

## MICROSATELLITE VARIATION IN DUTCH ROE DEER (*CAPREOLUS CAPREOLUS*) POPULATIONS

by

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### ABSTRACT

In this study we investigated microsatellite variation in Dutch roe deer (*Capreolus capreolus*) populations. We used 65 tissue samples from culled animals from three populations (Amsterdamse Waterleidingduinen, National Park Zuid-Kennemerland and Flevopolder). The first two are dune populations and are about 3.5 kilometers apart. In both populations, roe deer have been present since the early 1950's. In the Flevopolder roe deer were first observed in 1959. From theoretical predictions, a decrease in heterozygosity of 20% due to genetic drift could be expected in the small Amsterdamse Waterleidingduinen population, compared to the much larger Flevopolder population. However, the expected heterozygosity ( $H_e$ ) at seven loci was 0.56 on average with no significant differences in  $H_e$  between populations. The probability that a decrease of 20% or more did occur but that it went undetected is smaller than 2.2%. All populations were significantly differentiated from each other and showed positive  $F_{ST}$  and Rho values, suggesting limited gene flow between populations. The fact that the decrease of genetic variation was smaller than expected is probably due to gene flow between the Amsterdamse Waterleidingduinen and National Park Zuid-Kennemerland, effective population size at the time of introduction being larger than assumed, and/or because the animals which were used for stocking came from different populations. Considering the amount of variation still present in the different populations, negative effects of reduced genetic variation are currently not expected, but in both dune populations the decrease in the amount of variation will continue.

KEY WORDS: genetic variation, roe deer, *Capreolus capreolus*, microsatellites, differentiation.

### INTRODUCTION

Roe deer (*Capreolus capreolus*) numbers in the Netherlands have shown rapid growth during this century. According to field observations, most roe deer are part of a single, continuous population. There are, however, a few populations that are geographically isolated. Because roe deer are known to be very good colonizers and can travel long distances (DANILKEN & HEWISON, 1996), gene flow into these populations might still be possible.

Small isolated populations are expected to lose genetic variation relatively fast because of increased genetic drift. Examples of such populations might be those in the Amsterdamse Waterleidingduinen (AWD) (3400 ha) and in the National Park Zuid-Kennemerland (NPZK) (2500 ha), to the south and to the north of Zandvoort, respectively (fig. 1).

Various research groups have investigated genetic variability of roe deer. MÖRSCH & LEIBENGUTH (1994) fingerprinted German roe deer using minisatellites. RANDI *et al.* (1998) investigated mitochondrial polymorphism in Russian and Italian populations. FAKLER & SCHREIBER (1997) investigated the AWD and the, also Dutch, Flevopolder population using allozyme polymorphism. The level of population subdivision was lower than expected between Flevopolder and AWD, given the distance between both populations. These results might be influenced by the fact that they used allozyme electrophoresis. Using this method, the amount of detectable polymorphism is relatively low, and is therefore relatively insensitive in detecting population subdivision.

In this study we made use of microsatellites, a class of simple tandemly repeated sequences. They have proven to be sensitive markers for population genetic studies (ANDERSEN *et al.*, 1998; BROWN GLADDEN *et al.*, 1999; VAN HOOFT *et al.*, 1999) since they are highly polymorphic, codominant, abundant throughout the genome and amenable to PCR technology (ROED, 1998).

## MATERIAL AND METHODS

### *Populations*

The main functions of the AWD are water treatment and recreation. In the early 1950's the AWD population started off with the introduction of 11 roe deer from the eastern part of the Netherlands after a buck had been present for some years. Winter-feeding and the absence of competition led to high numbers of up to 500-600 roe deer in 1969. In that year, culling was introduced to avoid starvation due to overpopulation. During the following years, the population was maintained at 160-180 and later at 250 deer. In 1997, it was decided that culling should be cancelled for a period of at least 5 years because it was thought that density dependency would stabilize population size at an acceptable level. The present study is part of the extensive research that accompanies this experiment. Current numbers are estimated to be around 600 at maximum.

