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INVERTEBRATE MICROBIOLOGY

Bacteria of the Family 'Candidatus Midichloriaceae' in Sympatric Zones of *Ixodes* Ticks: Genetic Evidence for Vertical Transmission

Tatyana A. Mukhacheva[1] · Sergey Y. Kovalev[1]

Abstract *Ixodes* ticks transmit infectious agents and also harbor their own parasites and symbionts. The presumptive endosymbiont of *Ixodes ricinus*, 'Candidatus Midichloria mitochondrii', has a unique ability to invade mitochondria within tick ovarian cells and is transovarially transmitted with 100% efficiency. A closely related bacterium, provisionally named Montezuma (now 'Candidatus Lariskella arthropodarum'), was isolated from the *Ixodes persulcatus* ticks and human blood in 2004 as well as from *Ixodes pavlovskyi* in 2015. These microorganisms belong to the family 'Candidatus Midichloriaceae fam. nov.' and were detected not only in tick salivary glands, but also in animal blood. Nevertheless, the relative importance of vertical and horizontal routes for their transmission or maintenance in natural tick populations remains unclear. We analyzed the prevalence of *L. arthropodarum* and *M. mitochondrii* in two sympatric zones, where *I. persulcatus/I. ricinus* and *I. persulcatus/I. pavlovskyi* cohabit and produce interspecific hybrids. A specificity of the associations of *L. arthropodarum* with *I. persulcatus* (100%) and *M. mitochondrii* with *I. ricinus* (96.2%) was observed in the sympatric zone in Estonia, possibly showing poor contribution of the horizontal route to the overall prevalence of endosymbionts. *L. arthropodarum* was observed probably multiplying in *I. pavlovskyi* and also subjected to transovarial transmission, but much less efficiently compared to *I. persulcatus*. We revealed two new genetic variants of the rrl-rrf intergenic spacer of *L. arthropodarum*

isolated from *I. pavlovskyi* ticks that possibly could indicate an ongoing process of adaptation of the microorganism to a new host species.

Keywords *Ixodes* · *Midichloria mitochondrii* · *Lariskella arthropodarum* · Sympatric zone · Vertical transmission

Introduction

The *Ixodes* ticks transmit multiple infectious agents and also harbor their own parasites and symbionts. Since the discovery of the alphaproteobacterium 'Candidatus Midichloria mitochondrii', which has a unique ability to invade the mitochondria within ovarian cells of *Ixodes ricinus* L. [1–3], many studies have been focused on its biological features, evolution, and potential pathogenicity. In particular, 100% of *Ixodes ricinus* females have been reported to be infected with *M. mitochondrii*, whereas the prevalence of this microorganism in males did not exceed 44% [3, 4]. Moreover, tick sex determines the relative abundance of the presumptive endosymbiont, with females harboring 1000-fold more copies of *M. mitochondrii* genome than males [4, 5]. The observed differences between sexes led to the hypothesis that *M. mitochondrii*, although being transmitted from an infected female to both female and male eggs transovarially, can efficiently multiply only in females while being somehow lost during specialization of nymphs to adult males [3–5]. It is worth noting that *M. mitochondrii* has been found in a number of hard ticks [6, 7], tick salivary glands as well as in the blood of mammals being parasitized by ticks [8–10]. Thus, both vertical and horizontal routes of transmission have been shown for *M. mitochondrii*; however, their relative importance in overall prevalence is still to be elucidated.

✉ Sergey Y. Kovalev
  Sergey.Kovalev@urfu.ru

[1] Laboratory of Molecular Genetics, Department of Biology, Ural Federal University, Lenin Avenue 51, Yekaterinburg 620000, Russia

In 2004, Mediannikov et al. isolated a new bacterium, phylogenetically close to *M. mitochondrii*, from *Ixodes persulcatus* Schulze females, and human blood in the Russian Far East; its prevalence in adult *I. persulcatus* females was 97.1% [11]. This bacterium was provisionally named 'Montezuma'. Later, Montezuma was detected in the European part of Russia where its prevalence in adult females was shown to be from 8.4 to 51.9%, and in adult males from 2.5 to 16% [12, 13]. Although the prevalence of Montezuma in *I. persulcatus* females was shown to be lower than those of *M. mitochondrii* in *I. ricinus*, the hypothesis about the loss of endosymbiont during male development was extended to Montezuma in *I. persulcatus* as well [12]. This microorganism has been scarcely studied, and little is known about its biological features. Recently, the DNA of Montezuma has been detected in *Ixodes pavlovskyi* Pomerantsev females in a tick microbiome analysis [14], as well as in the salivary glands of *I. persulcatus* females in Japan [15]. Sequence analysis of the 16S rRNA gene, the only genetic marker used, showed almost no genetic diversity in this bacterium from different territories (Arkhangelsk, Novgorod Regions and St. Petersburg in the North West Russia; Russian Far East) [13].

