Genetic variation of ticks (*Ixodes ricinus* L.) in the Lithuanian and Norwegian populations

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Abstract: RAPD markers were used to measure the genetic diversity of 119 individuals of *Ixodes ricinus* collected from Lithuania and Norway. The samples were analysed within and also between the populations. We analysed 74 loci in each of 6 populations. Our results show high levels of diversity within the populations. The percentage of polymorphic loci of the six analysed populations: Birzai, Vilnius, Kretinga, Tjore, Kjosvik and Odderoya were 68.9%, 58.1%, 78.38%, 62.2%, 44.6% and 68.9%, respectively. The percentage of polymorphic loci in the Lithuanian populations was 93.2%, and in the Norwegian populations 81.08%. The genetic distance ranged from 0.019 to 0.079 within Norwegian populations and from 0.005 to 0.0967 within Lithuanian populations and between the countries from 0.022 to 0.146. The genetic variation of *I. ricinus* among Norwegian populations was lower than among Lithuanian populations. The highest part of genetic variation in *I. ricinus* ticks depends on variation within Kretinga (Lithuania) and Odderoya (Norway) populations situated in coastal areas where many migratory and sea birds are aggregated.

Keywords Tick - *Ixodes ricinus* - RAPD markers - Genetic variation

Introduction

Ixodes ricinus (L.) is a member of the *I. ricinus* species complex, which consist of 14 species that collectively have an almost worldwide distribution (Xu et al. 2003). *I. ricinus*, also known as the sheep tick or the castor bean tick, is common and widespread in Lithuania. In Norway *I. ricinus* distributed in a narrow zone along the southern coast between the Oslofjord and Jæren, and along the western coast in a relatively wide zone including the innermost regions of most of the fjords and neighbouring valleys (Mehl 1983; Mehl et al. 1987). Mehl studies of ticks in Norway do not show any expansion of the range of *I. ricinus* since 1940. But the density of the tick population has been rise in many parts of its range, especially on islands, due to changes in vegetation, and in the distribution and population densities of host animals.

Ixodes ricinus ticks are frequently the object of research in both countries, because of their medical importance. *I. ricinus* is known as the main vector in transmission of infective diseases which affect humans and livestock, like tick-born encephalitis, borreliosis (Lyme disease), babesiosis, and human anaplasmosis in both countries (Bagdonas et al. 2003; Ambrasiene et al. 2004; Stuen et al. 2002; Skarpaas et al. 2004).

The *I. ricinus* is a three-host tick where the immature stages are parasitizing small to medium-sized animals and birds and the adults parasitize large mammals or humans. Ticks are sensitive to desiccation and availability of suitable hosts during the non-parasitic phases of their life cycle (Gray 1998). The rates and patterns of dispersal are determined largely by host movements. Migratory

birds are capable of transporting ticks over large distances and are therefore of interest in the understanding of the tick population dynamics, and spreading of pathogens (Mehl et al. 1984). Local diversity of the ticks could influence genetic diversity of *Borrelia burgdorferi* sensu lato, the main causative agent of Lyme disease. Distribution of *B. burgdorferi* sensu lato genospecies differ in Europe (Kurtenbach et al. 2001) and could be associated with diversity of *I. ricinus*.

The application of molecular markers to the study of ticks has provided new insights into their population structures and taxonomic relationships. Ticks have been studied at individual, population and species level. Different methods, including random amplified polymorphic DNA (RAPD) have been used to study these organisms (Navajas and Fenton 2000). RAPD provides class of highly polymorphic markers. This technique uses a single primer with a very short sequence (8–10 base pairs) to amplify small regions of the genome. The minor variations in the DNA sequences among different isolates lead to distinct fingerprinting patterns that are discriminatory. In this way the RAPD analysis can provide a simple and reliable method for measuring genomic variation (Lynch and Milligan 1994). RAPD technique is ideal for DNA fingerprinting, with particular utility in the field of population genetics. In many instances, only a small number of primers are necessary to identify polymorphism within species. A single primer may often be sufficient to distinguish all of the sampled varieties (Williams et al. 1990).

