MICROBIOLOGY AND VIROLOGY

GENETIC HETEROGENEITY OF *RICKETTSIA HELVETICA* POPULATION

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ABSTRACT

Background. *To date, the genetic variability of Rickettsia helvetica has not been sufficiently studied.*
The aim. *To study the prevalence and genetic variability of R. helvetica in Ixodes spp. collected in Western Siberia and the Russian Far East.*
Materials and methods. *Ixodes spp. collected from rodents in the Omsk province, Western Siberia (n = 280) and collected by flagging on Putyatin and Russky Islands in Primorsky Krai, Russian Far East (n = 482) were analyzed for the presence of Rickettsia spp. All positive samples were genotyped for the gltA gene fragment. For a number of R. helvetica samples, fragments of the 16S rRNA, ompA, ompB, sca4, htrA, and groEL genes and 23S–5S intergenic spacer were additionally sequenced.*
Results. *Four Rickettsia species (R. helvetica, "Candidatus Rickettsia tarasevichiae", "Candidatus Rickettsia uralica", and "Candidatus Rickettsia mendelii") were found. Of them, R. helvetica was identified in 72.2 % of Ixodes apronophorus and 18.8 % of Ixodes trianguliceps from the Omsk province and in single Ixodes persulcatus from the Omsk province and Putyatin Island. This is the first finding of Rickettsia spp. in I. apronophorus. All known R. helvetica sequences from this study and the GenBank database belonged to four well supported monopheletic groups forming genetic lineages I–IV. Lineage I included European isolates from Ixodes ricinus, Western Siberian isolates from I. persulcatus, and some sequences from I. apronophorus. All R. helvetica sequences from I. trianguliceps from the Omsk province and I. persulcatus from the Komi Republic and one sequence from I. apronophorus were assigned to lineage II. Most sequences from I. apronophorus formed lineage III; all known R. helvetica sequences from I. persulcatus from the Far East formed genetic lineage IV.*
Conclusion. *The genetic heterogeneity of R. helvetica population was first demonstrated. Known isolates of R. helvetica are reliably assigned to four genetic lineages, but not in all cases association of different lineages with a specific tick species or specific territory was observed.*

Key words: *Ixodes apronophorus, Ixodes persulcatus, Ixodes trianguliceps, Rickettsia helvetica, sympatric areas, genetic lineages, phylogenetic analysis*

Received: 31.08.2023
Accepted: 24.11.2023
Published: 29.12.2023

For citation: Rar V.A., Igolkina Ya.P., Yakimenko V.V., Tikunov A.Yu., Nikitin A.Ya., Epikhina T.I., Tikunova N.V. Genetic heterogeneity of *Rickettsia helvetica* population. *Acta biomedica scientifica*. 2023; 8(6): 91-104. doi: 10.29413/ABS.2023-8.6.8

ГЕНЕТИЧЕСКАЯ ГЕТЕРОГЕННОСТЬ ПОПУЛЯЦИИ *RICKETTSIA HELVETICA*

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РЕЗЮМЕ

Обоснование. *Генетическая вариабельность Rickettsia helvetica недостаточно изучена.*
Цель исследования. *Изучить встречаемость и генетическую вариабельность R. helvetica в Ixodes spp., собранных в Сибири и на Дальнем Востоке.*
Методы. *На наличие риккетсий проанализированы клещи, снятые с грызунов в Омской области (n = 280) и собранные на флаг на островах Путятина и Русский в Приморском крае (n = 482). Для всех образцов риккетсий секвенированы фрагмента гена gltA, а для ряда образцов R. helvetica дополнительно секвенированы фрагменты 16S rRNA, ompA, ompB, sca4, htrA и groEL генов и 23S–5S межгенного спейсера.*
Результаты. *Всего было выявлено четыре вида риккетсий. Из них R. helvetica обнаружена в 72,2 % Ixodes apronophorus и 18,8 % Ixodes trianguliceps из Омской области и в единичных Ixodes persulcatus из Омской области и с острова Путятина. Это первое выявление риккетсий в I. apronophorus. На основании проведённого филогенетического анализа последовательности R. helvetica из данной работы и из базы данных GenBank отнесены к четырём генетическим линиям. Линия I включает европейские изоляты из Ixodes ricinus, изоляты из I. persulcatus из Западной Сибири и некоторые последовательности из I. apronophorus. Все последовательности R. helvetica из I. trianguliceps из Омской области и из I. persulcatus из Республики Коми, а также последовательность из I. apronophorus отнесены к линии II. Большинство последовательностей из I. apronophorus образуют линию III, а все последовательности R. helvetica из I. persulcatus с Дальнего Востока – линию IV.*
Заключение. *Впервые показана генетическая гетерогенность популяции R. helvetica. Известные изоляты R. helvetica надёжно отнесены к четырём генетическим линиям, однако ассоциация различных линий с определённым видом клеща или с определённой территорией наблюдается не во всех случаях.*

Ключевые слова: *Ixodes apronophorus, Ixodes persulcatus, Ixodes trianguliceps, Rickettsia helvetica, область симпатрии, генетические линии, филогенетический анализ*

Статья поступила: 31.08.2023
Статья принята: 24.11.2023
Статья опубликована: 29.12.2023

Для цитирования: Рар В.А., Иголкина Я.П., Якименко В.В., Тикунов А.Ю., Никитин А.Я., Епихина Т.И., Тикунова Н.В. Генетическая гетерогенность популяции *Rickettsia helvetica*. *Acta biomedica scientifica*. 2023; 8(6): 91-104. doi: 10.29413/ABS.2023-8.6.8

INTRODUCTION

Rickettsia helvetica Beati et al. 1993 [Rickettsiaceae; Rickettsiales], belonging to the spotted fever group (SFG), is one of the causative agents of rickettsioses in Eurasia. Rickettsiosis caused by *R. helvetica* is characterized by a wide range of clinical manifestations: fever is more often without rash, and cases of perimyocarditis, meningitis, and sarcoidosis have been described [1–5]. This infection is mainly diagnosed in European countries. In Russia, only one case of rickettsiosis caused by *R. helvetic*a has been described in a patient with signs of acute febrile illness from the Perm Territory [6].

