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Investigation genetic diversity of *Ixodes ricinus* based on four microsatellite loci

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Ixodes ricinus belongs to *Ixodes ricinus* complex composed of 14 species of ticks distributed worldwide and the most widely spread tick species in Europe *I. ricinus* is a major ixodid tick, involved in the transmission of a number of diseases to animals and humans in Eurasia. Genetic diversity of these tick populations may have implications on disease transmission. In order to investigate genetic diversity and to characterize the genetic structure of *I. ricinus* populations in the Baltic countries microsatellite markers described in literature were used. A total of 180 ticks (170 *I. ricinus* and 10 *I. persulcatus*) collected from 18 locations in Lithuania, Latvia and Estonia were examined based on four polymorphic microsatellite loci. Genetic diversity of six *I. ricinus* populations from Lithuania (n = 60), Latvia (n = 60), and Estonia (n = 60) were compared. The number of observed alleles between loci ranged from 5 to 11. Observed heterozygosities were lower than expected at most loci. Mean estimates of expected heterozigosities (He) over loci and populations varied from 0.621 to 0.800, and observed heterozygosity (Ho) from 0.304 to 0.634. *F* statistics were calculated to analyze the differences between observed and expected heterozygosities, and to obtain genetic distances between populations. Two loci deviated significantly from Hardy–Weinberg equilibrium. However, the highest observed heterozygosity (Ho) was found in coastal locations and lowest – in continental parts, which shows the importance of birds as hosts to tick population structure.

Key words: *I. ricinus*, Baltic countries, Lithuania, Latvia, Estonia

INTRODUCTION

Ixodes ricinus (Arthropoda, Acari, Ixodidae) is the main vector species in Europe, transmitting numerous human and livestock diseases including Lyme disease, tick-borne encephalitis, anaplasmosis and babesiosis (Stanek, 2009). Ticks of the genus *Ixodes* have a significant impact on the public health and rural economy in many parts of

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the world. At least 15 species of closely related ixodid ticks distributed throughout the world are united in the *Ixodes persulcatus* group or *Ixodes ricinus* complex (Fukunaga et al., 2000). *I. ricinus* distribute from Western Europe to Northern Africa, while *I. persulcatus* spread from Eastern Europe to Japan. *I. ricinus* can be found all over the Baltic countries: in Lithuania *I. ricinus* is widespread; in Latvia *I. ricinus* is spread in the western and central parts, and rarely occur in small numbers in the eastern part, while *I. persulcatus* dominate only in the eastern part of the country (Bormane et al., 2004). *I. ricinus* and *I. persulcatus* are distributed with different prevalence in different parts of Estonia (Jõgiste et al., 2004). The distribution of *I. persulcatus* in Eastern and Southern Estonia was confirmed by Geller et al. (2012), as well as mixed distribution of both species in the sympatric zone. Distribution range of *I. persulcatus* in the west is overlapped by a typically European distribution range of *I. ricinus*.

Efforts to understand the ecology of this tick in relation to disease transmission are complicated under natural conditions (Noel et al., 2012). This is particularly true for estimating patterns of dispersal and host use, two essential factors for understanding disease risk (McCoy, 2008). Indirect methods that employ genetic markers are currently one of the best options to overcome the inherent difficulty in studying parasitic organisms, but require certain assumptions in order to make robust inferences (De Meeûs et al., 2007).

During two last decades the application of molecular markers in the study of medically and veterinary important Ixodidae ticks has provided new essential information about their taxonomic relationships and population structures. Ticks have been studied at individual, population and species level using allozymes (Delaye et al., 1997; Kain et al., 1997; Healy et al., 2004; Radulović et al., 2006, 2010, 2012), RAPD (Paulauskas et al., 2006), mitochondrial and nuclear genome sequence analysis (Caporale et al., 1995; Rich et al., 1995; Norris et al., 1996; Delaye et al., 1998; Xu et al., 2003; De Meeûs et al., 2002, 2004a, 2004b; Røed et al., 2006; Casati et al., 2008; Hasle et al. 2008; Chitimia et al., 2010; Kempf et al., 2009, 2010, 2011; Noureddine et al., 2011).

16S rRNA and COI gene sequences are the most common genetic markers used to determine phylogenetic and taxonomy structure of tick populations. Caporale et al. (1995) used 16S rRNA gene sequences in order to analyze the ticks of *I. persulcatus* complex and found two distinct groups. Xu et al. (2003) analyzed 16S rRNA gene sequences in 11 species belonging to *I. persulcatus* complex and 16 other Ixodidea species. The analysis showed that *I. persulcatus* complex is paraphyletic (Xu et al., 2003).

Sequences of gene fragments of 28S (expansion segment D3) and mitochondrial 12S rRNA were analyzed in *I. persulcatus* ticks collected in various regions of Russia in 2007–2011. The sequences of the 28S rRNA D3 segment were identical for all ticks within the studied area. The analysis of the sequences of the mitochondrial 12S rRNA fragment revealed 4 haplotypes with one occurring at a frequency of 0.96. The authors conclude that among all the ticks of the genus Ixodes studied to date, *I. persulcatus* shows the lowest genetic variability, which is probably associated with the strong influence of glaciation on its habitat (Kovalev, Mukhacheva, 2012).

Control region and 12S rRNA sequences were used to determine phylogeographical structure of *I. scapularis* tick populiations in the USA. Both studies confirmed that *I. scapularis* has two distinct populiations, one of which is located in north-east and another – in south-east (Rich et al., 1995; Norris et al., 1996).

