



## Genetic variation in the Italian crested newt, *Triturus carnifex*, and the origin of a non-native population north of the Alps

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**Abstract.** Genetic variation over 40 protein loci and 46 populations representing three taxa of crested newts revealed moderate genetic distances between *Triturus carnifex carnifex*, *T. c. macedonicus* and *T. cristatus*. Two populations from the Geneva Basin (presumed to be introduced) were genetically similar to *T. c. carnifex* and dissimilar to *T. c. macedonicus* and *T. cristatus*, showing that they belong to *T. c. carnifex* and not to native *T. cristatus*. A significant pattern of spatial genetic variation was found within *T. c. carnifex* along a north to south axis, from Croatia to Calabria. The Genevan populations showed highest genetic similarity with *T. carnifex* from Tuscany, suggesting that the propagule originated from that area. Effects of a population genetic bottleneck associated with the introduction could not be documented. The observed high allelic variation in Genevan *T. c. carnifex* could not be directly explained by introgression from *T. cristatus*. Comparisons across the range, including zones of hybridization within the *T. cristatus* superspecies, indicated that some alleles typical for the Genevan population may represent the so-called ‘hybrizymes’.

**Key words:** geographical variation, hybrizyme, protein variation, species introduction, *Triturus*

### Introduction

Genetic variation is rarely uniformly or randomly distributed over time and space and often forms complex spatio-temporal patterns. Consequently, the study of spatial genetic variation can provide clues about the history of populations. Regional differentiation also means that local populations bear ‘genetic tags’ which provide a means of tracing the taxonomic and geographical origin of newly formed natural or artificial populations (Goldberg 1997; Goostrey et al. 1998) as well as populations known or suspected to have been introduced (Hauser et al. 1998; Rowe et al. 1998; Szymura 1998). Man-assisted colonizations are often detrimental to native biota and introduced species are increasingly recognized as an important contributor to the loss of biodiversity world-wide (Lever 1994; Fenner and Fantini 1999). One such introduction concerns an amphibian, the Italian crested newt – *Triturus carnifex*, in the Geneva area of Switzerland and adjacent France. The history of the introduction, the spread of *T. carnifex*, the decline of native *T. cristatus* (northern

crested newt) and genetic interactions between the species were described by Arntzen and Thorpe (1999). In the present paper, I describe genetic characteristics of *T. carnifex* and *T. cristatus* across their ranges. (*T. cristatus* and *T. carnifex*, together with *T. dobrogicus* and *T. karelinii* represent full species, organized in the *T. cristatus* superspecies (Arntzen and Wallis 1999); for a schematic distribution of crested newt species, see Figure 1). Aims of the study were: (i) to confirm the non-native nature of Genevan *T. carnifex*, (ii) to identify its geographical source, and (iii) to reconstruct its population genetic history. Conservation biologists, and indeed future geneticists, need to know about the history of wildlife movements by humans and its population genetic consequences.

### Material and methods

Crested newts were sampled from 44 localities over western, central and southern Europe. The sampling area covered a large part of the range of *T. cristatus* and the full range of *T. carnifex* (Arntzen 1995). Samples were identified as belonging to *T. cristatus* ( $N = 153$ , from 18 localities), *T. carnifex carnifex* ( $N = 67$ , from the 13 localities shown in Figure 2) or *T. carnifex macedonicus* ( $N = 94$ , from 13 localities),

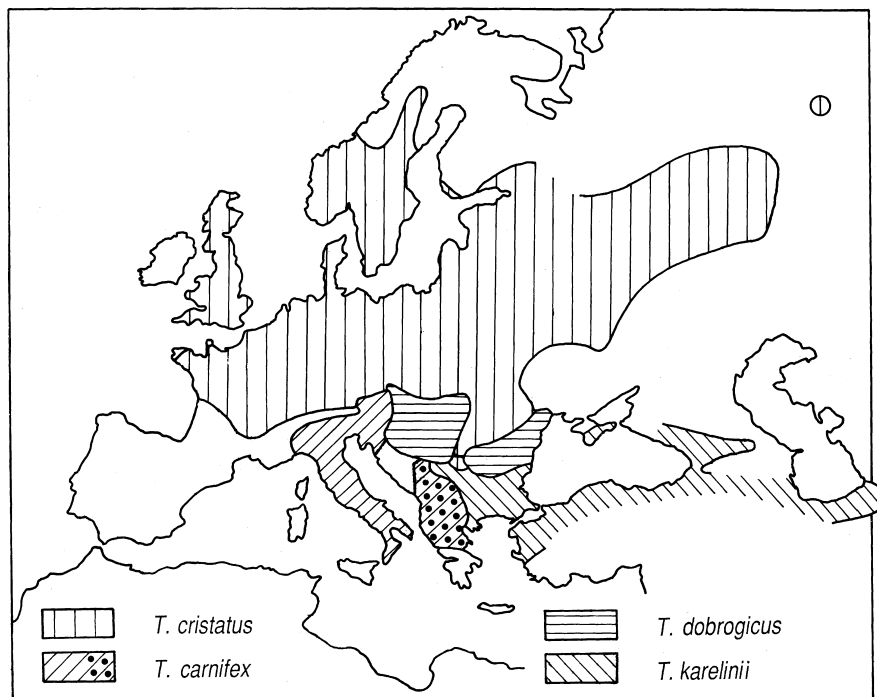


Figure 1. Schematic distribution of four species of crested newt in the *T. cristatus* superspecies (Arntzen 1995).

following morphological criteria presented by Arntzen and Wallis (1999). The identifications were in line with documented geographical distributions. Two more samples of *T. carnifex* were taken from the Basin of Geneva, north of the Alps, in Switzerland and adjacent France. Exact localities, sample size and voucher information are given in the Appendix.

