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ICHTHYOLOGY

Genetic Screening of Distribution Pattern of Roaches *Rutilus rutilus* and *R. lacustris* (Cyprinidae) in Broad Range of Secondary Contact (Volga Basin)

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Abstract—We investigated the distribution of mitochondrial lineages of two species—the common roach *Rutilus rutilus* and Ponto-Caspian roach *R. lacustris*—in the broadest zone of their secondary contact, the Volga basin. For the purpose of species identification, we applied the approach of multiplex PCR based on species-specific divergences in sequences of the first subunit of cytochrome oxidase (*COI*) of mtDNA. A total of 1120 samples from 82 localities are analyzed. The distribution of certain mitochondrial lineages and their sympatric co-occurrence clarified for the Volga basin. Our study shows that *R. rutilus* is significantly predominant in the Upper Volga, while the Middle and Lower Volga is dominated by *R. lacustris*. The various hypotheses of formation of the broad spatial pattern of secondary contact are discussed.

Keywords: roach, *Rutilus rutilus*, *R. lacustris*, genetic screening, secondary contact, phylogeography, Volga

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INTRODUCTION

The taxonomy of genus *Rutilus* Rafinesque, 1820 is controversial. According to various opinions, the genus includes 10 to 15 species with wide distribution in the Palaearctic (Kottelat and Freyhof, 2007; Fricke et al., 2020). During the 20th century, ichthyologists had recorded four *Rutilus* taxa in the Volga basin: the common roach *Rutilus rutilus* (Linnaeus, 1758), kutum *R. frisii* (Nordmann, 1840), the Caspian roach *R. caspicus* (Jakovlev, 1870), sometimes considered a subspecies of *R. rutilus,* and *R. rutilus fluviatilis* (Jakovlev, 1873) (Berg, 1949; Mironovsky and Kasyanov, 1986; Reshetnikov et al., 2003; Bogutskaya and Naseka, 2004). *R. rutilus* was thought to be distributed throughout the entire basin of the Volga River (the distribution range of the species extends from Great Britain to the Volga River basin (Berg, 1949)), while *R. caspicus,* as a semi-anadromous form widespread in the Caspian Sea, migrated up the Volga river no further than the delta (Berg, 1949; Mironovsky and Kasyanov, 1986). Kutum *R. frisii* is a valuable semi-anadromous fish that inhabits the Caspian Sea; its morphology, ecology, and genetics are different from all other aforementioned species (Kotlik et al., 2008). The validity of *R. rutilus fluviatilis* was significantly questioned (Mironovsky and Kasyanov, 1986).

Over the past 20 years, molecular genetic methods have become an important part of the studies of taxonomic diversity of fishes and their geographical distribution. Recent research on the phylogeny and phylogeography of *Rutilus* from the eastern part of the distribution range showed the presence of two well-differentiated mitochondrial lineages in the Volga (Levin et al., 2017): *R. rutilus* and *R. lacustris* (Pallas,

Abbreviations: PCR, polymerase chain reaction.

Fig. 1. External appearance of roaches: (a) *Rutilus rutilus* (Medveditsa River near the village of Il'goshi, Tver oblast); (b) *R. lacustris* (Kubnya River near the village of Malye Koshelei, Chuvashiya Republic).

1814). The latter includes four Ponto-Caspian nominal species and subspecies and one species from the Aegean Sea basin with no differentiation between them in mtDNA: *R. caspicus, R. heckelii* (Nordmann, 1840), *R. rutilus aralensis* Berg 1916, *R. schelkovnikovi* Derjavin 1926, and *R. stoumboudae* Bianco & Ketmaier 2014 (Levin et al., 2017). The contact zone of two mitochondrial lineages was recorded in the basins of the Aegean, White, Black, and Caspian seas, as well as the Sea of Azov. The largest contact zone, with a length of ~1700 km, was found in the Volga Basin (Levin et al., 2017). The morphological revision of these lineages requires further research; however, according to our preliminary data, the red color of the iris is more pronounced in *R. rutilus* compared to *R. lacustris* (Fig. 1).

To identify the distribution patterns of phylogenetic lineages of roach, we performed the genetic screening of mass material using multiplex PCR developed to identify the species *R. rutilus* and *R. lacustris* (Ermakov et al., 2017).

