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Genetic Differentiation of the Two Subspecies of the Smooth Newt Inhabiting Romania, *Triturus vulgaris vulgaris* and *T. v. ampelensis* (Urodela, Salamandridae) as Revealed by Enzyme Electrophoresis*

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Starch gel electrophoresis was applied to quantify genetic variation and divergence in samples from Romanian populations of the nominal form of the smooth newt *Triturus vulgaris* and those of the endemic Romanian subspecies *T. v. ampelensis*, a population from a parapatric area was additionally included. All the samples had similar levels of genetic variation measured by the mean heterozygosity, proportion of polymorphic loci, and mean number of alleles per locus. *T. v. ampelensis* samples were genetically clearly different from the nominal form samples, the mean genetic distance between the two subspecies was being estimated as $D_N = 0.114$. No fixed differences in allele composition between the two subspecies were found, although some of the alleles were found either exclusively in the nominal form (*Aat-1 a*) or in *T. v. ampelensis* (*Mpi a*). Other alleles at these loci together with *Mdh-1* differed markedly in frequency. The population from the parapatric area was intermediate in allelic composition, but grouped together with the *T. v. ampelensis* samples in a maximum likelihood tree (99.7% bootstrap support for this grouping). The data indicate that the two subspecies interbreed in a parapatric zone. The molecular clock applied to electrophoretic data indicates that these two forms split during the Pleistocene.

Key words: Smooth newt, *Triturus vulgaris ampelensis*, allozymes, speciation, hybridization.

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The smooth newt, *Triturus vulgaris* (L., 1758), is the most widespread of all *Triturus* species and ranges from Ireland in the west to the Altai Mts. in the east, and from the northern Scandinavia to the southern Peloponnese (KUZMIN & ZUIDERWIJK 1997). Most of this vast area is inhabited by the nominal form, *T. v. vulgaris*, whereas the southern margins of the range are occupied by populations which on the basis of the divergent body form were assigned a subspecific rank. At least 7 subspecies of *Triturus vulgaris* are currently recognised, all of which differ almost exclusively in male secondary sexual characters (RAXWORTHY 1990). One of the subspecies, *T. vulgaris ampelensis* FUHN, 1951, which inhabits the Transylvanian region of Romania, is morphologically similar to other southern subspecies but in contrast to them the area of its distribution is entirely surrounded by the populations of *T. v. vulgaris* (FUHN 1960; COGĂLNICEANU *et al.* 2000). In areas where populations of *T. v. ampelensis* are in close contact with the populations of the nominal form, phenotypically intermediate individuals have been found (COGĂLNICEANU *et al.* 2000). This indicates that there is some gene flow between these two subspecies in nature.

Genetic divergence among some of the southern subspecies of *T. vulgaris* relative to the nominal

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form has already been studied (KALEZIĆ 1983; KALEZIĆ 1984; KALEZIĆ & TUCIĆ 1984; KALEZIĆ *et al.* 1987). The genetic distinctness of *T. v. ampelensis* has not been studied and such data would be of special interest, since a long zone of intergradation which probably exists at the borders of the *T. v. ampelensis* distribution would provide, similarly as other zones of hybridization, a very valuable opportunity for the studies on speciation (ARNOLD 1992).

The aim of the present study was to establish how genetically different are the populations of *T. v. ampelensis* in relation to nearby populations of the nominal form. The authors also wanted to verify whether the genetic data could indicate that these two forms interbreed in parapatric locations. In the present work the evolutionary divergence of *T. v. ampelensis* was assessed by the enzyme electrophoresis as a preliminary step for further more comprehensive analysis.

Material and Methods

The animals were sampled from 7 populations in Romania. Four samples were collected within the area of *T. v. ampelensis*:

1. Cărpiniş, dep. Alba (CAR); 2. Izvorul Ampoiului, dep. Alba (IZV); 3. Zlatna dep. Alba (ZLA); 4. Deva dep. Hunedoara (DEV).
One sample was collected from the area of a putative intergradation:
5. Cîmpu lui Neag, dep. Hunedoara (CIM); and two samples from the area of the nominal subspecies distribution: 6. Reci dep. Covasna (REC); 7. Băneasa near Bucharest (BAN) (Fig. 1). The samples are referred to thereafter by their acronyms given in the parentheses. Numbers of specimens studied are given in Table 3.

