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Original article

Asian isolates of *Anaplasma phagocytophilum*: Multilocus sequence typing

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ABSTRACT

Anaplasma phagocytophilum is the bacterial agent of granulocytic anaplasmosis in humans and animals; it is widely distributed in Eurasia and North America and transmitted mainly by *Ixodes* ticks. Several approaches have been used to study genetic diversity in *A. phagocytophilum,* multilocus sequence typing (MLST) currently being the most reliable and comparable. The MLST method based on seven housekeeping loci, 2877 bp total length, has been used to create and maintain the MLST database available worldwide (https://pubmlst.org/aphagocytophilum/). Before this study, the database contained 150 sequence types (STs) and 418 isolates, 397 of them originating from Europe and 21 from the USA, with none from Asia. We typed 25 *A. phagocytophilum* isolated from *Ixodes* ticks collected in the Asian part of Russia and compared the results with the conventional 16S rRNA typing. Substantial variability in the primer binding sites was found, so we had to modify the original primers for six out of seven loci. None of the sequences obtained matched those from the database; 15 new STs and 39 new alleles were revealed. Russian isolates belonged to two clusters, cluster 1 (19 isolates) and 3 (6 isolates), in both of which they formed separate clades. For the first time, we found *A. phagocytophilum* isolates from *Ixodes persulcatus* and *I. pavlovskyi* to belong to cluster 3, previously containing only the strains from voles and shrews. Further research is needed to estimate the prevalence of two MLST clusters of *A. phagocytophilum* in ticks and vertebrate hosts in Asia.

1. Introduction

Anaplasma phagocytophilum is the bacterial agent causing human, canine, feline, equine granulocytic anaplasmosis and tick-borne fever in domestic ruminants; it is distributed in the USA, Europe and Asia (Silaghi et al., 2017). This pathogen is transmitted mainly by *Ixodes* ticks, namely *Ixodes scapularis* Say, 1821 and *Ixodes pacificus* Cooley & Kohls, 1943 in the USA, *Ixodes ricinus* L., 1758 in Europe, and *Ixodes persulcatus* Schulze, 1930 in Russia and Asia (Jin et al., 2012). Additionally, *A. phagocytophilum* has been detected in *Ixodes pavlovskyi* Pomerantsev, 1946 in Russia (Rar et al., 2017). The distribution range of *I. pavlovskyi* is disjoined into Western Siberian and Far Eastern parts, with this species being almost always sympatric with *I. persulcatus* (Ushakova et al., 1969). *A. phagocytophilum* epidemiological cycles are complex and involve different genetic variants circulating in a large range of hosts, including wild and domestic ruminants, rodents, dogs, horses, and some other species (Dugat et al., 2015).

Several approaches have been used to reveal genetic diversity of *A. phagocytophilum* isolated in different geographical regions or from different hosts: single loci analysis (16S rRNA, groESL, major surface proteins *msp2* and *msp4*, *ankA*) (Battilani et al., 2017; Rar and Golovljova, 2011), multiple-locus variable-number tandem repeat analysis, MLVA (Dugat et al., 2014a), multilocus sequence typing, MLST (Chastagner et al., 2014; Huhn et al., 2014), and whole-genome sequencing (Barbet et al., 2013; Dugat et al., 2014b). In the past, the 16S rRNA gene was used most often; the comparison of a variable fragment, 11 bps in length, revealed at least 15 genetic variants (Rar and Golovljova, 2011). Among them, two variants were described from mainland Russia, 2 (GenBank: AF093789) and 10 (DQ458805), and two from the Kaliningrad Region, 1 (U02521) and 3 (AY193887) (Katargina et al., 2012; Rar and Golovljova, 2011), with some minor variability observed in the rest of the gene (Rar et al., 2011). Further differentiation was difficult because the 16S rRNA gene is highly conserved with about 2% polymorphic sites (Huhn et al., 2014). Comparisons between single loci often showed contradictory results, which is why multi-locus approaches are preferable (Dugat et al., 2015).

