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## Italian Journal of Zoology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t741771159>

### Distribution and morphological characterization of the endemic Italian salamanders *Salamandrina perspicillata* (Savi, 1821) and *S. terdigitata* (Bonnaterre, 1789) (Caudata: Salamandridae)

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First published on: 10 November 2009

**To cite this Article** Romano, A., Mattoccia, M., Marta, S., Bogaerts, S., Pasmans, F. and Sbordoni, V. (2009) 'Distribution and morphological characterization of the endemic Italian salamanders *Salamandrina perspicillata* (Savi, 1821) and *S. terdigitata* (Bonnaterre, 1789) (Caudata: Salamandridae)', *Italian Journal of Zoology*, First published on: 10 November 2009 (iFirst)

**To link to this Article:** DOI: 10.1080/11250000802623995

**URL:** <http://dx.doi.org/10.1080/11250000802623995>

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## Distribution and morphological characterization of the endemic Italian salamanders *Salamandrina perspicillata* (Savi, 1821) and *S. terdigitata* (Bonnaterre, 1789) (Caudata: Salamandridae)

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(Received 8 May 2008; in final form 30 October 2008)

### Abstract

The Italian endemic genus *Salamandrina* was recently determined to be polytypic since two species, *Salamandrina perspicillata* and *S. terdigitata*, were recognized using both mitochondrial and nuclear genetic markers. However, data on their range is very scanty and no morphological traits are available to distinguish between these two taxa. A thorough field survey was carried out to detect and to sample new sites in areas in which *Salamandrina* remained unrecorded and to sample populations expected to occur in the documented range. Specimens were analyzed for variation of three mitochondrial DNA partial sequences of 12S and 16S ribosomal RNAs and cytochrome *b* genes. Attribution of individuals to either of the two species was therefore made on the basis of their haplotypes. Here we provide relevant information on the distribution of the two species, which were also found in areas where *Salamandrina* had previously been unrecorded, i.e. in the eastern Basilicata, in the region of Apulia and within the wide area which separated the two species. In the latter zone, a small area of sympatry between the two species was found. Furthermore, we analyzed the dorsal coloration pattern and size-related characters of the two species using Classification and Regression Tree analysis (CART), the results of which provided the first account of the morphological differences between *Salamandrina perspicillata* and *S. terdigitata*. The southern species, *S. terdigitata*, is smaller in size than *S. perspicillata* and differs in its coloration, mainly due to the red color on the dorsal surface of its tail.

**Keywords:** *Salamandrina*, distribution, contact zone, dorsal pattern, morphometry

### Introduction

The endemic Italian genus *Salamandrina* Fitzinger, 1826 ranges from the northern Apennines to the tip of Calabria. The majority of records are from water catchments on the western side of the Apennines that are tributaries to the Tyrrhenian Sea, while fewer records are available from those on the eastern side that flow toward the Adriatic Sea. Distribution data are rather sporadic, particularly in Molise, Basilicata and northern Campania, and no record is available for Sicily, Sardinia and Apulia (Barbieri & Pellegrini 2006; Angelini et al. 2007).

Within the genus *Salamandrina*, two species, the Southern spectacled salamander *S. terdigitata*

(Bonnaterre, 1789) and the Northern spectacled salamander *S. perspicillata* (Savi, 1821), have been recognized using both mtDNA and nuclear markers (Mattoccia et al. 2005; Nascetti et al. 2005; Canestrelli et al. 2006). However, in these papers a restricted number of samples were analyzed. All samples came from the Tyrrhenian side and there was a gap of 80–100 km between the nearest sample sites (Mattoccia et al. 2005).

In spite of the remarkable genetic divergence between the two species, no distinguishing morphological characteristics have so far been established. Indeed, a remarkable morphological homogeneity between the southern and the northern populations of the genus *Salamandrina* was previously supposed

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(e.g. Vanni 1980). This belief had probably hindered further taxonomic examination by means of genetic markers, which have been widely employed in other Italian amphibians. To clarify some open questions, we performed further studies (i) to determine the distribution of *S. perspicillata* and *S. terdigitata*; (ii) to check the possible occurrence of sympatry and syntopy between species; and (iii) to disclose any morphological differences that would distinguish between *S. perspicillata* and *S. terdigitata*.

