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Impact of anthropogenic pressure on the formation of population structure and genetic diversity of raccoon dog *Nyctereutes procyonoides*

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Abstract

The raccoon dog *Nyctereutes procyonoides* experienced an active introduction and acclimatization in the European part of Russia followed by its migration to and colonization in the neighboring countries. Eventually, it has spread rapidly into many European countries. *N. procyonoides* probably invaded Lithuania from the neighboring countries of Belarus and Latvia where the species was introduced. However, the data on genetic diversity and population structure of the raccoon dogs in the recently invaded territories are still scarce. The objectives of this study were to investigate genetic diversity of *N. procyonoides* in Lithuania after acclimatization, and to assess the impact of anthropogenic pressure on the formation of population structure. A total of 147 *N. procyonoides* individuals collected from different regions of Lithuania were genotyped using 17 microsatellite markers. The microsatellite analysis of raccoon dogs indicated high levels of genetic diversity within the population. The Bayesian clustering analysis in STRUCTURE identified 4 genetic clusters among sampled raccoon dogs that could not reveal a clear separation between subpopulations. The widespread distribution of raccoon dogs in Lithuania, high level of genetic variation observed within subpopulations, and low level of variation portioned among subpopulations suggest migration and gene flow among locations. The significant correlation between genetic and geographic distances indicated isolation that reflected the distance between locations. The fencing of highways and very intensive traffic could be barriers to gene flow between the western and eastern sampling areas of raccoon dogs.

Key words: *Nyctereutes procyonoides*, microsatellite loci, invasion, genetic diversity, Lithuania.

Currently, the raccoon dog *Nyctereutes procyonoides ussuriensis* is one of the most widespread alien carnivore species in Europe (Kauhala and Kowalczyk 2011). *N. procyonoides* had an active introduction and acclimatization in the European part of Russia followed by its migration and colonization in the neighboring countries (Korablev et al. 2011). Eventually, it has spread rapidly into many European countries. High adaptability of raccoon dogs to various environmental conditions and their successful expansion in Europe are explained by their natural tendency to quickly proliferate and migrate, by the high plasticity of the species (raccoon dogs are omnivorous) and their reproductive potential.

N. procyonoides probably invaded Lithuania from the neighboring countries of Belarus and Latvia where the species was introduced in 1936 and 1948, respectively. In Lithuania, raccoon dogs have

been noticed since 1948. Showing great plasticity in adaptation to various environmental and climatic conditions, they have spread into different areas of Lithuania and by 1960 had colonized the whole of the country (Prūsaitė et al. 1988). Rapid population growth during the first decade of the invasion is probably also related to the decreased number of wolves and lynx, the natural enemies of raccoon dogs, in 1940–1950 in Lithuania. In neighboring Estonia, raccoon dogs were found all over the country in the 1950s. However, their numbers remained low because of the presence of numerous wolf and lynx populations (Balčiauskas 1996). After this expansion, raccoon dogs prospered in Lithuania until the 1980s. The density of the Lithuanian population of raccoon dogs varies in different time periods, and according to the monitoring data from 1960 to 1970, it went up from 3,000 to 14,000 (data of the Ministry of Environment of the Republic of Lithuania, http://www.am.lt). Since 1970s, the raccoon dog has been declared as an invasive species in Lithuania and has been affected by an intense hunting pressure as well as by rabies. Nowadays, hunting of these animals is permitted throughout the year. The hunting statistics showed that during the past decade (2004–2014) 35,856 raccoon dogs were hunted, and the number of hunted animals ranged from 2,818 in 2006–2007 to 10,290 in 2010–2011. Regulating populations by hunting could affect the dispersal behavior and the population structure of this species.

Other aspects of anthropogenic activity such as road building and land development could have an effect on raccoon dog populations. Road networks and traffic fragment habitats create barriers, can hinder movement, prevent dispersal, and cause significant mortality of mid- to large-sized animals (Trombulak and Frissell 2000; Crooks 2002; Riley et al. 2006). The raccoon dog is one of the mammals most frequently killed on the roads of Lithuania (Balčiauskas 2009). During the period 2002–2013, 1,066 road-killed raccoon dogs were registered. The fencing of highways which began in 2004 has not reduced the number of wildlife–vehicle accidents associated with raccoon dogs (Balčiauskas and Balčiauskienė 2014). The road-kill index was significantly higher on main roads than on regional roads, and it was positively correlated with traffic intensity (Balčiauskas and Balčiauskienė 2014). There are a few highway stretches with the traffic volume of over 5,000 vehicles per day in Lithuania (Traffic 2014, Figure 1). From 2000 to 2014, traffic volumes on the main Lithuanian roads went up 1.91 times.

Although there are many studies devoted to the ecological impact of raccoon dogs, only a few studies investigating genetic and population characteristics have been conducted. The investigation into genetic diversity and the structure of the introduced populations can provide useful information on the dispersal patterns or on the range of expansion of the population and can help to understand the potential of the introduced species to become established and to

