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Allozyme evolution and the molecular clock in the *Lagomorpha*

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In order to provide a scale of genetic distances in the *Lagomorpha*, biochemical-systematic relationships among *Lepus europaeus*, *Lepus timidus*, *Oryctolagus cuniculus* (*Leporidae*) and *Ochotona rufescens* (*Ochotonidae*) were examined by horizontal starch gel electrophoresis of 38 isozyme systems. Nei's (1978) genetic distances were calculated over 58 presumptive structural loci and used for the construction of numerical dendrograms. The stability of clusters was examined by the jackknife method and by comparison to a Hennigian cladogram. All these procedures revealed a constant picture of lagomorph relationships, which is in accordance with the conclusions drawn from other evidence. Divergence times were estimated using two fundamentally different approaches. They were in good agreement with paleontological data (0.49myr between the *Lepus* species, 3.65myr between *Lepus* and *Oryctolagus*, 37.5myr between *Leporidae* and *Ochotonidae*), but only when calculated in different ways at low and at high taxonomic levels. The results suggest a temporal acceleration of the rate of allozyme evolution in the *Leporidae* due to rapid adaptive radiation of biochemically highly polymorphic taxa.

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Introduction

During the last two decades, protein electrophoretic data were increasingly used to evaluate biochemical-systematic relationships in mammals. However, apart from rodents and primates, which are favoured groups in all kinds of systematic investigations (the former probably because access to all kinds of material is comparatively easy, the latter because of their relationship to man), only two orders were examined more extensively for biochemical-systematic relationships above the level of congeneric species or closely related genera: The *Artiodactyla* (e.g. Baccus *et al.* 1983, Hartl *et al.* 1988a, 1990a, b; Georgiadis *et al.* 1990, Randi *et al.* 1991) and the *Carnivora* (e. g. Simonsen 1982, Wayne and O'Brien 1987, Hartl *et al.* 1988b, Goldman *et al.* 1989, Taylor *et al.* 1991). In some of these surveys, unequal rates of allozyme evolution were detected among taxa,

which can be interpreted either as the result of rapid cladogenesis (comp. Bonhomme *et al.* 1985, Hartl *et al.* 1990a, b) or by an increased rate of anagenetic change due to other factors than a previous radiation (Taylor *et al.* 1991, Hartl *et al.* 1992).

In the *Lagomorpha*, biochemical-systematic data are particularly scarce: The electrophoretic studies of Vergnes et al. (1974 – *Ochotona rufescens* / *Oryctolagus cuniculus*) and of Robinson and Osterhoff (1983 – *Lepus* spp. / *Pronolagus* spp.) are severely restricted in the numbers of isozyme loci examined. Genetic distance estimates are available only for three *Lepus* species from Spain (Bonhomme *et al.* 1986 – 42 loci) and for *Oryctolagus cuniculus* and *Lepus europaeus* (Hartl 1987 – 38 loci), which are based on very different sets of isozymes and are therefore not fully comparable (see Hartl *et al.* 1990a). It is the aim of the present paper, to provide a spectrum of genetic distances in the *Lagomorpha*, ranging from local populations over closely related species and genera up to the level of families. Based on a large number of loci screened, rates of enzyme evolution are evaluated and examined for their influence on both the reconstruction of phylogenetic relationships and the calculation of divergence times in the *Lagomorpha*.

Material and methods

Biochemical-systematic relationships among the brown hare (*Lepus europaeus*), the mountain hare (*Lepus timidus*), the old world rabbit (*Oryctolagus cuniculus*) (*Leporidae*), and the pika (*Ochotona rufescens*) (*Ochotonidae*) were examined, whereby the horse, *Equus przewalskii* f. caballus served as an outgroup. For direct comparison 6 brown hares (heterozygous carriers of the various allozymes detected by Hartl *et al.*, in preparation), 4 mountain hares (culled in Carinthia, Austria), 4 rabbits (heterozygous carriers of the various allozymes detected by Peterka and Hartl 1992), 4 pikas (breed of the Laboratoire de Pharmacologie et de Toxicologie Fondamentales, Toulouse, France) and 2 horses (slaughter-house St. Marx, Vienna) were used. Allele frequencies in the brown hare are based on 469 specimens from Austria screened by Hartl *et al.* (in preparation), in the rabbit on 204 specimens screened by Peterka and Hartl (1992).