Previous work on measuring the intraspecies genetic variation by using enzymes and cuticular hydrocarbons (Estrada-Peña et al. 1996) suggested that *I. ricinus* have high levels of variation among populations, comparable to that of the closely related North American species, *I. scapularis* (Ames et al. 2000). According to research results, *I. ricinus* members appear to have an unusually high degree of intraspecies genetic variation in comparison with other tick species (Ames et al. 2000).

The aim of our study was to investigate genetic diversity of *I. ricinus* at two distinct spatial scales: geographically separate regional populations and local populations within Norway and Lithuania using random amplified polymorphic DNA (RAPD) method.

Materials and methods

Tick collection

Ticks were collected from their microhabitats during April–June in 2004 using standard “flagging” method for collecting active ticks on vegetation. A cloth or blanket (1 m^2) is drawn over the vegetation and attached nymphs and adults were collected into sealed vials containing 70% ethanol and stored until processed. Adults were classified as male or female and were identified as *I. ricinus* by their morphological characteristics (Filippova 1977).

Study area

One hundred nineteen *I. ricinus* ticks were collected from six sampling sites located in inland and coastal areas in Lithuania (sites 1, 2, 3) and Norway (sites 4, 5, 6) (Table 1). In Lithuania two sites were situated in inland areas: the site 1 was located in the northern part of Lithuania (270 km from the Baltic Sea coast) in broad-leaf deciduous forest area, near Birzai town; the site 2 was located in the south-eastern part of Lithuania (310 km from the Baltic Sea coast) in coniferous forest near Vilnius city. The site 3 was located in a coastal area in the north-western part of Lithuania (15 km from the Baltic Sea) near Kretinga city in mixed conifer and broadleaf forest. This site is on the main birds migratory rout during the spring and the autumn (Patapavičius 1998, 2006). In Norway ticks were sampled from places situated in the southern part. Two sites were in coastal areas: the site 4 was located near Tjore in Aust Agder (5 km from the North Sea coast) in a mixed conifer and

broadleaf forest and the site 6 was located in Vest Agder on Odderoya peninsula. The site 5 was situated in a inland area at Kjosvik (40 km from North Seas coast) in boreal coniferous forest. This site is situated on a border of *I. ricinus* distribution area in Norway (Mehl 1983).

Table 1 Sampling locations, coordinates and sample sizes of *I. ricinus* in Lithuania and Norway

Sites	**Locations**	**Sample, *n***		**Latitude**	**Longitude**
		Adult	**Nymph**	**N**	**E**
1	Biržai	25	2	56° 13′	24°45′
2	Vilnius	20		54° 50′	25°30′’
3	Kretinga	23		55° 43′	21°08′
4	Tjore	16	2	58° 19′	08°31′
5	Kjosvik	7		59° 19′	9°16′
6	Odderoya	15	9	58° 08′	08°01′
	Total	106	13		

DNA isolation

Extraction of DNA was carried out by lyses of ticks in ammonium hydroxide (NH_4OH). We added 80 (for nymphs) −100 µl (for females and males) 2.5% ammonia solution to the sample in a microcentrifuge tube and heated at 99°C for 25 min. After a brief centrifugation, the tubes were opened and heated until half the initial volume had evaporated (about 10–15 min to remove ammonia). The lysates were stored at −20°C until use for PCR (Stańczak et al. 1999).

