Original article

Multilocus sequence analysis of *Borrelia burgdorferi* s.l. in Russia

Tatyana A. Mukhacheva, Sergey Y. Kovalev*

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

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ABSTRACT

Borrelia burgdorferi sensu lato is a species complex that includes the causative agents of Lyme borreliosis (LB). Classification of the complex was greatly influenced by the method of multilocus sequence typing (MLST), proposed in 2008 and analyzing the spatial distribution of sequence types (STs). Despite the fact that Russia is the largest natural focus of LB to date, it is represented by only 3 strains of the 1323 strains deposited in the MLST database. In this paper, we identified STs for 24 *B. burgdorferi* s.l. strains isolated from ticks from almost all regions of Russia, 16 of which have not been described so far. It has been shown that the Russian isolates of *B. afzelii* are of Asian origin and are characterized by a lack of territorial mixing of STs. In contrast, *B. garinii* and *B. bavariensis* showed ST mixing between different localities. Comparison of MLST data with the results of sequence analysis of rrf–rrl intergenic spacer led to the conclusion that the previously described genomic groups of *B. garinii* correspond to the genospecies according to the new classification: 20047 corresponds to *B. garinii*, and NT29 corresponds to *B. bavariensis*. The genomic group, ChY13p, characterized by an unusual PCR-RFLP profile, belongs to the species *B. garinii* (prototype strain 20047). Thus, the use of a reliable method to study the phylogeny and evolution of *Borrelia* based on MLST, helps to clarify the existing classification. The standardized research procedure and database created could become the basis for a global scientific cooperation in LB research.

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Introduction

Borrelia burgdorferi sensu lato is the causative agent of Lyme borreliosis (LB), a multisystem zoonotic disease. LB is registered in 72 regions of Russia with an annual incidence of 6400–9900 cases (Yastrebov et al., 2012). To date, at least 18 species (15 confirmed and 3 proposed) of *B. burgdorferi* sensu lato are known (Margos et al., 2011). They are differentiated by molecular genetic techniques. In particular, 2 genospecies (*B. bavariensis*, *B. kurtenbachii*) were proposed on the basis of multilocus sequence typing (MLST) and ecological traits (Margos et al., 2009, 2010). This method comprises the analysis of the nucleotide sequences of the fragments of conserved housekeeping genes and is widely used in the study of the genetic structure of populations and evolutionary processes within many bacterial pathogens (Enright and Spratt, 1999; Sullivan et al., 2005; Margos et al., 2008). The basic unit of analysis is a sequence type (ST), a sequence of 8 gene fragments that differ from each other by at least one nucleotide. In addition, there is an international database (<http://borrelia.mlst.net/>) containing the sequences of STs as well as epidemiological data and software for analysis. This makes MLST the most promising and objective method for studying *Borrelia* populations. However, among 1323 *B. burgdorferi* s.l. strains, whose sequences are deposited in the

database, only 3 originated from Russia, but were deposited by Japanese researchers (Takano et al., 2011). Thus, despite the fact that the taiga zone of Russia, where Europe meets Asia, is the biggest natural focus of LB, this approach in molecular epidemiology of LB is still not used. However, the researchers emphasized the need to study Russian strains of *Borrelia* which is a key point in understanding the evolution and spread of *Borrelia* through Eurasia (Margos et al., 2011).

Historically, PCR-RFLP of the rrf (5S)–rrl (23S) intergenic spacer (Postic et al., 1994) was the first technique extensively used for the differential diagnosis of *Borrelia* genospecies circulating in Russia (*B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*). This approach allowed the differentiation of the above genospecies as well as 3 genomic groups within *B. garinii*, differing by the profiles of restriction fragments (names are given according to the prototype strain): 20047, NT29, and ChY13p (Postic et al., 1994; Li et al., 1998) and 2 groups within *B. afzelii*: VS461 and NT28 (Masuzawa et al., 1996). With the development of the MLST scheme, a long-needed clarification of the taxonomic status of these groups can be accomplished and may help unifying the scientific research carried out in different countries.

