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Research Article

Genetic, chromosomal and phenotypic variation across a hybrid zone between two common vole species (*Microtus arvalis* and *M. obscurus*)

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Abstract

Research into gene flow across interspecies hybrid zones gives an opportunity to identify mechanisms involved in the formation and maintenance of a species. The section of the hybrid zone between *Microtus arvalis* and *M. obscurus* in the Lower Oka basin (East European plain, Russia) was analysed here. Clinal analysis of nuclear and mitochondrial DNA and karyotypic and phenotypic features revealed a cline for mitochondrial gene *Cytb*; this cline exceeded in width clines for nuclear gene *Tp53*, for male-specific gene *SMCY* and for chromosomal markers. The phenotypic cline is second in width after the *Cytb* cline. The centre of the *Cytb* cline was found to be shifted from centres of the other clines towards the *M. obscurus* geographic range. Centres of the nuclear and chromosomal clines nearly coincide. The width of the *Cytb* cline and the shift of its centre can be explained by expansion of *M. obscurus* males into the geographic range of *M. arvalis*. The wide phenotypic cline may be caused by a large number of loci of small effect that determine the phenotypic features. Despite the expected negative correlation between the level of genetic divergence of the contacting forms and the width of the hybrid zones between them, the *M. arvalis* x *M. obscurus* hybrid zone in the studied section is wider when compared with the hybrid zones between phylogenetic lineages of *M. arvalis* in Southwest and Central Europe. We suppose that this discrepancy results from an influence of specific landscape features on the parameters of the hybrid zones. An inversion in autosome No. 5, which is an exclusive feature of *M. obscurus*, was absent to the west of the centre of the hybrid zone. The limited distribution of this inversion is suggestive of incompatibility of this chromosomal rearrangement with some genes of *M. arvalis*.

Introduction

Application of karyological methods to the systematics of common voles of the *Microtus arvalis* group has made it possible to distinguish between two sympatric sibling species: the Eastern European vole *M. rossiaemeridionalis* (2N=54) and the common vole *M. arvalis* (2n=46) (Meyer et al., 1969, 1972). At the next stage of investigation of the latter species, two karyomorphs were ascertained (which differ in the ratio of small acrocentric and metacentric autosomes and in morphology of the Y chromosome): *arvalis* and *obscurus* (Vorontsov et al., 1984; Král and L'apunova, 1975; Malygin, 1974).

Thirteen pairs of small two-armed autosomes, four pairs of acrocentrics and the Y chromosome (representing the smallest acrocentric) are characteristic of the *arvalis* karyomorph (FN=84); seven small metacentric pairs, 10 acrocentric pairs and the medium-sized acrocentric Y chromosome are characteristic of the *obscurus* karyomorph (FN = 72). A polymorphism of autosome No. 5 has been demonstrated in *M. obscurus*; it can be a subtelocentric or acrocentric (Meyer et al., 1996). The acrocentric variant of autosome No. 5 originates from a pericentric inversion and probably from an increase in heterochromatin size in the inverted segment (Kozlovskii et al., 1988).

Before widespread application of molecular genetic methods, it had been supposed that *arvalis* and *obscurus* differ only in cytogenetic features (Bulatova et al., 2007). Afterwards, it has been demonstrated that these two forms possess highly diverged mitochondrial genomes (Fink et al., 2004; Haynes et al., 2003; Jaarola et al., 2004). Sequencing of the mitochondrial cytochrome b gene (*Cytb*) showed that the level of differentiation between *arvalis* and *obscurus* (4.6 % of nucleotide substitutions) is comparable with that between *M. rossiaemeridionalis* and *arvalis* (6.1 %) and between *M. rossiaemeridionalis* and *obscurus* (5.8 %) (Lavrenchenko et al., 2009). Furthermore, sequencing of nuclear gene *Tp53* has revealed some genetic differences between *arvalis* and *obscurus*; for this reason, identification of animals via electrophoresis of PCR-amplified fragments of the *Tp53* gene (without sequencing) has become possible (Bulatova et al., 2010a; Lavrenchenko et al., 2009).

Taking into account i) the level of genetic differentiation between *arvalis* and *obscurus* (comparable with the level of differentiation between good species of common voles), ii) their reciprocal monophyly (according to analyses of mitochondrial and nuclear markers) and iii) the stability of differences in chromosomal and molecular-genetic features throughout all their geographic ranges, these two forms were determined to be semi-species and were designated as independent species – *M. arvalis* and *M. obscurus* – in terms of their taxonomic status (Lavrenchenko et al., 2009). It should be emphasized that these two taxa are considered two distinct species in the most comprehensive taxonomic reviews on rodents (Kryštufek, 2017a,b; Kryštufek and Shenbrot, 2022). In accordance with this opinion, we designate these forms as independent species in the text below.

European populations of *M. arvalis* sensu stricto (s.s.) are rather homogeneous in cytogenetic features (2N=46, FN=84) but vary in mitochondrial and nuclear genes and in markers of the Y chromosome. Based on this differentiation, several European phylogenetic groups of

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M. arvalis (Balkan, Central, Eastern, Italian, North-Western and South-Western) have been defined (Stojak et al., 2016; Lischer et al., 2014; Bužan et al., 2010; Braaker and Heckel, 2009; Tougard et al., 2008; Heckel et al., 2005; Fink et al., 2004; Haynes et al., 2003).

Several hybrid zones between these European lineages have been described to date (Beysard et al., 2015; Beysard and Heckel, 2014; Sutter et al., 2013). Results of a multi-locus analysis (markers of *Cytb* and *SMCY11* and microsatellite loci) of the hybrid zones between Eastern and Central lineages (Bavaria) and between Central and North-Western lineages (Swiss Alps) led researchers to hypothesise a negative correlation between the level of genetic divergence of the contacting lineages and the width of the hybrid zone between them (Beysard and Heckel, 2014).

As for *M. obscurus*, a phylogeographic analysis of *Cytb* revealed apparent heterogeneity of this species (Tougard et al., 2013). Clearly distinct mitochondrial lineages have been described within *M. obscurus*, namely, the Sino-Russian lineage [divided into the Crimean sub-lineage and the Eurasian sub-lineage (Sibiryakov et al., 2018)] and the Middle Eastern lineage [divided into the South Caucasian sub-lineage and the Iranian sub-lineage (Sibiryakov et al., 2018; Mahmoudi et al., 2017)].

As a result of pilot genetic research, the contact zone between the Eastern lineage of *M. arvalis* and the Sino-Russian lineage (Eurasian sub-lineage) of *M. obscurus* has been found in the European part of Russia. At present, this hybrid zone is located in Vladimir Oblast (Golenishchev et al., 2001), Nizhny Novgorod Oblast (Baskevich et al., 2016) and Kursk and Lipetsk Oblasts (Baskevich et al., 2012). The boundary between geographic ranges of *M. arvalis* s.s. and *M. obscurus*, which spans middle taiga to dry steppes of the European part of Russia, is one of the longest contact zones among European mammals (Bulatova et al., 2010b).