Using routine electrophoretic techniques (Hartl and Höger 1986, Grillitsch 1990) 38 isozyme systems were screened. Enzyme names, abbreviations, E.C. numbers, tissues, buffer systems and references for staining procedures are given in Table 1.

The interpretation of electrophoretic patterns, the assessment of homology of the respective isozymes and the distinction between mitochondrial and cytosolic isozymes were performed according to Hartl et al. (1990a). The statistical evaluation of the data followed the various methods reviewed and compared empirically in the same paper.

Results

Screening of 38 isozyme systems in the *Lagomorpha* revealed a total of 62 presumptive structural loci. The alleles and their frequencies detected in the various taxa are listed in Table 2.

Genetic distances, calculated according to Nei (1978), are given in Table 3 – since four of the loci (*Pk-2*, *Pgm-3*, *Fdp-2*, and *Gpi-1*) were not scorable in all taxa,

Table 1: Isozyme systems screened (abbreviation and E.C. number in parentheses), tissues (L = liver, K = kidney, H = heart), buffer systems (P = continuous phosphate buffer, pH 7.4, TM = continuous tris-maleate buffer, pH 7.4 – Csaikl *et al.* 1980, TC = continuous tris-citrate buffer, pH 8.0 – Manlove *et al.* 1975) and references for staining procedures (1 = Thorup *et al.* 1961, 2 = Shaw and Prasad 1970, 3 = Brewer and Sing 1970, 4 = Selander *et al.* 1971, 5 = Siciliano and Shaw 1976, 6 = Harris and Hopkinson 1976, 7 = Qavi and Kit 1980, 8 = Richardson *et al.* 1986) used in the present study.

Isozyme system	Tissue	Buffer	Stain
α-glycerophosphate dehydrogenase (GDC, E.C. 1.1.1.8)	L	TC	2
sorbitol dehydrogenase (SDH, E.C. 1.1.1.14)	L	TC	2
lactate dehydrogenase (LDH, E.C. 1.1.1.27)	K	P	4
malate dehydrogenase (MDH or MOR, E.C. 1.1.1.37)	K	TC	4
malic enzyme (ME or MOD, E.C. 1.1.1.40)	K	TM	5
isocitrate dehydrogenase (IDH, E.C. 1.1.1.42)	K	TC	4
6-phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44)	K	P	4
glucose dehydrogenase (GDH, E.C. 1.1.1.47)	L	TC	6
glucose-6-phosphate dehydrogenase (GPD, E.C. 1.1.1.49)	K	TC	4
glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12)	L	TC	5
xanthine dehydrogenase (XDH, E.C. 1.2.3.2)	L	TC	2
glutamate dehydrogenase (GLUD, E.C. 1.4.1.3)	L	TC	3
NADH-diaphorase (DIA, E.C. 1.6.2.2)	H	TC	6
catalase (CAT or CE, E.C. 1.11.1.6)	K	TM	1
superoxide dismutase (SOD, E.C. 1.15.1.1)	K	P	4
purine nucleoside phosphorylase (NP, E.C. 2.4.2.1)	K	P	6
aspartate aminotransferase (AAT or GOT, E.C. 2.6.1.1)	K	TC	4
glutamate pyruvate transaminase (GPT, E.C. 2.6.1.2)	L	TC	6
hexokinase (HK, E.C. 2.7.1.1)	H	TC	6
pyruvate kinase (PK, E.C. 2.7.1.40)	H	TC	5
phosphoglycerate kinase (PGK, E.C. 2.7.2.3)	H	TC	5
creatine kinase (CK, E.C. 2.7.3.2)	H, K	TC	2
adenylate kinase (AK, E.C. 2.7.4.3)	H, K	TC	5
phosphoglucomutase (PGM, E.C. 2.7.5.1)	K	P	6
esterases (ES, E.C. 3.1.1.1)	K	P	2, 6
acid phosphatase (ACP, E.C. 3.1.3.2)	K	TC	2
fructose-1,6-diphosphatase (FDP, E.C. 3.1.3.11)	K	TC	8
ß-galactosidase (ß-GAL, E.C. 3.2.1.23)	L	P	6
ß-glucuronidase (ß-GUS, E.C. 3.2.1.31)	L	P	6
peptidases (PEP, E.C. 3.4.11)	K	P	5
aminoacylase 1 (ACY-1, E.C. 3.5.1.14)	L	TC	7
guanine deaminase (GDA, E.C. 3.5.4.3)	L	TC	6
adenosine deaminase (ADA, E.C. 3.5.4.4)	L, H	TC	6
aldolase (ALDO, E.C. 4.1.2.13)	H	TC	6
fumarate hydratase (FH, E.C. 4.2.1.2)	H	TC	5
aconitase (ACO, E.C. 4.2.1.3)	K	TC	6
mannosephosphate isomerase (MPI, E.C. 5.3.1.8)	K	P	5
glucosephosphate isomerase (GPI, E.C. 5.3.1.9)	K	P	4

