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Prevalence of *Borrelia burgdorferi* sensu lato and *Borrelia miyamotoi* in ixodid ticks in the Far East of Russia

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ABSTRACT

Borrelia burgdorferi sensu lato (s.l.) DNA was detected by PCR in *Ixodes persulcatus* Schulze, 1930, *Haemaphysalis concinna* Koch, 1844, *Haemaphysalis japonica douglasi* Nuttall et Warburton, 1915 and *Dermacentor silvarum* Olenev, 1932 ticks collected in the Amur region, the Jewish Autonomous region, the Sakhalin region and on the Khabarovsk territory. Infection rate of *I. persulcatus* with *B. burgdorferi* s.l. 10–69% exceeded the corresponding values of three other tick species in all examined regions during 1999–2014 despite different tick abundance and dominance structure. Bacterial loads estimated on the base of quantitative real time PCR varied from 10^2 to 10^9 genome-equivalents per a tick with maximal values for *I. persulcatus* and *H. japonica.* Phylogenetic analysis of 16S rRNA gene and 5S–23S rRNA intergenic spacer nucleotide sequences revealed two species: 1) *Borrelia garinii* of Asian type NT29 with several isolates of European type 20047; 2) *Borrelia afzelii* with identical sequences of the majority of studied isolates and VS461 reference strain in all regions except the Sakhalin Island where *B. afzelii* was not found. *Borrelia miyamotoi* of the relapsing fever group was detected as monoinfection or in combination with *B. burgdorferi* s.l. in 4.0 ± 0.9% and 4.8 ± 0.9% *I. persulcatus* ticks, respectively. Multiple locus sequence analysis of three fragments of 16S rRNA, glpQ and p66 genes proved that all the Far Eastern *B. miyamotoi* isolates belonged to the Asian type identical to FR64b strain (GenBank CP004217) from Japan. Wide distribution of *Borrelia* DNA in ticks, relative genetic homogeneity with similar sequences of the coding regions and the intergenic spacer of *Borrelia* wild isolates and temporal stability with high homology levels of the Far Eastern isolates of *B. garinii*, *B. afzelii* and *B. miyamotoi* with previously described spirochetes from the surrounding regions of Russia, China and Japan allowed us to suggest multiple ecological niches as the stability factor of the parasitic system.

1. Introduction

Lyme borreliosis is one of the most common vector-borne zoonotic bacterial diseases in the world. Tens of thousands of cases are reported annually in temperate regions of North America, Eurasia and Australia (Rosa et al., 2005). Lyme disease has both acute (with a transient inflammatory skin rash known as erythema migrans, arthritis, carditis, neuropathies) and persistent clinical manifestations with chronic arthritis, neuroborreliosis, small-point rash, erythema migrans and other skin problems. The prevalence of particular Lyme disease symptoms varies between North America and Europe, with arthritis more common in the United States and neurological and skin disorders more common in Europe. These distinct clinical manifestations of Lyme disease might reflect the geographical distribution of *Borrelia* genospecies. The bacteria that cause human Lyme disease belong to a clade of 16 named species called *Borrelia burgdorferi* sensu lato. Among these species, *Borrelia burgdorferi* sensu stricto (s.s.) *Borrelia garinii, Borrelia afzelii* and "*Borrelia bavariensis*" sp. nov. are well-known causes of Lyme disease in North America, Europe (Casjens et al., 2011) and Russia (Rar et al., 2017). *Borrelia finlandensis* isolated from an *Ixodes ricinus* tick in Finland is closely related to *B. burgdorferi* s.s. and is suggested as candidate for new-species status (Casjens et al., 2011). Infection by *B. burgdorferi* s.s. is frequently associated with arthritis, *B. garinii* with neurological disease and *B. afzelii* with chronic skin disorders, although the correlation

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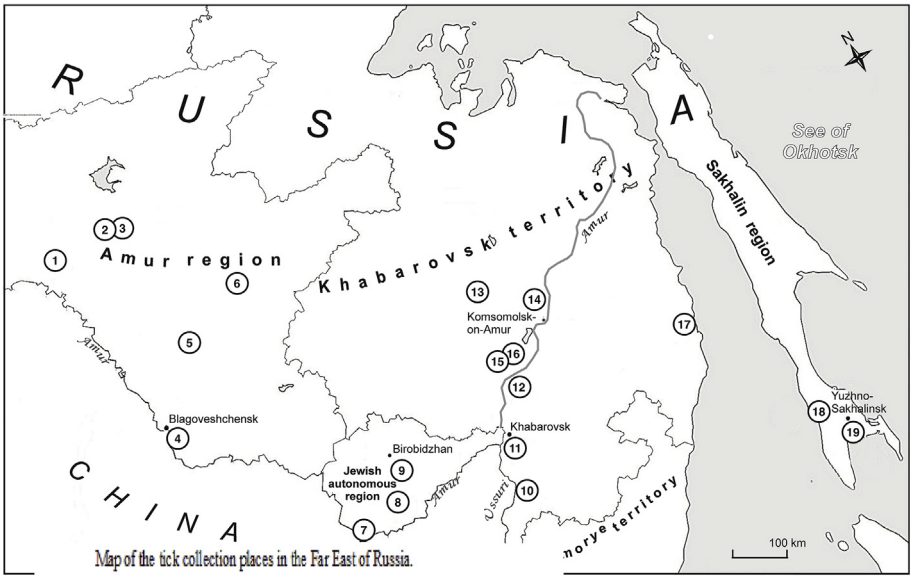


Fig. 1. Map of the tick collection places in the Far East of Russia.

is not absolute.

Besides *B. burgdorferi* s.l. another genetically distant *Borrelia* spp. are associated with hard ticks including *Borrelia miyamotoi* and *Borrelia* sp. HM, *Borrelia* sp. HF, *Borrelia* sp. HK, *Borrelia* sp. HL in *Haemaphysalis* spp. ticks (Furuno et al., 2017; GenBank accession numbers LC170019-LC170035). *B. miyamotoi* phylogenetically clusters with the relapsing fever *Borrelia* species (Krause et al., 2015). The distinctive feature of *B. miyamotoi* and other relapsing fever group species includes expression of a glycerophosphodiester phosphodiesterase (GlpQ) gene (Krause et al., 2015). Transovarial transmission is another biological feature that distinguishes relapsing fever *Borrelia* species including *B. miyamotoi* from *B. burgdorferi* (Krause et al., 2015). Human cases of *B. miyamotoi* infection were first reported in 2011 in Russia (Platonov et al., 2011) and subsequently in the United States, Europe, and Japan. The most common clinical manifestations of *B. miyamotoi* infection are fever, fatigue, headache, chills, myalgia, arthralgia, and nausea with possible severe sequellae, including meningoencephalitis (Krause et al., 2015).

Spirochetes represent a phylum of bacteria phylogenetically distinct from other main bacterial groups (Rosa et al., 2005). *B. burgdorferi* (B31 strain) was the third microbial genome ever sequenced (Fraser et al., 1997). Overall, *B. burgdorferi*'s genome consists of one megabase linear chromosome and a variety of circular and linear plasmids ranging in size from 9 to 62 kb. The chromosome, unlike many other eubacteria, has no relation to either the bacteria's virulence or to the host-parasite interaction (Fraser et al., 1997). Some of the plasmids are necessary for the *B. burgdorferi* life cycle in wild nature but not for propagation of the bacteria in culture. The genetic variations of *Borrelia burgdorferi* contribute to varying degrees of infection and dissemination (Theisen et al., 1995; Kurtenbach et al., 2006).

