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An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes, and mitochondrial DNA

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A total of 469 brown hares *Lepus europaeus* Pallas, 1778 from 20 sampling sites in Austria were examined for genetic diversity within and among populations by means of horizontal starch gel electrophoresis. Fourteen out of 54 presumptive structural loci were polymorphic, one of which was excluded from further population genetic analyses due to the occurrence of a null-allele. The mean proportion of polymorphic loci (P) was 15.3% (SD 2.2%), and mean expected average heterozygosity (H_e) was 4.6% (SD 0.5%). Both relative (F_{ST} = 5.4%) and absolute (mean Nei's 1978 D = 0.0016, SD 0.0016) genetic differentiation among populations were low, suggesting a generally high level of migration. Cluster analysis revealed some separation of brown hare populations in western and northern Austria from those in the east and in the south. In 131 individuals, mtDNA was digested with a battery of 16 restriction endonucleases. Besides the standard type I which occurred exclusively in most of the populations, five additional haplotypes, each of them deviating from type I by one base pair substitution, were detected. Together with rare alleles at allozyme loci, the distribution of variant haplotypes corroborated the spatial pattern obtained by allozyme distances and suggested considerable immigration of brown hares from the adjacent countries in the east and south. Twenty non-metric skull traits were scored in 443 individuals. Character variants were dichotomized (0/1) and the respective frequencies were used to calculate C. A. B. Smith's 'mean measure of divergence' (MMD) among five population groups. Morphological differentiation was in accordance with the major population genetic pattern as revealed by molecular techniques. MtDNA variation (nucleon diversity, nucleotide diversity) and morphological variation (mean of SD in single characters) within populations were not significantly associated with one another, and did not show a relationship with indices of genetic variation obtained by allozyme analysis. These findings suggest that variability in only one of these characters cannot be considered representative for overall gene pool diversity within populations.

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Key words: *Lepus europaeus*, morphology, non-metric skull traits, allozymes, mitochondrial DNA, population genetics

Introduction

The past decades have witnessed the utilization of a variety of morphological and molecular characters for investigating genetic diversity within and among populations of animals. However, compared with the considerable accumulation of data at each of those organismic levels, integrative approaches addressing the correspondence of population specific diversity patterns among the various characters are rare. Yet especially the comparison of the same individuals or populations as to differentiation at various organismic levels can be expected to provide insight into the biological significance of variation in particular characters. For example, Avise *et al.* (1987) reviewed some cases where highly divergent mitochondrial DNA (mtDNA) phylogeographic groupings also proved distinct in allozyme frequencies. In an other case, the American oyster *Crassostrea virginica*, a comparison of populations of the Atlantic with those inhabiting the Gulf of Mexico revealed distinctness in mtDNA and in anonymous single copy nuclear DNA. By contrast, there was striking homogeneity of allozyme frequencies, providing support for balancing selection (Karl and Avise 1992). A second example is based on studies on allozymes and morphology. Morphological variation has been suggested to serve as a valuable indicator for overall genetic variation (e.g. Soulé *et al.* 1973, Berry and Jakobson 1975). The results of comparisons of both non-metric (meristic) and metric variability with allozyme heterozygosity in a number of animals have made this hypothesis a very controversial issue (see Schnell and Selander 1981, Mitton and Grant 1984, Wayne *et al.* 1986, Yezerinac *et al.* 1992, for reviews). There is still the question as to what extent morphological variation reflects additive genetic variation or is attributable to developmental noise, and whether there is a difference in this respect between taxa (e.g. homeotherms *versus* poikilotherms) and assemblages of morphological traits (cf Soulé 1979, Allendorf and Leary 1986, Suchentrunk 1993).

In the brown hare *Lepus europaeus* Pallas, 1778, population genetic studies using allozymes have been conducted by Hartl *et al.* (1989, 1990, 1992). A small number of individuals was examined for mtDNA variation by Biju-Duval *et al.* (1991). Variability in non-metric dental traits was used for population studies by Suchentrunk *et al.* (in press), and was tested for a relationship with allozyme heterozygosity by Suchentrunk (1993). The present study aims at assessing correspondence of variability in non-metric skull traits, allozymes, and mtDNA in the brown hare. Furthermore, the three character sets are used for evaluating genetic relationships among local populations of this species in Austria in an integrative way.

Material and methods

Samples

A total of 469 brown hares from 20 different sampling sites (operationally called populations throughout the paper) in Austria (Fig. 1) were killed during the autumnal hunting season of 1988 and

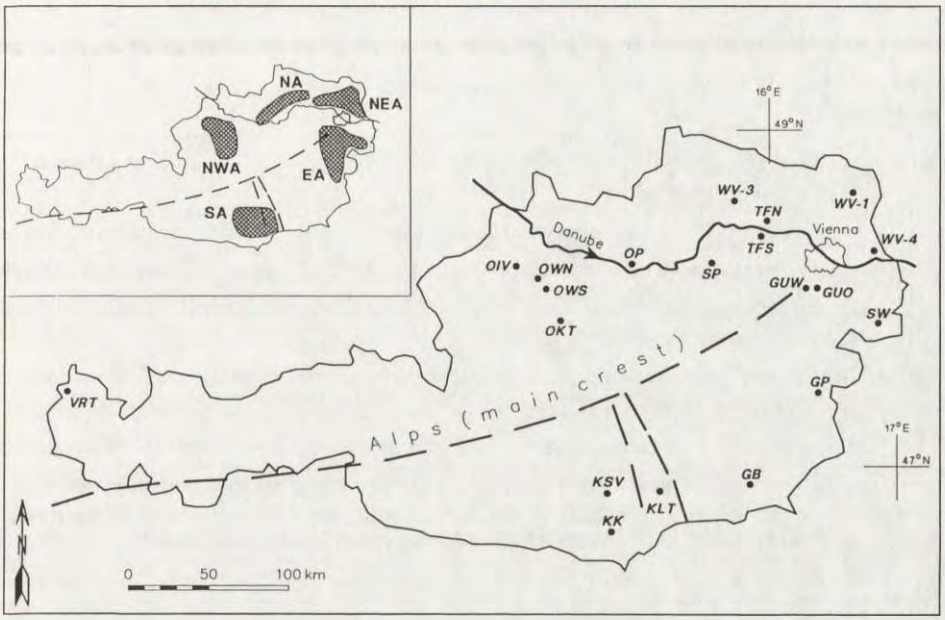

Fig. 1. Sampling sites of *Lepus europaeus* in Austria (districts in parentheses). GB – Gralla (Leibnitz), GP – Großpetersdorf (Oberwart), GUO and GUW – Guntramsdorf (Baden), KK – Grafenstein, Poggersdorf (Klagenfurt-Umg.), KLT – St. Margarethen/L. (Wolfsberg), KSV – St. Georgen, Goggerwenig, Mente, Shratt (St. Veit/Gl.), OIV – Wendling (Grieskirchen), OKT – Nußbach (Kirchdorf/Kr.), OP – Naarn (Perg), OWN – Edt/Lambach, Steinerkirchen (Wels-Land), OWS – Ried i. Trkr. (Kirchdorf/Kr.), SP – Haindorf, Markersdorf (St. Pölten-Land), SW – Illmitz (Neusiedl/S.), TFN – Bierbaum, Frauendorf (Tulln), TFS – Rust (Tulln), VRT – Fussach, Höchst, Gaißau (Bregenz), WV-1 – Bullendorf (Mistel- bach), WV-3 – Hohenwarth (Hollabrunn), WV-4 – Zwerndorf (Gänserndorf). The five geographic units (GUs) used in the present analysis are depicted in the insert. NWA – north- western Austria, NA – northern Austria, NEA – northeastern Austria, EA – eastern Austria, SA – southern Austria.

brought to the laboratory immediately after the hunts. There the animals were dissected and various morphological measurements were taken. Discrimination of juvenile and adult individuals was carried out by means of dry eye lens weight. When eye lens weight was lower than 275mg, the hares were considered as juvenile (i.e. born in the year of the hunt). When it was equal or higher than 275mg, the hares were considered as adult (see Suchentrunk *et al.* 1991). Liver, kidney, heart, and spleen were stored frozen at –20°C until electrophoresis.

Morphological study

A total of 443 brown hare skulls were used for morphological analyses. In a first attempt 53 different (uni- and bilateral) non-metric traits were identified using a dissecting microscope. Re-examination resulted in exclusion of 17 of those traits because of discordance of repeated scores. Another 16 traits were omitted from further analyses because of age- or/and sex-dependence of their character states (see below). A description of the remaining 20 skull characters and their respective dichotomized (0/1) character states is given in Table 1 and Fig. 2.

Table 1. Unilateral (U) and bilateral (B) non-metrical skull variants used for assessing morphological variability and differentiation of Austrian brown hare samples (see also Fig. 2).