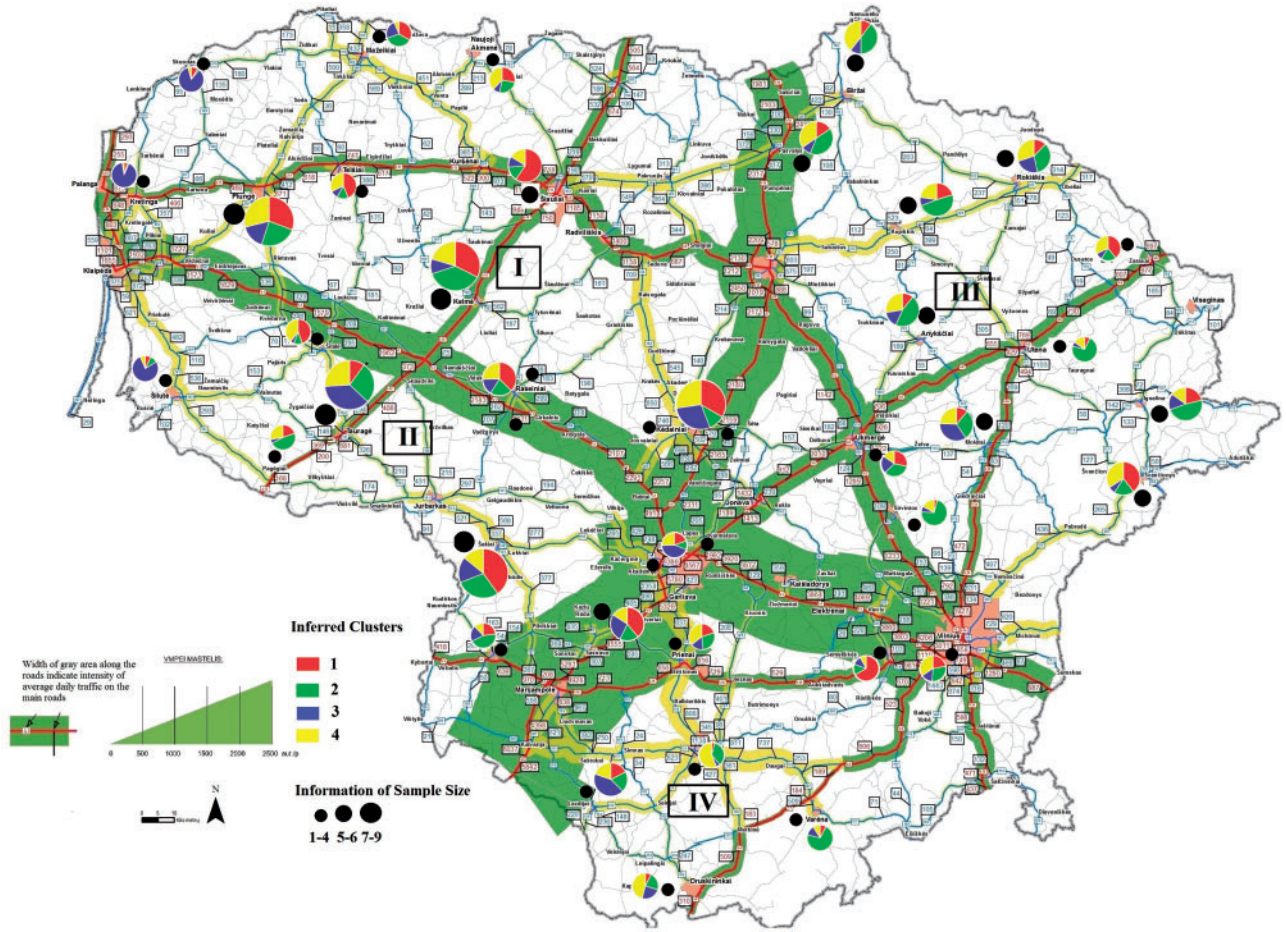

Figure 1. Map of sampling localities of the *N. procyonoides* in Lithuania. Black-filled circles show collecting localities of raccoon dogs (adapted from "The Lithuanian Road Administration under the Ministry of Transport and Communications of the Republic of Lithuania"; http://www.lakd.lt). Pie chart shows proportion of the STRUCTURE clusters at $K = 4$.

spread into new areas. In previous studies, genetic diversity, population structure, and phylogeography of raccoon dogs (Ślaska et al. 2008; Pitra et al. 2010; Korablev et al. 2011; Ślaska et al. 2011; Paulauskas et al. 2015) have been investigated based on such molecular genetic markers as mitochondrial DNA and Random Amplified Polymorphic DNA (RAPD). Canine-derived microsatellite markers were previously applied in 2 of 6 subspecies of *N. procyonoides*: *N. procyonoides procyonides* (Rogalska-Niznik et al. 2003; Ślaska et al. 2008) and *N. procyonoides viverrinus* (Hayashizono et al. 2010). Recently, Hong et al. (2013) have reported isolation and characterization of 12 novel polymorphic loci from *N. procyonoides koreensis* subspecies, and described the genetic structure of 5 endemic raccoon dog populations in South Korea. However, the data on genetic diversity and on the formation of population structure of the raccoon dogs in the recently invaded territories are still scarce.

The Lithuanian raccoon dog population could be used as a model: 1) to assess the genetic diversity of raccoon dogs that invaded a new territory from different locations of introduction and 2) to investigate the impact of anthropogenic pressure (intensive hunting, habitat fragmentation by major roads, intensive traffic) on the formation of population structure.

The objectives of this study were to investigate the genetic diversity and the population structure of *N. procyonoides* in Lithuania after acclimatization using microsatellite markers.

Material and Methods

Study sites and samples

This study was based on the specimens of raccoon dogs collected during a 2-year period (2011–2012) from 37 sampling sites across Lithuania (Figure 1). A total of 147 *N. procyonoides* individuals legally harvested by the hunters and found killed on the roads in different parts of Lithuania were investigated (Figure 1).