M. mitochondrii and Montezuma colonize closely related tick host species, yet they are not the closest relatives themselves. These two microorganisms are grouped as related alphaproteobacteria notably associated with a wide range of hosts, from amoebae to a variety of invertebrates [16–20]. Since *M. mitochondrii* was the first organism formally described for this phylogenetic group, it has been proposed to designate it as 'Midichloria and like organisms (MALOs)' [8], and then to elevate it to the family rank, 'Candidatus Midichloriaceae fam. nov.' within the order Rickettsiales [21]. Phylogenetic analysis revealed that the bacterium Montezuma is closer to endosymbionts isolated from stinkbugs of different families, as well as fleas, than it is to *M. mitochondrii*. It has been proposed that this clade, including Montezuma, forms a new species, '*Candidatus* Lariskella arthropodarum' [18]. *Lariskella arthropodarum* in stinkbugs was shown to be concentrated in oocytes in the ovaries of adult females, indicating the vertical transmission of the symbiont through the host generations. No significant difference in *L. arthropodarum* prevalence between females and males was previously shown [18].

The sympatric zones of overlapping distribution of two species represent an opportunity for the study of microorganism-host associations. Recently, we have shown that sympatric zones of *Ixodes* ticks are also zones of hybridization between these tick populations [22, 23]. Analysis of hybrid populations, i.e., populations containing individuals resulting from the breeding of two tick species, accompanied by the detection of associated symbionts could give clues on how they are transmitted in natural host populations. For example, genotyping of

Wolbachia, a well-known intracellular, maternally inherited endosymbiont of insects, accompanied by a parallel characterization of the associated host mitochondrial and nuclear DNA diversity, revealed a complex set of mechanisms of its transfer, including co-speciation, horizontal transfer, and hybrid introgression [24–26]. In the present work, we have estimated the prevalence and relative abundance of *L. arthropodarum* (previously Montezuma) in *I. persulcatus*, *I. ricinus*, and *I. pavlovskyi* ticks, as well as in their hybrids from the sympatric zones in Estonia and West Siberia. Discussion on the relative importance of vertical and horizontal routes of transmission for the establishment of *L. arthropodarum* and *M. mitochondrii* in natural populations of their *Ixodes* hosts is presented. Moreover, genetic analysis revealed new variants of the rrl-rrf intergenic spacer sequences of *L. arthropodarum* isolated from *I. pavlovskyi* ticks that could possibly indicate an ongoing process of adaptation of the symbiont to a new host species.

Materials and Methods

To determine the prevalence of *L. arthropodarum*, ticks were collected by flagging in the sympatric zone of *I. persulcatus*/*I. ricinus* in Estonia in 2006–2008 ($n = 250$) and in the sympatric zone of *I. persulcatus*/ *I. pavlovskyi* in the Tomsk region (Russia) in 2013–2014 ($n = 473$), as well as in two regions in Russia where only *I. persulcatus* was found: the Komi Republic (Syktyvkar) in 2014 ($n = 120$) and the Sverdlovsk region (Yekaterinburg) in 2015 ($n = 120$). All ticks were analyzed individually. Detailed information on the methods of sample preparation, DNA extraction, and detection of tick hybrids is given in previous papers [22, 23]. Briefly, interspecific tick hybrids were revealed by carrying out two real-time PCRs with species-specific probes for both mitochondrial (*cox1*) and nuclear (ITS2) markers, with subsequent identification of three groups of specimen: (1) 'pure' species with both mtDNA and nucDNA belonging to the same species; (2) hybrids with a mix of ITS2 alleles of two species; and (3) hybrids with mtDNA and nucDNA indicating different species (mitochondrial introgression).