The animals were killed by an overdose of the anaesthetic MS-222 (tricaine methanesulphonate), frozen, and kept at -85°C until electrophoresis. Small fragments of tail muscles were homogenised in water and subjected to standard starch-gel electrophoresis (MURPHY *et al.* 1996). Enzyme systems studied and buffers used are shown in Table 1. The loci and alleles were numbered consecutively beginning from the most anodal ones.

Allele frequencies and the measures of genetic variability (percentage of polymorphic loci using the 95% criterion, mean observed and expected heterozygosity, mean number of allelles per locus)

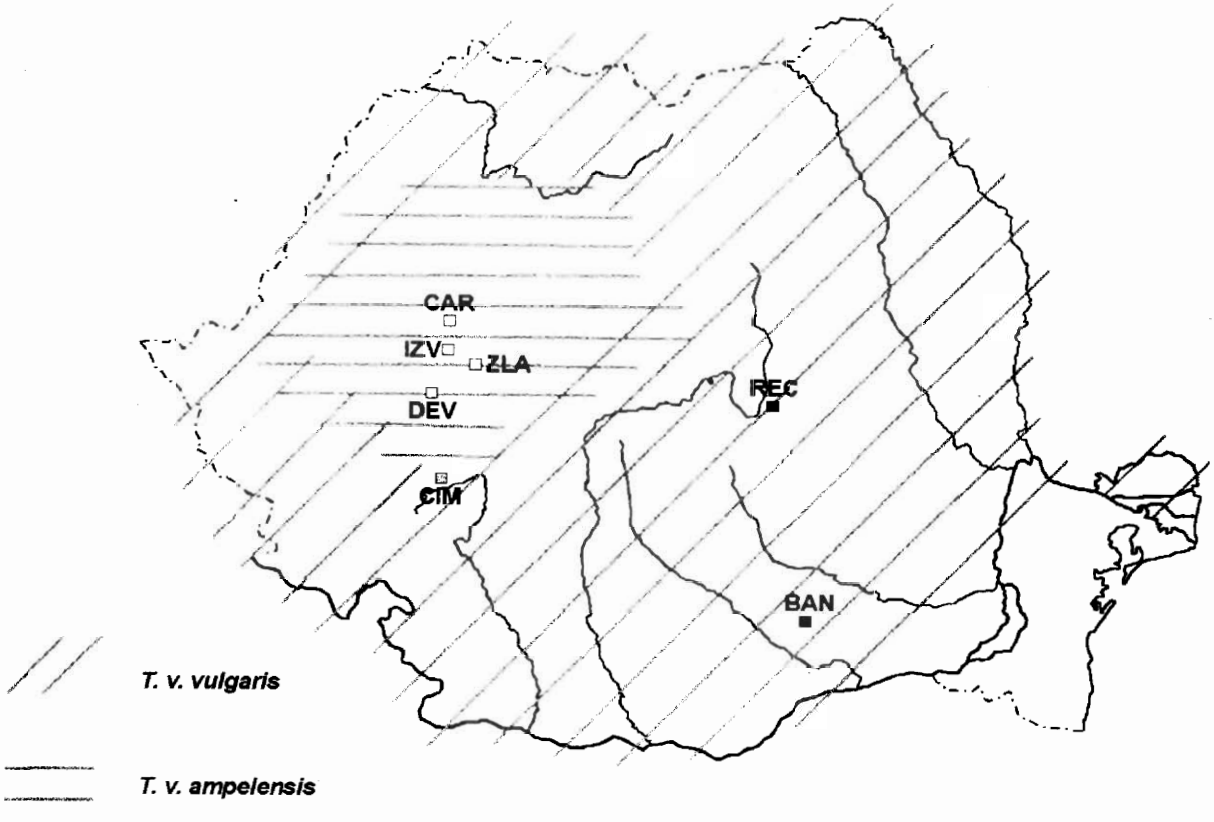

Fig 1. Distribution of the populations studied. Solid squares – *T. v. vulgaris*, open squares – *T. v. ampelensis*, shaded square – a sample from parapatric area. Abbreviations: CAR - Cărpiniş, dep. Cimpeni; IZV - Izvorul Ampoiului, dep. Alba; ZLA - Zlatna dep. Alba; DEV - Deva dep. Hunedoara; CIM - Cîmpu lui Neag, dep. Hunedoara; REC - Reci dep. Covasna; BAN - Băneasa near Bucharest.