After sequencing COI and ND5 genes of *D. marginatus, Haemaphysalis punctata, I. ricinus, Argas persicus* and *Haemaphysalis longicornis* ticks in Romania it was shown that interspecific variation (0.1–1.9%) was lower than that between species (COI genes 15.9–27.6%, ND5 – 20.3–42.4%) and concluded that these markers can be used for identification and characterization of species (Chitimia et al., 2010).

Casati et al. (2008) examined interspecific genetic variation among 26 *I. ricinus* ticks from different European countries (Switzerland, Italy, Austria, Denmark, Sweden and Finland) using 5 mitochondrial sequences – control region, 12S rDNA, *cytb*, COI and COII. The data showed a low genetic variability (1.6–5%). The authors concluded that *I. ricinus* ticks do not have phylogeographical structure in Europe (Casati et al., 2008).

Microsatellite markers have been previously described and applied to populations of *I. ricinus* (Delaye et al., 1998; De Meeûs et al., 2002, 2004a, 2004b; Røed et al., 2006; Kempf et al., 2009, 2010, 2011).

The first isolation and characterization of six microsatellite markers in *I. ricinus* complex were done by Delaye et al. (1997). However, the use of these markers in population analysis appeared to have limitations due to the reported locus duplication and their unusual patterns of high heterozygote deficit ascribed to the presence of null alleles and/or short allele dominance (De Meeûs et al., 2002, 2004).

Røed et al. (2006) reported the development and characterization of 17 new microsatellites in *I. ricinus*. These microsatellites significantly improve the available marker panel in *I. ricinus*, although the frequent non-Mendelian inheritances, as also reported for others by De Meeûs et al. (2002, 2004), call for prudence in interpretation when using such markers for analysis of genetic structure and dispersal patterns in these species (Røed et al., 2006).

Analysis of the genetic structure of *I. ricinus* ticks using 5 microsatellite markers showed differentiation between ticks collected in Switzerland and Tunisia, but there were no significant differentiation between ticks collected in different locations in Switzerland separated by Alps (Meeûs et al., 2002).

Analysing individual partners of *I. ricinus* microsatellite markers were used to determine multiple paternity (Hasle et al., 2008). After analysing copulating partners and offsprings it has been concluded that offsprings had different fathers.

Noel et al. (2012) isolated and characterized nine new polymorphic microsatellite markers for the tick *I. ricinus*. Nine new microsatellite loci were isolated from a microsatellite-enriched library according to Billotte et al. (1999) to improve the precision of population genetic estimates used to study the biological factors.

However, the studies of the diversity and genetic population structure of *I. ricinus* across Europe are still insufficient, the lack of genetic structure was reported by Noureddine et al. (2011) and Casati et al. (2008) who used mitochondrial and nuclear genome sequence analysis. The aim of this study was to investigate genetic diversity and to characterize the genetic structure of *I. ricinus* populations in the Baltic countries using microsatellite markers described in literature.

MATERIALS AND METHODS

Tick collection

A total of 1024 unfed Ixodes ticks were collected from the vegetation using standard "flagging" method in Lithuania, Latvia and Estonia during May of 2008, 2010, 2012. White cloth (1 m^2) was drawn over the vegetation. Ticks attached to the "flag" were picked with tweezers and placed into 1.5 ml tubes filled with 70% ethanol. Adults were classified as male or female and were identified as *I. ricinus* or *I. persulcatus* by their morphological characteristics (Filippova, 1977).

DNA isolation

DNA extraction was carried out by lysis of ticks in ammonium hydroxide (NH_4OH) (Stanczak, 1999). All ticks were analyzed individually. Tick lysates were either used directly for PCR or stored at –20 °C.

Amplification of microsatellite loci by PCR

In total 170 *I. ricinus* and 10 *I. persulcatus* ticks collected from 18 locations in the Baltic countries were investigated based on

Table 1. Locations of samples collected

No.	Locations	Sample, n	Latitude	Longitude
I	**Latvia**			
1.	Daugavpils	20	N55°54'9.39"	E26°23'7.87"
2.	Kraslava	5	N55°57'0.46"	E27°18'8.45"
3.	Vaidava	5	N57°25'8.45"	E25°22'9.73"
4.	Cesis	5	N57°13'2.70"	E25°13'0.00"
5.	Salaspils	20	N56°58'0.79"	E24°21'2.70"
6.	Ezernieki	5	N56°10'2.67"	E27°39'6.46"
II	**Estonia**			
7.	Võhma	20	N59°36'1.17"	E25°34'5.97"
8.	Varstu	5	N57°37'4.87"	E26°38'1.63"
9.	Palastvere1	5	N58°74'462"	E 25°70'212"
10.	Retla	5	N58°45'15.52"	E 25°40'43.43"
11.	Viisireiu	5	N58°5'43.08"	E 24°50'9.48"
12.	Kalita1	20	N58°7'48.44"	E 24°56'0.50"
III	**Lithuania**			
13.	Labanoras park	5	N55°13'7.65"	E25°39'9.33"
14.	Molėtai	5	N55°14'1.03"	E25°27'4.4"
15.	Dūkštas	5	N55°33'9.33"	E26°21'1.03"
16.	Šakiai district	5	N55°92'272"	E23°16'4.82"
17.	Kaunas (Botanical Garden)	20	N54°52'27.96"	E23°54'29.92"
18.	Juodkrantė	20	N55°33'10"	E21°07'30"

polymorphic microsatellite loci (Table 1). Ticks from two sites in each country were used for genetic diversity analysis of population structure. We chose 6 microsatellite markers designed and characterized by Røed et al. (2006). Four of them (IRN4, IRN7, IRN8, and IRN28) provided sufficient amplification products and were used for further analysis (Table 2).