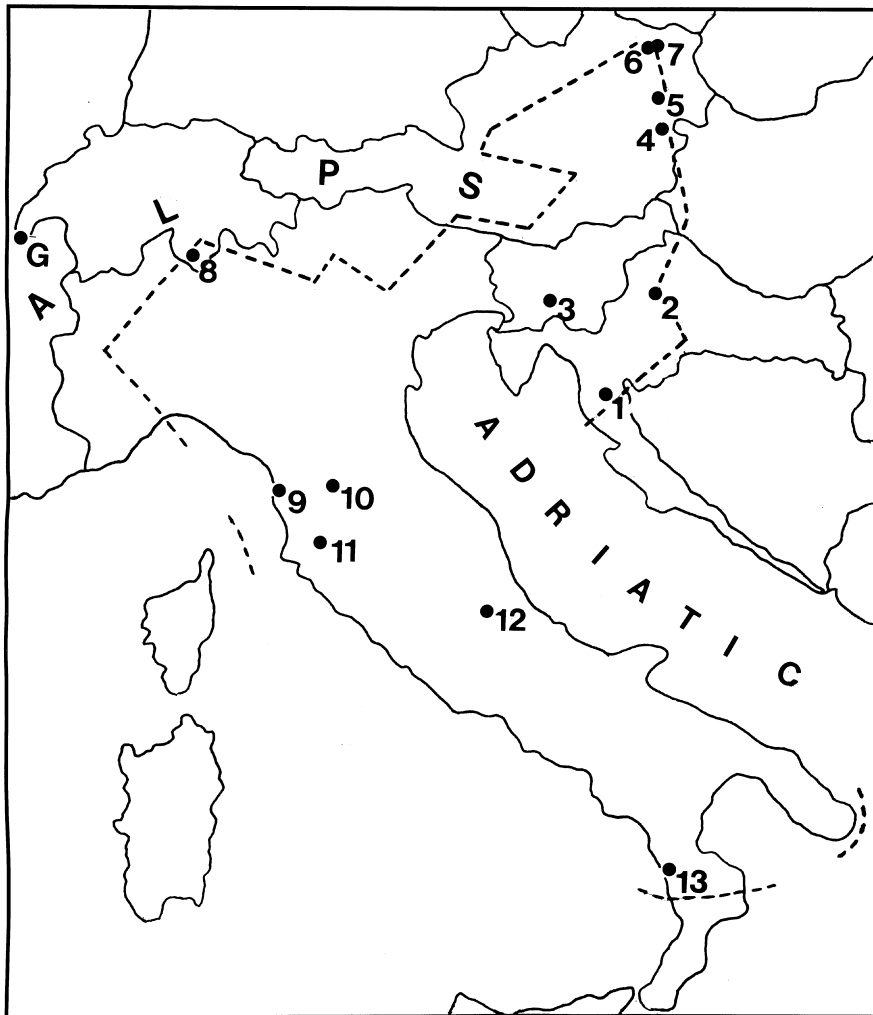


Figure 2. Sample localities of *T. c. carnifex* in Italy and adjacent countries: (1) Sinac and (2) Belovar Moravce, Croatia, (3) Kramplje, Slovenia, (4) Lackenback, (5) Haidlhof, (6) Kleinmeiseldorf and (7) Etzmannsdorf, Austria, (8) Locarno, Switzerland, (9) Pisa, (10) Florence, (11) Farma, (12) Bobinaco and (13) Fuscaldo, Italy; G = Geneva Basin, Switzerland and adjacent France (localities 7 and 17 in Arntzen and Thorpe 1999). The interrupted line connects records describing the edge of the subspecies range (from Arntzen, submitted). Note that locality 'Geneva' is north of the Alps and does not represent a natural occurrence of the taxon. For the distribution of related taxa see Arntzen (1995). The material studied and voucher information is listed in the Appendix.

A total of 27 protein systems representing 40 presumptive gene loci were studied by means of starch- and polyacrylamide-gel electrophoresis (Table 1). Laboratory and analytical protocols, genetic parameters evaluated ( $N_{\text{all}}$  – number of alleles per locus,  $H_e$  – heterozygosity expected under conditions of Hardy–Weinberg equilibrium,  $D$  – unbiased estimate of Nei’s genetic distance) and nomenclature of gene products were as described in Shaw and Prasad (1970), Harris and Hopkinson (1976), Swofford and Selander (1981) and applied by Arntzen and García-París (1995) and Arntzen et al. (1998). The genetic distance matrix was converted into a UPGMA-dendrogram, with jackknifing to evaluate the strength of support of the data to the topology (following Lanyon 1985). Neighbor joining clustering, in which the assumption of molecular change to be regular is relaxed, was also performed (PHYLIP; Felsenstein 1989).

*Table 1.* Electrophoretic conditions for 27 protein systems, corresponding to 40 loci, examined in three taxa of the *Triturus cristatus* superspecies. Starch buffers are A: Tris–citrate pH 6.0 (XIII); B: Tris–citrate pH 7.0 (I); C: Tris–citrate pH 8.0 (V); D: Lithium-hydroxide–Tris–citrate pH 8.1 (X); E: Tris–malate pH 7.4, electrode buffer is 0.22 M Tris, 0.10 M maleic acid, 0.01 M EDTA and 0.01 M MgCl<sub>2</sub>, gel buffer is electrode buffer diluted at 1: 10; F: Tris-EDTA-borate pH 8.9 (Ayala et al. 1972); G: Histidine-sodium-citrate pH 8.0 (Brewer 1970); H: discontinuous tris–citrate–borate pH 8.2–8.7 (Poulik 1957). Roman numerals refer to the buffer systems of Shaw and Prasad (1970). PAGE refers to acrylamide gels according to Maurer (1971; Table 4.1) for ‘general’ and plasma proteins (GP, ALB, TRF) and to Taggart et al. (1978) for Pepsinogen (PG). Tissues used are H: heart, L: liver, M: muscle, P: plasma, and S: stomach.

