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

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Keywords
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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 Podkarpackie and 32 from Warmia. From each animal a blood sample was taken from the chest, stored in a probe with K3EDTA and frozen. The serum was used to establish the genotype for transferrin 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 RT14-4-Falocus (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 (KALUZINSKI 1982), behavioral (BRESINSKI 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 (FRUZINSKI 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 – Czempin; Podkarpacie province – Rudnik on San; and Warmia province – Gierloz, 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$_2$EDTA 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-

| Marker | Primer sequences (5’-3’) | Annealing temperature (°C) |
|--------|--------------------------|---------------------------|
| RT1    | 5’TGCCCTTCTTTTCATCACCACA   | 54                        |
|        | 5’CATCTTCCCATCCTTTTAC     |                           |
| RT6    | 5’TCCCTCTTACTCATTTCTTG    | 50                        |
|        | 5’CGGATTTGGACACTGTTAC     |                           |
| RT7    | 5’CTGTGGCTTCATCTCTTCCT    | 56                        |
|        | 5’ACTTTTCACGGGGCACTGTTT   |                           |
| RT9    | 5’TGAATTTTAAATTCCACTCT    | 56                        |
|        | 5’CATGCACCTTCATCCCCACAT   |                           |
| RT13   | 5’GCCCATGTTAGGAAAGAAG     | 54                        |
|        | 5’ATCCCAAGAACAGGAGTGAG    |                           |
| RT23   | 5’CGGATTGGGCTAGTCTCC      | 54                        |
|        | 5’AGGCTCCCTGAGTGCTCT      |                           |
| RT27   | 5’CCAAGAAGCCAACAAGATG     | 56                        |
|        | 5’TGTAAACACAGCAAAAGCATT   |                           |
| RT30   | 5’CACTTGGCTTTTGGACTTA     | 54                        |
|        | 5’CTGTTGATATGATGACACT     |                           |
| NVHRT16| 5’ATTCTTAAAGCACCATAATCCT  | 54                        |
|        | 5’TCTAAGGGCTGTGCTCT       |                           |
| NVHRT21| 5’GCAGCGGAGGAGGACAAAAAG   | 54                        |
|        | 5’GGGAGGAGGCAGGGAAAATC    |                           |
| NVHRT48| 5’CGTGAATCTTAAACAGGTCT    | 52                        |
|        | 5’GGTCAGCTTCTTTAGAAAAC    |                           |
| NVHRT73| 5’CTTGCCCAATTAGTGTCTTCT   | 54                        |
|        | 5’TGCCTGTCAITGGATAGGAG    |                           |
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 Hbα allele was found, whereas in kakamega and kajiando breeds also the Hbα 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 1 class markers show low diversity. The homozygous genotype of transferrin 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 transferrin, however at the same time two alleles in albumin were present: A1α i A1β. Allele A1β 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...
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,

| Locus            | k | N  | HOb | HExp | PIC  | NE-1P | NE-2P | NE-SI | HW | F(Null) |
|------------------|---|----|-----|------|------|-------|-------|-------|----|---------|
| 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     |   |    |      |      |      | 0.024 | 0.002 | 0.001 |    |          |
| 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     |   |    |      |      |      | 0.085 | 0.010 | 0.003 |    |          |
| 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            |   |    |      |      |      | 0.067 | 0.008 | 0.004 |    |          |
| 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 | ND | -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 |    |          |
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

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

Table 3

Genetic distances between subpopulations derived by various methods

| 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 4a

Genetic differentiation for each population pair (exact G test)

| 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   |

Table 4b

Diversity indices for populations studied
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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