## Materials and methods

### Distribution

Field surveys were carried out from 2005 to 2007 to detect and sample new sites in areas in which *Salamandrina* remained unrecorded, and to sample populations occurring within the known range.

Potentially suitable habitats (mainly small streams with arboreal canopy cover) were searched using IGM maps (maps of the Istituto Geografico Militare, scale 1:25,000) and corresponding orthophotos (available from <http://www.pcn.minambiente.it/PCN/>). Salamanders were searched for under stones, litter and moss; larvae and spawning females were searched for in the water. Further useful information was obtained by local people (mainly from shepherds). In total, 65 localities were inspected and salamander tail tips were collected from 56 sampling sites (Table I and Figure 1). The geographical distribution of each species was inferred by analysis of the mtDNA sequences of 213 specimens. Tissue samples were examined for sequence variation of three mitochondrial DNA genes encoding the 12S and 16S ribosomal RNAs and cytochrome *b*. The final length of the alignment was 1345 bp (a 370 bp fragment of the 12S gene, 552 bp of the 16S gene and 423 bp of the *cytb* gene). The tissue sampling, DNA sources, PCR amplification and sequencing protocols are as in Mattoccia et al. (2005). Details on molecular diversity, phylogeographic structure and evolutionary history reconstruction of the two species are available in Mattoccia et al. (in prep.) and in Romano (2007).

### Comparative study of the dorsal pattern and morphometry

*Salamandrina* is a tiny salamander (8–9 cm; Zuffi 1999) with a white, whitish or grayish ventral region with dark gray to black spots. The underside of the tail and the feet, and frequently the distal part of the belly, are bright red. *Salamandrina* is usually deep brown or gray-blackish on the dorsal side of the body

and tail. The tail may be partially painted of reddish color on the dorsal side, as well. On the head, a V-shaped, more or less obvious, whitish or yellowish patch between the eyes, which thus forms a sort of “spectacles”, is the origin of the common name, Spectacled salamander.

We studied the variability in the dorsal chromatic patterns and in the morphometric features to disclose any differences that might distinguish between *S. perspicillata* and *S. terdigitata*. During the sampling, while the results of mtDNA analysis clarified the distributions of the two species, our field observations suggested that central–northern salamanders and southern ones could differ in their dorsal coloration and size. It was only from then onwards that the morphological features were also measured for each individual collected. For this purpose, genetic analyses and coloration and morphological analyses were performed on the same specimens, only for a very small subsample.

The salamanders for which morphological data were collected were divided into two different groups corresponding to the two species according to the geographical distributions inferred by the results of the mtDNA analyses. To avoid bias, we excluded from the morphological analyses all specimens originating from a wide zone located in the surroundings of the contact zone discovered between the species. Thus we could be sure that the specimens analyzed were either *S. perspicillata* or *S. terdigitata*.

Live animals were photographed in the field with a digital camera situated perpendicularly to the dorsal surface of the animal, a method often used to compare the dorsal pattern in salamanders (e.g. Bosch & López-Bueis 1994). Digital photographs of salamanders were imported into the ScionImage® software program to measure three features of the chromatic dorsal patterns (Figure 2):

- (i) the red color proportion on the dorsal and lateral sides of the tail (TAC) was calculated as the ratio between the extension of red on the tail (RTL) and the total tail length (TTL). Zero value was assigned to individuals with a completely black tail;
- (ii) the spectacle proportion (SP) was calculated as the ratio between the light area of the spectacles (SS) and the head surface, including both the spectacles and the black snout area in front of the spectacles (BS). The blackish spots included on the spectacles perimeter were added to the black area for the calculations. Zero value was assigned to individuals without spectacles;
- (iii) the reddish median dorsal line on the body (BDL) was evaluated as either “present” or “absent”.

Table I. Sampling localities and numbers of salamanders used in this study to establish the distribution of *Salamandrina terdigitata* and *S. perspicillata*.