Current number	Trait: morphological location and expressions
1 (B)	*Foramen condylare*: lateral of occip. condyle, mainly in dorsolateral position. 0 – absent, 1 – at least one *f.* present
2 (U)	*Foramen basioccipitale mediale:* medial on basioccipitale. 0 – absent, 1 – at least one *f.* present
3 (U)	*Foramen cavernosum*: medial on basisphaenoid. 0 – absent, 1 – present (sometimes subdivided)
4 (U)	Additional *foramen basisphaenoidale*: anywhere on basisphaenoid, usually small. 0 – absent, 1 – one or more *f.* present
5 (B)	Additional *foramen ethmoidale*: basio-rostral of *f. ethmoidale* (in proximity of *ethmoidale*). 0 – absent, 1 – at least one *f.* present
6 (U)	*Processus palatinus*: medial on *os palatinum*, pointing caudad. 0 – absent, 1 – clearly developed
7 (U)	*Processus maxillaris*: medial on *os maxillare*, pointing rostrad and reaching into area of *foramina incisiva*. 0 – rostral point of *p. m.* does not reach connecting line between mesial points of both P^2. 1 – rostral point exceeds connecting line between both P^2
8 (B)	*Foramen praemaxillare*: clearly visible *f.* on praemaxillare. 0 – absent, 1 – at least one *f.* present
9 (B)	*Foramen processus praemaxillaris*: tiny *f.*, situated in the rostral part of *p. praemax.* 0 – absent, 1 – at least one *f.* present
10 (B)	*Foramen zygomaticum anterius*: in the rostral groove of *os zygomaticum*. 0 – absent, 1 – at least one *f.* present (usually reticular expression)
11 (B)	*Processus nasalis ossis praemaxillaris*: length of *p.n.o.p.* that stretches dorso-caudad, parallel to *ossa nasalia*. 0 – shorter than *os nasale*, 1 – exceeds caudal points of *os nasale*
12 (B)	*Foramen frontale mediale*: para-saggital position on *os frontale*. 0 – absent, 1 – at least one *f.* present
13 (B)	*Foramen frontale postorbitale*: position in the caudal part of *os frontale*, in proximity of *os temporale* and *os parietale*. 0 – absent, 1 – at least one *f.* present
14 (B)	Additional upper molar. 0 – absent, 1 – present
15 (B)	Expression of upper M 3. 0 – absent, no or reduced alveole or reduced tooth size, 1 – present, regularly developed
16 (U)	*Os interparietale*. 0 – absent, 1 – present
17 (U)	Paramedian additional *processus maxillaris*: paramedian position in addition to normal *p. m.* 0 – absent, 1 – present
18 (B)	*Foramen basisphaenoideo-pterygoideum*: position between basal part of *os pterygoideum* and *os basisphaenoideum* in a nerve groove. 0 – absent, 1 – present
19 (B)	*Foramen temporale*: position in the rostral part of *os temporale* close to the base of *processus zygomaticus ossis temporalis*. 0 – absent, 1 – present
20 (U)	Wormian bone: *os suturale*, supernumerary bone occurring in the saggital suture as one or two bones. 0 – absent, 1 – present

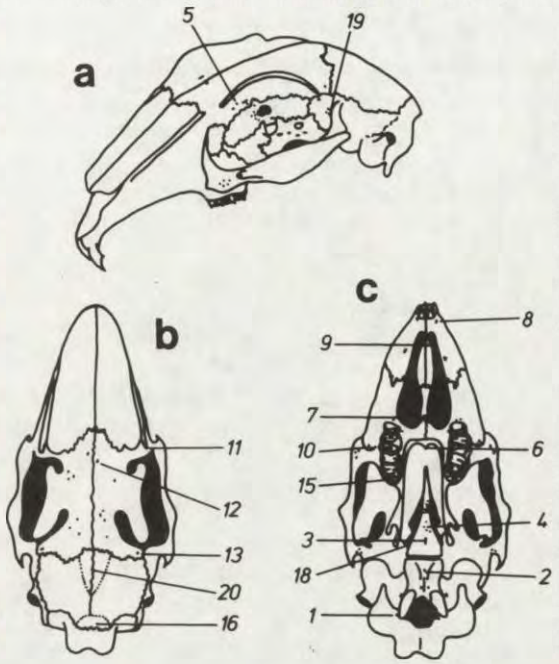

Fig. 2. Position of non-metric traits scored in the brown hare skull (for current numbers see Table 1). (a) dorso-lateral, (b) dorsal, (c) ventral view (characters 14 and 17 are not shown).

Electrophoretic study

In 469 brown hares, preparation of tissue extracts, electrophoretic and staining procedures were performed according to previously published methods (Hartl and Höger 1986, Hartl 1987, Grillitsch *et al.* 1992). The genetic interpretation of band-patterns was based on the principles outlined by Harris and Hopkinson (1976), and Harris (1980). The 35 enzyme systems screened and the loci scored are listed in Table 2. The most common allele in the sample from WV-1 (Fig. 1) was designated '*100*', variant alleles in the same or in other samples were designated according to the relative mobility of the corresponding allozymes (those already found in previous studies are defined in Hartl *et al.* 1990, 1992).

mtDNA study

A total of 131 brown hares from 18 sampling sites were examined for mtDNA variation. Apart from some modifications we followed the procedures outlined by Lansman *et al.* (1981). In brief: approximately 10 – 20 g of frozen liver were minced and homogenized in 3ml MSB-Ca^{++}/g tissue using a potter homogenizer (10 – 12 pistill strokes). Disodium EDTA (0.2 M, adjusted to pH 7.6 with NaOH) was then added to the homogenate to a final concentration of 10 mM. To remove nuclei and debris the homogenate was centrifuged twice for 5 min at 2,500 – 4,000 rpm (speed depending on the condition of the material) in a fixed-angle rotor (Heraeus Varifuge RF). Mitochondria were then pelleted at 14,000 rpm for 15 min, resuspended in 0.25 M sucrose (50 mM Tris, 10 mM EDTA, pH 7.6), recentrifuged as above, and resuspended in STE to a total of 5 ml. Mitochondria were SDS-lysed at room temperature (until this step all procedures were employed at 4°C). Then 1 g CsCl/ml lysate was added and centrifugation for 10 min at 6,000 rpm (Heraeus Labofuge I) allowed to eliminate the protein-SDS complex with a spatula. After adding 0.2 ml ethidium bromide (10 mg/ml) the lysate was centrifuged at 80,000 rpm and 20°C for 15 h (Sorvall RCM120, fixed-angle rotor RP80AT). The tube was inspected under UV light and the two fractions containing supercoiled and relaxed mtDNA were

Table 2. Enzyme systems studied and presumptive structural loci scored in the brown hare.

Enzyme system (abbreviation, E.C. number)	Locus
α-Glycerophosphate dehydrogenase (GDC, 1.1.1.8)	*Gdc*
Sorbitol dehydrogenase (SDH, 1.1.1.14)	*Sdh*
Lactate dehydrogenase (LDH, 1.1.1.27)	*Ldh-1, -2*
Malate dehydrogenase (MOR, 1.1.1.37)	*Mor-1, -2*
Malic enzyme (MOD, 1.1.1.40)	*Mod-1, -2*
Isocitrate dehydrogenase (IDH, 1.1.1.42)	*Idh-1, -2*
6-Phosphogluconate dehydrogenase (PGD, 1.1.1.44)	*Pgd*
Glucose dehydrogenase (GDH, 1.1.1.47)	*Gdh-2*
Glucose-6-phosphate dehydrogenase (GPD, 1.1.1.49)	*Gpd*
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH,1.2.1.12)	*Gapdh*
Xanthine dehydrogenase (XDH, 1.2.3.2)	*Xdh*
Glutamate dehydrogenase (GLUD, 1.4.1.3)	*Glud*
NADH-diaphorase (DIA, 1.6.2.2)	*Dia-1*
Catalase (CAT, 1.11.1.6)	*Cat*
Superoxide dismutase (SOD, 1.15.1.1)	*Sod-1, -2*
Purine nucleoside phosphorylase (NP, 2.4.2.1)	*Np*
Aspartate aminotransferase (AAT, 2.6.1.1)	*Aat-1, -2*
Hexokinase (HK, 2.7.1.1)	*Hk-1, -2, -3*
Pyruvate kinase (PK, 2.7.1.40)	*Pk-1*
Creatine kinase (CK, 2.7.3.2)	*Ck-1, -2*
Adenylate kinase (AK, 2.7.4.3)	*Ak-1, -2*
Phosphoglucomutase (PGM, 2.7.5.1)	*Pgm-1, -2, -3*
Esterases (ES, 3.1.1.1)	*Es-I, Es-d*
Acid phosphatase (ACP, 3.1.3.2)	*Acp-1, -2, -3*
Fructose-1,6-diphosphatase (FDP, 3.1.3.11)	*Fdp-1*
ß-Galactosidase (ß-GAL, 3.2.1.23)	*β-Gal*
Peptidases (PEP, 3.4.11)	*Pep-1, -2*
Aminoacylase-1 (ACY-1, 3.5.1.14)	*Acy-1*
Guanine deaminase (GDA, 3.5.4.3)	*Gda*
Adenosine deaminase (ADA, 3.5.4.4)	*Ada-2, -3*
Aldolase (ALDO, 4.1.2.13)	*Aldo*
Fumarate hydratase (FH, 4.2.1.2)	*Fh*
Aconitase (ACO, 4.2.1.3)	*Aco-1, -2*
Mannosephosphate isomerase (MPI, 5.3.1.8)	*Mpi*
Glucosephosphate isomerase (GPI, 5.3.1.9)	*Gpi-1, -2*

harvested by means of a Pasteur pipette. EtBr was removed by isoamyl alcohol extraction. Ethanol precipitation was performed at 4°C using 70% ethanol.

DNA was digested using a battery of 16 restriction enzymes (6 base cutters) purchased from Boehringer Mannheim and NEB: ApaI, BamHI, BclI, BglII, ClaI, DraI, EcoRI, EcoRV, HindIII, HpaI, PstI, PvuII, SacI, XbaI, XhoI, and XmnI. Fragments were separated electrophoretically in 0.7% agarose gels (0.5 × TBE buffer in gel and tank) containing 0.5 μg EtBr/ml gel and visualized under UV light. Fragment lengths were determined using Lambda phage DNA digested with HindIII as a

size standard. Using also the data of Biju-Duval *et al.* (1991), restriction maps of 10 mtDNA samples were constructed by means of double digestions.

Statistics

Morphological analyses of bilateral traits were based only on scores of the right body side. Whenever unscorable on the right side the respective left side score was used. Age (juveniles vs adults) and sex dependence of character states were examined using cross-tabs and χ^2-tests. Associations between character states among traits were tested by χ^2-tests (provided there were at least five cases in each cell) and quantified using Yule's Y index of association (cf Sjøvold 1977). Separately for each population, in each character the inter-individual variation was calculated as the standard deviation of the dichotomized character state values. The mean over the standard deviations of all characters (= IV) served as an index of population-specific inter-individual variability. Differences of IV between juveniles and adults were examined using the Wilcoxon test. Differences of IV across populations were tested by the Kruskal-Wallis test, based on standard deviations of single characters. Morphological differentiation among populations was investigated by means of C. A. B. Smith's mean measure of divergence (MMD, cf Sjøvold 1977). In order to achieve reasonable sample sizes of individuals it was necessary to pool the 20 single samples into 5 geographic units (GU) according to ecogeographic criteria and possible barriers to gene flow (Fig. 1). The measure of uniqueness (MU) was calculated as the sum of all MMDs involved in each GU, respectively. It gives an indication as to what extent a particular GU is separated from the others (Sjøvold 1977). A morphological phenogram was constructed using the UPGMA.