Protein	E. C. No.	Locus	Buffer system	Tissue extract
Acid phosphatase	3.1.3.2	<i>Acp-1, Acp-2</i>	A	L
Adenosine deaminase	3.5.4.4	<i>Ada</i>	F	L
Albumin	–	<i>Alb</i>	PAGE	P
Alcohol dehydrogenase	1.1.1.1	<i>Adh</i>	C	L
Aspartate transaminase	2.6.1.1	<i>Ata-1, Ata-2</i>	H	L
Esterase	3.1.1.-	<i>Est-1, Est-2</i>	C, D	L, P
General protein	–	<i>GP</i>	PAGE	M
Glucose dehydrogenase	1.1.1.47	<i>Gdh</i>	C	L
Glucose-6-phosphate dehydrogenase	1.1.1.49	<i>G-6-pd</i>	C	M
Glucose phosphate isomerase	5.3.1.9	<i>Gpi</i>	A	M
$\alpha$ -Glycerophosphate dehydrogenase	1.1.1.8	<i><math>\alpha</math>-Gly</i>	C	L
Isocitrate dehydrogenase	1.1.1.42	<i>Icd-1, Icd-2</i>	C	L
Lactate dehydrogenase	1.1.1.27	<i>Ldh-1, Ldh-2</i>	E	H, M
Leucine aminopeptidase	3.4.11.1	<i>Lap</i>	G	L
Malate dehydrogenase	1.1.1.37	<i>Mdh-1, Mdh-2</i>	B	M
Malic enzyme	1.1.1.40	<i>Me</i>	E	M
Mannose phosphate isomerase	5.3.1.8	<i>Mpi-1</i>	C	L
NADH dehydrogenase	1.6.99.2	<i>Nadh-1, Nadh-2</i>	H	L
Nucleoside phosphorylase	2.4.1.1	<i>Np</i>	G	L
Pepsine (Pepsinogen)	3.4.23.-	<i>Pg-1, Pg-2, Pg-3, Pg-4</i>	PAGE	S
Peptidase	3.4.11.-	<i>Pep-1, Pep-2</i>	D	S
Phosphoglucomutase	5.4.2.2	<i>Pgm-1, Pgm-2</i>	A	L
Phosphogluconate dehydrogenase	1.1.1.44	<i>6-Pgd</i>	C	M
Sorbitol dehydrogenase	1.1.1.14	<i>Sdh</i>	F	L
Superoxide dismutase	1.15.1.1	<i>Sod-1, Sod-2</i>	C	L
Transferrin	–	<i>Trf</i>	PAGE	P
Xanthine dehydrogenase	1.1.1.204	<i>Xdh</i>	C	L

Association between genetic distance and geographical distance of *T. c. carnifex* populations was tested with the Mantel-test (Manly 1996). Geographical distances were measured over land avoiding inhospitable terrain and as the crow flies, i.e., with and without considering barriers posed by the Adriatic Sea and the Alps.

Genetic similarity of individual newts was analyzed by principal coordinate analysis (PCA) with NTSYS 1.80 (Rohlf 1993). A binary data set was generated for each individual, in which the presence (1) or absence (0) of each allele at each locus was assumed to be independent (although in reality limited to a maximum of two scores of 1 per locus) and was defined as a separate character state. Homozygotes were not distinguished from heterozygotes (i.e., they were represented by a single score of 1). The subroutine SIMQUAL in NTSYS performs a comparison between all pairs of individuals. Jaccard's coefficient of association was chosen to represent the genetic similarity between individuals because this measure considers joint absences to be uninformative (Sokal and Sneath 1963). The subroutine DCENTER was used to transform the similarity matrix into scalar product form, after which it was factored using the subroutine EIGEN. Effects of a recent reduction in effective population size of the (introduced) Genevan population were tested for with the BOTTLENECK software (Cornuet and Luikart 1996; Piry et al. 1997).

## Results

The observed gene frequency data are presented in Table 2. The number of alleles ranged from 1 in the monomorphic loci *Ata-2*, *Gdh*, *Icd-1*, *Icd-2* and *Pgm-2*, to 8 at the *Gpi* locus. Loci monomorphic in *T. c. carnifex* were *Acp-1*, *Acp-2*, *Adh*, *Est-2*, *G-6-pd*,  $\alpha$ -*Gly*, *Ldh-2*, *Pg-2* and *Pg-3*. Heterozygosity (weighted mean across populations) was 0.050 for *T. cristatus*, 0.067 for *T. c. carnifex* and 0.091 for *T. c. macedonicus*, and 0.080 for the Genevan *T. carnifex*. Among *T. c. carnifex* populations  $H_e \pm SE$  ranged from  $0.022 \pm 0.013$  (Bobinaco) to  $0.105 \pm 0.029$  (Kramplje; Table 2). The genetic distance  $D \pm SE$  was  $0.10 \pm 0.05$  between *T. c. carnifex* and *T. c. macedonicus*,  $0.12 \pm 0.06$  between *T. c. carnifex* and *T. cristatus* and  $0.13 \pm 0.06$  between *T. c. macedonicus* and *T. cristatus*. The Genevan populations were genetically similar among themselves ( $D = 0.00 \pm 0.00$ ) and to *T. c. carnifex* ( $D = 0.04 \pm 0.03$ ) and dissimilar to *T. c. macedonicus* and *T. cristatus* ( $D = 0.16 \pm 0.07$ , respectively,  $D = 0.17 \pm 0.07$ ). Several groups were recognized in the UPGMA-dendrogram. Populations 3–12 formed a group at the level  $D = 0.06$ , representing all northwestern populations (Figure 3). The Genevan samples also fell into this group. The jack-knife test indicated that support for hierarchical relationships up to this level was mostly ambiguous. Population 13 from Calabria, southern Italy and populations 1 and 2 from Croatia clustered with the northwestern group at comparatively high levels ( $D = 0.10$ , respectively,  $D = 0.15$ ). The genetic differentiation within *T. c. carnifex* reached a level similar to that of inter(sub)specific comparisons (Figure 3).



