Appendix B. Allozyme variability in two populations of roe deer from The Netherlands

ALLOZYME VARIABILITY IN TWO POPULATIONS OF ROE DEER FROM THE NETHERLANDS

Arnd Schreiber and Peter Fakler
Zoologisches Institut I, Universität Heidelberg, Im Neuenheimer Feld 230, D-69120 Heidelberg, Deutschland

Abstract

Based on an electrophoretic study of 25 genetically-encoded loci in 30 roe deer from two populations in the Netherlands, three two-allelic polymorphisms are described. Another two loci are polymorphic but not amenable to genotyping at the present stage of the analysis, and another three proteins are variable and possibly polymorphic. The proportion of polymorphic loci in both populations does not differ grossly from typical populations in Eastern Central Europe, though heterozygosity is lower. Two out of three genotyped polymorphisms are based on very rare alleles, which are easily lost by genetic drift. Without exchange with additional populations, these polymorphisms are likely to be lost in isolated stocks.

1. INTRODUCTION

Although roe deer is the largest and most widespread mammalian herbivore in many European wildlife habitats and subject to numerous management decisions, its population genetic structure is still insufficiently understood, although at least seven publications are available on this subject. The most comprehensive published investigations are by G. HARTL and coworkers (1988, 1991) on populations from Austria, Hungary and Switzerland, the other papers being more restricted in scope, concerning both the number of sampled individuals, and of genetic loci (MAUGHAN & WILLIAMS 1967, LAWTON & SUTTON 1981, 1982, BACCUS et al. 1983, GYLLENSTEN et al. 1983). The overall results emerging from these studies are as follows:

- Roe deer is a particularly polymorphic species, with approximately 30% of electrophoretic loci being polymorphic in Eastern Central Europe.

- A portion of some 10% of the species's total genetic variation is due to interdemic differentiation, and the pattern of local allele frequencies suggest periodic bottlenecking and recolonization of local habitats.

Other evidence than electrophoretic work relevant to population differentiation, including socio-ecological studies, demographic analysis, etc. (review: KURT 1991) also suggest that *Capreolus* takes a position within the continuum connecting r- and k-strategists which lies towards the r-selected end of the spectrum, characterized by high population turnover, the potential of quick adaptation to local conditions, and marked colonizing abilities.

While the available population genetic evidence is in agreement of what can be deduced from the considerable ecological and behavioural knowledge on *Capreolus*, there are huge gaps in our knowledge concerning roe deer's population genetics. Multilocus-studies are essentially confined to Eastern Central Europe, which is particularly disturbing given the documented variability in roe deer social and demographic organization which ranges from populations built by philopatric, territorial specimens with different degrees of sociological stability to herd-formation in open habitats.

All genetic markers available from electrophoretic studies have been found in samples received from hunted specimens, with pedigrees remaining unknown. There appears never to have been attempts to verify the concluded codominant mode of inheritance of the alleles deduced from the electrophoretic phenotypes of the various proteins studied.

As a consequence, any study on roe deer cannot base on existing, well-documented genetic markers but has to begin literally at "point zero", i.e. studying as many proteins as possible, and to optimize the resolution any allozyme systems which

appear variable during the first pilot screens, so that genotyping becomes possible. Unfortunately, the group of researchers which published most data on roe deer genetics did not specify the methodic details of their electrophoretic analysis, and therefore subsequent workers cannot base on previous analytic experience. Equally problematic, there is little possibility to compare any alleles identified in subsequent investigations with what has been published before.

As a consequence, the point of departure for the present comparison of genetic variability between two populations of Dutch roe deer was the time- and resource-consuming necessity to optimize and develop electrophoretic systems for any allozyme suspected to be polymorphic during a first pilot screen of as many loci as possible.