To date, there are two MLST methods that were developed independently in 2014 (Chastagner et al., 2014; Huhn et al., 2014); both of them showed improved discriminatory power compared to single loci. The method proposed by Huhn et al. has been used for a greater number of samples isolated from more geographical regions; it consists of seven housekeeping loci, concatenated sequence 2877 bp in length. Importantly, the results obtained in different laboratories can be compared worldwide via the MLST database (https://pubmlst.org/

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aphagocytophilum/). Before this study, the database contained 150 sequence types (STs) and 418 isolates, 397 of them originating from Europe and 21 from the USA (November 2018). The loci contained from 10% to 20% polymorphic positions, thus allowing distinction of three major clusters. Cluster 2 consisted of isolates from deer, cluster 3 – from voles and shrews, and all other *A. phagocytophilum* strains from different hosts, including humans, belonged to the largest cluster 1 (Huhn et al., 2014). The limitations of this method are caused by the great genetic diversity of *A. phagocytophilum*. To amplify seven loci, it may require up to three combinations of primers for each round of nested PCR, the whole set including 63 primers. The authors mentioned that in 8 samples the DNA was depleted before completion, but the overall typeability was very high, 98% (Huhn et al., 2014). In the other study where this method was used, only 30 samples out of 56 (54%) were amplified across seven loci (Tveten, 2014). It could indicate different efficacies of this method depending on the origin of the strains.

The aim of this study was to reveal genetic diversity of *A. phagocytophilum* isolated in the Asian part of Russia using the MLST method proposed by Huhn et al., 2014, and to compare this method with the conventional 16S rRNA typing, previously used for Russian isolates.

2. Material and methods

2.1. Material collection and DNA extraction

DNA isolates of *A. phagocytophilum* were obtained directly from the ticks *I. persulcatus* and *I. pavlovskyi* collected from vegetation in several Asian regions of Russia in 2011–2018 (Fig. 1). Ticks were frozen in liquid nitrogen and homogenized manually in saline using sterile pestles (Silaghi et al., 2017). Nucleic acids were extracted using a commercial kit MAGNOsorb (InterLabService, Russia), with a Xiril robotic workstation (Xiril AG, Hombrechtikon, Switzerland). Reverse transcription reaction was performed using Reverta-L (InterLabService, Russia). In the Tomsk Region, a sympatric zone of *I. persulcatus* and *I. pavlovskyi,* tick species was determined as described previously (Kovalev et al., 2015).

2.2. A. phagocytophilum detection

A. phagocytophilum was detected according to Courtney et al. (2004), target gene – *msp2*, with minor modifications. Real-time PCR was performed on an ABI 7500 thermocycler (Applied Biosystems, USA). The relative abundance of *A. phagocytophilum* was estimated as a threshold cycle, Ct. Only the samples with $C_t < 31$ (empirically chosen threshold) were used for subsequent analysis because we could not amplify all loci for samples with lower DNA concentration, with neither nested nor one-round PCR.

2.3. PCR and sequencing

Sequences of a 554-bp fragment of 16S rRNA were obtained with the primers 16S8FE_mod and HGE2 (Table 1). PCR was performed with a reaction mixture 5X Mas^{CFE}TaqMIX – 2025 (DiaLat Ltd., Russia) on a Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) in total volume 12.5 mkl, including 1.5 mkl of DNA. The PCR conditions were as follows: 95 °C for 3 min; 45 cycles at 95 °C for 20 s, 64 °C for 20 s, and 72 °C for 40 s; 72 °C for 5 min. For the MLST, seven loci, *pheS, glyA. fumC, mdh, sucA, dnaN,* and *atpA,* were amplified with different combinations of the original primers (Huhn et al., 2014), and later with the final primers given in Table 1. The PCR conditions were as follows: 95 °C for 3 min; 50 cycles at 95 °C for 20 s, 54 °C or 60 °C (Table 1) for 20 s, and 72 °C for 40 s; 72 °C for 5 min. PCR products were separated by electrophoresis on 2% agarose gel, purified and sequenced bidirectionally on an ABI 3500 Genetic Analyzer using ABI PRISM BigDye™ Terminator Cycle 3.1 (Applied Biosystems, USA). The numbers of 16S rRNA variants were determined according to Rar and Golovljova (2011); allelic profiles and STs numbers were kindly assigned by Dr Friederike Loewenich, curator of the *Anaplasma phagocytophilum* MLST database.