Table 2. Alleles detected in four lagomorph taxa (in the case of polymorphism frequencies are given in parentheses). *L. e.* = *Lepus europaeus*, *L. t.* = *Lepus timidus*, *Or. c.* = *Oryctolagus cuniculus*, *O. r.* = *Ochotona rufescens*, *E. p.* = *Equus przewalskii*. X = locus not scorable.

Locus	*L. e.*	*L. t.*	*Or. c.*	*O. r.*	*E. p.*
1	2	3	4	5	6
Gdc	a	a	b	c	d
Sdh	a, b (.996/.004)	b	c	d	e
Ldh-1	a	a	a	b	c
Ldh-2	a, b (.997/.003)	a	c	d	a
Mdh-1	a	a	b	b	c
Mdh-2	a, b (.986/.014)	a	a	c	d
Me-1	a	a	b	b	c
Me-2	a	a	a	b	c
Idh-1	a	a	a	b	a
Idh-2	a, b, c (.959/.033/.008)	a	a	c	d
Pgd	a, b, c, d, e (.934/.025/.006/.015/.020)	a	a, f (.983/.017)	g	h
Gdh-1	a	a	a	a	b
Gdh-2	a	a	b	c	d
Gpd	a	a	a	b	b
Gapdh	a	a	b	a	a
Xdh	a	a	b	a	c
Glud	a	a	a	a	a
Dia-1	a	a	a	a	b
Dia-2	a	a	b	c	d
Cat	a	a	b	b	c
Sod-1	a	a	a	b	c
Sod-2	a	a	b	a	c
Np	a	a	a	b	c
Aat-1	a	a	a	b	a
Aat-2	a	a	a	b	c
Gpt	a	a	b	c	d
Hk-1	a	a	a	b	c
Hk-2	a	a	a	b	c
Hk-3	a	a	a	b	c
Pk-1	a	a	a	b	a
Pk-2	a	a	X	b	c
Pgk	a	a	a	b	c
Ck-1	a	a	a	b	c
Ck-2	a	a	b	a	a
Ak-1	a	a	b	a	c
Ak-2	a	a	b	a	c
Ak-3	a	a	b	a	c

