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GENIC VARIATION AND ITS EVOLUTIONARY IMPLICATIONS IN THE ITALIAN NEWT, *TRITURUS ITALICUS*

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ABSTRACT: Genic variation was studied in 103 individuals collected from 11 populations of the Italian newt, *Triturus italicus*, from peninsular Italy. Starch-gel and cellogel electrophoresis were used to study variation at 23 electromorphic loci, 14 of which were polymorphic. Geographically significant variation occurs at seven of the 14 loci, and the populations can be divided roughly into northern and southern units. Intermediate populations show increased heterozygosity and greater variability relative to more northern and more southern populations. The rather high value of F_{st} of 0.448 shows that extensive genetic subdivision exists, despite the relatively low genetic distances recorded among species (maximal value of Nei's $D = 0.19$). These results correlate well with studies of chromosomal polymorphism, and suggest that populations of the species were once separated and have come into relatively recent secondary contact. Results of this study are compared with a recent study of *T. vulgaris*.

Key words: Amphibia; Caudata; Salamandridae; *Triturus italicus*; Electrophoresis; Allozymes; Genic differentiation

THE Italian newt, *Triturus italicus*, ranges over the central and southern portions of the Italian Peninsula. This small newt, a member of the *vulgaris* group, or subgenus *Paleotriton*, lives either in standing water or in slow moving streams during the spring breeding season. Bucci-Innocenti et al. (1983) examined chromosomal and C-heterochromatin variation in the species. A combined chromosomal and C-heterochromatin polymorphism occurs in chromosome pair 12 (the smallest in the series, N = 12), and it has a discrete geographic distribution. The C-heterochromatin polymorphism appears to represent the loss of a portion of a proximal C-band. The chromosomal polymorphism involves two separate and independent inversions, both of which include the centromere and the proximal C-band. In the northern part of the range of the species, only subtelocentric pairs of chromosome 12 are found (ST/ST). To the south and east, metacentric chromosomes increase in frequency. Different metacentric chromosomes (M) are encountered, and the ST chromosomes become less frequent. In some localities, the ST are found only in heteromorphic states with M chromosomes, presumably because of the low frequency of ST. Finally, in southern Apulia, only M chromosomes are found. Approximately one-half of the total range of the species is a broad transitional belt that extends from north to south across the peninsula.

One possible explanation for the pattern of chromosomal variation in this species is that some historical event separated the species into two major units, which then evolved independently. According to this hypothesis, the zone of chromosomal heteromorphism results from recontact of the once separated segments of the species. If chromosomes diverged during the period of hypothesized isolation, one might also expect to see allozymic differentiation, and to find a zone of high allozymic heterozygosity and polymorphism in the area in which chromosomal heteromorphism occurs. To test this hypothesis, we selected samples from throughout the range of *Triturus italicus* and conducted starch-gel and cellogel electrophoretic analyses. In this paper, we present the results of that study.

Materials and Methods

Triturus italicus occurs throughout central and southern Italy, below a hypothetical line joining the Marches and the Gulf of Gaeta. Our material was collected from 11 populations scattered over the entire range of the species. The 103 individuals studied were as follows (abbreviations follow Bucci-Innocenti et al., 1983): Ge—Genga (Ancona, The Marches). Ponds at about 320 m elevation; 10 specimens collected April 1983; CSM—Colle San Marco (Ascoli-Piceno, The Marches). Four small, shallow ponds interconnected and receiving water from a small spring, about 800 m; nine specimens collected April 1983; Bu—Bucchianico (Chieti, Abruzzi). Reservoir at 300 m; 10 specimens collected April 1983; Vi—Vinchiaturo (Campobasso, Molise). Pond at 625 m; 10 specimens collected May 1983; MA—Maranola (or Monti Arunci, Latina, Lazio). Eleven artificial pools at about 750 m; four specimens collected May 1983; CV—Casal Velino (Salerno, Campania). Interconnected reservoirs, about 150 m; 10 specimens collected May 1983; FU—Foresta Umbra (Foggia, Apulia). Shallow, narrow canal connecting an artificial lake (Cutino d'Umbra) and a large pool (Lago di Otri), about 800 m; 10 specimens collected April 1983; CM—Cassano delle Murge (Bari, Apulia). Pond at about 340 m; 11 specimens collected late spring 1983; Co—Conversano (Bari, Apulia). Artificial tanks built inside so-called Laghi di Conversano, about 250 m; 10 specimens collected May 1983; Ru—Ruoti (Potenza, Basilicata). Pond at about 450 m; 10 specimens collected March 1983; Gr—Grisolia (Cosenza, Calabria). Pond at about 465 m; nine specimens collected spring 1983.