Although raccoon dogs prefer wet open habitats (damp meadows and forests, marshlands, and river valleys), they may occupy various habitats from continuous forests to open agricultural landscapes and suburban areas (Kauhala and Kowalczyk 2011). In Lithuania, raccoon dogs prefer moraines landscape and are more frequently observed in swamps (Baltrūnaitė 2010). The relative abundance of the raccoon dog was found to be significantly lower in the sandy plains landscape compared with clay plains and hilly morainic uplands (Baltrūnaitė 2010).

Raccoon dog samples were collected from different regions of Lithuania representing different landscapes. We arranged our sampling data in 4 groups (I, II, III, and IV subpopulations) also taking into account the fragmentation of country by main roads (E67, E85) with high volume of traffic (Figure 1). The first (I) and second (II) sampling areas are covered by mixed forests and grasslands; deciduous broad-leaved woods are dominant in the third (III) sampling area, and pine *Pinus sylvestris* forests are prevalent in the fourth (IV) sampling area.

DNA extraction

Muscle tissue samples were collected from raccoon dogs and preserved in 75% ethanol. Genomic DNA was extracted from tissue samples using "Thermo Scientific Genomic DNA Purification Kit" (Thermo Fisher Scientific Baltics, Vilnius, Lithuania; catalog No K0512). The concentration and the purity of isolated DNA were determined with Nanodrop 2000 Spectrophotometer (Thermo Scientific, DE, USA). DNA samples were stored frozen at −20 °C until later Polymerase Chain Reaction (PCR) amplification.

Microsatellite analysis

A total of 17 microsatellite markers, 5 described in canine genome (*FH2096, FH2054, FH2010* [Francisco et al. 1996], *PEZ17* [Kukekova et al. 2004], and *REN112I02* [Ślaska et al. 2005]) and 12 developed for *N. p. koreensis* (*Nyct1– Nyct12*; Hong et al. 2013; Table 1) were used in the present study.

The PCR analysis and polyacrylamide gel electrophoresis were performed to investigate the level of polymorphism at these loci and to evaluate their usefulness for the population genetic study of *N. p. ussuriensis*. The PCR mixture components and conditions for amplification of 5 canine microsatellite loci were as follows: 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 2 U Taq DNA polymerase (Thermo Fisher Scientific Baltics), 0.2 μM of each primer, and 20–60 ng of template DNA; an initial denaturation step (95 °C for 4 min) was followed by 30 cycles of 1 min at 94 °C, 25 s at the locus-specific annealing temperature (52 °C, 57 °C, 59 °C, 60 °C). PCR amplification of the other 12 microsatellite loci (Table 1) was carried out as described by Hong et al. 2013. The amplified products were separated on 8% denaturing polyacrylamide gel and then visualized by staining them with silver nitrate. Allele sizes were estimated in comparison with GeneRuler^{TM} 50 bp DNA ladder (Thermo Fisher Scientific Baltics). After determining the successfully amplified polymorphic loci, a subsequent multiplex PCR amplification with fluorescently labeled primers was carried out. We analyzed polymerase chain reaction products using capillary electrophoresis on the ABI PRISM 3100 Genetic Analyzer and the molecular size standard GeneScan^{TM} 500 LIZ (Applied Biosystems, CA, USA) following the protocol provided by the manufacturer. The alleles observed for each microsatellite were sized using GeneMapper software version 4.0 (Applied Biosystems). Output profiles were manually checked to confirm allelic size variants.

Statistical analysis

We estimated the average number of alleles per locus (*A*), observed heterozygosity (H_O), and expected heterozygosity (H_E) using GenAlEx version 6.5 (Peakall and Smouse 2006). Deviations from the Hardy–Weinberg equilibrium (HWE) were tested using GENEPOP version 4.0 (Raymond and Rousset 1995) with exact *P* values being estimated using the Markov chain algorithm with 10,000 dememorization steps 100 batches and 1,000 iterations. Significance levels were adjusted using a strict Bonferroni correction applied for multiple comparisons (Rice 1989)

MICROCHECKER version 2.2.3 (Oosterhout et al. 2004) set for 1,000 iterations and a 95% confidence interval, and checked for possible scoring errors and null alleles. The polymorphism information content (PIC) was calculated using MSTOOLS software (Park 2001). Allelic richness (A_R) was estimated using FSTAT software version 2.9.3 (Goudet 2001, updated from Goudet 1995), and private allelic richness (P_R) was obtained using HP-RARE version 1.1 via a rarefaction method (Kalinowski 2005).

To assess the level of population genetic structure, we calculated pairwise Nei's genetic distances (D_{Nei}; Nei 1973) between each pair of the sample sites using the software GenAlEx 6.5. We estimated F_{ST} values according to Weir and Cockerham's (1984) version of Wright's *F*-statistic with the use of FSTAT program package, followed by sequential Bonferroni correction for multiple tests.

The partitioning of total genetic diversity within and between the sample sites (without recurring genotypes) was estimated with

Table 1. Genetic diversity of raccoon dogs from Lithuania estimated based on polymorphisms in 16 microsatellite loci