Table 2 continued

1	2	3	4	5	6
Pgm-1	a	b	c	d	e
Pgm-2	a	a	b	b	c
Pgm-3	a	a	b	X	c
Es-d	a, b (.820/.180)	a, b (.500/.500)	a	c	d
Es-I	a, b, c, d (.021/.613/.353/.013)	a, b (.125/.875)	e, f (.815/.185)	g	h
Fdp-1	a	a	b	b	c
Fdp-2	a	a	X	a	b
Acp-1	a	b	c	d	d
Acp-2	a	a	b	c	d
Acp-3	a	a	b	a	c
β-Gal	a, b, c (.390/.522/.088)	b	b	d	e
β-Gus	a	b	a	c	a
Pep-1	a	a	b	c	d
Pep-2	a, b, c (.799/.193/.008)	b	d	e	f
Acy-1	a, b, c (.140/.537/.323)	b, c	c	a, b (.750/.250)	b (.500/.500)
Gda	a	a	b	c	a
Ada-1	a	a	b	b	c
Ada-2	a, b (.883/.117)	a, b, c (.375/.125/.500)	d	e	f
Aldo	a	a	a	a	a
Fh	a	a	a	a	b
Aco-1	a	a	a	b	b
Aco-2	a	a	a	b, c (.500/.500)	a
Mpi	a, b, c (.980/.018/.002)	a, b (.875/.125)	d, e, f (.423/.190/.387)	g, h (.500/.500)	e, i (.500/.500)
Gpi-1	a	a	a	b	X
Gpi-2	a	a	a	b	c, d (.750/.250)

they are based on 58 loci. Phylogenetic trees, constructed according to various methods (Nei's (1978) D/UPGMA – Fig. 1, Nei's D/Fitch-Margoliash tree – Fig. 2, Hennigian cladogram – Fig. 3) are in good agreement and also the maximum parsimony method (PHYLIP-programme package, Felsenstein 1985) revealed only one solution. The stability of clusters in relation to the composition of the isozyme loci scored was examined using the jackknife method (Hartl *et al.* 1990a). A majority rule consensus tree, constructed from 100 Nei's (1978) D/UPGMA dendrograms (in each of which 25% of the loci were randomly omitted), revealed an identical topology in all cases. Divergence times (t) were calculated using two

Table 3. Genetic identities – above the diagonal – and genetic distances – below the diagonal between the lagomorph taxa examined, calculated according to Nei (1978). *L. e.* = *Lepus europaeus*, *L. t.* = *Lepus timidus*, *Or. c.* = *Oryctolagus cuniculus*, *O. r.* = *Ochotona rufescens*, *E. p.* = *Equus przewalskii*.

	L. e.	L. t.	*Or. c.*	*0. r.*	*E. p.*
L. e.	–	0.9063	0.4905	0.2394	0.2059
L. t.	0.0984	–	0.4738	0.2386	0.1909
Or. c.	0.7123	0.7469	–	0.1942	0.1246
0. r.	1.4297	1.4329	1.6391	–	0.1326
E. p.	1.5805	1.6562	2.0824	2.0204	–

Table 4. Divergence times between the lagomorph taxa examined, calculated by two different methods. I = Nei (1975), II = Wayne and O'Brien (1987).

Taxa	Divergence time (myr)	
	Method I	Method II
Lepus europaeus/Lepus timidus	0.49	2.46
Lepus/Oryctolagus	3.65	18.24
Leporidae/Ochotonidae	7.50	37.52
Lagomorpha/Perissodactyla	9.18	45.87

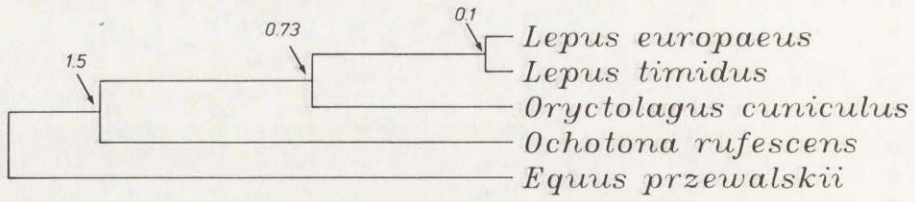

Fig. 1. Rooted dendrogram, showing genetic relationships in the *Lagomorpha* (Nei's 1978 D/UPGMA).