Statistical evaluation of electrophoretic data was supported by the BIOSYS-1 programme of Swofford and Selander (release 1.7, 1989). It was used for calculating allele frequencies, average heterozygosity (H), the proportion of polymorphic loci (P), F-statistics, Nei's (1978) unbiased D and modified Rogers distances (Wright 1978), for constructing a distance Wagner tree by midpoint rooting (Farris 1972), and to test for allele frequency homogeneity (Workman and Niswander 1970) and for deviations of genotypic proportions from Hardy-Weinberg equilibrium. Because the programme rounds values to only one decimal position, the mean number of alleles (A) was calculated by hand. For comparison with morphological variation, observed average heterozygosity (H_o) was calculated individually over 13 polymorphic loci, and averaged both over all individuals of each sample and separately over juveniles and adults. Sex dependence, age dependence, and differences of H_o among samples were tested by means of the Kruskal-Wallis test. The simultaneous influence of sample and either sex or age was tested simultaneously by means of two-way ANOVAs using arcsin transformed H_o-values. Differences in H_o between juveniles and adults across samples were further checked using the Wilcoxon test. Cross-tabs and χ^2-tests were used for detecting associations of genotypes among loci.

mtDNA variation within samples was quantified both by the measure of nucleon or haplotypic diversity (h) defined by Nei (1987), and by nucleotide diversity (π), which is the average over pairwise δ-values (δ – mean number of nucleotide substitutions per nucleotide site, Nei and Li 1979) within each sample. Sex and age dependence of haplotypes were checked by inspection of cross-tabs (the χ^2-test was not meaningful in our case due to the very low or missing occupation of several cells).

Associations among indices of genetic (allozymes, mtDNA) and morphological variation were examined by Spearman rank correlations across samples, taking into account possible dependencies of the respective indices on sample size. Checking for dependencies of indices on sample size was performed by Spearman rank correlations (values of respective index vs relevant sample sizes). If necessary, samples with very small or very large size, respectively, were excluded. Tests for associations between H_o and IV were performed both with and without separation of individuals into juveniles and adults.

Table 3. Percentage frequencies of character states of 20 non-metric skull variants in the five geographic units of *Lepus europaeus* in Austria used for comparison of morphological and genetic variation. Current numbers correspond to those in Fig. 2 and Table 1. Values for both body sides (right – r/left – l) are given in bilateral traits. 0/1 – character states (see Table 1), *n* – sample size (number of skulls).

Trait	Geographic unit														
	NWA			NA			NEA			EA			SA		
	n	0	1	*n*	0	1	*n*	0	1	*n*	0	1	*n*	0	1
1r	82	25.6	74.4	43	27.9	72.1	91	19.8	80.2	67	13.4	86.6	59	11.9	88.1
1l	79	27.8	72.2	41	29.3	70.7	91	20.9	79.1	69	15.9	84.1	56	16.1	83.9
2	84	90.5	9.5	41	90.2	9.8	92	91.3	8.7	71	91.5	8.5	56	85.7	14.3
3	87	5.7	94.3	43	4.7	95.3	90	7.8	92.2	71	5.6	94.4	59	3.4	96.6
4	87	17.2	82.8	43	16.3	83.7	90	21.1	78.9	70	22.9	77.1	58	20.7	79.3
5r	87	28.7	71.3	43	34.9	65.1	93	21.5	78.5	69	21.7	78.3	59	15.3	84.7
5l	86	24.4	75.6	42	28.6	71.4	92	32.6	67.4	71	26.8	73.2	58	20.7	79.3
6	87	87.4	12.6	42	81.0	19.0	94	91.5	8.5	71	93.0	7.0	58	87.9	12.1
7	88	20.5	79.5	43	9.3	90.7	94	8.5	91.5	69	4.3	95.7	58	19.0	81.0
8r	83	32.5	67.5	44	38.6	61.4	94	29.8	70.2	68	41.2	58.8	58	46.6	53.4
8l	85	32.9	67.1	42	33.3	66.7	94	36.2	63.8	68	45.6	54.4	58	50.0	50.0
9r	82	24.4	75.6	43	23.3	76.7	91	30.8	69.2	68	44.1	55.9	58	34.5	65.5
9l	83	24.1	75.9	44	34.1	65.9	92	32.6	67.4	68	54.4	45.6	58	44.8	55.2
10r	86	2.3	97.7	43	0.0	100	92	3.3	96.7	69	10.1	89.9	58	10.3	89.7
10l	87	2.3	97.7	40	5.0	95.0	91	3.3	96.7	68	13.2	86.8	58	8.6	91.4
11r	87	97.7	2.3	44	86.4	13.6	94	95.7	4.3	71	98.6	1.4	59	100	0.0
11l	87	97.7	2.3	43	86.0	14.0	94	95.7	4.3	71	97.2	2.8	59	98.3	1.7
12r	87	18.4	81.6	44	11.4	88.6	94	14.9	85.1	71	22.5	77.5	59	15.3	84.7
12l	87	16.1	83.9	44	29.5	70.5	93	18.3	81.7	71	15.5	84.5	59	23.7	76.3
13r	88	81.8	18.2	43	72.1	27.9	93	72.0	28.0	71	66.2	33.8	58	65.5	34.5
13l	88	85.2	14.8	42	69.0	31.0	92	71.7	28.3	70	68.6	31.4	57	64.9	35.1
14r	87	100	0.0	43	100	0.0	91	100	0.0	68	100	0.0	58	100	0.0
14l	87	100	0.0	42	97.6	2.4	92	100	0.0	69	100	0.0	57	100	0.0
15r	86	0.0	100	43	0.0	100	92	2.2	97.8	68	0.0	100	58	1.7	98.3
15l	88	1.1	98.9	42	0.0	100	94	2.1	97.9	70	0.0	100	59	3.4	96.6
16	84	91.7	8.3	42	76.2	23.8	93	86.0	14.0	69	91.3	8.7	56	78.6	21.4
17r	88	100	0.0	43	100	0.0	94	100	0.0	71	100	0.0	58	100	0.0
17l	88	100	0.0	42	100	0.0	94	100	0.0	70	100	0.0	58	98.3	1.7
18r	63	14.3	85.7	38	7.9	92.1	83	27.7	72.3	64	17.2	82.8	48	14.6	85.4
18l	63	14.3	85.7	40	10.0	90.0	80	22.5	77.5	63	15.9	84.1	49	14.3	85.7
19r	87	3.4	96.6	43	0.0	100	94	1.1	98.9	70	4.3	95.7	59	1.7	98.3
19l	85	1.2	98.8	42	0.0	100	93	4.3	95.7	70	4.3	95.7	59	0.0	100
20	85	94.1	5.9	44	95.5	4.5	93	96.8	3.2	70	97.1	2.9	59	93.2	6.8

Results

Morphological variation

Sex or/and age dependence was detected in 16 out of 36 non-metric traits ($p < 0.01$). Therefore they were not considered in subsequent morphological analyses. Among the remaining characters (Table 1), pairwise associations of character states were detected between traits 4/12, 5/13, 6/8, 6/11, and 7/18 ($p < 0.01$). However, since all coefficients of association were rather low (ranging from 0.23 to 0.36) and 6.7 cases of associations can be expected to reach significance at $p < 0.01$ by chance, no character was excluded. IV-values for each of the 20 populations are given in Table 10. They did not differ between the two age classes but showed a positive correlation with sample size ($r_S = 0.51$, $p = 0.01$). Frequencies of character states in the five geographic units are presented in Table 3. The MMD-matrix together with standard deviations and MU-values is shown in Table 4. A UPGMA-dendrogram of the five GUs is displayed in Fig. 3.

Table 4. Matrix of MMD-distances among the five geographic units of *Lepus europaeus* in Austria (standard deviations in parentheses). MU – measure of uniqueness, n – sample size (individuals), * – MMD significantly different from zero (i.e. larger than twice the standard deviation).

	n	NWA	NA	NEA	EA	SA	MU
NWA	88	–	0.0024 (0.0110)	0.0029 (0.0071)	0.0194* (0.0082)	0.0146 (0.0091)	0.0393
NA	44		–	0.0083 (0.0107)	0.0377* (0.0118)	0.0258* (0.0127)	0.0742
NEA	94			–	0.0004 (0.0079)	0.0079 (0.0088)	0.0195
EA	71				–	0.0000 (0.0099)	0.0575
SA	59					–	0.0483

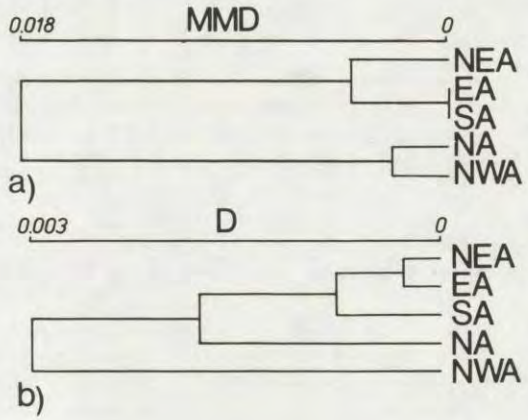

Fig. 3. (a) Morphological relationships among the five population groups (GUs) of the brown hare (MMD/UPGMA). (b) Genetic relationships among the five population groups (GUs) of the brown hare (Nei's 1978 D/UPGMA).