Results of studies on different sections of the *M. arvalis* × *M. obscurus* hybrid zone indicate the following features of this zone: a deficiency of F1 hybrids (according to analyses of karyological markers) and asymmetric introgression of mitochondrial gene *Cytb* from *M. arvalis* to *M. obscurus* (Baskevich et al., 2012, 2016; Bulatova et al., 2010a; Lavrenchenko et al., 2009). It should be noted that available information about the hybrid zone between *M. arvalis* and *M. obscurus* is fragmentary, and the number of collected samples is not sufficient even for approximate description of the zone and is suitable only for determining its location.

The purpose of this work was to investigate genetic, chromosomal and phenotypic variation along an extended transect across a section of the *M. arvalis* × *M. obscurus* hybrid zone located in the central part of European Russia.

The main aim was an analysis of parameters of *Cytb*, *Tp53*, *SMCY*, chromosomal and phenotypic clines with a focus on the genetic factors and specific landscape features that may affect hybrid zone structure. Taking into consideration that an inversion in autosome No. 5 is specific to *M. obscurus* and that the frequency of this inversion significantly varies among different populations, we also determined the distribution of this chromosomal rearrangement along the transect.

Materials and Methods

The study area

The studied section of the hybrid zone between the two species of common voles is located in the central part of the Eastern European Plain (Fig. 1). It lies approximately between the Oka River and its tributary (Klyazma River), in Vladimir Oblast (close to the city of Murom) and Nizhny Novgorod Oblast. The area belongs to a zone of mixed and broad-leaved forests. Open habitats favourable for common voles are mainly anthropogenic in origin: field and fallow lands and sometimes water meadows (the right bank of the low current of the Oka River). The territory under study is rather mosaic: open areas and forest patches are adjacent, and the fields are sown mainly with fodder crops (annual and perennial leguminous plants, bluegrass and motley grass) and with monocultures of cereals (wheat, rye, barley and oats). Fallow lands suitable for common voles are unused arable lands that are overgrown with annual and perennial herbs. This vegetation persists in fallow lands for 3–4 years after ploughing; subsequently, these fallow lands are overgrown with deciduous and coniferous trees.

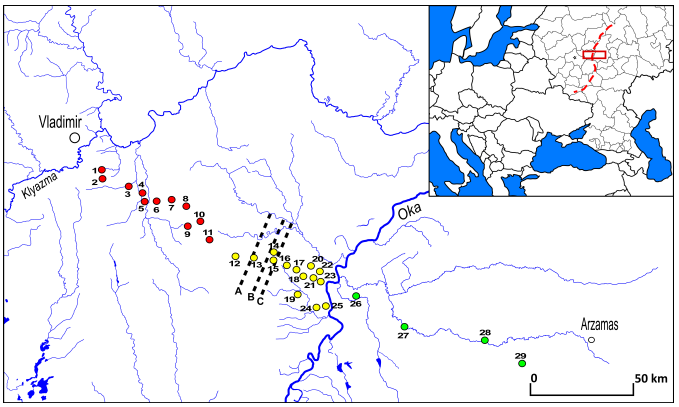

Figure 1 – Geographical locations and positions of the sampling sites across the analysed hybrid zone between common voles *M. arvalis* and *M. obscurus*. Red circles denote the sites where only 'pure' *M. arvalis* individuals were found, green circles indicate the sites where only 'pure' *M. obscurus* were found, and yellow circles the sites where some specimens revealed admixture of genetic markers of both species. A: a centre of clines for *Tp53, SMCY* and chromosomes, B: a centre of the phenotypic cline, C: a centre of the cline for *Cytb*. The map was prepared using GIS software MapInfo 16.0. The map of Eastern Europe in the upper right corner shows the location of the study area (in a red frame) and the general location of the hybrid zone (the dashed red curve).

Sample collection

The material was collected in July–October 2015 and 2016; voles were caught with live traps by the trap-line method. Oatmeal flakes with vegetable oil and chopped carrot or white cabbage were used as bait. Thirty or 50 traps were placed along lines at a distance of seven metres one from another. Live traps were set in the evening and checked in the morning; exposition time was 8–10 h/day. The transect goes across the hybrid zone of *M. arvalis* and *M. obscurus*; the total length of the transect reaches 200 km (Fig. 1). A total of 736 common voles (*M. arvalis* sensu lato [s.l.]) were collected at 29 sites (Table 1). All the sampling sites were projected onto the transect, which crosses the hybrid zone perpendicularly, and connect its first (site No. 1) and terminal (site No. 29) points. The position (distance) of a point relative to the beginning (site No. 1; '0 km') of the transect was calculated according to its projected position on the transect (in kilometres).

In western sampling sites (from No. 1 to No. 11), the found chromosomal and molecular markers were specific to *M. arvalis* exclusively, and in eastern sites (from No. 26 to No. 29), they were specific to *M. obscurus* (Tables 1 and S1; Fig. 1); for this reason, 14 sites (No. 12 to No. 25) are hereafter referred to as the 'hybrid zone'.

Molecular genetic analysis

DNA from tissue samples fixed in 96 % ethanol was extracted using the DNA-Extran-2 Tissue Kit (Syntol, Russia). A mixture of species-specific primers was used to identify sibling species of common voles (*M. arvalis* s.l.) by means of mitochondrial *Cytb* and nuclear *Tp53* markers.

Fragments of *Cytb* were amplified using a mixture of three forward primers – cbMA842F (5'-GGGGTTTACTATGGCTCA-3'), cbMO604F (5'-CCTTTCACTTTATTCTACCT-3') and cbMR469F (5'-CAGTCAAAGACTTCTTAGGG-3') – with reverse primer H15915-SP (5'-TTCATTACTGGTTTACAAGAC-3'). Lengths of the obtained fragments were as follows: 842 bp for *M. arvalis*, 604 bp for *M. obscurus* and 469 bp for the sibling species *M. rossiaemeridionalis* (Fink et al., 2004; Bulatova et al., 2010a).

Fragments of nuclear gene *Tp53* were amplified using a mixture of forward primer tp53D (5'-CGGTTCATGGCCCCCCATGC-3') (DeWoody, 1999; Bulatova et al., 2010a) with two reverse primers – tp53MAF (5'-CTCCGATGGTGGATGGTGGAGTACCCA-3') and tp53MOF (5'- CGACGGTGGATGGTGGAGTTCCCG-3') –

Table 1 – Frequencies of each marker examined in this study. D: distance along the transect; N: the number of studied tissue samples; Chr: chromosomes; A: *M. arvalis*; O: *M. obscurus*.