Specific vectors of *R. helvetica* are ticks of the genus *Ixodes*. *Rickettsia helvetica* is widely prevalent in ticks *Ixodes ricinus* (Linnaeus, 1758) in various European countries [7, 8]. In Russia, *R. helvetica* is observed in different regions; however, in most regions the occurrence of this species was rather low and did not exceed 5–8 %. Thus, *R. helvetica* was found in 4.6 % of the taiga tick (*Ixodes persulcatus* (Schulze, 1930)) in the Komi Republic [9], in 1.9 % of *I. persulcatus* in the Omsk region [10], in 5.1 % of *Ixodes* spp. in the Altai Territory [11], as well as in 8.1 % of *Ixodes persulcatus* (Pomerantzev, 1946) and in 6.9 % of interspecies hybrids of *I. persulcatus*/*I. pavlovskyi* in the Altai Republic [12]. In the mainland of the Far East, the occurrence of *R. helvetica* in taiga ticks was also low and amounted to 3.8–4.3 % in some areas of Khabarovsk Territory, and 2.4 % in the south of the Kamchatka Peninsula [13, 14]. The exception is Sakhalin Island, where the occurrence of *R. helvetica* in *I. persulcatus* exceeded 60 % [14].

In addition to *I. persulcatus* and *I. pavlovskyi* attacking humans, the nidicolous ticks *Ixodes trianguliceps* (Birula, 1895) and Ixodes *apronophorus* (Schulze, 1924), all developmental stages of which feed on small mammals, inhabit various areas of Western Siberia. Both species have an extensive but mosaic range. The tick *I. apronophorus* is a moisture-loving species inhabiting wetlands; one of its main hosts is the European water vole (*Arvicola amphibius* (Linnaeus, 1758)) [15–18].

Many works have been devoted to the study of *I. persulcatus* for the presence of various species of rickettsia; most often, "*Candidatus* Rickettsia tarasevichiae" is detected in *I. persulcatus* ticks (up to 90 % of infected ticks), and in rare cases – *R. helvetica*, *Rickettsia heilongjiangensis*, *Rickettsia raoultii* and *Rickettsia sibirica* [10, 12, 14, 19]. The tick *I. trianguliceps* is significantly less studied; in our previous studies, a new candidate species "*Candidatus* Rickettsia uralica" was found in *I. trianguliceps* ticks, as well as *R. helvetica* and "*Candidatus* R. tarasevichiae" [19]. Data on the infection of *I. apronophorus* ticks with rickettsiae were not available at the beginning of this study.

In the southern taiga and sub-taiga of Western Siberia there are areas of sympatry of three tick species that belong to the genus *Ixodes*: *I. persulcatus*, *I. trianguliceps* and *I. apronophorus*. In these areas of sympatry, the preimaginal stages of *I. persulcatus* and all developmental stages of *I. trianguliceps* and *I. apronophorus* can feed on the same small mammals, which may result in the transmission of any rickettsial species/gene variants from one tick species to another. In preliminary studies, we have discovered sites in the sympatric regions of *I. apronophorus*/*I. persulcatus*/*I. trianguliceps* in the Omsk region with the high abundance of all three tick species [20]. Islands in the Far East are also of great interest for study, as one of these islands (Sakhalin Island) had an unexpectedly high infection rate of the taiga tick with *R. helvetica* [14].

THE AIM OF THE STUDY

To study the occurrence and genetic variability of *R. helvetica* in different species of ticks of the genus *Ixodes* in remote areas of the Omsk region and the Far East.

MATERIALS AND METHODS

Collecting ticks

The material was collected on the territory of Bolsheukovsky district (site Om-Bo, 56° 46′ N, 72° 03′ E) and Znamensky district (site Om-Zn, 57° 23′ N, 73° 40′ E) of the Omsk Region, as well as on the territory of Putyatin Island (site Put 42° 50′ N, 132° 25′ E) and Russky Island (site Rus 43° 00′ N, 131° 50′ E), located in Peter the Great Bay of the Sea of Japan in Primorsky Territory (Fig. 1). *Ixodes* spp. ticks collected from rodents in the Omsk region and ticks collected by flagging in Primorsky Territory were included in the study.

Rodents were captured in the Omsk region at the Om-Bo site in June 2016 and at the Om-Zn site from June through September 2014–2015. Animals were examined for the presence of attached ticks (larvae, nymphs and adults), which were removed with tweezers. The species and developmental stage of ticks were preliminarily determined using a stereomicroscope MC-800 (Micros, Austria), according to morphological keys [21]. Some of the engorged and nearly engorged larvae and nymphs were stored at 10–15 °C for 1–2 weeks and then transported to the laboratory for metamorphosis. The remaining ticks were placed in sealed plastic tubes that were stored in liquid nitrogen until DNA extraction.

Questing ticks were collected from vegetation by flagging on Putyatin Island in 2021 and on Russky Island in 2019 and 2021. The species and developmental stage of ticks were preliminarily determined based on morphological criteria; only ticks of the genus *Ixodes* were included in further study.

Carrying out metamorphosis of ticks under laboratory conditions

Partially engorged larvae and nymphs were fed on laboratory white mice to repletion to successfully undergo metamorphosis. Each engorged tick was placed in an individual glass tube and kept in the dark at 100 % relative humidity at 24–26 °C until moulting was completed. The ticks that underwent metamorphosis after 4 weeks were individually frozen and stored at –70 °C until DNA isolation.

