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GENETIC EVIDENCE FOR TWO DISTINCT SPECIES WITHIN THE ITALIAN ENDEMIC *SALAMANDRINA TERDIGITATA* (BONNATERRE, 1789) (AMPHIBIA: URODELA: SALAMANDRIDAE)

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Genetic variation in 12 populations of the Italian endemic spectacled salamander *Salamandrina terdigitata* was investigated through the analysis of 29 allozyme loci. Two well-differentiated population groups were identified, one ranging from the Tusco-Emilian Apennine to southern Latium, the other comprising populations from central Campania to Calabria. Nine diagnostic and four highly differentiated loci led to an average genetic distance of D_{Nei}=0.47 between the two groups, while within them D_{Nei} ranged from 0.00 to 0.05. The observed genetic structure strongly suggests that two distinct species have so far been included within *Salamandrina terdigitata*. The names *Salamandrina perspicillata* (Savi, 1821) and *S. terdigitata* (Bonnaterre, 1789) are here proposed for the species from central and southern Italy respectively.

Key words: Caudata, cryptic species, Italy, molecular taxonomy

INTRODUCTION

Since their emergence, biochemical and molecular techniques have allowed the study of the genetic structure of populations, providing evidence for the existence of cryptic biodiversity that was previously unsuspected. For amphibians, which are generally conservative in their morphological evolution (Cherty *et al.*, 1978; Hass *et al.*, 1995; Richards & Moore, 1996), the routine use of these tools has led to the identification of an astonishing number of morphologically "cryptic" species (e.g. Duellman, 1993; Nascetti *et al.*, 1996; Hanken, 1999; Frost, 2002), even in the well-studied European batrachofauna, as recently reviewed by Veith (1996) and Borkin (1999). In fact, the number of amphibian species recognized for the European area has almost doubled during the last four decades (Mertens & Wermuth, 1960; Frost, 2002), but it should be borne in mind that several species have not yet been investigated. Among these, *Salamandrina terdigitata* constitutes an interesting case study. It is a stream-breeding species endemic to peninsular Italy, mainly distributed on the western side of the Apennine chain from 200 m to 900 m a.s.l. (Lanza, 1983; Mazzotti *et al.*, 1999; Corsetti & Angelini, 2000; see Fig. 1). It is the only known representative of a genus which, according to Titus & Larson (1995), is an ancient lineage that separated from other newt lineages very shortly after the split between newts and true salamanders. The species is protected by international and regional laws (it is listed in Annexes II and IV of the EU Council Directive for the Conservation of Natural Habitats and of Wild Fauna and Flora), and it is of particular concern to Italian zoologists because it is the only Italian endemic terrestrial vertebrate genus (Lanza, 1988). In spite of this, its geographic variation and genetic population structure have been investigated only recently (Nascetti *et al.*, 2005). According mainly to mitochondrial DNA sequence data, that study suggested the existence of two distinct species within the spectacled salamander. However, the use of cytoplasmic markers (i.e. mitochondrial or chloroplast DNA) alone to recognize species has been criticized, mainly because in diverging populations they become reciprocally monophyletic much faster than even a single nuclear locus, and very much faster than a set of nuclear loci (e.g. Hudson & Coyne, 2002). Therefore, in this paper we provide further data on the genetic population structure of the spectacled salamander, as assessed by means of 29 nuclear (allozyme) loci.

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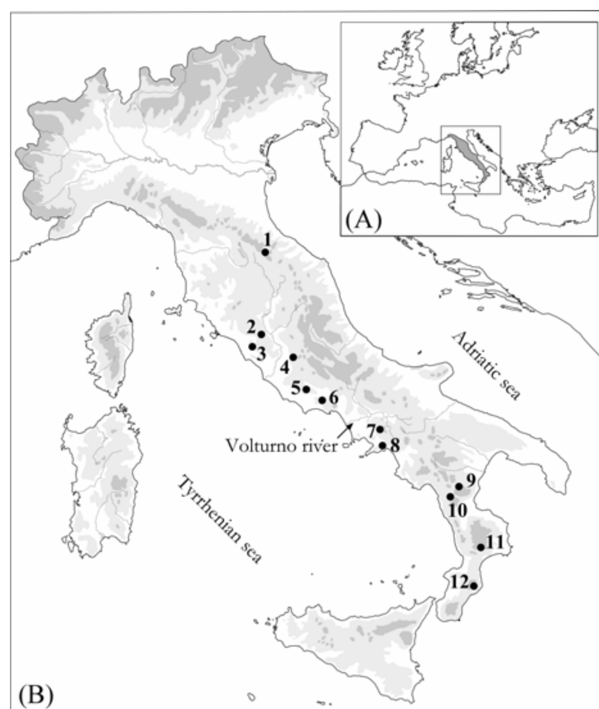