Map No.	D (km)	Latitude	Longitude	*Cytb* A	*Cytb* O	N	*Tp53* A	*Tp53* O	N	*SMCY* A	*SMCY* O	N	Chr A	Chr O	N
1	0	56.035528°	40.559167°	1	0	27	1	0	27	1	0	7	1	0	20
2	2	56.008925°	40.569447°	1	0	5	1	0	5	1	0	1	1	0	5
3	11	55.986083°	40.716000°	1	0	3	1	0	3	-	-	0	1	0	3
4	19	55.959389°	40.832778°	1	0	3	1	0	3	1	0	1	1	0	3
5	22	55.916778°	40.848611°	1	0	10	1	0	10	-	-	0	1	0	6
6	23	55.940744°	40.892128°	1	0	52	1	0	52	1	0	10	1	0	23
7	24	55.927333°	41.015556°	1	0	24	1	0	24	1	0	6	1	0	9
8	37	55.907472°	41.123083°	1	0	20	1	0	20	1	0	5	1	0	13
9	44	55.826556°	41.102389°	1	0	15	1	0	15	1	0	7	1	0	12
10	45	55.879306°	41.163361°	1	0	13	1	0	13	1	0	2	0.94	0.06	7
11	52	55.781750°	41.279361°	1	0	15	1	0	15	1	0	5	1	0	11
12	67	55.710917°	41.452083°	0.68	0.32	19	0.82	0.18	19	1	0	5	0.87	0.13	13
13	73	55.711889°	41.586222°	0.78	0.22	9	0.39	0.61	9	0.5	0.5	2	0.17	0.83	4
14	80	55.725750°	41.709806°	0.43	0.57	21	0.24	0.76	21	0	1	6	0.08	0.92	8
15	83	55.697167°	41.736806°	0.31	0.69	16	0.31	0.69	16	-	-	0	-	-	0
16	87	55.676056°	41.793556°	0.40	0.60	142	0.19	0.81	142	0	1	7	0.04	0.96	67
17	90	55.651000°	41.834472°	0.38	0.62	13	0.12	0.88	13	-	-	0	0	1	3
18	93	55.666667°	41.900000°	0.50	0.50	16	0.28	0.72	16	-	-	0	0.07	0.93	8
19	94	55.581611°	41.853694°	0.40	0.60	5	0.40	0.60	5	-	-	0	0.07	0.93	5
20	95	55.684583°	41.943111°	0.44	0.56	16	0.16	0.84	16	-	-	0	0	1	1
21	96	55.629167°	41.899444°	0.41	0.59	39	0.08	0.92	39	0	1	7	0.02	0.98	21
22	100	55.623167°	41.997528°	0.26	0.74	11	0.15	0.85	11	0	1	2	0	1	3
23	101	55.631361°	41.954889°	0.36	0.64	31	0.27	0.73	31	0	1	9	0.01	0.99	13
24	105	55.499611°	41.981306°	0.2	0.8	5	0	1	5	-	-	0	0	1	2
25	111	55.491667°	42.073417°	0.25	0.75	4	0	1	4	-	-	0	0	1	2
26	117	55.563639°	42.233861°	0	1	65	0	1	65	0	1	8	0	1	18
27	142	55.457861°	42.579917°	0	1	70	0	1	70	0	1	7	0	1	23
28	173	55.389139°	43.071250°	0	1	49	0	1	49	0	1	1	0	1	35
29	195	55.297694°	43.369222°	0	1	18	0	1	18	0	1	13	0	1	7

developed by us. Lengths of the obtained fragments were 788 bp in *M. arvalis* and 1003 bp in *M. obscurus*. All *Tp53* fragments from *M. obscurus* included an insertion of type B2 Mm2 (short interspersed repetitive element B2: SINE B2) (Bulatova et al., 2010a).

PCR typing of fragments of mitochondrial gene *Cytb* and nuclear gene *Tp53* by means of their size allowed us to identify *M. arvalis* and *M. obscurus* individuals and heterozygotes for the nuclear gene. Additionally, by this method, we excluded allied species (*M. rossiaemeridionalis*, *M. agrestis* and *M. oeconomus,* which can share habitats with *M. arvalis*) from further analysis.

A fragment of the paternally (Y chromosome) inherited *SMCY* gene was investigated in addition to the maternally inherited mitochondrial gene *Cytb* so that we could estimate sex-specific gene flow between *M. arvalis* and *M. obscurus*. We selected primers specific for *M. arvalis* and *M. obscurus* on the basis of primers SMCY11-f (5'-CTGCCCTGYRCCATGCAT-3') and SMCY11-r (5'-TCCACCTGTTSMAGRACAT-3') (Hellborg and Ellegren, 2003; Sutter et al., 2013).

Primers SMCY11m-F (5'-GGAATTGTAAGAACCTGATT-3') and SMCY11m-R (5'-GGATTACTTAATACATACTT-3') were used for PCR amplification under the following conditions: denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min. A final extension step at 72°C for 5 min was added at the end. The obtained fragment was sequenced on automatic analyser ABI PRISM 310 with the ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). Nucleotide sequences of intron 11 of gene *SMCY* (length of 640 bp) were obtained for 111 males from 20 sampling sites (Table S1). Previously, the five single-nucleotide polymorphisms (SNPs) that are characteristic of Central, Western and Italian evolutionary lineages of *M. arvalis* were described (Sutter et al., 2013). To determine whether there are any SNPs distinguishing the Eastern lineage of *M. arvalis* from *M. obscurus*, we compared sequences of intron 11 of gene *SMCY* from 25 male specimens of 'pure' *M. arvalis* from sites No. 1–7 and those from 29 male specimens of 'pure' *M. obscurus* from sites No. 26–29 (no signs of hybridisation were noted in these individuals by means of the other markers used). We found only one bi-allelic SNP (position 295) showing fixation of alternative alleles in both species: all the *M. arvalis* individuals are characterised by cytosine (C), while all the *M. obscurus* individuals have thymine (T) at this position. All the obtained *SMCY* sequences from 111 males (from both 'pure' and hybrid populations) are available in GenBank under accession numbers OQ630767–OQ630877.

Karyotyping

Chromosome preparations were obtained from bone marrow cells according to Ford and Hamerton (1956), with modifications. The procedures included *in vivo* injection of a 0.05 % colchicine solution for 50 min, hypotonic treatment of the cell suspension with a 0.56 % KCl solution (37°C, 10–15 min) and fixation with a 3:1 mixture of methanol and glacial acetic acid. Air-dried slides of spread cells were subjected to Giemsa staining.