2.4. Phylogenetic analysis

The sequences obtained were edited in SeqScape 3 (Applied

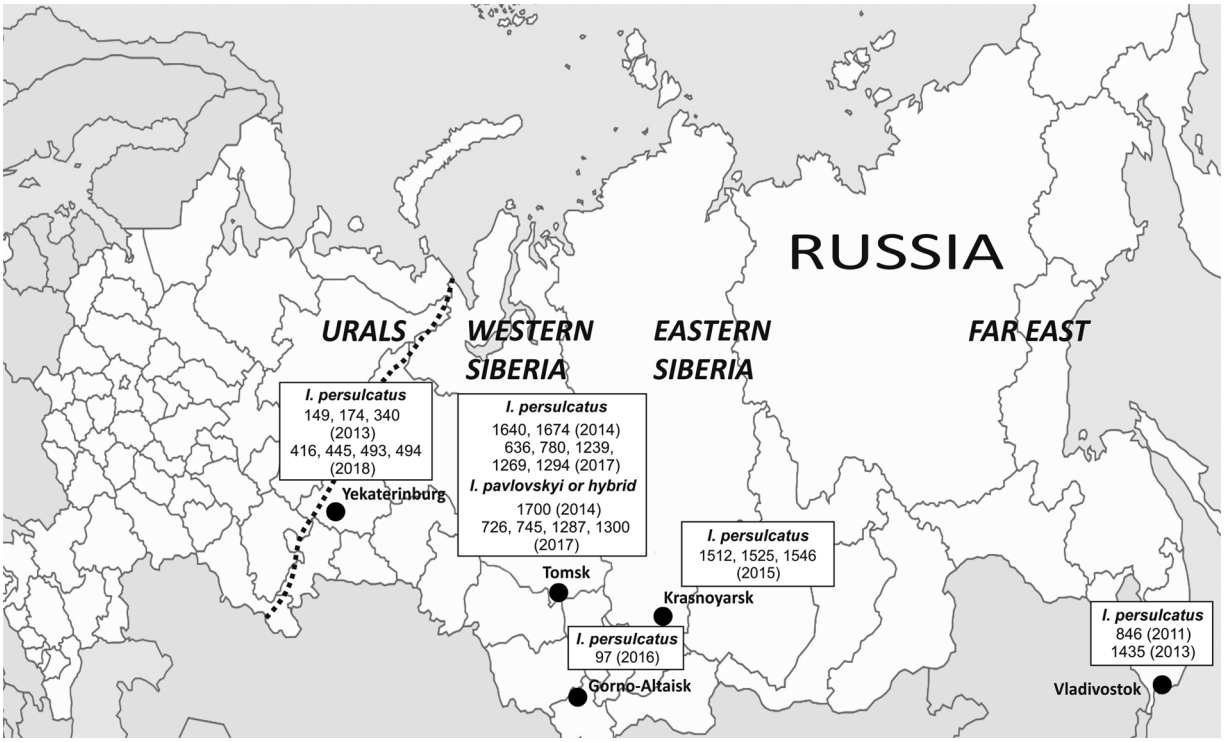

Fig. 1. *A. phagocytophilum* isolates obtained in this study and their geographical origin. Dotted line indicates the approximate border between European and Asian parts of Russia.

Table 1
Primer sequences, annealing temperatures (Ta), and PCR product lengths.