2. ANALYSIS

A total of 30 roe deer, 14 from the populations managed by the Amsterdam Drinking Water Company (called "dune population" in the following), and 16 from Flevoland, were analyzed as to 25 loci (Tables 1., 2.) of protein-encoding genes. Kidney and liver isoenzymes were compared for each specimen of which both tissues were available. Thus, 750 genotypes were resolved for both populations, plus another 90 more for those enzymes in which liver and kidney displayed different isoenzyme patterns: SOD, PGM1, EST (substrate: methyl-umbelliferyl acetate). However, since we are unaware of possible non-genetic modifications of isoenzymes between different organs, which may pretend the existence of different gene products expressed variably between tissues, we did not include these tissue-specific enzymes as additional genotypes.

For each deer and each tissue, samples were homogenized in 50 mM Tris/HCl-buffer pH 7.5, and the lysates run on 1 mm thin horizontal agarose gels (Multiphor electrophoresis chambers, LKB Pharmacia). After electrophoresis, enzymes were specifically stained using the protocols described in detail elsewehere (SCHREIBER 1991, RIFFEL & SCHREIBER 1993).

Polymorphic systems, and those variable allozymes which we found difficult to resolve by agarose gel electrophoresis were analyzed additionally by isoelectric focusing. This technique is based on ultra-thin polycarylamide gels (0,3 mm) which contain ampholines, i.e. substances which behave as acids and as alkalines at the same time. During the electrophoresis, these ampholines migrate to their isoelectric points and rest there, thus creating a gradient of pH-values acrosss the gel. Any proteins migrating in this field rest at the position of their isoelectric point. This behaviour results in very sharp resolution. Details of the analytic methods, and the statistical procedures to compute parameters for the characterization of populations have been described previously (SCHREIBER 1991, RIFFEL & SCHREIBER 1993).

3. RESULTS

3.1 Descriptive genetics

Electrophoretic conditions were established for 17 allozyme system from *Capreolus* which represented 27 expressed genetic allozyme loci (Tables 1,2). Of these, 23 were sufficiently resolved and scorable. In addition, the non-enzymatic haemoglobin could be resolved from blood cells contained in the samples, increasing the number of screened loci to 25. Sixteen of the allozyme loci were identical (monomorphic) within and between populations: ACP (possibly two loci), ADH, GLUDH, GOT-1, GOT-2, GDH-1, GDH-2, DIA-1, DIA-2, MDH-1, MDH-2, ME-1, ICD-1 (ICD-2 not scorable), LDH-1, PGM-1. Both haemoglobin-forming protein chains, Hb alpha and Hb beta, were also monomorphic, bringing non-variable loci to sixteen.

Variability was encountered in the following seven proteins: MPI, SOD, LDH-2, AK, PGM-2, PEP-2, and EST (substrate: methyl-umbelliferyl acetate). Some care is required when interpreting variable systems as genetically determined polymorphisms in a species whose genetics has been studied as little as in roe deer, and we classify the variants into three groups: (i) allelic polymorphism, (ii) probable polymorphisms which cannot be genotyped in all cases, and (iii) protein variation probably due to non-genetically determined variability. The first group is useful for population analysis, the second group for calculation of percentages of polymorphic loci but not of heterozygosity, while the third group has to be omitted from further considerations until pedigreed families of *Capreolus* are available for tracing the inheritance of possible alleles.

3.1.1 Polymorphisms

Three allelic polymorphisms were encountered:

MPI: MPI produced a single-banded zymogram in all specimens with the exception of one individual which was a heterozygote containing a second allele, equally single-banded. The relative activity of each of the MPI-bands in the double-banded heterozygous pattern was reduced, as is expected in a codominantly expressed polymorphism.

SOD: Superoxide dismutase activity was observed in several gels stained for formazan appearance. However, best resolution was obtained in focusing gels with a narrow pH-gradient of 5-7. In this system, another allele, second to the main type encountered in the majority of deer, was seen in one individual.

LDH-2: The activity of the more cathodally-migrating locus of LDH was considerably weaker than of LDH-1 but still clear enough for population screening. Two patterns were evident on the gels, one obviously the heterozygous combination of

the major type with a second, rarer allele.