FIG. 1. Species range (A) and geographic location of the 12 populations of *S. terdigitata* sampled (B). Localities are numbered as in Table 1.

TABLE 1. Geographic origin and sample size (*n*) of the 12 populations of *S. terdigitata* studied.

Sample code	Locality	Altitude (m a.s.l.)	Region	*n*
1	Bagno di Romagna	460	Emilia-Romagna	20
2	Barbarano Romano	340	Latium	9
3	Tolfa	480	Latium	7
4	Percile	575	Latium	11
5	Bassiano	560	Latium	12
6	M.te San Biagio	140	Latium	10
7	Serino	630	Campania	18
8	Amalfi	15	Campania	12
9	S. Severino Lucano	880	Basilicata	14
10	Viggianello	560	Basilicata	8
11	Taverna	670	Calabria	18
12	Stilo	520	Calabria	10

MATERIALS AND METHODS

We collected 149 specimens of *Salamandrina terdigitata* from 12 populations covering almost the entire range of the species (Fig. 1). The geographical origin of the samples and sample sizes are presented in Table 1. Each specimen was anaesthetized in the field with 3-aminobenzoic acid ethyl ester (MS222) and tail-clipped (about 2 cm) before being released in the same place. Tail samples were transported to the laboratory in liquid nitrogen containers and stored at –80 °C until further analyses. In order to adjust technical procedures and score liver-active enzymes, five specimens from each sampling site were euthanased with an excess of MS222. Samples of skeletal muscle and liver were then obtained and stored at -80 °C.

Tissues from each specimen were crushed in 0.1 ml of distilled water and adsorbed onto chromatography paper labels. Horizontal electrophoresis was carried out onto 10% starch gels. We studied electrophoretically 20 enzymes encoded by 29 presumptive loci (see Table 2 for description of systems and electrophoretic conditions). Isozymes were numbered in order of decreasing mobility from the most anodal one (*Ldh-1* and *Ldh-2* correspond to *Ldh-A* and *Ldh-B* respectively). Alleles at each locus were designated by their mobility (in mm, standardized conditions) relative to the most common one (100) in the reference population (Taverna, Calabria).

TABLE 2. Enzymes studied in *S. terdigitata*, their commission number (EC), encoding loci, buffer systems and tissues used in electrophoresis (M = skeletal muscle, L = liver). Buffer systems: 1) Discontinous Tris/Citrate pH 8.7 (Poulik, 1957); 2) Continous Tris/Citrate pH 8.0 (Selander *et al*., 1971); 3) Tris/Versene/Borate pH 8.0 (Brewer & Sing, 1970); 4) Tris/Maleate pH 7.4 (Brewer & Sing, 1970); 5) Phosphate-Cytrate pH 6.3 (Harris, 1966); 6) Histidine/Citrate pH 7 (Cheliak & Pitel, 1984); 7) Lithium-borate pH 8.3 (Soltis *et al*., 1983).