Taking into account population numbers since introduction and assuming an approximate relationship between the effective and the numerical population sizes of  $N_e = 0.3N$  (Princée in FAKLER & SCHREIBER,

1997) ( $N_e$  = effective population size,  $N$  = census size), using  $H_t/H_0 = (1 - 1/2N_e)^t$  with  $N_e = 25$  and  $t = 11$ , a decrease in heterozygosity of approximately 20% could be expected, in the absence of gene flow, relative to the large population in the Flevopolder. This is in accordance with estimations by Princée (in FAKLER & SCHREIBER, 1997).

The nearby NPZK population has a similar history as the one in the AWD. Roe deer have existed there since 1954. Animals probably came from the eastern part of the country and the earlier mentioned population in the AWD. By culling, a population size of several hundred individuals is maintained. Theoretically, gene flow between the AWD and NPZK is possible. The distance between both areas is 3.5 kilometers and they are separated by a railway, fences and two busy regional roads with buildings.

The Flevopolder was colonized by roe deer in 1959. The fact that colonization occurred naturally and soon after the polder had fallen dry in 1957, suggests that the roe deer in the Flevopolder are part of a larger population that stretches out to the middle and the east of the Netherlands. The Flevolder population is assumed to be isolated from both dune populations taking into account the distance and the nature of the area between both populations (50 kilometers and highly urbanized) (fig. 1).

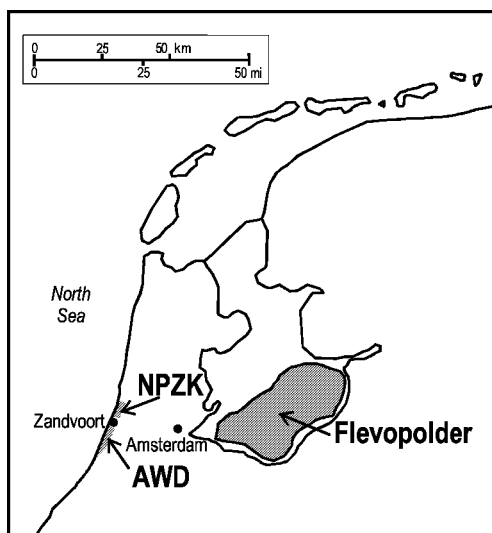


Fig. 1. Location of the Amsterdamsche Waterleidingduinen (AWD), National Park Zuid Kennemerland (NPZK) and the Flevopolder population.

### *Collection of DNA samples*

Spleen and muscle tissue samples were collected from female roe deer, shot in 1998. Despite the abolition of hunting in the AWD, every year 20 roe deer are shot for scientific research. A total of 65 samples were collected in AWD ( $n = 20$ ), NPZK ( $n = 21$ ) and Flevopolder ( $n = 22$ ). AWD and NPZK samples were stored in 80% ethanol and 1 mM EDTA (pH 8.0) at room temperature. Flevopolder samples were kept at  $-20^{\circ}\text{C}$ . DNA was isolated from the samples using a Puregene DNA Isolation Kit (Gentra Systems), following manufacturer's instructions.

### *Microsatellite analysis*

In related species, microsatellites, together with their unique flanking sequence, are preserved to some degree. This allows primers to be used across species (ROED, 1998). The microsatellites used in this study were developed in reindeer (*Rangifer tarandus*). They were selected for the amount of polymorphism shown in Norwegian roe deer. We used the following microsatellites: NVHRT16, -21, -24, -30, -48, -71 and -73 (ROED & MIDTHJELL, 1998).

The polymerase chain reaction (PCR) was carried out on 50 ng of genomic DNA in a 12  $\mu\text{l}$  reaction mixture which contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200  $\mu\text{M}$  dNTPs, 1.5 mM  $\text{MgCl}_2$ , 1 mM tetramethyl-ammonium-chloride, 0.1% Triton x-100, 0.01% gelatin, 46.9 pmol of the primers for NVHRT16, -30 and -73, 93.8 pmol of the primers for NVHRT21, -24, -48 and -71 and 0.25 U Taq-polymerase (Goldstar). Amplification was completed using a PTC-100 machine (M.J. Research), in 35 cycles with annealing temperatures of  $50^{\circ}\text{C}$  for NVHRT48 and -71, and  $45^{\circ}\text{C}$  for NVHRT16, -21, -24, -30 and -73.