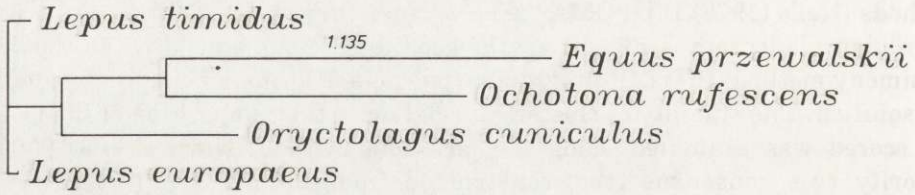

Fig. 2. Unrooted dendrogram, showing genetic relationships in the *Lagomorpha* (Nei's 1978 D/Fitch-Margoliash tree).

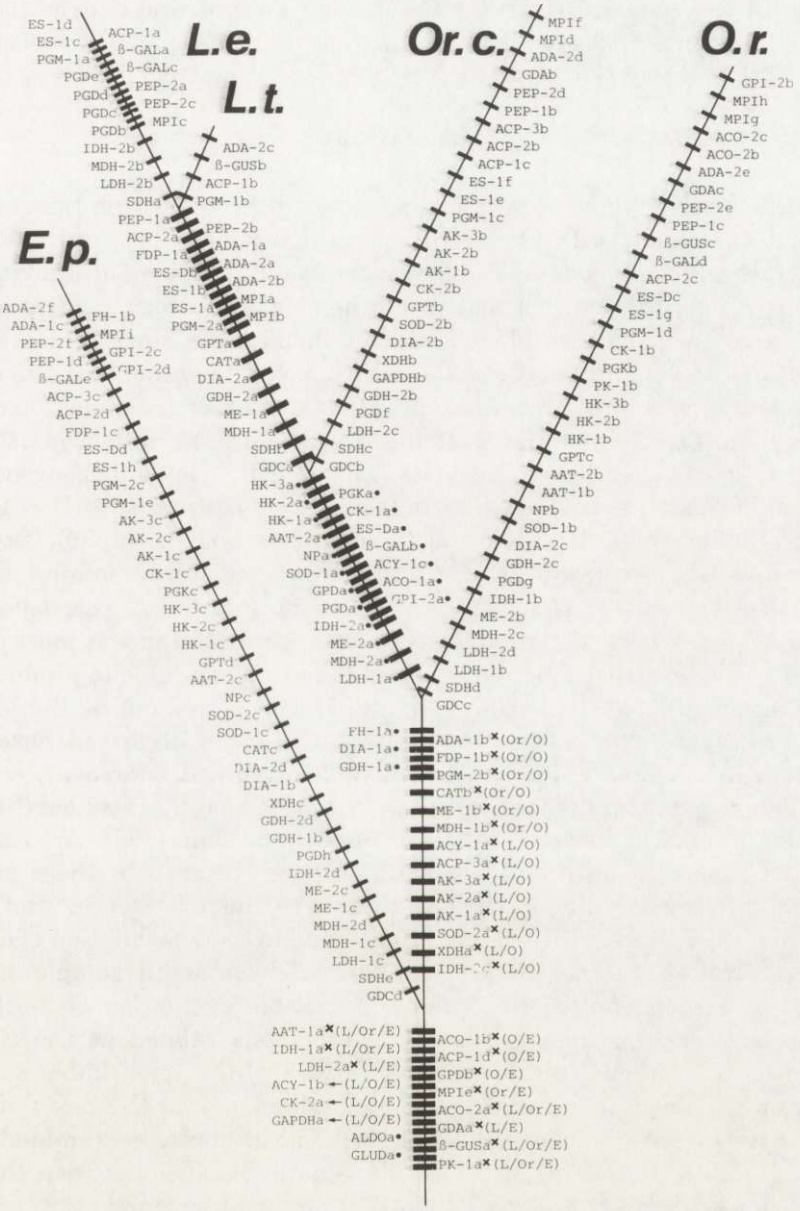

Fig. 3. Hennigian cladogram, showing genetic relationships in the *Lagomorpha* (constructed according to the criteria outlined in Hartl et al. 1990a). Isozymes = characters, allozymes = character states, dot = character state symplesiomorphic or synapomorphic, arrow = character state likely to be symplesiomorphic or synapomorphic, cross = character state possibly convergent (criteria are defined in Hartl *et al.* 1990a). *L. e.* = *Lepus europaeus*, *L. t.* = *Lepus timidus*, *Or. c.* = *Oryctolagus cuniculus*, *O. r.* = *Ochotona rufescens*, *E. p.* = *Equus przewalskii*. The higher number of autapomorphies in *L. e.* in comparison to *L. t.* is probably due to the much larger number of individuals examined.