	Nyct1	*Nyct3*	*Nyct4*	*Nyct5*	*Nyct6*	*Nyct7*	*Nyct8*	*Nyct9*	*Nyct10*	*Nyct11*	*Nyct12*	Mean	*FH2054*	*FH2010*	*PEZ17*	*REN112I02*	*FH2096*	Mean
	I ($N = 41$)																	
A	8	6	9	10	8	6	9	11	4	9	4	7.64	15	4	12	9	4	8
H_E	0.800	0.651	0.761	0.769	0.737	0.688	0.667	0.818	0.499	0.589	0.544	0.684	0.882	0.569	0.878	0.788	0.603	0.703
H_O	0.805	0.561	0.659	0.854	0.805	0.707	0.537	0.805	0.317	0.610	0.488	0.650	0.949	0.683	0.854	0.854	0.902	0.712
P	0.821	0.017	0.193	0.217	0.243	0.680	0.022	0.424	0.000*	0.464	0.223		0.314	0.053	0.135	0.581	0.000*	0.929
	II ($N = 36$)																	
A	9	6	9	9	9	6	11	10	3	5	4	7.36	14	6	10	9	4	7.8
H_E	0.776	0.574	0.761	0.762	0.768	0.664	0.723	0.816	0.526	0.598	0.545	0.683	0.874	0.640	0.865	0.789	0.663	0.709
H_O	0.800	0.529	0.743	0.750	0.743	0.778	0.833	0.743	0.486	0.528	0.528	0.678	0.968	0.750	1.000	0.861	0.943	0.749
P	0.994	0.009	0.489	0.063	0.016	0.954	0.098	0.025	0.001*	0.004	0.458		0.089	0.076	0.009	0.145	0.000*	0.995
	III ($N = 56$)																	
A	9	7	7	5	9	5	6	7	2	4	4	5.91	12	5	10	10	5	6.7
H_E	0.775	0.601	0.744	0.629	0.746	0.635	0.590	0.750	0.436	0.525	0.474	0.628	0.829	0.596	0.855	0.734	0.659	0.661
H_O	0.741	0.625	0.643	0.625	0.714	0.661	0.661	0.679	0.536	0.554	0.482	0.629	0.889	0.691	0.818	0.818	0.852	0.687
P	0.006	0.979	0.023*	0.635	0.328	0.951	0.235	0.000*	0.0845	0.992	0.524		0.424	0.058	0.860	0.022	0.000*	0.999
	IV ($N = 14$)																	
A	7	4	6	5	7	4	4	5	2	4	3	4.64	10	5	8	7	5	5.4
H_E	0.778	0.566	0.707	0.666	0.778	0.541	0.635	0.742	0.477	0.594	0.349	0.621	0.832	0.622	0.827	0.755	0.735	0.663
H_O	0.500	0.500	0.643	0.857	0.714	0.357	0.571	0.786	0.643	0.357	0.429	0.578	0.929	0.571	0.786	0.857	0.857	0.647
P	0.002*	0.279	0.166	0.084	0.250	0.048	0.299	0.568	0.273	0.005	0.506		0.776	0.469	0.386	0.637	0.776	0.004
	Total ($N = 147$)																	
A	10	9	9	10	10	7	11	14	4	11	4	9	15	6	12	11	6	–
H_E	0.782	0.598	0.743	0.706	0.757	0.632	0.653	0.781	0.485	0.577	0.478	0.654	0.854	0.607	0.856	0.767	0.665	–
H_O	0.711	0.554	0.672	0.771	0.744	0.626	0.651	0.753	0.495	0.512	0.482	0.634	0.933	0.674	0.864	0.848	0.889	–
P	0.000*	0.000*	0.026	0.427	0.114	0.013	0.236	0.000*	0.999	0.000*	0.615		0.163	0.002*	0.684	0.062	0.000*	–
	Hong et al. 2013 ($N = 104$)																	
A	10	13	8	10	10	12	9	9	4	6	4	8.7	–	–	–	–	–	–
H_E	0.783	0.689	0.776	0.787	0.807	0.804	0.739	0.793	0.515	0.696	0.574	0.723	–	–	–	–	–	–
H_O	0.692	0.702	0.653	0.689	0.624	0.767	0.598	0.667	0.375	0.65	0.466	0.619	–	–	–	–	–	–
P	0.005	0.00*	0.008	0.00*	0.00*	0.001*	0.004	0.002*	0.002*	0.426	0.011		–	–	–	–	–	–

N: number of samples, A: number of alleles, (H_O) and (H_E): observed and expected heterozygosities, and P: values for HWE exact test for heterozygote deficiency/excess. Significant HWE deviation values marked by * (Bonferroni correction, adjusted $P = 0.003$).

the hierarchical analysis of molecular variance (AMOVA) using both R_{ST} (SMM—stepwise mutation model) and F_{ST} (IAM—infinite-allele model) values in GenAlEx version 6.5.

We used a Mantel test (Mantel 1967) with 999 permutations in the software GenAlEx to test for evidence of isolation by distance (IBD).

Factorial correspondence analysis (FCA) on the microsatellite data for individual raccoon dogs was performed using GENETIX version 4.05.2 (Belkhir et al. 2004).

Bayesian clustering analyses were performed with STRUCTURE version 2.3.4 (Pritchard et al. 2000) to determine the number of genetic clusters (K) and to assign individuals to their likely origin. STRUCTURE was run 10 times for each value of K ranging from $K = 1$ to $K = 9$ with an initial burn-in period consisting of 200,000 replications and a run length of 100,000 Markov chain Monte Carlo (MCMC) iterations of the total dataset. Because significant gene flow was expected, admixture and allele frequency correlated models were applied. We used StructureHarvester version 0.6.9.3 to determine the most likely number of clusters by calculating ΔK, which is based on the rate of change of the "estimate likelihood" between successive K values (as described in Evanno et al. 2005).

The output of genetic clustering for all the individuals in 37 sampling sites and for each of pre-defined sampling area was visualized by calculating the average per cluster assignment values (Figure 1).