Code	Sampling site		N
	Region, Province, Municipality		
1	Liguria, Genova, Genova		5
2	Liguria, Genova, Lorsica		3
3	Emilia Romagna, Bologna, Casalecchio di Reno		5
4	Emilia Romagna, Forlì, Bagno di Romagna		4
5	Tuscany, Lucca, Stazzema		4
6	Tuscany, Lucca, Stazzema		5
7	Marche, Macerata, Visso		2
8	Latium, Viterbo, Canino		2
9	Umbria, Perugia, Piegara		3
10	Umbria, Terni, Terni		6
11	Latium, Rieti, Poggio Catino		5
12	Latium, Roma, Cesano		2
13	Latium, Roma, Jenne		3
14	Abruzzi, Chieti, Fara San Martino		2
15	Abruzzi, Chieti, Palena		2
16	Latium, Roma, Ciciliano		4
17	Abruzzi, Chieti, Rosello		6
18	Molise, Campobasso, Casacalenda		6
19	Campania, Caserta, Castello del Matese		6
20	Apulia, Foggia, San Marco La Catola		7
21	Campania, Benevento, San Bartolomeo in Galdo		8
22	Molise, Campobasso, Guardiaregia		6
23	Molise, Campobasso, Guardiaregia		1
24	Molise, Campobasso, Guardiaregia		2
25	Latium, Latina, Roccamassima		4
26	Latium, Latina, Cisterna di Latina		7
27	Latium, Latina, Norma		3
28	Latium, Roma, Gorga		3
29	Latium, Roma, Gorga		6
30	Latium, Frosinone, Supino		4
31	Latium, Latina, Patrica		3
32	Latium, Latina, Roccagorga		2
33	Latium, Latina, Maenza		3
34	Latium, Latina, Monte San Biagio		3
35	Latium, Latina, Monte San Biagio		2
36	Latium, Latina, Itri		5
37	Latium, Frosinone, Esperia		1
38	Latium, Frosinone, San Biagio Saracinisco		2
39	Campania, Caserta, Mondragone		7
40	Campania, Caserta, Caserta		5
41	Campania, Benevento, Cusano Mutri		5
42	Campania, Benevento, Morcone		6
43	Campania, Benevento, Buonalbergo		4
44	Campania, Avellino, San Martino Valle Caudina		4
45	Campania, Salerno, Acerno		1
46	Campania, Salerno, Cava de' tirreni		4
47	Basilicata, Potenza, Rionero in Vulture		3
48	Basilicata, Potenza, Rionero in Vulture		2
49	Basilicata, Matera, Accettura		2
50	Basilicata, Matera, Cirigliano		1
51	Basilicata, Potenza, Calvello		2
52	Basilicata, Potenza, San Severino Lucano		5
53	Calabria, Cosenza, San Sosti		4
54	Calabria, Vibo Valentia, Serra San Bruno		3
55	Calabria, Reggio Calabria, San Luca		5
56	Calabria, Reggio Calabria, Samo		3

The occurrence of any interrupted red line (i.e. reduced to a series of aligned red points or segments) was evaluated as “presence of BDL”.

The total length (TOTL: distance from the tip of the snout to the tail tip) was measured in the field on living specimens. We also measured the distance from the tip of the snout to the anterior end of the vent (SVL). The difference between TOTL and SVL was the tail length (TL). All variables were measured to 0.1 mm with a dial caliper. Because TOTL and SVL are not fully independent, we performed the analyses using only one of them (TOTL) and the ratio (TL/SVL). TOTL was used because preliminary analyses suggested that this character was a better discriminator between species than SVL (data not shown).

Coloration and morphometry were measured in 167 salamanders. Based on their distinguishing haplotypes or on the geographic location of their sampling sites, 75 individuals were attributed to *S. terdigitata* and 92 were attributed to *S. perspicillata* (Table II). The Classification Tree (CT) was used to identify the most important variables (predictors) for distinguishing between species. The algorithm used here for constructing the CT was the algorithm used for the classification and regression trees (CART) developed by Brieman et al. (1984). The CART-style exhaustive search for univariate splits is nonparametric, therefore numerical data can be analyzed without transformations and it is not influenced by outliers (e.g. Brieman et al. 1984; De'ath & Fabricius 2000; Vayssie'res et al. 2000). This analysis is a powerful method for splitting the data into conditional subsets that best explain the variation in the response variable; that is, it works by dividing the data at the point that maximizes the homogeneity of the dependent variable in the resulting two groups (e.g. De'ath & Fabricius 2000). The CART algorithm uses the Gini index (which is the Gini coefficient, a measure of statistical dispersion, expressed as a percentage), as its splitting criterion (Brieman et al. 1984). If not stopped, the tree algorithm will ultimately extract all information from the data, including information that is not and cannot be predicted in the population with the current set of predictors. The general approach to addressing this issue is first to stop generating new split nodes when subsequent splits only result in very little overall improvement of the prediction. Pruning of the misclassification error was selected as a stopping rule and, therefore, minimal cost-complexity cross-validation pruning was performed. Pruning of the misclassification error uses the costs as a measure of the prediction error. These costs correspond to the proportion of misclassified cases,