The PCR amplifications were carried out in a 25 µl final volume containing: 10 × PCR buffer (2.5 µl), 25 mM MgCl (1.5 µl), 4 mM dNTPs (0.2 µl), 0.5 µM of each primer (10 pmol/µl), 5 U

Table 2. Characterization of six microsatellites in tick, *Ixodes ricinus.* Listed below are microsatellite locus designation, repeat motif, GenBank Accession no., primer sequences, PCR annealing temperatures (Ta) (according to Røed et al., 2006)

Locus	Repeat motif	GenBank Accession	Primer Sequence (5'–3')	Ta (°C)
IRN-4	(CA)14	DQ482826	F:GCCATTTTATGTGCCGTTTT R:CTTTGAGTGCGTGCGTGT	51
IRN-7	(CA)13	DQ482827	F:CGGATGATCAATAGTCGATTCC R:CCTAGTCACAAACTCTACCAAGTTA	51
IRN-8	(CA)15	DQ482828	F:CGCTTCGAAGACGACTAAACA R:TGCGAACAATGACAAACAGA	51
IRN-28	(CA)23	DQ482835	F:AGCCACGCTAGTTCTGAGA R:CCTGTTGTGTTTTGTTGGTC	51
IRN-30	(GT)6(GC)4(GT)3(GT)6	DQ482836	F:GCAATTGCTATTCTTTGT R:AGTCTACTAAATCGTCACCA	45
IRN-37	(GT)19TT(GT)2CT(GT)2	DQ482841	F:CGGGGCGTTTTTCTTTATTCT R:GAAGCGTCAGACTCCGTAACAG	45

Taq DNA polymerase (0.1 µl) (Thermo Fisher Scientific, Lithuania), ddH_2O (18.7 µl) and 1 µl of a tick DNA sample. PCR amplifications were performed in 0.2 ml tubes using a 30-cycle programme. Thermocycling parameters after denaturation at 94 °C in 2 min, 95 °C for 30 sec, the annealing temperature (45–51 °C) for 30 sec and 72 °C for 1 min followed by 10 min at 72 °C (according to Roed et al., 2006).

Microsatellite analysis

First amplified DNA was separated by electrophoresis in 1.5% agarose gels with 0.5·Tris–Borate–EDTA (pH 8.2) as running buffer for 2.5 h at 115 V (Fig. 1). The DNA bands were stained with ethidium bromide and visualized by UV transillumination. DNA fragment sizes were assessed by comparison with GeneRulerTM 50 bp DNA Ladder (Thermo Fisher Scientific, Lithuania) (Rosenberg and Nordborg, 2002). Electrophoresis in agarose gel was used for interpretation of the patterns on the size and detection of the presence or absence of amplified DNA bands (Williams, 1990).

The second step in microsatellite analysis was the preparation of the polyacrylamide electrophoresis gels (Fig. 2). Both glass plates were washed with liquid soap and cleaned with absolute ethanol using a paper towel. After two min of drying one glass was cleaned with Silane solution (3 µl of γ-methacryloxypropyltrimethoxysilane (Bind Silane, M-6514, Sigma), 950 µl of absolute ethanol and 50 µl of 10% acidic acid). The other glass was cleaned with "TurtleWax" (window liquid, Rain Repellent). After two min of drying both glass plates were cleaned with a paper towel moistured with absolute ethanol to remove the excess and left to dry.

Acrylamide solution (40%) with 38 g of acrylamide and 2 g of N, N'-methylene bisacrylamide was prepared in 100 ml distilled water and stored at 4 °C.

APS (ammonium persulfate) was prepared: 5 g amonium persulfate was dissolved in distilled water to a final volume of 50 ml, and stored at –20 °C in the fridge.

Polyacrylamide gel (8%) was prepared by mixing 36.5 ml distilled water, 2.5 ml 10x TBE buffer, 10 ml of acrylamide solution, 0.5 ml of ammonium persulfate and 41.5 µl of TEMED (Sigma). Gel solution was applied to the assembled gel plates (1.5 mm thick) using S2 sequencing gel electrophoresis apparatus (Invitrogen).

The running conditions were 999 V, 37 mA, 80 W for 2–5 hrs, after a pre-run of the gels for 15 min. The electrophoresis running buffer (TBE 1x) contained 10 mM Trizma, 8.9 mM boric acid, 2 mM Na2 EDTA.