FIG. 1.
Tick collection sites

DNA isolation

Frozen ticks were homogenized with a MagNA Lyser Instrument using MagNa Lyser Green Beads (Roche Diagnostics, Switzerland). Total DNA was isolated using the Proba NK kit (DNA-Technology, Russia) according to the manufacturer's protocol.

Determination of tick species by molecular genetic methods

The species of *I. persulcatus*, *I. trianguliceps*, and *I. apronophorus* ticks was determined by multiplex polymerase chain reaction (PCR) using species-specific primers for the ITS2 intergenic spacer fragment as previously described [20]. *I. persulcatus*, *I. pavlovskyi* and interspecific hybrids were differentiated based on the determination of mitochondrial (cox1) and nuclear (ITS2) loci as described previously [12].

Identification and genotyping of *Rickettsia helvetica*

Rickettsia DNA was detected using nested PCR in the presence of genus-specific primers from the *glt*A gene region in the first round and primers specific for "*Candidatus* R. tarasevichiae" and specific for SFG rickettsiae in the second round, as described previously [14]. The obtained *glt*A gene fragments were sequenced for all positive rickettsiae samples from the SFG. For a number of positive samples containing *R. helvetica* DNA, gene fragments of 16S rRNA, *omp*A, *omp*B, *sca*4, and *htr*A, as well as a fragment of the *gro*ESL operon and the intergenic spacer 23S-5S rRNA (23S-5S IGS) were additionally amplified in the presence of primers indicated in Table 1 for subsequent sequencing. In addition, the same fragments of seven genetic loci were amplified for samples of *R. helvetica* revealed earlier on Sakhalin Island (site Skh) and in Khabarovsk Territory (site Khab) [14]. The identified nucleotide sequences have been deposited in the GenBank database under accession numbers OQ092468-OQ092487, OQ102487-OQ102493, OQ271213-OQ271221, OQ275007-OQ275011, OQ675828-OQ675832, OQ861252, OQ866612-OQ866624.

Sequencing and phylogenetic analysis

PCR products were purified using GFX columns (Amersham Biosciences, USA). The Sanger sequencing reaction was performed using BigDye Terminator v. 3.1 Cycling Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Sequencing products were purified using CentriSep columns (Princeton Separations, USA) and analyzed using ABI 3500 Genetic Analyzer (Applied Biosystems, USA). Sequence analysis was performed using BlastN [29]. Phylogenetic analysis was performed with the MEGA 7.0 phylogenetic software package [30] using the maximum likelihood (ML) method [31].

RESULTS

Species identification of collected ticks

The species identity of all ixodid ticks collected in the Omsk Region and Primorsky Territory was determined using molecular genetic methods. Ticks of three species (*I. apronophorus*, *I. persulcatus* and *I. trianguliceps*) were found in two sites in the Omsk region, but the pro-

TABLE 1

PRIMERS USED FOR *R. HELVETICA* AMPLIFICATION

Locus	Organism	Round	Primer	Sequence 5′-3′	T (°C)	Ref.
16S gene rRNA	*Rickettsia* spp.	I	16S1	gacgggtgagtaacacgtggg	56	[14]
			16S2	gtcttttagggatttgctccac		
		II	16S3	gatggatgagcccgcgtcag	60	
			16S4	gcatctctgcgatccgcgac		
ompA gene Fragment I	*Rickettsia* spp.		Rr190.70p	atggcgaatatttctccaaaa	55	[22]
			190-701	gttccgttaatggcagcatct		
ompA gene	*Rickettsia* spp.	I	Afnw_1	ggcacaaatactttaacattacc	52	#
			190-6808	cacgaactttcacactacc		[22]
		II	Afnw_3	aagcctactcctaaagagaatg	53	#
			Afnw_4	cgacagtctctagtgccg		
	Rickettsia spp.	I	A1	taacattacaagctggaggaagcc	58	#
			A2	ttcagagcctgaccaccgg		
		II	A5	caagtgctggtgatgttacta	56	
			A6	tagttacatttcctgcacctac		
	R. helvetica	I	Afn1_helv	gtaatactagcatcaccgaaatcc	55	#
			190-6808	cacgaactttcacactacc		[22]
		II	190-5125	gcggttactttagccaaagg	54	
			Afnw_4	cgacagtctctagtgccg		#
ompB gene	*Rickettsia* spp.	I	M59 F	ccgcagggttggtaactgc	55	[23]
			120-1497m*	cctatatcgccggtaattgtagc		
		II	BR1	gttactaatggatttattcaagt	53	#
			BR2	gcataaacttgtccagcgat		
	Rickettsia spp.	I	B2f_5	taaacttgctgacggtacag	56	#
			B2f_2	cgattatgccgttatcgcttccaag		
		II	B2f_3	gtagcctaacaaatgctcaaac	52	
			120-2399	cttgtttgtttaatgttacggt		
	R. helvetica	I	B2f_3	gtagcctaacaaatgctcaaac	52	[23]
			B2f_2	cgattatgccgttatcgcttccaag		
		II	B2f_1helv	cagtacaattcgctcacaacac	55	#
			120-2399	cttgtttgtttaatgttacggt		
	Rickettsia spp.	I	B1	atatgcaggtatcggtact	56	#
			B2	ccatataccgtaagctacat		
		II	B3	gcaggtatcggtactataaac	56	
			B4	aatttacgaaacgattacttccgg		

TABLE 1 (*continued*)