Enzyme	EC	Encoding loci	Buffer systems	Tissue
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3pdh*	5	M
Lactate dehydrogenase	1.1.1.27	*Ldh-1*	4	M
		Ldh-2	4	M
Malate dehydrogenase	1.1.1.37	*Mdh-1*	5	M
Malate dehydrogenase (NADP⁺)	1.1.1.40	*Mdhp-1*	2,5	M, L
		Mdhp-2	2,5	M, L
Isocitrate dehydrogenase	1.1.1.42	*Icdh-1*	6	M
		Icdh-2	6	M
6-phosphogluconate dehydrogenase	1.1.1.44	*6Pgdh*	5	M
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6pdh*	1	M
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	*Gapdh*	6	M
Superoxide dismutase	1.15.1.1	*Sod-1*	3	M
Aspartate transaminase	2.6.1.1	*Aat-1*	5	L, M
		Aat-2	5	L, M
Alanine transaminase	2.6.1.2	*Alat*	2, 7	L
Creatine kinase	2.7.3.2	*Ck*	2	L
Adenylate kinase	2.7.4.3	*Adk*	2	L
L-leucylglycylglycine peptidase	3.4.13	*Pep-B1*	7	M, L
		Pep-B2	7	M, L
L-phenylalanyl-L-proline peptidase	3.4.13.9	*Pep-D1*	2, 7	M, L
		Pep-D2	2, 7	M, L
Carbonic anhydrase	4.2.1.1	*Ca-2*	3	M
Triose-phosphate isomerase	5.3.1.1	*Tpi*	2	L
Mannose phosphate isomerase	5.3.1.8	*Mpi*	3	M
Glucose phosphate isomerase	5.3.1.9	*Gpi*	3	M
Phosphoglucomutase	5.4.2.2	*Pgm-1*	4	M
		Pgm-2	4	M
		Pgm-3	4	M
		Pgm-4	4	M

TABLE 3. Allele frequencies and estimates of genetic variability at 16 polymorphic loci for the 12 populations sampled. P_{95}=percentage of polymorphic loci with the most common allele not exceeding 95%. *A*=mean number of alleles per locus. H_e= average expected heterozygosity assuming Hardy-Weinberg equilibrium. H_o=average observed heterozygosity. Standard errors in parentheses.

		Population											
Locus		1	2	3	4	5	6	7	8	9	10	11	12
Ldh1	100	0.79	0.83	0.90	0.95	0.93	1.00	0.17	-	0.23	0.06	0.97	0.80
	110	0.21	0.17	0.10	0.05	0.07	-	-	-	-	-	-	-
	112	-	-	-	-	-	-	0.83	1.00	0.77	0.94	0.03	0.20
Mdhp1	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	102	0.17	-	-	-	-	-	-	-	-	-	-	-
	104	0.83	1.00	1.00	1.00	0.36	0.25	-	-	-	-	-	-
	108	-	-	-	-	0.64	0.75	-	-	-	-	-	-
Mdhp2	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	112	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
Icdh2	88	1.00	1.00	1.00	1.00	1.00	1.00	-	-	0.27	0.42	0.50	0.44
	100	-	-	-	-	-	-	1.00	1.00	0.73	0.58	0.50	0.56
Gapdh	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	110	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
Aat1	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	120	0.50	1.00	0.88	1.00	1.00	0.25	-	-	-	-	-	-
	130	0.28	-	0.12	-	-	0.75	-	-	-	-	-	-
	140	0.22	-	-	-	-	-	-	-	-	-	-	-
Alat	70	0.10	-	-	-	-	-	-	-	-	-	-	-
	80	0.90	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
PepB1	95	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
PepD2	100	-	-	-	-	-	-	1.00	1.00	0.94	0.88	1.00	1.00
	105	0.17	-	-	0.06	-	-	-	-	-	-	-	-
	110	0.83	1.00	0.88	0.56	0.50	0.85	-	-	-	-	-	-
	115	-	-	-	-	-	-	-	-	0.06	0.12	-	-
	120	-	-	0.12	0.38	0.50	0.15	-	-	-	-	-	-
Ca2	90	-	-	-	-	-	-	-	-	-	0.19	0.25	0.40
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.81	0.75	0.60
Tpi	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	105	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-
Mpi	95	0.70	0.69	0.60	0.70	0.83	0.85	-	-	-	-	-	-
	100	0.30	0.31	0.40	0.30	0.17	0.15	1.00	1.00	1.00	1.00	1.00	0.95
	106	-	-	-	-	-	-	-	-	-	-	-	0.05
Gpi	95	0.15	0.44	0.30	0.23	0.29	0.25	-	-	-	-	-	-
	100	0.85	0.56	0.70	0.77	0.71	0.75	1.00	1.00	1.00	1.00	1.00	1.00
Pgm2	100	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	103	-	-	-	-	0.07	0.30	-	-	-	-	-	-
	106	1.00	1.00	1.00	1.00	0.93	0.70	-	-	-	-	-	-
Pgm3	90	-	-	-	-	-	-	-	-	-	-	0.03	-
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00
	108	-	-	-	-	-	-	-	-	-	-	0.03	-
Pgm4	90	-	-	-	-	-	-	-	-	-	-	0.04	-
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.92	1.00	0.89	0.95
	106	-	-	-	-	-	-	-	-	0.08	-	0.07	0.05
	P_{95}	24.1	10.3	17.2	10.3	20.7	20.7	3.4	0.0	13.8	13.8	13.8	17.2
	A	1.3	1.1	1.2	1.2	1.2	1.2	1.0	1.0	1.1	1.1	1.2	1.2
		(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.0)	(0.0)	(0.1)	(0.1)	(0.1)	(0.1)
	H_e	0.08	0.04	0.06	0.05	0.07	0.08	0.01	0.00	0.04	0.04	0.04	0.06
		(0.03)	(0.02)	(0.03)	(0.03)	(0.03)	(0.03)	(0.01)	(0.00)	(0.02)	(0.02)	(0.02)	(0.03)
	H_o	0.09	0.04	0.06	0.03	0.07	0.07	0.01	0.00	0.04	0.03	0.03	0.06
		(0.03)	(0.02)	(0.03)	(0.02)	(0.03)	(0.03)	(0.01)	(0.00)	(0.02)	(0.02)	(0.01)	(0.03)