The aim of this study is to determine the distribution boundaries of the two roach species marked by mtDNA lineages, identify the zone of sympatry of these species that originated as a result of secondary contact, and discuss hypotheses of the formation of their current distribution pattern.

MATERIALS AND METHODS

The study used contemporary (2014–2017) and historical (1956) material. Contemporary material was collected in the Volga Basin (n = 812 from 52 localities) and from the upper reaches of adjacent basins (n = 179 from 12 localities) of the Northern Dvina, Onega, Ob, Dnieper, Don, and Ural rivers, as well as endorheic Lake Saryshyganak (Table 1). Some data (83 individuals from 18 localities) was obtained from the GenBank NCBI genetic database (www.ncbi.nlm.nih.gov). Historical material obtained from the collection of the Papanin Institute for Biology of Inland Waters of the Russian Academy of Sciences and is represented by dry scales from archive scale books (Rybinsk Reservoir, n = 46). A total of 1120 individuals were analyzed.

We used fin fragments fixed in 96% ethanol or scales preserved by drying in scale books for DNA extraction. DNA was isolated using the salt method in combination with lysis by proteinase K (Aljanabi and Martinez, 1997). To identify the species, we used a multiplex PCR test system based on species-specific differences in the sequences of the mtDNA *COI* gene

Table 1. Localities and sampling size

No.	Localities	Source of data (Genbank Acc. Nos.)	Sample size	Lat	Long
	Upper Volga				
1	Lake Beloe	Authors data	35	60.168	37.636
2	Rybinsk Reser.	The same	55	58.083	38.278
3	Volga R. (Uglich. Res.), mouth of Nerl' River	KX583840-844	5	57.130	37.650
4	Volga R. (Ivankovo Res.), near the city of Konakovo	Authors data	24	56.714	36.748
	Middle Volga				
5	Unzha River, Manturovo	Authors data	20	58.380	44.881
6	Yaiva River, Volodin Kamen' vill.	KX583891	1	59.270	56.730
7	Mulyanka River, Bolshoe Savino vill.	Authors data	24	57.931	56.008
8	Lyp River, Kez settl.	The same	12	57.861	53.705
9	Votkinsk Res., Konakovo Nytva City	″	2	57.830	55.420
10	Pond on Malinovka River	″	4	57.804	55.881
11	Cheptsa River, Chepyk vill.	″	5	57.726	53.621
12	Volga River (Gorky res.), Kostroma City	″	20	57.722	40.961
13	Sylva River, Kungur City	″	21	57.469	56.891
14	Volga River (Gorky Res.), Utes vill.	″	19	57.432	41.609
15	Volga River (Gorky Res.), Elnat'	″	7	57.369	42.870
16	Lake Nero	KX583865-70	6	57.180	39.400
17	Tulva River, Krylovo vill.	Authors data	14	57.155	55.573
18	Volga River (Gorky res.), Novlenskoe vill.	The same	20	57.153	43.014
19	Kama River, Obvinsky bay	″	18	58.625	55.967
20	Vetluga River, Lysitsa vill.	″	21	57.059	45.271
21	Lukh River, Myt settl.	″	22	56.854	42.280
22	Kerzhenets River, Bydreevka vill.	″	8	56.839	44.636
23	Volga River (Gorky Res.), Chkalovsk City	″	17	56.736	43.211
24	Votkinsk Res., Chaikovskiy City	″	5	56.735	54.256
25	Kamenka River	″	1	56.720	59.188
26	Volga River (Gorky Res.), Zubovo settlement	″	24	56.728	43.356
27	Lake Galichskoe	″	3	58.443	42.394
28	Volga River, near the city of Balakhna	″	13	56.553	43.525
29	Volga River (Cheboksary Res.), mouth of Vetluga River	KX583892-94	3	56.320	46.370
30	Izh River, Tuba vill.	Authors data	6	56.311	52.972
31	Volga River (Cheboksary Res.), mouth of Parat River	The same	27	56.209	46.906
32	Klyazma River, Fryazino vill.	″	21	56.043	40.283
33	Volga River (Kuybyshev Res.), Zvenigovo City	″	8	55.961	48.003
34	Volga River (Kuybyshev Res.), Zelenodol'sk City	″	24	55.828	48.500
35	Volga River (Kuybyshev Res.), mouth of Svijaga River	″	14	55.783	48.693
36	Yuryzan River, Idelbaevo 2 vill.	″	5	55.418	57.824
37	Yuryzan River, Mechetlino vill.	″	20	55.354	57.988
38	Vorya River, Tupicheno vill.	KX583874-78	5	55.250	35.070
39	Volga River (Kuybyshev Res.), Kamskoe Ustye settl.	Authors data	24	55.200	49.401
40	Aktai River, Vozhi vill.	The same	3	55.115	49.571
41	Lake Zyuratkul	″	20	54.915	59.226
42	Skniga River, Serpukhov City	″	2	54.870	37.400
43	Oka River, Perovo vill.	″	21	54.833	41.457
44	Sura River, Alatyr City	″	5	54.825	46.624
45	Sim River, Tikeevo vill.	″	20	54.821	56.903