<i>Gp</i>	d	0.20 c	c	0.75 c	0.50 c	0.13 c	0.33 c	0.41 c	0.90 c	0.35 c	0.25 c	0.80 c	0.25 c	0.846 c	0.993 c
<i>Gpi</i>	0.25 b	0.80 d	0.50 d	0.25 d	0.50 d	0.88 d	0.67 d	0.59 d	0.10 d	0.65 d	0.75 d	0.20 d	0.75 d	0.154 d	0.007 d
	0.75 e	0.26 b	e	e	e	e	0.08 b	e	0.50 e	0.65 e	0.75 e	e	e	0.016 a	0.010 c
	0.72 e	0.72 e	0.83 e	0.08 g	0.50 g	0.83 e	0.68 g	0.25 g	0.50 g	0.35 g	0.25 g	0.904 c	0.954 e	0.005 b	0.020 d
	0.02 h		0.08 g									0.074 e	0.003 g	0.904 c	0.003 c
<i>G-6-pd</i>	b	b	b	b	b	b	b	b	b	b	b	b	b	0.803 a	0.007 a
<i>α-Gly</i>	a	a	a	a	a	a	a	a	a	a	a	a	a	0.984 a	0.007 a
<i>Ldh-1</i>	c	0.02 b	c	c	c	c	c	0.18 a	0.70 c	0.70 b	0.50 b	0.20 b	0.63 b	0.835 b	0.003 b
	0.98 c	0.98 c						0.73 c	0.30 d	0.25 c	0.50 d	0.80 c	0.38 c	0.101 c	0.997 c
<i>Ldh-2</i>	d	d	d	d	d	d	d	0.09 d	d	d	d	d	d	0.064 d	0.023 b
														0.282 b	0.003 c
														0.718 d	0.974 d
<i>Lap</i>	b	b	b	b	b	b	b	0.91 b	b	b	b	b	b	0.989 b	0.003 a
								0.09 c						0.005 c	0.993 b
<i>Mdh-1</i>	b	b	b	b	b	0.13 a	b	b	b	0.95 b	b	b	0.88 b	0.005 d	0.003 d
	0.88 b	0.88 b	0.88 b	0.88 b	0.88 b	0.88 b	0.88 b	0.77 b	b	b	b	b	0.13 d	0.973 b	0.984 b
	0.13 c							0.23 d	b	b	b	b	b	0.027 d	0.016 d
<i>Me</i>	c	c	c	c	c	c	c	c	c	0.05 b	c	c	c	0.979 b	0.997 b
														0.021 c	0.003 c
														0.112 b	0.977 b
<i>Mpi</i>	c	0.02 b	c	c	c	c	c	0.18 b	c	c	c	c	c	0.851 c	0.013 c
		0.98 c						0.82 c						0.037 d	0.010 d
														0.005 a	c
														0.50 b	0.005 a
														0.037 b	c
														0.50 c	0.037 b
														0.957 c	0.957 c



<i>Pep-2</i>	0.13 a	0.02 a	b	b	b	b	b	b	b	b	b	b	b	b	0.50 a	0.005 a	0.082 a
	0.88 b	0.98 b	b	b	b	b	b	b	b	b	b	b	b	b	0.50 b	0.995 b	0.918 b
<i>Pgm-1</i>	b	b	b	b	b	b	b	b	0.75 b	b	b	b	b	b	0.016 a	0.984 b	b
	d	d	d	d	0.05 d	0.10 c	d	d	0.25 c	d	d	d	d	0.38 d	0.005 c	0.010 b	
<i>6-Pgd</i>					0.96 g	0.90 d								0.63 g	0.590 d	0.827 c	
															0.404 e	0.163 d	
<i>Sdh</i>	b	0.07 a	b	0.17 a	b	0.10 a	0.14 a	b	b	0.10 a	b	0.10 a	b	b	0.154 a	0.033 a	
		0.94 b		0.83 b		0.90 b	0.86 b			0.90 b					0.846 b	0.905 b	
<i>Sod-1</i>	0.50 a	0.59 a	b	0.08 a	b	b	b	0.10 a	b	b	b	b	b	b	0.016 a	0.010 a	
	0.50 b	0.41 b		0.92 b	0.25 a	0.17 a	b	0.10 a	b	0.90 b				0.984 b	0.990 b		
<i>Sod-2</i>	b	0.07 a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	
		0.94 b															
<i>Trf</i>	0.38 c	0.59 c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	0.007 b
	0.63 d	0.41 d															0.951 c
																	0.003 d
<i>Xdh</i>	0.25 b	0.04 b	c	b	0.50 c	0.38 b	b	0.55 b	c	c	0.15 b	c	0.40 b	b	c	0.085 b	0.007 b
	0.75 c	0.67 c			0.50 d	0.63 c		0.46 c			0.70 c		0.60 c			0.846 c	0.810 c
		0.28 d									0.15 d					0.069 d	0.183 d
Average $N_{\text{ait}}$	1.2	1.4		1.1	1.1	1.1	1.4	1.4	1.2	1.3	1.4	1.2	1.2	1.2			
SE	0.1	0.1		0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1			
$H_c \pm SE(\%)$	7.4	8.1		2.2	4.3	3.5	7.3	6.7	6.7	7.1	10.5	5.8	7.1	7.1			
SE	2.5	2.6		1.3	1.9	1.7	2.3	2.3	2.3	2.4	2.9	2.2	2.6	2.6			