Table 5. Allele frequencies in the 20 *Lepus europaeus* populations studied. Deviations of observed genotype frequencies from Hardy-Weinberg expectations are indicated by probability value on the

Locus	Allele	Brown hare populations								
		VRT	OWN	OWS	OIV	OKT	OP	WV-3	WV-1	WV-4
	n	12	31	23	30	13	16	28	30	41
Sdh	*100*	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.933	1.0
	300	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.067	0.0
Ldh-2	*100*	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	83	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mor-2	*100*	0.833	0.984	0.978	0.967	0.885	1.0	1.0	1.0	1.0
	79	0.167	0.016	0.022	0.033	0.115	0.0	0.0	0.0	0.0
Idh-2	*100*	1.0	0.984	0.826	1.0	1.0	1.0	1.0	1.0	1.0
	130	0.0	0.016	0.174	0.0	0.0	0.0	0.0	0.0	0.0
	83	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pgd	*100*	1.0	0.952	0.957[5]	1.0	0.923	0.906	0.786	0.850	0.915
	170	0.0	0.016	0.022	0.0	0.0	0.0	0.018	0.017	0.0
	129	0.0	0.0	0.021	0.0	0.0	0.0	0.017	0.083	0.049
	117	0.0	0.0	0.0	0.0	0.0	0.0	0.036	0.0	0.036
	64	0.0	0.032	0.0	0.0	0.077	0.094	0.143	0.050	0.0
Hk-2	*100*	1.0	0.952	1.0	0.950	1.0	1.0	1.0	0.967	0.976
	67	0.0	0.048	0.0	0.050	0.0	0.0	0.0	0.033	0.024
Es-I	*−100*	0.625	0.672	0.717	0.650	0.615	0.625	0.732	0.633	0.462
	−108	0.0	0.0	0.0	0.050	0.038	0.0	0.0	0.017	0.038
	−75	0.375	0.328	0.283	0.300	0.347	0.375	0.268	0.333	0.500
	−42	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.017	0.0
Es-d	*100*	0.833	0.887	0.783	0.783	0.692	0.875	0.839	0.833	0.817
	141	0.167	0.113	0.217	0.217	0.308	0.125	0.161	0.167	0.183
β-Gal	*100*	0.125	0.283	0.333	0.345[5]	0.308	0.281	0.423[1]	0.650[1]	0.645[1]
	117	0.0	0.0	0.048	0.0	0.077	0.063	0.115	0.0	0.079
	83	0.875	0.717	0.619	0.655	0.615	0.656	0.462	0.350	0.276
Pep-2	*100*	0.792	0.750	0.804	0.783	0.885	0.875	0.768	0.917	0.768
	114	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	104	0.208	0.250	0.196	0.217	0.115	0.125	0.232	0.083	0.232
Acy-1	*100*	0.292	0.367	0.370	0.367	0.500	0.344	0.268[5]	0.224	0.268
	81	0.542	0.400	0.543	0.500	0.385	0.562	0.696	0.690	0.634
	66	0.166	0.233	0.087	0.133	0.115	0.094	0.036	0.086	0.098
Ada-2	*100*	0.727	0.855	0.870	0.950	0.409	0.867	0.911	0.900	0.897
	121	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	75	0.273	0.145	0.130	0.050	0.591	0.133	0.089	0.100	0.103
Ada-3	*100*	0.773	0.700	0.696	0.783	0.750	1.0	0.946	0.983	0.897
	111	0.227	0.300	0.304	0.217	0.250	0.0	0.054	0.017	0.103
Mpi	*100*	1.0	0.952[5]	1.0	1.0	1.0	1.0	1.0	1.0	0.988
	126	0.0	0.048	0.0	0.0	0.0	0.0	0.0	0.0	0.012
	77	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

right hand side of the most common allele (5 – $p < 0.05$, 1 – $p < 0.01$, exact probability; in most cases there was an excess of homozygotes, p-value in italics – excess of heterozygotes).

Brown hare populations										
TFN	TFS	SP	GUW	GUO	SW	GP	GB	KSV	KLT	KK
30	20	30	20	15	29	7	29	17	33	15
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.955	1.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.045	0.0
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.985	0.967
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.015	0.033
0.933	1.0	0.950	0.975	0.967	1.0	1.0	0.732	0.941	0.955	1.0
0.067	0.0	0.050	0.0	0.0	0.0	0.0	0.232	0.059	0.0	0.0
0.0	0.0	0.0	0.025	0.033	0.0	0.0	0.036	0.0	0.045	0.0
1.0	0.825	0.967	1.0	0.867	0.948	0.929	0.966	0.912	0.970	1.0
0.0	0.0	0.0	0.0	0.0	0.0	0.071	0.0	0.0	0.015	0.0
0.0	0.075	0.017	0.0	0.033	0.052	0.0	0.034	0.029	0.015	0.0
0.0	0.100	0.0	0.0	0.100	0.0	0.0	0.0	0.059	0.0	0.0
0.0	0.0	0.016	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.833	0.650	0.550	0.450	0.567	0.569	0.357	0.534	0.676	0.667	0.433
0.0	0.025	0.067	0.0	0.0	0.0	0.143	0.086	0.0	0.0	0.0
0.167	0.325	0.383	0.550	0.433	0.414	0.500	0.242	0.324	0.303	0.567
0.0	0.0	0.0	0.0	0.0	0.017	0.0	0.138	0.0	0.030	0.0
0.750	0.850	0.733	0.800	0.867	0.672^{5}	0.857	0.983	0.824	0.833^{5}	0.967
0.250	0.150	0.267	0.200	0.133	0.328	0.143	0.017	0.176	0.167	0.033
0.535^{1}	0.553	0.300	0.842^{5}	0.700^{5}	0.444^{1}	0.833	0.810^{5}	0.688^{1}	0.672^{1}	0.733^{5}
0.086	0.158	0.167	0.053	0.0	0.130	0.0	0.190	0.250	0.156	0.067
0.379	0.289	0.533	0.105	0.300	0.426	0.167	0.0	0.062	0.172	0.200
0.776	0.925	0.833	0.800	0.900	0.862	0.857	0.672	0.618	0.758	0.767
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.138	0.0	0.0	0.0
0.224	0.075	0.167	0.200	0.100	0.138	0.143	0.190	0.382	0.242	0.233
0.300	0.300	0.300	0.375	0.233	0.328	0.286	0.397	0.412	0.303	0.300
0.550	0.500	0.517	0.575	0.634	0.396	0.643	0.413	0.559	0.470	0.633
0.150	0.200	0.183	0.050	0.133	0.276	0.071	0.190	0.029	0.227	0.067
0.690	0.875	0.897	0.850	0.767	0.768^{5}	0.857	0.833	0.882	0.848	0.867
0.0	0.025	0.017	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.310	0.100	0.086	0.150	0.233	0.232	0.143	0.167	0.118	0.152	0.133
1.0	0.900	0.810	0.947	0.917	0.880	0.929	1.0	0.941	0.970	0.767
0.0	0.100	0.190	0.053	0.083	0.120	0.071	0.0	0.059	0.030	0.233
0.983	0.950	1.0	0.925	1.0	0.914	1.0	1.0	0.971	1.0	0.900
0.017	0.050	0.0	0.075	0.0	0.086	0.0	0.0	0.029	0.0	0.033
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.067

Electrophoretic variation

Screening of 35 enzyme systems representing a total of 54 presumptive structural loci revealed polymorphism in the following isozymes: SDH, LDH-2, MOR-2, IDH-2, PGD, HK-2, ES-I, ES-D, ß-GAL, PEP-2, ACY-1, ADA-2, ADA-3, and MPI. Some of these polymorphisms are described in Hartl (1987, 1991), together with family studies to demonstrate the Mendelian inheritance of allozymes. Allele frequencies at the corresponding loci in each of the 20 samples are listed in Table 5. Except for *Hk-2*, *Es-d*, and *Acy-1* there was significant heterogeneity of allele frequencies among populations ($p < 0.05$ for *Ldh-2*, $p < 0.001$ for all other loci). There was no sex and age dependence of particular genotypes and of heterozygosity at single loci. At the *β-Gal* locus, genotypic proportions showed a consistent excess of homozygotes with respect to Hardy-Weinberg expectations (Table 5). This is demonstrated also by *F*-statistics for single loci (Table 6). The excess of homozygotes observed may be due to the occurrence of a null-allele, which is supported by the complete lack of any ß-GAL activity in several specimens (= presumptive homozygotes for the null-allele). Since misscoring of potential heterozygotes for a null-allele prevents an accurate estimation of allele frequencies, the *β-Gal* locus was excluded from further population genetic analyses. For comparison with morphological variability, overall observed heterozygosity (H_o) was calculated over 13 polymorphic loci (Table 10). H_o was not sex and age dependent, and there were no associations of genotypes among loci. H_o was significantly heterogeneous among populations ($p < 0.05$). For characterizing allozyme variation within populations, expected average heterozygosity (H_e), the mean number of alleles per locus (A), and the proportion of polymorphic loci (P) were calculated over 53 loci and are

Table 6. Summary table of *F*-statistics by locus for the 20 brown hare populations studied.

Locus	F_{IS}	F_{IT}	F_{ST}
Sdh	−0.071	−0.003	0.188
Ldh-2	−0.048	−0.002	0.043
Mor-2	−0.124	−0.020	0.092
Idh-2	0.165	0.265	0.119
Pgd	0.090	0.141	0.056
Hk-2	−0.044	−0.008	0.034
Es-I	−0.029	0.021	0.034
Es-d	−0.052	−0.010	0.039
Pep-2	0.041	0.076	0.037
β-Gal	0.514	0.606	0.188
Acy-1	−0.177	−0.141	0.030
Ada-2	0.081	0.165	0.092
Ada-3	−0.006	0.089	0.095
Mpi	0.014	0.060	0.047
Mean:	0.055	0.129	0.078
Mean (*β-Gal* excluded):	−0.034	0.022	0.054

Table 7. Nei's (1978) unbiased genetic distances (above diagonal) and modified Rogers distances (Wright 1978, below diagonal) between the 20 brown hare samples studied. Both distances are calculated over 53 loci (*β-Gal* excluded).