Morphometric analysis

The material available for the morphometric analysis consisted of 107 skulls. The specimens are stored in collections of the Laboratory of Mammalian Microevolution at A.N. Severtsov Institute of Ecology and Evolution, the Russian Academy of Sciences (Moscow, Russia). Age of the specimens was determined according to the craniological criteria of Bashenina (1953) and Kropacheva et al. (2013). Juvenile specimens were excluded from the analysis. Each skull was scanned in the ventral view using an Epson Perfection V30 (Epson, Japan) tablet scanner at a resolution of 2400 dpi. The ventral view was described by a configuration of 17 landmarks (Fig. S2). All the landmarks were digitised using the TPSdig2 software (Rohlf, 2008). Centroid size (CS) and relative warps (RWs) were calculated by standard methods (Zelditch et al., 2004; Bookstein, 1989) using the TPSrelw software (Rohlf, 2007) with α parameter of −1, which indicates small scale differences (Rohlf, 1993). Comparative analysis of RWs was performed to discriminate three groups: 'pure' *M. arvalis*, 'pure' *M. obscurus* and their hybrids (identified by molecular and/or chromosomal analyses). The voles that had the molecular markers (*Cytb*, *Tp53* and *SMCY11*) specific for both *M. arvalis* and *M. obscurus* and/or an intermediate number of small acrocentrics, which varied from nine to 19, were designated as hybrids. One-way ANOVA was carried out to test statistical significance of differences in size (CS) and shape (RW1) between the 'pure' species (for *M. arvalis*: N = 87, sites No. 1–9; for *M. obscurus*: N = 20, sites No. 26–29). Jack-knifed statistic Q_{ST} was used to measure differentiation between the two groups of 'pure' species residing on either side of the hybrid zone:

$$Q_{ST} = (n-1-k)\mathrm{SSB}/(k-1)(\mathrm{SSW}+\mathrm{SSB}),$$

where SSB is the sum of squared distances between group means and the total mean, SSW is the sum of squared distances between individuals and their group mean, n is sample size, and k is the number of groups (Polly, 2007). An unbiased estimate of Q_{ST} and its standard error were obtained by the jack-knife procedure of Weir and Cockerham (1984). One thousand jack-knife iterations were performed in which a random member of each group was dropped and the Q_{ST} statistic was recalculated (Manly, 1991). The analysis was performed using the R package *boot*, version 1.3-28 (Canty and Ripley, 2021).

Clinal analysis

To describe patterns of genetic, chromosomal and phenotypic variation across the hybrid zone, geographic cline analysis was performed. Five features were objects of the clinal analysis, namely, the nuclear DNA not linked with sex (*Tp53*), sex-linked Y-specific nuclear DNA (*SMCY11*), the mitochondrial (mt)DNA (*Cytb*), the chromosome set (karyotype) and cranial morphology. Frequencies of *Tp53* and *Cytb* were calculated for all 29 capture localities (i.e. sampling sites), frequencies of chromosomal markers were calculated for 28 sites (excluding sampling site No. 15 because of poor quality of all chromosome slides obtained there), and frequencies of *SMCY11* were calculated for 20 sites only (because of small numbers of captured/sequenced males at some sites). Frequencies varied from 0 to 1, where 'pure' *M. obscurus* was designated as 0 and 'pure' *M. arvalis* as 1. As for the *Tp53* gene, which is diploid and occurs in homo- and heterozygous states, the numbers of parental alleles were added up, and the sum was divided by total allele number (2N).

Regarding karyotypes, the calculation scheme was based on the following premises. *M. arvalis* and *M. obscurus* (2n = 46 in both species) differ in morphology of 12 small autosomes: there are eight small acrocentrics in *M. arvalis* and 20 in *M. obscurus.* Hybrid voles are characterised by an intermediate number of small acrocentrics varying from nine to 19. Because the number of intermediate variants of chromosomes is 11, the 'hybrid coefficient' is 1/11 (0.091). Above, we assigned 1 to 'pure' *M. arvalis* and 0 to 'pure' *M. obscurus*, and consequently hybrid voles are characterised by intermediate frequencies of small acrocentrics: nine acrocentrics: 0.916, 10 acrocentrics: 0.833, 11 acrocentrics: 0.75, …, and 19 acrocentrics: 0.083. Detailed information about frequencies of all intermediate karyotypes is available in Supplemental Materials (Table S1).

Next, 107 specimens subjected to the morphometric analysis were grouped by site for cline fitting. Only 21 sites were used in this analysis because broken skulls and small numbers of skull specimens made some of those sites unsuitable for the morphometric analysis. For each sampling site, the average RW1 was calculated.

We fitted cline models by means of the R package HZAR (Derryberry et al., 2014) to the aforementioned genetic, chromosomal and phenotypic markers or traits. Clines were modelled for each genetic locus on the basis of allele frequency. For the chromosomal data, the cline was modelled using the hybrid index (see above). For the morphometric data, the cline was modelled via the average RW1. We compared the fit of 15 possible models to the three genetic loci and to one chromosomal marker and the fit of seven possible models to morphological traits, as described in detail by Derryberry et al. (2014). For each characteristic, the best model was chosen based on the lowest Akaike information criterion; the parameters for cline width and centre estimates were extracted from this model. We assessed concordance between clines using ±2-log-likelihood confidence intervals from the best cline model for each marker or trait.

Results

Species assignment, marker mismatches and spatial patterns of introgression

Out of the 736 common voles that were identified as *M. arvalis* s.l. by means of genes *Cytb* and *Tp53*, 211 individuals were found to be 'pure' *M. arvalis*, and 349 'pure' *M. obscurus*. As for hybrid genotypes, combinations of *M. arvalis* and *M. obscurus* nuclear and mtDNA genes seemed to be different. Eighty-four specimens bearing *Cytb* haplotypes of *M. arvalis* are homozygotes for *Tp53* alleles characteristic of *M. obscurus*. Fourteen individuals possessing mtDNA of *M. obscurus* were found to be homozygotes for *Tp53* alleles specific to *M. arvalis*. Thirty-five voles carrying mtDNA of *M. arvalis* and 43 voles with mtDNA of *M. obscurus* were found to be heterozygotes for the alleles of the *Tp53* gene. The complete data on molecular-genetic markers in DNA samples from different sites are presented in Supplementary Materials (Table S1).

Although the patterns of introgression typically were consistent among different markers, there were certain differences related to the width of areas, where we found individuals carrying both (*M. arvalis* and *M. obscurus*) traits (Fig. 2). For instance, for mitochondrial marker *Cytb*, we found both (*M. arvalis* and *M. obscurus*) haplotypes at sites No. 12 to No. 25, which thus form an extended zone of admixture with a length of 44 km. For nuclear marker *Tp53*, the zone of admixture including both *M. arvalis* and *M. obscurus* as well as their hybrids (heterozygous individuals) was found to be somewhat shorter in the eastern part (sites No. 12–23), covering 34 km in total. By contrast, male-linked genetic marker *SMCY* showed a sharp transition, with a single population (site No. 13) harbouring both haplotypes.