Table 1

Enzyme system studied and the buffers used. LiOH – lithium-borate buffer pH 8.1/8.4, TC7 – tris-citric acid buffer pH 7.0, TC8 – tris-citric acid buffer pH 8.0.

Enzyme	EC. No	Locus	Buffer
Adenylate Kinase	2.7.4.3	*Ak*	TC7
Aspartate Aminotransferase	2.6.1.1	*Aat-1, Aat-2*	LiOH
Creatine Kinase	2.7.3.2	*Ck*	TC7
Glucose-6-phospahte Isomerase	5.3.1.9	*Gpi*	TC7
Glutamate Dehydrogenase	1.4.1.3	*Gdh*	TC8
Isocitrate Dehydrogenase	1.1.1.42	*Idh-2*	TC8
L-Lactate Dehydrogenase	1.1.1.27	*Ldh-1, Ldh-2*	TC7
Malate Dehydrogenase	1.1.1.37	*Mdh-1, Mdh-2*	TC7
Mannose-6-phosphate Isomerase	5.3.1.8	*Mpi*	LiOH
Phosphoglucomutase	5.4.2.2	*Pgm*	TC7

were computed using BIOSYS-1 (SWOFFORD & SELANDER 1989). Also tested was the concordance of the genotypic frequencies with Hardy-Weinberg expectations and the linkage disequilibria using permutation tests as implemented in GENEPOP (RAYMOND & ROUSSET 1995). To assess genetic relationships among populations two methods were used. First, a maximum likelihood (ML) tree was constructed from the allele frequencies data using program CONTML in PHYLIP (FELSENTEIN 1993). The robustness of the topology was tested with 10,000 bootstrap replicates with SEQBOOT in PHYLIP. Secondly, the matrix of NEI (1972, 1978) genetic distances between the populations was computed. It was decided that NEI's measures of genetic distances be used because they had been applied in the previous studies on *T. vulgaris* evolutionary divergence and permitted comparisons with the former data. Besides ML tree two-dimensional scaling of genetic distances was applied to show the relationships among populations in the plane. Multidimensional scaling techniques, in contrast to the agglomerative clustering, do not impose hierarchical structure on data and are useful in clarifying the position of intermediates (LESSA 1990).

The time of divergence between *T. v. vulgaris* and *T. v. ampelensis* was estimated using two calibrations of the molecular clock based on allozymes. One calibration for the Aegean water frogs gave the $D_N = 0.10$/Myr (BEERLI *et al.* 1996) while the other obtained for plethodontid salamanders with a slightly slower rate of $D_N = 0.07$/Myr (AVISE & AQUADRO 1982).

Results

Out of the 13 enzyme-coding loci surveyed, 7 were monomorphic in all samples: *Ak*, *Aat-2*, *Ck*, *Gdh*, *Idh-2*, *Ldh-2*, *Mdh-2*. Three loci, *Idh-1*, *Pgdh* and *Pgm-2* were polymorphic but since the variation was not scorable in all samples they were excluded from further analyses. Frequencies of alleles at 6 variable loci are presented in Table 2. After applying the sequential Bonferroni correction neither significant departures from Hardy-Weinberg equilibrium nor linkage disequilibria between any of the loci were found.

Table 2

Allele frequencies at the variable loci. Abbreviations of population names – see Fig. 1.