Table 1. (Contd.)

No.	Localities	Source of data (Genbank Acc. Nos.)	Sample size	Lat	Long
46	Ugra River, Yukhnov City	KX583871-73	3	54.750	35.150
47	Moksha River, Mordovian Nature Reserve	Authors data	20	54.727	43.151
48	Ugra River, Plyuskovo vill.	The same	21	54.693	35.527
49	Moksha River, Kadom City	″	10	54.511	42.510
50	Ik River, Kyzyl-Yar vill.	″	7	54.393	53.405
51	Volga River, Usinskiy bay	″	20	53.283	49.111
52	Sura River, Ukhtinka vill.	″	2	53.270	45.042
53	Sura River, Sosnovoborsk settl.	″	5	53.261	46.247
54	Varezhka River	″	23	53.232	43.974
55	Sursk Res.	″	4	52.996	45.310
	Lower Volga				
56	Volga River (Saratov Res.), Alexeevka settl.	KX583899-906	8	52.300	48.050
57	Volga River (Volgograd Res.)	Our data	22	51.259	45.854
58	Volga River, Volgograd City	Our data	24	48.525	44.511
59	Volga–Akhtuba system	KX583907-17	11	48.480	45.480
60	Akhtuba River	KX583918-19	2	47.430	47.170
61	Volga River, Ikryanoe vill.	Our data	20	46.091	47.740
	Northern Dvina				
62	Lake Sol	KX583754-57	4	62.570	41.470
63	Yug River, Veliky Ustyug City	KX583758-63	7	60.720	46.330
64	Luza River	Authors data	19	60.421	48.560
65	Sukhona River, Tot'ma City	The same	13	59.969	42.786
66	Lake Kubenskoe	″	24	59.623	39.493
67	Sukhona River, Sokol City	″	28	59.445	40.170
	Onega				
68	Lake Vozhe	″	23	60.687	38.970
	Ob				
69	Turya River (Andriushinskoe res.), Karpinsk City	″	10	59.815	59.790
70	Tura River, Karelino settl.	″	13	58.734	60.262
71	Lake Bolshie Allaki	″	19	55.963	60.891
	Dnieper				
72	Vyaz'ma River, Leontyevo vill.	KX583772-77	6	55.220	33.850
	Don				
73	Krutets River, bridge on Kolyshley	Authors data	10	52.887	44.591
74	Khoper River, Bekovo settl.	The same	9	52.451	43.721
75	Don River, Faustovo vill.	″	8	52.440	38.560
76	Vorona River, Korostelevo vill.	″	3	51.841	42.425
77	Usmanka River, Venevitinskiy settl.	KX583796-805	10	51.820	39.380
78	Savala River, Troitskoe vill.	KX583807-08	2	51.270	41.470
79	Don River, Stupino	KX583826-27	2	50.620	39.920
80	Don River, Golubinskaya	KX583806	1	48.830	43.530
	Ural				
81	Lake Shalkar	KX583940-43	5	50.630	51.750
	Inland basin				
82	Lake Saryshiganak	KX583945-46	2	49.450	49.880