Locus	Primer name	Sequence	Ta	PCR product length	Reference
16S rRNA	16S8FE_mod	GAGAGTTTGATCCTGGCTCAG	64 °C	554 bp	Schouls et al., 1999 with modifications
	HGE2	CTTACCGAACCGCCTACATG			Rar et al., 2010
pheS	pheS_f	GACGCTTTAAA**Y**ACACCTGT**R**CATCA	54 °C	498 bp	This study
	pheS_r	CTCAACACCCATTCCGAAAGCAAA			
glyA	glyA_f	TAAGGTCGGC**R**GTATTTCCTGG	54 °C	492 bp	This study
	glyA_r	CCCTAACAAGAG**W**AGCAAC**Y**TCTTC			
fumC	fumC_f	CTTTACCAGCTTGCACAAGG	54 °C	554 bp	This study
	fumC_r	AGCGATGC**M**CC**Y**AGCAAAG			
mdh	mdh_f	AGGAG**Y**TGGTATTCGT**W**GATGT	60 °C	462 bp	This study
	mdh_r	GCAACATAAG**R**TCACC**R**TGC			
sucA	sucA_3fa	AGACGTTGTGATAGATGTGG	60 °C	560 bp	Huhn et al., 2014
	sucA_4 ra	CATGGCGGTGCGAGAAAGT			
dnaN	dnaN_f	GTTAGGTTCAAACTTAATGATGGCA	54 °C	559 bp	This study
	dnaN_r	GGTC**Y**ACTGCAC**Y**CTGCAA			
atpA	atpA_f	ATTGCTGTAGT**Y**TACGGGTTGC	60 °C	530 bp	This study
	atpA_r	GAAGAATTCTTCTGCCCTATCGC			

Biosystems, USA), aligned and analysed using MEGA 6 (Tamura et al., 2013). Intra- and inter- group distances were calculated using the Maximum Composite Likelihood model. The concatenated sequences representing each ST were downloaded from the *Anaplasma phagocytophilum* MLST database and used for phylogenetic tree reconstruction. The tree was obtained using the Neighbor-joining algorithm with the evolutionary distances computed using the Maximum Composite Likelihood method. The analysis involved 175 nucleotide sequences, each 2877 bp in length.

3. Results

3.1. MLST optimization

Multilocus sequence typing of Russian isolates was firstly performed according to the original MLST method (Huhn et al., 2014) using nested PCR and combinations of primers proposed by the authors. Unfortunately, we could not always amplify all the loci even if we selected the samples with a greater relative concentration of *A. phagocytophilum* DNA ($C_t < 31$), nor could we use all the primer combinations because the samples were depleted. To overcome this problem, we amplified the longer PCR products for several samples. Additionally, we made a BLAST search of the NCBI Whole-Genome Shotgun (WGS) database that revealed from 24 to 26 contigs of *A. phagocytophilum* genome containing one of seven loci; we aligned them together with our sequences. The comparison between sequences of primers and our isolates revealed variability up to 6 nucleotide substitutions per one primer (Table 2). Several primers (pheS 3f b, pheS 4 r, glyA 3f b, fumC 3f a, sucA 3f a, sucA 4 r a, dnaN 3f, dnaN 3f b, and dnaN 4 r) matched all our sequences, but only one pair, sucA 3f a and sucA 4 r a, contained no substitutions compared to WGS sequences as well. For six out of seven loci, we designed twelve degenerated primers based on our sequences (Table 1) and used them in one-round PCR.

In total, nucleotide sequences of seven loci, total length 2877 bp, were identified for 25 isolates out of 47 selected isolates (53% typeability overall). 14 samples were not amplified across all loci, and 8 samples showed double peaks in chromatograms in one or more loci indicating multiple strains; all of these were excluded. The sequences were submitted to the *Anaplasma phagocytophilum* MLST database and GenBank (*pheS* MK251251 - MK251275, *glyA* MK251276 - MK251300, *fumC* MK251301 - MK251325, *mdh* MK251326 - MK251350, *sucA* MK251351 - MK251375, *dnaN* MK251376 - MK251400, *atpA* MK251401 - MK251425).

Table 2
Variable positions within the original primers (Huhn et al., 2014) revealed by their comparison with the sequences of Russian isolates (highlighted in bold italics) and those from the WGS database (bold underlined).