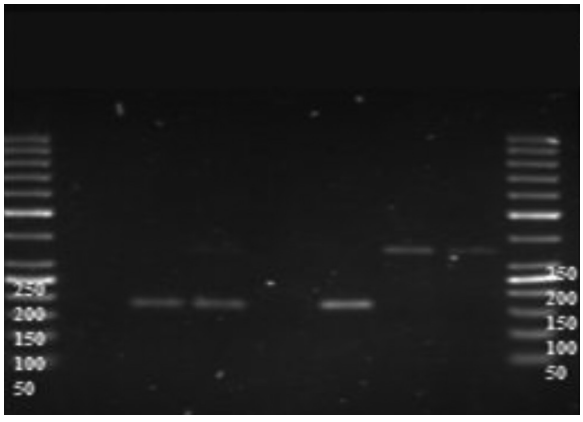

Fig. 1. Electrophoregram of agarose gel with IRN-7H and IRN7R marker

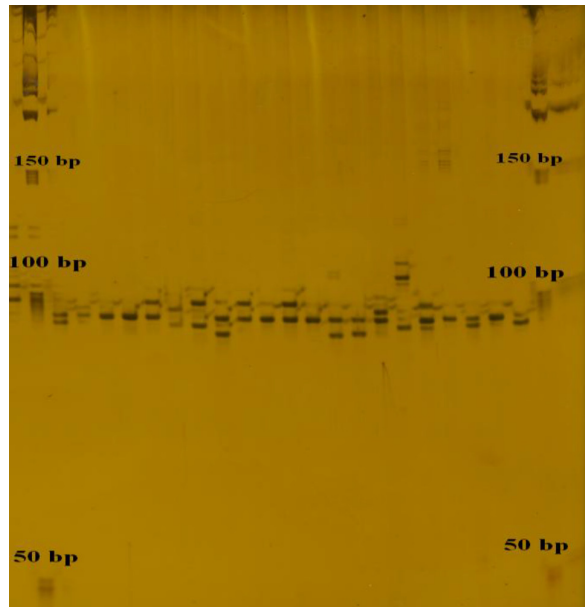

Fig. 2. Electrophoregram of polyacrylamide gel with IRN7F and IRN-7R marker

Table 3. Estimators of F-statistics at each locus of *I. ricinus* population

All Pops.	IRN8	IRN4	IRN28	IRN7	Mean
F_{IS}	0.549	0.334	0.468	0.200	0.387
F_{IT}	0.580	0.397	0.518	0.291	0.446
F_{ST}	0.068	0.095	0.094	0.115	0.093

Gel staining

After electrophoresis, gels were washed for 5 min in cold (10–12 °C) fixing solution (10% absolute ethanol, 0.5% acetic acid). Washed gels were soaked for 6–7 min at room temperature in a solution of 0.15% $AgNO_3$, 2 ml 37% HCOH. Gels were rinsed quickly (10–15 sec) once with distilled H_2O. Bands were developed by soaking the gel at room temperature in a developing solution (1.5% NaOH, 3 ml 37% HCOH) until the bands appeared with a sufficient intensity (3–5 min). When the desired intensity was achieved the development was stopped by saturating gel in a 2 000 ml stop solution (10% absolute ethanol, 0.5% acetic acid) for 2 min. Gels were dried at room temperature and DNA bands were viewed directly with the aid of a white light box, and then scanned. The length of microsatellite alleles was estimated according to 50 bp DNA ladder (Thermo Fisher Scientific, Lithuania).

Data analysis

The allele frequencies, mean effective number of alleles per locus (Ne), average number of alleles per locus (Na), Shannon index (I), population mean observed (Ho) and expected heterozygosities (He), estimation of Wright's fixation index (F_{IT}, F_{IS} and F_{ST}) (Weir and Cockerham, 1984), Nei's standard genetic distances among populations (Nei, 1978), molecular analysis of the genetic diversity (AMOVA, Excoffier et al., 1992), principal coordinates analysis (PCA) were calculated with the GenAlEx 6b4 (Peakall & Smouse, 2006).

RESULTS AND DISCUSSION

Microsatellite loci polimorphism of *I. ricinus* from 18 different locations in Lithuania, Latvia, and Estonia and *I. persulcatus* from 2 different locations in Latvia and Estonia were investigated.

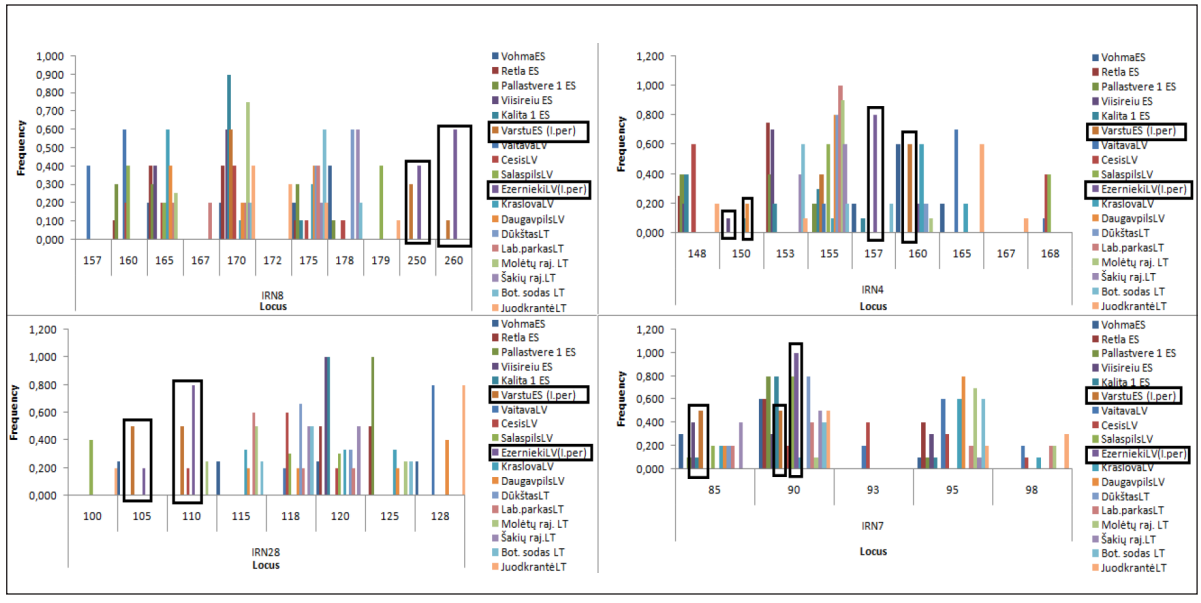

Fig. 3. Distribution of *I. ricinus* and *I. persulcatus* allele in each locus (outlined *I. persulcatus* alleles)

The microsatellite analysis showed that the number of alleles per locus in *I. persulcatus* was from 1 to 2, and in *I. ricinus* varied from 5 to 11. The highest number of alleles was observed in IRN8 locus (11 alleles), then in IRN4 (9 alleles), IRN28 (8 alleles), and the lowest number – in IRN7 locus (5 alleles). Allele sizes varied in different loci: IRN7 from 85 to 98 bp, IRN8 from 157 to 260 bp, IRN4 from148 to168 bp and IRN28 from 100 to 128 bp (Fig. 3).