	Rickettsia spp.	I	120-3462	ccacaggaactacaaccatt	52	[23]
			120-4879m*	tagaagtttacacggacttttagag		
		II	B3f_3f	gctggacctgaagctggagc	55	#
			120-4879m*	tagaagtttacacggacttttagag		[23]
*sca*4 gene	*Rickettsia* spp.	I	D1f	atgagtaaagacggtaacct	52	[24]
			D1876rm*	tagtttgttccgccgtaatc		
		II	sc1f_3	gatgtaggtgatgaactctg	52	#
			D1390r	cttgcttttcagcaatatcac		[24]
		I	sc4-1	atgtctctgaattaagcaatgc	52	#
			Rj2837r	cctgatactacccttacatc		[25]
		II	sc4-5	ccggcacaacaacaattgatg	50	#
			sc4-6	cctttaccagctcatctactt		
		I	sc4-3	aattattaggctctgtattaaaga	52	#
			D3069r	tcagcgttgtggaggggaag		[24]
		II	sc4-5	ccggcacaacaacaattgatg	52	#
			sc4-7	ctctcttttaataggtgttgatt		#
*htr*A gene	*Rickettsia* spp.		17k-5	gctttacaaaattctaaaaaccatata	55	[26]
			17k-3	tgtctatcaattcacaacttgcc		
23S-5S IGS	*Rickettsia* spp.		RCK/23-5-F	gataggtcrgrtgtggaagca	55	[27]
			RCK/23-5-R	tcgggaygggatcgtgtgtttc		
groESL Operon	*Rickettsia* spp.	I	Ric-ESL-F1	ggtaaatgggcaggyaccgaa	60	[28]
			Ric-ESL-R1	gaagcaacrgaagcagcatctt		
		II	Ric-ESL-F2	atcgttatgaaagaaagcgayg	58	
			Ric-ESL-R2	agwgcagtacgcactactttagc		

Note. T (°C) – annealing temperature; m* – modified primer; # – this study.

portion of ticks of different species on these sites differed significantly. At the Om-Bo site, 67 (40.6 %) *I. apronophorus*, 73 (44.2 %) *I. persulcatus* and 25 (15.1 %) *I. trianguliceps* were identified among 145 ticks removed from 29 rodents and 20 ticks removed from rodents and underwent metamorphosis in laboratory conditions, and at the Om-Zn site among 115 ticks removed from rodents and molted in the laboratory, 5 (4.4 %) *I. apronophorus*, 87 (75.6 %) *I. persulcatus* and 23 (20.0 %) *I. trianguliceps* were found (Table 2).

In the Far East, on Putyatina Island, 56 *I. persulcatus,* 4 *I. pavlovskyi* and one *I. persulcatus*/*I. pavlovskyi* interspecies hybrid were identified among unfed adults collected from vegetation by flagging, and on Russian Island – 190 *I. persulcatus,* 199 *I. pavlovskyi* and 32 interspecies hybrids were found (Table 2).

Identification of *Rickettsia* spp. in *Ixodes* spp. ticks in the Omsk region

Among ticks from the Om-Bo site collected from rodents but not molted *R. helvetica* DNA was identified in 45 (72.5 %) *I. apronophorus* of all development stages (including 1 case of *R. helvetica* and "*Candidatus* R. tarasevichiae" mixed infection) and in 9 (37.5 %) *I. trianguliceps* but was not identified in *I. persulcatus.* Notably, all infected *I. trianguliceps* were larvae (Table 2). Additionally, "*Candidatus* R. tarasevichiae" DNA was found in 48 (81.4 %) *I. persulcatus* and 1 (1.6 %) *I. apronophorus* larvae, and "*Candidatus* R. uralica" DNA was found in 7 (29.2 %) *I. trianguliceps*. "*Candidatus* R. tarasevichiae" with *Rickettsia raoultii* was detected in one larva (1.6 %) of *I. persulcatus*.

Among the molted ticks, 3 out of 5 are *I. apronophorus* from the Om-Bo site and 4 of 5 *I. apronophorus*

TABLE 2

IDENTIFICATION OF *R. HELVETICA* IN *IXODES* SPP. TICKS

Sites/ analyzed ticks	Tick species	Stage	Tick population	Ticks containing *R. helvetica* DNA, abs. (%)
Om-Bo/ collected from rodents	*I. apronophorus*	Larvae	47	33
		Nymphs	5	4
		Adults	10	8
		All stages	**62**	**45 (72.5)**
	I. persulcatus	Larvae	55	0
		Nymphs	4	0
		All stages	**59**	**0**
	I. trianguliceps	Larvae	17	9
		Nymphs	4	0
		Adults	3	0
		All stages	**24**	**9 (37.5)**
Om-Bo/ molted	*I. apronophorus*	Nymphs	2	1
		Adults	3	2
		All stages	**5**	**3 (60)**
	I. persulcatus	Nymphs	3	0
		Adults	11	0
		All stages	**14**	**0**
	I. trianguliceps	Nymphs	1	0
		All stages	**1**	**0**
Om-Zn/ molting	*I. apronophorus*	Nymphs	4	3
		Adults	1	1
		All stages	**5**	**4 (80)**
	I. persulcatus	Nymphs	23	0
		Adults	64	1
		All stages	**87**	**1 (1.1)**
	I. trianguliceps	Nymphs	10	0
		Adults	13	0
		All stages	**23**	**0**

TABLE 2 (*continued*)

Put/ collected by flagging	*I. persulcatus*	**Adults**	**56**	**1 (1.8)**
	I. pavlovskyi	**Adults**	**4**	**0**
	hybrids	**Adults**	**1**	**0**
Rus/ collected by flagging	*I. persulcatus*	**Adults**	**190**	**0**
	I. pavlovskyi	**Adults**	**199**	**0**
	hybrids	**Adults**	**32**	**0**

Note. Combined data for all stages examined for each tick species are shown in bold.

from the Om-Zn site contained *R. helvetica* DNA. In addition, *R. helvetica* was revealed in one molted *I. persulcatus* as a mixed infection with "*Candidatus* R. tarasevichiae" (Table 2). "*Candidatus* R. tarasevichiae" DNA was revealed in 80 (79.2 %) *I. persulcatus* and 2 (8.3 %) *I. trianguliceps*, while "*Candidatus* R. uralica" DNA was detected in 3 (12.5 %) *I. trianguliceps*. A mixed infection "*Candidatus* R. uralica" with *Rickettsia* sp. was revealed in 1 (1 %) nymph *of I. persulcatus*.