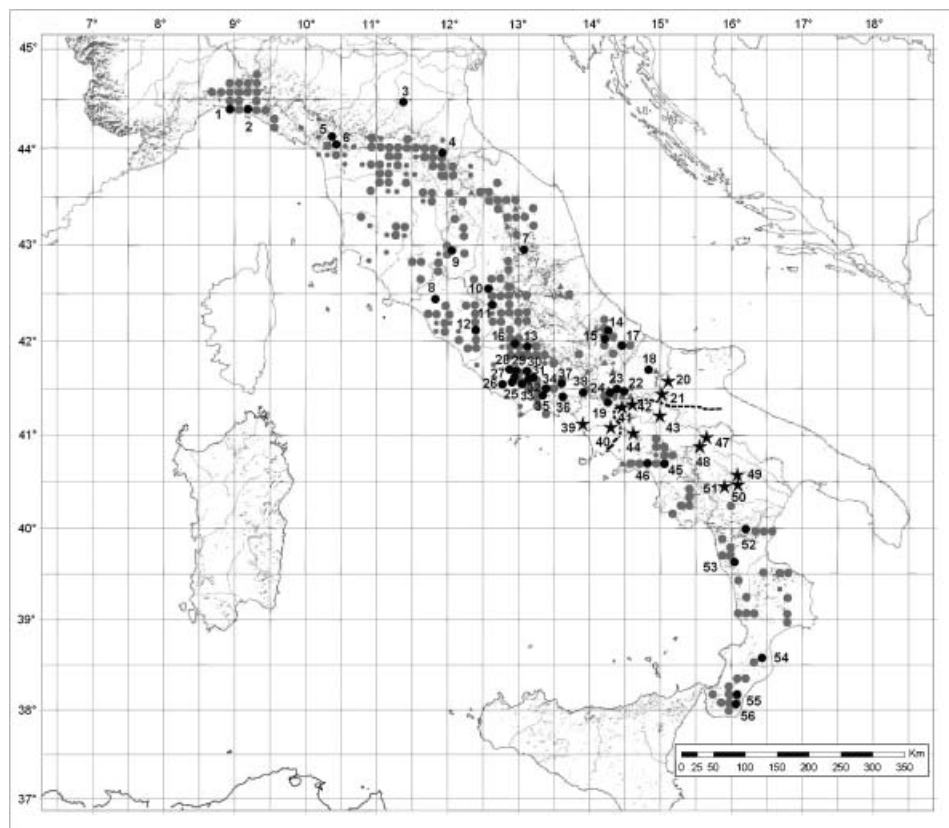


Figure 1. Distribution map of the localities of origin of the *Salamandrina* samples used in this study. The numbers correspond to the sampling sites listed in Table I. Gray circles: sites reported in the distribution map of *Salamandrina* in Barbieri and Pellegrini (2006). Black circles: samples collected within the documented range of *Salamandrina* (Barbieri & Pellegrini 2006). Black stars: samples from areas in which *Salamandrina* was unrecorded. Black dashed line: boundary between the two species of *Salamandrina* that resulted from this study (see the text). Italian map modified from Sindaco et al. (2006).

which in turn equals the misclassification rate when priors are estimated and misclassification costs are equal. The least classification error was determined using 10-fold cross-validation, as recommended by Brieman et al. (1984). In general, “cross-validation” refers to the process of assessing the predictive accuracy of a model in a test sample relative to its predictive accuracy in the learning sample from which the model was developed. The V-fold cross-validation available in CART (in our case  $V=10$ ) is a type of cross-validation which is useful when no test sample is available and the learning sample is too small to have the test sample taken from it. V determines the number of random subsamples, as equal in size as possible, that are formed from the learning sample. Each subsample is used  $(V - 1)$  times in the learning sample and just once as the test sample. An extensive literature is available which provides evidence that using a V-fold cross-validation of 10–20 gives better results than using a smaller number (e.g. Brieman et al. 1984; Kohavi 1995; Moon et al. 2006). CART analyses were carried out using Statistica® ver. 6.0 (Statistica package, Statsoft Inc., USA).

