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Inter and Intra Subpopulation Genetic Variability of Roe Deer (*Capreolus capreolus* L.) Assessed by I and II Class Genetic Markers

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The material was collected in three regions of Poland and consisted of 105 randomly chosen individuals killed during hunts (49 males, 56 females), out of which 51 were from Wielkopolska, 22 from Podkarpacie and 32 from Warmia. From each animal a blood sample was taken from the chest, stored in a probe with K_2EDTA and frozen. The serum was used to establish the genotype for transferin and albumin whereas the samples with erythrocytes provided information on hemoglobin genotype. DNA was isolated from samples from each individual. Characteristics of eight (from among twelve studied) microsatellite loci and genetic distances were estimated by the use of standard computer package programs. Generally, monomorphism in blood proteins was registered. For the microsatellite loci the number of alleles ranged from 3 in the RT27-6-Fa locus (effectively two as the third allele was present only in two subpopulations with a very low frequency) to 10 in RT1-VI. Five loci showed heterozygosity of 0.5 or above which suggests their usefulness in parentage control. Considerable genetic distances (corresponding to geographical mileages) between the subpopulations were observed based on microsatellite markers.

Key words: Biodiversity, microsatellites, roe deer.

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Roe deer (*Capreolus capreolus*) is the most common representative of big free living mammals in Poland. Its population size in 2007 was estimated at about 706000 individuals. The density of the species shows high variability within the country. The highest frequency is registered in western Poland, which is a consequence of high numbers of roe deer in forests as well as the presence of so called field roe deer (KAMIENIARZ & PANEK 2008). This ecological form developed in central Europe probably at the turn of the 19th and 20th century and is characterized by living in open agricultural areas avoiding forests (PIELOWSKI 1999). Consequently, anatomical (KAŁUZIŃSKI 1982), behavioral (BRESIŃSKI 1982) and physiological (MAJEWSKA *et al.* 1982) differences between ecotypes have been observed.

Roe deer live across the whole area of Poland in very diverse environments. As a consequence subpopulations are formed differing in body weight and quality of antlers (FRUZIŃSKI *et al.* 1982; PIELOWSKI 1999). The diversification of the subpopulation is increased by migration barriers such as fenced highways. The length of high speed roads in Poland will increase in the coming years because of modernization and extension of infra-

structure within the Trans-European Transportation Network. As a result, fragmentation of areas occupied by wild animals will increase, even leading to the isolation of some populations. Passages for animals built over highways and express roads are often incorrectly situated and defectively constructed which restricts or even prevents animals from using them (JĘDRZEJEWSKI *et al*. 2006).

The objective of the study was to estimate inter- and intra- subpopulation genetic diversity of roe deer including both ecological forms by the use of blood protein polymorphism and microsatellite markers.

Material and Methods

Animals

The study was undertaken on subpopulations of roe deer from three breeding centers located in different provinces: Wielkopolska province – Czempiń; Podkarpacie province – Rudnik on San; and Warmia province – Gierłoż, denoted as pop1, pop2 and pop3, respectively. The material consisted of 105 randomly chosen individuals killed during hunts (49 males, 56 females), out of which 51 were from Wielkopolska, 22 from Podkarpacie and 32 from Warmia.

From each animal a blood sample was taken from the chest, stored in a probe with K_2EDTA and frozen. In the next step DNA was isolated from the samples. If there was a possibility of analysis without freezing, two additional blood samples were taken to test I class markers. The sample from a sterile probe was used to extract serum, and blood conserved with 6% sodium citrate was a source of erythrocytes. In this way material from 46 random individuals (11 males, 35 females) was obtained (20 from Wielkopolska, 17 from Podkarpacie and 9 from Warmia).

Blood markers

Horizontal electrophoresis on starch gels was used in the analysis of first class markers (SMITHIES 1955). The serum was used to establish the genotype for transferin and albumin whereas the samples with erythrocytes provided information on hemoglobin genotype.