Combining data obtained for all examined ticks from the Omsk region, *R. helvetica* was revealed in 75.0 % of *I. apronophorus*, 18.8 % of *I. trianguliceps* and 1.9 % of *I. persulcatus*, indicating a close ecological relationship between *R. helvetica* and *I. apronophorus*.

Identification of *Rickettsia spp.* in *Ixodes* spp. ticks in the Primorsky Territory

R. helvetica DNA was revealed in only 1 of 56 *I. persulcatus* from Putyatin Island, but was not revealed in any other tick species from the same site, nor in any of 421 ticks of the genus *Ixodes* from Russky Island (Table 2). "*Candidatus* R. tarasevichiae" DNA was revealed in 42 (75.0 %) *I. persulcatus*, in 1 of 4 *I. pavlovskyi* and in the single interspecies hybrid from Putyatin Island, and in 73.7 % of *I. persulcatus*, 5.3 % of *I. pavlovskyi* and 31.3 % of inter-species hybrids from Russky Island. DNA of "*Candidatus* Rickettsia mendelii" was revealed in 2 *I. pavlovskyi* on Russky Island (2016).

R. helvetica genotyping

Sequences of the *glt*A gene fragment (840 nucleotide pairs) were determined for all *R. helvetica* isolates, and 6 sequence variants were identified based on analysis of these sequences. Fragments of *omp*B (1255 bp), *sca*4(783 bp), and 16S rRNA (684 bp) genes were additionally sequenced for a number of samples with different variants of the *glt*A gene. Comparative analysis of the sequences obtained revealed 7 sequence variants of *R. helvetica* differing from each other by 2–8 nucleotide substitutions; all detected sequence variants differed from the sequence of the prototypical strain C9P9 (AICO01000001) (Fig. 2a).

Phylogenetic analysis based on comparison of the concatenated sequences *glt*A-*omp*B-*sca*4 with a total length of 2259 bps showed that the sequences obtained belong to four genetic lineages (Fig. 3). The samples belonging to lineage I (European lineage) formed a common cluster together with the prototype strain *R. helvetica* C9P9 isolated from the tick *I. ricinus* from Switzerland. Lineage I sequences were revealed only at the Om-Zn site in three *I. apronophorus* and one *I. persulcatus* (Table 3); these sequences differed from the *R. helvetica* C9P9 sequences by single substitutions at the *gltA* or *ompB* genes (Fig. 2a). Lineage II (*I. trianguliceps* line) included all samples of *R. helvetica* from *I. trianguliceps* ticks and one sample from molted *I. apronophorus* from the Om-Bo site. The lineage II sequences determined in this study were identical to previously determined sequences from two nymphs of *I. trianguliceps* taken from rodents from another area of Omsk region. Lineage III (*I. apronophorus* line) was the most numerous and included sequences of 48 *I. apronophorus*, mainly from the Om-Bo site. Samples from this lineage were genetically heterogeneous – one sample differed by a single substitution in the *glt*A gene from the others; notably, 5 tick samples taken from the same rodent had the same substitution in the 16S rRNA gene. Lineage IV (Far Eastern lineage) included all determined sequences of *R. helvetica* from *I. persulcatus* from the Far East: Putyatin Islands, Sakhalin and Khabarovsk Territory (Table 3).

Since most *R. helvetica* isolates from the GenBank database have been characterized using only the *omp*B gene, we used this locus to compare the *R. helvetica* sequences obtained in this study with those published previously. Phylogenetic analysis based on comparison of the sequences of the *omp*B gene, 2684 bps in length, showed the presence of the same four genetic lineages. Based on the analyses conducted, the European lineage (lineage I) additionally contained a number of specimens of *R. helvetica* from *I. ricinus* originated from Germany and one specimen from *I. persulcatus* from the Novosibirsk region, and the *I. trianguliceps* linage (lineage II) additionally included 32 specimens of *R. helvetica* from *I. persulcatus* from the Komi Republic (Fig. 4). Sequence analy-

Isolates	Tick species	Region	Lineage	*gltA*					*ompB*					*sca4*		*16S*
				175525	175576	175847	176092	176156	381079	381549	381958	382176	382239	812583	812147	523727
C9P9	*I. ricinus*	Europe	I	G	C	T	C	C	G	C	G	G	G	G	C	T
Om-74_Iapr_m	*I. apronophorus*	Siberia	I	.	.	.	.	.	.	.	.	T	.	.	.	.
Om-20_Iper_m	*I. persulcatus*	Siberia	I	.	.	.	T	.	nd	nd	nd	nd	nd	.	.	nd
Om-75_Itr	*I. apronophorus*	Siberia	II	A	.	.	.	T	A	.	A	T	.	.	.	.
Om-145_Iapr	*I. apronophorus*	Siberia	III	.	T	.	.	.	.	T	A	T	A	.	T	.
Om-79_Iapr	*I. apronophorus*	Siberia	III	.	T	.	.	.	.	T	A	T	A	.	T	G
Om-103_Iapr	*I. apronophorus*	Siberia	III	.	T	A	nd	nd	.	T	nd	nd	nd	.	T	nd
Skh-7_Iper	*I. persulcatus*	Far East	IV	.	.	.	.	.	.	.	A	T	.	A	.	.
Put-117_Iper	*I. persulcatus*	Far East	IV	.	.	.	.	.	.	.	A	T	.	A	.	.