Of each primer pair, one was end-labeled with the fluorescent dye TET, 6-FAM or HEX. PCR samples were genotyped on a 6% sequencing gel (Ultrapure Sequagel-6) with Genescan<sup>TM</sup> 350-Tamra as internal standard using a 373 automatic DNA sequencer (Applied Biosystems).

Data were collected, analyzed and genotyped using ABI Prism<sup>TM</sup> 373 collection (version 1.1), Genescan<sup>TM</sup> analysis (version 1.2.2) and Genotyper<sup>TM</sup> (version 1.1) software (Applied Biosystems).

### *Statistical analysis*

Fisher's combined probability test was applied to analyze whether the different populations were significantly differentiated from each other (SOKAL & ROHLF, 1995; RAYMOND & ROUSSET, 1995a). This test was performed using TFGA version 1.3 (MILLER, 1997).

Fisher's exact test for the estimation of linkage disequilibrium was performed using Genepop version 3.1 (updated version of version 2.1, described in RAYMOND & ROUSSET, 1995), using a Markov chain (1000 dememorizations, 50 batches and 1000 iterations per batch).

The amount of genetic diversity present in each population was measured as the mean number of alleles per locus ( $N_A$ ), the observed heterozygosity ( $H_o$ ) and expected heterozygosity under Hardy-Weinberg equilibrium ( $H_e$ ) using GDA version 1.0 (d12) (LEWIS & ZAYKIN, 1999). Assuming linkage equilibrium, standard errors of  $H_o$  and  $H_e$  were calculated using within locus variance according to WEIR (1996).

Significance levels of deviations from Hardy-Weinberg equilibrium were estimated with the score test (ROUSSET & RAYMOND, 1995) using a Markov chain (1000 dememorizations, 50 batches, 2000 iterations per batch). This was done with Genepop version 3.1.

With TFGA version 1.3 Wright's  $F_{ST}$  was estimated by an analysis of variance (WEIR & COCKERHAM, 1984). Confidence intervals (95%) were estimated by bootstrapping (10,000 iterations).

$R_{ST}$  is an analogue of  $F_{ST}$  but, in contrast to  $F_{ST}$ , is specially adapted to microsatellite data and assumes a stepwise mutation model instead of an infinite alleles model.  $R_{ST}$  was estimated using  $R_{ST}Calc$  (GOODMAN, 1997). Confidence intervals (95%) were estimated by bootstrapping (10,000 iterations).

## RESULTS

All seven microsatellites were polymorphic in all populations (see Appendix). No significant linkage equilibrium was observed between the loci used, suggesting an absence of genetic linkage between these loci. There were no significant differences between  $H_o$  and  $H_e$ .

Averaged across loci the number of alleles per locus was 3.9 across all populations (table 1).  $H_e$  was  $0.547 \pm 0.025$  for the AWD population,  $0.557 \pm 0.018$  for the NPZK population and  $0.574 \pm 0.019$  for the Flevopolder population.  $H_o$  varied between 0.503 for the AWD population and 0.546 for the Flevopolder population (table 1). There were no significant differences in both  $H_e$  and  $H_o$  between different populations (ANOVA,  $p \gg 0.05$ ).

In all populations allele frequencies have significantly differentiated from each other ( $p < 0.0001$ ). For all pairs of populations, values of  $F_{ST}$  were significantly greater than zero ( $p < 0.05$ ).  $F_{ST}$  values were 0.036 (0.0037-0.071 ( $p < 0.05$ )) for the AWD and the NPZK population, 0.058 (0.022-0.092 ( $p < 0.05$ )) for AWD and Flevopolder and 0.11 (0.041-0.21 ( $p < 0.05$ )) for NPZK and Flevopolder.  $R_{ST}$  was only significantly

TABLE 1

Genetic diversity of Dutch roe deer populations in the Amsterdamse Waterleidingduinen (AWD), in National Park Zuid-Kennemerland (NPZK) and in the Flevopolder. NA, mean number of alleles per locus;  $H_e$ , expected heterozygosity with standard error based on within locus variance;  $H_o$ , observed heterozygosity with standard error based on within locus variance.