Regarding chromosomal variation, hybrid recombinants with karyotypes 18-19A (very similar to *M. obscurus*) predominated in the studied section of the hybrid zone. A single karyotype, 14A (similar to F1), was noted at site No 12 close to the centre of the hybrid zone. Intermediate chromosome variants 15A and 17A were rare, and karyotypes 12A, 13A and 16A were not detected. Overall, there seemed to be some correspondence between the chromosomal and nuclear markers. Voles possessing alleles of the *Tp53* gene and *SMCY* haplotypes specific to *M. obscurus* carry chromosomal sets identical or similar to the karyotype of this species (18–20 small acrocentrics). As for combinations

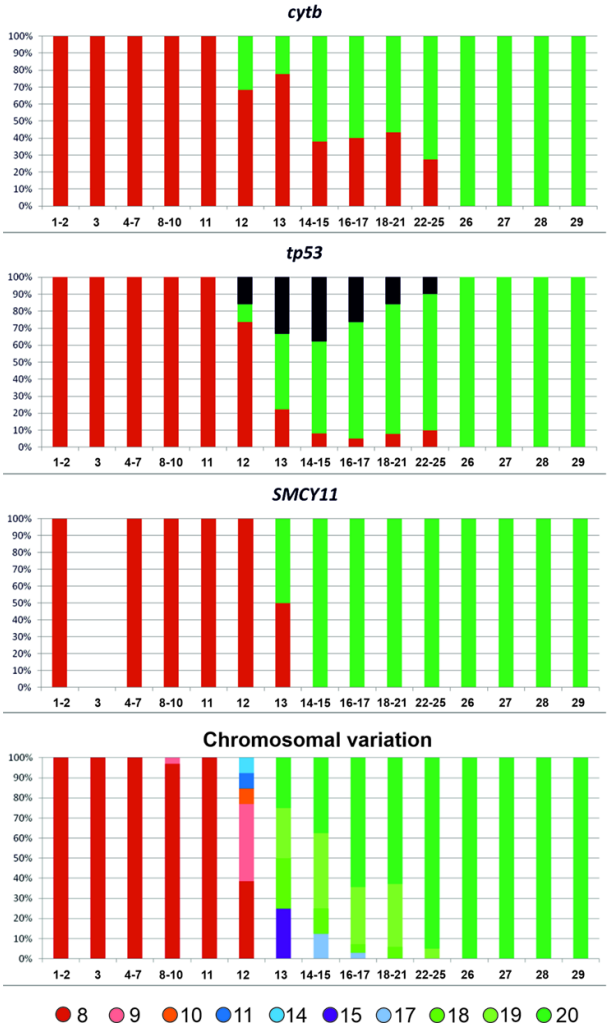

Figure 2 – Frequency variations estimated for the molecular-genetic markers and small acrocentrics. 1–29: sampling sites across the transect (see Fig 1). Due to pronounced mosaic structure of suitable habitats in the hybrid zone (and hence the occasionally nonuniform sampling), we merged the sampling sites located in close proximity to each other. The red colour indicates *M. arvalis*, green *M. obscurus*, and black *Tp53* heterozygotes. Coloured circles (8–20) denote counts of small acrocentrics used for estimation of the chromosomal variation.

of mitochondrial and nuclear genes and karyotypes, some individuals carrying alleles of the *Tp53* gene, *SMCY* haplotypes and chromosomal sets of *M. obscurus* are characterised by *Cytb* of *M. arvalis* (Fig. 2, Table S1).

The morphometric analysis

Considering all individuals, RW1 and RW2 explain 27.9 % and 12.4 % of total variance, respectively (Fig. S2). 'Pure' *M. arvalis* and 'pure' *M. obscurus* are separated clearly in the plot of RW1 and RW2 with some overlap between them, and the hybrids occupy an intermediate position. The shape difference between 'pure' *M. arvalis* and 'pure' *M. obscurus* was found to be statistically significant; the size difference between them is nearly non-existent (Table 2). The two 'pure' species and hybrids are best distinguished by RW1, which accounts for most of the explained variance (Fig. S2).

Table 2 – Phenotypic differentiation between 'pure' *Microtus arvalis* and 'pure' *M. obscurus*; df (between groups) = 1, df (within groups) = 105.

	Q_{ST}	F	P
Shape (RW1)	0.408±0.007	72.45	0.00
Size (CS)	0.001±0.001	0.06	0.81

The clinal analysis

We found different patterns of concordance for genetic, chromosomal and phenotypic clines (Table 3, Fig. 3). The centre of the cline for *Cytb* is shifted along the transect south-eastwards (into the *M. obscurus* geographic range) in comparison with the centres of clines for *Tp53*, *SMCY11* and chromosomes; its two log-likelihood low and high support limits (2LL low-high) do not overlap with limits for the centre of the three other markers. Positions of the clines for three markers (*Tp53*, *SMCY11* and chromosomes) nearly coincide; their 2LL low-high limits overlap significantly. When projected onto the transect, centres of the clines for these three markers are located approximately 72–73 km away from the initial point of the transect, i.e. they are close to site No. 13. In contrast to three above-mentioned markers, the centre of the *Cytb* cline is shifted south-eastwards approximately by 11–12 km. The centre of this cline is located at the 84 km point, close to site No. 15. The centre of the phenotypic cline (located at the 81 km point) occupies an intermediate position between that of the *Cytb* cline and centres of the *Tp53*, *SMCY11* and chromosomal clines.

The phenotypic cline and the *Cytb* cline significantly differ from other clines in width (37 and 46 km, respectively), exceeding this parameter of the *Tp53* cline (15 km) and of the chromosome cline (20 km). The cline for Y-linked marker *SMCY11* is the narrowest one; its width is smaller than half a kilometre (Table 3; Fig. 3).