Materials and methods

Both *Borrelia* strain cultures and uncultured isolates from *Ixodes persulcatus* (Schulze, 1930) ticks were used in the study (Table 1). Eleven strains were obtained from ticks collected in the

* Corresponding author. Tel.: +7 343 2616826; fax: +7 343 3507401.

E-mail address: Sergey.Kovalev@usu.ru (S.Y. Kovalev).

Table 1
Information about the strains used for the analysis.

No.	Name	ST	Species	Origin ^a	Strain/isolate	Year of isolation
1	SPb8-08	438	<i>B. afzelii</i>	Saint-Petersburg, north-western Russia (1)	Isolate	2008
2	Krg1090-09	128	<i>B. bavariensis</i>	Kurgan, West Siberia (9)	Isolate	2009
3	Ekb166-10	441	<i>B. bavariensis</i>	Sysert', Yekaterinburg region, Middle Urals (8)	Isolate	2010
4	Ekb701-11G	431	<i>B. garinii</i>	Yekaterinburg, Middle Urals (7)	Strain	2011
5	Ekb704-11G	364	<i>B. garinii</i>	Yekaterinburg, Middle Urals (7)	Strain	2011
6	Ekb706-11G	431	<i>B. garinii</i>	Yekaterinburg, Middle Urals (7)	Strain	2011
7	Ekb709-11	431	<i>B. garinii</i>	Yekaterinburg, Middle Urals (7)	Isolate	2011
8	Ekb712-11G	154	<i>B. garinii</i>	Yekaterinburg, Middle Urals (7)	Strain	2011
9	Ekb767-11A	432	<i>B. afzelii</i>	Severouralsk, Yekaterinburg region, Middle Urals (6)	Strain	2011
10	Prm7564-11G	433	<i>B. bavariensis</i>	Cherdyn', Perm region, Middle Urals (4)	Strain	2011
11	Prm7565-11A	434	<i>B. afzelii</i>	Cherdyn', Perm region, Middle Urals (4)	Strain	2011
12	Prm7567-11A	434	<i>B. afzelii</i>	Cherdyn', Perm region, Middle Urals (4)	Strain	2011
13	Prm7569-11G	374	<i>B. bavariensis</i>	Cherdyn', Perm region, Middle Urals (4)	Strain	2011
14	Prm7570-11A	435	<i>B. afzelii</i>	Cherdyn', Perm region, Middle Urals (4)	Strain	2011
15	Alt730-11	439	<i>B. afzelii</i>	Altai region, South Siberia (10)	Isolate	2011
16	Krv750-11	436	<i>B. bavariensis</i>	Omutninsk, Kirov region, European part of Russia (3)	Isolate	2011
17	Alt756-11	436	<i>B. bavariensis</i>	Altai region, South Siberia (10)	Isolate	2011
18	Alt763-11	440	<i>B. bavariensis</i>	Altai region, South Siberia (10)	Isolate	2011
19	Vld836-11	437	<i>B. afzelii</i>	Vladivostok, Far East (11)	Isolate	2011
20	Arh948-12	443	<i>B. afzelii</i>	Kotlas, Arkhangelsk region, north-western Russia (2)	Strain	2012
21	Arh974-12	444	<i>B. afzelii</i>	Kotlas, Arkhangelsk region, north-western Russia (2)	Isolate	2012
22	Arh976-12	445	<i>B. bavariensis</i>	Kotlas, Arkhangelsk region, north-western Russia (2)	Isolate	2012
23	Arh997-12	446	<i>B. afzelii</i>	Kotlas, Arkhangelsk region, north-western Russia (2)	Isolate	2012
24	Prm7050-12	442	<i>B. afzelii</i>	Kungur, Perm region, Middle Urals (5)	Isolate	2012

ST, sequence types.

^a The numbers in parenthesis correspond to numbers of collection sites shown in Fig. 1.

surroundings of Yekaterinburg (Sverdlovsk region) and Cherdyn (Perm region), by culturing them in BSK-H medium. Additionally, sequences of 16 *Borrelia* isolates were obtained directly from ticks collected in St. Petersburg, Arkhangelsk, Kirov, Sverdlovsk, Altai, and Primorye regions (Fig. 1). Multilocus typing was performed according to the common MLST scheme (Margos et al., 2008) with minor modifications. Nucleotide sequences of 8 housekeeping gene fragments, total length 4785 bp, were identified. Sequences of rrf–rrl intergenic spacer were obtained for the same strains and isolates according to the standard procedure (Postic et al., 1994).