Population	Mean sample size	NA	$H_e$	$H_o$
AWD	16.6	4.0	0.547±0.025	0.503±0.044
NPZK	19	4.0	0.557±0.018	0.533±0.037
Flevopolder	17	3.57	0.574±0.019	0.546±0.046

TABLE 2

$F_{ST}$  and  $R_{ST}$  values for the different pairwise comparisons of the Amsterdamse Waterleidingduinen (AWD), National Park Zuid-Kennemerland (NPZK) and the Flevopolder populations (95% confidence interval). Values marked with \* are significantly greater than zero.

	AWD-NPZK	AWD-Flevopolder	NPZK-Flevopolder
$F_{ST}$	0.0362* (0.0037-0.0713)	0.0581* (0.0221-0.0920)	0.1083* (0.0413-0.2080)
$R_{ST}$	0.00335 (-0.0066-0.0959)	0.03284 (-0.0028-0.1578)	0.07760* (0.0381-0.1868)

positive between NPZK and Flevopolder. Values of  $F_{ST}$  and  $R_{ST}$  did not differ significantly from each other but  $R_{ST}$  showed larger 95% confidence intervals, except between NPZK and Flevopolder (table 2).

## DISCUSSION

Allele frequencies in all populations are significantly differentiated from each other. According to HARTL & CLARK (1989) populations will be strongly differentiated if the number of reproductively successful immigrants per generation ( $Nm$ ) is much smaller than one. If  $Nm$  is larger than four, they will behave as one single panmictic unit. The fact that all populations are significantly differentiated might indicate that gene flow is limited to four or fewer migrants per generation between AWD, NPZK and Flevopolder. To quantify the level of differentiation, one can use  $F_{ST}$  and  $R_{ST}$  values.  $F_{ST}$  and  $R_{ST}$  will probably overestimate the amount of gene flow because migration-drift equilibrium is reached only after  $1/m$  generations. Besides, in this study estimated migration rates based on  $F_{ST}$

and  $R_{ST}$  using  $F_{ST}$  (or  $R_{ST}$ ) =  $1/(4Nm + 1)$  were only slightly or not informative because of the large confidence intervals for both values and were therefor not included in this paper.

Differentiation between both dune populations and the Flevopolder was expected because of the large geographical distance between these areas. The significant differences in allele frequencies and positive  $F_{ST}$  and  $R_{ST}$  values between NPZK and AWD were more surprising because of the earlier mentioned possibility for gene flow between both areas. For instance, MÖRSCH & LEIBENGUTH (1994) found no significant differentiation between populations 20 to 40 kilometers apart and separated by fenced motorways and densely populated areas. The fact that we did find a significant differentiation of allele frequencies between populations might be caused by the history of both populations. We will go further into this later.

When compared to the Flevopolder population, genetic variation in both isolated dune populations was not significantly lower due to genetic drift. The probability that we would not have detected a decrease of 20% or more due to large standard errors is very small (power analysis,  $\beta < 0.0222$ ). The estimation of the decrease of the amount of genetic variation contains some assumptions about both populations, which could explain the discrepancy between the observed and the expected decrease of heterozygosity.

The first assumption made is that no gene flow takes place between AWD and NPZK. When gene flow between both populations is substantial, one might ask whether they could, genetically speaking, be considered as a single population. If roe deer in both areas really belong to genetically the same population, population size will increase and the effect of genetic drift will decrease. According to HARTL & CLARK (formula 6.28, 1989) a migration rate of only a few percent will cut down the estimated decrease of heterozygosity in the AWD population compared to the Flevopolder population within observed values by the exchange of alleles.