a

Isolates	Lineage	*gltA*			*ompA*			*ompB*												*sca4*						*htrA*	*16S*		*IGS*	
		175525	175576	176156	209675	208648	208557	380060	380249	380592	380622	381079	381549	381958	382176	382239	382562	382824	382895	383024	813613	813472	813341	812583	812147	811867	195041	523727	523907	1187522	1187463
C9P9	I	G	C	C	C	G	T	T	A	C	T	G	C	G	G	G	G	T	C	G	A	A	C	G	C	G	T	T	A	T	G
Novosibirsk08-5	I	.	.	.	nd	nd	nd	.	.	.	.	.	.	.	.	.	.	.	.	.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Komi	II	.	.	T	nd	nd	nd	.	G	.	C	A	.	A	T	.	A	.	.	.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Om-74_Iapr_m	I	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Om-75_Itr	II	A	.	T	T	.	.	.	G	T	C	A	.	A	T	.	A	.	.	.	C	G	.	.	.	A	.	.	.	C	.
Om-145_Iapr	III	.	T	.	.	.	.	G	.	.	C	.	T	A	T	A	.	C	T	.	.	.	.	.	T	.	G	.	G	.	.
Om-79_Iapr	III	.	T	.	.	.	.	G	.	.	C	.	T	A	T	A	.	C	T	.	.	.	.	.	T	.	G	G	G	.	.
Skh-7_Iper	IV	.	.	.	.	A	C	.	.	.	C	.	.	A	T	.	.	C	.	.	.	.	T	A	.	.	.	.	.	C	A
Put-117_Iper	IV	.	.	.	.	A	.	.	.	.	C	.	.	A	T	.	.	C	.	A	.	.	T	A	.	.	.	.	.	C	A

b

FIG. 2.

a *– condensed alignment based on gene sequences of gltA (840 bp), ompB (1255 bp), sca4 (783 bp) and 16S rRNA (684 bp) sequence variants of R. helvetica;* ***b*** *– condensed alignment based on gene sequences gltA (1037 bp), ompA (1417 bp), ompB (3100 bp), sca4 (2398 bp), htrA (499 bp), 16S rRNA (1070 bp) genes and the intergenic spacer 23S-5S rRNA (23S-5S IGS) (489 bp) of R. helvetica genetic lines. Polymorphic sites are listed according to the sequence of R. helvetica, strain C9P9 (AICO01000000). Non-synonymous substitutions are marked in green color*

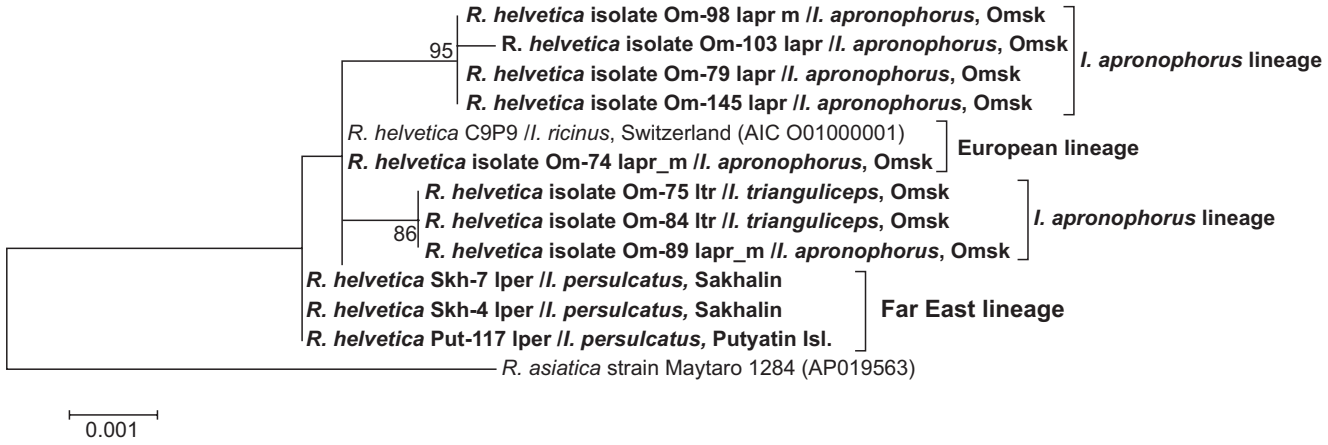

FIG. 3.

Dendrogram constructed by ML method based on concatenated sequences of gltA-ompB-sca4 gene fragments (2259 bps). Sequences obtained in this study are highlighted in bold font

TABLE 3

PREVALENCE OF DIFFERENT GENETIC LINEAGES OF *R. HELVETICA* IN *IXODES* SPP.

Site	Tick species	Analyzed ticks	Number of genotyped *R. helvetica* samples	Number of *R. helvetica* specimens belonging to the lineage			
				I	II	III	IV
Om-Bo	*I. apronophorus*	Not molted	45	0	0	45	0
		Molted	3	0	1	2	0
		Total	**48**	**0**	**1**	**47**	**0**
	I. trianguliceps	Not molted	9	0	9	0	0
Om-Zn	*I. apronophorus*	Molted	4	3	0	1	0
	I. persulcatus	Molted	1	1	0	0	0
Put	*I. persulcatus*	Collected by flagging	1	0	0	0	1
Khab	*I. persulcatus*	Collected by flagging	1	0	0	0	1
Skh	*I. persulcatus*	Collected by flagging	4	0	0	0	4