Nucleic acid was extracted using a Ribo-Sorb kit for RNA/DNA extraction (Interlabservis, Russia), and reverse transcription was performed using the Reverta-L kit (Interlabservis), both according to the manufacturer's instructions. Detection of *Borrelia* in ticks was performed by real-time PCR on an ABI PRISM 7500 (Applied Biosystems, USA) according to the method by Ornstein and Barbour (2006). The amplification reaction for subsequent sequencing was performed in a Veriti® 60-Well Thermal Cycler (Applied Biosystems). PCR products were separated by electrophoresis on 2% agarose gel, and determination of the sequences of fragments was performed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences obtained for 10 strains and 14 isolates were deposited in GenBank under the numbers JX971230–JX971421

(MLST data) and KC261424–KC261447 (rrf–rrl intergenic spacer). Sequences from the international databases GenBank and *Borrelia* MLST (borrelia.mlst.net/) were included in the analysis. Phylogenetic analysis was performed using the software, MEGA v.5.5 (Tamura et al., 2011).

Results

Of 27 *Borrelia*-positive samples selected for MLST, 3 samples showed the presence of mixed-base peaks [intra- ($n=2$) or inter-specific ($n=1$) mixed infections] and were excluded from the further analysis. Thus, we compared phylogenetic information present in genome fragments of 8 housekeeping genes (MLST) and the rrf–rrl intergenic spacer for 24 *Borrelia* strains and isolates from different regions of Russia, from St. Petersburg to Vladivostok (Fig. 1, Table 1, detailed information in Supplemental Table 1). Each of the obtained sequences belonged to one of 3 *Borrelia* genospecies, *B. afzelii* (11 strains), *B. garinii* (5), and *B. bavariensis* sp. nov. (8). Sixteen MLST STs obtained had not been present in the database *Borrelia* MLST and were newly described (STs 431–446). The sequences of 4 strains belonged to existing STs – ST128 (China, Japan, Mongolia), ST154 (China), ST364 (Japan), ST374 (Russia, Moscow region) (Fig. 2, Table 1). Genetic distances between the sequences obtained and those of type strains are given in Supplemental Table 2.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.ttbdis.2013.02.004.

Phylogenetic trees were constructed using rrf–rrl intergenic spacer sequences and concatenated sequences of housekeeping genes (Fig. 3). Comparative analysis indicates that the 2 different approaches result in similar topologies, but bootstrap values for the tree obtained with MLST data are greater, proving it to be more reliable (Fig. 3). Generally, the previously described genomic groups of *B. garinii* correspond to genospecies according to the new classification: 20047 indicates *B. garinii*, and NT29 clusters with *B. bavariensis* sp. nov. The most significant difference between the phylogenies was the position of the genomic group ChY13p, whose specific PCR-RFLP sequence was more similar to those of *B. afzelii* (Livanova et al., 2003). However, analysis of the housekeeping genes (MLST) proved

Fig. 1. Number of *B. burgdorferi* s.l. strains from different countries (shown by color, data are obtained from the MLST Database). Collection sites of samples obtained in the present study are indicated with circles and those of samples deposited in the database *Borrelia* MLST, with squares. Locations are numbered according to Table 1.

Fig. 2. Phylogenetic trees constructed on the basis of concatenated sequences of 8 gene fragments used in an MLST scheme (4785 bp). Sequence types from the international MLST database and those obtained in the present study were used for the construction. The trees were obtained using the Neighbor-joining algorithm implemented in MEGA5. STs, isolated in Asia, Europe, and Russia (mostly in the present study) are shown in different colors.

it to cluster with *B. garinii* 20047 (Fig. 3), but not to form a separate clade.