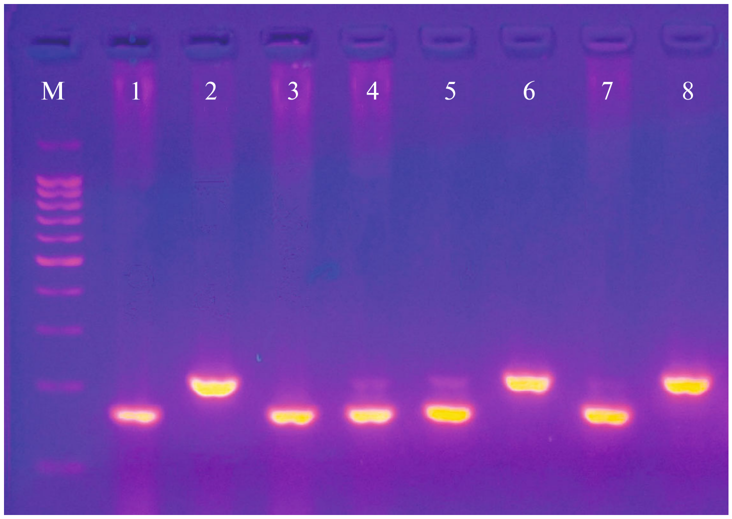

Fig. 2. Electropherogram of products of multiplex PCR with species-specific primers for mtDNA *COI* gene during the identification of two mitochondrial lineages of *Rutilus*: wells 1, 3–5, 7: *R. lacustris*; 2, 6, 8: *R. rutilus*; M, 100 bp DNA ladder.

fragment (Ermakov et al., 2017). The system uses one common forward primer (*COI* D-Ru 5'-ATT CGG CAA CTG ACT CGT CC-3') and two species-specific reverse primers (*COI* R-Rl 5'-GCG GGT ATA CTG TTC ATC CT-3' for *R. lacustris* and *COI* R-Rr 5'-GTT AAA TCT ACT GAT GCC CCG-3' for *R. rutilus*). As a result of the PCR (amplification conditions are as follows: 94°C for 30 s, 61°C for 30 s, 72°C for 30 s, and 30 cycles), fragments of the *COI* gene with different lengths are amplified: 161 bp for *R. lacustris* and 207 bp for *R. rutilus*. The difference of 46 nucleotides is satisfactory for a visual identification of the species based on the electrophoresis in agarose gel (Fig. 2).

We used a generally accepted division of the Volga basin into three sections: the dam of the Rybinsk Reservoir was considered the border between the Upper and Middle Volga; the dam of the Kuybyshev Reservoir was considered the border between the Middle and Lower Volga (*Volga* ..., 1978).

The ArcGIS 10.8 geoinformation system was used to construct a distribution map with the inclusion of HydroATLAS data (Linke et al., 2019) and GADM v. 3.6 (www.gadm.org).

The distance from a locality to the Volga River headwaters was measured based on the HydroATLAS data. The relationship between the portion of the species in the total sample and the distance from the headwaters to the locality was assessed using the Pearson correlation coefficient r (STATISTICA 6.0).

To compare the distribution frequencies of mitochondrial lineages in different regions of the Volga basin, we used the χ^2 criterion for the fourfold tables (Sokal and Rohlf, 1981).

RESULTS

Both lineages of roach are widespread in the Volga River basin (Fig. 3). *Rutilus rutilus* is predominant in the Upper Volga reaching 95% of the total sample in this section (sample size $n = 119$); *R. lacustris* is predominant in the Middle Volga (72% of the sample, $n = 650$) and Lower Volga (92%, $n = 87$) (Table 2). Differences in the distribution frequencies of the mtDNA lineages between the Upper Volga and the other two regions (Middle and Lower Volga) have statistical support ($\chi^2 = 82.1$, $p < 0.0001$ and $\chi^2 = 124.9$, $p < 0.0001$, respectively); no differences were found between the frequency distribution in the Middle and Lower Volga ($\chi^2 = 2.4$, $p > 0.05$).

Both species were found in most localities (74%, $n = 61$). The portion of localities represented by only species is much smaller: 4% for *R. rutilus* ($n = 3$) and 22% for *R. lacustris* ($n = 18$). For the total sample from the Volga basin, individuals with *R. lacustris* mtDNA account for 65%; individuals with *R. rutilus* mtDNA account for 35%.

A comparison of the historical and contemporary data on the Rybinsk Reservoir shows that the ratio of species was stable over the past 60 years. For example, the ratio recorded in 1956 was 93% *R. rutilus* vs. 7% *R. lacustris* ($n = 46$); in 2016, the ratio was 91% *R. rutilus* vs. 9% *R. lacustris* ($n = 55$). It should be noted that, by 1956, approximately 10 years had passed since the creation of the reservoir; therefore, the frequency ratio of the lineages basically had not changed during the entire existence of the reservoir.