Allele frequencies and estimates of genetic variability – mean observed and expected heterozygosity (H_o and H_e), percentage of polymorphic loci (P) and average number of alleles per locus – were calculated for each population using the software BIOSYS-2 (Swofford & Selander, 1999). Exact significance tests for Hardy-Weinberg equilibrium (HW) were conducted for each locus and sample, then the Bonferroni correction for multiple tests was applied (Rice, 1989).

Genetic distances between populations were calculated with Nei's (1972) standard genetic distance matrix, which was then used to build an UPGMA phenogram. We ran 1000 bootstrap pseudoreplicates over loci to test the reliability of the UPGMA phenogram with the BOOTDIST option in BIOSYS-2. The consensus UPGMA was then obtained using the subroutines NEIGHBOR and CONSENSE in the software PHYLIP 3.5c (Felsenstein, 1993). The cophenetic correlation coefficient, which measures the correlation between distance values calculated during tree building and the observed distance, was also computed. In addition, a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was carried out in order to partition total genetic variance into covariance components due to differences within populations, among populations within groups and between groups, using ARLEQUIN 2.000 (Schneider *et al.*, 1999).

RESULTS

Thirteen out of 29 loci analysed (*G3pdh, Ldh2, Mdh1, Icdh1, 6Pgdh, G6pdh, Sod1, Aat2, Ck, Adk, PepB2, PepD1,* and *Pgm1*) were monomorphic in all populations surveyed. Allele frequencies at the sixteen polymorphic loci are presented in Table 3. None of the tests for HW equilibrium was significant after Bonferroni correction. Based on allele frequencies at polymorphic loci, the samples can be classified into two well-differentiated groups, one comprising samples from the Tusco-Emilian Apennine to southern Latium (samples 1–6), and the other including those from central Campania to southern Calabria (samples 7–12). This subdivision is reflected by the two main clusters in the UPGMA phenogram showed in Fig. 2 (cophenetic correlation coefficient CCC=0.995). Nine loci (*Mdhp1, Mdhp2, Gapdh, Aat1, Alat, PepB1, PepD2, Tpi, Pgm2*) were fully diagnostic between both groups of populations, while at the other four loci (*Ldh1, Icdh2, Mpi, Gpi*) distinct alleles were found at moderate to high frequencies in only one of these groups (see Table 3). Genetic distances – D_{Nei} – between the two population groups ranged from 0.41 to 0.52, with an average value of 0.47 (SD=0.03), whereas within each of the two groups D_{Nei} varied from 0.00 to 0.05, with an average value of 0.02 (SD=0.02). Within the southern group, populations 11 and 12 (Calabria) were the most differentiated, with high bootstrap support (88%) in the UPGMA analysis and presenting an average genetic distance D_{Nei}=0.03 (SD=0.01) with respect to the other southern samples. When populations were grouped into the two main groups identified by the UPGMA analysis, the results of the AMOVA analysis (Table 4) indicated that up to 91% of the total genetic variability found in our dataset can be attributed to differences among groups.

