Acta Zoologica Academiae Scientiarum Hungaricae 53 (1), pp. 75–87, 2007

ALLOZYME VARIABILITY OF BROWN HARES (*LEPUS EUROPAEUS*) FROM THE VOJVODINA (SERBIA), COMPARED TO CENTRAL AND SOUTHEASTERN EUROPEAN POPULATIONS

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To assess genetic variability of brown hares (*Lepus europaeus* PALLAS, 1778) from the Vojvodina province (Serbia) and to reveal gene pool relationships with central and southeastern European populations, 33 hares were screened for allozymic variation at 40 putative structural gene loci and directly compared to earlier data sets of 20 Austrian and eight Bulgarian populations. Seven loci (17.5% rate of polymorphism) were polymorphic in the Vojvodinian hares. All indices of genetic diversity of the Vojvodina population were within the ranges found for the Austrian and Bulgarian populations, indicating no loss of genetic diversity. Pairwise genetic distances revealed low levels of divergence among all compared populations. Low bootstrap values provided little support for allocating the Vojvodina hares to either the central European or southeastern European populations. Multidimensional scaling (MDS) of the pairwise Rogers´ distance matrix and multivariate analysis of variance of population coordinates for three dimensions revealed significant differentiation between central and southeastern European populations. Stepwise discriminant analysis of population coordinates from MDS classified the Vojvodina population with 69.0% into the group of central European populations.

Key words: allozymes, brown hare, electrophoresis, population structure, Vojvodina

INTRODUCTION

The brown hare (*Lepus europaeus*, PALLAS 1778) occurs throughout large parts of Europe and constitutes an important game species in agricultural areas, open woodland and grassland up to 1500m. At the end of the 1950s, it was the most numerous game species in the Vojvodina (i.e., northern province of Serbia), with numbers between 400,000 and 500,000. Since then, environmental changes caused a rapid population decline to about 200,000 hares within only ten years. Both anthropogenic effects on habitat quality, such as urbanization, agricultural techniques, and traffic development, as well as increasing numbers of hunters and the

appearance of various infectious diseases ("hare plague") are likely responsible for this decline (VAPA & SELMIC 1997). In 1971 the hare number decreased below 200,000 and the "Hunters Association of the Vojvodina" banned hunting for two years. According to the latest data published of the "Hunters Association of Serbia", brown hares in the Vojvodina numbered around 300,000 in 1996 (VAPA & SELMIC 1997).

In the face of the marked environmental changes for hares and their regional population declines in large parts of Europe, maintenance of genetic resources of locally adapted populations is considered important for the long-term development of this species. Hares do not only play an important role for the hunting economy of the Vojvodina (VAPA & SELMIC 1997), but they also represent a significant prey species particularly in the agrosystem of the region, and they contribute to the flow of organic matter and nutrients: for instance, assuming a density of 50 hares per 100 ha, we might expect over one ton of dry weight of dung per year.

Studies on genetic variability of brown hares based on large sets of allozyme loci for populations from central and southeastern Europe as well as Britain and Anatolia (e.g., HARTL *et al.* 1990, 1992, 1993, 1994, SUCHENTRUNK *et al.* 2000, 2001, 2003, SERT *et al.* 2005) reveal a variety of rare or regionally restricted alleles and a tendency of decreasing genetic diversity from Anatolia and southeastern Europe towards central Europe and Britain. In spite of relatively little overall gene pool differentiation across long geographic distances, the various regionally restricted alleles occurring at low frequencies suggest gene pool variation on the smaller geographic scale (see e.g. SERT *et al*. 2005, BEN SLIMEN *et al*. 2005, 2006*a, b*) for brown hares and hares commonly considered belonging to cape hares.

In the present study we investigate the allozyme composition of brown hares from the Vojvodina to assess their level of genetic diversity in relation to southeastern (Bulgarian) and central European (Austrian) populations. Under the assumption that the southern and central Balkans likely has functioned as a Late Glacial refugium for brown hares (KASAPIDIS *et al.* 2005), we might expect novel alleles, so far not known from other regions of Europe. This might indicate gene pool distinction from central and southeastern European brown hares. Alternatively, Vojvodina hares might merely form a stepping stone population connecting central and southeastern European populations, with higher genetic similarity either to the further or the latter.