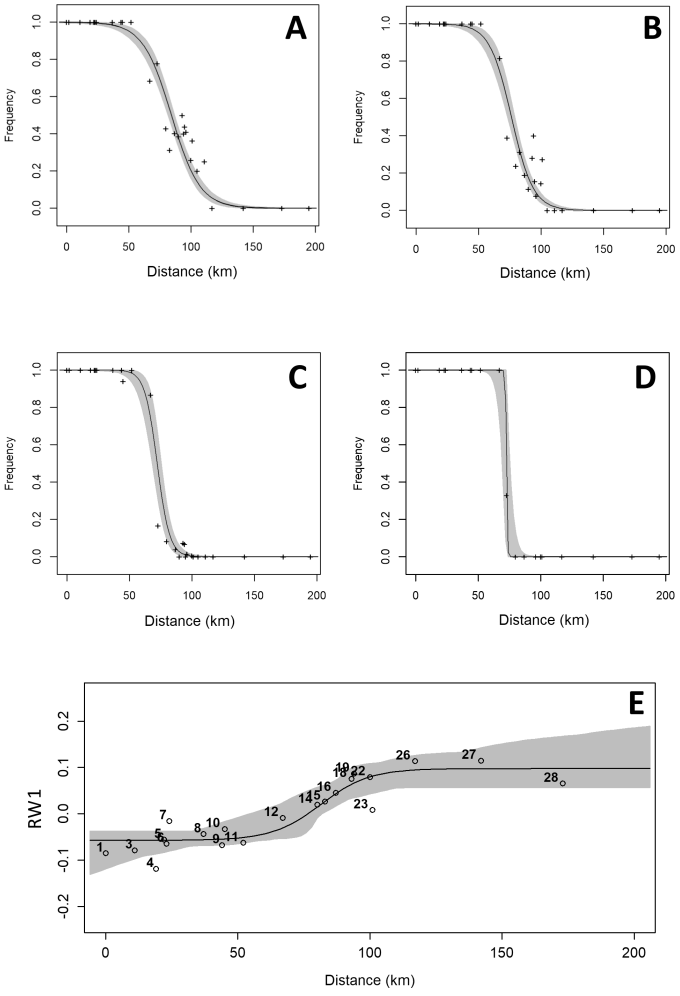

Figure 3 – The plot of allele frequencies, of the chromosomal index and of cranial shape along the transect. A: mtDNA (*Cytb*), B: nuclear DNA (*Tp53*), C: chromosomes, D: Y-linked gene *SMCY*, and E: cranial shape (RW1). The shaded area represents the 2 log likelihood confidence interval for each cline.

Frequency of the autosome No. 5 inversion

This inversion was detected at eight sites of the hybrid zone: No. 16 (two individuals), No. 21 (two), No. 22 (one), No. 23 (one), No. 25 (one), No. 27 (one), No. 28 (two) and No. 29 (two individuals;

Table 3 – Best-fit cline models for genetic markers, for the chromosomal index and for cranial shape (RWI) in the hybrid zone between *M. arvalis* and *M. obscurus*. The centre and width are given for each cline with two log-likelihood credible intervals.

Marker/Trait	Best model (scaling, tails)	Centre estimates (km from site No. 1)	Width estimates (km)
Cytb	No scaling, no tails	84.31 (81.46-86.86)	45.87 (37.84-55.74)
Tp53	No scaling, right tail	72.35 (70.34-76.94)	15.17 (9.43-29.72)
SMCY11	No scaling, no tails	72.91 (69.06-75.42)	0.49 (0.10-14.65)
Chromosomes	No scaling, no tails	72.23 (68.11-75.68)	19.81 (14.52-27.25)
Cranial shape	No scaling, no tails	81.41 (61.75-87.28)	36.7 (19.99-136.70)

Table S1). Out of the 12 voles having the inversion that were collected in the hybrid zone, seven individuals (one male and six females) possess a complete set of markers corresponding to 'pure' *M. obscurus*, and four individuals (three males and one female) are characterised by the *Tp53* gene of *M. obscurus*, although they carry the *Cytb* haplotype of *M. arvalis*. Only one animal (female) with the *Cytb* haplotype specific to *M. arvalis* was found to be heterozygous for the *Tp53* gene. In all the analysed voles carrying the inversion in autosome No. 5, this mutation was found only in the heterozygous state.

Therefore, because the numbers of collected tissue samples (i.e. captured voles) varied significantly among different sampling sites, we calculated the frequency of the inversion in autosome No. 5 for a combined group from the section of the hybrid zone on the left bank of the Oka River (sites No. 16 and 21–23, 25) and in populations of 'pure' *M. obscurus* on its right bank (sites No. 27–29; Table S1; Fig. 1). The frequencies of the inversion were low in both sections of the transect; this parameter was 1.6 % on the left bank and 1.5 % on the right bank of the Oka River. Overall, the frequency of the inversion varied from 0.7 % (site 16) to 12.5 % (site 25) (the total sample size for sites where the inversion was present was 209; Table S1).

Discussion

Coincidence of the clines: width and positions of centres

We revealed an overall match between genetic, chromosomal and morphological clines in the hybrid zone investigated here. All estimated cline centres are located in an interval between kilometre 72 and kilometre 84 of the transect. Nevertheless, patterns of variation in the estimates of a cline centre and width indicated some differences in cline shapes. Width of the *Cytb* cline exceeding the width of the *Tp53* and chromosome clines as well as the very narrow cline for the *SMCY* gene are expected findings. Strongly male-biased dispersal has been documented for *M. arvalis* s.l. (Hahne et al., 2011; Gauffre et al., 2009). As demonstrated for many species (Petit and Excoffier, 2009), markers associated with the least-dispersing sex are more introgressed. In particular, most of studied mammals with male-biased dispersal share the following pattern: mtDNA markers are more introgressed than nuclear markers, and the latter are more introgressed than Y-chromosome markers [see Table 1 in ref. (Petit and Excoffier, 2009)].

The very narrow *SMCY* cline revealed in our study can be also explained by reduced fitness of male hybrids [in accordance with Haldene's rule (Haldane, 1922)]. In laboratory hybridisation between *M. arvalis* and *M. obscurus*, sterility of F1 males has been noted; the sterility is specific exclusively to the crossing 'female *M. obscurus* x male *M. arvalis*' (Sablina and Golenishchev, 2015). *M. arvalis* of the Eastern lineage and *M. obscurus* of the South Caucasian lineage were used in that experiment. The South Caucasian phylogenetic lineage is considerably different from the Eurasian lineage (Mahmoudi et al., 2017), and its representatives are distinguished from other lineages of the species by nine pairs of acrocentric autosomes (instead of 10) and by the metacentric Y chromosome (Meyer et al., 1996). Here we examined the natural hybrid zone between the Eastern lineage of *M. arvalis* and the Sino-Russian lineage (Eurasian sub-lineage) of *M. obscurus*, and when comparing our data with the above-mentioned results, we must be cautious. It should be noted that Safronova et al. (2011) failed to detect meiotic aberrations in F1 hybrids between *M. arvalis* (Zvenigorod vicinity; Eastern lineage) and *M. obscurus* (Voronezh Oblast; Eurasian sub-lineage).

Furthermore, we documented a significant shift of the centre of the mtDNA (*Cytb*) cline from centres of *Tp53*, *SMCY11* and chromosome clines. Such asymmetric gene introgression can be explained more parsimoniously by range expansion of one of the interacting species. Recent simulation studies predicted that range expansion can lead to massive introgression of local genes into the genome of an invader (Excoffier et al., 2009; Currat et al., 2008). In particular, growing evidence suggests that mtDNA introgression is often asymmetric between hybridising lineages and mostly proceeds from a local species to a colonising species (Mastrantonio et al., 2016; Toews and Brelsford, 2012). This phenomenon can be explained in the following way. When a rapid expansion of a species from a small population (for example, from a glacial refugium) occurs, the resulting small populations pass through the bottleneck many times; this process leads to a drop of genetic variability and to disequilibrium between male and female numbers. If such a small population and a population of the local species come into contact, low density and the skewed male:female ratio often lead to interspecies hybridisation. When numbers and densities of the populations increase, the selection acting against heterozygotes becomes stronger and leads to a drop of heterozygote frequency, while the frequency of selectively neutral mitochondrial genes changes more slowly. As a consequence of the rapid population expansion together with the elimination of heterozygotes, considerable introgression of mtDNA into the area of the colonising species takes place (Hewitt, 1999, 2000, 2001).