We developed new primers and TaqMan probes to detect and estimate the abundance of *L. arthropodarum* and *M. mitochondrii* DNA by real-time PCR (Table 1). Primers and probes were designed based on the alignment of the 16S rDNA sequences of *L. arthropodarum* (uncultured Rickettsiales bacterium Montezuma GenBank AF493952) and *M. mitochondrii* (GenBank CP002130). The mitochondrial 16S rRNA gene fragment, amplified for all three *Ixodes* species with the same primers and probe [27], was used as a reference gene to assess the quality of DNA extraction and amplification. Two PCR reactions were performed separately on the ABI 7500 thermocycler (Applied

Table 1 Primers and probes for qPCR and sequencing of *L. arthropodarum* and *M. mitochondrii* rDNA loci

Oligonucleotide name	Sequence (5'-3')	Method, target	Start position[a]	End position[a]	T_a	PCR amplicon size, bp[b]
MM-RT_F MM-RT_R	TACCCTGGTAGTCCACGCAG TTAATCTTGCGACCGTACTCCC	Real-time qPCR, 16S rDNA	198,074 198,164	198,093 198,185	68 °C	111–112
Probe-Mtz (*L. arthropodarum*)	FAM————————**BHQ1** **TTC**TTCAGTGTGTCGCAGCT AACGCGTTAAG[c]		198,124	198,152		
Probe-Midi (*M. mitochondrii*)	ROX————————**BHQ2** **AAGT**CTCAGTGTCGCAGCT AACGCATTAAG		198,123	198,152		
Midi-5S-F Midi-23S-R2	GCGATGGAGCTCTTCACTTC GGACGTACCACTGGTGTACC	Sequencing, 5S-23S IGS (nested PCR; outer)	787,083 787,442	787,102 787,461	53 °C	405 (*L. arthropodarum*) 379 (*M. mitochondrii*)
Mtz-5S-F Mtz-23S-R	GGATCAGGTGTTTCATCTCT TTGTTCCGCCAGGAGCAGCG	Sequencing, 5S-23S IGS (nested PCR; inner)	787,116 787,420	787,135 787,439	61 °C	350 (*L. arthropodarum*)

[a] Position numbers are given according to the whole-genome sequence of *M. mitochondrii* GenBank Acc. No. CP002130

[b] Expected PCR amplicon length for each symbiotic bacterial species indicated in parenthesis

[c] The nucleotides differing between two probes are underlined and marked in bold

Biosystems™, USA). The PCR conditions were as follows: 95 °C for 15 min; 40 cycles at 95 °C for 15 s, 68 °C (for the bacterial target gene) or 60 °C (for the *Ixodes* target gene) for 1 min. The relative abundance of *L. arthropodarum* was estimated as a ratio between threshold cycles C_t of an analyzed and a reference gene using the formula *Lariskella*/mitochondria ratio = $2^{\wedge}(C_{t\ \text{tick 16S rDNA}} - C_{t\ \text{Lariskella 16S rDNA}})$. Statistical analysis and generation of a boxplot graph were carried out with Microsoft Excel (Microsoft™ Corp., USA) and Statistica software v.13. (StatSoft™, USA). The prevalences of *L. arthropodarum* and *M. mitochondrii* in different tick species were estimated using 95% confidence interval (95%CI) and compared with *t* test for the comparison of percentages (Tables 2 and 3). *Lariskella*/mitochondria ratios between different tick species, sexes, and geographical origins were compared using Mann-Whitney U test (Fig. 1). *P* values less than 0.05 were considered to be significant.

Primers for the amplification of rrl (23S rDNA) - rrf (5S rDNA) intergenic spacer (rrl-rrf IGS) of *L. arthropodarum* were designed in two stages. In the first stage, we designed the primers Midi-5S-F and Midi-23S-R2 based solely on the annotated whole-genome sequence of *M. mitochondrii*; they were used to amplify the corresponding genome fragment of *L. arthropodarum* isolated from *I. persulcatus* (Table 1). In the second stage, the obtained sequence, 405 bp in length, was used to design *L. arthropodarum*-specific primers Mtz-5S-F and Mtz-23S-R. They were used subsequently to amplify and sequence rrl-rrf IGS fragments for 62 samples of *I. persulcatus* and *I. persulcatus*/ *I. pavlovskyi* hybrids (with mtDNA of *I. persulcatus*) from different geographical regions (25 samples from Tomsk region, 7—Estonia, 7—Krasnoyarsk, 8—Vladivostok, 5—Komi Republic, 4—Urals, 2—Altai, 2—Tyva Republic, 2—Arkhangelsk region), as well as for 23 *I. pavlovskyi* and *I. persulcatus*/*I. pavlovskyi* hybrids (with mtDNA of *I. pavlovskyi*). For the samples with low abundance of *L. arthropodarum*, where no amplification was detected, a nested PCR was performed using two pairs of primers (Table 1). PCR reactions were performed on the Veriti® 60-well Thermal Cycler (Applied Biosystems™, USA) under the thermocycling conditions as follows: initial denaturation at 94 °C for 1 min; 42 cycles at 94 °C for 20 s, 53 °C or 61 °C for 20 s, 72 °C for 40 s; final elongation at 72 °C for 5 min.