Results

Genetic diversity

A total of 147 raccoon dogs were successfully genotyped at 16 microsatellite loci, and altogether 149 alleles were detected (Table 1). One locus (*Nyct2*), either failed to amplify or amplified alleles were difficult to score. The size of alleles varied according to each locus and ranged from 84 bp to 266 bp (Table 2). The number of alleles for each locus (A) ranged from 4 to 15 with an average over all loci and all sample sites of 9.31 (Table 1). Over all loci, the average number of alleles per sample site (A) varied from 5.4 (IV) to 8 (I) (Table 1) with an average of 6.975.

Allelic richness (A_R) values ranged from 2.88 to 9.77. Estimates of allelic richness (A_R) varied slightly among sampling areas, with the highest value being obtained in sampling locality I (6.14) (Table 2).

Private alleles were found in all sampling areas. The greatest average number of unique alleles was detected in the sampling areas II and I (0.65 and 0.59, respectively) (Table 2).

The PIC index ranged from 0.40 to 0.87, with the average of 0.68 (Table 2). The high PIC values indicated suitability of these loci for genetic studies of the invaded *N. procyonoides* population in Lithuania. MICROCHECKER detected the possible presence of null alleles or scoring errors in *Nyc3* and *Nyc10* loci in some samples of raccoon dogs. However, the Brookfield frequency indicates that the heterozygote deficiency is very low (0.0938 and 0.1123) and that there was no evidence for linkage disequilibrium (data not shown).

The values of observed (H_O) and expected (H_E) heterozygosities across all loci ranged from 0.482 to 0.933 and from 0.485 to 0.856, respectively (Table 1). Five loci (*FH2054, FH2010, PEZ17, REN112I02*, and *FH2096*) demonstrated a higher number of observed heterozygosities than expected. However, a significant deviation was detected only in 2 of them. A significantly higher number of expected than observed heterozygosities was detected in 4 loci (*Nyct1*, *Nyct3*, *Nyct9*, and *Nyct11*) (Table 1).

Table 2. Summary of genetic variation analysis of raccoon dogs: A_R, P_R, and PIC

Locus	Size (bp)		I			II			III			IV			Mean	
		A_R	P_R	PIC	A_R	P_R	PIC	A_R	P_R	PIC	A_R	P_R	PIC	A_R	P_R	PIC
Nyc1	184–196	7.06	0.23	0.77	7.53	0.85	0.75	7.06	0.60	0.75	7.00	0.05	0.74	7.43	0.43	0.77
Nyc3	233–255	5.08	0.38	0.60	4.95	0.66	0.54	5.55	0.92	0.57	4.00	0.00	0.52	5.25	0.49	0.58
Nyc4	227–249	6.57	0.24	0.73	6.72	0.32	0.73	5.68	0.39	0.71	6.00	0.41	0.67	6.20	0.34	0.72
Nyc5	158–178	7.09	0.71	0.74	6.95	1.14	0.73	4.55	0.00	0.57	5.00	0.28	0.62	6.10	0.53	0.68
Nyc6	146–172	5.77	0.07	0.70	6.48	0.86	0.73	6.96	0.37	0.71	7.00	0.22	0.74	6.50	0.38	0.73
Nyc7	124–148	5.19	0.57	0.63	4.271	0.63	0.61	4.11	0.00	0.57	4.00	0.13	0.50	4.55	0.33	0.61
Nyc8	107–131	6.12	0.99	0.62	6.49	1.66	0.68	4.06	0.15	0.53	4.00	0.30	0.57	5.28	0.78	0.60
Nyc9	157–200	7.99	1.65	0.80	8.75	1.69	0.79	5.12	0.59	0.71	5.00	0.16	0.69	7.19	1.02	0.77
Nyc10	181–188	3.30	0.45	0.44	3.44	0.04	0.44	2.00	0.00	0.34	2.00	0.00	0.36	2.88	0.12	0.40
Nyc11	90–117	4.85	2.06	0.51	4.36	0.34	0.51	2.69	0.44	0.41	4.00	0.54	0.51	4.08	0.85	0.48
Nyc12	161–170	3.77	0.12	0.49	4.28	0.02	0.47	3.27	0.00	0.41	3.00	0.00	0.31	3.67	0.03	0.44
FH2054	134–164	11.0	0.82	0.87	11.0	1.15	0.86	8.61	0.27	0.81	10.0	0.30	0.81	9.77	0.64	0.85
FH2010	218–238	3.80	0.00	0.51	4.94	0.39	0.59	4.11	0.00	0.54	5.00	0.17	0.59	4.37	0.14	0.56
PEZ17	186–218	9.89	0.68	0.87	8.48	0.16	0.85	8.59	0.00	0.84	8.00	0.36	0.81	9.22	0.30	0.87
REN112I02	240–266	7.05	0.40	0.76	6.89	0.55	0.76	6.77	0.00	0.69	7.00	0.01	0.72	6.96	0.24	0.74
FH2096	84–106	3.70	0.00	0.53	3.93	0.00	0.60	4.16	0.26	0.59	5.00	1.00	0.69	4.13	0.32	0.59
Total	–	6.14	0.59	0.66	6.12	0.65	0.67	5.20	0.29	0.61	5.38	0.25	0.62	–	–	–

Table 3. Pairwise F_{ST} (above diagonal) and Nei's genetic distance D_{Nei} (below diagonal) between Lithuanian raccoon dogs in 4 sampling areas

Populations	I	II	III	IV
I		0.0008	0.0027*	0.0068
II	0.034		0.0031*	−0.0007
III	0.027	0.029		0.0009
IV	0.070	0.053	0.047	

*Significant difference following Bonferroni correction (adjusted critical $P = 0.008333$).