To estimate differences in size between species, we implement the biometric data measured for the CART analyses pooling measures of SVL and TOTL of 40 more specimens of *S. perspicillata* that were sampled on the ground near Rosello (Chieti province, Abruzzi region, Central Italy). Both SVL and TOTL of this total sample (75 specimens of *S. terdigitata* and 132 individuals of *S. perspicillata*) were processed using the unequal variance *t*-test (Welch’s test) as performed in SISA online software. This test should always be used in preference to Student’s *t*-test or the Mann–Whitney U test if the central tendency of two populations based on samples of unrelated data is being compared under conditions similar to those here reported (see Moser et al. 1989; Ruxton 2006).

The chromatic and morphometric variables measured on salamanders in terrestrial activity (i.e. unsexed salamanders) were used in the CART and in Welch’s test to estimate the differences between the species. Live *Salamandrina* males and females are in fact morphologically nearly indistinguishable (Lanza 1983; Brizzi et al. 1989). Furthermore, in three sites we also measured the biometry of 49 *S.*

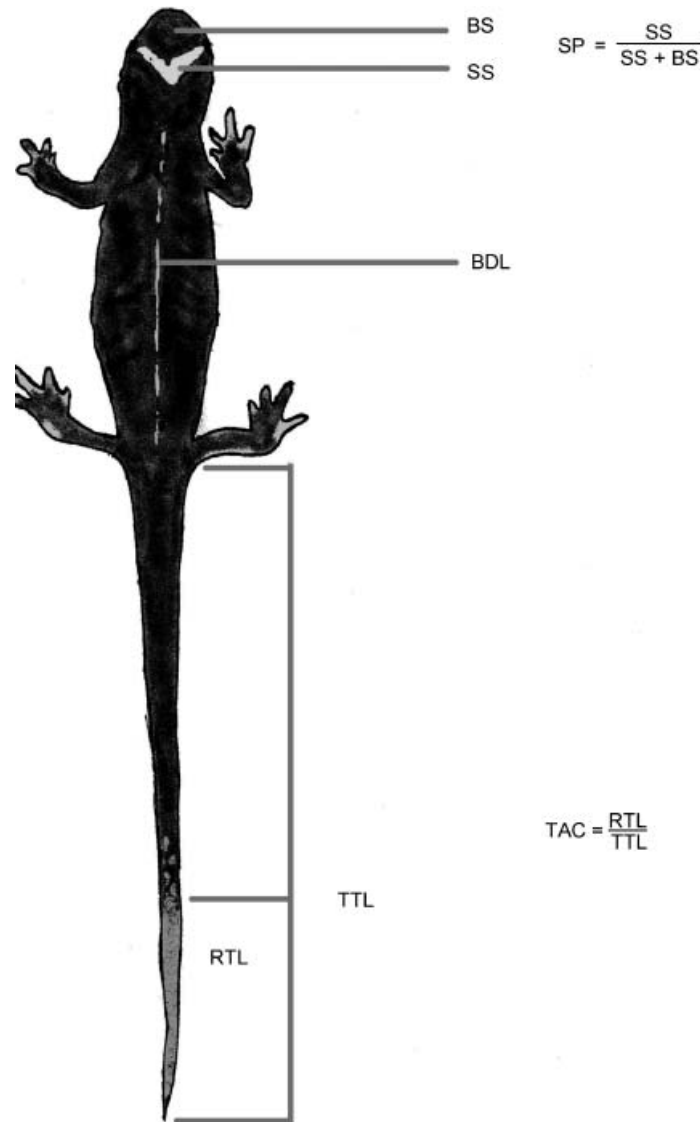


Figure 2. Schematic representation of the coloration characters used in the CART analysis. RTL=red color on the dorsal and lateral sides of the tail, TTL=total length tail, SS=area of the spectacles, BS=black snout area in front of spectacles, BDL=reddish median dorsal line. The ratios SP and TAC are calculated as reported in the figure. BDL was evaluated either as “present” or “absent”.