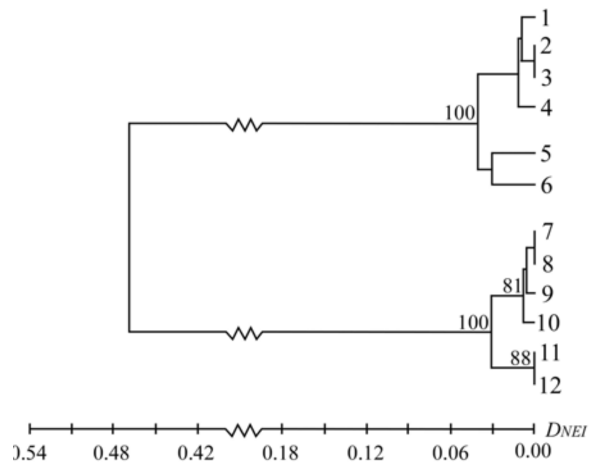

FIG. 2. UPGMA tree showing genetic relationships among the 12 populations of *S. terdigitata*, based on Nei's (1972) standard genetic distance values. Bootstrap values >70% over 1000 pseudoreplicates are indicated. Populations are numbered as in Table 1.

Estimates of genetic diversity are presented in Table 3. Expected heterozygosity varied from 0.00 to 0.08, with the highest values in populations from the central group (populations 1, 5 and 6). Within the southern group, the population from Amalfi completely lacks genetic diversity, and that from Serino was polymorphic at only one locus (*Ldh1*), with H_e=0.01. Other measures of genetic variability (observed heterozygosity, percentage of polymorphic loci and average number of alleles per locus) exhibited the same geographical pattern as H_e.

DISCUSSION

We observed a particularly significant pattern of genetic differentiation among the studied populations, mainly due to the existence of two well-defined clusters of closely related samples, one comprising populations from central Italy (samples 1 to 6), and the other those from southern Italy (samples 7 to 12). The pattern of genetic divergence between the two groups (leading to an average D_{Nei} of 0.47) was found to be unexpectedly high considering the restricted distribution of this species (it is an Apennine endemic), as well as its apparent morphological homogeneity. The level of divergence observed resembles that found between several congeneric species of salamanders (see, for example, Macgregor *et al.*, 1990; Lanza *et al.*, 1995; Nascetti *et al.*, 1996). When also considering the existence of nine fully diagnostic loci between the two groups, as well as the full concordance with the mitochondrial DNA data (Nascetti *et al.*, 2005), the observed overall genetic pattern indicates that the two well-differentiated

TABLE 4. AMOVA results for *S. terdigitata* data obtained using ARLEQUIN 2.000 (Schneider *et al.*, 1999). Groups were defined as the two main clusters identified by the UPGMA cluster analysis (Fig. 2).

Source of variation	Percentage of variation	Fixation indices	*P*-values
Among groups	91.38	$F_{ct} = 0.914$	<0.01
Among populations within groups	1.47	$F_{sc} = 0.171$	<0.01
Within populations	7.14	$F_{st} = 0.929$	<0.01

evolutionary lineages found within the spectacled salamander might actually represent two distinct species (see below).

Substantial genetic homogeneity was found within both population groups, notwithstanding the fact that within the southern group the populations from Calabria showed some distinctiveness according to the UPGMA analysis. For several populations, the levels of genetic diversity resemble the average values reported by Nevo & Beiles (1991) for representatives of the family Salamandridae (H_e=0.058; P=24.0%). However, the sample from Amalfi lacks genetic diversity at the studied loci, and the sample from Serino showed almost no genetic diversity. This could be a result of either human-driven depletion such as that caused by habitat destruction, population isolation, etc. (e.g. Haig, 1998), or the marginal position of these populations within the species range (Ledig, 1986), or from a combination of both factors. At this time we are unable to distinguish between these possible causes, even if the poor conservation status and fragmentation of the habitats in the underlying geographic area are likely to have played some role.