Nucleotide sequences of the rrl-rrf IGS were determined on the ABI PRIZM 3130 Genetic Analyzer (Applied Biosystems™, USA). Sequence alignment and analysis were performed using the software MEGA 6 [28]. Positions of the 23S and 5S rRNA genes flanking the intergenic spacer were determined compared to the corresponding bacterial rRNA sequences from the Rfam database (http://rfam.xfam.org).

Table 2 The prevalence of *L. arthropodarum* and *M. mitochondrii* in *Ixodes persulcatus*, *I. ricinus*, and their hybrids

Species/sex, stage	Number of ticks	*L. arthropodarum*		*M. mitochondrii*	
		Positive ticks (%)[a]	95% confidence interval	Positive ticks (%)	95% confidence interval
I. persulcatus					
females	72	**62 (86.1)**	**77.9–94.3**	0	N/A[b]
males	54	14 (25.9)	13.8–38.0	2 (3.7)	1.5–8.9
nymphs	18	14 (77.8)	56.5–99.1	0	N/A
I. ricinus					
females					
males					
nymphs					
	25	0	N/A	**22 (88.0)**	**74.3–100**
	27	0	N/A	11 (40.7)	20.9–60.5
	33	0	N/A	18 (54.5)	36.6–72.4
			Tick hybrids		
Hybr[perc]					
females	2	**2 (100)**	N/A	0	N/A
males	1	**1 (100)**	N/A	0	N/A
Hybr[ric]					
females	5	0	N/A	**5 (100)**	N/A
males	7	0	N/A	1 (14.3)	N/A
nymphs	6	0	N/A	4 (66.7)	N/A

[a] Absolute number of ticks (%); values more than 80% are highlighted in bold

[b] *N/A* the data were not statistically analyzed due to the small number of samples

[c] Hybr[per] are the hybrids with mtDNA of *I. persulcatus* and nucDNA of a mix of *I. persulcatus* and *I. ricinus*; Hybr[ric] are the hybrids with mtDNA of *I. ricinus* and nucDNA of a mix of *I. ricinus* and *I. persulcatus*
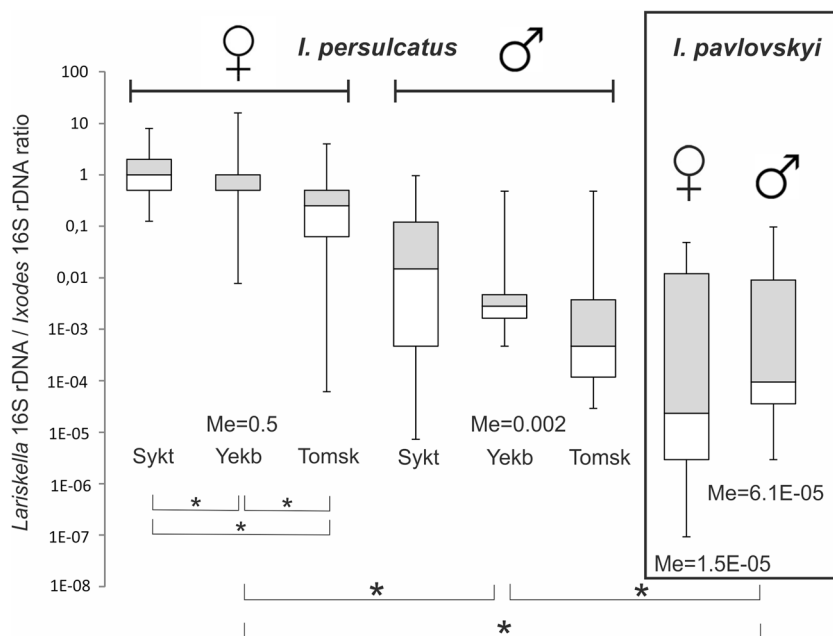
Table 3 The prevalence of *L. arthropodarum* in *Ixodes persulcatus*, *I. pavlovskyi*, and their hybrids