MATERIAL AND METHODS

Allozyme diversity

Liver tissue samples of 33 hares were obtained from eight sampling localities in the Vojvodina Province (northern Serbia). Preparation of tissue extracts as well as electrophoretic and staining procedures were performed according to previously published methods (HARTL & HÖGER 1986, HARTL 1987, GRILLITSCH *et al.* 1992). Twenty-seven isozyme systems encoded by 40 putative structural gene loci were assayed for allozymic variation by horizontal starch gel electrophoresis. All examined gene loci were previously studied in Austrian (HARTL *et al.* 1993) and Bulgarian (SUCHENTRUNK *et al.* 2000) brown hares. The geographical distribution of these latter two population groups are also given in the two papers cited above.

For resolving allelic variants, direct side-by-side comparison of migrating allozymes was carried out, including samples of the Austrian brown hares studied earlier on the same gel. Genotypes at polymorphic loci were determined following the principles given in ROTHE (1994). All variant alleles were named alphabetically with increasing anodal electrophoretic mobility. However, genotypes could not be determined for the entire set of loci in several individuals due to insufficient resolution producing ambiguous interpretations.

The following isozyme systems were examined (isozyme/-system, abbreviation, E.C. number and corresponding structural gene loci in parenthesis): 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); Isocitrat dehydrogenase (IDH, 1.1.1.42, Idh-1, -2); 6-phosphogluconate dehydrogenase (PGD, 1.1.1.44, Pgd-1); 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); Catalase (CAT, 1.11.1.6, Cat); Superoxid dismutase (SOD, 1.15.1.1, Sod-1, -2); Purine nucleoside phosphorilase (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); Pyruvate kinase, (PK, 2.7.1.40, Pk); 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 (EST, 3.1.1.1, Es-I, -D); Fructose-1,6-diphosphatase (FDP, 3.1.3.11, Fdp-1); Peptidases (Pep, 3.4.1.1, Pep-1, -2); Guanine deaminase (GDA, 3.5.4.3, Gda); Fumarate hydratase (FH, 4.2.1.2, Fh); Aldolase (ALDO, 4.2.1.3, Aldo); Mannosephosphat isomerase (MPI, 5.3.1.8, Mpi).

Statistical analyses

The BIOSYS-1 computer package, release 1.7 (SWOFFORD & SELANDER 1989) was used to calculate allele frequencies, average heterozygosity (H_o – observed; H_e – expected), proportion of polymorphic loci (P), mean number of alleles per locus (A), F-statistic parameters, Nei's unbiased genetic distances (NEI 1978), and modified Rogers' distances (WRIGHT 1978). All these calculations were carried out with the inclusion of the enzyme data sets of 469 Austrian (HARTL *et al.* 1993) and of 157 Bulgarian brown hares (SUCHENTRUNK *et al.* 2000), produced in the same laboratory, and by adjusting them to the presently assayed 40 loci. The same program was used to construct a Wagner dendrogram (FARRIS 1972) based on pairwise modified Rogers' distances, to summarize genetic relationships among the Vojvodina population, the eight Bulgarian, and the 20 Austrian populations. A Fortran program developed by R. WILLING (Vienna) was used to calculate bootstrap support values for the Wagner dendogram by 100 random resampling runs of allele frequencies. The FSTAT pro-

gram, version 2.9.3 (GOUDET 2001, see also GOUDET 1995) was used to test for significant deviations of genotype frequencies from Hardy-Weinberg (HW) expectations and linkeage disequilibrium (LD) between pairs of polymorphic loci, separately for each population. It was also used to calculate overall and population-specific WEIR and COCKERHAM (1984) estimators of $F_{IS}(f)$ and $F_{ST}(\theta)$ and associated significance levels for difference from zero by randomization tests and for calculation of population-specific allelic richness values (Rs), with a rarefaction approach that corrects for unbalanced sample sizes.