NOMENCLATURE DESIGNATION

As mentioned by Lanza (1988), the spectacled salamander was originally described by Lacépède (1788) with the name *Salamandra ter-digitata* on the basis of a single specimen collected by M. le Comte de Milly from Vesuvius. Fifteen years later, the species was also described (under the name *Salamandra tridactyla*) by Daudin (1803), who based his description on a specimen collected by De Nesle in the same locality. Later, another description was published by Savi (1821), who described the new species *Salamandra perspicillata* from cool and shady sites of Tuscan Apennine and particularly Mugello. He believed that he had found a new salamander species, because Lacépède's salamander was described as having four toes on the hind feet, but only three toes on the front feet. Later studies indicated that the two salamanders belonged to the same species, the original description by Lacépède being based on a poorly preserved specimen. Following the principle of priority of the International Code of Zoological Nomenclature, the species was given the name chosen by Lacépède, changed into *terdigitata* by removing the hyphen. Finally, Fitzinger (1826) proposed that the species belonged to a distinct monotypic genus, which he named *Salamandrina*. Other names have subsequently been proposed for the species, but they were all later synonymized (e.g. *Salamandra imperati* Costa, 1828; *Salamandra savi* Cuvier, 1829).

A recent decision of the International Commission on Zoological Nomenclature (Opinion 2104, based on the application of Savage, 2003) states that the work by Lacépède (1788) is no longer available as a source. As also pointed out by Savage (2003), this decision does not affect the name for *Salamandrina terdigitata* and other Latinized vernacular names from the Lacépède's (1788) work, because they "...were given proper binomials based on Lacépède's names in Bonnaterre's (1789–1790) binomial work". Nevertheless, this decision affect the authorship and year of the species' description. With the rejection of Lacepede's work, the correct author for *Salamandrina terdigitata* is now Bonnaterre (1789).

According to the principle of chronological priority and the geographic origin of our samples, we suggest that the name *Salamandrina perspicillata* (Savi, 1821) should be used for the species from central Italy, while the name *Salamandrina terdigitata* (Bonnaterre, 1789) should be retained for the southern species. However, this new nomenclatural arrangement will need to be further discussed, depending on the identity of the type specimens being confirmed. The analyses of type specimens will follow the completion of morphological analyses, now in progress, to identify potential morphological differences of diagnostic value between the two species. At present the two species can be diagnosed based on their genetic divergence, as assessed at diagnostic allozyme loci (*Mdhp1, Mdhp2, Gapdh, Aat1, Alat, PepB1, PepD2, Tpi, Pgm2*) and mitochondrial DNA sequences (Nascetti *et al.*, 2005). Moreover, the geographic origin of specimens is also of diagnostic value, although the species assignment of populations from the central portion of the Volturno river drainage basin still needs to be assessed (see Fig. 1).

CONCLUSIONS

The main outcome of this study has been the recognition of two distinct species within the Italian endemic *Salamandrina terdigitata*. Future efforts will be focused on two main fields: morphological variation, and genetic analyses of intermediate populations.

In many amphibians, previously undescribed morphological variation has often been assessed after species recognition in genetic studies (e.g. Nascetti *et al.*, 1988; Nascetti *et al.*, 1995). Since no studies to date have investigated morphological variation within the spectacled salamander, future studies will attempt to identify potential differences of diagnostic value between *S. terdigitata* and *S. perspicillata* at the level of chromatic, morphological and osteological characters.

However, preliminary observations suggest that some chromatic differences do exist between the species. The ventral surface of the tail exhibits a bright red in *S. terdigitata*, whereas it looks reddish to brownish-orange in *S. perspicillata*. In addition, the demarcation between the dorsal and ventral coloration of the tail is sharper in *S. terdigitata* than in *S. perspicillata*.

With respect to genetic studies, further sampling will be focused on potential contact zones, such as mid-altitude areas of the Volturno river drainage basin (see Fig. 1). The main objectives of the study of these populations will be: (1) to define the reciprocal distribution of the two species (i.e. if allopatric or parapatric); (2) to ascertain whether a contact zone does exist and, if so, to delimit it and to address what kind of genetic and ecological interactions are occurring between both species in this area.

Finally, the split of the former *S. terdigitata* into two distinct species with restricted geographical distributions strongly suggests the necessity for a careful evaluation of their conservation status, as well as the relevance of including them in the main international lists of threatened species, such as the IUCN Red List.

NOTE ADDED IN PROOF

Recently, a further paper has also been published describing mitochondrial DNA variation within the spectacled salamander (Mattoccia *et al*., 2005). Since the data presented within this paper substantially confirms the previous findings of Nascetti *et al*. (2005), they will not be further discussed here.