Genetic diversity was compared in six *I. ricinus* tick populations from Lithuania (Kaunas (n = 20), Juodkrantė (n = 20)), Latvia (Salaspils (n = 20), Daugavpils (n = 20)) and Estonia (Kalita1 (n = 20), Vohma (n = 20)).

I. ricinus tick population differentiation examined by fixation indexes F_{IT}, F_{IS} and F_{ST} for each of the four analyzed loci are given in Table 3. Mean estimates of F-statistics obtained over loci (Weir, 1990) were: F (F_{IT}) = 0.446 ± 0.13, (F_{IS}) = 0.387 ± 0.13, and (F_{ST}) = 0.093 ± 0.039.

Mean estimates of expected heterozygosities (He) of all loci and populations varied from 0.621 to 0.800 and observed heterozygosity (Ho) from 0.304 to 0.634 (Table 4).

Private alleles were detected in Juodkrantė (LT), Kalita1(EE) and Salaspils (LV) populations – 0.250 and Vöhma (EE) – 0.750 (Table 4).

Effective number of alleles varied from 2.805 (Kalita1) to 5.081 (Vöhma). The average number of alleles varied from 5.000 (Kalita1) to 7.250 (Vöhma) among populations (Table 4).

The matrix of Nei's standard genetic distances (D_S) among *I. ricinus* tick populations is presented in Table 5. The highest genetic distance was between Kaunas (LT) and Kalita1 (EE) (0.713), the lowest genetic distance was between Kaunas (LT) and Daugavpils (LV) (0.117) populations.

The highest F_{ST} distances between *I. ricinus* tick populations ranged from 0.018 for Kaunas

Table 4. Mean values of allelic patterns and heterozygosity across six *I. ricinus* populations in Estonia, Latvia and Lithuania

Mean values	Kaunas (LT)	Juodkrantė (LT)	Vöhma (EE)	Kalita1 (EE)	Daugavpils (LV)	Salaspils (LV)
Na	5.750	6.500	7.250	5.000	6.000	6.250
Na Freq. >=5%	5.000	4.250	6.750	4.000	5.000	5.500
Ne	4.406	4.123	5.081	2.805	4.195	3.963
I	1.529	1,511	1.776	1.204	1.563	1.534
No. Private Alleles	0.000	0.250	0.750	0.250	0.000	0.250
No. LComm Alleles (<=25%)	0.000	0.000	0.000	0.000	0.000	0.000
No. LComm Alleles (<=50%)	2.250	2.000	1.750	1.000	1.250	1.500
He	0.750	0.734	0.800	0.621	0.755	0.738
Ho	0.304	0.559	0.485	0.393	0.303	0.634

Table 5. Nei's standard genetic distances between the analyzed *I. ricinus* tick populations

Kaunas (LT)	Juodkrantė (LT)	Vöhma (EE)	Kalita1 (EE)	Daugavpils (LV)	Salaspils (LV)	
0.000						Kaunas (LT)
0.518	0.000					Juodkrantė (LT)
0.436	0.417	0.000				Vöhma (EE)
0.713	0.343	0.373	0.000			Kalita1 (EE)
0.117	0.448	0.438	0.616	0.000		Daugavpils (LV)
0.394	0.300	0.330	0.253	0.409	0.000	Salaspils (LV)

Table 6. Estimates of pairwise F_{ST} distances between the analyzed *I. ricinus* tick populations

Kaunas (LT)	Juodkrantė (LT)	Võhma (EE)	Kalita1 (EE)	Daugavpils (LV)	Salaspils (LV)	
0.000						Kaunas (LT)
0.067	0.000					Juodkrantė (LT)
0.050	0.050	0.000				Võhma (EE)
0.107	0.065	0.064	0.000			Kalita1 (EE)
0.018	0.059	0.049	0.097	0.000		Daugavpils (LV)
0.053	0.043	0.040	0.052	0.053	0.000	Salaspils (LV)

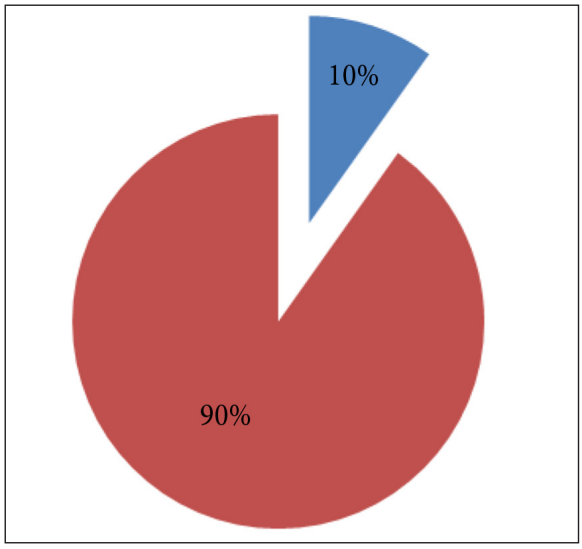

Fig. 4. Molecular variance of *I. ricinus* ticks within and among populations

(LT) and Daugavpils (LV) to 0.107 for Kaunas (LT) and Kalita1 (EE) (Table 6).

Molecular variance (AMOVA) analysis showed that genetic diversity of *I. ricinus* within sampling locations yielded 90%, and among locations 10% of the total genetic diversity (Fig. 4).