	VRT	OWN	OWS	OIV	OKT	OP	WV-3	WV-1	WV-4	TFN	TFS	SP	GUW	GUO	SW	GP	GB	KSV	KLT	KK	
VRT	–	0.000	0.000	0.000	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.003	0.001	0.000	0.000	VRT
OWN	0.037	–	0.000	0.000	0.004	0.002	0.002	0.003	0.002	0.003	0.001	0.001	0.002	0.002	0.001	0.002	0.003	0.001	0.001	0.001	OWN
OWS	0.043	0.036	–	0.000	0.004	0.002	0.002	0.002	0.002	0.002	0.001	0.000	0.002	0.002	0.002	0.002	0.003	0.001	0.002	0.002	OWS
OIV	0.039	0.029	0.032	–	0.005	0.001	0.001	0.002	0.001	0.002	0.001	0.000	0.001	0.001	0.001	0.001	0.003	0.001	0.001	0.001	OIV
OKT	0.058	0.074	0.075	0.081	–	0.005	0.007	0.007	0.006	0.003	0.005	0.004	0.005	0.004	0.002	0.005	0.008	0.006	0.005	0.006	OKT
OP	0.048	0.051	0.054	0.041	0.081	–	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.001	0.000	0.002	0.000	0.000	0.001	OP
WV-3	0.055	0.056	0.052	0.044	0.091	0.031	–	0.000	0.001	0.002	0.001	0.001	0.002	0.001	0.003	0.001	0.004	0.000	0.001	0.003	WV-3
WV-1	0.054	0.060	0.057	0.047	0.090	0.025	0.030	–	0.001	0.002	0.000	0.001	0.001	0.000	0.002	0.000	0.004	0.002	0.001	0.002	WV-1
WV-4	0.045	0.050	0.052	0.038	0.086	0.034	0.041	0.036	–	0.003	0.001	0.000	0.000	0.000	0.001	0.000	0.003	0.001	0.001	0.000	WV-4
TFN	0.051	0.059	0.055	0.054	0.069	0.047	0.049	0.053	0.061	–	0.002	0.002	0.003	0.002	0.002	0.003	0.003	0.001	0.001	0.005	TFN
TFS	0.047	0.044	0.048	0.038	0.080	0.028	0.040	0.031	0.039	0.052	–	0.000	0.001	0.000	0.001	0.000	0.003	0.001	0.000	0.002	TFS
SP	0.039	0.037	0.034	0.023	0.076	0.038	0.047	0.042	0.030	0.054	0.034	–	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.001	SP
GUW	0.048	0.055	0.057	0.045	0.080	0.034	0.051	0.045	0.023	0.060	0.045	0.036	–	0.000	0.001	0.000	0.003	0.001	0.001	0.000	GUW
GUO	0.041	0.052	0.053	0.048	0.073	0.028	0.042	0.030	0.031	0.051	0.029	0.039	0.037	–	0.001	0.000	0.003	0.001	0.000	0.001	GUO
SW	0.044	0.047	0.053	0.044	0.062	0.046	0.060	0.053	0.046	0.050	0.039	0.032	0.042	0.044	–	0.001	0.004	0.002	0.001	0.003	SW
GP	0.052	0.060	0.062	0.050	0.085	0.037	0.052	0.041	0.023	0.068	0.045	0.039	0.028	0.033	0.052	–	0.002	0.001	0.001	0.000	GP
GB	0.067	0.062	0.062	0.063	0.094	0.055	0.066	0.066	0.062	0.062	0.060	0.061	0.062	0.061	0.068	0.064	–	0.002	0.002	0.004	GB
KSV	0.051	0.048	0.047	0.039	0.087	0.041	0.036	0.051	0.040	0.047	0.050	0.045	0.043	0.051	0.056	0.055	0.054	–	0.000	0.002	KSV
KLT	0.041	0.041	0.048	0.035	0.079	0.029	0.040	0.041	0.039	0.036	0.034	0.035	0.041	0.038	0.039	0.048	0.047	0.034	–	0.002	KLT
KK	0.045	0.048	0.056	0.049	0.088	0.050	0.059	0.057	0.033	0.077	0.054	0.046	0.037	0.045	0.061	0.038	0.069	0.055	0.055	–	KK

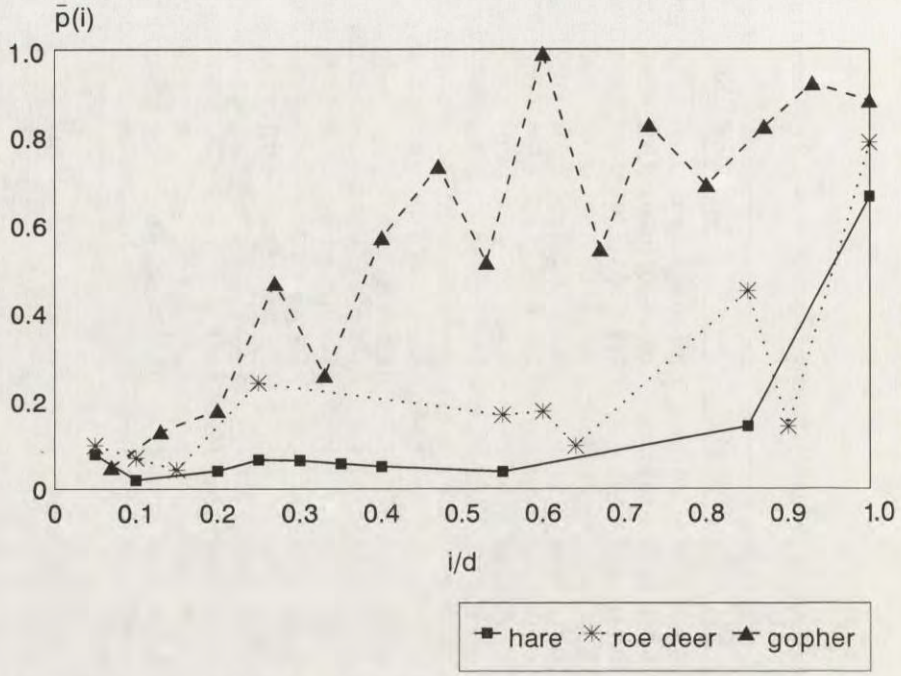

Fig. 4. Conditional average allele frequencies p(i) in the brown hare (Slatkin 1981). For comparison with species showing more separation among populations, data from roe deer (*Capreolus capreolus*, Hartl *et al.* 1991a, sampling area largely overlapping with that of the brown hare) and the pocket gopher (*Thomomys umbrinus*, Patton and Feder, 1978) are also presented. d – number of locations sampled (20 in the brown hare, 20 in the roe deer, and 15 in the pocket gopher).

given in Table 10. In contrast to H_o, H_e, and F_{IS}, P and A showed a significant positive correlation with sample size ($r_s = 0.50$, $p = 0.012$ and $r_s = 0.63$, $p = 0.001$, respectively). There was no significant association of overall heterozygosity (H_o, H_e) and F_{IS} with either P or A, even when the effect of sample size on P and A was eliminated. Relative genetic differentiation among populations (F_{ST} in Table 6 is equivalent to Nei's 1973 G_{ST}) was 5.4%, yielding an estimated Nm (Slatkin and Barton 1989) of 4.4. Calculated by means of Slatkin's (1985) private allele approach we obtained a Nm of 1.2 ($\bar{p}(1) = 0.079$, Table 5, Fig. 4). Since our mean sample size of 23 was very similar to that used in Slatkin's model no correction for sample size was necessary. Genetic distances – Nei's (1978) unbiased D and the Rogers distance modified according to Wright (1978) – are given in Table 7. A distance Wagner tree (Farris 1972), displaying genetic relationships among populations is shown in Fig. 5.

mtDNA variation

Screening of mtDNAs in 131 individuals with 16 restriction enzymes yielded a total of 44 fragments which could be assigned to 6 different haplotypes.

Table 8. Numerical maps of the 6 brown hare mtDNA haplotypes, and of the type described previously. * – Starting site for mapping defined in Biju-Duval *et al.* (1991), B.D. – Biju-Duval *et al.* 1991, ns – not studied by these authors. PstI and XmnI are omitted because there were no cutting sites (PstI – same result in B.D., XmnI – ns).

Restriction enzyme	Cutting* sites (kb)	Haplotypes						
		I	II	III	IV	V	VI	B.D.
ApaI	2.8	+	+	+	+	+	+	ns
	3.9	+	+	+	+	+	+	
	15.9	+	+	+	+	+	+	
BamHI	5.0	+	+	+	+	+	+	+
BclI	2.3	+	+	+	+	+	+	+
	3.0	+	+	+	+	+	+	+
	3.4	+	+	+	+	+	+	+
	11.2	+	+	+	+	+	+	+
	11.9	+	+	+	+	+	+	+
BglII	7.3	+	+		+	+	+	ns
ClaI	0.0							+
	0.6	+	+	+	+	+	+	+
DraI	1.4	+	+	+	+	+	+	ns
	2.6	+	+	+	+	+	+	
	6.7	+	+	+	+	+	+	
	8.0	+	+	+	+	+	+	
	12.0	+	+	+	+	+	+	
	15.5	+	+	+	+	+	+	
	17.1	+	+	+	+	+	+	
EcoRI	1.7	+	+	+	+	+	+	+
EcoRV	13.1	+	+	+	+	+	+	ns
HindIII	0.6	+	+	+	+	+	+	+
	3.5	+	+	+	+	+	+	+
	5.5	+	+	+	+	+	+	+
	7.2	+	+	+	+	+	+	+
	11.0	+	+	+	+	+	+	+
	15.4					+		
HpaI	1.9	+	+	+	+	+	+	+
	5.1	+	+	+	+	+	+	+
	6.6	+	+	+	+	+	+	+
	8.6	+	+	+	+	+	+	+
	14.1	+	+	+	+	+		+
PvuII	5.8	+	+	+	+	+	+	+
	7.0				+			
	13.0	+	+	+	+	+	+	+
SacI	2.6	+	+	+	+	+	+	+
	6.3	+	+	+	+	+	+	+
	7.0	+	+	+	+	+	+	+
	7.2	+	+	+	+	+	+	
	9.2	+	+	+	+	+	+	+
XbaI	2.3	+	+	+	+	+	+	+
	3.7	+	+	+	+	+	+	+
	7.6	+	+	+	+	+	+	+
	9.6	+	+	+	+	+	+	+
XhoI	11.2	+		+	+	+	+	ns

Table 9. Distribution of mtDNA haplotypes among the 18 brown hare populations studied.