3.1.2. Polymorphisms which require further electrophoretic analysis.

In addition to the mentioned two-allele systems, the following proteins produced variable patterns but even when using different buffer systems, and applying isoelctric focusing both in the wide (pH 3,5-10) and narrow (pH 5-7) pH ranges, we failed to resolve this variation sufficiently for genotpying. Polymorphism of these proteins has been published (HARTL et al. 1991) but the publications lack the methodic details required for attempting their resolution conditions, as well as a desription or illustration of the patterns. On the basis of our gels we prefer not to interprete these patterns as genotypes until either pedigree populations become available, or tissues with possibly less complex allozyme patterns (e.g., red blood cells) can be investigated. Unfortunately, it is AK and PGM which may constitute useful systems for comparing the two populations because both of them looked highly variable. Quite evidently, heterozygosity values would increase if these systems could be included.

AK: In agarose gel electrophoresis, AK migrated slowly, and the low molecular sieve effect of agarose gels did not contribute to increased resolution of those activity spots remaining close to the origin of sample application on the gel. Isoelectric focusing produced so many closely packed bands (>20) that any interpretation would be premature without a better knowledge of the formal genetics of this system in *Capreolus*. Presumably, two loci contributed to the AK patterns, both of which may be variable, and which overlap on the gel. Quite probably, more than two alleles are involved.

PGM: In focusing gels with narrow (pH 5-7) and wide (pH 3,5-10) pH-gradients, two PGM-isoenzymes appeared. One, named PGM-1, was monomorphic, while the other was variable. This variation in the focusing pattern was not as extensive as the PGM-polymorphism which we described previously from a bovid (Schreiber et al. 1993) but rather resembled the minor variants found when "subtyping" human PGM.

3.1.3. Further variation

Three more systems showed variation in electrophoretic pattern but we are not definitely certain as to the genetic determination.

ME: Malic enzyme produced variable zymograms. However, we believe that this variation has a non-genetic background, possibly biochemical modification of the

molecule after genetic expression. Again, pedigree material would be preferable. HARTL & REIMOSER (1988) interpreted this system as polymorphic but HARTL et al. (1991) omitted it from their more recent analysis for not being consistently scorable. Irrespective of this variation, the other of the two ME-isoenzymes was monomorphic.

Another two systems cannot be finally judged: One locus with esterase activity (substrate: methyl-umbelliferyl acetate) was variable and initially 13 roe deer could be allocated to either one of three patterns which resembled a two-allele polymorphism. However, the patterns were difficult to recognize in several samples, and the system was obviously sensitive to ageing of the tissues. Therefore, we prefer to omit this system from any interpretation, in the knowledge that we possible may lose information. The same conclusion was reached concerning PEP-2, since again this protein produced patterns which were difficult to reproduce.

3.2 Genetic variability in Dutch roe deer

In summary, the screening of unpedigreed 30 roe deer from two Dutch populations revealed three clearly interpretable two-allele systems, plus another two enzymes definitely subject to genetic variation (although we consider any genotyping doubtful at the present stage of the analysis), plus another three loci which may represent either polymorphism or rather be subject to non-genetic variarion.

Omitting the doubtful variants, we have five genetic polymorphisms (three of which could be genotyped) from a locus sample of 25 protein-coding loci. This yields a proportion of genetic polymorphism (P) of 20,0 % which lies in the upper range of P-value among cervid species in general: P = 31,6% in white-tailed deer, 20,6% in red deer, 2,0% in fallow deer (review: HARTL et al. 1991). HARTL and cowokers (1988, 1991) found a mean P for their Eastern European populations of 15,8%, and if all studies on roe deer are combined, polymorphism has been found at 30% of the loci ever studied electrophoretically. The Dutch metapopulation thus ranges above the mean found in East Central Europe.