As an alternative approach to visualize genetic relationships among the studied populations, we applied multidimensional scaling on the matrix of modified Rogers' distances, using a three-dimensional Euclidian distance model with a maximum of 30 iterations, as implemented in SPSS 10.0.1. The resultant population-specific stimulus coordinates of the three dimensions were subjected to a MANOVA, to test for differences between Austrian and Bulgarian populations. Furthermore, because of the low bootstrap support of the Wagner dendogram (see Results), which gives only a week image of genetic relationships among populations, we performed a stepwise discriminant analysis with equal a priori-probabilities on the normally distributed population-specific stimulus coordinates with Austrian vs. Bulgarian population as grouping variable. The resultant discriminant function was used to allocate the Vojvodina hares.

RESULTS

The screening of the Vojvodina hares for 27 enzyme systems representing a total of 40 presumptive structural loci revealed polymorphism at seven loci (P = 17.5%, 99% criterion), with two to fours alleles per locus (Table 1). Allele frequencies of polymorphic loci are listed in Table 1 for the Vojvodina hares along with respective ranges of allele frequencies of polymorphic loci in the compared eight Bulgarian and 20 Austrian sampling regions. The alleles Ldh-2^{83}, Idh-2^{130}, Pgd^{170}, Hk-2^{67}, Es-1^{-100} Es-1^{-42} and Mpi^{77}, that occurred in Bulgarian and Austrian populations at low frequencies, were not found in the Vojvodina sample. Significant deviation from HW proportions of genotypes (heterozygote deficits) was found at the Sdh and Pgd loci in the Vojvodina population. Population-specific indices of genetic variability (H_o, H_e, P, A, Rs) and f values are also given in Table 1. The rate of polymorphism in the Vojvodina population (P = 17.5%, 99% criterion) was within the ranges of the Bulgarian (12.5–17.5%) and Austrian (10–20%) populations. The same was true for the mean number of alleles per locus (*A*) and for allelic richness (Rs) (Table 1). Heterozygosity (H_0, H_e) was slightly higher in the Vojvodina hares compared to Austrian hares but within the range found for Bulgarian populations (Table 1). No *f* value differed significantly from zero in any population.

Pairwise values of absolute (genetic distances) and relative (θ values) genetic differentiation between the Vojvodina hares and the Austrian or Bulgarian populations were generally of the same low or moderate magnitude as for population

Table 1. Allele frequencies at polymorphic loci of Vojvodina (VOJ) brown hares and respective ranges of allele frequencies of 8 Bulgarian (BL) and 20 Austrian (AT) populations. Asterisks at the respective 100-allele denote loci with significant deviations from Hardy-Weinberg equilibrium in the Vojvodinian sample. Mean number of hares (averaged over all loci scored) are given in parentheses for each population. H_o = observed heterozygosity (standard error in parenthesis); H_e = expected heterozygosity; P(99%) = rate of polymorphism (99% criterion) (variance in parenthesis); A = mean number of alleles per locus (standard error in parenthesis); Rs = allelic richness; f – WEIR and COCKERHAM's (1984) estimators of F_{IS}.

Locus	Allele	VOJ	BL	AT
		(32.0)	(15.0–24.8)	(7.0–41.0)
Sdh	100	0.939*	0.975–1.000	0.933–1.000
	300	0.061	000–0.025	0.000–0.067
Ldh-2	100	1.000	0.969–1.000	0.955–1.000
	83	0.000	0.000–0.031	0.000–0.045
Mor-2	100	1.000	1.000	0.833–1.000
	79	0.000	0.000	0.000–0.167
Idh-2	100	0.939	0.500–1.000	0.732–1.000
	130	0.000	0.000–0.500	0.000–0.232
	83	0.061	0.000	0.000–0.045
Pgd	100	0.924*	0.455–0.900	0.786–1.000
	170	0.000	0.000–0.273	0.000–0.083
	129	0.000	0.000	0.000–0.071
	117	0.045	0.100–0.438	0.000–0.100
	64	0.030	0.000–0.071	0.000–0.143
Hk-2	100	1.000	0.974–1.000	0.950–1.000
	67	0.000	0.000–0.026	0.000–0.050
Es-1	–100	0.000	0.000–0.079	0.000–0.143
	–108	0.417	0.083–0.450	0.357–0.833
	–75	0.583	0.450–0.794	0.167–0.567
	–42	0.000	0.000–0.139	0.000–0.138
Es-D	100	0.712	0.781–1.000	0.672–0.983
	141	0.288	0.000–0.219	0.017–0.328
Pep-2	100	0.727	0.725–0.938	0.618–0.925
	104	0.273	0.063–0.275	0.075–0.382
	114	0.000	0.000	0.000–0.138
	94	0.000	0.000–0.056	0.000
Mpi	100	0.985	0.806–0.977	0.900–1.000
	126	0.015	0.023–0.194	0.000–0.086
	77	0.000	0.000–0.025	0.000–0.067