Samples of liver, stomach, intestine and heart were extracted from freshly killed specimens and stored at −76 C for later use. Carcasses are preserved in the herpetological collections of the Museum of Vertebrate Zoology, University of California, Berkeley. Homogenized tissue extracts were pooled from each animal and were analyzed by standard techniques of horizontal starch-gel electrophoresis (Ayala et al., 1972; Harris and Hopkinson, 1976; Selander et al., 1971) using Electrostarch (Lot 83F-D162). One locus (AK) was run on Cellogel (Chemetron).

The following gel/buffer systems were used.

Tris citrate pH 8.0 (1:30 dilution of electrode buffer for gel) for phosphoglucomutase (Pgm), aconitase (Acon, stained with 1% agar overlay), sorbitol dehydrogenase (Sordh), lactate dehydrogenase (Ldh), and alpha-glycerophosphate dehydrogenase (alpha-Gpd);

Tris citrate pH 8.0 (1:30 dilution of electrode buffer for gel, plus NADP) for isocitrate dehydrogenase (Icd), malate dehydrogenase (Mdh), and glutamate dehydrogenase (Glud, stained with 1% agar overlay);

Tris citrate pH 8.0 (1:30 dilution of electrode buffer for gel, plus NAD and alpha-mercaptoethanol) for glyceraldehyde-phosphate dehydrogenase (Gapdh);

Tris maleic EDTA (1:10 dilution of electrode buffer for gel, plus NADP) for 6-phosphogluconate dehydrogenase (6-Pgd) and Malic enzyme (Me);

Lithium hydroxide A+B pH 8.2 (lithium hydroxide A pH 8.1 for gel) for protein-A (Pt A) and peptidases utilizing the following substrates: phenyl alanyl proline (Pap, stained with 1% agar overlay), L-leucyl glycyl glycine (Lgg, stained with 1% agar overlay), L-leucyl-L-alanine (La), stained with 1% agar overlay;

Tris citrate pH 7.0 (1:15 dilution of electrode buffer for gel) for erythrocytic acid phosphatase (Eap) and glutamine oxalacetic transaminase (Got);

Pgi phosphate (1:20 dilution of electrode buffer for gel) for glucosephosphate isomerase (Gpi, stained with 1% agar overlay), and mannose-6-phosphate isomerase (Mpi, stained with 1% agar overlay);

Tris citrate EDTA pH 7.5 for adenylate kinase (Ak), analyzed using Cellogel.

Estimates of genic heterozygosity were obtained from direct-counts for each local population (mean heterozygosity is the number of heterozygous genotypes recorded in the sample divided by the product of the number of individuals and the number of proteins surveyed). Estimates

TABLE 1.—Allozyme frequencies at variable loci in *Triturus italicus*. The following loci are monomorphic: Icd-1, Icd-2, Acon-1, Acon-2, Sordh, Ldh, Got-2, Glud and Pt A. Letters refer to variants in relation to rate of migration, with a being the most rapid.

	Population										
Locus	Ge	CSM	Bu	Vi	MA	CV	FU	CM	Co	Ru	Gr
(*n*)	10	9	10	10	4	10	10	11	10	10	9
6-Pgd	a	a	a	a	a	a	a	d	d	a (0.75) b (0.15) c (0.05) d (0.05)	c (0.11) d (0.89)
Mdh-2	a	a	a	a	a	a	a	a	a	a	a (0.94) b (0.06)
Gapdh	c	c	a (0.05) c (0.95)	c	c	b (0.05) c (0.95)	c	c	c	c	c
Pgm	a (0.55) b (0.45)	a	a (0.75) b (0.25)	a (0.90) b (0.10)	a	a (0.75) b (0.25)	a	a	a	a (0.95) b (0.05)	a (0.89) b (0.11)
Gpd	b	b	b	b	b	a (0.10) b (0.90)	b	b	b	b	a (0.06) b (0.94)
La	a	a	a	a	a	a (0.90) b (0.10)	a	a	a	a (0.90) b (0.10)	a
Lgg	a	a	a	a	a	a (0.85) c (0.05) d (0.10)	a	a (0.59) c (0.41)	a (0.70) c (0.20) d (0.10)	a (0.85) c (0.15)	a (0.11) b (0.17) c (0.22) d (0.44) e (0.06)
Pap	a	a	a	a	a	a (0.95) b (0.05)	a	a	a	a	a

TABLE 1.—Continued.