	BAN	REC	CIM	ZLA	CAR	IZV	DEV
Aat-1							
a	0.735	0.386	0.000	0.000	0.000	0.000	0.000
b	0.147	0.568	0.925	0.864	0.804	0.792	0.833
c	0.118	0.045	0.075	0.136	0.196	0.208	0.167
Gpi							
a	0.176	0.159	0.000	0.114	0.000	0.146	0.167
b	0.118	0.000	0.000	0.000	0.000	0.000	0.000
c	0.588	0.841	1.000	0.886	0.911	0.833	0.833
d	0.118	0.000	0.000	0.000	0.089	0.021	0.000
Ldh-1							
a	0.000	0.000	0.275	0.023	0.000	0.021	0.017
b	0.029	0.000	0.000	0.000	0.089	0.021	0.000
c	0.971	1.000	0.725	0.977	0.875	0.917	0.933
d	0.000	0.000	0.000	0.000	0.036	0.042	0.000
e	0.000	0.000	0.000	0.000	0.000	0.000	0.050
Mdh-1							
a	0.147	0.182	0.375	0.636	0.732	0.688	0.633
b	0.853	0.818	0.625	0.364	0.268	0.313	0.367
Mpi							
a	0.000	0.000	0.225	0.023	0.018	0.063	0.067
b	0.088	0.136	0.350	0.886	0.768	0.854	0.900
c	0.882	0.841	0.425	0.091	0.214	0.083	0.033
d	0.029	0.023	0.000	0.000	0.000	0.000	0.000
Pgm							
a	0.000	0.000	0.000	0.000	0.000	0.021	0.000
b	0.000	0.045	0.175	0.136	0.125	0.396	0.067
c	0.971	0.932	0.825	0.818	0.821	0.583	0.750
d	0.029	0.023	0.000	0.045	0.054	0.000	0.183

The most polymorphic loci were: *Ldh-1* with a total of 5 alleles as well as *Gpi*, *Mpi*, and *Pgm-1*, with 4 alleles each. Every polymorphic locus was variable in each of the samples, although allele distribution and frequency differed among them. The samples all showed similar levels of genetic variation as measured by the number of alleles per locus (ranging from 1.5 in CIM to 1.8 in BAN and IZV), proportion of the polymorphic loci (from 30.8 in BAN to 46.2 in CAR, IZV and DEV), and mean heterozygosity (the observed values ranged from 0.112 in REC to 0.158 in CIM) (Table 3).

Table 3

Measures of genetic variability in the populations studied. N – number of individuals; * – mean expected unbiased heterozygosity of NEI (1978); standard errors in parentheses. Abbreviations of population names – see Fig. 1.

Population	N	No alleles/locus	% polymorphic loci	Mean heterozygosity	
				observed	expected*
BAN	17	1.8 (0.3)	30.8	0.149 (0.068)	0.127 (0.055)
REC	22	1.6 (0.2)	38.5	0.112 (0.050)	0.118 (0.049)
CIM	20	1.5 (0.2)	38.5	0.158 (0.065)	0.153 (0.064)
ZLA	22	1.6 (0.2)	38.5	0.115 (0.043)	0.115 (0.044)
CAR	28	1.7 (0.2)	46.2	0.143 (0.050)	0.138 (0.046)
IZV	24	1.8 (0.3)	46.2	0.154 (0.054)	0.154 (0.053)

Samples representing *T. v. vulgaris* were the most clearly differentiated from those of *T. v. ampelensis* by the allelic composition at *Aat-1*, *Mdh-1*, and *Mpi* (Table 2). *Aat-1 a* was present exclusively, and in relatively high frequencies, in samples of the nominal form (0.735 in BAN and 0.386 in REC). *Mpi d* was found only in the nominal form, in contrast to *Mpi a* which was present in *T. v. ampelensis* samples (ZLA, CAR, IZV and DEV) and in the sample CIM. The last sample came from the population in which morphological intermediates between *T. v. ampelensis* and *T. v. vulgaris* were observed. Another allele which was found in high frequencies in *T. v. ampelensis* samples was *Mpi b* (from 0.768 in CAR to 0.900 in DEV). The frequencies of this allele were much lower in *T. v. vulgaris* samples (0.088 in BAN and 0.133 in REC). On the contrary *Mpi c* was the most frequent *Mpi* allele in *T. v. vulgaris* samples (0.841 and 0.882 in REC and BAN, respectively), in *T. v. ampelensis* samples its frequency ranged from the maximum of 0.214 in CAR to 0.033 in DEV. At the *Mdh-1* locus *a* allele predominated in *T. v. ampelensis* samples, whereas the alternative allele, *Mdh-1 b*, occurred in high frequencies in the *T. v. vulgaris* samples. Intermediate frequencies of *Mdh-1 a* and *b* alleles, as well as *Mpi b* and *c*, were detected in the sample CIM located in the area of putative intergradation (Table 2). There was very little differentiation in allelic frequencies at *Gpi*, *Ldh-1*, and *Pgm-1* among all the samples surveyed. In some populations unique alleles were found, usually occurring in low frequencies (*Ldh-1 e* in sample DEV and allele *Pgm-1 a* in sample IZV); an exception was allele *Gpi b* which was found in BAN only, and at the same time in a relatively high frequency (0.118).