However, as will be evident from the preceding text, any direct comparison of the polymorphism enountered in the Duth roe deer with the considerable data body from East Central Europe is premature because of differences between the analytic approaches chosen. This affects both the identity of loci studied, since the tendency to evolve polymorphism differs widely between loci, and the exact composition of the locus sample is of decisive importance for P-values. Differences between biochemical methods can also be expected, particularly the chemical composition of the electrophoresis buffers which have not been indicated in the numerically comprehensive study by HARTL and others. In addition, all our genotyped polymorphisms are based on rare alleles, as is evident from Table 3 which lists

allele frequencies: MPI polymorphism rests on a single heterozygous individual from the dune population, and SOD polymorphism on a single heterozygote from Flevoland. LDH-2 is exceptional in having two alleles in both populations. I.e. P for either the dune or the Flevoland populations are merely 17,4%, and thus closer to the single-population average found in Austria, Switzerland, and Hungary.

Heterozygosity (percentage of heterozygous genotypes related to all measured genotypes) is considerably lower in our sample than reported by HARTL and coworkers. They found a mean of 4.6% heterozygote loci patterns for single populations, and a derived 5.6% if the idealized metapopulation of all studied stocks has been considered. If we neglect those loci which were polymorphic but where we failed to differentiate all genotypes (i.e. 19 loci remaining), heterozygosity (H) in the dune population measured 1,1%, and in Flevoland roe deer 0,98%, However, all systems unambiguously phenotyped by us rest on rare alleles which contribute but little to overall heterozygosity, and AK and PGM with their putatively more balanced frequencies could not be interpreted on the basis of the present material.

Allele frequencies and genotypes encountered in both populations are listed in Table 3. Nei's standard genetic distance between both populations amounts to 0.0016 which is much lower than the mean value of 0.0064 found for the more heterozygous stocks from East Central Europe. Genetic identity (I) was 0.9998 (mean value for Austria: 0.9937). There are no obvious deviations in genotype frequencies from Hardy-Weinberg-relations which would suggest sub-structures within either populations.

The occurrence of rare alleles in small sample sizes is partly determined by chance alone. Therefore, any conclusions as to differences of polymorphism between both populations should be treated cautiously. Pairwise comparisons of allele frequencies did not reveal significant differences between populations. This means that the genetic distance derived may be distorted by sample size effects, although this possibility is not a necessity.

4. CONCLUSIONS

One may conclude that in principle the overal pattern found for other populations of roe deer also holds true for the two Dutch populations covered by the present study: a relatively high proportion of polymorphic loci, the presence of rare alleles and therefore a comparatively low heterozygosity (considering the number of polymorphisms). Quite evidently, stochastic factors of genetic drift play a prominent role in roe deer population genetics. This pattern is typical of a very polymorphic species with somewhat unstable demographic conditions, i.e. a colonizer. While bottle-necking may be a factor in the natural lifestyle of the species, continued isolation of small populations of *Capreolus* will see the loss of alleles at a faster rate than if allele frequencies were more balanced. Heterozygosity is lower in both Dutch populations than typical in Austria, Switzerland, and Hungary, although we have omitted two systems which may increase that value. While our results do show remaining genetic variation in both populations, the lower heterozygosity may indicate that a process of variability reduction is going on in the Dutch populations.

Bottle-necks and colonization events in man-dominated and fragmented habitats may be entirely compatible with the natural lifestyle of the species. However, a faster rate of genetic differentiation between isolated stocks can be postulated for *Capreolus* than for red deer (cf. HARTl et al. 1991), given the predominance of rare alleles, and if those rare alleles are to be preserved in isolated populations at the long-term, the imitation of natural patterns of gene flow should be considered.

5. OUTLOOK

Efforts to resolve AK and PGM variation described here to the level of individual alleles continue. After resolution, final values for heterozygosity can be quantified. Additional studies into genetic differentiation of roe deer in man-dominated landscapes which are strongly modified when compared to the species's natural habitats would be scientifically rewarding, and of interest of applied wildlife biology. The authors try to organize a follow-up study, including populations from Germany and France, and would welcome continued support from the Dutch colleagues to understand more efficiently the seemingly complex population structure of the commonest large mammal species remaining in most parts of Northwest Europe. Of particular importance for practice would be the comparison of roe deer demes subject to different ways of social organization.

REFERENCES

BACCUS, R., RYMAN, N., SMITH, M.H., REUTERWALL, C., CAMERON, D. (1983). Genetic variability and differentiation of large grazing mammals. J. Mamm. 64: 109-120.