The distribution of phyletic lineages in the Volga basin is uneven and represents a wedgelike shift of dominance from *R. rutilus* to *R. lacustris* from the upstream to the downstream, which is observed both in the basin as a whole and along the Volga River channel

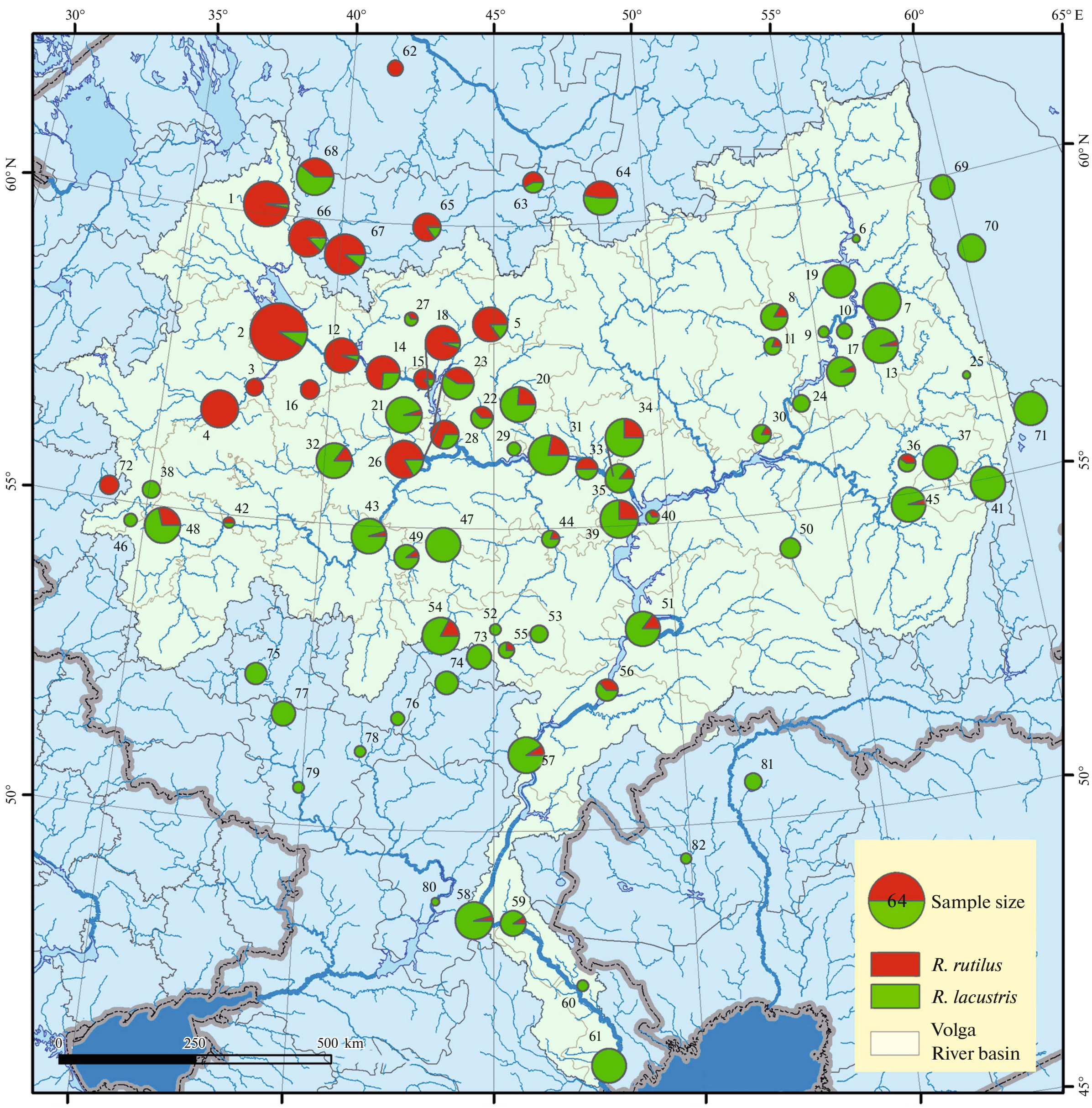

Fig. 3. Ratio of two roach species in localities of the Volga basin and upper reaches of adjacent basins. Locality numbers correspond to those in Table 1. Circle sizes correspond to sample sizes.