Gene	Name	PCR round	Direction	Sequence (5' – 3')
pheS	pheS 2r	First	Reverse	TAA CCA GTC AAT GCG ***ACT*** AC
	pheS 3f	Second	Forward	***C***AC ACC TGT ***A***CA TCA TCC ***A***GC ***C***
	pheS 3f b	Second	Forward	GGT TTG CTA CTG TTC GTG G
	pheS 4 r	Second	Reverse	CAC CCA TTC CGA AAG CAA A**A**C C
	pheS 4 r a	Second	Reverse	CTG AG***G*** TCT CC***A*** AT***G*** TC***G*** T
glyA	glyA 3f	Second	Forward	TGT AAT AGC ***G***GC ***G***AA AGC ***G***G
	glyA 3f b	Second	Forward	TGT AAT AGC AGC AAA AGC AG
	glyA 4 r	Second	Reverse	AGC AAC **C**TC TTC ***T***C***T*** AAC C***C***C ***C***
fumC	fumC 1f	First	Forward	CTT ACA AGA TGC ***T***AC TCC ***G***T
	fumC 1f a	First	Forward	AC***T*** GGC TC***C*** GGA AC***G*** CAG
	fumC 3f	Second	Forward	TGT CCG TTT GTG AC***A*** GCA GAG
	fumC 3f a	Second	Forward	GTG GTG AGA TAG GAA GTA AGT
	fumC 3f b	Second	Forward	AGA GGT TG**T** AGA GGG ***C***AG ***A***T
	fumC 4 r	Second	Reverse	TC***A*** **G**CG ATG C***A***C C**T**A GCA AAG
mdh	mdh 2r	First	Reverse	CCA CA***G*** CTT ATA AG***G*** TCT GA
	mdh 3f	Second	Forward	TGT G***CC*** ***A***CG **G**GG G***A***A ***G***TT ***A***TT G
	mdh 4 r	Second	Reverse	***A***GG CAA CAT AAG ***G***TC ACC ***G***TG C
	mdh 4 r a	Second	Reverse	***C***AC ATC ***C***TC A***G***A ACT CA***G*** AC
sucA	sucA 3f	Second	Forward	ATG AGC CTA TGT TTA C***G***C AGC
	sucA 3f a	Second	Forward	AGA CGT TGT GAT AGA TGT GG
	sucA 4 r	Second	Reverse	TCT TCA CCA GAT A***GA*** CG***C*** ACC C
	sucA 4 r a	Second	Reverse	CAT GGC GGT GCG AGA AAG T
dnaN	dnaN 2r	First	Reverse	C***A***A TTG A***T***A C***A***C T**A**T T**AC** C***G***C A
	dnaN 3f	Second	Forward	TAG TGG CGT GTG GAA ATG CGA G
	dnaN 3f a	Second	Forward	TAG TGG CGT GTG GAA ATG CG***C*** G
	dnaN 3f b	Second	Forward	AGC GGA CGT TAG GTT CAA AC
	dnaN 4 r	Second	Reverse	GTC TGG AAA CGT ACC **G**TC CAA **C**
	dnaN 4 r a	Second	Reverse	ACT AC***T*** GA***T*** ACA CGG TC***T*** AC
atpA	atpA 3f	Second	Forward	ATG GAG CGG GAT TTG G***A***G AGA C
	atpA 4 r	Second	Reverse	TTA TCC TTC TCA CT***TGCC*** GC**A** C
	atpA 4 r a	Second	Reverse	A**G**T C***T***G AC***G*** CAC C***A***G TAG C

3.2. MLST analysis and 16S rRNA typing

None of the sequences obtained matched those from the database; 15 new STs (ST279-ST293) and 39 new alleles were revealed (Table 3).

Table 3
16S rRNA variants, allelic profiles, and sequence-types of *A. phagocytophilum* isolates obtained in this study.