Monomorphic loci are *Ata-2*, *Gdh*, *Icd-1*, *Icd-2*, *Pgm-2*.

Alleles not mentioned here are subject to a future publication on enzyme variation in the *T. cristatus* superspecies plus *T. marmoratus*.

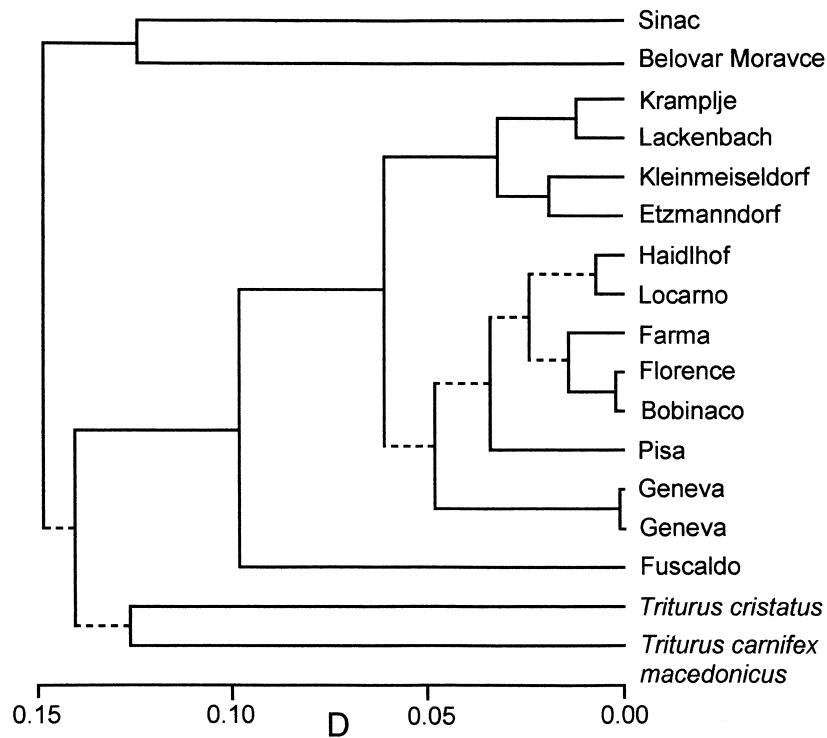


Figure 3. UPGMA-dendrogram for 13 native *T. c. carnifex* populations, two crested newt populations from Geneva, and related taxa, based upon Nei's genetic distance (*D*). Note that the Genevan populations are grouped with *T. c. carnifex*, confirming earlier identifications (Arntzen and Thorpe 1999).

In the neighbour joining dendrogram, both Genevan populations clustered with the population from Farma (results not shown).

Loci fully diagnostic for the three taxa were not observed. Some loci, however, were partially diagnostic: *T. cristatus* differed from *T. c. carnifex* on account of *Ata-1* and *Nadhdh-2* allele frequencies and from *T. c. macedonicus* on account of *Ldh-1*, *G-6-pd* and *Gpi*, and from both these taxa on account of *6-Pgd*, *Est-1* and *Me*. *T. c. carnifex* differed from *T. c. macedonicus* on account of the loci *G-6-pd*, *Gpi* and *Ata-1*. Loci discriminating *T. c. carnifex* from *T. c. macedonicus* and *T. cristatus* were also discriminating the Genevan populations from the latter two taxa. The *Ata-1* locus at which a single allele (*Ata-1<sup>b</sup>*) was shared at low frequency was especially informative (Table 2).

Character loadings derived from individual genetic profiles described three clusters of data points, corresponding to *a priori* defined groups, with no overlap between clusters across the first- and second-principal coordinate axis (Figure 4a). Taxon similarity, as judged from the position of the cluster centroids, was higher among *T. c. carnifex* and *T. c. macedonicus* than for either of these taxa and *T. cristatus*. Data points representing the Genevan newts all fell within the *T. c. carnifex* cluster. Their