Sample	Type I	Type II	Type III	Type IV	Type V	Type VI
VRT	3					
OWN	6					
OWS	4					
OIV	4	1				
OKT	4	2				
OP	---------- not studied ----------					
WV-3	6					
WV-1	4					
WV-4	10			1	1	
TFN	1		2			
TFS	7		5	2	3	
SP	8		2		1	1
GUW	2					
GUO	5					
SW	12			1		
GP	3					
GB	8					
KSV	---------- not studied ----------					
KLT	16					
KK	6					

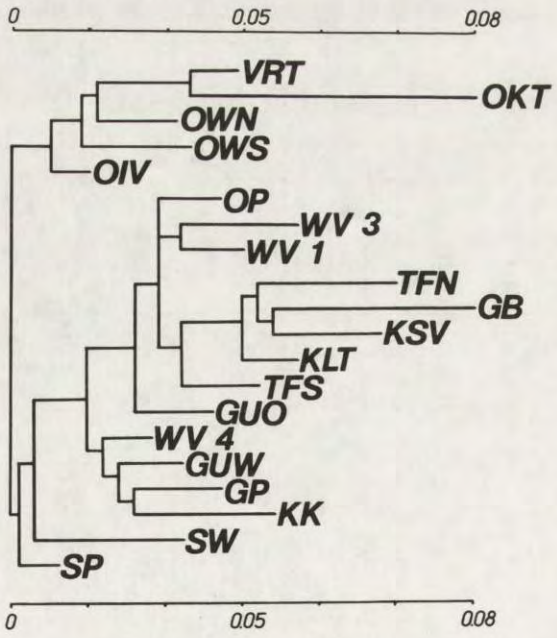

Fig. 5. Genetic relationships among the 20 *Lepus europaeus* populations studied (modified Rogers distance (Wright 1978) / distance Wagner tree with midpoint rooting (Farris 1972). Cophenetic correlation coefficient = 0.85.

Table 10. Allozyme, mtDNA, and morphological variation in the brown hare. n_1 – sample size for electrophoresis, H_o – direct count heterozygosity (over 13 polymorphic loci), H_e – expected heterozygosity (over 53 loci), F_{IS} – inbreeding coefficient (over 53 loci), A – mean number of alleles (over 53 loci), P – proportion of polymorphic loci (over 53 loci), n_2 – sample size for mtDNA, h – nucleon diversity (Nei 1987), π – nucleotide diversity (Nei 1987), n_3 – sample size for morphology, IV – inter-individual variation (= SD over 20 traits).

Sample	n_1	H_o (%)	H_e (%)	F_{IS}	A	P (%)	n_2	h (%)	π (%)	n_3	IV
VRT	12	22.2	5.3	−0.019	1.15	13.2	3	0.0	0.0	12	0.262
OWN	31	21.4	5.1	−0.039	1.24	20.8	6	0.0	0.0	31	0.267
OWS	23	19.1	5.2	0.096	1.21	17.0	4	0.0	0.0	17	0.300
OIV	30	18.5	4.5	0.0	1.19	15.1	5	32.0	0.03	30	0.292
OKT	13	26.8	5.8	−0.155	1.19	15.1	6	44.4	0.04	10	0.192
OP	16	15.6	3.6	−0.056	1.13	11.3	–	–	–	18	0.198
WV-3	28	14.8	4.0	0.075	1.21	13.2	6	0.0	0.0	26	0.329
WV-1	30	14.6	3.9	0.077	1.26	17.0	4	0.0	0.0	30	0.277
WV-4	41	18.6	4.4	−0.046	1.23	17.0	12	29.2	0.02	35	0.268
TFN	30	15.4	4.2	0.095	1.15	13.2	3	44.4	0.04	30	0.306
TFS	20	17.3	4.4	0.0	1.23	15.1	17	69.9	0.06	20	0.331
SP	30	18.3	4.8	0.062	1.23	15.1	12	51.3	0.04	28	0.323
GUW	20	17.4	4.3	0.0	1.17	15.1	2	0.0	0.0	22	0.294
GUO	15	17.2	4.4	0.046	1.19	15.1	5	0.0	0.0	15	0.253
SW	29	21.4	5.1	−0.039	1.19	15.1	13	14.2	0.01	26	0.317
GP	7	18.7	4.3	−0.070	1.17	13.2	3	0.0	0.0	7	0.188
GB	29	21.2	4.9	−0.082	1.23	13.2	8	0.0	0.0	27	0.301
KSV	17	20.4	4.6	−0.087	1.21	17.0	–	–	–	15	0.253
KLT	33	16.8	4.4	0.068	1.24	18.9	16	0.0	0.0	31	0.326
KK	15	18.0	4.4	0.0	1.19	15.1	6	0.0	0.0	13	0.275
Mean	23	18.7	4.6		1.20	15.3	7	15.8	0.01	22	0.278
SD	9	2.9	0.5		0.03	2.2	4	23.0	0.02	8	0.044

Numerical restriction maps for all haplotypes are given in Table 8. The distribution of the 6 mtDNA types in the various populations studied is shown in Table 9. Nucleon (haplotype) diversity (h) and nucleotide diversity (π) for each population are presented in Table 10. In both h and π there was no statistically significant influence of sample size.

Relationships between molecular and morphological variation

MtDNA diversity did not show any relationship with overall electrophoretic or morphological variation. Of the various indices of electrophoretic variation (Table 10) only the inbreeding coefficient (F_{IS}) was statistically significantly correlated with IV ($r_s = 0.48$, $p = 0.022$, $n = 18$). The correlation was even higher when F_{IS} was calculated using H_e-values not corrected for sample sizes ($r_s = 0.51$, $p = 0.015$, $n = 18$). Regarding differentiation among populations, the distribution of mtDNA haplotypes was largely concordant with that of some enzyme alleles (Fig. 6). The

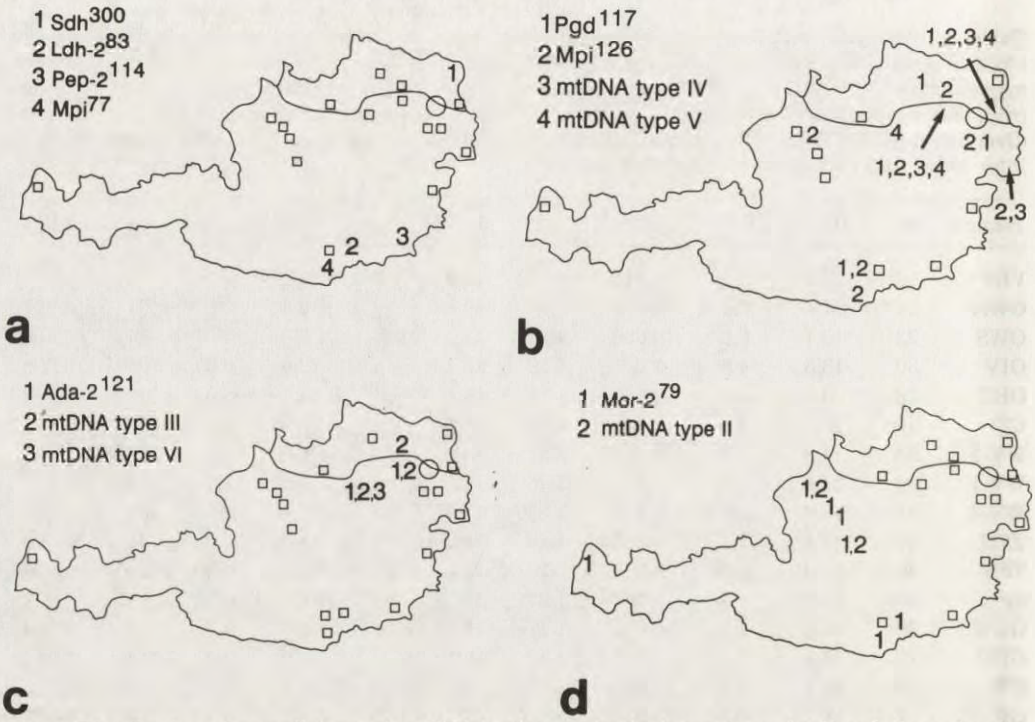

Fig. 6. Distribution of rare alleles and mtDNA haplotypes in the brown hare. (a) Populations showing 'private alleles'. (b) and (c) Distribution of rare alleles and haplotypes suggesting gene flow along the Danube valley. (d) Rare alleles and haplotypes supporting a separation of western populations from those in the east. Of all rare variants shown in this figure, only the incidence of haplotypes IV (p = 0.018) and V (p = 0.03, Kruskal-Wallis test) showed a dependence on sample size. For further explanation see text.

UPGMA-dendrogram of *GU*s based on non-metric traits (MMD) was topologically rather similar to a corresponding UPGMA-dendrogram based on allozyme data (Nei's 1978 *D*, Fig. 3).