Mechanisms controlling the *Borrelia* selection are multiple-niche polymorphism and negative frequency-dependent selection (Kurtenbach et al., 2006; Samuels and Radolf, 2010). Multiple-niche polymorphism is maintained within a population due to the varying amount possible ecological niches such as various biotops, reservoir hosts and vectors. Both genetic diversity, various gene expression patterns and protein polymophrism are required for *B. burgdorferi* adaptation to phylogenetically divergent arthropods and vertebrate hosts. In negative frequency-dependent selection, *B. burgdorferi* rare low-frequency variants may have a selective advantage because of lack of an immunological response of arthropod hosts.

Despite high prevalence of Lyme borreliosis in the Far East of Russia (3.3–5.45 confirmed cases per 100,000 population) little is currently known about *Borrelia* biodiversity (Sato et al., 1996; Mediannikov et al., 2005; Liu et al., 2012). High prevalence of *B. burgdorferi* s.l. (Korenberg et al., 1993), predominance of *B. garinii* over *B. afzelii* (Li et al., 1998; Mediannikov et al., 2005) and absence of *Borrelia burgdorferi* sensu stricto or *Borrelia japonica* isolates (Li et al., 1998; Morozov and Morozova, 2012 and references therein) were thoroughly examined. Meanwhile, genetic diversity, geographic distribution, natural hosts and vectors of *B. miyamotoi* are at the beginning of study (Mukhacheva et al., 2015). Our aim was the comparative analysis of *Borrelia* species in ixodid ticks collected from various biotopes in the Far East of Russia.

2. Materials and methods

2.1. Ticks

Were collected from vegetation by using 60 × 100 cm cotton cloths (flag), ticks were removed from the "flag" and placed in cotton wet bandage and cotton bag. Average number of ixodid ticks was calculated as "ticks per one flag - hour" is the score of relative abundance of ticks that is calculated as the number of unfed adult ticks averaged per 1 h, $\frac{\sum \text{all collected tricks}}{\sum \text{hours}}$ $\frac{\sum \text{all collected tricks}}{\sum \text{hours}}$ $\frac{\sum \text{all collected tricks}}{\sum \text{hours}}$. Ticks species were determined on the base of their morphological traits according to (Filippova; 1985). Adult questing ticks were flagged from vegetation during May and June (when ticks are the most active in the region) 1999–2014 in the Far East (Amur region, Jewish Autonomous region, Khabarovsk territory and Sakhalin region) of Russia in sampling sites 1–19 differing in their geographic location, climatic conditions, vegetation and anthropogenic pressure (Fig. 1 and Table 2). Sites 1–6 are located in the Amur region; sites 7–9 - in the Jewish Autonomous region, 10–17 on the Khabarovsk territory; and sites 18 and 19 - in the Sakhalin region. A few premature ticks can also be collected from vegetation but we focused on adult ixodid ticks due to their epidemiological importance.

2.2. Borrelia DNA detection

Arthropod suspensions were prepared from individual ticks in disposable tubes with pestles in liquid nitrogen. Since 2013 «TissueLyser LT» (Qiagen, Germany) kit was used.

Total nucleic acids were isolated from individual tick suspensions using phenol-chloroform deproteinization with subsequent alcohol

precipitation ("Vector Best", Russia) during 1999–2009 and later in 2010–2014 it was replaced with adsorption of nucleic acids on silica by using "Ribosorb" ("InterLabService", Russia).

PCR with primers SL (Demaerschalck et al., 1995) and mastermix ("Syntol", Russia) with subsequent electrophoresis was performed in 1999–2006; test-system for *Borrelia burgdorferi* s.l. detection ("IzoGen", Russia) was used during 2007–2009. Real time PCR detection and quantitation were began to use in 2010 initially with the kit "AmpliSens *B. burgdorferi* sensu lato - FL" and later in 2011–2014 – with "AmplySens *TBE, B. burgdorferi, A. phagocytophila, E. muris/E. chaffensis* - FL" ("InterLabService", Russia). *Borrelia miyamotoi* DNA was detected by PCR with subsequent electrophoresis using primers specific to p66 gene (Fomenko et al., 2010) in 2011–2013 and by real time PCR in 2014 with the kit "Vecto *Borrelia miyamotoi* - FL" (Vector Best, Russia) according to the manufacturer's instructions.

2.3. Bacteirial loads

Bacteirial loads were estimated using quantitative real time PCR as described above with calibration curve of dependence between quantities of *Borrelia burgdorferi* s.s. strain B31 DNA with the known concentrations and threshold cycles (Ct) of fluorescence as previously published (Morozova et al., 2011).

2.4. Nucleotide sequences

Nucleotide sequences of PCR products were determined using primers (Fomenko et al., 2008, 2010), BigDye 3.1 Terminator Cycle Sequencing Kit and DNA analyzer ABI 3500 (Applied Biosystems, USA). GenBank (http://www.ncbi.nlm.nih.gov) accession numbers of the *Borrelia* spp. determined in our study are shown in Table 1. Phylogenetic analysis was performed using MEGA 6.06 (5 alternative algorithms, 1,000 replications) (Tamura et al., 2013). Reference nucleotide sequences were the following: *B. afzelii* VS461 (NR164748), *B. afzelii* VS461 (L30135), *B. afzelii* HLJ01 (CP003882), *Borrelia* sp. Tokachi-J-IP21f (EF160140); *B. garinii* CZ (CP007564), *B. garinii* BqVir (CP003151), *B. garinii* 20047 (NR043413), *B. garinii* N337 (AB035398), *B. garinii* N341 (AB035395), *B. garinii* F632 (AB035403), *B. garinii* NT29 (L30130), *B. garinii* 20047 (L30119), *B. garinii* SZ 8-1 (JX570876), *B. garinii* Itn-4150 (AM748054), *B. bavariensis* PBi (CP000013), *B. burgdorferi* B31 (CP009656), *B. finlandensis* SV1 (NZ_ABJZ02000005), *B. valasiana* Tom4006 (CP009117); *B. miyamotoi* FR64b (CP004217), *B. miyamotoi* Izh-5 (CP024205), *B. miyamotoi* Nsk57 (EF488992), *B. miyamotoi* 14T114 (KU749376), *B. miyamotoi* Lipetzk-09(1)-IR (JF951382), *B. miyamotoi* tik485 (KJ412189), *B. miyamotoi* CT14D4 (CP010308), *B. miyamotoi* LB-2001 (CP006647), *B. miyamotoi* Khabarovsk-1 (KU845210), *B. miyamotoi* 57Nsk (FJ940729), *Borrelia* sp. Seq3-tick (AB824855), *B. miyamotoi* M24FT2 (KX885477), *B. miyamotoi* 57Nsk (EU645995), *B. miyamotoi* tik508 (KJ425365), *B. miyamotoi* An80 NX1 (KF054069); *Borrelia* sp. HM (LC170034), *Borrelia* sp. HM (LC170035), *B. recurentus* A1 (CP000993). Coincidence of topologies of 5 phylogenetic trees constructed by means of 5 alternative algorithms (Tamura et al., 2013) for each locus of *Borrelia* genomes and reasonable bootstrap support indexes of cladistic groups above 70 were considered as significant.

2.5. Statistical comparisons

Were carried out using percentages with the Standard Error of the Percentage (SEP) (Sheskin, 2011). Continuous variables were compared using Student's t-test. P values < 0.05 were assumed to be significant.