Locus	Population										
	Ge	CSM	Bu	Vi	MA	CV	FU	CM	Co	Ru	Gr
Pgi	e	a (0.17) e (0.83)	a (0.25) e (0.75)	a (0.20) e (0.80)	e	a (0.25) b (0.05) c (0.05) d (0.05) e (0.60)	a (0.20) e (0.80)	a (0.18) e (0.82)	a (0.10) e (0.90)	a (0.25) d (0.15) e (0.60)	a (0.44) e (0.56)
Mpi	a (0.95) b (0.05)	a	a	a	a	a	a	a	a	a	a
Me	a (0.80) b (0.15) c (0.05)	a (0.83) b (0.17)	a (0.70) b (0.30)	a (0.80) b (0.20)	a	a (0.20) b (0.70) c (0.10)	b	a (0.95) b (0.05)	a	a (0.55) b (0.45)	a (0.56) b (0.44)
Eap											
(*n*)	10	9	8	8	4	10	10	9	9	8	8
Eap-1	a	a	a	a	a	a	a	a (0.11) b (0.89)	a (0.67) b (0.33)	a (0.75) b (0.25)	a (0.38) b (0.62)
Eap-2	a	a	a	a	a	a	a	a	a	a (0.75) b (0.25)	a
Ak											
(*n*)	5	6	6	4	4	6	6	2	6	7	6
	a	a	a	a (0.88) c (0.12)	a	a (0.75) c (0.25)	a	c	a (0.42) c (0.58)	a (0.21) b (0.07) c (0.72)	a (0.67) b (0.08) c (0.25)