The PCA analysis did not reveal distinct clustering for samples from different locations. Samples of different origin showed varying overlap (Fig. 5).

Microsatellite markers have been previously described and applied to populations of *I. ricinus* (Delaye et al., 1998; De Meeûs et al., 2002, 2004a, 2004b; Røed et al., 2006; Kempf et al., 2009, 2010, 2011; Noel et al., 2012). However, the analysis using these markers has revealed significant deviations from Hardy-Weinberg proportions within populations. Hypotheses to explain these heterozygote deficits are numerous and not mutually exclusive: null alleles, short allele dominance, Wahlund effects or homogamy (Kempf et al., 2009).

Our results are consistent with the previous studies on *I. ricinus* showing heterozygote deficits and revealed significant deviations from Hardy-Weinberg proportions (De Meeûs et al., 2002, 2004a; Røed et al., 2006; Kempf et al., 2009b; Dharmarajan et al., 2011).

Hardy-Weinberg proportions in four of six analyzed populations were observed using IRN7, locus IRN4 showed Hardy-Weinberg proportions in two of six analyzed populations. Loci IRN28 and IRN8 demonstrate significant deviation from HW equilibrium (Ho were much lower than expected) for five of six tested populations.

Other researchers (De Meeûs et al., 2002, 2004a; Røed et al., 2006) proposed two possible hypotheses to explain heterozygote deficits at the population level. First, technical problems such as null alleles and short allele dominance that can produce false homozygote and blank (null homozygote) genotypes and are known to occur in *I. ricinus*.

Next, a possible Wahlund effect was examined to explain heterozygote deficits within populations. Host and tick behaviour, life stage, social system and other habits may influence the relative inbreeding of individuals from the same subsample (for example, larvae from the same clutch are known to be clustered in the environment). The cause of this linkage is probably of a demographic nature (e. g., reproductive mode, Wahlund effect, small

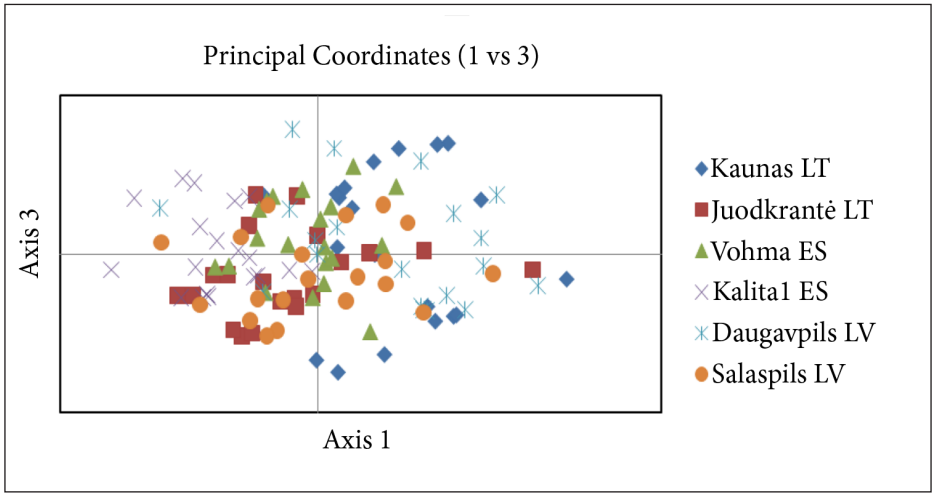

Fig. 5. Principal Coordinates Analysis (PCA)

populations) (De Meeûs et al., 2002, 2004a; Røed et al., 2006; Kempf et al., 2009b).

Dharmarajan et al. (2011) oppose other authors by contending that biological factors can also lead to patterns that have usually been interpreted as being caused by technical issues (for example, null alleles) when analyzing tick population structure using microsatellite markers. The authors revealed that two of the explored hypotheses were statistically significant: null alleles and kin structure. The high variance in F_{IS} across loci at the scale of the component population (CP) and intrapopulation (IP) is suggestive of the presence of null alleles. On the other hand, three lines of evidence suggest that null alleles were unlikely to be the main cause of deviation from HW equilibrium: (1) levels of missing data did not support the null allele hypothesis at the IP or CP scales; (2) analyses provided strong statistical support for the presence of kin structure in a majority of CPs examined, and analysis of genetic patterns in randomly generated kin-structured populations revealed that pooling kin groups could lead to a pattern usually considered to be a signature of null alleles: high levels of F_{IS} as well as high variance in F_{IS} among loci and (3) finally, null alleles are expected to lead to an underestimation of relatedness between individuals. The authors conclude that the results indicate that it is important to take such biological factors into consideration when addressing heterozygote deficits in natural systems, particularly because loci that deviate from Hardy-Weinberg proportions are likely to reflect the effects of real biological processes (Dharmarajan et al., 2011).

In the present study the highest observed heterozygosity (Ho) was found in Juodkrantė (Lithuania) and Salaspils (Latvia). Both of these sites are located close to the sea. The lowest observed heterozygosity (Ho) was found in Daugavpils (Latvia) and Kaunas (Lithuania) that are located in the continental parts of these countries. These results support our previous study where RAPD marker analysis was used to determine *I. ricinus* population structure and showed that genetic diversity is higher in *I. ricinus* population located close to the sea and bird migratory routes, while populations in the continental parts remain genetically less diverse (Paulauskas et al., 2006).

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References

1. Benbouza H, Jacquemin J-M, Baudoin J-P, Mergeai G. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechn Agr Soc Envir 2006; 10(2): 77–81.