3. Results

3.1. Distribution of ixodidae ticks in the Far East of Russia

Ixodid ticks were collected in the Far East of Russia both in the mainland part (the Amur, the Jewish Autonomous regions and the Khabarovsk territory) and on Sakhalin Island during 1999–2014 (Fig. 1, Table 2). Totally, 2,158 individual ixodid ticks were collected, identified and examined by means of PCR for *Borrelia burgdorferi* s.l. detection. In 2011–2014 part of them (519 *I. persulcatus*) was also assayed to detect *B. miyamotoi* DNA. The ixodid tick populations in the continental collection places included four prevalent tick species: *Ixodes persulcatus* P. Schulze, 1930; *Haemaphysalis concinna* Koch, 1844; *Haemaphysalis japonica douglasi* Nuttall et Warburton, 1915 and *Dermacentor silvarum* Olenev, 1932 in different proportions, while on the Sakhalin Island only *I. persulcatus* ticks were found (Fig. 1, Table 2). In populous southern and central parts of the Amur region and on the Khabarovsk territory, where agrolandscapes replaced the original forests, *H. concinna* was predominant, and average tick density in deciduous and broadleaved forests (sites 4–6, 10, 12, 15) was essentially less than in larch-birch and pine-birch forests (site 1, 3) or in the coniferous forest landscapes with *I. persulcatus* prevalence on the Khekhtzir mountains (Bolshekhekhtzirsky wildlife reserve, site 11) and in Solnechny district (site 14). In the Jewish Autonomous region in coniferous–broadleaved forest (site 9) predominant *I. persulcatus* ticks reached the maximum population density (Table 2). Structure of tick populations of the Sakhalin Island differed from mainland, *I. persulcatus* was the only detected tick species (Table 2). Humidity of soil seems to be a limited factor for ixodid ticks development and is especially crucial parameter for *I. persulcatus* (Filippova; 1985). Therefore, the species remains abundant and predominant in coniferous–broadleaved forests far away from towns and villages. Other tick species including *Haemaphysalis* spp. can survive in less humid conditions and gradually replace the former prevailing *I. persulcatus*.

3.2. PCR detection of Borrelia DNA

PCR detection of *B. burgdorferi* s.l. in *I. persulcatus* ticks was successful in all explored biotops and areas. *Borrelia* DNA detection rate reached 69.0 ± 4.6% in larch-small-leaved forest (with *I. persulcatus* domination) on the Khabarovsk territory (site 17) and 68.8 ± 6.8 in larch-birch forest (with *H. concinna* domination) in the Amur region (site 1) with low anthropogenic pressure and relatively high tick population density. *Borrelia* DNA detection rate 0–28.6% for other tick species was significantly below the corresponding values for *I. persulacatus* collected from the same regions ($P < 0.01$) (Table 3). For comparison, totally in the Far East of Russia the average infection rate of *I. persulcatus* during 1999–2014 was 36.4 ± 1.2%, whereas *H. japonica* - 14.3 ± 5.1%, *D. silvarum* - 6.9 ± 4.8% and *H. concinna* -

Table 1
Accession numbers of nucleotide sequences of *Borrelia* isolates determined in our study.

Borrelia species	sequence			
	16S rRNA	5S−23S IGS	p66	glpQ
B. garinii	KY312010 - KY312015, KY312118, KY346888 - KY346892, KY346970 - KY346973, KY348800,	KY924779, KY937676 - KY937682, KY963154 - KY963161		
B. afzelii	KX622580, KX622581, KX622852, KX688604	KX685726 - KX685729		
B. miyamotoi	KX769848 - KX769851		KX812709 - KX812712	KX898133

Table 2
Geographic locations of collection places and ratio of the ixodid tick species.

Site №	District	Collection place (or exact location)	Biotop	Geographic coordinates of the site		Year	Average number (ticks per 1 flag-hour)	Tick species ratio (%)			
				N	E			*Ixodes persulcatus* P. Schulze, 1930	*Haemaphysalis concinna* Koch, 1844	*Haemaphysalis japonica douglasii* Nuttall et Warburton, 1915	*Dermacentor silvarum Olenev,* 1932
Amur region											
1	Magdagachinsky	Ductui	larch-birch forest	53°22′	126°08′	2011	85.3	33.6	**65.6** *	0	0.8
2	Zeisky	Zeya	pine –birch forest	53°44′	127°15′	2010	N/A**				
3	Shimanovsky	Belovezh	larch-birch forest	52°19′	127°24′	2011	122.0	4.9	**95.1**	0	0
4	Blagoveshchensky	Raduga camp	deciduous forest	50°40′	127°42′	2007	41.2	15.5	**84.5**	0	0
	Blagoveshchensky	Raduga camp	deciduous forest	50°40′	127°42′	2008	13.8	41.4	**58.6**	0	0
5	Svobodnensky	Kosmodrom	broadleaved forest	51°53′	128°20′	2011	30.0	8.9	**88.9**	0	2.2
6	Selemdzhinsky	Norsk	mixed deciduous forest	52°20′	129°53′	2010	16.0	0	**100**	0	0
Jewish Autonomous region											
7	Oktyabrsky	Stolbovoye	broadleaved forest	47°55′	131°03′	2013	19.3	6.9	**51.7**	24.1	17.3
8	Leninsky	Churki	coniferous–broadleaved forest	48°04′	132°39′	2013	14.3	**38.0**	**44.0**	18.0	0
9	Birobidzhansky	Birshosse, 17th km	coniferous–broadleaved forest	48°41′	132°48′	2013	71.4	**54.0**	35.2	10.8	0
Khabarovsk territory											
10	Lazo	Kiinsk	deciduous forest	47°59′	134°49′	2014	38.3	2.6	**93.0**	4.4	0
11	Khabarovsky	Khekhtzir	coniferous–broadleaved forest	48°15′	135°00′	1999–2014	182.9	**88.9**	2.5	5.4	3.2
12	Nanaisky	Troitzkoe	mixed broadleaved forest	49°22′	136°36′	2014	13.8	40.6	**52.2**	7.2	0
13	Solnechny	Gorny	coniferous–broadleaved forest	50°45′	136°27′	2009	62.0	**100**	0	0	0
14	Solnechny	Solnechny	mixed coniferous–small-leaved forest	50°26′	136°58′	2014	103.5	**99.0**	1.0	0	0
15	Komsomolsky	Khurba	secondary fine-leaved forest	50°24′	136°52′	2009	25.0	18.0	**72.0**	0	0
16	Komsomolsky	Taezhny	coniferous–broadleaved forest	50°35′	136°54′	2014	4.0	**90.0**	10.0	0	0
17	Vaninsky	Toki	larch-small-leaved forest	49°07′	140°18′	2011	57.0	**100**	0	0	0
Sakhalin region											
18	Kholmsky	Pionery	mixed coniferous–small-leaved forest	47°16′	142°02′	2011	39.0	**100**	0	0	0
19	Yuzhno-Sakhalinsky	Isvestkovy	mixed coniferous–small-leaved forest	46°50′	142°56′	2011	60.9	**100**	0	0	0

Notes: * –the dominant ixodid species percents are marked in bold; **- average numbers of ixodid ticks were not calculated.

Table 3
PCR detection of the *Borrelia burgdorferi* sensu lato DNA in the individual ixodid ticks collected from vegetation.