RAPD-PCR amplification

After a screening of 10 oligonucleotide primers (synthesized in MBI Fermentas, Lithuania) for polymorphisms we chose five that revealed consistent profiles: OPA-01 (5′-CAGGCCCTT-<C>-3′), OPA-02 (5′-AATCGGGCT-<G>-3′), OPA-05 (5′-AGGGGTCTT-<G>-3′), OPA-07 (5′-GAAACGGGT-<G>-3′) and OPA-09 (5′-GGGTAACGC-<C>-3′) (Radzijevskaja et al. 2005). PCR was performed in a final volume of 25 µl containing 12.5 µl 2× PCR Master Mix (0.05units/µl *Taq* DNA polymerase in reaction buffer; 4 mM $MgCl_2$ 0.4 mM of each dNTPs), 2 µl 10-oligonucleotide primer (stock 10 pmol/µl) (MBI Fermentas, Lithuania), 6.5 µl double distilled water and 4 µl DNA of tick sample. All reactions were carried out in an Eppendorf PCR system “*Mastercycler personal”* thermal cycler. The samples were initially denatured for 1 min at 94°C. Subsequent cycles were at 94°C for 30 s (denaturation), 35°C for 30 s (primers annealing), and 72°C for 1 min (extension). Thirty-nine cycles were performed. Final extending was at 72°C for 3 min. PCR for each sample were replicated three times.

RAPD fingerprinting analysis

Amplified DNA was separated by electrophoresis in 1.5% agarose gels with 0.5×Tris–Borate–EDTA (pH 8.2) as running buffer and electrophoresed for 2.5 h at 115 V.

The DNA bands were stained with ethidium bromide and visualized by UV transillumination (EASY Win32, Herolab, Germany). DNA fragment sizes were assessed by comparison with GeneRuler^{TM} 100bp DNA Lader Plus (MBI Fermentas, Lithuania). Polymorphism data included the genotypes of many individuals sampled at one or more loci. We considered a locus to be polymorphic if two or more distinct types were observed, regardless of their frequencies (Rosenberg and Nordborg 2002).

In order to detect the genetic homogeneity as well as diversity of the *I. ricinus* individuals that were examined, the RAPD fingerprints were analyzed. Interpretation of the patterns was based on the size and on the presence or absence of amplified DNA bands. For each sample, the DNA fingerprinting patterns obtained with each primer were combined. These combined patterns were used for the similarity estimation and cluster analysis.

The Nei and Li algorithm (Nei and Li 1979) contained in the TREECON computer package program (Van de Peer and De Wacher 1994) were used to evaluate the genetic distances between the individuals of *I. ricinus*. The dendrogram were constructed by UPGMA (Unweighted Pair Group with Arithmetic Mean) method.

We used the PopGen32 program (Yen and Boyle 1997) to calculate genetic distances and genetic identity between populations (Nei 1978). Nei's gene diversity (Nei 1973) and Shannon's information index (Lewontin 1972) were used to calculate and describe diversity within and among populations.

Results

The number of fragments and the amount of intraspecies polymorphism varied between the primers, between the sites and between the countries (Table 2). Of the 74 RAPD bands isolated 41 were common in both the Norwegian and Lithuanian populations. The country-specific bands occurred at low frequencies with 21 bands in the Lithuanian and 12 in the Norwegian population, respectively. Similarities between RAPD patterns were based on a fraction of identical fragment for every possible pair of the DNA samples.

Table 2 Number of polymorphic RAPD bands found per primer in each population of *I. ricinus*

	Population	**OPA-01**	**OPA-02**	**OPA-05**	**OPA-07**	**OPA-09**
Lithuania	Birzai	8	13	4	18	8
	Vilnius	7	11	6	13	6
	Kretinga	12	12	12	13	9
Norway	Tjore	11	7	6	12	8
	Kjosvik	9	6	1	12	4

	Population	OPA-01	OPA-02	OPA-05	OPA-07	OPA-09
	Odderoya	9	12	3	15	11

We analyzed 74 loci per 6 population and all of them were polymorphic (100%). The percentage of polymorphic loci of the six analyzed populations: Birzai, Vilnius, Kretinga, Tjore, Kjosvik and Odderoya were 68.92%, 58.11%, 78.38%, 62.16%, 44.59% and 68.92%, respectively. The polymorphic loci in the Lithuanian populations was 93.24%, and in the Norwegian populations 81.08%.