Table 1 (continued)

Locus	Allele	VOJ	BL	AT
H_o		0.042 (0.019)	0.032–0.056	0.027–0.041
H_e		0.043 (0.019)	0.033–0.058	0.028–0.042
R_s		1.134	1.117–1.185	1.092–1.295
P		17.50 (0.36)	12.50–17.50	10.00–20.00
A		1.20 (0.07)	1.17–1.25	1.10–1.23
f		0.165	0.056–0.356	–0.260–0.240

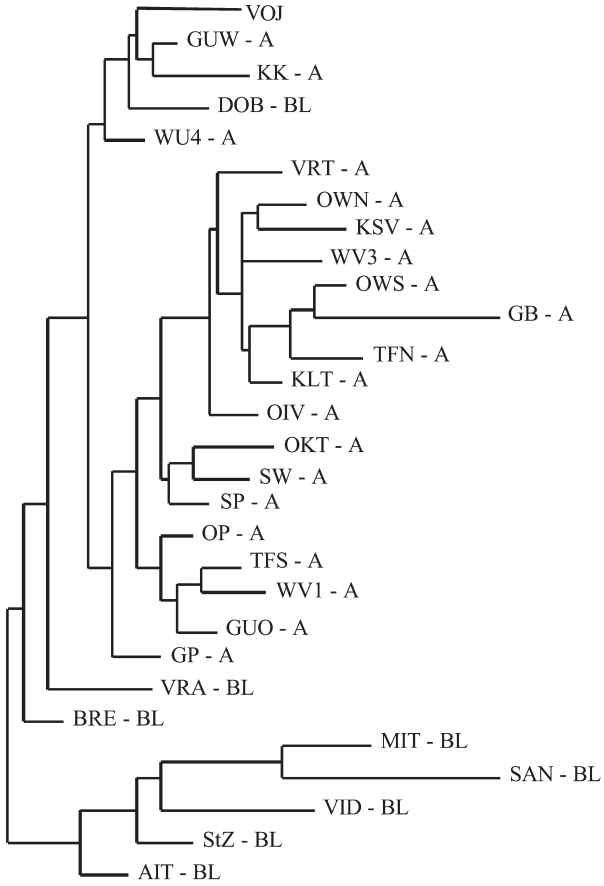

Fig. 1. Unrooted Wagner dendogram based on modified Roger's distances (WRIGHT 1978), representing genetic relationships among brown hares from the Vojvodina (VOJ), 20 Austrian (population acronyme – A), and eight Bulgarian (population acronyme – BL) populations. For acronymes of the Austrian populations see HARTL *et al.* (1993) and for Bulgarian populations see SUCHENTRUNK *et al.* (2000).

Table 2. Genetic differentiation between Vojvodina hares (VOJ), 8 Bulgarian (BL) and 20 Austrian populations (AT). Mean values of NEI's (1978) distances, corrected for small sample sizes (first row), and modified Rogers' distances (second row) are given above the diagonal. Means and ranges of pairwise WEIR and COCKERHAM's (1984) θ values of relative genetic differentiation (analogous to WRIGHT's (1978) F_{ST}) are given below the diagonal.

	VOJ	BL	AT
VOJ	–	0.0045	0.00185
		(0.000–0.014)	(0.000–0.004)
		0.069	0.049
		(0.036–0.120)	(0.026–0.074)
BL	0.0817	–	0.0055
	(0.006–0.220)		(0.000–0.023)
			0.0892
			(0.026–0.136)
AT	0.03385	0.1227	–
	(–0.010–0.101)	(–0.0237–0.369)	

comparisons within Austria or Bulgaria (Table 2). θ values (Table 2) were slightly lower between the Vojvodina and the Austrian populations than between the Vojvodina and the Bulgarian populations. Applying Bonferroni corrections to account for multiple tests (RICE 1989), 50% of the θ values differed significantly from zero in pairwise comparisons between hares from the Vojvodina and the Bulgarian populations, whereas only 25% differed significantly from zero in comparisons between the Vojvodina and the Austrian populations (result details not shown).