Site No	Year	District	The *Borrelia burgdorferi* sensu lato infection rate in ixodid ticks											
			Ixodes persulcatus Schulze, 1930			*Haemaphysalis concinna* Koch,1844			*Haemaphysalis japonica douglasi* Nuttall et Warburton,1915			*Dermacentor silvarum* Olenev,1932		
			Number of studied ticks	Ticks with the Borrelia DNA	Rate (%)	Number of studied ticks	Ticks with the Borrelia DNA	Rate (%)	Number of studied ticks	Ticks with the Borrelia DNA	Rate (%)	Number of studied ticks	Ticks with the Borrelia DNA	Rate (%)
Amur region														
1	2011[1]	Magdagachinsky	48	33	68.8 ± 6.8	51	2	3.9 ± 2.7	0			1	0	
2	2011[1]	Shimanovsky	9	2	22.2 ± 14.7	89	4	4.5 ± 2.2	0			2	0	
3	2011[1]	Svobodnensky	12	2	16.7 ± 11.2	85	3	3.5 ± 2.0	0			3	0	
4	2010[1]	Selemdzhinsky	0			56	0	0	0			0		
5	2010[1]	Zeisky	82	34	41.5 ± 5.5	0			0			17	2	11.8 ± 8.1
6	2007[2]	Blagoveshchenky	30	8	26.7 ± 8.2	70	0	0	0			0		
6	2008[2]	Blagoveshchenky	27	12	44.4 ± 9.7	73	0	0	0			0		
1–6	2007 -2011[1,2]		208	91	**43.8 ± 3.4**	424	9	**2.1 ± 0.7**	0			23	2	**8.7 ± 6.0**
Jewish Autonomous region														
7	2013[1]	Oktyabrsky	20	2	10.0 ± 6.9	2	0	0	0	–	–	5	0	0
8	2013[1]	Leninsky	23	6	26.1 ± 9.4	0	–	–	12	0	0	0		
9	2013[1]	Birobidzhansky	50	27	54.0 ± 7.1	13	0	0	0		0	0		
7–9	2013[1]		**93**	**35**	**37.6 ± 5.1**	**15**	**0**	**0**	**12**	**0**		**5**	**0**	0
Khabarovsk territory														
10	2014[1]	Lazo	15	3	20.0 ± 10.7	21	1	4.8 ± 4.8	13	0		1	0	
11	1999–2009[2]	Khabarovsky (Khekhtzir)	530	152	28.7 ± 2.0	0			0			0		
11	2010–2014[1]	Khabarovsky (Khekhtzir)	250	136	54.4 ± 3.2	0			0			0		
12	2014[1]	Nanaisky	26	9	34.6 ± 9.5	21	4	19.0 ± 8.8	3	1	33.3 ± 33.3	0		
13	2009[2]	Solnechny	40	10	25.0 ± 6.9	0			0			0		
14	2014[1]	Solnechny	66	17	25.8 ± 5.4	0			0			0		
15	2009[2]	Komsomolsky	50	10	20.0 ± 5.7	0			0			0		
16	2014[1]	Komsomolsky	21	7	33.3 ± 10.5	0			21	6	28.6 ± 10.1	0		
17	2011[1]	Vaninsky	100	69	69.0 ± 4.6	0			0			0		
10–17	1999 -2014[1,2]		**1098**	**413**	**37.6 ± 1.5**	**42**	**5**	**11.9 ± 5.1**	**37**	**7**	**18.9 ± 6.5**	**1**	**0**	
Sakhalin region														
18	2011[1]	Kholmsky	100	19	19.0 ± 3.9	0			0			0		
19	2011[1]	Yuzhno-Sakhalinsky	100	24	24.0 ± 4.3	0			0			0		
18, 19	2011[1]		**200**	**43**	**21.5 ± 2.9**									
Far East														
1–19	1999 -2014[1,2]		**1599**	**582**	**36.4 ± 1.2**	**481**	**14**	**2.9 ± 0.8**	**49**	**7**	**14.3 ± 5.1**	**29**	**2**	**6.9 ± 4.8**

Notes:[1] – results of real time PCR, [2] – data of PCR with subsequent electrophoresis.

Table 4
PCR detection of the *Borrelia miyamotoi* DNA in individual *Ixodes persulcatus* ticks collected from vegetation.

Site No	Year	District	The *Borrelia miyamotoi* DNA detection in *I. persulcatus* ticks					
			Number of studied ticks	*B. miyamotoi* monoinfection		*B. miyamotoi* + *B. burgdorferi* s. l.		*B. miyamotoi* in total	
				Ticks with the *Borrelia* DNA	Rate (%)	Ticks with the *Borrelia* DNA	Rate (%)	Ticks with the *Borrelia* DNA	Rate (%)
Amur region									
1	2011[1]	Magdagachinsky	48	0	0	0	0	0	0
Jewish Autonomous region									
7	2013[2]	Oktyabrsky	20	0	0	2	10.0 ± 6.9	2	10.0 ± 6.9
8	2013[2]	Leninsky	23	0	0	1	4.3 ± 4.2	1	4.3 ± 4.2
9	2013[2]	Birobidzhansky	50	0	0	5	10.0 ± 4.3	5	10.0 ± 4.3
7–9	2013[2]		**93**	**0**	**0**	**8**	**8.6 ± 2.9**	**8**	**8.6 ± 2.9**
Khabarovsk territory									
11	2013[2]	Khabarovsky	50	0	0	1	2.0 ± 2.0	1	2.0 ± 2.0
11	2014[2]	Khabarovsky	50	5	10.0 ± 4.3	4	8.0 ± 3.9	9	18.0 ± 5.5
10	2014[2]	Lazo	15	4	26.7 ± 11.8	1	6.7 ± 6.7	5	33.3 ± 12.6
12	2014[2]	Nanaisky	26	2	7.7 ± 5.3	1	3.8 ± 3.8	3	11.5 ± 6.4
14	2014[2]	Solnechny	66	3	4.5 ± 2.6	3	4.5 ± 2.6	6	9.1 ± 3.6
16	2014[2]	Komsomolsky	21	1	4.8 ± 4.8	2	9.5 ± 6.6	3	14.3 ± 7.8
17	2011[1]	Vaninsky	50	0	0	1	2.0 ± 2.0	1	2.0 ± 2.0
10-12, 14, 16, 17	2013–2014[1,2]		**278**	**15**	**5.4 ± 1.4**	**13**	**4.7 ± 1.3**	**28**	**10.1 ± 1.8**
Sakhalin region									
18	2011[1]	Kholmsky	50	3	6.0 ± 3.4	2	4.0 ± 2.8	5	10.0 ± 4.3
19	2011[1]	Yuzhno-Sakhalinsky	50	3	6.0 ± 3.4	2	4.0 ± 2.8	5	10.0 ± 4.3
18, 19	2011[1]		**100**	**6**	**6.0 ± 2.4**	**4**	**4.0 ± 2.0**	**10**	**10.0 ± 3.0**
Far East									
1–19	2011–2014[1,2]		**519**	**21**	**4.0 ± 0.9**	**25**	**4.8 ± 0.9**	**46**	**8.9 ± 1.3**

Notes: [1] – data of PCR with electrophoresis detection; [2] – results of real-time PCR.

Table 5
Threshold cycles (Ct) of quantitative real time PCR and deduced estimations of *Borrrelia burgdorferi* sensu lato DNA amounts in ixodid ticks in the Far East of Russia.

