Microtus arvalis* in Ukraine. I. Distribution of *Microtus subarvalis, Vestn. Zool.*, 1986, no. 3, pp. 34–40.

Zagorodnyuk, I.V., Karyotypic variability of 46-chromosome forms of voles of the *Microtus arvalis* group (Rodentia): taxonomic assessment, *Vestn. Zool.*, 1991a, no. 1, pp. 36–46.

Zagorodnyuk, I.V., Systematic position of *Microtus brevirostris* (Rodentiformes): data on taxonomy and diagnostics of the "arvalis" group, *Vestn. Zool.*, 1991b, no. 3, pp. 26–35.

Zhigarev, I.A., Changes in the population density of murid rodents under the influence of recreational pressure in the south of Moscow oblast, *Zool. Zh.*, 1993, vol. 72, no. 12, pp. 117–137.

Zhigarev, I.A., *Melkie mlekopitayushchie rekreatsionnykh i estestvennykh lesov Podmoskov'ya* (Small Mammals of Recreational and Natural Forest of Moscow Oblast), Moscow: Prometei, 2004.

Zhigarev, I.A., Lapkovskii, V.V., Alpatov, V.V., and Malygin, V.M., Identification of sibling species *Microtus arvalis* sensu lato and their hybrids by electrophoresis, in *Mezhdunarodnoe soveshchanie "Teriofauna Rossii i sopredel'nykh territorii"* (Proc. Int. Conf. "Theriofauna of Russia and Adjacent Countries"), Moscow: KMK, 2011, p. 165.

Translated by N. Smolina

SPELL: 1. OK