fundamentally different approaches – comp. Hartl *et al.* 1990a, 1992 : I) $t = D/(2\alpha)$, whereby $\alpha = 10^{-7}$ (Nei 1975), II) 0.1 D = 2.5myr (Wayne and O'Brin 1987). The respective divergence times between the taxa examined are shown in Table 4.

Discussion

The biochemical-systematic relationships among the lagomorph taxa examined are in good agreement with the picture known from conventional systematics (comp. Diersing 1984, Nowak 1991). The use of small numbers of individuals for constructing numerical dendrograms in biochemical-systematic studies has been criticized, e. g. by Archie et al. (1989), but in our case fluctuations in allele frequencies due to sampling error could have some influence only on the distance between *L. europaeus* and *L. timidus*. All other distances are almost exclusively due to fixed alleles, which is confirmed by the remarkable consistency of dendrograms constructed by various methods and by the jackknife consensus tree.

The genetic distance between *L. europaeus* and *L. timidus* (D = 0.098) is somewhat smaller than that detected by Bonhomme *et al.* (1986) between *L. europaeus* and *L. granatensis* (D = 0.135) and considerably smaller than the D-values described by the same authors for *L. europaeus* / *L. castroviejoi* (D = 0.355) and *L. castroviejoi* / *L. granatensis* (0.334). This difference is most probably not related to the fact, that *L. europaeus* and *L. timidus* are able to produce fertile hybrids (Angermann 1983, Schröder *et al.* 1987). As pointed out by Bonhomme *et al.* (1986), allozyme genetic distances within the scope discussed here are no reliable indicator for the ability or inability to interbreed. Moreover, occasional hybridization also among the Iberian species is not to be fully excluded (Schneider and Leipoldt 1983). The larger D-values of Bonhomme *et al.* (1986) are rather due to the larger proportion of rapidly evolving proteins screened by these authors – among the differential diagnostic loci there are the transferrin and several esterases not considered in the present study – and to their calculation of distances according to Nei (1972), which are not corrected for small sample sizes and commonly yield somewhat higher values. A reliable evaluation of biochemical-systematic relationships among taxa being as closely related as the European *Lepus* species will be particularly valuable in view of the remarkable karyotypic conservatism (Robinson *et al.* 1983), but is possible only on the basis of a large and – most importantly – identical set of biochemical markers examined.

With a D-value (Nei 1978) of 0.712, the genetic distance between the rabbit and the hare was found to be larger than that obtained by Hartl (1987), which is based on 38 loci and shows a D-value (Nei 1972) of 0.487. This discrepancy can be fully explained by the fact, that – in contrast to Hartl (1987) – the isozymes SDH, XDH, CAT, AK-2, AK-3, PGM-2, and ADA were interpreted to show slightly different mobilities due to allelic differences at the corresponding loci between both genera in the present survey. The genetic distances of *Ochotona* from both

Lepus and *Oryctolagus* are very similar, which is in agreement with the evolutionary conclusions drawn by Stock (1976), Corbet (1983), and Diersing (1984) from cytogenetic and morphological evidence. The horse was chosen as an outgroup, because among various mammalian orders discussed to share a common origin with the *Lagomorpha*, except for rodents, there is most evidence in favour of the *Artiodactyla* (or *Ungulata* in general – see Grillitsch 1990, for review). Regarding the genetic distances from the lagomorph taxa, a certain amount of bias due to problems in assessing the homology of loci and to homoplasy (comp. Fig. 3) must be considered (Buth 1984).