Region/territory	No of tick collection site	Ixodidae tick species	Number of samples with the *Borrelia* DNA	Ct range (min-max)	Average Ct	Average quantity of genome-equivalents per a tick
Amur	1	*I. persulcatus*	33	11.47–31.28	22.84 ± 7.05	1.46 × 10(5)
Amur	2	*I. persulcatus*	2	15.05–17.35	16.20 ± 1.63	1.46 × 10(7)
Amur	3	*I. persulcatus*	2	19.43–19.73	19.58 ± 0.21	1.40 × 10(6)
Amur	5	*I. persulcatus*	34	18.93–30.32	24.73 ± 2.70	5.93 × 10(5)
Jewish Autonomous	7	*I. persulcatus*	2	12.44–14.74	13.59 ± 1.63	5.35 × 10(9)
Jewish Autonomous	8	*I. persulcatus*	6	11.24–15.15	12.84 ± 1.41	8.75 × 10(9)
Jewish Autonomous	9	*I. persulcatus*	27	10.68–17.44	13.41 ± 1.85	6.06 × 10(9)
Khabarovsk	10	*I. persulcatus*	3	14.65–30.04	19.90 ± 8.79	6.72 × 10(7)
Khabarovsk	11	*I. persulcatus*	136	10.62–31.84	20.03 ± 6.42	6,16 × 10(7)
Khabarovsk	12	*I. persulcatus*	9	10.42–23.73	18.48 ± 6.33	1.81 × 10(8)
Khabarovsk	14	*I. persulcatus*	17	10.79–25.97	17.97 ± 5.59	2.57 × 10(8)
Khabarovsk	16	*I. persulcatus*	7	11.01–22.53	14.58 ± 4.01	2.69 × 10(9)
Khabarovsk	17	*I. persulcatus*	69	9.33–33.91	22.56 ± 7.03	1.07 × 10(7)
Sakhalin	18	*I. persulcatus*	19	11.71–31.64	19.28 ± 7.57	1.04 × 10(8)
Sakhalin	19	*I. persulcatus*	24	11.27–31.05	20.07 ± 7.20	5.99 × 10(7)
In total		***I. persulcatus***	**390**	**9.33–33.91**	**20.16 ± 0.18**	**5.63 × 10(7)**
Amur	1	*H. concinna*	2	29.49–34.20	31.85 ± 3.33	2.84 × 10(2)
Amur	2	*H. concinna*	4	29.98–33.21	32.02 ± 1.41	2.52 × 10(2)
Amur	3	*H. concinna*	3	30.30–33.01	31.29 ± 1.49	4.18 × 10(2)
Khabarovsk	10	*H. concinna*	1	30.69	30.69	3.81 × 10(4)
Khabarovsk	12	*H. concinna*	4	24.42–26.48	24.95 ± 0.04	2.88 × 10(6)
In total		***H. concinna***	**14**	**24.42–34.20**	**26.53 ± 3.60**	**1.13x10(4)**
Khabarovsk	12	*H. japonica*	1	13.96	13.96	4.14 × 10(9)
Khabarovsk	16	*H. japonica*	6	20.47–21.86	21.22 ± 0.48	4.50 × 10(5)
In total		***H. japonica***	**7**	**13.96–21.86**	**20.18 ± 2.78**	**5.55x10(7)**
Amur	5	***D. silvarum***	**2**	**28.35–28.94**	**28.65 ± 0.42**	**2.61x10(3)**

2.9 ± 0.8%.

B. miyamotoi DNA was detected in 2.0–33.3% *I. persulcatus* ticks collected from various biotopes of the Jewish Autonomous region, the Sakhalin region and the Khabarovsk territory (Table 4). *B. miyamotoi* was not found in the Amur region (Table 4) despite availability of *I. persulcatus* on the examined site 1 (Table 2). On an average, *B. miyamotoi* was detected in 8.9 ± 1.3% *I. persulcatus* ticks (as monoinfection in 4.0 ± 0.9% or as mixed infection with *B. burgdorferi* s.l. in 4.8 ± 0.9%) with reduced rate in comparison with *B. burgdorferi* s.l. (Tables 3 and 4).

3.3. Quantitative estimations of Borrelia DNA in ixodid ticks

Average threshold cycles (Ct) of real time PCR with the analyzed *B. burgdorferi* s.l. DNA significantly differed among four examined tick species with the maximal deduced bacterial loads for *I. persulcatus* and

Table 6
Quantitative comparative analysis of *Borrelia miyamotoi* and *Borrelia burgdorferi* sensu lato Ct and bacterial loads in *Ixodes persulcatus* ticks collected in the Far East of Russia.

District	Number of tick collection site	*Borrelia miyamotoi* monoinfection				Mixed infection with *Borrelia miyamotoi* and *B. burgdorferi* s. l.			
		Number of samples with the *Borrelia* DNA	Ct range (min-max)	Average Ct	Average quantity of genome-equivalents per a tick	Number of samples with the Borrelia DNA	Ct range (min-max) *B. miyamotoi*/ *B. burgdorferi* s. l.	Average Ct *B. miyamotoi*/ *B. burgdorferi* s. l.	Average quantity of genome-equivalents per a tick *B. miyamotoi*/*B. burgdorferi* s. l.
Jewish Autonomous region									
Oktyabrsky	7	0				2	32.89–33.79/ 12.44–14.74	33.34 ± 0.64/13.59 ± 1.63	3,03 × 10(3)/5.35 × 10(9)
Leninsky	8	0				1	35.25/12.87	35.25/12.87	8.07 × 10(2)/8.81 × 10(9)
Birobidzhansky	9	0				5	33.66–36.02/ 10.84–13.84	34.92 ± 1.17/12.20 ± 1.16	1,01 × 10(3)/1.40 × 10(10)
Khabarovsk territory									
Lazo	10	4	33.94–37.24	35.55 ± 1.54	6.56 × 10(2)	1	37.31/30.04	37.31/30.04	1.94 × 10(2)/5.98 × 10(4)
Khabarovsky	11	5	27.74–31.55	30.27 ± 1.73	2.55 × 10(4)	5	17.53–30.80/ 11.92–28.85	25.37 ± 6.51/21.62 ± 7.60	7.61 × 10(5)/2.05 × 10(7)
Nanaisky	12	2	35.34–38.58	36.96 ± 2.29	2.47 × 10(2)	1	33.80/11.17	33.80/11.17	2.21 × 10(3)/2.86 × 10(10)
Solnechny	14	3	22.78–33.25	29.75 ± 6.03	3.65 × 10(4)	3	32.54–35.00/ 12.46–25.97	33.92 ± 1,26/17.57 ± 7.33	2,03 × 10(3)/3.39 × 10(8)
Komsomolsky	16	1	18.72		7.64 × 10(7)	2	21.62–36.19/ 11.01–12.68	28,91 ± 10.30/11,85 ± 1.18	6.54 × 10(4)/1.79 × 10(10)
In total	**7–12, 14, 16**	**15**	**18.72–38.25**	**31.69 ± 5.36**	**9.52 × 10(3)**	**20**	**21.62–36.19/ 10.84–30.04**	**31.70 ± 5.72/16.37 ± 6.34**	**9.56 × 10(3)/7.95 × 10(8)**

H. japonica whereas those for *H. concinna* and *D. silvarum* were essentially lower (Table 5). Bacterial loads estimated on the base of quantitative real time PCR varied in a wide range from 10^2 to 10^9 genome-equivalents per a tick (Table 5). Based on the calibration curve of Ct from quantities of *B. burgdroferi* B31 genome-equivalents (Morozova et al., 2011) and the Lukyanov-Matz equation, one might estimate the average number of genome-equivalents per a tick 5.63×10^7 for *I. persulcatus*, 5.55×10^7 for *H. japonica,* 1.13×10^4 for *H. concinna* and 2.61×10^3 for *D. silvarum* (Table 5). The highest bacterial loads for *I. persulcatus* along with infection rate with *B. burgdorferi* s.l. exceeded the corresponding values of other tick species in all the examined regions during the whole period of observations (1999–2014) despite different tick abundance and dominance structure revealed the leading role of the taiga ticks in transmission of *Borrelia*. Similar quantitations for *B. miyamotoi* in *I. persulcatus* revealed the average bacterial load near 10^4 genome-equivalents per a tick in mono- and mixed infections (Table 6) that were in 10–1000 times less compared to amounts of *B. burgdroferi* s.l. ($p < 0.001$).