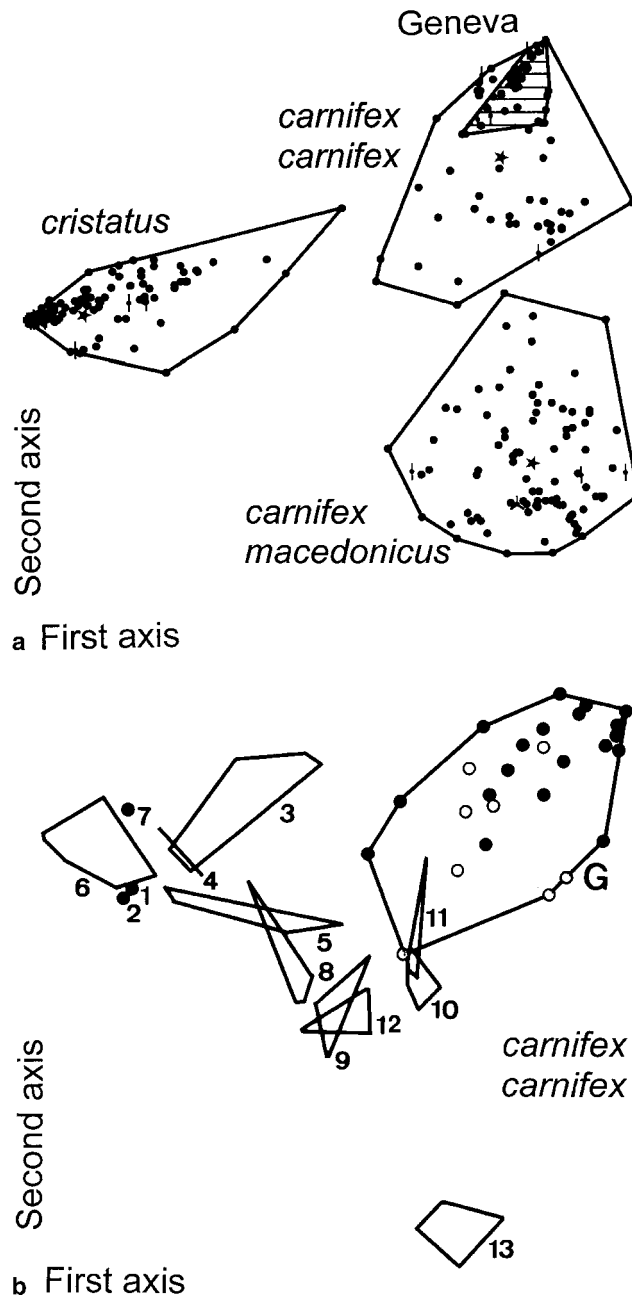


Figure 4. Bivariate scatterplot of the character loadings derived by principal coordinate analysis of individual allozyme profiles, with convex outline polygons for: (a) *T. cristatus*, *T. carnifex carnifex* and *T. carnifex macedonicus*; Taxon means indicated by asterisks. (b) *T. c. carnifex* (population numbers as in Figure 2). Individuals from Geneva with and without inferred 'hybrizyme' alleles are shown by solid and open dots, respectively; details see text.

similarity to *T. cristatus* and *T. c. macedonicus* was lower than that shown by the average *T. c. carnifex*. A similar analysis confined to *T. c. carnifex* revealed relatively tight clusters of data points, indicating contrasting levels of intra- and inter-population genetic variation (Figure 4b). Visual inspection of the data plot revealed: (i) that geographically adjacent populations tend to be genetically similar, (ii) a correspondence between genetic differentiation and geography along a north to south axis, and (iii) that the southernmost Italian population is distinct from other *T. c. carnifex*. Furthermore, the populations from Geneva: (iv) display an unusual amount of genetic variation, (v) are positioned away from the observed north to south axis, and (vi) are most similar to populations 10 (Firenze) and 11 (Farma) from Tuscany. Excluding small ( $N < 4$ ) and non-native samples, a significant association was observed between genetic distance with both measures of geographical distance (Mantel-test,  $P < 0.01$ ). Partial Mantel tests showed a strong association of genetic distance with the geographical distance that avoids barriers to dispersal ( $P < 0.01$ ) and not with 'as the crow flies' geographical distance ( $P > 0.05$ ). Excluding the southern Italian population on basis of the argument that it might represent a different taxon, the association between genetic distance and geographical distance remained significant ( $P < 0.01$ ).

Two near-diagnostic loci (*Ada* and *Pep-1*) contributed strongly to the distinctness of the southernmost Italian population. Additionally, three low frequency private alleles were observed at the loci *Alb<sup>b</sup>*, *Ldh-1<sup>a</sup>* and *Np<sup>a</sup>*. Loci diagnostic for the Genevan populations were not observed. However, unexpected alleles were found at some loci (*Gpi<sup>h</sup>*, *Mdh-2<sup>c</sup>*, *Sod-2<sup>a</sup>* and *Trf<sup>d</sup>*). Individuals possessing these alleles were placed further away from the north to south axis extracted by PCA than those without them (Figure 4b, *t*-test,  $P < 0.05$ ). Single private alleles were observed in two populations (*Mdh-1<sup>a</sup>* in Farma and *Pgm-1<sup>c</sup>* in Lackenbach). The comparative analysis of  $N_{all}$  and  $H_e$  did not reveal a significant effect of genetic bottlenecks in the Geneva populations (BOTTLENECK,  $P > 0.05$ ).

## Discussion

I observed a substantial amount of genetic differentiation within *T. c. carnifex*, describing a north to south clinal pattern of variation. The association of genetic distance with overland geographical distance indicated that isolation-by-distance was operating between populations. Populations representing the extreme northeastern and southern fringes of the subspecies range were different from more centrally located ones. Unfortunately, populations at the range edges were numerically (Croatia) and spatially (southern Italy) poorly sampled. The documentation of more or less abrupt changes in spatial genetic variation in *T. c. carnifex*, as observed in central Italy for the Italian newt, *T. boscai* (Ragghianti and Wake 1986) and in the northern Adriatic coastal area for the Italian treefrog, *Hyla intermedia* (Nascetti et al. 1995), awaits a

spatially detailed study. Comparison with recently published data indicates that genetic differentiation along a latitudinal axis is most pronounced at c. 42° N, in between Bobinaco (population 12 at 42°21' N) and three populations at up to 41°43' N (Lenola, Avezzano and Monte Sant' Angelo; Scillitani and Picariello 2000).