(Table 2). The dominance of *R. rutilus* is very noticeable in the Upper Volga in localities up to 1500 km from its headwaters along the channel, which is statistically supported by a strong negative correlation (–0.74) between the proportion of *R. rutilus* and the distance from Volga River headwaters (Fig. 4).

In adjacent basins, *R. rutilus* was recorded in river systems drained by the Valdai Upland: in the Baltic Sea basin and in the basins of the Severnaya Dvina, Onega, and Upper Dnieper rivers (Levin et al., 2017), which have a common watershed with the Upper Volga. In basins of other rivers bordering the Volga basin, *R. rutilus* was recorded only in the lower reaches of the Don River (Levin et al., 2017).

DISCUSSION

Secondary contact between closely related species is an extremely interesting phenomenon from an evolutionary point of view and has an environmental aspect to it as well. Upon contact of the previously isolated but still closely related species or populations, the introgressive hybridization often occurs, leading to a variety of consequences: from the genome heterogenization of the contacting parts of the populations with

Table 2. Ratio of *Rutilus rutilus* and *R. lacustris* in various regions of Volga River basin and reservoirs.

Region	Number of specimens	*R. rutilus, %*	*R. lacustris, %*
Regions of Volga basin			
Upper Volga	119	95	5
Middle Volga	650	28	72
Lower Volga	87	8	92
Reservoirs of Volga River			
Ivankovo	24	100	0
Uglich	5	100	0
Rybinsk:			
data for 2014–2017	55	91	9
data for 1956	46	93	7
Gorky	126	83	17
Cheboksary	43	35	65
Kuybyshev	90	23	77
Saratov	8	38	63
Volgograd	22	9	91
Volga River below the city of Volgograd	57	4	96

the preservation of both contacting species and the formation of mitonuclear dissonance (mitochondrial introgression) to the hybrid swarm and displacement of one species by another (Harrison, 1993; Lajbner et al., 2009; Abbott et al., 2016; Sousa-Santos et al., 2018; Levin et al., 2019a, 2019b). In some cases, heterogeneous genomes resulting from ancient hybridization can serve as the basis for adaptive radiation and further species diversification (for example, Meier et al., 2017).

Having no nuclear genome data to date, in order to assess the degree of hybridization of the two *Rutilus* species, let us consider the historical and geographical aspects of the formation of the contact zone.

An extensive zone of secondary contact of two *Rutilus* species in the Volga basin can be the result of both anthropogenic activity and the result of natural processes. We consider both hypotheses in more detail.

One may assume that the zone of secondary contact of *Rutilus* species in the Volga basin arose as a result of anthropogenically determined entry of *R. rutilus* from the basins of the Baltic and White seas along the Volga-Baltic invasion corridor (Slynko et al., 2010) and its further spread along the Volga River up to the Volga-Akhtuba floodplain during the last two or three centuries. The Volga River basin is connected to the Baltic Sea basin by three artificially constructed

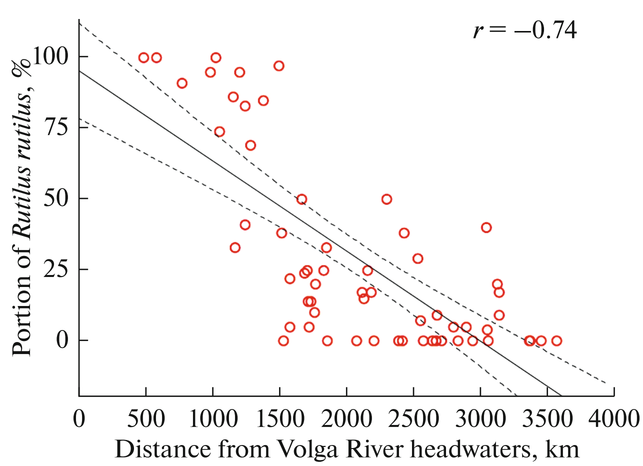

Fig. 4. Portion of *Rutilus rutilus* at various distances from Volga River headwaters.

canal systems: Vyshnevolotskaya (through the Tvertsa River, since 1709), Tikhvinskaya (through the Mologa river, since 1811), and Mariinskaya (through the Sheksna river, since 1810); it is connected to the White Sea basin with the North Dvinsky Canal (through the Sheksna River, since 1828) (Nizovtsev et al., 2009). The last three systems are located in the area of the Rybinsk reservoir and the Vyshnevolotskaya system is located further up the Volga River (near the city of Tver). The latter was created earlier and there 100% dominance of *R. rutilus* recorded. In the area of the Tikhvinskaya, Mariinskaya, and Severo-Dvinskaya water systems, which built later, few representatives of *R. lacustris* detected. However, the vast size of the contact zone and the absence of changes in the ratio of mitochondrial lineages in the Rybinsk Reservoir over the past 60 years provide little support for hypothesis of the human induced secondary contact.