3.4. Phylogenetic analysis of Borrelia species diversity in the Far East of Russia

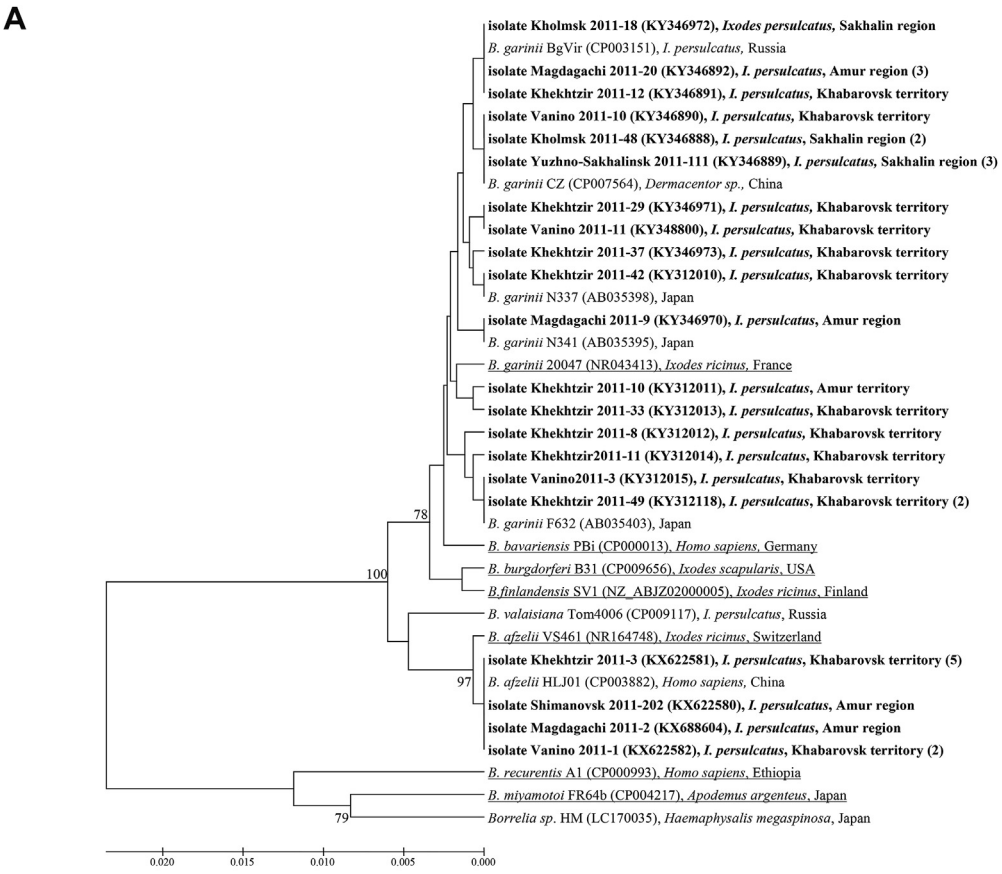
Genetic diversity of *Borrelia* in the Far East of Russia (Fig. 2) was similar to surrounding area of Eurasia (Kurtenbach et al., 2006) including sequence from a tick collected from migrating bird in Japan (AB015911). Sequences of 16S rRNA gene and 5S–23S rRNA intergenic spacer (ITS) of *B. garinii* corresponding to Asian type with prototype NT29 and European type with reference strain 20047 were found in all the examined regions of the Far East. The majority of *B. afzelii* isolates from ixodid ticks was identical to *B. afzelii* HLJ01 strain (CP003882) from China and clustered with strain VS461 (GenBank accession number NR164748) from Switzerland (Fig. 2) with the single nucleotide polymorphism (SNP) in 5S−23S ITS of *Borrelia* isolate Vanino 2011-2 (KX685729) similar to *Borrelia* sp. isolate Tokachi-J-IP21f (EF160140) from Japan. One should note that the only *B. garinii* (9 isolates) were found on the Sakhalin Island despite the simultaneous circulation of both *B. garinii* (23 isolates) and *B. afzelii* (12 isolates) in the continental part of the Far East of Russia. Observed fluctuations of ratios between *B garinii* and *B. afzelii* in two areas of the Khabarovsk territory with prevalence of *B. garinii* over B. *afzelii* (18:8, respectively) and in two collection places of the Amur region with the corresponding ratio species between *B. garinii* and *B. afzelii* near 1 (5:4) were not significant due to small sampling sizes (Fig. 2). Phylogenetic analysis of nucleotide sequences of both 16S rRNA gene and 5S–23S rRNA ITS does not permit to distinguish between *B. garinii* and *Borrelia bavarensis* (Fig. 2). Both species belong to the same clade with good bootstrap indexes (Fig. 2).

3.5. MLSA analysis of B. miyamotoi

Multiple locus sequence analysis (MLSA) of *B. miyamotoi* nucleotide sequences of three fragments of 16S rRNA, glpQ and p66 genes showed similar patterns (Fig. 3). Isolates of *B. miyamotoi* from the Far East of Russia belong to the Asian group previously found in Japan, China, Siberia and Ural (Fig. 3). All our nucleotide sequences of 16S rRNA and p66 genes were identical to each other and to the strain FR64b (CP004217) from Japan, the only glpQ gene sequence includes the SNP identical to Siberian strain 57Nsk (FJ940729).

4. Discussion

In the Far East of Russia, 22 hard tick species of the family Ixodidae have been identified, among them 11 species: *Ixodes maslovi* Emelyanova and Kozlovskaya, 1967; *Ixodes uriae* White, 1852; *Ixodes lividus* Koch, 1844; *Ixodes signatus* Birula, 1895; *Ixodes angustus* Neumann, 1899; *Ixodes pavlovskyi* Pomerantsev, 1946; *Ixodes*