The genetic data indicate that the Genevan samples represent *T. c. carnifex*, confirming identifications based on morphology and three enzyme genetic markers (Arntzen and Thorpe 1999). Genetic variation within *T. c. carnifex* reaches a high level and is geographically structured. This demonstrates the dissimilarity of the Genevan sample with all but a few of the sampled natural populations. Their similarity to the Florence and Farma populations suggests that the source of the introduced population may have been in the Tuscany area. However, the genetic profiles are not identical. In particular, the high level of genetic variation of the Genevan population is surprising.

Hypotheses to explain the presence of particular alleles and comparatively high  $H_e$  in Genevan *T. c. carnifex* can be divided into two categories, involving and not involving the native *T. cristatus*, respectively. Firstly, the joint processes of mutation and drift may have increased genetic variation and differentiation. Genetic drift may have been considerable, especially if the source population was small. However, with under 30 generations and known low mutation rates for protein loci, the time span since introduction has been too short to generate an appreciable amount of new genetic variation. A more likely explanation in this category is that the Genevan *T. c. carnifex* were derived from an as yet unidentified, but genetically variable source population. Furthermore, the genetic diversity of founding populations will increase with the number of introductions (Wade and McCauley 1988; Whitlock and McCauley 1990) and it is conceivable that two (or more) *T. carnifex* introductions have taken place, prior to 1908 and in the post-war period, respectively (Arntzen and Thorpe 1999).

Secondly, the gene pool of Genevan *T. c. carnifex* may have been enriched through introgressive hybridization with *T. cristatus*. In its simple form this hypothesis has to be rejected for two reasons: (i) an extensive survey showed inter-specific gene flow to be limited in the Geneva Basin (Arntzen and Thorpe 1999) and (ii) contrary to expectations, Genevan *T. c. carnifex* are *less* similar to *T. cristatus* than are average *T. c. carnifex*. However, in many hybrid zones certain alleles have been observed that are absent in the parental taxa. Such unexpected alleles ('hybrizymes', Woodruff 1989), representing novel genetic variation, are common to hybrid zones (Barton and Hewitt 1985; Schilthuizen et al. 1999). The alleles *Gpi*<sup>h</sup>, *Mdh-2*<sup>c</sup>, *Sod-2*<sup>a</sup>, *Trf*<sup>d</sup> represent potential hybrizymes, because they were found in (Genevan) populations subject to hybridization and not in native *T. c. carnifex*. The presence of *Mdh-2*<sup>c</sup> and *Trf*<sup>d</sup> in closely related taxa would support the hypothesis that they represent ancient polymorphisms at their respective loci. However, while indeed present in sister-taxa, both alleles were found exclusively at the very edge of the *T. c. macedonicus* and *T. cristatus* ranges, in populations

known or assumed to be in genetic contact with yet other crested newt species (Table 2; Arntzen and Wallis 1999; J.W. Arntzen, unpublished data). I suggest that these alleles are genuine hybridzymes that originated in different combinations of hybridizing species ( $Mdh-2^c$  and  $Trf^d$  in *T. c. carnifex* × *T. cristatus* in the Geneva Basin, Table 2;  $Mdh-2^c$  in *T. c. macedonicus* × *T. karelinii* in Probištip and Stanišinci and in *T. cristatus* × *T. karelinii* in Milanovac and  $Trf^d$  in *T. cristatus* × *T. dobrogicus* in Virfuri, J.W. Arntzen, unpublished data; for localities see the Appendix, for detailed species distributions, see Arntzen and Wallis 1999). Hybridzymes have previously been observed within a single hybrid zone at distances much larger than dispersal distances, indicating not to represent common ancestry, but parallel evolution (Schilthuizen and Gittenberger 1994). Evidence is accumulating that their existence at detectable frequencies results from selective advantage (Bradley et al. 1993; Schilthuizen et al. 1999).

Colonization events, including man-assisted introductions, often entail severe reduction in population size and permanent isolation from a larger parental population. The genetic consequences of the founding event may include rapid alteration and loss of genetic variation (Nei et al. 1975; Watterson 1984). Both theoretical and experimental results emphasize the importance of propagule size and the rate of population growth (McCommas and Bryant 1990; Leberg 1992). The smaller the initial population and the longer it remains small, the more variation will be lost. However, the signature of a genetic bottleneck was not observed in the Genevan population. The main reasons for this observations could be: (i) lack of statistical power; (ii) the rapid growth of the introduced population directly after the passage through the bottleneck; (iii) a sufficient length of time since the introduction for a  $N_{all}-H$  disequilibrium to be restored, and (iv) added genetic variation (alleles) through introgressive hybridization and hybridzyme formation. Although a rapid population growth following introduction is plausible given the former presence of large *T. carnifex* populations in the area (Arntzen and Thorpe 1999), the mutation rate at protein loci and time since introduction are insufficient to explain the result. Unfortunately, the propagule size of Genevan *T. carnifex* is unknown and the introduction site not well documented, precluding further analysis.

The assignment of a sample to its source may be difficult when the level of variation is low, as often the case for spatial genetic variation at the protein level (Ward et al. 1992; Merilä et al. 1995). The spatial distribution of genetic variation in *T. c. carnifex* allowed the approximate identification of the geographical source of the non-native Genevan population. Precision and power gained through the use of hypervariable DNA markers (e.g., microsatellite loci – Hauser et al. 1998; Rowe et al. 1998) could be used to test the conclusions reached from the protein data. Other introductions of *T. carnifex* in São Miguel, Azores and Surrey, United Kingdom (Machado 1997; Brede et al. 2000) have not yet been subject to genetic analysis. It would be interesting to see whether these populations would have the same provenance as the Genevan population.