Microsatellite markers

Primers (Table 1) were designed based on bovine sequence. For each primer set, amplification of microsatellite loci was carried out in 10-μl reactions containing: 10 pmol of each primer, 0.4U Taq polymerase (Super-Therm Polymerase, Qia-

Table 1

Information for 12 microsatellite loci

Marker	Primer sequences (5'-3')	Annealing temperature (^{o}C)
RT1	5'TGCCTTCTTTCATCCAACAA 5'CATCTTCCCATCCTCTTTAC	54
RT6	5'TTCCTCTTACTCATTCTTGG 5'CGGATTTTGACACTGTTAC	50
RT7	5'CCTGTTCTACTCTTCTTCTC 5'ACTTTTCACGGGCACTGGTT	56
RT9	5'TGAAGTTTAATTTCCACTCT 5'CAGTCACTTTCATCCCACAT	56
RT13	5'GCCCAGTGTTAGGAAAGAAG 5'CATCCCAGAACAGGAGTGAG	54
RT23	5'GGCCATTGGGTAGTCTCC 5'AGCCTCCCTGAGTGCTCT	54
RT27	5'CCAAAGACCCAACAGATG 5'TTGTAACACAGCAAAAGCATT	56
RT30	5'CACTTGGCTTTTGGACTTA 5'CTGGTGTATGTATGCACACT	54
NVHRT16	5'ATTCTAAGCCCAAATAATCTT 5'TCTAAGGGGTCTGTGTCTT	54
NVHRT21	5'GCAGCGGAGAGGAACAAAAG 5'GGGGAGGAGCAGGGAAATC	54
NVHRT48	5'CGTGAATCTTAACCAGGTCT 5'GGTCAGCTTCATTTAGAAAC	52
NVHRT73	5'CTTGCCCATTTAGTGTTTTCT 5'TGCGTGTCATTGAATAGGAG	54

gen) and 1 μl of DNA template (ca. 50 ng). Cycling conditions for each locus were as follows: initial denaturation at 95°C for 10 min, followed by 30 cycles at 95°C for 30 s, annealing temperature for 1 min, 72°C for 1 min and final extension at 65 C for 45 min. Annealing temperatures for each of twelve loci are presented in Table 1. Before electrophoresis, the PCR mixture was diluted with water (from 1:10 to 1:100) and pooled. The amplified alleles were separated by ABI PRISM 3130XL capillary electrophoresis (Applied Biosystems) using Genescan 600 LIZ size standard from the same manufacturer. Alleles were scored manually, using Peak Skanner Software v1.0 (Applied Biosystems).

Statistical analysis

Each locus was characterized by a number of alleles detected in the population (subpopulations) and the heterozygosity observed. The informativeness of loci was described by expected heterozygosity under Hardy-Weinberg equilibrium, polymorphic information content (PIC) and average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex, for a candidate parent pair, for identity of two unrelated individuals and non-exclusion of sibs. Hardy-Weinberg equilibrium was tested for each locus using a chi-square test including Yates correction for classes with low expected frequencies and Bonferroni correction for multiple testing. As there may exist some alleles which are not detected using a given set of primers, each locus was tested for the presence of so called null alleles. The CERVUS program (MARSHALL *et al*. 1998) was used for the above listed computations. Genic differences, allele size based covariance (rhoIS) and diversity (MSD: mean squared allele size difference) were calculated using GENEPOP (ROUSSET 2008). Various genetic distance measures were calculated using MICROSAT (MINCH *et al*. 1997). Phylogenic trees were constructed using Nei's genetic distance (NEI 1972), Cavalli-Sforza's chord measure (CAVALLI-SFORZA & EDWARDS 1967) and REYNOLDS *et al*. (1983) genetic distance distances in PHYLIP (FELSENSTEIN 1989) and edited in TREEVIEW (PAGE 1996).

Results and Discussion

Class I markers

In this study, low variability was present only in the hemoglobin (only one heterozygous individual was registered). This corresponds with results obtained by other authors for both wild animals and livestock populations. HARTL *et al.* (1991) found monomorphism for several loci of roe deer in three central Europe countries (Austria, Hungary and Switzerland). On the other hand, some differentiation in the populations has been observed. Furthermore, for different local livestock breeds, the variability of hemoglobin is low or absent. In Kenyan sheep: kwale, makueni and siaja only Hb^B allele was found, whereas in kakamega and kajiando breeds also the Hb^A allele was present with a respective frequency of 0.006 and 0.017 (MWACHARO *et al*. 2002). Negligible variation of biochemical markers has been reported for roe deer from five populations in Austria (HARTL & REIMOSER 1988). Unfortunately, the results of the present study confirmed low usefulness of the so-called blood markers in genetic analysis of wild animal populations.