According to the unrooted Wagner dendogram derived from modified Rogers' distances (Fig. 1), the Vojvodina hares showed a slightly closer genetic affinity to some Austrian populations than to Bulgarian hares. However, except for one, bootstrap values for internal nodes were below 50%, and this indicated little support for the tree topology. Therefore, we employed multidimensional scaling (MDS) as an alternative approach to resolve genetic relationships among the Vojvodina, the Austrian, and the Bulgarian hares. MDS (3-dimensional model) of the Rogers' distance matrix yielded a sufficient stress value of 0.178 and an RSQ value (squared correlation coefficient between Rogers' distances and disparities) of 0.852. The stimulus coordinates of all populations are plotted for the first and second dimensions in Figure 2A and for the first and third dimensions in Figure 2B. They differed significantly between the Austrian and the Bulgarian populations (Hotelling's trace = 2.305, $F = 18.439$, $p < 0.0005$, MANOVA). Subsequent stepwise discriminant analysis (DA) of population-specific stimulus coordinates from MDS with the grouping variable "Austrian vs. Bulgarian populations"

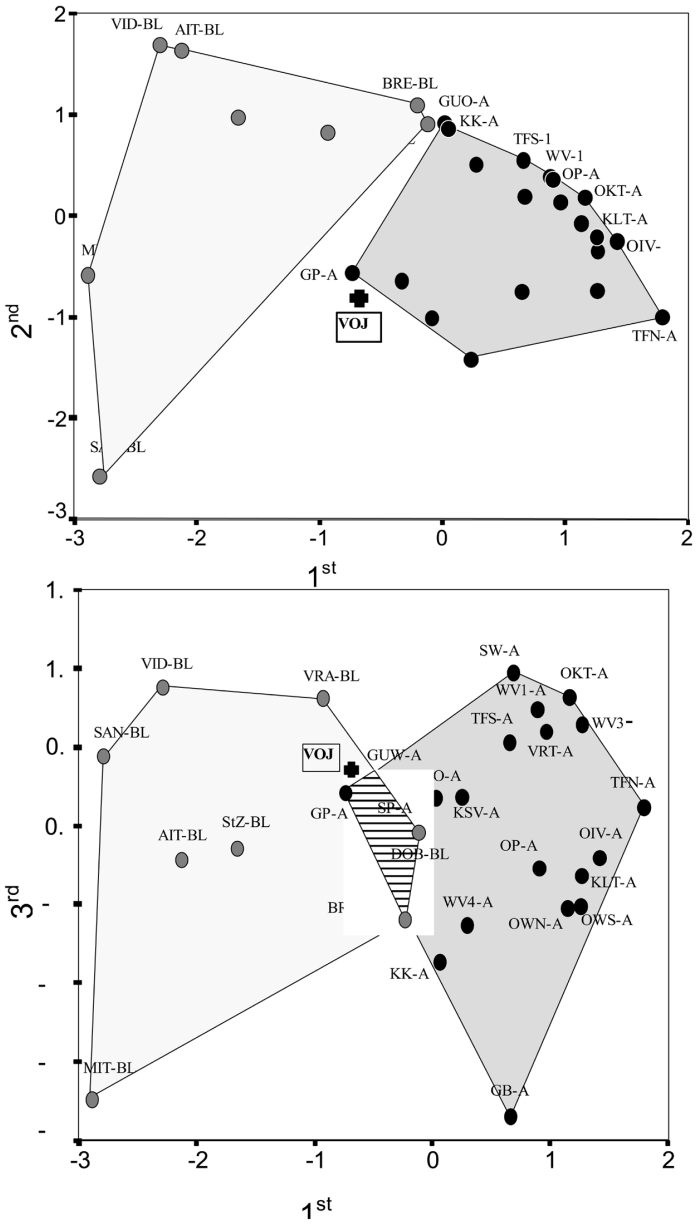

Fig. 2. Scatterplots of population-specific dimension stimulus coordinates as obtained from multidimensional scaling (three-dimensional model); A (up): plot of first and second dimensions, B (down): plot of first and third dimensions. Convex polygons encompass the 20 Austrian (black circles and light grey area) and the eight Bulgarian (white circles and dark grey area) populations, respectively. Black cross indicates the Vojvodina (VOJ) population; for acronymes of Austrian populations see HARTL *et al.* (1993) and of Bulgarian populations see SUCHENTRUNK *et al.* (2000).