Apparently, the zone of secondary contact existed long before the connection of the basins by human constructed channels and could have arisen in the postglacial time. It is likely that *R. lacustris* inhabited the Volga basin earlier than *R. rutilus,* judging by its predominance in the Volga river system and by this lineage being the only one present in all other parts of the Caspian Sea basin. However, the starlike structure of the haplotype network of cytochrome *b* gene (Cyt *b*) sequences and the relatively low haplotype diversity of this species in the Volga basin (Levin et al., 2017) indicates a relatively young age of the Volga *R. lacustris* population.

It is possible that *R. rutilus* entered the Volga basin through the periglacial lakes that had formed as a result of the melting of the last glacier 20000–17000 years ago; the connection of the Upper Volga with the surrounding basins of the Baltic and White Seas and the Dnieper River through them is well known (Kvasov, 1975; Mangerud et al., 2004; Svendsen et al., 2004). Periglacial lakes played an important role in the migration of freshwater ichthyofauna between Europe and Asia (Kusznierz et al., 2011; Borovikova et al., 2013). The significant predominance of the *R. lacustris* lineage in the Kama River basin at similar latitudes to the Upper Volga basin (dominated by the *R. rutilus* lineage) is likely due to the remote location of the Kama basin from the watershed with the Baltic and the Upper Dnieper, where *R. rutilus* occurs. In addition, a significant part of the Kama basin watershed borders the Ob basin, where only *R. lacustris* was recorded (Levin et al., 2017).

The noticeable similarity in the geography of distribution of both roach lineages according to the data of genetic screening with the taxonomic concepts of Berg (1949) is quite remarkable. In particular, Berg believed that *R. rutilus rutilus* inhabits the Upper and Middle Volga to the mouth of the Kama River, while *R. rutilus fluviatilis* inhabits the Kama as well as Middle and Lower Volga. Moreover, he also referred to the morphological similarity of the Siberian roach (*R. lacustris*) and Aral roach (*R. rutilus aralensis*), which, according to a recent molecular genetic study (Levin et al., 2017), represent a single mitochondrial *R. lacustris* lineage along with other taxa. Previously, *R. lacustris* was called the Siberian roach, but due to the addition of new data (extremely wide distribution and inclusion of a number of other taxa in the species), the name Ponto-Caspian roach is proposed (Levin et al., 2017).

Remarkably a similar frequency distribution of mitochondrial haplotypes in the Volga basin is typical not only for fishes, but also for two cryptic forms of the marsh frog: Central European *Pelophylax ridibundus* (Pallas, 1771) and Anatolian *Pelophylax* cf. *bedriagae* (Camerano, 1882). Haplotypes of the southern Anatolian frog are dominant in the Lower and Middle Volga regions, and haplotypes of the northern (Central European) frog are dominant in the Upper Volga region; however, the entire territory of the Volga basin is a zone of sympatry and hybridization of these two forms (Ermakov et al., 2014; Lyapkov et al., 2018; Ivanov, 2019; Litvinchuk et al., 2020).

In conclusion, we note that additional morphological and genetic studies are required to identify more accurate directions of the dispersal of the phyletic lineages in the Volga and adjacent basins, as well as to determine the degree of hybridization of lineages in the contact zone.

CONCLUSIONS

The sympatric zone of *Rutilus rutilus* and *R. lacustris* includes almost the entire Volga River basin with the exception of the most upper reaches of the Volga River (where only *R. rutilus* was recorded), as well as the eastern (pre-Ural part of the Kama basin) and southern (delta and adjacent area of the Volga-Akhtubinskaya floodplain) parts of the basin, where only *R. lacustris* was recorded. The degree of predominance of *R. rutilus* decreases in a wedgelike manner from the Upper Volga to the Middle and Lower Volga, where *R. lacustris* is predominant. It appears that the Volga basin was initially inhabited by *R. lacustris*, and the entry of *R. rutilus* occurred during the postglacial period through the northwestern watershed with the White Sea, Baltic, and Dnieper basins.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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