The mean values of the observed and expected heterozygosities estimated across the sampling areas were $H_O = 0.699$ (ranged from 0.647 to 0.749) and $H_E = 0.684$ (ranged from 0.661 to 0.709), respectively.

Genetic differentiation and population structure

We investigated the genetic differentiation among subpopulations designated a priori (that corresponding with sampling areas in different geographical regions according to fragmentation by 2 main roads of Lithuania) (Figure 1). Nei's genetic distances (D_{Nei}) and F_{ST} analysis indicated a low genetic differentiation between all pairs of subpopulations (Table 3). Significant F_{ST} values were found between subpopulation I and III, and II and III (Table 3). Pairwise values between other combinations were not significant (Table 3).

The AMOVA based on R_{ST} estimated that 8% of the genetic variation is attributable to differences among the sampled locations and 91% of the genetic variation—within the locations, whereas based on F_{ST} it estimated 1% of the genetic variation among the sampled locations.

The FCA analysis, as shown in Figure 2, did not reveal distinct clustering for the samples from different locations. The subpopulations partially overlap and are not clearly separable from each other. However, a tendency of separation of individuals from different sampling localities is also observed (Figure 2).

The Mantel test based on correlation between the pairwise geographic and genetic distances observed across all collection localities revealed a weak but significant ($r = 0.229$, $P = 0.001$) correlation (Figure 3). A relatively high regression coefficient and significant association between genetic and geographic distances was obtained for the individuals from combined subpopulations I and II (western part; $r = 0.432$, $P = 0.001$), and a weak but significant ($r = 0.188$, $P = 0.029$) correlation for the individuals from combined subpopulations I and III. However, there was no significant association between genetic and geographic distances for the individuals of the combined subpopulations III and IV (eastern part; $r = 0.081$, $P = 0.250$). This result demonstrated west–east direction in observed correlation between genetic and geographical distances.

Bayesian population structure

We used Bayesian clustering analyses to determine the hidden population structure of raccoon dogs in Lithuania, and to find out whether the geographical grouping of samples corresponded with genetic groups. The ΔK values were the highest when 4 genetically distinct clusters were identified. Plots of mean estimates of log-likelihood probability of data (Ln $P[K]$) and the ΔK (mean ($|L''[K]|$/sd($L[K]$)) versus K values generated by STRUCTURE simulations of the data from 4 sample areas are shown in Figure 4A and B, respectively. A sharp peak of ΔK at 9.69 for $K = 4$ indicated 4 genetic clusters according to the genotypic variations. However, these 4 clusters did not correspond to the 4 a priori designated subpopulations (Figure 4). Although a single cluster dominates in some sampling localities from the coastal area (Figures 1 and 4), no clear geographical clustering of raccoon dogs was observed. All genetic clusters with low to high confidence were found in most locations, and each of the 4 subpopulations had more than 1 cluster (Figures 1 and 4).

Discussion

In the present study, the genetic diversity and the population structure of invasive *N. p. ussuriensis* in colonized areas in the Baltic region were for the first time investigated using microsatellite markers. Our investigations show that the invasive raccoon dogs in Lithuania exhibit a high level of genetic diversity. The mean values