The highest genetic distance (0.1460) was between the Kjosvik (Norwegian) and the Kretinga (Lithuanian) populations (Table 3). The most similar populations (0.0049) were Vilnius and Birzai. The genetic distance ranged from 0.0191 to 0.0791 within Norwegian populations and from 0.0049 to 0.0967 within Lithuanian populations and between the countries ranged from 0.0219 to 0.1460. Genetic distance within the Lithuanian populations was higher than within Norwegian populations.

Table 3 Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between Lithuanian and Norwegian populations of *I. Ricinus*

Population	Birzai	Vilnius	Kretinga	Tjore	Kjosvik	Odderoya
Birzai	0.000	0.9951	0.9228	0.9783	0.9503	0.9596
Vilnius	0.0049	0.000	0.9079	0.9750	0.9409	0.9493
Kretinga	0.0803	0.0967	0.000	0.9026	0.8642	0.9422
Tjore	0.0219	0.0254	0.1024	0.000	0.9811	0.9621
Kjosvik	0.0509	0.0610	0.1460	0.0191	0.000	0.9239
Odderoya	0.0413	0.0520	0.0595	0.0386	0.0791	0.000

Combined data for six analysed populations (Table 4) shows that the genetic variation in *I. ricinus* is high. The genetic variation within each of population is lower. The genetic variation of *I. ricinus* among Norwegian populations was lower than among Lithuanian populations (Table 4).

Table 4 Genetic variation in the Lithuanian and Norwegian populations of *I. ricinus*

	Lithuanian population				**Norwegian population**				**Combined data for *I. ricinus***
	Birzai	**Vilnius**	**Kretinga**	**Combined data**	**Tjore**	**Kjosvik**	**Odderoya**	**Combined data**	
Shannon's Information Index	0.2812	0.2193	0.3243	0.3358	0.2315	0.2019	0.2953	0.3040	0.3418
Nei's gene diversity	0.1783	0.1380	0.2063	0.2096	0.1428	0.1297	0.1922	0.1904	0.2108
Observed mean number of alleles	1.6892	1.5811	1.7838	1.9324	1.6216	1.4459	1.6892	1.8108	2.0000
Effective mean number of alleles	1.2828	1.2211	1.3333	1.3250	1.2231	1.2119	1.3242	1.2989	1.3196

The dendrogram for 119 individual of *I. ricinus* constructed by cluster analysis using RAPD-based genetic distance is presented in Fig. 1. On the dendrogram ticks from each site not clearly separated, except ticks from Kretinga and Odderoya. The ticks from Lithuania as well as ticks from Norway appeared in four different main groups. Ticks from two Lithuanian sites Birzai (site 1) and Vilnius (site 2) according their genotypes are similar and formed one group. Another group formed *I. ricinus* ticks from two Norwegian site: Tjore (site 4) and Kjosvik (site 5). Separate groups formed ticks collected in Lithuanian coastal area Kretinga (site 3) and Norwegian coastal area Oderroya (site 6) (Fig. 1). Some of ticks are genetically different from four main groups and formed separate small clusters or branches. The data illustrated that the geographic distance and the large bodies of water, such as Baltic Sea, do not limited *I. ricinus* movement to new areas.