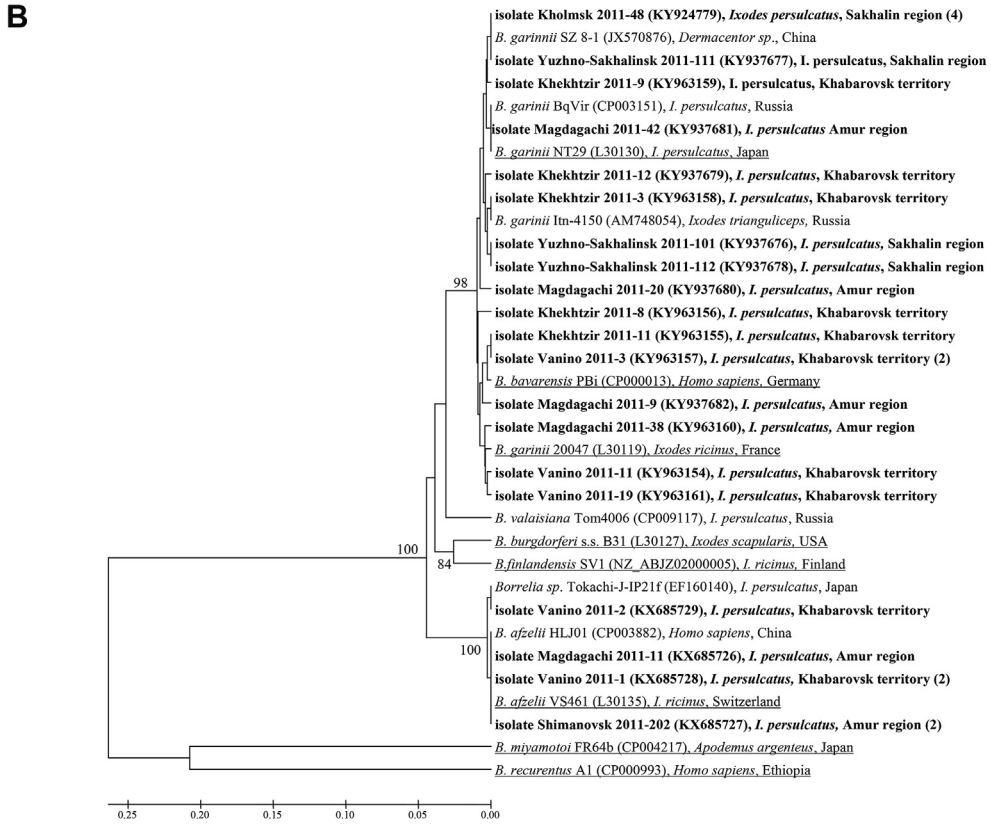
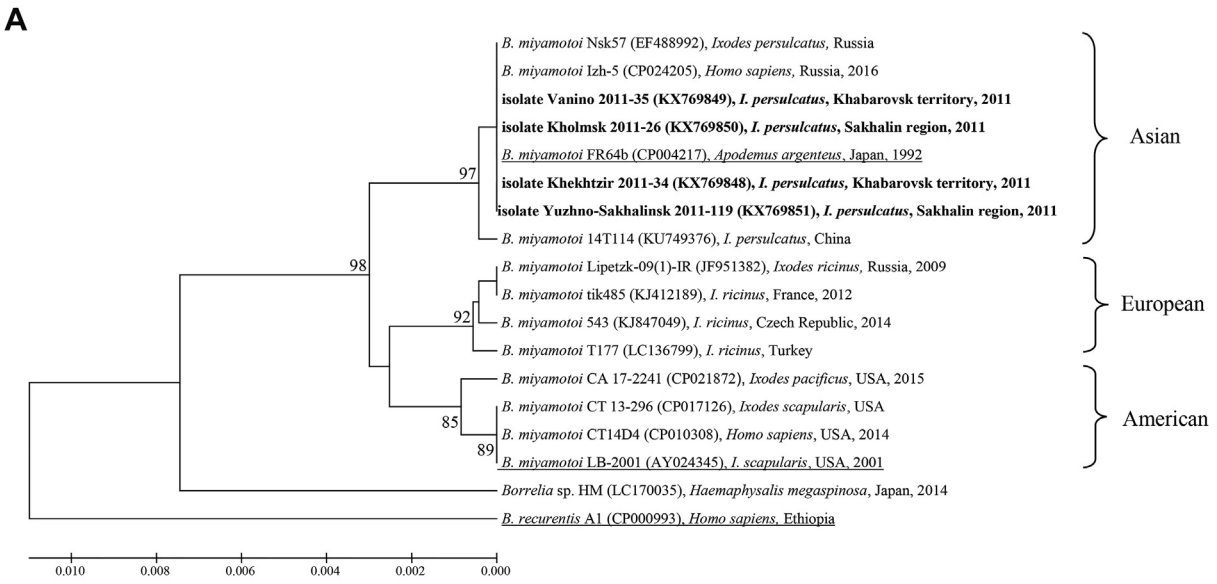
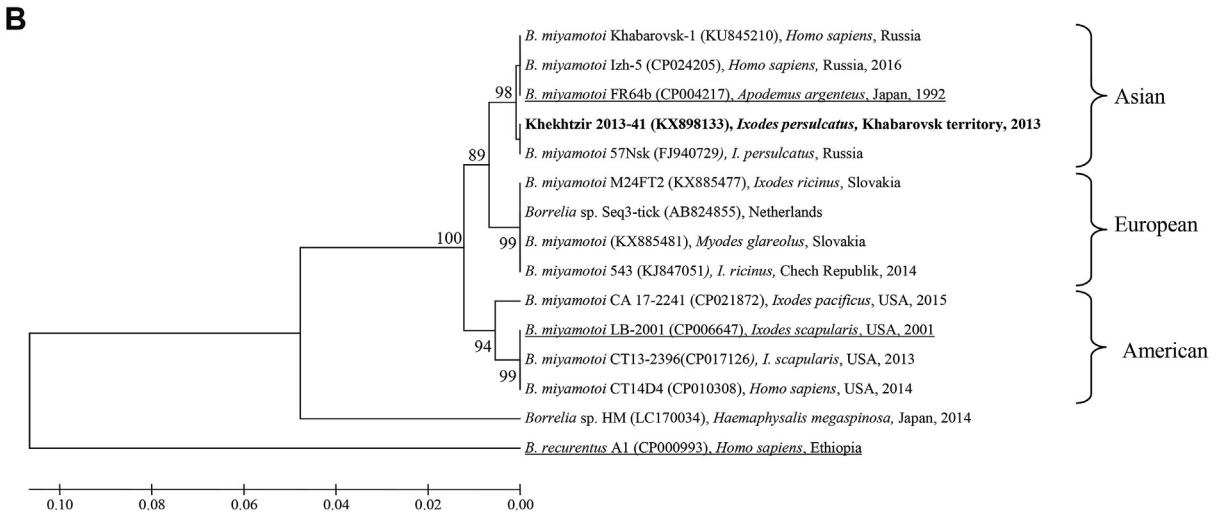


Fig. 2. Phylogenetic analysis of the nucleotide sequences of *Borrelia* 16S rRNA gene fragment of 733 bp long (**A**) and 5S−23S ITS of 211–218 bp long (**B**) using Mega 6.06 software, UPGMA algorithm and 1,000 replications. Phylogenetic trees constructed by means of 5 alternative algorithms (Maximum likelihood, Neighbor-Joining, Minimum-Evolution, UPGMA and Maximum Parsimony) show similar topologies and reasonable bootstrap support. Nucleotide sequences of *Borrelia* isolates determined in our study are shown in bold. The number of identical sequences of *Borrelia* isolates determined in these ixodid ticks is shown in parentheses. Branches corresponding to the reference strains are underlined.