Second, the assumption that  $N_e = 0.3N$  might not be correct, especially for a population as small as 12 animals at the moment of introduction. Because both populations are relatively young and population sizes have been relatively high, the initial founder effect accounts for the largest part of the reduction of the amount of variation. A small increase of  $N_e/N$  at the moment of introduction would cut down the expected decrease.

The third assumption made was that all animals introduced in the AWD as well as in NPZK directly or indirectly originated from genetically the same population. If the roe deer from which each population was stocked came from multiple, genetically different populations, the amount

of variation in the founder population would be higher than expected. This constitutes an underestimation of the amount of variation at the moment of introduction by the current amount of variation in the Flevopolder population.

If the AWD and NPZK population were each stocked from different populations, they might have been differentiated at the moment of introduction already. The amount of differentiation we observed in this study could be a reflection of the differentiation that existed between both founder populations. Even if founders came from the same population, a founder effect could have caused differentiation between both populations at the moment of introduction (HEDRICK, 1999). But, if gene flow was substantial (about 10%), differentiation between the AWD and the NPZK population would have disappeared after 10 generations. Also, if differentiation at the moment of introduction was the case, it would enlarge the effect of gene flow on genetic variation.

This shows that a population's history should be taken into account when studying genetic variation and population structure. Especially in young populations, statements should be made with great care.

## CONCLUSIONS

Gene flow between both dune populations occurs but is limited to less than four animals per generation. Although decrease of genetic variation in the isolated populations has probably taken place, the amount of variation is still substantial. Taking into account a current population size of 600 roe deer, a decrease in heterozygosity of 0.5% per generation due to genetic drift could be expected. Because little gene flow probably occurs, this decrease will be even smaller. However, in the long run the decrease will be larger. To prevent the eventual negative effects of low genetic variation in the long run, the number of barriers between the isolated populations should be reduced and the introduction of animals from other populations could be considered.

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## APPENDIX I

Tables of allelic frequencies for each locus and each population.

## NVHRT16

Population	Alleles						2N
	162	164	168	172	174	180	
AWD	0.275	0.075	0.100	0.075	0.350	0.125	40
NPZK	0.237	0.158	0.026	0.316	0.105	0.158	38
Flevopolder	0.167	0.119	0.000	0.429	0.286	0.000	42

## NVHRT21

Population	Alleles									2N
	161	163	165	167	173	177	181	183	185	
AWD	0.083	0.042	0.000	0.333	0.000	0.125	0.125	0.208	0.083	24
NPZK	0.233	0.000	0.000	0.233	0.033	0.000	0.167	0.167	0.167	30
Flevopolder	0.000	0.000	0.214	0.143	0.000	0.214	0.000	0.143	0.286	14

## NVHRT24

Population	Alleles					2N
	133	145	147	149	153	
AWD	0.050	0.900	0.000	0.000	0.050	20
NPZK	0.125	0.825	0.000	0.000	0.050	40
Flevopolder	0.028	0.806	0.139	0.028	0.000	36

## NVHRT30

Population	Alleles					2N
	145	155	157	159	167	
AWD	0.000	0.063	0.031	0.375	0.531	32
NPZK	0.025	0.000	0.125	0.300	0.550	40
Flevopolder	0.000	0.000	0.000	0.553	0.447	38

## NVHRT48

Population	Alleles				2N
	87	93	95	97	
AWD	0.000	0.275	0.725	0.000	40
NPZK	0.000	0.452	0.548	0.000	42
Flevopolder	0.023	0.205	0.727	0.045	44

## NVHRT71

Population	Alleles			2N
	123	125	127	
AWD	0.778	0.222	0.000	36
NPZK	0.972	0.028	0.000	36
Flevopolder	0.474	0.474	0.053	38

## NVHRT73

Population	Alleles					2N
	237	257	259	261	271	
AWD	0.525	0.000	0.225	0.125	0.125	40
NPZK	0.275	0.300	0.125	0.225	0.075	40
Flevopolder	0.385	0.000	0.000	0.115	0.500	26