GYLLENSTEN, U., REUTERWALL, C., RYMAN, N., STAHL, G. 1980. Geographical variation of transferrin allele frequencies in three deer species from Scandinavia. Hereditas 92: 237-241.

HARTL, G.B., REIMOSER, F. 1988. Biochemical variation in roe deer (*Capreolus capreolus*): are r-strategists among deer genetically less variable than k-strategists? Heredity 60: 221-227.

HARTL, G.B., REIMOSER, F., WILLING, R., KÖLLER, J. 1991. genetic variability and differentiation in roe deer (*Capreolus capreolus*) of Central Europe. Genet. Sel. Evol. 23: 281-299.

KURT, F. 1991. Das Reh in der Kulturlandschaft. Sozialverhalten und Ökologie eines Anpassers. Hamburg, Parey.

LAWTON, M.E., SUTTON, J.G. 1981. Species identification of deer blood by isoelectric focusing. Deer 5: 285-288.

LAWTON, M.E., SUTTON, J.G. 1982. Species identification of deer blood by isoelectric focusing. J Forensic Sci. 22: 361-366.

MAUGHAN, E., WILLIAMS, J.B. 1967. Haemoglobin types in deer. Nature 215: 404-450.

RIFFEL, M., SCHREIBÉR, A. 1993. Genetische Inventarisierung fragmentierter Fischpopulationen als Basis für ihr Erhatungsmanagement. Veröff. Projekt Angewandte Ökologie, Karlsruhe.

SCHREIBER, A. 1991. Molekulare Individualität und Naturschutz. Populationsgenetische Beiträge zur Erhlatung bedrohter Arten. PhD Thesis, University of Heidelberg.

SCHREIBER, A., NÖTZOLD, G., HELD, M. 1993. molecular and chromosomal evolution in anoas. Ztschr. Zoolog. Syst. Evolutionsforschung 31: 64-79.

Tab. 1. Proteins studied in Dutch roe deer. Systems marked with an asterisk did not reveal informative patterns.

Protein	*E.C. Number*
Acid phosphatase (ACP)	3.1.3.2
Adenylate kinase (AK)	3.5.4.4
*Alcaline phosphatase (ALP)	3.1.3.1
Alcohol dehydrogenase (ADH)	1.1.1.1
Carbonic anhydrase (CA)	4.2.1.1
Esterase (EST)	3.1.1.1
Glucose dehydrogenase (GDH)	1.1.1.47
* Glucose phosphate isomerase (GPI)	5.3.1.9
Glutamate dehydrogenase (GLUDH)	1.4.1.3
Glutamate oxalacetate transaminase (GOT)	2.6.1.1
Haemoglobin (Hb)	
Isocitrate dehydrogenase (ICD)	1.1.1.42
Lactate dehydrogenase (LDH)	1.1.1.27
Malate dehydrogenase (MDH)	1.1.1.37
Malic enzyme (ME)	1.1.1.40
Mannose phosphate isomerase (MPI)	5.3.1.8
NADH-Diaphorase (DIA)	1.6.2.2
Peptidase (PEP)	3.4.11
* Phosphogluconate dehydrogenase (PGD)	1.1.1.44
Phosphoglucomutase (PGM)	2.7.5.1
* Sorbitol dehydrogenase (SDH)	1.1.1.14
Superoxide dismutase (SOD)	1.15.1.1

Tab.2. Electrophoretic conditions chosen to study allozymes in roe deer. AGE = agarose gel electrophoresis, IEF = isoelectric focusing, el. = electrode.