(Wilk's Lambda = 0.303, chi square = 29.879, d.f. = 2, $p < 0.0005$) assigned all Austrian populations correctly and only one (12.5%) Bulgarian population incorrectly; thus, 96.6% of all Austrian and Bulgarian populations were assigned correctly, indicating a high degree of confidence of this procedure. The Vojvodina population was assigned to the group of Austrian populations ($p = 0.69002$) by DA. The distributions (box plotts) of discriminant scores of the Austrian, the Bulgarian, and the Vojvodina populations are displayed in Figure 3.

DISCUSSION

The hares from the Vojvodina (Serbia) are genetically very similar to the presently compared central European (Austria) and southeastern European (Bulgaria) populations, as revealed by their allele composition and frequencies. Their allele composition is also very similar to those of brown hares from other parts of central Europe (HARTL *et al.* 1990, 1992, 1994) and Greece (SUCHENTRUNK *et al.* 2003). Compared to all these studies, we did not find any novel allele in the Vojvodina hares, and most common alleles of the Austrian and Bulgarian populations were also common in the Vojvodina sample. The absence of certain alleles

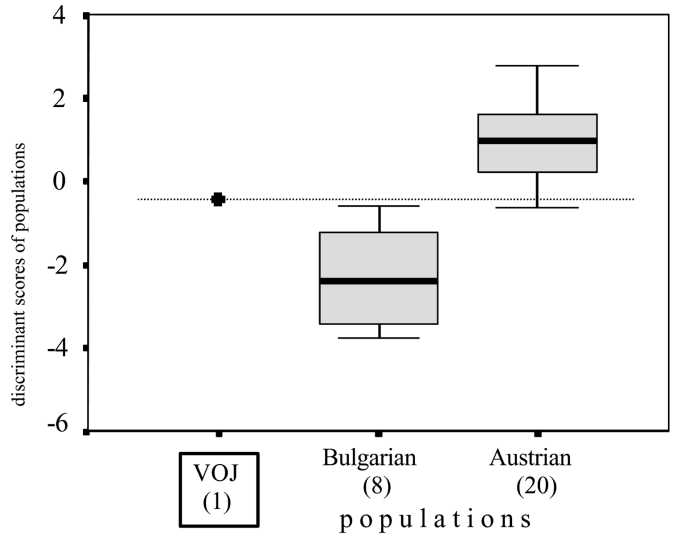

Fig. 3. Stepwise discriminant analysis (DA): box plots of discriminant scores for the Austrian and Bulgarian populations as well as the discriminant score of the initially unclassified Vojvodina population (cross, VOJ), as obtained from DA of stimulus coordinates from the multidimensional scaling (three-dimensional model). The stippled horizontal line indicates classification of VOJ to the Austrian populations. Population numbers in parentheses.

that were present in some Austrian and/or Bulgarian populations at low frequencies is probably due to the small size of the Vojvodina sample, relative to the Bulgarian and Austrian samples. In fact, several rare alleles, namely Ldh-2^{83}, Mor-2^{79}, Idh-2^{130}, and Hk^{67}, which were not found presently, were revealed recently in another sample of Vojvodina hares at very low frequencies (VAPA *et al.* 2002, DAVIDOVIC 2003).

Genetic variability of the Vojvodina hares was within the range found for the central European populations (this study and HARTL *et al.* 1990, 1992, 1994) as well as the Bulgarian and the Greek (SUCHENTRUNK *et al.* 2003) populations. Basically, the levels of genetic diversity of all these populations comply with the mean genetic diversity reported for a wide range of terrestrial mammal species (e.g., TIEDEMANN *et al.* 1996) and do not provide any hint of possible strong regional drift or inbreeding effects. The significant deviations from HW expectations for two loci in the Vojvodina population might indicate a slight gene pool substructuring (demic structure) rather than inbreeding effects, because in the latter case we should observe heterozygote deficits or a general trend for such in the other polymorphic loci, too.