Species/geographical origin	Total No. of ticks	Females			Males		
		Number of ticks	*Lariskella* positive (%)[a]	95% confidence interval	Number of ticks	*Lariskella* positive (%)	95% confidence interval
I. persulcatus							
Syktyvkar (Sykt)	120	60	**53 (88.3)**	**79.9–96.7**	60	25 (41.7)	28.8–54.6
Yekaterinburg (Yekb)	120	60	**54 (90.0)**	**82.2–97.8**	60	16 (26.7)	15.2–38.2
Tomsk	120	60	**53 (88.3)**	**79.9–96.7**	60	13 (21.7)	11.0–32.4
Total	360	180	**160 (88.9)**	**84.3–93.5**	180	54 (30.0)	23.2–36.8
I. pavlovskyi							
Tomsk	248	109	21 (19.3)	11.8–26.8	139	19 (13.7)	7.9–19.5
			Tick hybrids				
Hybr[perb]	20	9	**8 (88.9)**	**63.2–100**	11	4 (36.4)	2.5–70.3
Hybr[pav]	85	47	8 (17.0)	5.8–28.2	38	3 (7.9)	0–16.9

[a] Absolute number of ticks (%); values more than 80% are highlighted in bold

[b] Hybr[per] are the hybrids with mtDNA of *I. persulcatus* and nucDNA of either a mix of both species or *I. pavlovskyi*; Hybr[pav] are the hybrids with mtDNA of *I. pavlovskyi* and nucDNA of either a mix of both species or of *I. persulcatus*

Fig. 1 The *L. arthropodarum* 16S rDNA/*Ixodes* sp. mitochondrial 16S rDNA ratios between different sexes and species of ticks. The number of samples for each group is given in Table 3. The *boxes* represent the 25th and 75th percentiles of the values, with the *line* inside the boxes marking the median (Me). The *whiskers* indicate the maximum and minimum values. Statistically significant differences between tick species and geographical territories (Mann-Whitney test, $p < 0.05$) are shown by an asterisk

Results

Prevalence of *L. arthropodarum* and *M. mitochondrii* in Ticks in the *I. persulcatus*/*I. ricinus* Sympatric Zone

Genetic features of the detected tick hybrids, where DNA analysis shows a mix of two species, and their proportion relative to 'pure' species in natural populations have been described previously [22, 23]. The prevalence of *L. arthropodarum* and *M. mitochondrii* in the 'pure' species *I. persulcatus* and *I. ricinus* as well as in their hybrids are presented in Table 2. Tick hybrids were grouped based on the species identified by the analysis of mtDNA, in other words, based on their maternal species; no hybrids with a mix of mitochondria were detected. Although *I. persulcatus* and *I. ricinus* in the sympatric zone probably feed on the same animals, a clear association between *M. mitochondrii* and *I. ricinus* and between *L. arthropodarum* and *I. persulcatus* was observed. Only two *I. persulcatus* males harbored the DNA of *M. mitochondrii* and no sample of *I. ricinus* infected with *L. arthropodarum* was detected. The prevalence of *L. arthropodarum* in *I. persulcatus* females and *M. mitochondrii* in *I. ricinus* females was 86–88% (Table 2); in males it was significantly lower (Table 2). Detection of both endosymbionts in nymphs revealed intermediate values, 77.8 and 54.5%, respectively. Association of *L. arthropodarum* and *M. mitochondrii* with their hosts was observed for the tick hybrids as well, but because of their small number statistical analysis was not performed.

Prevalence and Relative Abundance of *L. arthropodarum* in Ticks *I. persulcatus*, *I. pavlovskyi*, and their Hybrids

The prevalence of *L. arthropodarum* was determined for adult *I. persulcatus* and *I. pavlovskyi* ticks of both sexes collected in the Tomsk region, a sympatric and hybridization zone, as well as for *I. persulcatus* ticks from two regions where only *I. persulcatus* is found (Table 3). The prevalence of *L. arthropodarum* in *I. persulcatus* females did not depend on their origin and was about 90% (95%CI = 84.3–93.5%), 3-fold the observed in males with 30% on average (95%CI = 23.2–36.8%). The percentage of *L. arthropodarum*-infected ticks was significantly higher for *I. persulcatus* males than for both females and males of *I. pavlovskyi* (t test, $p < 0.05$). At the same time, no statistical difference between the prevalence of *L. arthropodarum* in females and males of *I. pavlovskyi* was found. *I. persulcatus*/ *I. pavlovskyi* hybrids were divided into two groups as follows: (1) Hybr[per]—the hybrids with mtDNA of *I. persulcatus* and nuclear DNA of either a mix of both species or *I. pavlovskyi* (hybrids with mitochondrial introgression); (2) Hybr[pav]—the hybrids with mtDNA of *I. pavlovskyi* and nuclear DNA of either a mix of both species or of *I. persulcatus*. Statistical analysis of the two groups of hybrids revealed the same pattern as shown for the 'pure' species, namely, that *L. arthropodarum* is much more frequently found in Hybr[per] females than in males and less frequently in both females and males of Hybr[pav] than in Hybr[per] males (Table 3).