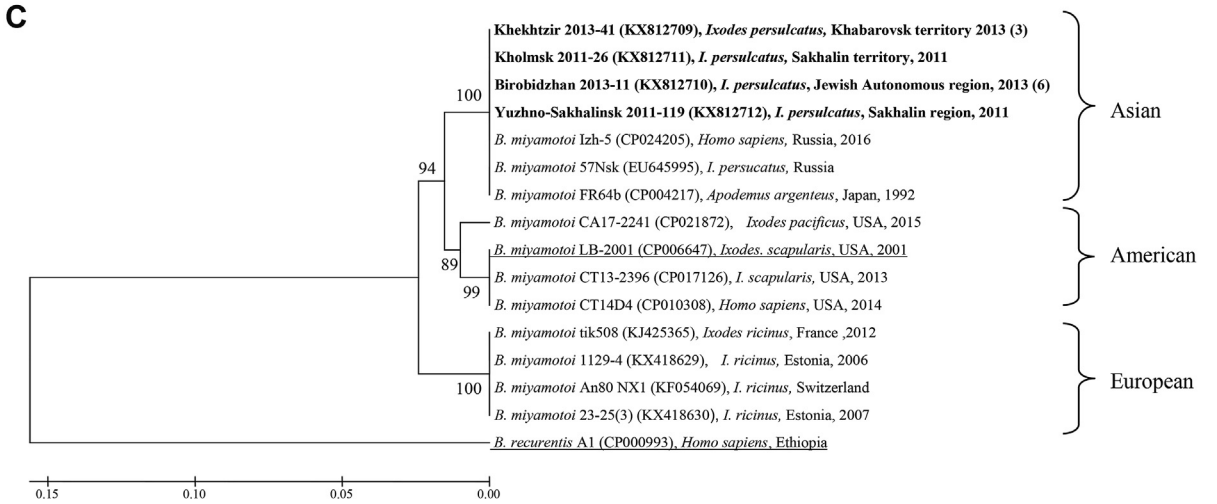


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Fig. 3. Multiple locus sequence analysis (MLSA) of *Borrelia miyamotoi* 16S rRNA gene fragment of 1248 bp (**A**), glpQ gene fragment 536 bp (**B**) and p66 gene fragment 526 bp long (**C**) using Mega 6.06 software, UPGMA algorithm and 1,000 replications. Phylogenetic trees constructed by means of 5 alternative algorithms (Maximum likelihood, Neighbor-Joining, Minimum-Evolution, UPGMA and Maximum Parsimony) show similar topologies and reasonable bootstrap support. Nucleotide sequences of *Borrelia* isolates determined in our study are shown in bold. The number of identical sequences of *Borrelia* isolates determined in these ixodid ticks is shown in parentheses. Branches corresponding to the reference strains are underlined.

persulcatus P. Schulze, 1930; *Haemaphysalis phasianna* Saito and Wassef, 1974; *Haemaphysalis concinna* Koch, 1844; *Haemaphysalis japonica* Nuttall et Warburton, 1915 and *Dermacentor silvarum* Olenev, 1932 were found on the Khabarovsk territory. The four tick species including *I. persulcatus*, *H. concinna*, *H. japonica*, *D. silvarum* predominate and have epidemiological importance (Volkov, 2005). During our period of observation 1999–2014 the four prevailing tick species - *I. persulcatus*, *H. concinna*, *H. japonica*, *D. silvarum* were found in all examined regions. Minor tick species were not observed, therefore, they were not analyzed. Monodominant populations of ixodids with the only species *I. persulcatus* remain on the Sakhalin Island and on Kamchatka Peninsula (Pukhovskaya et al., 1991, 2010). Tick numbers and dominance structure depend on anthropogenic influence. In afforestation biotopes both total number of ixodid ticks and proportion of taiga ticks dissipate (Volkov, 2005). Wide distribution of *Borrelia* DNA in the ixodid ticks of different species collected from all the studied areas of the Far East of Russia including Kamchatka Peninsula (Pukhovskaya et al., 2010) was shown (Tables 2–4). Nevertheless, maximal values of *Borrelia* DNA detection rate and bacterial loads were observed for *I. persulcatus* that together with its wide distribution throughout forest zone of Eurasia suggest its leading role as vector of *B. burgdorferi* s.l. Our comparative analysis confirmed the previous observation of principal role of *I. persulcatus* to cause Lyme disease in China and at the border with Russia (Liu et al., 2012).

Phylogenetic analysis of 16S rRNA gene and 5S−23S rRNA intergenic spacer nucleotide sequences revealed two species of *B. burgdorferi* s.l. complex, *Borrelia garinii* of prevailing Asian type NT29 with a few isolates of European type 20047 and *Borrelia afzelii* close toVS461 reference strain in ixodid ticks collected from all the examined regions besides the Sakhalin Island with the *B. garinii* only. One should note that currently according to BLAST homology search among GenBank deposited nucleotide sequences *Borrelia* DNA genetic variability in *I. persulcatus* ticks remains low: 1) for 16S rRNA gene - 0% for *B. afzelii*, 0–1% for *B. garinii*, 0–2% for *B. miyamotoi*; 2) for 5S−23S ITS - 0–2% for *B. garinii* and 1–2% for *B. afzelii*; 3) for glpQ – 0–4% for *B. miyamotoi*; 4) for p66 – 0–5% for *B. miyamotoi.* One should note that variability of *B. miyamotoi* glpQ and p66 gene fragments slightly exceeded nucleotide change levels of 16S rRNA gene and 5S−23S ITS. The remarkable genetic stability of *Borrelia* might suggest epigenetic regulation for adaptation to evolutionary divergent invertebrate vectors and vertebrate.

Molecular typing of *Borrelia* on the base of a single fragment of coding region or intergenic spacer as well as numerous attempts to use random set of several genetic loci were not always successful (Baranton and Postic, 2006). It may be caused by orthologous and paralogous genes localized on numerous plasmids with varying copy numbers in wild populations and possible loss during laboratory passaging. Therefore, description of natural diversity of *Borrelia* by analysis of laboratory strains or single locus (Sato et al., 1996) and especially fragment of the plasmid gene encoding outer surface protein A (Mediannikov et al., 2005) can lead to misrepresentations and wrong interpretations. MLSA of wild isolates (without a laboratory passage) with similar topologies of phylogenetic trees with reasonable bootstrep support (Figs. 2 and 3) provided the convincing evidences of spatial homogeneity and temporal stability of species.

Differences exist between *Borrelia* isolates according to tick vector and geographic region, but little genetic difference has been found between isolates within a given geographic area or with the same tick vector association (Figs. 2 and 3) (Morozov and Morozova, 2012 and references therein). Despite detection of *Borrelia valaisiana* in China (Hao et al., 2011) they were not detected in the Far East of Russia (Fig. 2). Low-frequency *Borrelia* variants were not found in ixodid ticks surviving without immune system at ambient temperatures (Fig. 2, 3). Genetic similarity was observed for *Borrelia* isolates from invertebrate vectors and vertebrate reservoir hosts as well as from patients (Figs. 2 and 3). The available data support concept of multiple ecological niches (such as various vectors, reservoir hosts and biotops) with minimal genetic polymorphism. But further study of *Borrelia* adaptation to divergent arthropods and vertebrate hosts is required. Rich ixodofauna of the Far East of Russia along with multiple vertebrate reservoir hosts (Volkov, 2005) provide multiple ecological niches not only for tick-borne borrelia with high spacial and temporal genetic stability but even for relative stable wild populations of RNA-containing tick-borne encephalitis virus (Pukhovskaya et al., 2018).

However, recent study revealed that in Siberia main part of borrelioses was caused by *B. miyamotoi* but not *B. burgdorferi* s.l. as previously believed (Titkov et al., 2018). The recently described infection with *B. miyamotoi* that is transmitted by the same vector - *I. persulcatus* ticks and proceeds as fever with unspecific symptoms without erythema and relatively rare serious complications (Krause et al., 2015). Despite lower bacterial loads and decreased infection rate of ticks with *B. miyamotoi* compared to *B. burgdorferi* s.l. the epidemiological significance is emerging. High abundance of ixodid ticks in the Far East of Russia, high infection rate of different tick species with *Borrelia* spp. with enormous bacterial loads and absence of vaccines may be reasons of stable and high Lyme disease rate.

5. Conclusion

Comparative analysis of both infection rate and bacterial loads of four prevailing species of ixodid ticks with *Borrelia burgdorferi* s.l. revealed the leading role of *Ixodes persulcatus* in the Far East of Russia. Genetic homogeneity and temporal stability of *B. garinii*, *B. afzelii* and *B. miyamotoi* suggest multiple ecological niches as mechanism providing their stability.

Conflicts of interest

There are no conflicts to declare.

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