Isolate	16S rRNA variant	MLST								
		pheS	*glyA*	*fumC*	*mdh*	*sucA*	*dnaN*	*atpA*	ST	Cluster
1674-2014	**10**	138	97	101	64	130	90	66	**279**	1
780-2017	**10**	138	97	101	64	130	90	66	**279**	1
1239-2017	**10**	138	97	101	64	130	90	66	**279**	1
1269-2017	**10**	138	97	101	64	130	90	66	**279**	1
1640-2014	**10**	139	98	86	63	130	90	67	**280**	1
1700-2014	**10**	139	98	86	63	130	90	67	**280**	1
636-2017	**10**	139	98	86	63	130	90	67	**280**	1
745-2017	**10**	139	98	86	63	130	90	67	**280**	1
1287-2017	**10**	139	98	86	63	130	90	67	**280**	1
1294-2017	**10**	139	98	86	63	130	90	67	**280**	1
1300-2017	**10**	139	97	85	60	130	90	66	**281**	1
340-2013	**10**	139	97	86	63	130	90	66	**282**	1
1435-2013	**10**	148	116	86	64	130	90	66	**283**	1
149-2013	**10**	140	97	85	60	130	90	66	**284**	1
1512-2015	**10**	141	97	86	64	130	90	67	**285**	1
1546-2015	**10**	141	97	86	64	130	90	67	**285**	1
1525-2015	**2**	142	114	102	65	131	101	78	**286**	3
174-2013	**10**	143	97	85	64	130	90	67	**287**	1
416-2018	**2**	144	115	102	65	131	101	77	**288**	3
445-2018	**2**	145	115	102	66	133	101	77	**289**	3
493-2018	**2**	145	114	102	65	131	101	77	**290**	3
494-2018	**2**	145	114	102	65	131	101	77	**290**	3
726-2017	**2**	146	114	102	65	131	101	78	**291**	3
846-2011	**10**	147	97	86	67	132	90	79	**292**	1
97-2016	**10**	149	113	86	63	130	90	66	**293**	1