Discussion

Genetic diversity within populations

With mean P = 15.3% (SD = 2.2%) and mean H_e = 4.6% (SD = 0.5%), values of allozyme polymorphism and heterozygosity are similar to those reported in previous population genetic studies on the brown hare (Hartl *et al.* 1990, 1992). A detailed comparison of allozyme variation in the brown hare with that detected in the other lagomorph species studied so far is given in Hartl *et al.* (1990). Based on largely the same set of enzymes, *P*- and *H*-values in the brown hare are comparable to those in the rabbit (cf Hartl 1987, Peterka and Hartl 1992), the only other lagomorph species studied more extensively for mtDNA variation within local populations (Ennafaa *et al.* 1987, Biju-Duval *et al.* 1991). The number of

mtDNA haplotypes detected in the rabbit by Biju-Duval *et al.* (1991) is three times larger than that found in the hare in the present study. However, the apparently big difference in intraspecific variation between both species must be evaluated in the light of the geographical range surveyed. The French study included populations from France, Spain, and Tunisia, and covered two subspecies of the rabbit. Our study on the hare covered only a very restricted geographical sample of a widely distributed species. Moreover, data from a large scale screening of allozyme differentiation (Hartl *et al.* 1990) did not suggest any taxonomic differences among Central European brown hare populations. Generally, mean nucleotide diversity in the hare (mean $\pi = 0.01\%$, SD = 0.02%) is within the range commonly found in mammals (Avise and Lansman 1983, Nevo *et al.* 1993). The general lack of a correlation between allozyme and mtDNA variation (in terms of various indices – Table 10) within populations may be a result of the migration pattern described in the next section.

Genetic differentiation among populations

One objective of the present study was to check the effects of various possible natural (rivers, mountains, forests) and anthropogenic (motorways, settlements) barriers on patterns of gene flow in the hare. Generally, no restriction of gene flow was suggested by our data. *Nm* was high and both relative (in terms of F_{ST}) and absolute (in terms of Nei's 1978 D) genetic differentiation among populations were low (cf Hartl *et al.* 1990, 1992). The high level of gene flow in the hare is demonstrated also in Fig. 4, where the conditional average frequency ($\bar{p}(i)$, Slatkin 1981) for each allele is plotted against the number of populations in which the respective allele was present ('occupancy number'). Although hares are generally able to pass even broad rivers (Schneider 1978) and in spite of the presence of some bridges, we initially expected the river Danube to separate the Austrian brown hare population into a northern and a southern cluster. Moreover we expected some separation between local populations east and west of Vienna and the pre-alpine forests south of this town. The Carinthian populations (KLT, KSV, KK) could have formed a separate cluster because they are inhabiting geographically rather closed alpine valleys. In contrast to these hypotheses, the major separation was found between brown hare samples of western Austria (VRT, OWN, OWS, OIV, OKT) and all the other populations (Fig. 5). Within the 'eastern cluster' some of the subclusters were consistent with geographic distribution (e.g. OP, WV-3, WV-1, and KSV, KLT, GB) whereas most of the samples appeared at unexpected positions in the tree. This result may to some extent be due to unequal sample sizes and to generally low genetic distances. However, according to the incidence of rare alleles and of mtDNA haplotypes, the pattern of genetic differentiation among hare populations seems to be considerably influenced by immigration of brown hares from the neighbouring countries.

As can be seen in Fig. 6a, 'private' alleles occurred exclusively in samples from populations situated at the very margin of our study area. The allele Sdh^{300} in

WV-1 has already been detected in Polish and Czechian populations by Hartl (1991) and Hartl *et al.* (1992). The alleles *Ldh-2*^{83}, *Pep-2*^{114}, and *Mpi*^{77} are likely to have been introduced by immigrants from Slovenia or Italy, but to date no relevant studies are available for testing this assumption. Also the high allelic diversity in KLT in spite of low population density (Margl 1982) as well as deviations from Hardy-Weinberg equilibrium in SW and KLT may be explained by immigration. The distribution of rare alleles and of haplotypes in Figs. 6b and c and that of some more alleles in Table 5 strongly suggest the Danube valley to be a major route for immigration and gene flow. An alternative explanation for the pattern in Fig. 6c would be a release of brown hares from abroad, but no introductions are reported in the protocols of local hunting authorities. Also the distribution of *Mor-2*^{79} and haplotype II in Fig. 6d corroborates the major clustering of the distance Wagner tree (Fig. 5) and suggests some influx of genetic variants from Germany as well. Interestingly, several of the rare alleles otherwise restricted to eastern or western populations are also found in one or more of the populations in the south. However, unless data from adjacent populations in Italy, Slovenia, and Hungary are available it remains speculative whether this is due to immigration from the same source gene pools or to gene flow along alpine riverine valleys. The same holds for the origin of one or the other mtDNA haplotype (Fig. 6c, d) by mutation, especially as all variant haplotypes (Table 8) can be derived from the standard haplotype I by only one base pair substitution (cf Avise *et al.* 1987).

Relationships between genetic and morphological variation

Relationships between biochemical-genetic and non-metric (meristic) morphological variability have been examined in a number of animals. Two fundamentally different concepts emerge from the studies available. The first one is based on the hypothesis that morphological variation is positively related to genetic variation (see Schnell and Selander 1981, for review). Based on the argument that the underlying genetic basis covers a larger fraction of the whole genome, some authors stated that morphological variation may be an even better estimator of overall genetic diversity than a set of allozymes (e.g. Soulé *et al.* 1973, Berry and Jakobson 1975). The second concept is based on the hypothesis that the more heterozygous individuals have an increased developmental stability (Lerner 1954). As a measure for developmental stability, not only fluctuating asymmetry (FA), (see Novak *et al.* 1993 for review) but also inter-individual morphological variation (IV) has been used by various authors (cf Suchentrunk 1993). Comparisons of morphological variation among groups of individuals differing in average heterozygosities (H) revealed a significant negative relationship between the variation in morphological traits and H (e.g. Mitton 1978, Fleischer *et al.* 1983, Yezerinac *et al.* 1992). However, an approximately equal number of studies failed to detect an association between heterozygosity and inter-individual morphological variation (e.g. Handford 1980, McAndrew *et al.* 1982, Kieser and Groeneveld 1991). The results of our

study do not support a relationship between IV and allozyme heterozygosity or mtDNA diversity in the brown hare. Regarding allozymes, this holds also for non-metric dental characters in the same individuals (Suchentrunk 1993). Nevertheless, increased IV as a consequence of reduced developmental homeostasis is suggested by the positive correlation with F_{IS}. But since F_{IS}-values are not remarkably deviating from zero and concerning the relatively high number of correlation tests performed (at the 0.05 level a significant result is expected to occur by chance in 1.4 out of 28 tests) the positive correlation between F_{IS} and IV could be spurious as well. In order to verify this result, morphological variability is being examined in relation to inbreeding coefficients in a long-term study on a brown hare breed (cf Hartl *et al.* 1991b).

Regarding morphological differentiation among GUs only 30% of all MMD--values differed significantly from zero (Table 4). This suggests that morphological differentiation as indicated by non-metric skull traits is generally rather poor. Although some MMD-values reached the average level reported for other mammalian populations (see Sjøvold 1977 for red foxes *Vulpes vulpes*, Berry *et al.* 1978 for house mice *Mus musculus*, Sikorski 1982 for striped field mice *Apodemus agrarius*, Sikorski and Bernshtein 1984 for bank voles *Clethrionomys glareolus*, Markowski and Markowska 1988, and Zima 1989 for roe deer *Capreolus capreolus*), they are generally lower than those reported in the studies quoted above. However, in all those studies MMDs were calculated after excluding non-metric traits whose frequencies did not differ significantly between at least two populations. That analytical procedure was reasoned because non-metric characters without significant geographical variation of character frequencies are considered to contribute no additional information to patterns of spatial differentiation (Sjøvold 1977). However, an exclusion of geographically invariant traits means an alteration within the sample of morphological characters directed towards an increased absolute differentiation among populations. MMD-values are increased whereas standard deviations are not affected, which raises the chance for MMDs to become statistically significant. If the objective of a non-metric morphological analysis is merely to reveal relative differentiation among samples, excluding traits without significant differences in frequencies of character states is an adequate approach. Presently, however, we were interested in comparing the absolute degree of differentiation in skull traits among populations with patterns of overall molecular differentiation. Since there were some (weak) intercorrelations among non-metric character states, morphological differentiation among GUs as revealed through non-metric skull traits might be even somewhat lower than indicated by the MMDs given in Table 4. Nevertheless, the significant morphological separation of GUs in the north-western part of Austria (NWA, NA) from those in the south and in the east corresponds very well to molecular differentiation as revealed by overall genetic distances (Figs 3 and 5), and by single enzyme alleles and mtDNA haplotypes, respectively (Fig. 6).

Conclusion

In the present study three character systems (allozymes, mtDNA, morphology) were used for resolving genetic diversity within and among populations of a mammal, rather continuously distributed within a geographically restricted, albeit geomorphologically and climatically heterogeneous area. Both with respect to overall indices of variation and differentiation, and to the distribution of single genetic variants the allozymes yielded the most informative results: Average heterozygosity was significantly different among populations and a hypothesis on spatial distribution and migration patterns emerging from overall genetic distances could be worked out in more detail utilizing the distribution of rare alleles. Based on the incidence of rare haplotypes, the mtDNA data provided valuable support for the above hypothesis, but would not have contributed considerably to an assessment of genetic diversity in the brown hare without additional information from allozymes. Given the high degree of panmixia in this species, in spite of several possible barriers to gene flow our sampling area obviously was too small for obtaining relevant phylogeographic patterns (cf Avise *et al* 1987, Nevo *et al.* 1993). The spatial pattern of morphological differentiation roughly portrayed the main separation among populations as suggested by molecular data. However, a fine-grained analysis of morphological differentiation based on single populations was prevented by too small sample sizes for obtaining reasonable MMD-values and leveling out possible environmental influences on non-metric variants (Sjøvold 1977).

Altogether, the three character systems studied showed some concordance in displaying spatial differentiation among populations and migration patterns. However, according to our results each of them considered separately is not representative for overall gene pool diversity within populations.

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References

Allendorf F. W. and Leary R. F. 1986. Heterozygosity and fitness in natural populations of animals. [In: Conservation biology – The science of scarcity and diversity. M. E. Soulé, ed]. Sinauer Associates, Inc. Publ., Sunderland, Massachusetts: 57 – 76.

Avise J. C. and Lansman R. 1983. Polymorphism of mitochondrial DNA in populations of higher animals. [In: Evolution of genes and proteins. M. Nei and R. K. Koehn, eds]. Sunderland, Massachusetts: 147 – 164.

Avise J. C., Arnold J., Ball R. M., Bermingham E., Lamb T., Neigel J. E., Reeb C. A. and Saunders N. C. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18: 489 – 522.