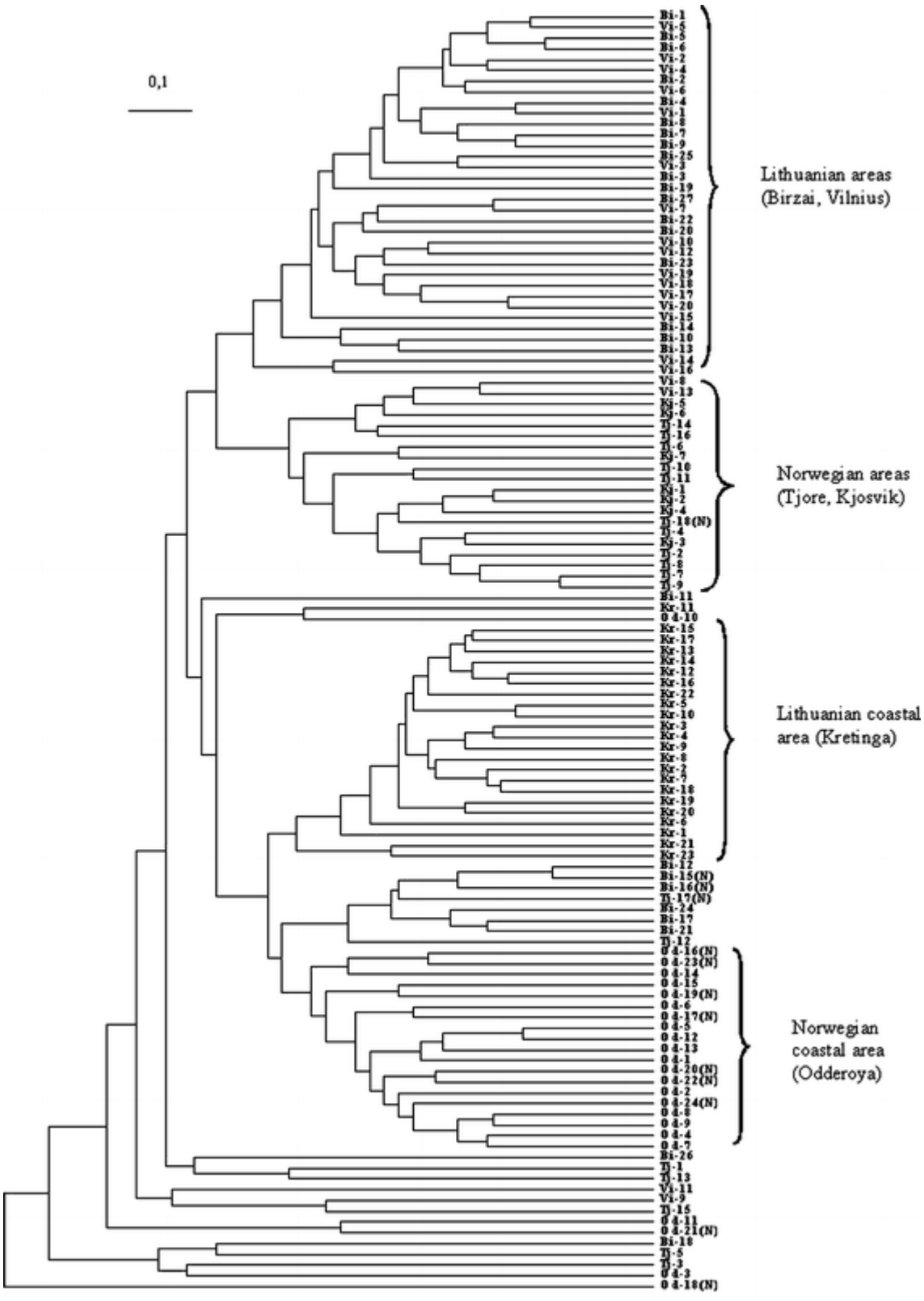

Fig. 1 The phylogenetic tree for 119 individual of *I. ricinus* constructed on the basis of RAPD data. Bi—ticks collected in Birzai; Vi—Vilnius; Kr—Kretinga; Tj—Tjore; Kj—Kjosvik; Od—Odderoya. (N)—nymph

Discussion

Morphological description has been widely used for quantifying and classifying diversity in acarology and has given rise to much successful research (Navajas and Fenton 2000). The generally techniques was direct observation of phenotypic differences between organisms. Studies of tick and mite chromosomes have led to an interesting hypothesis that ticks and mites have distinct origins (Oliver 1977), an observation that is a suitable challenge for a molecular phylogenetic analysis. Ticks are usually identified with microscopic and morphometric analysis. But, there are multiple difficulties associated with accurate tick identification (Poucher et al. 1999). For example separate keys must be used for larvae, nymphal, and female and male adult forms. Besides, tick mouthparts and adjacent structures that are usually essential for identification may become damaged during the removal of ticks from its host. Consequently, these difficulties could be resolved by using keys based on molecular genetic markers (Poucher et al. 1999).