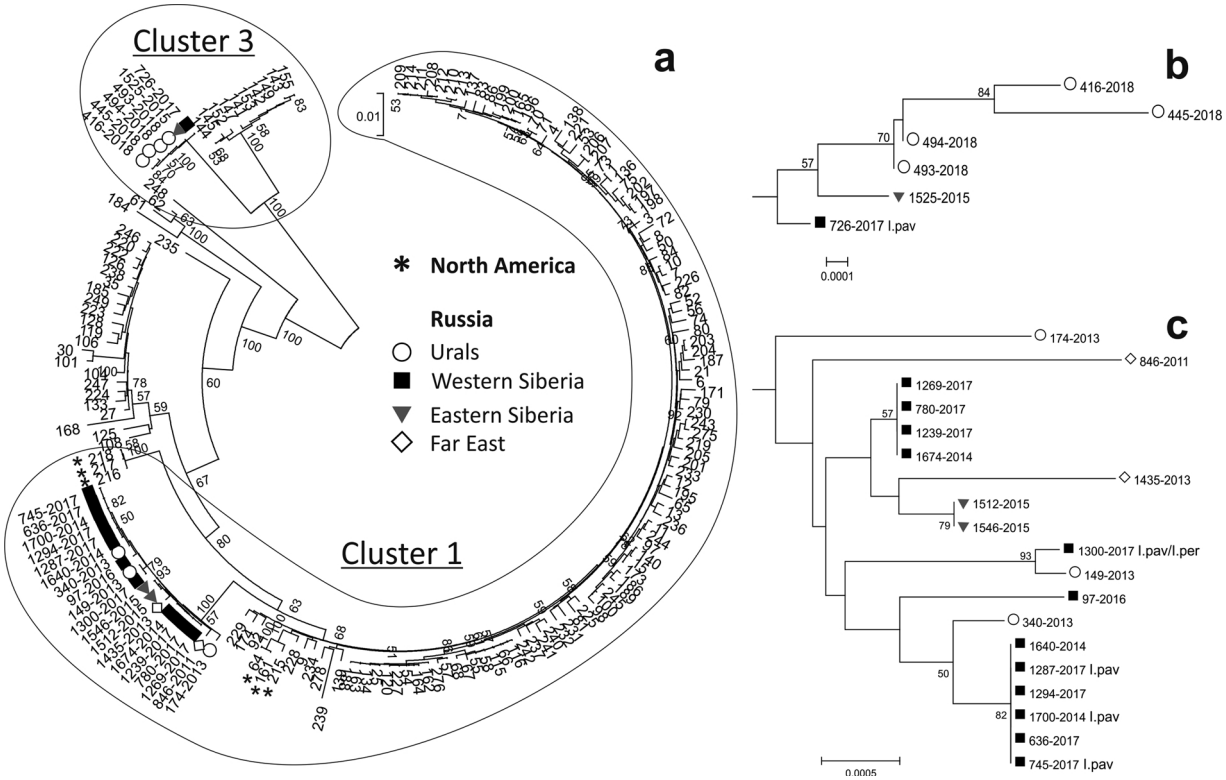

Fig. 2. a Phylogenetic tree based on the concatenated sequences of 7 loci (2877 bp). Downloaded STs from the MLST database and those obtained in the present study were used for tree construction. **b** The clade formed by Russian isolates within cluster 3. **c** The clade formed by Russian isolates within cluster 1. The tree was obtained using the Neighbor-joining algorithm, with 500 bootstrap replicates (bootstrap values below 50 are hidden). Russian isolates from different geographical regions are marked by different symbols; North American – by an asterisk.

The percentage of polymorphic sites depended on the locus and varied from 9.3% (*mdh*) to 13.9% (*pheS*), with an average of 11.1% for the concatenated sequences. The phylogenetic tree based on the concatenated sequences obtained both in this study and from the database revealed that the Russian isolates belong to two clusters, 1 and 3; in both they form separate clades (Fig. 2a). In cluster 1, the estimated mean distance between the Russian and all the other isolates was 0.02 which is significantly greater than the mean distances within any of

these groups (0.002 and 0.007, respectively). In cluster 3, the difference was even more dramatic – 0.04 compared to 0.001 and 0.002 within the groups.

16S rRNA typing revealed two variants, 2 (6 isolates) and 10 (19) (Table 3). All the isolates with 16S rRNA variant 2 belonged to MLST cluster 3, and those with variant 10 – to cluster 1 (Table 3).

4. Discussion

The MLST method proposed by Huhn et al. (2014) proved to be highly discriminatory, comparable and reliable; it was tested on a great number of *A. phagocytophilum* isolates from different sources. Nevertheless, when we applied it to the samples isolated from ticks in Asian regions of Russia, we were confronted with two difficulties. Firstly, a sophisticated scheme of several nested PCRs for each of seven loci is time-consuming and requires a large amount of the sample, which is not always available. Secondly, the primers proposed by the authors were tested mostly on European samples and were not always suitable to amplify DNA fragments of the Asian isolates.

When possible, we amplified and sequenced the fragments longer than required for MLST analysis and detected nucleotide substitutions in the primer binding sites, at least for the inner primers (Table 2). Since the outer primers may also contain nucleotide substitutions and work inefficiently, we designed degenerated primers that, in theory, could amplify all the known samples thus making the method simpler to use and requiring smaller amounts of samples. Also, we included the whole-genome sequences that were not available when the method was proposed. Our modifications have their limitations: (i) we did not test our primers on the isolates belonging to cluster 2 circulating in deer; (ii) one-round PCR may be sensitive to the DNA concentration, so we needed to select samples using real-time PCR. We did not calculate and compare the typeability with the original and our primers since the amounts of samples available were not sufficient to type them with both sets of primers. The overall typeability was 53% which means the method needs further improvement. The low percentage of typed samples is also related to the presence of multiple strains in one sample (17% of the samples); this problem has been mentioned previously, especially for isolates from ticks (Huhn et al., 2014).