Berry R. J. and Jakobson M. E. 1975. Ecological genetics of an island population of the house mouse (*Mus musculus*). J. Zool., Lond. 175: 523 – 540.

Berry R. J., Jakobson M. E. and Peters J. 1978. The house mice of the Faroe Island: a study of microdifferentiation. J. Zool., Lond. 185: 73 – 92.

Biju-Duval C., Ennafaa H., Dennebouy N., Monnerot M., Mignotte F., Soriguer R. C., El Gaaied A., El Hili A. and Mounolou J.-C. 1991. Mitochondrial DNA evolution in lagomorphs: Origin of systematic heteroplasmy and organization of diversity in European rabbits. J. Mol. Evol. 33: 92 – 102.

Ennafaa H., Monnerot M., El Gaaied A. and Mounolou J.-C. 1987. Rabbit mitochondrial DNA: preliminary comparison between some domestic and wild animals. Genetics, Selection, Evolution 19: 279 – 288.

Farris J. S. 1972. Estimating phylogenetic trees from distance matrices. Am. Nat. 106: 645 – 668.

Fleischer R. C., Johnston R. F. and Klitz W. J. 1983. Allozymic heterozygosity and morphological variation in house sparrows. Nature 304: 628 – 630.

Grillitsch M., Hartl G. B., Suchentrunk F. and Willing R. 1992. Allozyme evolution and the molecular clock in the *Lagomorpha*. Acta theriol. 37: 1 – 13.

Handford P. 1980. Heterozygosity at enzyme loci and morphological variation. Nature 286: 261 – 262.

Harris H. 1980. The principles of human biochemical genetics. North Holland, Amsterdam: 1 – 554.

Harris H. and Hopkinson D. A. 1976. Handbook of enzyme electrophoresis in human genetics. North Holland, Amsterdam (unnumbered pages).

Hartl G. B. 1987. Biochemical differentiation between the wild rabbit (*Oryctolagus cuniculus* L.), the domestic rabbit and the brown hare (*Lepus europaeus* Pallas). Z. zool. Syst. Evolut.-forsch. 25: 309 – 316.

Hartl G. B. 1991. Genetic polymorphism of sorbitol dehydrogenase in the brown hare and the distribution of the variation in Central Europe. Bioch. Gen. 29: 49 – 54.

Hartl G. B. and Höger H. 1986. Biochemical variation in purebred and crossbred strains of domestic rabbits (*Oryctolagus cuniculus*). Gen. Res., Camb. 48: 27 – 34.

Hartl G. B., Markowski J., Kovacs G., Grillitsch M. and Willing R. 1990. Biochemical variation and differentiation in the brown hare (*Lepus europaeus*) of Central Europe. Z. Säugetierk. 55: 186 – 193.

Hartl G. B., Markowski J., Świątecki J., Janiszewski T. and Willing R. 1992. Genetic diversity in the Polish brown hare *Lepus europaeus* Pallas, 1778: implications for conservation and management. Acta theriol. 37: 15 – 25.

Hartl G. B., Reimoser F., Willing R. and Köller J. 1991a. Genetic variability and differentiation in roe deer (*Capreolus capreolus* L.) of Central Europe. Genetics, Selection, Evolution 23: 281 – 299.

Hartl G. B., Suchentrunk F., Willing R. and Grillitsch M. 1989. Biochemisch-genetische Variabilität und Differenzierung beim Feldhasen (*Lepus europaeus*) in Niederösterreich. Wien. tierärztl. Mschr. 76: 279 – 284.

Hartl G. B., Vodnansky M., Suchentrunk F., Steineck T., Willing R., Tataruch F. and Oberwalder U. 1991b. Fortpflanzungsstörungen in einer Feldhasenzucht – Folge einer Inzuchtdepression? Akad. Verl. Berlin, Verh. Ber. Erkrkg. Zootiere 33: 17 – 26.

Karl S. A. and Avise J. C. 1992. Balancing selection at allozyme loci in oysters: Implications from nuclear RFLPs. Science 256: 100 – 102.

Kieser J. A. and Groeneveld H. T. 1991. Fluctuating odontometric asymmetry, morphological variability and genetic monomorphism in the cheetah *Acinonyx jubatus*. Evolution 45: 1175 – 1183.

Lansman R. A., Shade R. O., Shapira J. F. and Avise J. C. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Mol. Evol. 17: 214 – 226.

Lerner I. M. 1954. Genetic homeostasis. Oliver and Boyd, London: 1 – 134.

Margl H. 1982. Die Abschüsse von Schalenwild, Hase und Fuchs in Beziehung zum Wildstand und Lebensraum in den politischen Bezirken Österreichs. Mitt. Forstl. Bundesversuchsanst. Wien, Österr. Agrarverlag, Wien, 146: 1 – 42.

Markowski J. and Markowska M. 1988. Non-metrical variation in three populations of roe deer. Acta theriol. 33: 519 – 536.

McAndrew B. J., Ward R. D. and Beardmore J. A. 1982. Lack of relationship between morphological variance and enzyme heterozygosity in the plaice, *Pleuronectes platessa*. Heredity 48: 117 – 125.

Mitton J. B. 1978. Relationship between heterozygosity for enzyme loci and variation of morphological characters in natural populations. Nature 273: 661 – 662.

Mitton J. B. and Grant M. C. 1984. Associations among protein heterozygosity, growth rate, and developmental homeostasis. Ann. Rev. Ecol. Syst. 15: 479 – 499.

Nei M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA 70: 3321 – 3323.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583 – 590.

Nei M. 1987. Molecular evolutionary genetis. Columbia University Press, New York: 1 – 512.

Nei M. and Li W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269 – 5273.

Nevo E., Honeycutt R. L., Yonekawa H., Nelson K. and Hanzawa N. 1993. Mitochondrial DNA polymorphisms in subterranean mole-rats of the *Spalax ehrenbergi* superspecies in Israel, and its peripheral isolates. Mol. Biol. Evol. 10: 590 – 604.

Novak J. M., Rhodes O. E., Jr, Smith M. H. and Chesser R. K. 1993. Morphological asymmetry in mammals: genetics and homeostasis reconsidered. [In: Ecological genetics in mammals. G. B. Hartl and J. Markowski, eds]. Acta theriol. 38, Suppl. 2: 7 – 18.

Patton J. L. and Feder J. 1978. Genetic divergence between populations of the pocket gopher, *Thomomys umbrinus* (Richardson). Z. Säugetierk. 43: 17 – 30.

Peterka M. and Hartl G. B. 1992. Biochemical-genetic variation and differentiation in wild and domestic rabbits: On the significance of genetic distances, dendrograms and the estimation of divergence times in domestication studies. Z. zool. Syst. Evolut.-forsch. 30: 129 – 141.

Schnell G. D. and Selander R. K. 1981. Environmental and morphological correlates of genetic variation in mammals. [In: Mammalian population genetics. M. H. Smith and J. Joule, eds]. University of Georgia Press, Athens, GA.: 60 – 99.

Schneider E. 1978. Der Feldhase – Biologie, Verhalten, Hege und Jagd. BLV München: 1 – 198.

Sikorski M. D. 1982. Non-metric divergence of isolated populations of *Apodemus agrarius* in urban areas. Acta theriol. 27: 169 – 180.

Sikorski M. D. and Bernshtein A. D. 1984. Geographical and intra-population divergence in *Clethrionomys glareolus*. Acta theriol. 29: 219 – 230.

Sjøvold T. 1977. Non-metrical divergence between skeletal populations. Ossa 4, Suppl. 1: 1 – 133.

Slatkin M. 1981. Estimating levels of gene flow in natural populations. Genetics 99: 323 – 335.

Slatkin M. 1985. Rare alleles as indicators of gene flow. Evolution 39: 53 – 65.

Slatkin M. and Barton N. H. 1989. A comparison of three indirect methods for estimating average levels of gene flow. Evolution 43: 1349 – 1368.

Soulé M. E. 1979. Heterozygosity and developmental stability: another look. Evolution 33: 396 – 401.

Soulé M. E., Yang S. Y., Weiler M. G. W. and Gorman G. C. 1973. Island lizards: The genetic-phenetic variation correlation. Nature 242: 191 – 193.

Suchentrunk F. 1993. Variability of minor tooth traits and allozymic diversity in brown hare *Lepus europaeus* populations. [In: Ecological genetics in mammals. G. B. Hartl and J. Markowski, eds]. Acta theriol. 38, Suppl. 2: 59 – 69.

Suchentrunk F., Willing R. and Hartl G. B. 1991. One eye lens weight and other age criteria of the brown hare (*Lepus europaeus* Pallas, 1778) Z. Säugetierk. 56: 365 – 374.

Suchentrunk F., Willing R. and Hartl G. B. 1993. Non-metrical polymorphism of the first lower premolar (P3) in Austrian brown hares (*Lepus europaeus*): a study on regional differentiation. J. Zool., Lond. (in press).

Swofford D. L. and Selander R. B. 1989. BIOSYS-1. A computer program for the analysis of allelic variation in popular genetics and biochemical systematics. Release 1.7. User's manual. Illinois Natural History Survey, Champaign.

Wayne R. K., Forman L., Newman A. K., Simonson J. M. and O'Brien S. J. 1986. Genetic monitors of zoo populations: Morphological and electrophoretic assays. Zoo Biology 5: 215 – 232.

Workman P. L. and Niswander J. D. 1970. Population studies on southwestern Indian tribes. II. Local genetic differentiation in the Papago. Am. J. Human Genet. 22: 24 – 49.

Wright S. 1978. Evolution and the genetics of populations. Vol. 4. Variability within and among natural populations. University of Chicago Press, Chicago.

Yezerinac S. M., Lougheed S. C. and Handford P. 1992. Morphological variability and enzyme heterozygosity: Individual and population level correlations. Evolution 46: 1959 – 1964.

Zima J. 1989. Non-metrical variability in the skull of the roe deer (*Capreolus capreolus*). Folia zool. 38: 119 – 137.

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