The genetic divergence among the populations was summarised and represented as a matrix of Nei genetic distances (Table 4). Mean genetic distance (D_N, as estimated by the NEI 1972 formula) between *T. v. vulgaris* and *T. v. ampelensis* samples was 0.114 (range 0.078-0.152), mean distance among *T. v. ampelensis* samples (ZLA, CAR, IZV and DEV) was 0.006, and the genetic distance between two *T. v. vulgaris* samples was 0.018. CIM sample, when compared with *T. v. vulgaris* samples, gave $D_N = 0.066$, and when compared with *T. v. ampelensis* samples $D_N = 0.036$. All *T. v. ampelensis* samples clustered together in the maximum likelihood tree (bootstrap support 81.0 %) (Fig. 2), the sample from Cîmpu lui Neag (CIM) is closer to *T. v. ampelensis* than to two samples representing the nominal form (bootstrap support for this group 99.7%). The intermediate position of the CIM sample is even clearer in the two-dimensional scaling of D_N plot (Fig. 3); the very low stress value (0.000005) indicates that scaling reflects almost perfectly genetic distances between the populations studied.

Table 4

Genetic distances between populations studied. Above diagonal – unbiased NEI (1978) distance, below diagonal – NEI (1972) genetic distance. Abbreviations of population names – see Fig. 1.

	BAN	REC	CIM	ZLA	CAR	IZV	DEV
BAN	—	0.014	0.089	0.135	0.127	0.148	0.141
REC	0.018	—	0.035	0.079	0.075	0.093	0.087
CIM	0.093	0.038	—	0.029	0.027	0.037	0.035
ZLA	0.138	0.082	0.033	—	0.001	0.003	.001
CAR	0.131	0.078	0.031	0.004	—	0.006	0.004
IZV	0.152	0.097	0.041	0.007	0.009	—	0.005
DEV	0.144	0.089	0.039	0.002	0.007	0.008	—

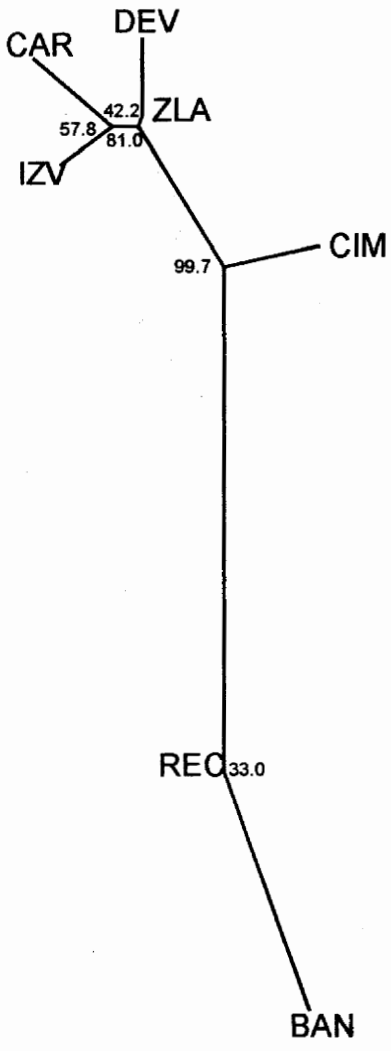

Fig 2. Maximum likelihood tree constructed from the allele frequencies. Numbers – bootstrap support for particular nodes. Abbreviations of population names – see Fig. 1.

As the average D_N between populations representing *T. v. vulgaris* and *T. v. ampelenesis* was 0.114, the divergence time of these two subspecies could be estimated as about 1.1 – 1.6 Myr using calibration for Aegean water frogs (BEERLI *et al.* 1996) and plethodontid salamanders (AVISE & AQUADRO 1982) respectively.