Relative abundance of *L. arthropodarum* in ticks was performed by qPCR determination based on the C_t ratio for the *Lariskella*/tick target genes (Fig.1). The median *Lariskella*/

mitochondria ratio in *I. persulcatus* females was 0.5, 250-fold greater than those in *I. persulcatus* males (0.002), which, in turn, was significantly greater than the median value in *I. pavlovskyi*, $1.5 \cdot 10^{-5}$ in females and $6.1 \cdot 10^{-5}$ in males (Fig. 1). It is interesting that the comparison of *Lariskella*/mitochondria ratios between *I. persulcatus* females originating from different regions showed statistically significant differences: the values are maximal in female ticks from Syktyvkar (North-West Russia), decreased in Yekaterinburg (Urals) and minimal in Tomsk (Fig. 1). No statistical difference between *Lariskella*/mitochondria ratios in *I. persulcatus* males from the same regions, as well as between females and males of *I. pavlovskyi*, was found (Fig. 1). Comparison between hybrids was not performed because of the small number of Hybr[per] samples.

Genetic Diversity of *L. arthropodarum* in *I. persulcatus* and *I. pavlovskyi* Ticks

Since low genetic diversity of 16S rDNA sequences has been shown for *L. arthropodarum* isolated from *I. persulcatus* [13], we decided to use another, presumably more variable genetic marker, the rrl-rrf intergenic spacer. Design of the primers for its amplification was complicated by the absence of a whole-genome sequence of *L. arthropodarum*. In the present study, we obtained a new sequence of rrl-rrf IGS of *L. arthropodarum* isolated from *Ixodes* ticks (GenBank Acc. Nos. KX578087–KX578171). The length of the spacer was 120 bp, 21 bp longer than in *M. mitochondrii* with 99 bp. Alignment of the 62 rrl-rrf IGS sequences of *L. arthropodarum* from *I. persulcatus*, including those collected in the sympatric zone, and Hybr[per] showed 100% identity, i.e., all samples harbored the same IGS variant, designated here as Per1 (GenBank Acc. No. KX578110). However, analysis of *I. pavlovskyi* and Hybr[pav] ticks revealed two new IGS variants in 18 out of 23 samples; they were designated as Pav1 (KX578087) and Pav2 (KX578104). The former had a nucleotide substitution T200C (position numbers are given according to KX578087) and was found in 17 samples; the latter had two substitutions compared to Per1, T200C and T227C, and was observed in only one sample (Fig. 2).

Discussion

Two main routes of transmission of microorganisms from one tick to another are known: vertical (transovarial), from female to its progeny, and horizontal, either by feeding on an infected animal or by co-feeding with infected ticks simultaneously on the same host [29]. Once a tick acquires a microorganism, two scenarios are possible. The first one assumes that this microorganism is unable to multiply in tick tissues and is consequently digested in the tick gut, although even in this case the

microorganism's DNA can be detected by sensitive molecular genetics methods. For example, animal DNA, equally exposed to digestion, can be detected in ticks several months after the bloodmeal [30, 31]. An alternative scenario is that the microorganism multiplies in the tick tissues, thus undergoing natural selection imposed by a host.

Nowadays, it is well known that *M. mitochondrii* can be efficiently transmitted transovarially. However, its DNA has been detected in animal blood which suggests the possibility of horizontal transmission [6]. As an example, horizontal transmission of a phylogenetically related microorganism, *Anaplasma phagocytophilum*, by co-feeding has been demonstrated experimentally [32–34]. Since DNA of *L. arthropodarum* has been detected in human blood [11] and *I. persulcatus* salivary glands [15], we suggest horizontal transmission for this bacterium as well.