The observed asymmetric introgression is consistent with the assumption that *M. obscurus* colonises the geographic range of *M. arvalis*. Behavioural differences between *M. arvalis* and *M. obscurus* revealed under experimental conditions indirectly indicate that *M. obscurus* migrated to the territory of *M. arvalis* rather than *vice versa*. In the open field test, *M. obscurus* males explore a new territory actively, whereas *M. arvalis* males stand still and avoid contact with the heterospecific individuals (Sablina and Belozertseva, 2012). In the partition test, *M. obscurus* males in the presence of heterospecific individuals show higher locomotor activity than *M. arvalis* males do (Sablina et al., 2017).

It is worth noting that the aforementioned suggestions can explain the observed clines of the studied hybrid zone satisfactorily without invoking positive selection. Nevertheless, asymmetric introgression of mtDNA is often attributed to a selective advantage of mtDNA belonging to one of contacting species. Meanwhile, this idea is supported only by a few research articles involving 3D modelling of proteins coded by mitochondrial genes and an analysis of selection pressure on mitochondrial genes of some mammalian taxa participating in interspecific hybridisation (Awadi et al., 2021; Bartáková et al., 2021; Boratyński et al., 2014). Currently, we cannot exclude an effect of a selective advantage of some mitochondrial genes on the asymmetric introgression of mtDNA in the studied *M. arvalis* × *M. obscurus* hybrid zone.

The overall match between the positions of the phenotypic and genetic/chromosomal clines and relatively large width of the former (comparable with that of the *Cytb* cline) are expectable too. Skull shape in mammals is commonly polygenic (including autosomal genes). As

shown in recent genomic studies (Pallares et al., 2014, 2016), many loci of small effect determine skull shape differences between *Mus musculus musculus* and *M. m. domesticus*, and this polygenic effect is responsible for smooth transition in the hybrid zone between these subspecies of the house mouse. Such skull shape clines therefore indicate gross differentiation in terms of the autosomal genome. Therefore, the observed phenotypic cline and its overall match with other clines suggest that the hybrid zone analysed here is acting as a well-formed but still permeable barrier to gene flow.

Formation of the recent hybrid zone between *M. arvalis* and *M. obscurus*

Localisation and parameters of hybrid zones are determined by spreading of ancestral populations from refugia after the last, Valdai, glaciation. Ranges of the common vole and Altai vole have formed via their recent invasion into the Eastern European Plain. Taking into account results of an analysis of mtDNA (*Cytb*) and microsatellite markers (diversity of alleles and expected heterozygosity), Stojak et al. (2015, 2016) propose that the Eastern *M. arvalis* phylogroup, which inhabits Eastern Europe and the European part of Russia, survived during the Last Glacial Maximum in the Carpathian refugium. As potential glacial refugia of the Sino-Russian lineage of *M. obscurus*, the Crimean Peninsula and northern slopes of the Altai Mountains were analysed recently (Tougard et al., 2013). Taking into consideration the division of this lineage into the Crimean sub-lineage and Eurasian sub-lineage (Sibiryakov et al., 2018), we can suggest the northern Altai as a Last Glacial Maximum refugium for the latter.

The boundary between the Eastern *M. arvalis* phylogroup and the Eurasian sub-lineage of the Sino-Russian lineage of *M. obscurus* crosses the European part of Russia: Kirov, Kostroma, Nizhny Novgorod, Vladimir, Ryazan, Tambov, Lipetsk, Voronezh, Kursk and Belgorod Oblasts (Baskevich et al., 2009, 2012, 2016; Bulatova et al., 2010b) and continues to eastern Ukraine (Zagorodnyuk, 1993). Keeping in mind the data on dynamics of ecosystems on the territory where the *M. arvalis* × *M. obscurus* hybrid zone lies, after the Valdai (Wurm) glaciation, these species probably came into contact in various sections of the hybrid zone at different time points. The zone of meadow-steppes has formed on the present-day territory of the Central Black Earth Region after the retreat of the glaciers. Forests occupy small local plots in flood-lands, and a considerable part of the territory represents typical habitats of common voles.

In the north, in the zone of mixed and broad-leaved forests (including the Low Oka basin) and in the zone of south taiga, the spread of voles was restricted by forests. A necessary condition for the expansion of voles northwards – and consequently for the contact between *M. arvalis* and *M. obscurus* and for the formation of a stable hybrid zone – was the existence of open plots.

Despite substantial predominance of forests on the given territory, there is evidence of two large open areas in the post-glaciation period (the Vladimirskoye Opolje and Muromskoye Opolje), where meadow steppes and south flora have prevailed (Seregin, 2014). Apparently, *M. arvalis* occupied all available open habitats on the left bank of the Oka River, including plots of meadows in flood-lands of the river. MtDNA of the *M. arvalis* that occurs on the left bank of Oka right up to the river supports this notion. The Oka River, or strictly speaking, the large area of a forest on the right bank and marsh-ridden plots in the flood-lands of the river, occupies the eastern natural boundary of *M. arvalis*. 'Pure' populations of *M. obscurus* were registered on the right bank of Oka; no signs of *M. arvalis* were noted on the right bank.

It was shown previously that the spread of *M. arvalis* and *M. obscurus* is not limited by rivers, including major rivers in the region, the Volga and the Don (Bulatova et al., 2010b). Riverine barriers may affect location and shape of a hybrid zone in different ways. Tension zone theory predicts that moving hybrid zones easily become trapped at population density troughs or local physical barriers (including rivers), implying that they can move only short distances before they stabilise (Barton and Hewitt, 1985). A river may both contribute to shrinking of cline width (Narain and Fredga, 1996) and increase the width of a cline because of the 'pocket effect' (Moska, 2003). The centre of the studied section of the hybrid zone between *M. arvalis* and *M. obscurus* is located 43 km away from the Oka River, and it is unlikely that the latter can significantly affect the shape and width of the zone. Nevertheless, we can theorise that the Oka River represents some barrier to gene flow between common vole populations inhabiting opposite banks of the river. The absence of *M. arvalis*'s genetic markers on the right bank of the Oka River can be regarded as a consequence of the isolating effect.

Does the width of the hybrid zone correlate with the divergence level of contacting taxa?