Discussion

The present study shows that *T. v. ampelensis* is clearly distinct from the Romanian populations of the nominal form at the allozyme level. *T. v. ampelensis* differs from the nominal subspecies mainly in allelic frequencies (Table 2), but some alleles are diagnostic for each of the forms. At least for the populations studied the allele *Aat-1 a* and *Mpi d* could be treated as diagnostic markers of *T. v. vulgaris* and *Mpi a* as a marker of *T. v. ampelensis*. These two subspecies can also be characterised by different frequencies of other alleles at *Aat* and *Mpi*, as well as different frequencies of two alleles at *Mdh-1*. The sample from the population Cîmpu lui Neag (CIM), which is situated close to the area inhabited by the nominal form and which contained individuals in external appearance intermediate between *T. v. ampelensis* and *T. v. vulgaris*, was also intermediate in the allelic composition at such loci as *Mdh-1* and *Mpi*. The intermediate genetic status of this population is evident upon the inspection of the multidimensional scaling diagram (Fig. 3), although in the maximum likelihood tree the CIM sample is closer to those of *T. v. ampelensis* than to those of the nominal form (Fig. 2).

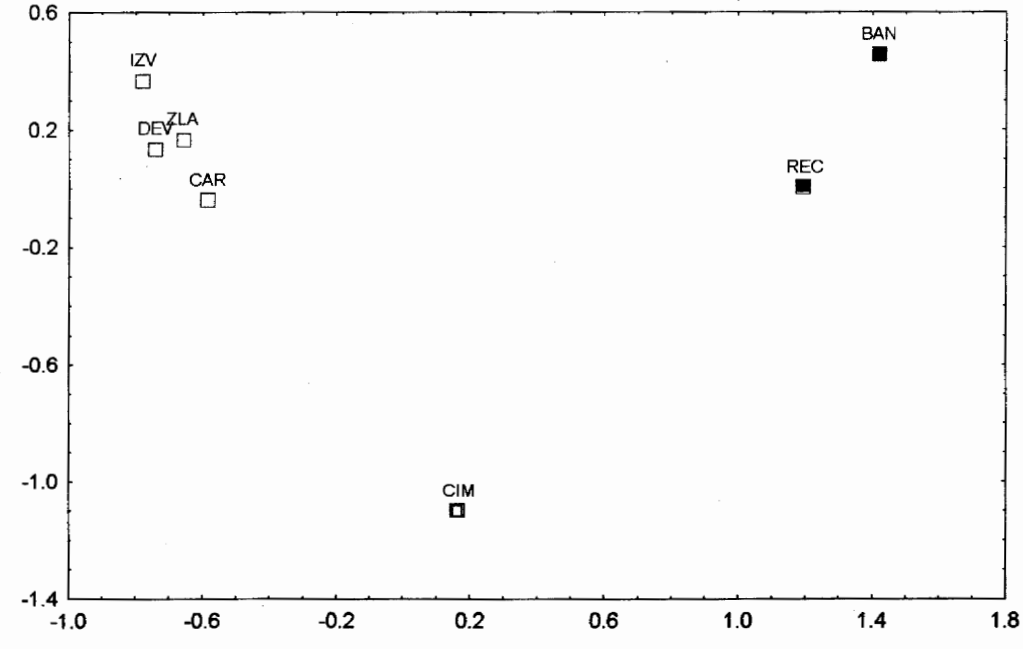

Fig 3. Two-dimensional scaling of the matrix of NEI (1972) genetic distances (stress = 0.000005). Solid squares – *T. v. vulgaris*, open squares – *T. v. ampelensis*, shaded square – a sample from parapatric area. Abbreviations of population names – see Fig. 1.

These data indicate strongly that the two subspecific taxa interbreed in the areas of parapatry. We presume that a zone of hybridization might surround the enclave populated by *T. v. ampelensis*, although the geographical position of such an intergradation zone remains to be explored by detailed geographic and genetic sampling.