B. afzelii isolates belonged mainly to the group VS461, homogeneity within the group reaching 99.5% and 94.1% based on MLST and IGS data, respectively. One isolate (Prm7050-12) belonged to the NT28 group, which was also originally discriminated on the basis of PCR-RFLP of *rrf-rrl* intergenic spacer (Masuzawa et al., 1996). As no strain of *B. afzelii* NT28 group has been typed using MLST scheme so far, performing a comparative analysis was not feasible. Despite the specific sequence of *rrf-rrl* IGS of *B. afzelii* NT28 group, it can be concluded that the distance between VS461 and NT28 groups is minimal based on the analysis of conserved housekeeping genes.

All MLST profiles of *B. afzelii* from Russia were obtained for the first time and do not refer to a previously known ST. *B. afzelii* from all regions were most closely related to strains from China and Japan. *B. afzelii* from the European part of Russia and Siberia formed a separate branch in the phylogenetic tree, while the Far Eastern isolate joined the clade formed by Chinese and Japanese strains (Fig. 2A). Strains that are close to the European *B. afzelii* were not found.

B. bavariensis was isolated mainly in Asia, with the exception of a few strains from Europe, including the European part of Russia (Fig. 2B). In our study, *B. bavariensis* strains were isolated in all studied regions of the country without forming a separate group of strains within the genospecies. Fig. 2B shows that they joined different clades within *B. bavariensis* regardless of geographic origin. The situation is similar for the *B. garinii* strains. Despite the fact that the strains of this species were found only in the city limits of Yekaterinburg and isolated from ticks collected in the same area, some of them are phylogenetically very distinct from each other (Fig. 2C).

Discussion

In Russia, Lyme disease was serologically confirmed for the first time in 1985, and in 1991, it was included in the official list of infectious diseases. Since that time, many studies have aimed to elucidate the clinical variants of the disease, and the features of the pathogen. By serological and molecular genetic methods, it was shown that 2 *Borrelia* genospecies are clinically significant in Russia: *B. afzelii* and *B. garinii*, with the latter being the most heterogeneous group. Firstly, *B. garinii* was divided into 5 serotypes (3–7) (Wilske et al., 1993), and later into 3 genomic groups (20047, NT29, and ChY13p) based on the PCR-RFLP (Postic et al., 1994; Li et al., 1998). In 2009, after the development of an MLST scheme, *Borrelia* serotype 4 was proposed to be a separate genospecies, *B. bavariensis* (Margos et al., 2009), which is also pathogenic to humans and associated with neurological manifestations of borreliosis. Doubtless, due to the introduction of a new classification, the question about the correlation between genomic groups and *Borrelia* genospecies was raised. Thus, in Russian scientific literature concerning the subject, *B. bavariensis* does not appear, although the name of this species is widely used in foreign publications, and it is recorded in the NCBI Taxonomy database as a separate species. However, the question is still under discussion, and *B. bavariensis* is absent in the conventional nomenclature of microorganisms as being an accepted species (Nolte, 2012). The analysis of *Borrelia* STs from Asia allows us to conclude that the majority of them are phylogenetically closer to *B. bavariensis* than to *B. garinii* 20047. For example, Takano et al. (2011) clearly distinguished 2 types within *B. garinii*, which clustered phylogenetically as clade A and clade B, the latter group including the prototype strain *B. bavariensis* PBi. In 2012, Margos et al. (2012) finally suggested that the genospecies *B. bavariensis* not only included PBi-like strains, but also strains of the NT29 genomic

Fig. 3. Comparison of phylogenetic trees constructed on the basis of rrf-rrl intergenic spacer (A) and MLST data (B) by the method of Neighbor-joining. Five hundred bootstrap replicates, insertion/deletions were removed by pairwise comparison of sequences. Names of prototype strains (in the absence of ST for the strain ChY13p, ST154 for the strain N161, belonging to the same group, was taken in the analysis) are given in bold. *B. burgdorferi* s.s. B31 was used as an outgroup.

group. Moreover, the formation of 2 subgroups within *B. bavariensis* appears to be a result of the adaptation to different vectors; i.e. PBI-like strains, which are found only in Europe, seem to be transmitted by *Ixodes ricinus* (L., 1758) while *Borrelia* group NT29 is common in Asia and is transmitted mainly by *I. persulcatus* (Postic et al., 1997). The fact of a greater genetic diversity of Asian *B. bavariensis*, probably indicates an Asian origin of this species as recently proposed (Scholz et al., 2012). It was hypothesized that *B. bavariensis* was separated from a common ancestor with *B. garinii* by adaptation to a new host (rodents) and then adapted to *I. ricinus* in Europe (Margos et al., 2012).