Protein	*Alleles*	*Technique*	*Buffer*
ACP	1	AGE	el: 0.15M tridsodium citrate/0.24M sodium dihydrogen phosphate, pH 8.0; gel: 1:40 dil. of el. buffer
ADH	1	AGE	el.: 0.47 M tris/citrate, pH 8.6 gel: 1:20 dil. of electrode buffer
ALP	?	AGE	el.: boric acid/NaOH, pH 8.0 gel: 0.076 M tris/0.007 M citric acid, pH 8.6
AK	?	IEF	
CA	1	AGE	el.: 1:14 dil. of 0.9M tris/0.5M boric acid/0.02M EDTA gel : 1: 40 dil. of the above stock solution
DIA-1,-2	1+1	AGE	el.: 1.3 M tris/0.07 M boric acid/0.003 M EDTA gel: 0.09M tris/ 0.05 M boric acid/ 0.002 M EDTA
EST	?	AGE	el.: 0.1 M tris/0.1 M maleic anhydride, pH 7.2 gel: 1: 10 dil. of el. buffer
GDH-1,-2	1+1	AGE	el.:0.687 M tris/0.157 M citric acid, pH 8.0 gel: 1:30 dil. of el. buffer
GLUDH	1	AGE	el.: 0.135 M tris/citrate pH 7.0 gel: 1:4 dil. of el. buffer
GOT-1,-2	1+1	AGE	el.:0.687 M tris/0.157 M citric acid, pH 8.0 gel: 1:30 dil. of el. buffer
GPI	?	AGE	el.: 0.25 M tris/ 0.057 M citric acid, pH 8.0 gel: 0.017 M tris/0.0023 M citric acid, pH 8.0
Hb	1+1	IEF	
ICD-1,-2	1+1	AGE	el.: 0.245 M NaH,PO4/0.15 M citric acid, pH 5.9 gel: 1:40 dil. of el. buffer
LDH-1	1	AGE	el.:0.687 M tris/0.157 M citric acid, pH 8.0 gel: 1:30 dil. of el. buffer
LDH-2	2	AGE	as LDH-1
MDH-1,-2	1+1	AGE	el.: 0.135 M tris/citrate pH 7.0 gel: 1:4 dil of el. buffer
ME-1	?	AGE	el.:0.687 M tris/0.157 M citric acid, pH 8.0 gel: 1:30 dil. of el. buffer
ME-2	1	AGE	as ME-1
MPI	2	AGE	el.: 0.05 M tris/0.05 M NaH2P04, pH 8.3
PEP	?	AGE	el.: 0.1 M tris/0.1 M NaH2PO4, pH 7.4 gel: 1: 20 dil. of el. buffer
PGD	?	AGE	el.: 0.1 M phosphate, pH 7.0 gel: 1:10 dil. of el. buffer
PGM	?	IEF	

SDH	?	AGE	el.: 0.15 M Triethanolamine/HCl, pH 8.6 gel: 1:5 dil of el. buffer
SOD	2	AGE	el.: 0.1 M phophate, pH 7.0 gel: 0.01 M phosphate, pH 7.0

Tab. 3. Allele frequencies and genotypes of polymorphic proteins from roe deer.

MPI

dunes
n = 14 roe deer

$p_{MPIa} = 0,96$
$q_{MPIb} = 0,04$ (single heterozygote)

Flevoland
n = 16 roe deer

$p_{MPIa} = 1,00$

Genotype distribution:

	AA	AB	BB	AA	AB	BB
genotypes observed	13	1	0	16	0	0
genotypes expected	12, 88	1,12	< 0,002	16	0	0

SOD

dunes
n = 14 roe deer

$p_{SODa} = 1,00$

Flevoland
n = 16 roe deer

$p_{SODa} = 0,97$
$q_{SODb} = 0,03$ (single heterozygore)

Genotype distribution:

	AA	AB	BB	AA	AB	BB
genotypes obs.	14	0	0	15	1	0
genotypes exp.	14	0	0	15,05	0,93	<0,0009

LDH-2

dunes
n = 14 roe deer

$p_{LDH\text{-}2a} = 0,89$
$q_{LDH\text{-}2b} = 0.11$

Flevoland
n = 16 roe deer

$p_{LDH\text{-}2a} = 0,94$
$q_{LDH\text{-}2b} = 0,06$

Genotype distribution:

	AA	AB	BB	AA	AB	BB
genotypes obs.	11	3	0	14	2	0
genotypes exp.	11,08	2,7	0,16	14,1	1,8	0,05