The relatively low values of absolute genetic differentiation among the Vojvodina hares and the populations from central Europe and the southeastern Balkans suggest generally shallow gene pool divergence among the studied populations (see also HARTL *et al.* 1990, 1992, 1994 for further central European comparisons and MAMURIS *et al.* 2001, 2002 for Greek populations). However, relative genetic differentiation (F-statistics) suggests moderately reduced gene flow across longer geographic distances. Contrary to the relatively shallow nuclear gene pool divergence observed on the large geographic scale, mtDNA differentiation is obviously much more pronounced between populations from the southern and southeastern Balkans and central European populations (MAMURIS *et al.* 2001, KASAPIDIS *et al.* 2005, see also BEN SLIMEN *et al*. 2005, 2006*a, b* for many *Lepus* taxa). More pronounced mtDNA divergence relative to geographic differentiation in nuclear markers was also observed on a smaller geographic scale for brown hares from Germany (FICKEL *et al.* 1999, 2005).

The Wagner dendrogram suggests a slightly closer nuclear gene pool affinity of the Vojvodina hares to the central European populations, but the generally low bootstrap support values for internal nodes do not allow a clear decision. To circumvent this ambiguity, we combined various multivariate statistical techniques as an alternative approach to standard cluster analyses for assessing genetic relationships and classifying the Vojvodina population. Specifically, multidimensional scaling (MDS) of the Rogers' distance matrix enabled to graphically allocate the Vojvodina population in the context of the central European (Austrian)

and southeastern European (Bulgarian) populations. The MANOVA of the respective population coordinates from MDS clearly demonstrated a significant nuclear gene pool separation between the Austrian and Bulgarian populations. This corresponded to the results obtained from the analysis of relative genetic differentiation among Austrian and Bulgarian hares (θ values; see also SUCHENTRUNK *et al.* 2000), which yielded tentatively a higher proportion of fixation indices significantly differing from zero for pairwise comparisons between Austrian and Bulgarian populations than between pairwise comparisons within Austria or Bulgaria. Concordantly, the θ values indicated tentatively little gene pool differentiation within Austrian and Bulgarian hares, respectively, but a moderate level of differentiation between these two regions. However, because of non-independent data sets for these comparisons, a statistical test could not be performed. To overcome this shortcoming and to classify the Vojvodina population into either the Austrian or the Bulgarian population group, we performed a stepwise discriminant analysis of the population coordinates resulting from the statistically independent values of the MDS of the Rogers' distance matrix. In this way all Austrian and 87.5% of the Bulgarian populations could be classified correctly. The overall probability of 96.6% of classifying these populations correctly into a geographically defnined of group of populations indicated a high level of reliability of this procedure, which allocated the Vojvodina hares closer to the Austrian than to the Bulgarian hares. However, the somewhat reduced probability (0.69002%) of allocating the Vojvodina population to the Austrian population group indicates a more or less intermediate position of the Vojvodina population, which corresponds to geographical relations. This resultant classification complies with the pairwise distance data for the Vojvodina hares and the combined Austrian (Rogers' distance = 0.042) and Bulgarian (Rogers' distance = 0.063) populations, respectively. The present statistical approach which is based on a combination of the transformation of the matrix of pairwise population distances into statistically independent principle coordinate values (multi-dimensional scaling values) for several dimensions, that are necessary to explain the original distance matrix, and their subsequent disrcimination by standard discriminant analysis represents an alternative option to the various recently developed assignment test procedures (implemented e.g. in the GENECLASS software, PIRY *et al*. 2004); its general power relative to assignment tests will be examined in a later paper to be publised elsewhere.

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Acknowledgements – We would like to thank Mrs. ANITA HAIDEN (Vienna) for valuable help with the laboratory work, the hunters from Serbia for collecting samples, and Mr. R. WILLING (Vienna) for statistical advises. The Austrian Academic Exchange Service (ÖAD, Vienna) provided financial support for MD.

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Received February 8, 2006, accepted July 4, 2006, published February 28, 2007