Since it is virtually impossible to distinguish between microorganisms of the same species acquired either transovarially or horizontally, we have analyzed as a model the sympatric zone of two tick species, each of them associated with its own endosymbiont, *I. ricinus* with *M. mitochondrii* and *I. persulcatus* with *L. arthropodarum*. The sympatric zone of these tick species is located in the East European Plain and the Baltic countries; ticks not only co-habit the same area but also produce interspecific hybrids [23]. Our assumption was that the horizontal route, which is generally accidental, would be seen as detection of endosymbionts in non-specific hosts. Nevertheless, analysis of 144 *I. persulcatus* and 86 *I. ricinus* ticks, as well as a small number of their hybrids from Estonia revealed almost complete specificity of tick-symbiont association. Only in two *I. persulcatus* males, which consist of 1.4% of the total number of ticks of this species, did we detect the non-specific endosymbiont *M. mitochondrii*; this value probably reflects the relative contribution of the horizontal transmission route in the overall prevalence. No *I. ricinus* tick harbored DNA of *L. arthropodarum*. Therefore, we are inclined to think that the vertical transmission route is dominant for *M. mitochondrii* and *L. arthropodarum* in natural tick populations, while the horizontal route does not contribute much to their prevalence in ticks or could not be detected by the methods used in this study.

Taken together, if a significant prevalence of *M. mitochondrii* or *L. arthropodarum* in ticks is detected, it is probably due to a specific vertical transmission route accompanied by multiplication of endosymbionts in host reproductive organs. It is a situation that was observed for the second sympatric zone, that of *I. persulcatus* and *I. pavlovskyi*. The distribution range of *I. pavlovskyi* is disjoined into Western Siberian and Far Eastern parts, which almost completely overlaps with that of *I. persulcatus*. In this paper, we studied only the Western Siberian part, where interspecific hybrids had been previously detected [22]. The prevalence of *L. arthropodarum* was evenly high, about 90%, in
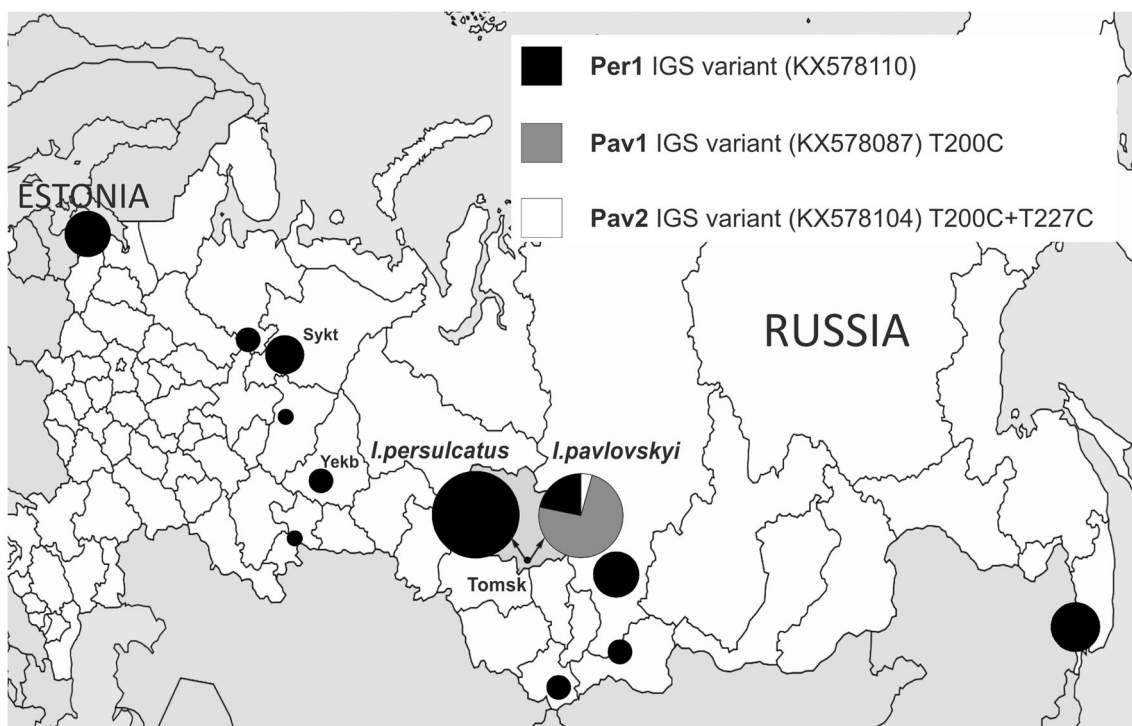
Fig. 2 Distribution of *L. arthropodarum* rrl-rrf IGS variants between different localities and tick species. The size of a circle corresponds to the number of the *L. arthropodarum* sequences obtained (85 in total). Tomsk region, a sympatry zone where both tick species were sampled together, is shadowed in *gray*; samples from other regions are presented by *I. persulcatus* only. The *L. arthropodarum* rrl-rrf IGS variants are indicated by *colors*; GenBank accession numbers and the sequences' variation are indicated