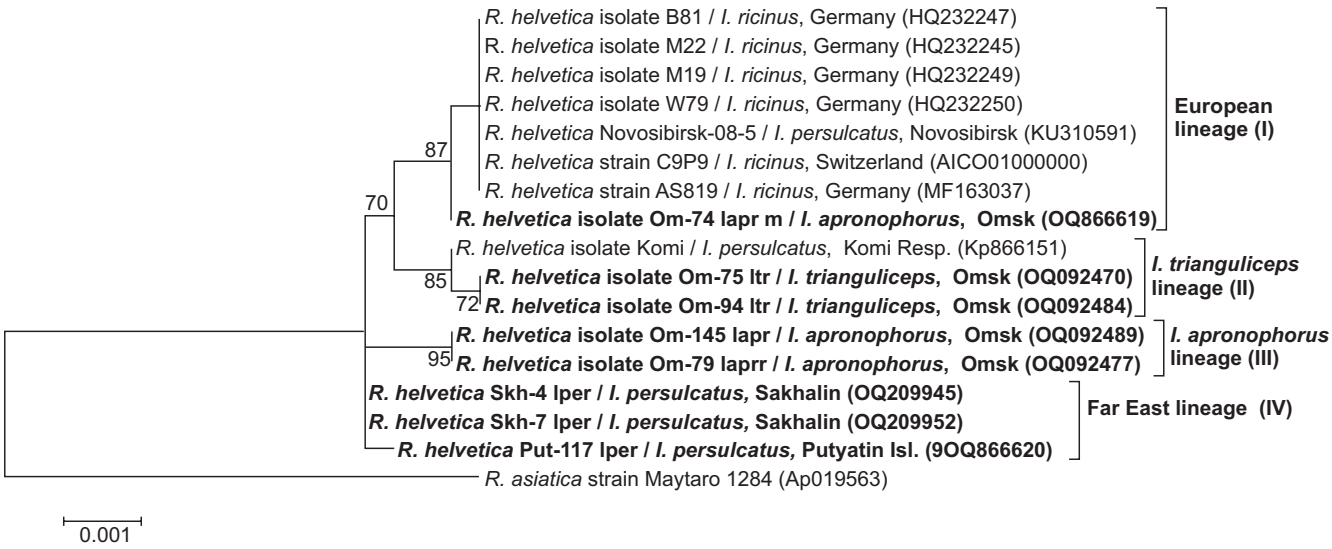

FIG. 4.

Dendrogram constructed by ML method based on sequences of the ompB gene fragment (2684 bps) of R. helvetica. R. asiatica sequence was used as an outgroup. Sequences obtained in this study are highlighted in bold font

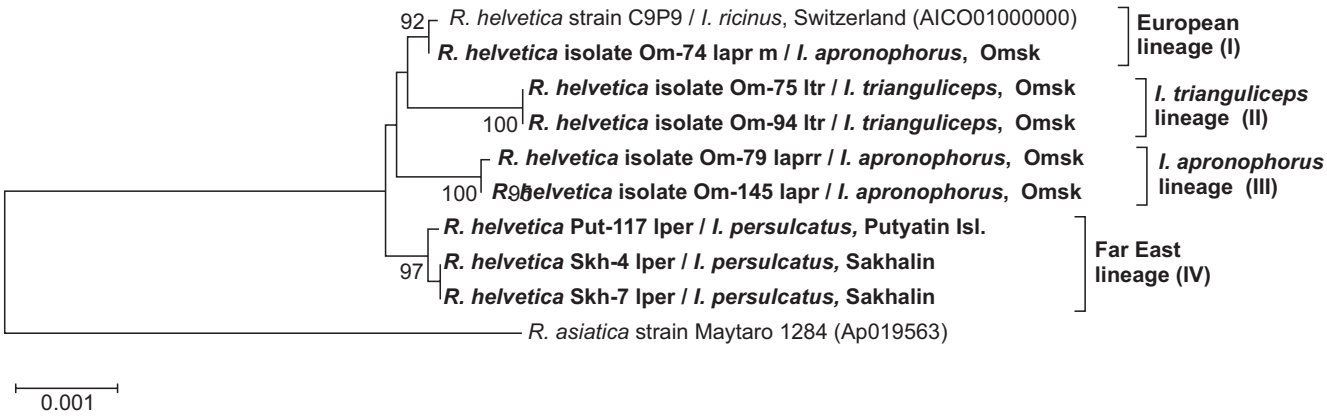

FIG. 5.

A dendrogram constructed by the ML method based on concatenated sequences of fragments of seven loci (gltA – ompA – ompB – sca4 – htrA – 16S rRNA – IGS) (9840 bp). Sequences obtained in this study are highlighted in bold font

sis of the *sca*4 gene revealed that the Far Eastern lineage also includes *R. helvetica* specimens from *I. persulcatus* from Japan [25].

For more detailed genotyping of the 16S rRNA gene sequence (1070 bp), *glt*A (1037 bp), *omp*A (1417 bp), *omp*B (3100 bp), *sca*4 (2398 bp), *htr*A (499 bp), *GroEL* (1528 bp), as well as 23S-5S IGS (489 bp) were determined for eight samples of *R. helvetica* belonging to different genetic lineages. All *gro*EL gene sequences were identical. The remaining genetic loci had polymorphic sites; of these, the *omp*B gene was the most variable. Among the coding sequences, nucleotide substitutions at 15 of the 25 polymorphic sites were nonsynonymous (Fig. 2b). Phylogenetic analysis based on a comparison of the concatenated sequences 16S – *glt*A – *omp*A – *omp*B – *sca*4 – *htr*A – IGS (9840 bps) also revealed with a high level of support the presence of four clusters that corresponded to the genetic lineages identified based on the analysis of shorter sequences (Fig. 5). It should be noted that within each genetic lineage, *R. helvetica* samples differed among themselves by 1–2 nucleotide substitutions. In the case of genetic lines I and II, samples from the Omsk region differed from samples from other regions, and in the case of genetic lineage IV, the sample from Putyatin Island differed by two substitutions from samples from Sakhalin.

DISCUSSION

Different species of rickettsiae tend to be associated with certain species of ticks. *Rickettsia helvetica* is closely related to ticks of the genus *Ixodes* and is the dominant species of rickettsia in *I. ricinus* in Europe and *I. persulcatus* in some regions of Russia (Sakhalin Island and Komi Republic) [7, 9, 14].