As stated above, the studies on genetic variability in roe deer in Poland based on I class markers show low diversity. The homozygous genotype of transferin confirms previous studies by HERZOG *et al*. (1993), who reported a lack of genetic variability for this locus in a German population of roe deer. The authors suggested that monomorphism was caused by selection rather than by drift. Studies carried out in Brazil on 147 marsh deer (*Blastocerus dichotomus*) living in three subpopulations showed monomorphism of transferin, however at the same time two alleles in albumin were present: Al^A i Al^B. Allele Al^B was detected only in one of three subpopulations with a frequency of 0.079 (de OLIVEIRA *et al*. 2005). On the other hand, some authors obtained considerable polymorphism for the hemoglobin locus, for instance in Indian Zebu cattle and Indian buffalo (SEN *et al*. 1966). One must therefore be cautious in making any generalization in animal population studies.

In the case of Polish roe deer monomorphism was also detected in the albumin locus but considering the low frequency of alternative alleles in the related species (*Blastocerus dichotomus*), this finding could have been caused by a small amount of available data.

Microsatellite markers

Nine (out of twelve) chosen bovine microsatellite markers were successfully amplified for roe deer. However, one of them was basically monomorphic (only one heterozygous individual was registered). Hence, this locus has been omitted in the present study. Finally, eight loci were analysed. The description of allele frequencies and a measure of their informativeness is included in Table 2. The number of alleles ranged from 3 in

Table 2

Intrapopulational variability based on microsatellites

Locus	k	N	HObs	HExp	PIC	NE-1P	NE-2P	NE-SI	HW	F(Null)
Population 1										
NVHRT48-VI	4	46	0.457	0.512	0.448	0.867	0.733	0.572	NS	0.061
RT27-6-Fa	3	46	0.522	0.405	0.342	0.920	0.816	0.654	NS	-0.144
NVHRT16-VI	6	46	0.870	0.740	0.698	0.663	0.481	0.411	NS	-0.091
NVHRT21-NE	8	46	0.848	0.843	0.813	0.501	0.330	0.345	NS	-0.007
RT7-6-Fa	9	44	0.750	0.832	0.800	0.522	0.349	0.352	NS	0.050
RT1-VI	9	45	0.867	0.870	0.845	0.441	0.281	0.329	ND	-0.008
NVHRT73-NE	5	46	0.565	0.580	0.531	0.820	0.655	0.519	NS	0.003
RT13-PE	9	48	0.792	0.845	0.818	0.487	0.318	0.343	NS	0.035
Combined						0.024	0.002	0.001		
Population 2										
NVHRT48-VI	4	32	0.438	0.454	0.409	0.897	0.756	0.613	NS	-0.015
RT27-6-Fa	3	32	0.344	0.298	0.265	0.957	0.858	0.735	ND	-0.089
NVHRT16-VI	6	31	0.710	0.753	0.697	0.667	0.491	0.407	NS	0.025
NVHRT21-NE	7	32	0.906	0.761	0.712	0.648	0.469	0.400	*	-0.112
RT7-6-Fa	7	30	0.633	0.682	0.636	0.725	0.544	0.451	NS	0.022
RT1-VI	8	30	0.700	0.770	0.722	0.631	0.453	0.395	NS	0.034
NVHRT73-NE	5	31	0.387	0.563	0.513	0.831	0.668	0.533	NS	0.195
RT13-PE	7	30	0.933	0.800	0.753	0.599	0.421	0.376	NS	-0.088
Combined						0.085	0.010	0.003		
Population 3										
NVHRT48-VI	4	20	0.250	0.315	0.291	0.951	0.833	0.720	ND	0.085
RT27-6-Fa	2	20	0.400	0.328	0.269	0.949	0.866	0.718	ND	-0.110
NVHRT16-VI	6	20	0.600	0.695	0.650	0.713	0.526	0.444	NS	0.031
NVHRT21-NE	8	20	0.900	0.862	0.820	0.490	0.321	0.341	ND	-0.036
RT7-6-Fa	5	20	0.650	0.709	0.634	0.734	0.569	0.442	NS	0.023
RT1-VI	9	20	0.900	0.871	0.831	0.467	0.301	0.336	ND	-0.030
NVHRT73-NE	4	20	0.250	0.235	0.220	0.973	0.878	0.786	ND	-0.059
RT13-PE	9	21	0.762	0.763	0.712	0.640	0.461	0.402	NS	-0.020
Combined						0.067	0.008	0.004		
Total										
NVHRT48-VI	4	98	0.408	0.458	0.414	0.892	0.753	0.606	NS	0.052
RT27-6-Fa	3	98	0.439	0.354	0.306	0.938	0.838	0.690	NS	-0.119
NVHRT16-VI	6	97	0.763	0.740	0.703	0.657	0.475	0.408	NS	-0.018
NVHRT21-NE	8	98	0.878	0.844	0.820	0.490	0.320	0.341	NS	-0.024
RT7-6-Fa	9	94	0.691	0.778	0.743	0.602	0.424	0.384	NS	0.058
RT1-VI	10	95	0.821	0.860	0.840	0.450	0.288	0.331	NS	0.020
NVHRT73-NE	5	97	0.443	0.524	0.495	0.847	0.676	0.553	NS	0.083
RT13-PE	9	99	0.828	0.819	0.793	0.530	0.356	0.357	NS	-0.006
Combined						0.033	0.003	0.001		