Unexpectedly we did not find much differences in the level of genetic variation within *T. v. ampelensis* and *T. v. vulgaris* samples (Table 3). Reduced genetic variation could be expected in *T. v. ampelensis*, a relic form (see later) with a limited distribution, as it had been found for the southern populations of *Rana arvalis* (RAFIŃSKI & BABIK 2000). Allozymic studies of the genetic variation in *T. vulgaris* subspecies from the former Yugoslavia showed that the populations of the nominal form, sampled throughout the territory of Croatia and Serbia, were more variable than the samples of the three southern subspecies, *T. v. meridionalis*, *T. v. dalmaticus*, and *T. v. graecus* (KALEZIĆ 1983; KALEZIĆ & TUCIĆ 1984). However, all the samples of southern subspecies were collected from the mediterranean-type habitats which are much less suitable for the *Triturus* than more mesic northern sites populated by *T. v. vulgaris*. The low genetic variation of subspecies living in the southern areas might be a result of much lower demographic stability of newt populations in the mediterranean-type habitats.

Mean genetic distance between *T. v. ampelensis* and *T. v. vulgaris* samples was found to be D_N = 0.114. Similar D_N values were obtained for other *T. vulgaris* subspecies pairs (KALEZIĆ 1984). D_N = 0.113 was found for the pair *T. v. vulgaris*/*T. v. dalmaticus*; while even smaller values were found for other subspecies pairs, *T. v. vulgaris*/ *T. v. meridionalis* (D_N = 0.049) and *T. v. vulgaris*/*T. v. graecus* (D_N = 0.070). The small genetic divergence among *T. vulgaris* subspecies suggests that they all diverged relatively recently. Our estimates for *T. v. ampelensis* and *T. v. vulgaris* based on the molecular clock for allozymes indicate that they diverged most probably during the Pleistocene as earlier suggested on biogeographical grounds by COGĂLNICEANU & VENCZEL (1992). The extensive Pleistocene glaciations restricted the distribution of many plant and animal species to a number of refugia in southern Europe where they diverged genetically in allopatry (HEWITT 1996). Available data on genetic divergence among *T. vulgaris* subspecies, together with their present distributional pattern indicate that they most probably diverged during Pleistocene in isolated southern refugia, the nominal form being most successful in colonizing formerly glaciated northern areas. The distribution of *T. v. ampelensis* is something of a mystery, since the area populated by this subspecies lies within that of the nominal form. The most plausible explanation is that *T. v. ampelensis* represents a group of relic populations which was later surrounded by the colonising populations of the nominal form. Similarly, the existence of the enclaves of the yellow-bellied toad (*Bombina variegata*) in northern Croatia, Serbia, central Romania, and southern Hungary was explained by the later arrival of the fire-bellied toad (*B. bombina*) from the southern refuges (ARNTZEN 1978).

Genetic divergence among the subspecies of *T. vulgaris*, as measured by values of D_N, is below the mean value of D_N = 0.16 reported for the closely related amphibian species (THORPE 1982). What is more, our data indicate that the two studied taxa interbreed in nature. Wide zones of intergradation were also hypothesised for other *T. vulgaris* subspecies on grounds of electrophoretic (KALEZIĆ 1984) and morphological data (SCHMIDTLER & SCHMIDTLER 1983). Taken together the data suggest that *T. vulgaris* subspecies have not reached the level of genetic divergence characterising fully differentiated species.

Morphologically *T. vulgaris* subspecies differ almost exclusively in male secondary sexual characters (tail-fin height and shape, presence/absence of tail-tip filament, dorso-lateral ridges, toe flaps, and details of nuptial coloration) (RAXWORTHY 1990). However no comprehensive morphometric studies on both males and females are available and the extent of differences in body proportions between sexes and subspecies remains to be studied. The situation in *T. vulgaris* contrasts sharply with that found in *Triturus cristatus* superspecies. Several subspecies were first described within *T. cristatus* on the basis of minor differences in body proportions and coloration not related to sexually dimorphic traits (WOLTERSTORFF 1923; for more recent data see ARNTZEN & WALLIS 1999). Subsequent molecular studies revealed that these taxa showed a substantial degree of evolutionary divergence reflected by relatively high values of genetic differentiation and limited intergradation in parapatric situations (WALLIS & ARNTZEN 1989) and, as a consequence, the *T. cristatus* subspecies were raised to the specific rank. The subspecies of the *T. vulgaris* seem to represent an alternative route to speciation which have primarily involved the divergence of secondary sexual traits functioning in the mate recognition. To establish at what stage of speciation subspecific taxa of *T. vulgaris* are at present, both further genetic studies on the extent

of intergradation in parapatric populations and laboratory studies on the strength of the behavioural reproductive isolation between subspecies are needed.

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