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REFERENCES

Bonnaterre, P.-J. A. (1789). *Erpétologie*. xxviii, 71 pp. Paris: Panckoucke Lib.

Borkin, L. J. (1999). Distribution of amphibians in North Africa, Europe, Western Asia, and the former Soviet Union. In: *Patterns of Distribution of Amphibians: A Global Perspective*, 329–420. Duellmann, W. E. (Ed.). Baltimore: The John Hopkins University Press.

Brewer, G. J. & Sing, C. F. (1970). *An Introduction to Isozyme Techniques*. New York & London: Academic Press.

Cheliak, W. M. & Pitel, J. A. (1984). *Techniques for Starch Gel Electrophoresis of Enzymes from Forest Tree Species*. Petawawa National Forestry Institute, Information Report P1-X-42. Chalk River, Ontario: Canadian Forestry Service, Agriculture.

Cherty, L. M., Case, S. M. & Wilson, A. C. (1978). Frog perspective on the morphological difference between humans and chimpanzees. *Science* **200**, 209–211.

Corsetti, L. & Angelini, C. (2000). *Salamandrina terdigitata*. In *Anfibi e Rettili del Lazio*, 38–39. Bologna, M. A., Capula, M. & Carpaneto, G. M. (Eds.). Roma: Palombi editore.

Daudin, F. M. (1803). *Histoire Naturelle, Générale et Particulière des Reptiles*, vol. VIII. Paris: de l'Imprimerie de F. Dufart.

Duellman, W. E. (1993). *Amphibian Species of the World: Additions and Corrections*. Lawrence, KS: University of Kansas Printing Service.

Excoffier, L., Smouse, P. E. & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479–491.

Felsenstein, J. (1993). *PHYLIP (Phylogenetic Inference Package) and Manual, version 3.5c*. Seattle: Department of Genetics, University of Washington.

Fitzinger, L. J. (1826). *Neue Classification der Reptilien nach ihren natürlichen Verwandschaften nebst einer Verwandtschaften-Tafel und einem Verzeichnisse der Reptilien-Sammlung des K. K. Zoologischen Museums zu Wien*. Vienna: J. G. Huebner.

Frost, D. R. (2002). *Amphibian Species of the World: An Online Reference (V2.21)*. Electronic database available at http://research.amnh.org/herpetology/amphibia /index.html.

Haig, S. M. (1998). Molecular contributions to conservation. *Ecology* **79**, 413-425.

Hanken, J. (1999). Why are there so many new amphibian species when amphibians are declining? *Trends in Ecology and Evolution* **14**, 7-8.

Harris, H. (1966). Enzyme polymorphism in man. *Proceeding of the Royal Society London, Series B* **164**, 298–310.

Hass, C. A., Dunski, J. F. & Maxson, L. R. (1995). Divergent lineages within the *Bufo margaritifera* complex (Amphibia: Anura; Bufonidae) revealed by albumin immunology. *Biotropica* **27**, 238–249.

Hudson, R. D. & Coyne, J. A. (2002). Mathematical consequences of the genealogical species concept. *Evolution* **56**, 1557–1565.

Lacépède, B. G. E. (1788). *Histoire naturelle des quadrupèdes ovipares et des serpens*, Vol. 1. Paris: Imprimerie du Roi (Hôtel de Thou).

Lanza, B. (1983). Anfibi e Rettili. *Guide per il Riconoscimento delle Specie Animali delle Acque Interne Italiane* **27**, 64–70.

Lanza, B. (1988). *Salamandrina terdigitata* (Lacépède, 1788): emblem of the Unione Zoologica Italiana. *Bollettino di Zoologia* **55**, 1–4.

Lanza, B., Caputo, V., Nascetti, G. & Bullini, L. (1995). Morphologic and genetic studies of the European plethodontid salamanders: taxonomic inferences (genus *Hydromantes*). *Monografie* **XVI**, Museo Reg. Sc. Nat., Torino.

Ledig, F. T. (1986). Heterozygosity, heterosis, and fitness in outbreeding plants. In C*onservation Biology: The Science of Scarcity and Diversity*, 77–104. Soulé, M. E. (Ed.). Sunderland, MA: Sinauer Associates.