The results obtained in the present study lend support to this hypothesis. Thus, the comparison of phylogenetic trees (Fig. 3) revealed that the genomic group NT29, formally differentiated by the characteristic restriction profile, corresponds to *B. bavariensis*. In this case, using PCR-RFLP (newly obtained and unregistered data), we can estimate the spatial distribution and prevalence of the 'new' genospecies, which is needed for phylogenetic and epidemiological studies. For example, the study of 227 *B. garinii*, isolated from 16 regions of Russia (Kaliningrad to Sakhalin) and analyzed by PCR-RFLP showed that 117 (51.6%) belonged to the group 20047 (*B. garinii*); 108 (47.5%) to a subgroup of NT29 (*B. bavariensis*); and 2 (0.9%) to a subgroup of ChY13p (Nefedova et al., 2010). So, *B. garinii* and *B. bavariensis* in Russia are found in almost equal proportions. The latter genospecies was found in Russia almost from the beginning of LB epidemiology research (Postic et al., 1997).

Whilst *B. garinii* and *B. bavariensis* are found in Russia in almost equal proportions, the genomic group ChY13p, by contrast, is characterized by a very low prevalence. Despite the fact that the sequences of these strains form a distinct characteristic IGS

PCR-RFLP profile, MLST data show that they obviously belong to *B. garinii* 20047 (Fig. 3). There is a similar situation in the group *B. afzelii* NT28, whose IGS sequences have features in common with both *B. afzelii* and *B. garinii* (Li et al., 1998). Despite the fact that the analysis of IGS sequences allows us to consider the groups *B. afzelii* NT28 and *B. garinii* ChY13p as intermediate forms between 2 genospecies (Fig. 3), the MLST data do not support this hypothesis. Thus, we can assume that the sequence variations in the intergenic spacer do not reflect, objectively, evolutionary processes in *Borrelia* genospecies and cannot be the only criterion for dividing them into genomic groups. The causes of the emergence and fixation of specific mutations in the IGS and the possibility of recombination require further research.

Interesting questions about the ecology and geographic distribution of *Borrelia* genospecies were raised. Among the 6 *B. garinii* serotypes only one (serotype 4 or *B. bavariensis*) is ecologically associated with rodents, while the others prefer birds as their main host. Thus, the preferential association with small mammals was shown for *B. afzelii* and *B. bavariensis* while birds seem to be the main reservoir host for *B. garinii* (Kurtenbach et al., 2002). Taking into account the much lower mobility of rodents, we can expect *B. afzelii* and *B. bavariensis* to have more distinct phylogeographic structures than *B. garinii* due to less active spatial mixing of strains. Our data generally support this hypothesis. In particular, it was shown that the *B. afzelii* populations showed pronounced structuring on the European scale and an almost complete absence of mixing of sequence types between different territories (Vollmer et al., 2011). The same was also observed for the Russian strains of *B. afzelii*, the Far Eastern strain Vld836-11 clustering with the Chinese and Japanese strains, and all other strains forming a separate

clade (as previously no sequence type of *B. afzelii* from Russia was obtained), though related to the Asian strain. Thus, we suggest the Asian rather than European origin of *B. afzelii* isolated in Russia.

The 2 other species studied, *B. bavariensis* and *B. garinii*, showed evidence of spatial mixing of STs between geographic regions, which is most characteristic for *B. garinii* (Fig. 2B and C). This fact was revealed earlier (Margos et al., 2011; Vollmer et al., 2011).

Despite the relatively small sampling of strains used in this study, we were able to reveal some phylogeographic features of *Borrelia* in Russia, which was previously terra incognita. In order to better understand the phylogeography, evolution, and distribution of *Borrelia*, more isolates, including those from Russia, have to be obtained. The use of a reliable method to study the phylogeny and evolution based on MLST, clarifies and complements the existing classification as well as the standardized study protocol, and web resources created could become a basis for a global collaboration of scientists in studying the epidemiology of borreliosis.

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