RT27-6-Fa (effectively two as the third allele was present only in two subpopulations with a very low frequency) to 10 in RT1-VI. Five loci (NVHRT16-VI, NVHRT21-NE, RT7-6-Fa, RT1-VI, RT13-PE) exhibited a heterozygosity of 0.5 or above, which suggests their usefulness in parentage control. Similar levels of heterozygosity were estimated within subpopulations despite a higher number of alleles segregating in subpopulation 2. The non-exclusion probability was high for single loci, however, if the information was combined across loci, reliable information about parentage was obtained for both the total population and subgroups. No deviations from Hardy-Weinberg equilibrium were estimated at the population level, the significant results in subpopulation 2 were caused by the presence of some rare gene variants. The mean frequency of private alleles was equal to 0.125,

Table 3

Genetic distances between subpopulations derived by various methods

Measurement od distance	pop1-pop2	pop1-pop3	pop2-pop3
D1: average square	23.949	21.110	19.752
Gst - Nei standard transgormed by ln	0.073	0.019	0.062
Gst - Nei standard transgormed by 1-Gst	0.071	0.019	0.060

Table 4a

Genic differentiation for each population pair (exact G test)

Locus	Population pair	P-value	S.E.
NVHRT48-VI	pop1-pop2	0.7177	0.0039
NVHRT48-VI	pop1-pop3	0.0656	0.0022
NVHRT48-VI	pop2-pop3	0.3114	0.0037
RT27-6-Fa	pop1-pop2	0.3403	0.0045
RT27-6-Fa	pop1-pop3	0.3637	0.0023
RT27-6-Fa	pop2-pop3	0.3781	0.0023
NVHRT16-VI	pop1-pop2	0.0935	0.0030
NVHRT16-VI	pop1-pop3	0.6813	0.0045
NVHRT16-VI	pop2-pop3	0.0694	0.0024
NVHRT21-NE	pop1-pop2	0.0000	0.0000
NVHRT21-NE	pop1-pop3	0.2168	0.0051
NVHRT21-NE	pop2-pop3	0.0150	0.0012
RT7-6-Fa	pop1-pop2	0.0017	0.0004
RT7-6-Fa	pop1-pop3	0.0146	0.0013
RT7-6-Fa	pop2-pop3	0.0423	0.0020
RT1-VI	pop1-pop2	0.0000	0.0000
RT1-VI	pop1-pop3	0.1295	0.0043
RT1-VI	pop2-pop3	0.0002	0.0001
NVHRT73-NE	pop1-pop2	0.0008	0.0003
NVHRT73-NE	pop1-pop3	0.0091	0.0007
NVHRT73-NE	pop2-pop3	0.0425	0.0018
RT13-PE	pop1-pop2	0.0098	0.0010
RT13-PE	pop1-pop3	0.3043	0.0058
RT13-PE	pop2-pop3	0.1295	0.0037