Divergence between *M. arvalis* and *M. obscurus* significantly exceeds the divergence between European phylogroups of *M. arvalis* (Haynes et al., 2003; Bulatova et al., 2010a; Stojak et al., 2016). It is logical to expect that reproductive isolation between *M. arvalis* and *M. obscurus* is greater when compared with phylogenetic lineages of *M. arvalis* and that the width of a hybrid zone is inversely proportional to reproductive isolation between the crossing forms.

Beysard and Heckel (2014), Sutter et al. (2013) provided data on the width of the individual clines, but information about the width of the hybrid zone is not available. For this reason, we compared the width of *Cytb* and autosome clines in the studied section of the hybrid zone between *M. arvalis* and *M. obscurus* and between North-Western, Central, Eastern and Italian lineages of *M. arvalis* (it is admissible to compare the *Tp*53 cline and the cline of autosomal microsatellite markers).

Contrary to our expectations, the width of the *Cytb* cline in Vladimir Oblast (46 km) significantly exceeds the width of this cline in the following hybrid zones: North-Western–Central, Jura-1, Jura-2 and Jura Mountain northeast of Lake Geneva (16, 4 and 17 km, respectively); Central-Italian, Grisons (39 km) and Central-Eastern, Bavaria (23 km) (Sutter et al., 2013; Beysard and Heckel, 2014). As for autosome clines, the width of the *Tp53* cline in Vladimir Oblast (15 km) exceeds the width of the autosomal microsatellites' cline in Jura-1 and Jura-2 (9 and 13 km, respectively); meanwhile, the width of the autosomal microsatellites' cline in Bavaria is 29 km (Beysard and Heckel, 2014).

It should be noted that landscape features are significantly different among the above-mentioned hybrid zones. The Jura 1 transect crosses a valley characterised by several potential geographical barriers (lakes, a river and forest) and is surrounded by uninhabitable swamps. The Jura 2 transect is located between two lakes, where the territory had been marsh-ridden (i.e. impassable for common voles) until it was drained artificially in 1868–1876. The Bavaria transect was oriented along a broad corridor of farmlands between two forest blocks without a potential geographical barrier for common voles in this contact zone (Beysard and Heckel, 2014). The studied section of the hybrid zone between *M. arvalis* and *M. obscurus* represents a complex mosaic of farming landscapes and forests. Obviously, common vole populations inhabiting separated agricultural fields (or clusters of fields) are isolated from each other to different degrees. The Oka River is the only geographical barrier existing in the studied section of this hybrid zone (see above about its possible isolating effect). We propose that the field-forest mosaic feature of the area has an influence on the dispersion pattern of common voles and consequently on parameters of the hybrid zone.

Taking into consideration information on hybrid zones North-Western–Central in three sections of this zone, Central-Italian and Central-Eastern (Beysard and Heckel, 2014; Sutter et al., 2013), we can conclude that characteristics of the hybrid zone are more likely determined by landscape factors than by the degree of divergence between contacting lineages. It should be emphasised that landscape characteristics in every hybrid zone and individual sections of the zone are unique. We analysed only one section of the long hybrid zone between *M. arvalis* and *M. obscurus.* To compare in detail this hybrid zone with hybrid zones between different phylogenetic lineages of *M. arvalis* adequately, it is necessary to investigate characteristics of the *M. arvalis* × *M. obscurus* hybrid zone in several sections of this zone and to estimate the influence of specific landscape features on hybrid zone parameters.

The pericentric inversion in autosome No. 5 appears to have not introgressed across the hybrid zone

A specific feature of the studied section of the hybrid zone is occurrence of the pericentric inversion in autosome No. 5. Radjabli and Grafodatsky (1977) discovered this inversion; afterwards, it was noticed in Russia: in the Caucasus, in the Central Black Earth Region, in the Volga region, in the South Urals and Trans Urals (Gileva et al., 1996; Koval'skaya et al., 2007) and in Eastern Kazakhstan (Vorontsov et al., 1984; Kozlovskii et al., 1988; Akhverdian et al., 1999; Baskevich et al., 2005, 2016). Among different territories, the frequency of acrocentric chromosome No. 5 varies. Bulatova et al. (2007, 2010) did not find this rearrangement in hybrid populations from the Vladimir Oblast (Kovrov district). Near Arzamas (Nizhny Novgorod Oblast), the frequency of the inversion varies from 8.5 % to 12.5 % and overall equals 8.9 % (Baskevich et al., 2016). Meanwhile, in the analysed section of the hybrid zone in Vladimir and Nizhny Novgorod Oblasts, the frequency varies among eight sites from 0.71 % to 12.5 %, and overall was found to be 1.5 %. As readers can see, the frequency of the inversion in the hybrid zone under study is lower than that in Nizhny Novgorod Oblast. The variation in the inversion frequency may be attributed to gene drift in small populations. On the other hand, it cannot be ruled out that ecological conditions influence the occurrence of this rearrangement (Baskevich et al., 2016; Gileva et al., 2005).

In the analysed section of the hybrid zone under study, the inversion in autosome No. 5 is present exclusively in voles showing the prevalence of cytogenetic (18–20A) and molecular-genetic features of *M. obscurus*. This pattern may be caused by incompatibility between this chromosomal mutation and some genes of *M. arvalis*.

An analogous situation has been noted in the hybrid zone between *M. m. musculus* and *M. m. domesticus* in Denmark (Nance et al., 1990; Fel-Clair et al., 1996). In this zone, *M. m. musculus* and one of chromosomal races of *M. m. domesticus*, which is characterised by multiple Robertsonian translocations, come into contact. Introgression of certain Robertsonian rearrangements from *M. m. domesticus* to *M. m. musculus* was not found. Analysis of the allozyme genes linked with different Robertsonian translocations indicates the incompatibility of some pericentric regions with genes of *M. m. musculus* (Nance et al., 1990; Fel-Clair et al., 1996).

Conclusion

This work provides evidence of an overall match among genetic, chromosomal and morphological clines in a relatively narrow hybrid zone where the overall barrier to gene flow is strong but incomplete. The revealed difference in introgression among paternal, maternal and biparental molecular markers is rather typical for mammals, where many species show male-biased dispersal. The observed asymmetrical mtDNA introgression is more parsimoniously explained by a recent invasion of *M. obscurus* into the geographic range of *M. arvalis*. Furthermore, we found that the pericentric inversion in autosome No. 5 (specific for *M. obscurus*) does not pass through the centre of the hybrid zone; this result can be attributed to incompatibility between this chromosomal mutation and certain genes of *M. arvalis*. It is clear that the studied hybrid zone represents a suitable model for future investigation of the mechanisms allowing diverging species to maintain their integrity despite gene flow. A comparative analysis of genomic variation along several transects through presumably differentially aged parts of this long hybrid zone is an interesting avenue of further research (this project is in progress).

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Dataset.

Figure S2 Geometric landmarks used in the study.