2. Bormane A, Lucenko I, Duks A, Mavtchoutko V, Ranka R, Salmina K, Baumanis V. Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993–2002. Int J Med Microbiol 2004; 293 (Suppl. 37): 36–47.
3. Billotte N, Lagoda PJL, Risterucci AM, Baurens FC. Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits 1999; 54: 277–88.
4. Caporale DA, Rich SM, Spielman A, Telford SR, Kocher TD. Discriminating between ixodes ticks by means of mitochondrial DNA sequences. Mol Phylog Evol 1995; 4: 361–5.
5. Casati S, Bernasconi MV, Gern L, Piffaretti J. Assessment of intraspecific mtDNA variability of European *Ixodes ricinus* sensu stricto (Acari: Ixodidae). Infec Genet Evol 2008; 2: 152–8.
6. Chitimia L, Lin RQ, Cosoroaba I, Wu XY, Song HQ, Yuan ZG, Zhu XQ. Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial cox1 and nad5 genes. Exp Appl Acar 2010; 3: 305–11.
7. De Meeûs T, Beati L, Delaye C, Aeschlimann A, Renaud F. Sex-biased genetic structure in the vector of Lyme disease, *Ixodes ricinus*. Evolution 2002; 56(9): 1802–7.
8. De Meeûs T, Humair P-F, Grunau C, Delaye C, Renaud F. Non-Mendelian transmission of alleles at microsatellite loci: an example in *Ixodes ricinus*, the vector of Lyme disease. Int J Parasit 2004; 34: 943–50.
9. De Meeûs T, McCoy KD, Prugnolle F, Chevillon C, Durand P, Hurtrez-Boussès S, Renaud F. Population genetics and molecular epidemiology or how to "débusquer la bête". Infect Genet Evol 2007 Mar; 7(2): 308–32.
10. Delaye C, Aeschlimann A, Renaud F, Rosenthal B, de Meeûs T. Isolation and characterization of microsatellite markers in the *Ixodes ricinus* complex (Acari: Ixodidae). Mol Ecol 1998; 7: 360–1.
11. Delaye C, Béati L, Aeschlimann A, Renaud F, de Meeûs T. Population genetic structure of *Ixodes ricinus* in Switzerland from allozymic data: no evidence of divergence between nearby sites. Int J Parasitol 1997 Jul; 27(7): 769–73.
12. Dharmarajan G, Beasley JC, Rhodes OE Jr. Heterozygote deficiencies in parasite populations: an evaluation of interrelated hypotheses in the raccoon tick, *Ixodes texanus*. Heredity 2011 Feb; 106(2): 253–60.
13. Excoffier L, Smouse P-E, Quattro J-M. Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitrochondrial DNA restriction sites. Genetics 1992; 131: 479–1.
14. Filippova N. Ixodovye kleshi podsem. Ixodinae (Ixodid ticks of the subfamily Ixodinae). Fauna SSSR. Nauka, Leningrad 1977.
15. Fukunaga M, Yabuki M, Hamase A, Oliver JH Jr. et al. Molecular phylogenetic analysis of ixodid ticks based on the ribosomal DNA spacer, internal transcribed spacer 2, sequences. J Parasit 2000; 86: 38–43.
16. Geller J, Nazarova L, Katargina O, Järvekülg L, Fomenko N et al. Detection and Genetic Characterization of Relapsing Fever Spirochete Borrelia miyamotoi in Estonian Ticks. 2012, PLoS ONE 7(12): e51914. doi:10.1371/journal.pone.0051914.
17. Hasle G, Roed KH, Leinaas HP. Multiple paternity in *Ixodes ricinus* (Acari: Ixodidae) assessed by microsatellite markers. J Parasit 2008; 2: 345–7.
18. Healy JA, Cross TF, Healy A. The alpha-Gpdh polymorphism in the tick *Ixodes ricinus*: similar diurnal trends in genotypic composition in Irish and Swedish population samples. Exp Appl Acarol 2004; 32(1–2): 111–8.
19. Kempf F, De Meeus T, Arnathau C, Degeilh B, McCoy K-D. Assortative Pairing in *Ixodes ricinus* (Acari: Ixodidae), the European Vector of Lyme Borreliosis. J Med Entomol 2009; 46: 471–4.
20. Kempf F, De Meeûs T, Vaumourin E, Noel V, Taragel'ová V, Plantard O, Heylen D, Eyraud C, Chevillon C, McCoy KD. Host

races in *Ixodes ricinus*, the European vector of Lyme borreliosis. Infect Gen Evol 2011; 11: 2043–8.