Table 4b

Diversity indices for populations studied

Taxon	Fst Het	Avg Het	Tot Het	Avg var	Tot Var	Avg All	Tot All	Avg Ran	Tot Ran	Avg Max	Tot Max	Avg Ent	Tot Ent
pop1	0.27	0.696	0.954	12.287	1017	6.625	39	11.25	115	102	155	0.617	0.701
pop2	0.33	0.625	0.938	11.326	1008	5.875	38	10.75	115	102	155	0.536	0.650
pop3	0.38	0.582	0.939	7.125	1010	5.875	38	8.88	115	100	155	0.586	0.655
Average	0.33	0.634	0.944	10.246	1012	6.125	38	10.29	115	102	155	0.580	0.669

which when corrected for population size gives an estimate of 0.338 migrants between populations. A 114bp allele in the RT7-6-Fam locus was the only taxon (pop1) specific allele.

Estimates of genetic distances are listed in Table 3. Although genetic diversity was observed within the subpopulations, the results of paired subpopulation comparisons were considerably affected by the criteria used. The methodological aspects are not discussed in the present study. It should be stressed that the phylogenetic tree based on a standard Nei method (NEI 1972) indicates the largest genetic distance between subpopulation 1 and 2 (see Fig. 1). Phylogenetic trees were similar for the

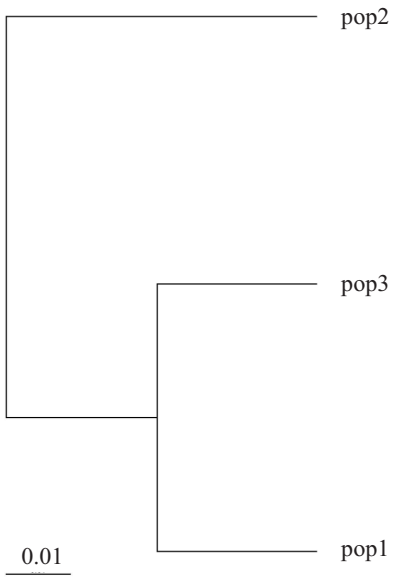

Fig. 1. Phylogenic tree using NEI (1972) distance.

three distance measures. Across all loci each pair of subpopulations showed highly significant intra loci differentiation, for within locus differentiation. The P-values for paired groups are listed in Table 4a. For five loci differentiation in the analyzed populations was highly significant ($P<0.01$). This concerned seven pairwise subpopulation combinations. In the case of subpopulations 1 and 2, differences for all five loci were significant whereas differences between population 1 and 3, as well as 2 and 3, were significant within locus NVHRT73-NE and RT1-VI, respectively.

Generally, the obtained results indicate a relatively large similarity of these subpopulations (Table 4b). Despite some natural barriers and geographic distance, gene flow between these groups was possible, ensuring genetic variation. By contrast to some species of livestock (e.g. LEMUS-FLORES *et al*. 2001), roe deer do not tend to differentiate genetically in one geographic region. This is likely to be connected with directional selection with controlled mating. On the other hand some authors (VERNESI *et al*. 2002; ROYO *et al*. 2007) using molecular (microsatellites and mitochondrial) markers reported relatively large genetic variability in roe deer in western and southern Europe. Also ZACHOS *et al*. (2006) reported the results of a genetic analysis of roe deer populations in different European countries. Relatively small genetic differentiation of the species can be explained by the demographic history of roe deer in some parts of Europe. In the 19th century, roe deer populations were nearly driven to extinction through relentless persecution (ZACHOS *et al*. 2006). A similar historical background for the populations in Poland can be hypothesized. The animals recorded from these three regions are relatively distant. However, they can cross rivers and a number of industrial barriers.

Comprehensive knowledge of genetic diversity is the first step for conservation of a given population (LI *et al*. 2008). The results of the present work indicate a similarity of the subpopulations. However, it should be emphasized that this conclusion was based on only twelve loci. Further study should cover more loci, including mitochondrial ones as well.

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