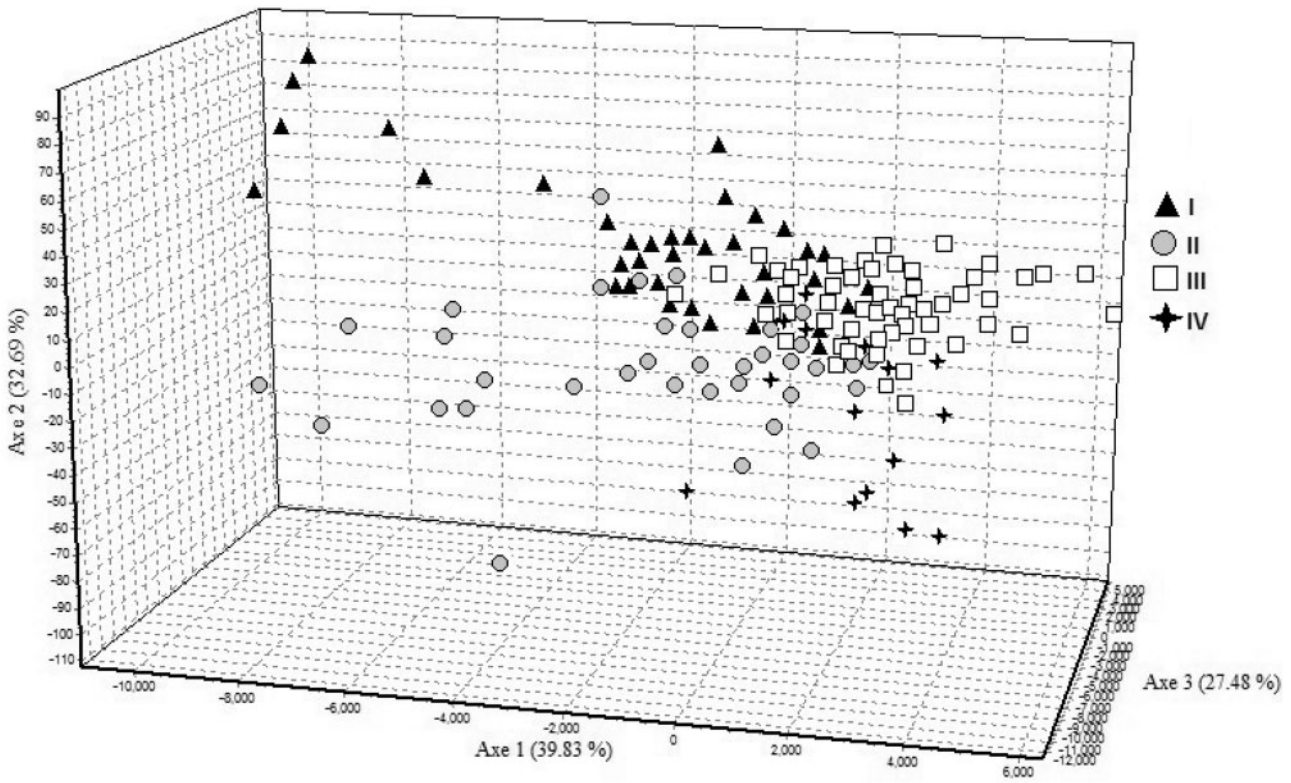

Figure 2. Spatial presentation of the distribution pattern of *N. procyonoides* from 4 sampling areas obtained by FCA.

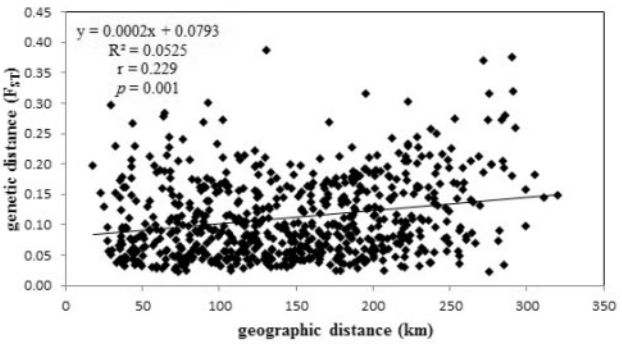

Figure 3. The relationship between geographic (in kilometer) and genetic distances (F_{ST}) among the 37 sample sites.

of the observed and expected heterozygosities estimated across the sampling areas were 0.699 and 0.684, respectively,

In order to compare the genetic diversity of raccoon dogs from their recently expanded range (in Lithuania) and natural range (in South Korea), we analyzed polymorphisms in 11 microsatellite loci developed for *N. p. koreensis* by Hong et al. (2013). We have detected similar genetic variation patterns in the investigated loci of raccoon dogs as compared with previously published on South Korean dogs (Hong et al. 2013). The number of alleles per locus (*A*) ranged from 4 to 14 and from 4 to 13 in the invasive *N. p. ussuriensis* and the endemic *N. p. koreensis* (Hong et al. 2013), respectively. The average number of alleles over all loci was slightly higher in Lithuanian raccoon dogs (Table 1). The mean value of observed heterozygosity was higher in Lithuanian populations, whereas the mean value of expected heterozygosity was higher in South Korean raccoon dogs' populations. Allele size ranges in the raccoon dog populations in South Korea were narrower (excluding 1 locus—*Nyct12*) than those of raccoon dog populations in Lithuania (Table 2).

Our previous study (Paulauskas et al. 2015) has showed a higher genetic variation of the mitochondrial DNA of raccoon dogs from Lithuania compared with those from Northern and Western Europe (Pitra et al. 2010), but lower genetic diversity compared with raccoon dogs from South Korea (Korablev et al. 2011; Paulauskas et al. 2015). Phylogenetic analysis indicates different invasion corridors of the raccoon dog in Lithuania.

Multiple introductions in different years and from different locations as well as the admixture and the subsequent intraspecific hybridization of invasive populations could explain the high genetic diversity in microsatellite loci of the Lithuanian raccoon dog population observed in the present study.

In the present study, higher heterozygosity levels were reported in subpopulations I and II situated in the western part of country, than in subpopulation from the eastern part (III and IV) (Table 1; Figure 1). The observed heterozygosities (H_O) were higher than the expected (H_E) in 3 subpopulations (I, II, and III) (Table 1). However, in subpopulation IV the observed heterozygosity (H_O) was estimated to be lower than the expected heterozygosity (H_E) (Table 1).

The genetic differentiation among subpopulations based on Nei's genetic distances and F_{ST} analysis was very low. Significant F_{ST} values were observed between the western (sampling areas I and II) and eastern subpopulations (III) (Table 3). According to R_{ST} estimates (AMOVA), only 8% of the genetic variation was attributable to differences among the sampled locations. Although STRUCTURE and FCA analyses did not reveal a clear separation between subpopulations, it indicated a difference in genotype and allele frequencies