The study of tick infectivity with infectious agents in areas of sympatry is of particular interest because it allows us to compare the pathogen-tick association for different tick species in the same area. This study included ticks collected at two sites in the areas of sympatry *I. apronophorus*/*I. persulcatus*/*I. trianguliceps* in the Omsk region; at the Om-Bo site the abundance of all three tick species was high, while at the Om-Zn site *I. persulcatus* was dominant and the abundance of *I. apronophorus* was low. In this study, rickettsiae were first revealed in *I. apronophorus*. *Rickettsia helvetica* was revealed in 60–80 % of ticks at different developmental stages from both sites, indicating a close association of *R. helvetica* with *I. apronophorus* (Table 2).

In the Omsk region, *R. helvetica* was also revealed in single *I. persulcatus* and in 38 % of *I. trianguliceps* collected from rodents from the Om-Bo site. It should be noted that *R. helvetica* was revealed only in *I. trianguliceps* larvae, but not in nymphs and adults (Table 2). Considering that all larvae infected with *R. helvetica* were collected from only two voles, the observed discrepancy can be explained by the insufficient number of *I. trianguliceps* examined and the uneven distribution of infected and uninfected larvae, being offspring from different females. This uneven distribution of larvae can also explain the fact that all *R. helvetica* samples with a unique substitution in the 16S rRNA gene were revealed only in larvae (but not in adults) of *I. apronophorus* collected from the same vole.

In addition to the areas of sympatry in the Omsk region, ticks collected on two islands in Primorsky Territory were also included in the study. Since an unexpectedly high level of infection of taiga ticks with *R. helvetica* was previously observed on Sakhalin Island, it could be expected that other islands would also show an atypical distribution of *Rickettsia* spp. in different tick species. Notwithstanding, on the surveyed Putyatin and Russky islands, as well as on the mainland of the Far East and in Western Siberia [10, 12–14, 19], "*Candidatus* R. tarasevichiae" was significantly dominant in *I. persulcatus* ticks, and *R. helvetica* was revealed only in one *I. persulcatus* on Putyatin Island (Table 2).

Rickettsia helvetica is a highly variable species. Analysis of the sequences of this species determined in this study

and available in the GenBank database resulted in the assignment of *R. helvetica* isolates to four genetic lineages, but the association of different lineages with a particular tick species or territory was not observed in all cases. Thus, genetic lineage I combined all genotyped specimens from *I. ricinus* from Europe, a number of specimens from *I. apronophorus* from the Omsk region and specimens from *I. persulcatus* from the Omsk and Novosibirsk regions. The genetic lineage of *I. trianguliceps* (lineage II) was found in two different species of *Ixodes* spp. in distant regions: in *I. trianguliceps* in the Omsk region and *I. persulcatus* in the Komi Republic. At the same time, the genetic lineage of *I. apronophorus* (lineage III) was revealed only in *I. apronophorus* in the Omsk region; and the Far Eastern genetic lineage (lineage IV) was revealed only in *I. persulcatus* in the Far East. Thus, three genetic lineages of *R. helvetica* were revealed in samples from the Omsk region, and only one lineage was identified in samples from the Far East.

The observed high genetic heterogeneity of the *R. helvetica* population may be associated with a wide range of their vectors: *I. ricinus*, *I. pavlovsky*, *I. persulcatus*, *I. apronophorus*, *I. trianguliceps*, *Ixodes hexagonus* (Leach 1815), *Ixodes arboricola* (Schulze & Schlottke, 1929), *Ixodes ovatus* (Neumann, 1899) and *Ixodes monospinosus* (Saito, 1968) [7, 9, 12, 14, 32]. It should be noted that in *I. trianguliceps* only one sequence variant belonging to genetic lineage II was revealed, whereas in *I. apronophorus* five sequence variants belonging to three genetic lineages were revealed (Table 3). Sequences belonging to three lineages were identified in *I. persulcatus* ticks: lineage I in ticks from Western Siberia, lineage II in ticks from the Komi Republic, and lineage IV in ticks from the Far East. Such inconsistency may be associated with the significantly higher genetic variability of *I. apronophorus* and *I. persulcatus* ticks compared to *I. trianguliceps* [20].

At present, there is no reliable data to support the presence of co-feeding transmission of rickettsiae in ticks. Although such transmission can occur under artificial conditions (in the case of *Rickettsia rickettsia* (Wolbach 1919) Brumpt 1922)), its impact on pathogen transmission in nature appears to be negligible [33]. Our study of larvae collected from rodents revealed that there was no effective contained *R. helvetica* DNA transmission of *R. helvetica* between different species of *Ixodes* spp. during simultaneous feeding on small mammals. Actually, all *I. persulcatus* larvae collected from rodents were not infected with *R. helvetica*, *I. apronophorus* larvae contained *R. helvetica* DNA only from lineage III, and *I. trianguliceps* larvae only from lineage II (Tables 2, 3). Notably, the association between tick species and *R. helvetica* variants was observed even when larvae of different species were fed on the same rodent.

It should be noted that the data on genetic variability of *R. helvetica* are limited to a small number of studied regions and include mainly samples from Germany, the Komi Republic, the Omsk and Novosibirsk Regions, and the Far East. Further genotyping of *R. helvetica* specimens from other regions and different tick species is needed to assess the prevalence of different genetic lineages of this species. It is possible that different genetic lineages of *R. helvetica* may differ in their pathogenic properties.

CONCLUSION

In conclusion, a high level of infection of *I. apronophorus* ticks with the pathogenic for humans *R. helvetica* was revealed for the first time. High genetic variability was observed for *R. helvetica* samples from the Omsk region. For the first time, it was revealed that *R. helvetica* isolates could be reliably assigned to four genetic lineages, but no strict association of different *R. helvetica* lineages with a particular tick species or territory was observed.

Conflict of interest

The authors of this article declare no conflicts of interest.

Financing

This work was supported by the Russian state-funded project for the Institute of Chemical Biology and Fundamental Medicine SB RAS (No. 121031300043-8).

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