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## Appendix

Material studied, locality, sample size (*N*), site coordinates and voucher information (University of Amsterdam, ZMA Herp.).

*T. c. carnifex*: Austria: Etzmandorf, nr. Eggenburg (*N* = 1) (48°39' N, 15°45' E) (ZMA Herp. 9105), Haidlhof, nr. Baden (*N* = 5) (47°58' N, 16°10' E) (ZMA Herp. 9117), Kleinmeiseldorf (*N* = 11) (48°40' N, 15°45' E) (ZMA Herp. 9129), Lackenbach (*N* = 2) (47°36' N, 16°27' E) (ZMA Herp. 9133); Croatia: Belovar Moravce (*N* = 1) (45°51' N, 16°10' E) (ZMA Herp. 9089), Sinac (*N* = 1) (44°49' N, 15°22' E) (ZMA Herp. 9155); France: Basin of Geneva (*N* = 23) (46°15'30'' N, 6°18'10'' E, locality 16 in Arntzen and Thorpe 1999) (ZMA Herp. 9110); Italy: Bobinaco, nr. l'Aquila (*N* = 6) (42°21' N, 13°24' E) (ZMA Herp. 7553), Farma, Torniella (*N* = 4) (43°08' N, 11°10' E) (ZMA Herp. 9106), Firenze (*N* = 6) (43°47' N, 11°15' E) (ZMA Herp. 9107), Fuscaldò (*N* = 11) (39°25' N, 16°02' E) (ZMA Herp. 9108), Pisa, Italy (*N* = 4) (43°43' N, 10°24' E) (ZMA Herp. 9145); Slovenia: Kramplje, nr. Nova Vas (*N* = 10) (45°44' N, 14°30' E) (ZMA Herp. 9132); Switzerland: Locarno (*N* = 5) (46°10' N, 08°48' E) (ZMA Herp. 9136), Geneva (*N* = 4) (46°10'50'' N, 6°00'50'' E, locality 7 in Arntzen and Thorpe 1999) (ZMA Herp. 9109).

*T. carnifex macedonicus*: Bosnia-Herzegovina: Donja Čadjavica – Gornja Čadjavica (*N* = 2) (44°48' N, 19°02') (ZMA Herp. 9100), Gornja Čadjavica (*N* = 9) (44°45' N, 19°05' E) (ZMA Herp. 9112), Tavna Monastire (*N* = 9) (44°36' N, 19°04' E) (ZMA Herp. 9162), Višegrad (*N* = 15) (43°47' N, 19°20' E) (ZMA Herp. 9168); Greece: Ano Kaliniki (*N* = 11) (40°52' N, 21°26' E) (ZMA Herp. 9085), Livadia, Greece (*N* = 1) (41°17' N, 23°01' E) (no voucher); Macedonia: Probištip (*N* = 5) (41°59' N, 22°10' E) (ZMA Herp. 9147); F.R. Yugoslavia: Divčibare, (*N* = 11) (44°06' N, 19°56' E) (ZMA Herp. 9098), Grčak (*N* = 8) (43°28' N, 20°57' E) (ZMA Herp. 9114), Karan (*N* = 6) (43°54' N, 19°52' E) (ZMA Herp. 9122 and 9123), Karbinci (*N* = 1) (41°46' N, 22°14' E) (ZMA Herp. 9124), Lučane (*N* = 12) (42°25' N, 21°43' E) (ZMA Herp. 9137), Stanišinci (*N* = 4) (43°31' N, 20°53' E) (ZMA Herp. 9158).

*T. cristatus*: Austria: Ottenstein, nr. Zwettl (*N* = 1) (48°28' N, 14°17' E) (ZMA Herp. 9143); France: Mayenne (*N* = 49) (48°18' N, 00°37' W) (ZMA Herp. 8048), St Lô (*N* = 1) (49°07' N, 01°05' W) (no voucher); Switzerland: Biel (*N* = 5) (47°09' N, 07°16' E) (ZMA Herp. 8056 and 9091); Poland: Limanowa, (*N* = 10) (49°43' N, 20°25' E) (ZMA Herp. 9135); Romania: Cîmpeni (*N* = 10) (46°23' N, 23°05' E) (ZMA Herp. 9094), Šebiš (*N* = 1) (46°22' N, 22°06' E) (ZMA Herp. 9152), Sinaia (*N* = 10) (45°20' N, 25°33' E) (ZMA Herp. 9156), Tîrgoviste (*N* = 10) (44°56' N, 25°27' E) (ZMA Herp. 9163), Videle (*N* = 11) (44°16' N, 25°31' E) (ZMA Herp. 9166), Virfuri (*N* = 9) (46°17' N, 22°28' E) (ZMA Herp. 9167); United Kingdom: Canterbury (*N* = 5) (51°17' N, 01°05' E) (ZMA Herp. 9126), Peterborough (*N* = 5) (52°35' N, 00°15' W) (ZMA Herp. 9144); F.R. Yugoslavia: Bor (*N* = 4) (44°02' N, 22°08' E) (ZMA Herp. 9093), Jabukovac (*N* = 5) (44°20' N, 22°24' E) (ZMA Herp. 9119), Klokočevac (*N* = 5) (44°20' N, 21°12' E) (ZMA Herp. 9130), Milanovac (*N* = 7) (44°11' N, 21°36' E) (ZMA Herp. 9139), Štubik (*N* = 5) (44°16' N, 22°22' E) (ZMA Herp. 9159).

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