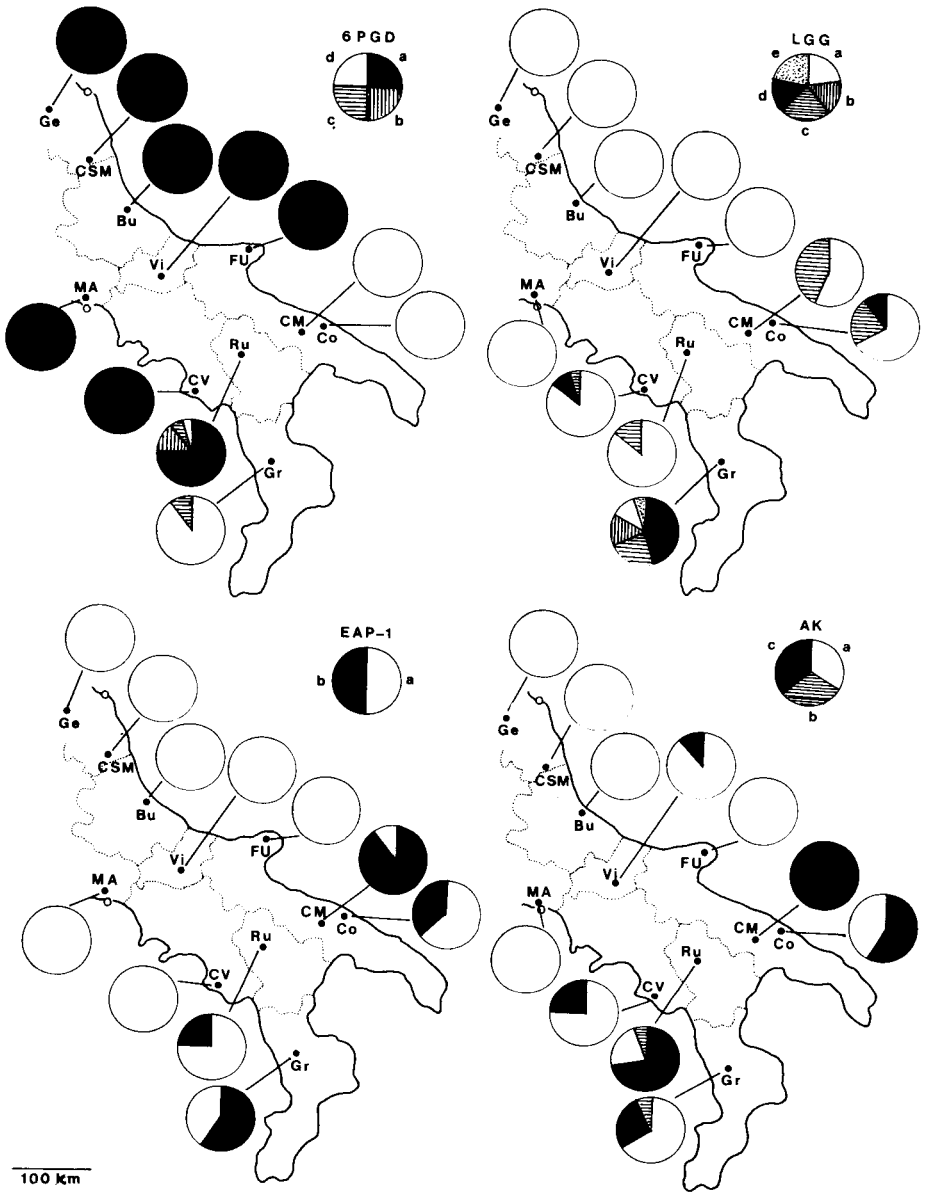

FIG. 1.—Distribution of variation at four electromorphic loci in *Triturus italicus* in the southern part of the Italian peninsula. For abbreviations of localities, see text. The proportion of the circle filled indicates the frequency of the indicated allelomorph in each population.

of polymorphism are based on the number of proteins having two or more variants, divided by the total number of proteins. Estimates of genetic distance are based on Rogers (1972) (for genetic distance, D) and Nei (1972) (for Nei's genetic distance D_N). Levels of genetic subdivision within *T. italicus* were estimated using the standardized variance in gene frequency, F_{st} (Wright, 1965, 1978). Computation was by means of the BIOSYS program (Swofford and Selander, 1981).

The populations of *T. italicus* were analyzed by the method of Slatkin (1981)

TABLE 2.—Frequencies of Nei's genetic distance (above diagonal) and Rogers genetic distance (below diagonal) for populations of *Triturus italicus* studied.

Population	Population										
	Ge	CSM	Bu	Vi	MA	CV	FU	CM	Co	Ru	Gr
Ge	—	0.011	0.006	0.008	0.010	0.028	0.042	0.158	0.085	0.054	0.116
CSM	0.031	—	0.004	0.001	0.002	0.025	0.031	0.144	0.073	0.041	0.102
Bu	0.030	0.022	—	0.002	0.009	0.015	0.025	0.153	0.082	0.041	0.100
Vi	0.034	0.013	0.021	—	0.004	0.019	0.030	0.134	0.069	0.032	0.099
MA	0.030	0.014	0.037	0.027	—	0.038	0.047	0.143	0.071	0.050	0.113
CV	0.080	0.074	0.055	0.062	0.088	—	0.011	0.156	0.098	0.030	0.095
FU	0.066	0.038	0.046	0.045	0.052	0.059	—	0.190	0.122	0.051	0.113
CM	0.179	0.149	0.170	0.150	0.153	0.191	0.186	—	0.024	0.077	0.052
Co	0.129	0.105	0.127	0.107	0.099	0.146	0.143	0.056	—	0.056	0.037
Ru	0.121	0.098	0.098	0.090	0.112	0.092	0.108	0.131	0.103	—	0.079
Gr	0.166	0.147	0.141	0.135	0.162	0.138	0.158	0.107	0.096	0.129	—

to analyze patterns of gene flow. The relationship between the conditional average frequency of an allele ($\bar{p}_i$) and the fraction of the total population in which it occurs (i/d) was examined following the method of Slatkin (1981): (1) The conditional average frequency ($\bar{p}_i$) was calculated for each protein variant scored. (2) Protein variants were grouped according to the fraction of the total populations studied in which they were observed (i/d). (3) The average value of ($\bar{p}_i$) was calculated for protein variants having the same value of i/d. (4) These values were plotted with i/d on the abscissa and ($\bar{p}_i$) on the ordinate.

For comparison of empirical curves with theoretical curves, see Slatkin (1981) and Larson et al. (1984).

Estimates of the gene flow parameter of Wright (1965), Nm, are derived from F_{st} using the formula $F_{st} = 1/(4Nm + 1)$.

The method of Nei (1975) was used to estimate the maximum possible value of m (migration rate), using the formula $I = m/(m + v)$, where I = Nei's normalized coefficient of genetic identity and v = mutation rate (value used for mutation rate is 2×10^{-6} per locus per generation). A minimum estimate of the average effective size of individual populations is derived by dividing Nm by m.