The divergence times between the *Lepus* species (0.49myr) and between *Lepus* and *Oryctolagus* (3.65myr) are in good agreement with divergence times obtained from other evidence (paleontological – Erbajeva 1981, Dawson 1981, mtDNA – Biju-Duval *et al.* 1991), but only when they are calculated according to method I. Also the divergence time between the *Leporidae* and the *Ochotonidae* (37.5myr) fits well to estimates obtained from the literature (myoglobin sequencing – Dene *et al.* 1982, paleontology – Diersing 1984), but in this case only when calculated according to method II. The latter result suggests, that the estimate of 0.1D = 2.5myr used by Wayne and O'Brien (1987) in the *Canidae* gives a good estimate of time divergence also in the *Lagomorpha*, but only at a high taxonomic level. Among confamilial genera the estimates are much too high (Table 4), suggesting a generally more rapid rate of allozyme evolution in the *Lagomorpha* than in the *Canidae*. (The validity of this interpretation is corroborated by the very similar number and composition of isozyme loci screened in both surveys).

Apart from differences in allozyme evolutionary rates among mammalian orders, our data suggest considerable non-linearity of rates of allozyme substitution within one order. Similar results have been obtained already in the *Artiodactyla* and the *Rodentia* (comp. Hartl 1990, Hartl *et al.* 1990a, 1992). This non-linearity could be an artifact due to homoplasy, but could be also the result of accelerated rates of molecular evolution at low taxonomic levels. Homoplasy is to be expected mainly in rapidly evolving isozymes (comp. classification in Hartl *et al.* 1990a), which – with the exception of ACY-1 – show different alleles in *Leporidae* and *Ochotonidae* (see Fig. 3). Furthermore, the jackknife dendrograms confirm the topology of Figs. 1 – 3 without a single exception. Thus, albeit not to be fully excluded, we do not think that homoplasy is sufficient to explain the observed non-linearity of divergence times.

As described by Hartl *et al.* (1990a, b) rapid cladogenesis in the presence of high levels of allozyme polymorphism within species could lead to the fixation of alternative alleles in the derived taxa and, thus, to a temporal acceleration of allozyme evolutionary rates by overriding the normal rate of codon substitution governed by mutation and genetic drift (Kimura 1987). In contrast to the *Ochotonidae*, the *Leporidae* are a thriving family with considerable rates of cladogenesis during the late Miocene and the Pliocene (Diersing 1984). However, in contrast to the predictions of Hartl *et al.* (1990a, b), according to which derived

taxa should exhibit higher distances and less variation than basal forms, comprehensive population genetic investigations in the brown hare (Hartl *et al.* 1989, 1990c, 1992, in preparation) and the rabbit (Richardson *et al.* 1980) revealed extensive allozyme polymorphism in both species. Moreover – at least in the brown hare – genetic distances among populations are close to zero. Nevertheless, in animals with high polymorphism, once reproductive isolation is established, considerable divergence can accumulate within a short period of time. This is corroborated by genetic distances among breeds of the domestic rabbit, reaching values which are higher than those found among subspecies of artiodactyls (comp. Peterka and Hartl 1992, Hartl and Markov, in press) only after several decades or centuries of separation (Peterka and Hartl 1992). Furthermore, in animals with rapid population growth after genetic bottlenecks, such as rodents and lagomorphs, genetic variation in derived species is not necessarily expected to be dramatically reduced (comp. Nei *et al.* 1975): in fact all lagomorphs studied so far are comparatively highly polymorphic (see Hartl *et al.* 1990c, for review).

In conclusion, an acceleration of molecular evolutionary rates during the radiation of the *Leporidae* seems to be possible and this may be the reason, why the estimation of divergence times by method I, being much less dependent on rate constancy among taxa than method II (Hartl 1990), yields the more reliable values at the species and genus level. A combined investigation of biochemical genetic variation and differentiation in the *Leporidae*, based on largely identical sets of isozymes is necessary for testing this hypothesis by tracing the evolutionary pathways of particular allozymes in a large number of subspecies, species and genera.

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