Towards the end of the 20th Century the advent of molecular techniques has generated the potential to investigate DNA at the individual base pair. This is clearly a much more direct way of measuring and quantifying the genetic variation within and between species (Hoy 1994). Detailed analysis of population structures has also been possible (Navajas and Fenton 2000).

The knowledge of the genetic diversity of the Ixodidae has been considerably enhanced by the application of molecular techniques. Protein electrophoresis has been an effective technique for the detection of genetic polymorphism for over three decades (Navajas and Fenton 2000). Enzymatic polymorphism detected by electrophoresis has been widely used on ticks. In order to investigate genetic structure of *I. ricinus* populations in Switzerland, 18 loci were analysed and 2 appeared polymorphic. This shows the low allozymic variability displayed by *I. ricinus* (Delaye et al. 1997). The researches conclude that more powerful genetic markers as microsatellite could be used (Delaye et al. 1998) in study of these ticks. In a sample of 50 individuals of *I. ricinus*, five of the six isolated loci were polymorphic. Three of these loci were highly polymorphic: they had more than 16 alleles in each.

Healy (1979) measuring phosphoglucomutase polymorphism in *I. ricinus* detected 10 variants of phosphoglucomutase. Allelic proportions in Irish tick samples indicated that both spatial and temporal genetic differentiations existed. It was suggested that this polymorphism may be useful as a marker for studying the relationships of *I. ricinus* populations in Europe and may act to maintain an array of individuals in each population with varying developmental rates and longevities.

Kain et al. (1999) have used the cytochrome oxidase III (COIII) gene to assess population structure of the tick *I. pacificus* from the USA. In a study of the tick *I. scapularis* Say, Norris et al. (1996) adopted a novel strategy to detect genetic variation that involves the use of single strand conformation polymorphism (SSCP) analysis. Using the SSCP approach to detect variation in a region of the 16S mitochondrial ribosomal DNA, Norris et al. (1996) estimated the frequency of haplotypes in various regions of the United States and determined the greatest variation in the North American *I. scapularis* among population in regions and less within the populations. A similar study of genetic variation of *I. ricinus* (Ames et al. 2000) based on analysis of 16S mitochondrial rDNA, concluded that there is significant intraspecies diversity in *I. ricinus* from different countries, but that genetic variation within countries is negligible.

RAPD methods were used to create genetic linkage map for the *I. scapularis* ticks (Ullman et al. 2003). Yang et al. (2004) used RAPD methods in investigations genetic distance of 7 species of Ixodidae ticks. The amplified products of the 7 species of ticks by RAPD all showed their specific DNA band. The authors conclude, that RAPD could differentiate between species of Ixodidae ticks. Lan et al. (1996) used RAPD to detect polymorphisms in *Boophilus microplus* (Ixodidae), but found that RAPD markers were only able to distinguish species of ticks.

We assumed that RAPD analyses could be used to assess the amount and structure of genetic diversity within and between natural populations of *I. ricinus* ticks. Previous study (Norris et al.

1996), which was analyzed the diversity of RAPD markers in *I. scapularis*, did not find variation between specimens from geographically separated sites of the United States. Only a small proportion of overall genetic variation in *I. scapularis* could be apportioned regionally (Norris et al. 1996). The present study of genetic diversity of 119 individuals of *I. ricinus* showed high levels of diversity of RAPD markers. The analysed populations differed in their polymorphism. The less polymorphic population of *I. ricinus* was in Kjosvik. Low genetic variation of *I. ricinus* in Kjosvik may be determined by location of this site on a border of *I. ricinus* distribution area in Norway. The most polymorphic was the population from Kretinga (Lithuania). The highest part of genetic variation in *I. ricinus* ticks depends on variation within Kretinga and Odderoya population. The occurring significant genetic variation in *I. ricinus* might be explained by aggregation of many migratory and sea birds in these places situated in the coastal areas and on the main birds migratory rout during the spring and the autumn (Mehl et al. 1984; Patapavičius 1990; Žalakevičius et al. 1995). As demonstrated previous study (Olsen 2003) birds may play an important role for *I. ricinus* wide distribution and affect the tick population dynamics and structure.