I. persulcatus females and about 30% in males. Both high prevalence and relative abundance of *L. arthropodarum* in females proves the hypothesis assuming that this bacterium efficiently multiplies in *I. persulcatus* females but is probably lost in males during ontogenesis [12]. DNA of *L. arthropodarum* has been previously detected in *I. pavlovskyi* females from the Tomsk region [14]; however, no systematic studies to estimate its prevalence or characterize its genetic diversity have been made. We showed that, unlike *I. persulcatus*, there is no statistical difference between the prevalence of *L. arthropodarum* in *I. pavlovskyi* females (19.3%) and males (13.7%); the relative abundance of this bacterium is also almost the same and about 100-fold smaller than those in *I. persulcatus* males. Moreover, tick hybrids grouped according to their maternal species showed a similar pattern. It could be suggested that the relatively low prevalence and quantity of *L. arthropodarum* in *I. pavlovskyi* is probably due to its accidental uptake with animal blood during tick feeding, but the results obtained for the *I. ricinus*/*I. persulcatus* sympatric zone do not support this hypothesis. Therefore, *L. arthropodarum* is most probably able to multiply in *I. pavlovskyi* and can be transmitted transovarially, but much less efficiently than in *I. persulcatus*, based on both the lower quantity and prevalence of the endosymbiont.

Since multiplying microorganisms necessarily undergo natural selection imposed by a host, it would be logical to

assume that *I. pavlovskyi* ticks harbor an isolated population of *L. arthropodarum*, even at the genetic level. To test this assumption, we estimated genetic diversity of this microorganism isolated from *I. persulcatus* outside the sympatric zone, as a control, and from both species within the sympatric zone of the Tomsk region. The rrl-rrf intergenic spacer was used as a genetic marker. Although this marker is not used as widely as 16S rRNA gene, it has been shown to be twice as variable as rRNA genes [35]. It has also been used to study bacteria of the genus *Rickettsia* [36]. In this study, for the first time, we obtained the sequences of rrl-rrf IGS of *L. arthropodarum*, isolated from *Ixodes* ticks. They were 46.7% identical to the rrl-rrf IGS sequence of *M. mitochondrii*; the identity of the flanking 174 bp fragment of the 23S rDNA with the corresponding sequence of *M. mitochondrii* was 85.6%. The most interesting thing is that all studied *I. persulcatus* ticks collected in different regions in Russia and Estonia harbored the same variant of the rrl-rrf IGS (Fig. 2), while in 78% of *I. pavlovskyi* samples we found a specific nucleotide substitution, T200C. Another variant, with an additional substitution, T227C, was found in one *I. pavlovskyi* female. Formally, greater diversity of *L. arthropodarum* in *I. pavlovskyi*, yet minimal, could lead to the conclusion that *I. pavlovskyi* has been associated with this bacterium for longer time period, and that one particular strain then invaded *I. persulcatus* and spread to fixation.

However, we think such a scenario to be unlikely because the prevalence and transmission efficiency of *L. arthropodarum* in *I. persulcatus* exhibit a strong symbiotic relationship, while its association with *I. pavlovskyi* is more likely to indicate an initial stage of adaptation. In fact, it has been shown previously that the sympatric zone between *I. persulcatus* and *I. pavlovskyi* in the Tomsk region is relatively recent and had been formed due to the spread of *I. pavlovskyi* northward and even its dominance near Tomsk city [37, 38]. The absence of new genetic variants of *L. arthropodarum* in *I. persulcatus* argues for its association with *I. pavlovskyi* and against the possibility of its accidental uptake with bloodmeal.

In conclusion, we can say that the biological features of *L. arthropodarum* in ticks, namely preferential multiplication in females, capacity for vertical transmission through the host generations and, probably, its high efficiency, resemble those of *M. mitochondrii*. Detection of new genetic variants of *L. arthropodarum* in *I. pavlovskyi*, even with a minor difference from the variant in *I. persulcatus*, could indicate an ongoing process of adaptation and formation of symbiotic relationships.

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