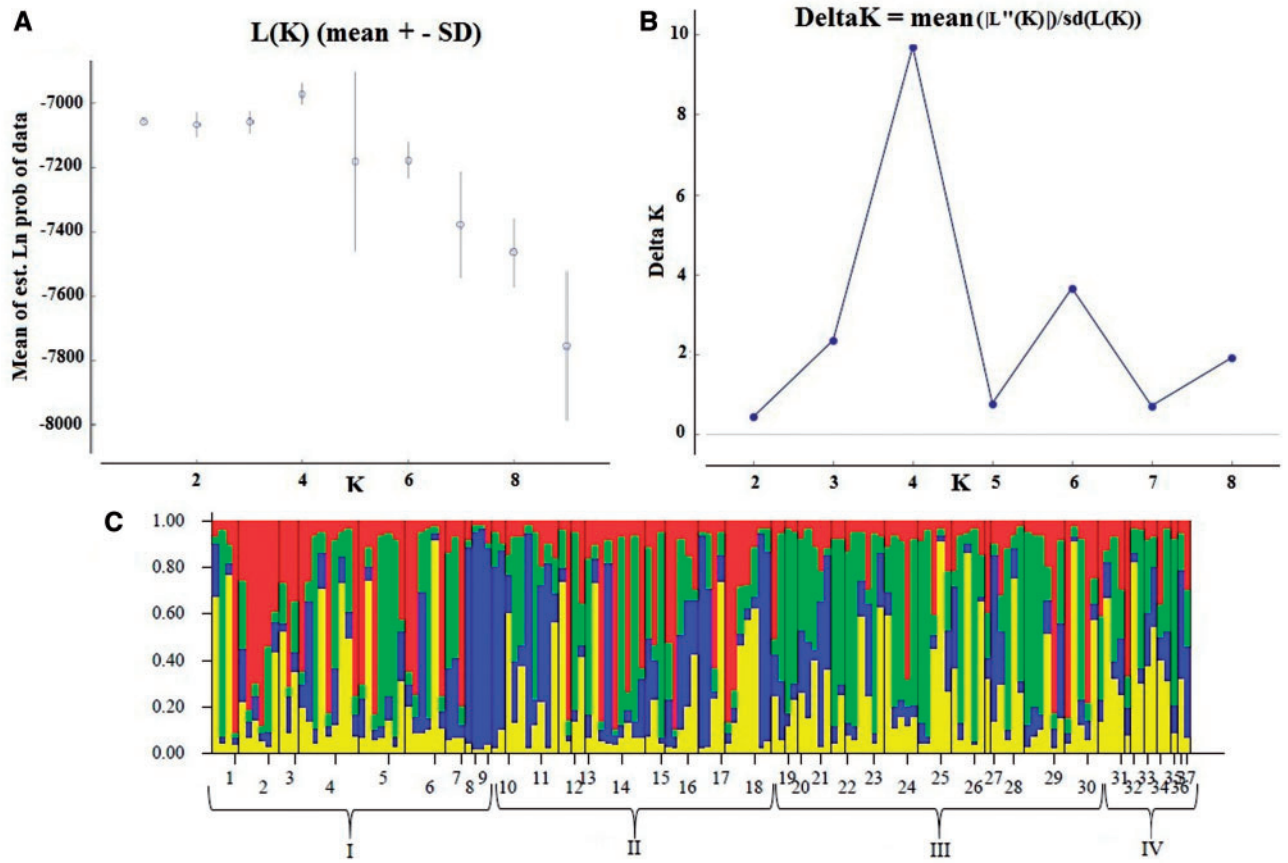

Figure 4. Clustering analysis of raccoon dog genotypes from Lithuania using Bayesian assignment. (A) Mean likelihood [$L(K) \pm$ SD] over 10 runs assuming K clusters ($K = 1$–8). (B) ΔK, where the modal value of the distribution is considered as the highest level of structuring. (C) Individual assignment to the $K = 4$ clusters. Each individual is represented by a bar, with colored sections indicating the likelihood of assignment to the corresponding cluster.

between the western and eastern subpopulations (Figures 2 and 4). The IBD test confirmed this structure (Figure 2).

The widespread distribution of raccoon dogs in Lithuania, high level of genetic variation within the raccoon dog subpopulations and low level of variation portioned among subpopulations suggest migration and gene flow among locations and may give the appearance of a lack of population structure. However, the pattern of IBD indicated weak population structure and demonstrated west–east direction in the observed correlation between the genetic and geographical distances.

As it was suggested by the previous population study on raccoon dog conducted using mtDNA markers (Pitra et al. 2010), the power of correlation between pairwise genetic divergence and pairwise geographic distance could depend on the length of time because a territory was colonized. So, the impact of distance on the genetic structure of Lithuanian raccoon dog population could become more pronounced in the future. The result of Mantel test obtained for combined subpopulations (I and II; and III and IV) supported the genetic differentiation between western (I, II) and eastern (III, IV) subpopulations.

Anthropogenic pressure and behavior may play a more significant role in the formation of the raccoon dog population structure. The raccoon dog has a high dispersal capability that is a necessary precursor for the gene flow. Raccoon dogs have colonized a territory of 1.4 million km within 50 years (1935–1984) in Europe (Sutor 2008). Generally, raccoon dogs disperse when they have reached sexual maturity at the age of 8–10 months, but according to some investigations, it seems that dispersal could take place at an earlier age (Sutor 2008). There is also evidence that not only juveniles but even unmated adults disperse (Sutor 2008). However, a species' ability to disperse does not depend only on its ability and propensity to move from one site to another. Physical barriers may also hinder dispersal and prevent gene flow. The obtained moderate genetic differentiation among the raccoon dog populations in South Korea occurs due to such geographical barriers as high mountain ranges, which separated the populations, and geographical distances between the populations (Hong et al. 2013). In Lithuania, no such natural geographical barriers exist and other barriers, created due to anthropogenic activity, such as main roads with the high volume of traffic, could reduce gene flow and affect the formation of population structure of raccoon dogs. The effect of geographic distance on genetic differentiation and significant genetic differences observed between the eastern and western subpopulations could occur due to the habitat fragmentation by the "Via Baltica" highway that stretches from the south to the north of Lithuania (Figure 1) with a traffic volume of 4,000–8,000 vehicles and more than 2,500 heavy vehicles' per day, and by the fenced Vilnius–Kaunas highway.

The high level of genetic diversity of the raccoon dog populations in Lithuania could be one of the factors that led to the high population viability, which can be realized through resistance to diseases, physical condition of an individual, low embryonic mortality rate, and many other features. The results of the present study provide a

foundation for subsequent studies on the partitioning of genetic variation across Lithuanian populations.

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