Macgregor, H. C., Sessions, S. K. & Arntzen, J. W. (1990). An integrative analysis of phylogenetic relationships among newts of the genus *Triturus* (family Salamandridae), using comparative biochemistry, cytogenetics and reproductive interactions. *Journal of Evolutionary Biology* **3**, 329–373.

Mattoccia, M., Romano, A. & Sbordoni, V. (2005). Mitochondrial DNA sequence analysis of the spectacled salamander, *Salamandrina terdigitata* (Urodela: Salamandridae), supports the existence of two distinct species. *Zootaxa* **995,** 1-19.

Mazzotti, S., Caramori, G. & Barbieri, C. (1999). Atlante degli anfibi e dei rettili dell'Emilia-Romagna (aggiornamento 1993/1997). *Quaderni della Stazione Ecologica del Museo Civico di Storia Naturale di Ferrara* **12**, 1–121.

Mertens, R. & Wermuth, H. (1960). *Die Amphibien und Reptilien Europas* (Dritte Liste, nach dem Stand vom 1. Januar 1960). Frankfurt/M: Kramer.

Nascetti, G., Androne, F., Capula, M. & Bullini, L. (1988). A new *Salamandra* species from southwestern Alps (Amphibia, Urodela, Salamandridae). *Bollettino del Museo Regionale di Scienza Naturali di Torino* **6**, 617-638.

Nascetti, G., Lanza, B. & Bullini, L. (1995). Genetic data support the specific status of the Italian treefrog (Amphibia: Anura: Hylidae). *Amphibia-Reptilia* **16**, 215–227.

Nascetti, G., Cimmaruta, R., Lanza, B. & Bullini, L. (1996). Molecular taxonomy of European plethodontid salamanders (Genus *Hydromantes*). *Journal of Herpetology* **30**, 161–183.

Nascetti, G., Zangari, F. & Canestrelli, D. (2005). The spectacled salamanders, *Salamandrina terdigitata* Lacépède, 1788 and *S. perspicillata* Savi, 1821: 1) genetic differentiation and evolutionary history. *Rendiconti Lincei: Scienze Fisiche e Naturali* **16**, 159–169.

Nei, M. (1972). Genetic distance between populations. *The American Naturalist* **106**, 283–292.

Nevo, E. & Beiles, A. (1991). Genetic diversity and ecological heterogeneity in amphibian evolution. *Copeia* **1991**, 565–592.

Poulik, M. D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. *Nature* **180**, 1477.

Rice, W. R. (1989). Analysing tables of statistical tests. *Evolution* **43**, 223–225.

Richards, C. M. & Moore, W. S. (1996). A phylogeny for the African treefrog family Hyperoliidae based on mitochondrial DNA. *Molecular Phylogenetics and Evolution* **5**, 522–532.

Savage, J. M. (2003). Case 3226. Lacépède, B. G. E. de la V., 1788, Histoire Naturelle des Quadrupèdes Ovipares: proposed rejection as a non-binominal work. *Bulletin of Zoological Nomenclature* **60**, 138–140.

Savi, P. (1821). Descrizione (inedita) di una nuova specie di Salamandra terrestre, *Salamandra perspicillata* Nob. *Biblioteca italiana (Giornale di Letteratura, Scienze ed Arti)* **22**, 228–230.

Schneider, S., Roessli, D. & Excoffier, L. (1999). *ARLEQUIN ver 2.0: Software for Population Genetic Data Analysis.* Geneva, Switzerland: University of Geneva Genetics and Biometry Laboratory.

Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E. & Gentry, J. B. (1971). Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse. University of Texas Publications **7103**, 49–90.

Soltis, D. E., Haufler, C. H., Darrow, D. C. & Gastony, G. J. (1983). Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *American Fern Journal* **73**, 9–27.

Swofford, D. L. & Selander, R. B. (1999). *BIOSYS-2: A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics (Release 2.0).* Urbana-Champaign, IL: University of Illinois.

Titus, T. A. & Larson, A. (1995). A molecular phylogenetic perspective on the evolutionary radiation of the salamander family Salamandridae. *Systematic Biology* **44**, 125–151.

Veith, M. (1996). Molecular markers and species delimitation: examples from the European batrachofauna. *Amphibia–Reptilia* **17**, 303–314.

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