RESULTS

Of the 23 proteins that could be scored, nine show no variation in our sample (Icd-1, Icd-2, Acon-1, Acon-2, Sordh, Ldh, Got-2, Glud, and Pt A). Divergence is minimal among the populations for seven of the 14 polymorphic proteins; a single variant of each predominates in all populations (Table 1). The variants present in low frequency are unique to single populations (Mdh-2, Gapdh, Pap, Mpi, Eap-2) or are shared between two populations (Gpd, La). The remaining polymorphic proteins (6 Pgd, Pgm, Lgg, Pgi, Eap-1, Me, Ak) show differences in frequency among populations and most have different variants predominating in different populations and geographic groupings of populations (Fig. 1).

For purposes of discussion, the samples are divided into geographic quadrats, as follows: Northwest, MA; Northeast, Ge, CSM, Bu, Vi, FU (all of the above are northern); Southeast, CM, Co; Southwest, CV, Ru, Gr (the last two groups are southern).

Populations in the north and in the south differ in distribution of protein variants (Fig. 1, Table 1). In particular, the southern and southeastern populations CM, Co, and Gr stand out from the rest, with the southwestern Ru being somewhat transitional, having variants from both groups of populations. This is a weak generalization, however, and is based primarily on 6 Pgd d, LGG c (also present in CV), and EAP 1 b. These are the only concordantly varying loci.

Populations in the south (Ru, CV and Gr) are the most variable, all being polymorphic for 39.1% of proteins sampled.

Heterozygosity ranged from 0 (the small sample from MA) to 9%, with the highest heterozygosities being found in the most polymorphic populations.

The maximal value of D_N among the 11 populations is 0.19, between FU and CM, two geographically close populations (Table 2). Populations CM, Co, and Gr have a maximum genetic distance of 0.052 among them, and the population most similar to these three is Ru (0.056–0.079). The six northern populations and the southwestern CV have a maximum genetic distance of 0.047 among them, and again it is Ru that is the remaining population least differentiated from them (minimum D_N = 0.030). Ignoring Ru for the moment, the minimum value of D_N between CM, Co, and Gr in the south and southeast and the seven populations just discussed is 0.069 (the distance between Co and Vi). Thus, although genetic differentiation as measured with the summary statistics is relatively small among the populations sampled, the greatest distances are measured from the northern and western to the southern and eastern groups of populations, with a central population, Ru, being somewhat intermediate between the two weakly defined units.

The calculated value of F_{st} (Wright, 1965) is 0.448, an intermediate level for salamanders but very high for vertebrates in general. The value is a little below the average of 0.53 (ranges 0.13–0.80) reported for 22 species by Larson et al. (1984). As is the case for most species of salamanders studied to date, *Triturus italicus* is comprised of populations that are strongly isolated from each other, even though the species has a restricted geographic range.

Using the average value for Nei's genetic identity (I) of 0.941, we obtain an estimate of $m = 3.2 \times 10^{-5}$. By dividing the value of Nm (0.3075) derived from F_{st} by the calculated value of m, we obtain an estimate of N of approximately 9600. This value assumes equilibrium conditions. Although this figure appears to be high, it is exceeded by 17 of the 22 species of plethodontid salamanders surveyed by Larson et al. (1984). Slatkin (1981) presented theoretical curves based on the relationship between the conditional average frequency of an allele and the fraction of the total populations in which it occurs. The curves illustrated by him correspond to values of Nm of 0.025, 0.125, 0.25, and 1.25. The curve that we obtained corresponds more closely to the island model simulation of Slatkin (1981) with a value of $Nm = 0.125$ than to that for $Nm = 0.25$.

Discussion

Our initial goal in this study was to determine whether patterns of genic differentiation are concordant with those of chromosomal differentiation in the newt species *Triturus italicus* of peninsular Italy. Although the amount of genic differentiation in the species is not great compared to other species of urodeles, there is substantial differentiation and a general correlation with the patterns of chromosomal differentiation. Populations in the southwest (Gr, Ru, CV) are more polymorphic than remaining populations. The level of genic similarity is high among three populations in the south and southeast (Co, CM, Gr), and the seven populations in the north (Ge, CSM, Bu, MA, Vi, FU) and southwest (CV), with the southwestern population Ru being somewhat intermediate. This last population is from an area in which a high degree of chromosomal polymorphism was found by Bucci-Innocenti et al. (1983). It may be significant that this population is intermediate in allozyme frequencies and in having high polymorphism and high heterozygosity, as well as having high chromosomal polymorphism. More specifically, if segments of the species were separated geographically and have come into secondary contact, the populations in the vicinity of the secondary contact would be expected to show increased values of polymorphism relative to the species as a whole.