21. Kain DE, Sperling FA, Lane RS. Population genetic structure of *Ixodes pacificus* (Acari: Ixodidae) using allozymes. J Med Entomol 1997 Jul; 34(4): 441–50.
22. Kempf F, McCoy K-D, De Meeûs T. Wahlund effects and sex-biased dispersal in *Ixodes ricinus*, the European vector of Lyme borreliosis: New tools for old data. Infect Genet Evol 2010; 10: 989–97.
23. Kovalev SY, Mukhacheva TA, Phylogeographical structure of the tick *Ixodes persulcatus*: A novel view. Ticks and Tick-borne Diseases 2012; 3: 212–8.
24. Jõgiste A, Barotov O, Kutsar K, Bersenyeva G, Varjas J. Lyme Disease in Estonia. A Cooperation Project for Communicable Disease Control in Northern Europe, Epinorth. 2004.
25. McCoy KD. The population genetic structure of vectors and our understanding of disease epidemiology. Parasite 2008; 15: 444–8.
26. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 1978; 89: 583–90.
27. Noel V, Leger E, Gómez-Díaz E, Risterucci A-M, Mccoy KD. Isolation and characterization of new polymorphic microsatellite markers for the tick *Ixodes ricinus* (Acari: Ixodidae). Acarologia 2012; 52(2): 123–8.
28. Norris DE, Klompen JS, Keirans JE, Black WC. Population genetics of *Ixodes scapularis* (Acari: Ixodidae) based on mitochondrial 16S and 12S genes. J Med Entom 1996; 1: 78–89.
29. Noureddine R, Chauvin A, Plantard O. Lack of genetic structure among Eurasian populations of the tick *Ixodes ricinus* contrasts with marked divergence from north-african populations. Int J Parasit 2011; 2: 183–92.
30. Paulauskas A, Radzijevskaja J, Rosef O, Turcinaviciene J, Ambrasiene D, Makareviciute D. Genetic variation of ticks (*Ixodes ricinus* L.) in the Lithuanian and Norwegian populations. Exp Appl Acarol 2006; 40(3–4): 259–70.
31. Peakall R, Smouse PE. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 2006; 6: 288–95.
32. Radulović Z, Milutinović M, Andelković M, Vujcić Z, Tomanović S, Bozić N, Marinković D. Allozyme polymorphism of Mdh and alpha-Gpdh in *Ixodes ricinus* populations: comparison of borreliae-infected and uninfected ticks. Exp Appl Acarol 2006; 40(2): 113–21.
33. Radulović Z, Milutinović M, Tomanović S, Mulenga A. Exon variability of gene encoding glycerol-3-phosphate dehydrogenase of *Ixodes ricinus* ticks. Parasite 2010 Dec; 17(4): 363–8.
34. Radulović Z, Milutinović M, Tomanović S, Mihaljica D, Cakić S, Stamenković-Radak M, Andelković M. Seasonal and spatial occurrence of glycerol-3-phosphate dehydrogenase variability in *Ixodes ricinus* (Acari: Ixodidae) populations. J Med Entomol 2012 May; 49(3): 497–503.
35. Radzijevskaja J, Indriulyte R, Paulauskas A, Ambrasiene D, Turcinaviciene J. Genetic polymorphism study of *Ixodes ricinus* L. populations in Lithuania using RAPD markers Act Zool Lituan 2005; 15(4): 341–8.
36. Roed K-H, Hasle G, Midthjell V, Skretting G, Leinaas H-P. Identification and characterization of 17 microsatellite primers for the tick, *Ixodes ricinus*, using enriched genomic libraries. Mol Ecol Notes 2006; 6: 1165–7.
37. Rich SM, Caporale DA, Telford SR, Kocher TD, Hartl DL, Spielman A. Distribution of the *Ixodes ricinus*-like ticks of eastern North America. Proceedings of the National Academy of Sciences of the United States of America 1995; 14: 6284–8.
38. Stanczak J, Racewicz M, Kubica-Biernat B, Kruminis-Łozowska W, Dabrownski J, Adamczyk A, Markowska M. Prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks (Acari: Ixodidae) in different Polish woodlands. Annals Agr Env Medic 1999; 127–32.

39. Stanek G. Pandora's Box: pathogens in *Ixodes ricinus* ticks in Central Europe. Wien Klin Wochenschr 2009; 121: 21–22.

40. Weir BS, Cockerham CC. Estimating F-statistics for the analysis of population structure. Evolution 1984; 38: 1358–70.

41. Williams J, Kubelik A, Livak K, Rafalski J, Tingey S. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990; 18(22): 6531–5.

42. Xu G, Fang QQ, Keirans JE, Durden LA. Molecular phylogenetic analyses indicate that the *Ixodes ricinus* complex is a paraphyletic group. J Parasit 2003; 3: 452–7.

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IXODES RICINUS ERKIŲ GENETINĖ ĮVAIROVĖ, PAREMTA MIKROSATELITŲ ŽYMENIMIS

Santrauka

Ixodes genties erkės priklauso *Ixodes ricinus* kompleksui, kurį sudaro 14 rūšių, paplitusių visame pasaulyje, taip pat ir Europoje. *I. ricinus* erkės parazituoja įvairių rūšių stuburinius gyvūnus ir yra įvairių infekcinių ligų sukėlėjai. Kadangi patogenų paplitimas erkėse ir šeimininkų specifiškumas priklauso nuo erkių genetinės struktūros, svarbu ištirti iksodinių erkių genetinę įvairovę. Šio tyrimo tikslas – įvertinti *I. ricinus* erkių genetinę įvairovę ir populiacijų genetinę struktūrą Baltijos šalyse panaudojant mikrosatelitų žymenis. Iš viso buvo ištirta 180 erkių (170 *I. ricinus* ir 10 *I. persulcatus*), kurios buvo surinktos 18-oje skirtingų Lietuvos, Latvijos ir Estijos vietovių. Šešios populiacijos, po dvi iš kiekvienos šalies, buvo naudotos genetinės įvairovės tyrimams. Atlikus mikrosatelitinių lokusų analizę buvo nustatyta, kad alelių skaičius tirtuose lokusuose svyravo nuo 5 iki 11. Tikėtinas heterozigotiškumas *I. ricinus* populiacijose svyravo nuo 0,621 iki 0,8, o stebimas heterozigotiškumas svyravo nuo 0,304 iki 0,634, ir tai rodo didelį nuokrypį nuo Hardy-Weinberg pusiausvyros. Didžiausias heterozigotiškumo lygis buvo pajūrio vietovėse, kur didelę įtaką erkių genetinei įvairovei daro migruojantys paukščiai.

Raktažodžiai: *I. ricinus*, Baltijos šalys, Lietuva, Latvija, Estija