It was found that regional differences existed in the epidemiology of tick-borne diseases in *I. ricinus* populations (Gray 2002). There are differences in the readiness of adult female to bite humans in different *I. ricinus* populations. A greater proportion of the nymphal *I. ricinus* population feeds on wood mice (*Apodemus sylvaticus*) in some European regions than in others. Variations in susceptibility of different tick populations to tick-borne pathogens may also occur. As described Estrada-Pena et al. (1996) in a study on the susceptibility to *B. afzelii* of larvae derived from Spanish, Irish and German *I. ricinus* females, the Spanish ticks were more susceptible than either German or Irish *I. ricinus*. Surprisingly, the German ticks were the least susceptible to this German *B. afzelii* strain, which was from the same region. Regional differences in the epidemiology of tick-borne diseases are often explained in terms of pathogen behaviour, but it may be related with variations in tick populations (Gray 2002).

In Lithuania and Norway the largest wildlife biomass is found in deciduous and mixed forest and the highest species diversity occurs in ecotonal areas, such as where forest and wetlands meet (Mehl 1983; Balčiauskas et al. 1999). In these types of landscapes the primary and secondary hosts for ticks are dominant and include yellow necked mouse (*Apodemus flavicollis*), bank vole (*Clethrionomys glareolus*), red squirrel (*Sciurus vulgaris*), and roe deer (*Capreolus capreolus*), moose (*Alces alces*) and blue hare (*Lepus timidus*) are also present (Balčiauskas et al. 1999; Mehl 1983). In inland and coastal areas nesting migratory birds like redstart (*Phoenicurus phoenicurus*), thrush nightingale (*Luscinia luscinia*), song thrush (*Turdus philomelus*) and ground-feeding birds like blackbirds, *Turdus merul*a, and robins, *Erithacus rubecul*a occur and are also parasitized by ticks (Mehl et al. 1984; Žalakevičius et al. 1995). Comstedt et al. (2006) study demonstrated that migratory passerine birds constitute an epidemiologically important alternative reservoir of Lyme disease.

As described Mehl et al. (1984), in study of ticks on migratory birds in Norway, the most important and most heavily infested hosts for *I. ricinus* were *Turdus* spp., *Erithacus rubecula*, *Phoenicurus phoenicurus*, *Prunella modularis*, *Anthus trivalis* and *Luscinia svecica.* It is impossible to determine the place of origin for the ticks that are transported to Norway with migratory birds. They could by originate from a very large area, because the species of birds have different overwintering areas, different migratory routes, and migrate during different periods during spring and autumn. The dominant direction of migration during spring in Northern Europe is from southwest towards northeast, and the opposite direction during autumn.

In Lithuania are two dominant birds' migratory routes (Patapavičius 1998, 2006). During the spring the birds migrate from southwest towards northeast through Vilnius, Birzai, Daugavpils. This route passes through inland area, 200 km from the Baltic Sea. During the autumn the birds migrate from northeast towards southwest through Baltic Sea coastal area. On this route are located Kretinga and two birds ringing station: Ventes Ragas Ornithological Station, situated on the eastern coast of Curonian Lagoon and Neringa Birds Ringing Station located in the Curonian Spit which is a narrow

land of 97 km length between the Baltic Sea and Curonian Lagoon. A huge amount of birds from North Europe migrated via these stations. Some reports were got from Norway about catching birds ringed in Lithuania and some reports were got in Lithuania about birds ringed in Norway during the 1979–2003 (Patapavičius 1990, 1998).

Our results support the hypothesis that *I. ricinus* has migrated into new areas by host-mediated dispersal, primarily on avian hosts. So birds could play an important role for the long-range migration of *I. ricinus* from Eastern and Central Europe to Scandinavian countries, affect the tick population dynamics and structure and transporting infecting ticks. Also short-range migration of *I. ricinus* on host, including mammals and birds, could explain the similarity of ticks from some Lithuanian and also from some Norwegian sites.

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