There are some instances of lack of correspondence between chromosomal and allozymic data sets. For example, the southwestern CV population is monomorphic for chromosome type ST, but it is the most variable in allozymes. The northeastern Vi population has all of the chro-

mosomal types, but it has only moderate allozymic variability.

Our results do not lead to a rejection of the hypothesis that the zone of chromosomal heteromorphism described by Bucci-Innocenti et al. (1983) is the result of secondary contact. The patterns of chromosomal and protein variation extant in the species might have resulted from isolation of at least two major populational units during Pleistocene times, when much of peninsular Italy was under water and terrestrial vertebrates were forced to move to higher, insular areas. This species occurs today in lowland sites that were inundated during pluvial periods of the Pleistocene, and it also occurs in highland localities (over 1500 m, Bruno, 1973). In support of this scenario is the relatively high value estimated for F_{st} for the species, which implies, especially in view of the relatively low genetic distances measured in the species (cf. Larson et al., 1984; Larson, 1984), that different parts of the species have been isolated with little or no gene flow for extensive periods of time. Further support comes from the pattern of genic differentiation, which reaches its highest level between the relatively nearby populations FU and CM, in the eastern part of the range. If this scenario is correct, there is a zone of secondary contact in the vicinity of Ruoti in which both chromosomal and protein polymorphism is high and genetic distances to more northern and more southern groups are about equal.

Bucci-Innocenti et al. (1983) postulated that the ST/ST karyotype in the northern and western portions of the range of the species was the plesiomorphic condition, and that the various M/M combinations in the south and east were derived, even though M/M conditions prevail in most species of urodeles. The higher levels of genic polymorphism and heterozygosity in the south and west and the greater number of alleles in this area argue in favor of their being the older populational unit (following Nei, 1975), although the evidence is not strong.

Our results can be compared with those recently presented for another member of the *Paleotriton* group of *Triturus*, *T. vulgaris* (Kalezic and Tucic, 1984). That study included 27 populations of four subspecies of *T. vulgaris* in Yugoslavia, occupying a total territory of substantially greater area than the range of *T. italicus* (see map in Kalezic, 1983). Surprisingly, the genic differentiation among these four subspecies is substantially less than that which we measured in *T. italicus*. The value of F_{st} for all 27 populations recorded by Kalezic and Tucic (1984) was 0.217, and for 15 populations of *T. vulgaris vulgaris* it was 0.096. We cannot explain this difference; one possibility is that the populations in Italy are more completely separated by inhospitable terrain than are those in Yugoslavia, and accordingly gene flow is reduced. Another is that the populations in Italy are older, despite their lower morphological differentiation.

Both our study and that of Kalezic and Tucic (1984) recorded relatively high values of F_{st} (cf. Larson et al., 1984; Wright, 1978). These values are sufficiently high as to suggest that there has been no gene flow throughout these species (at the level of population structure analyzed) for a long time. The Yugoslavian populations of *T. vulgaris* have the lower value of F_{st}, but there is much greater morphological differentiation (four subspecies are represented in the sample) in the region studied than there is in our sample of *T. italicus*. In contrast, the less morphologically diverse *T. italicus* is more subdivided genetically. We interpret levels of genetic differentiation as being the result of different histories, and reflecting the relative recency (as opposed to the regularity) of gene flow between populations studied. Under this interpretation, morphological differentiation may have been more rapid in *T. vulgaris* than in *T. italicus*. However, until a detailed analysis of interspecific variation throughout the range of *T. vulgaris* is completed, this idea must remain hypothetical.

Triturus italicus is yet another species of urodele in which there is a high degree of genetic fragmentation (cf. Larson et al., 1984; Larson, 1984). Compared with vertebrates in general, there is a great range

of intraspecific genetic differentiation in urodeles (Larson, 1984; cf. Avise and Aquadro, 1982). Presumably the species that are well differentiated are old as well, notwithstanding their low degree of intraspecific and interspecific morphological differentiation (cf. Wake et al., 1983).

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