MLST proposes two types of analysis, allele-based and sequence-based. The former is based on allelic profiles, not sequences themselves, and groups STs into clonal complexes; the latter assumes conventional phylogenetic trees and allows estimation of evolutionary relationships between isolates. In this work, we did not apply allele-based analysis since all the sequences obtained were new, and no connection with the allelic profiles from the database could be found. The sequence-based analysis showed Russian isolates from ticks to belong to two clusters out of three previously described, 1 and 3 (Fig. 2a). We did not find any samples of cluster 2 containing isolates from roe deer, red deer, and ticks.

19 out of 25 Russian isolates belonged to the most prominent cluster 1 formed by samples from different sources, including humans, hedgehogs, domestic, farm and large wild animals, and ticks. Since all the isolates from the database are of European or North American origin, it is not surprising that Asian samples formed a separate clade (Fig. 2c). The small number of samples analysed (19) did not allow reliable estimation of geographical structure; the samples isolated in a particular geographical region were genetically heterogeneous. No clear pattern was observed; at the same time, we found no samples originating from different regions to have the same sequence-type (Fig. 2c). Four STs were detected in the Tomsk Region but three isolates from *I. pavlovskyi* belonged to only one of them, ST280. It is not clear whether it is an effect of small sample size or whether *I. pavlovskyi* ticks could transmit particular genetic variants of *A. phagocytophilum*.

Six Russian isolates belonged to cluster 3, previously containing only the strains from voles and shrews (Huhn et al., 2014). As has been shown, these strains are predominately transmitted by the nest-living tick, *Ixodes trianguliceps* Birula, 1895, resulting in a distinct zoonotic cycle, at least in Europe (Bown at al., 2009; Blaňarová et al., 2014). This is the first time isolates from ticks have been found to belong to this cluster, probably indicating that *Ixodes* ticks participate in the circulation of this variant in Asia. The mean genetic distance between Russian and European isolates in cluster 3 was greater than in cluster 1 (0.04 vs 0.02); moreover, some geographical clustering within Russian isolates was observed. Generally, MLST has been shown to differentiate between European and American strains; also, some geographical pattern has been observed for *A. phagocytophilum* from ticks in Europe (Tveten, 2014). Despite the number of samples in cluster 3 being only six (or due to it), four isolates from the Urals clustered together; two isolates from Western and Eastern Siberia formed separate branches, too (Fig. 2b). We can only hypothesize that such clustering may be due to a narrower range of vertebrate hosts and so fewer possibilities for spreading of genetic variants. Previously, cluster 3 has been shown to have the lowest amount of genetic diversity in Europe, probably resulted from one or more bottlenecks and consequent genetic drift (Aardema and von Loewenich, 2015). More samples are needed to properly assess genetic diversity of *A. phagocytophilum* within cluster 3 in Asia.

Comparison between MLST and 16S rRNA typing revealed that all the samples with 16S rRNA variant 2 belong to cluster 3 while those with variant 10 belong to cluster 1. The 16S rRNA variant 2 has been previously detected in a wide range of hosts in Russia, Europe, USA, South Korea (Chae et al., 2000; Oh et al., 2009; Portillo et al., 2011; Rar et al., 2011); variant 10 has been reported in Russia, Europe, and Asia (Lee et al., 2016; Portillo et al., 2011; Rar et al., 2011; Zhan et al., 2008). Although we observed a correspondence between 16S rRNA variants and MLST clusters, it does not seem to be true for all the samples (Huhn et al., 2014), which can be explained a much lower variability of 16S rRNA.

5. Conclusions

In this study, we first applied a modified MLST method for Asian isolates of *A. phagocytophilum* and analysed their genetic diversity in comparison with the isolates from Europe and North America. Further research is needed to estimate the prevalence of two MLST clusters in ticks and vertebrate hosts in Asia and to reveal the phylogeographical structure of *A. phagocytophilum* using the MLST method. We believe that using such a reliable and comparable method will facilitate global collaboration of scientists in studying the phylogeny and evolution of *A. phagocytophilum*.

Declarations of interest

The authors declare no conflict of interest.

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