Table II. List of samples of *Salamandrina* used in the morphological analyses.

Species	Region	Number of sampling sites	Number of specimens
<i>S. perspicillata</i>	Liguria	1	20
	Tuscany	1	20
	Latium	1	12
	Molise	1	40
	Abruzzi*	1	40
<i>S. terdigitata</i>	Basilicata	5	33
	Calabria	2	14
	Campania	3	28

\*The sample from Abruzzi was used only in the morphometric study. In all other samples, both the chromatic pattern and the morphometric variables were analyzed and processed by CART analyses.

*terdigitata* that had been captured in water bodies (one in Basilicata, one in Campania and one in Calabria). These salamanders can be considered females because in *Salamandrina* only females enter the water (Lanza 1983; Zuffi 1999).

## Results and discussion

### Distribution

*Salamandrina* was found at 56 of the surveyed potential sites (12 of them had already been reported in Mattocchia et al. 2005). Detailed information on the sampling localities is shown in Figure 1 and Table I. In 40 sampling sites we found only haplotypes corresponding to *S. perspicillata* (samples

1–40), and in 14 sampling sites we found only haplotypes corresponding to *S. terdigitata* (samples 43–56). In two sites (41 and 42), haplotypes characterizing *S. perspicillata* or, alternatively, *S. terdigitata*, were found in syntopy.

Before this study, the updated distribution map of *Salamandrina* showed the largest populations mainly concentrated in Tuscany and Latium (Central Italy), while distribution records were more sporadic in Marche, Umbria, Molise, Campania and Basilicata, with populations mostly localized on the principal mountainous reliefs (Barbieri & Pellegrini 2006). Furthermore, there was a wide distribution gap corresponding to the boundaries between the two species ranges, i.e. the northern Campania region (cf. Mattoccia et al. 2005; Nascetti et al. 2005; Canestrelli et al. 2006), and no records were available for a large portion of Basilicata and the whole of Apulia (see Barbieri & Pellegrini 2006; Angelini et al. 2007, and map therein). On the basis of these data, *Salamandrina* was considered absent from the whole Adriatic side of southern Italy.

The new distribution data reported here (see Figure 1) shows that *Salamandrina* is also widely distributed in northern Campania and eastern Basilicata. Moreover, site 20, on the northern Daunian Apennine, is the first record of *Salamandrina* being present in Apulia. In fact, the old records from the Salento (southernmost part of Apulia) and from the Gargano massif (Scarzia 1893; Ballaben et al. 1978, respectively) have been excluded or found to be unconfirmed by recent reviewers (cf. Scillitani et al. 1996; Fattizzo & Marzano 2002; Barbieri & Pellegrini 2006; Angelini et al. 2007).

Another notable contribution to determining the *Salamandrina* distribution was provided by site 39 (close to the town of Mondragone, Campania) because it is a low altitude site (about 90 m a.s.l.) located close to the coast (3.3 km) and in a broad area where salamanders so far had been unrecorded (Barbieri & Pellegrini 2006; Angelini et al. 2007).

As shown by mtDNA data (Romano 2007; Mattoccia et al. in prep.), the northernmost portion of Campania represents the northern boundary of *S. terdigitata* and the southern boundary of *S. perspicillata*. Furthermore, haplotypes of both species were found in two populations close to each other in the province of Benevento (sites 41 and 42), thus revealing a contact zone between the two species. The nearest sites where single haplotype lineages occur are only a few kilometres away. In fact, *S. perspicillata* populations have been recorded in sites 19 and 40 (12.5 km and 25.4 km distant from site 41, respectively), while the closest sites of *S. terdigitata*

were sites 43 and 44 (28.5 km and 33.7 km distant from site 42, respectively). This contact zone appears to be rather small, although a larger number of sampling sites, and larger numbers of individuals per sampling site in the area surrounding this zone, are needed to clarify the actual breadth of the contact zone.

Here we report the range limit of both species. For *S. perspicillata*, we also report the limits on the Tyrrhenian Italian side (TYS) and Adriatic side (ADS) separately because the Apennine ridge traverses Italy from north-north-west to south-south-east, thus separating the Italian peninsula in two fully distinguishable parts.

*Range of Salamandrina perspicillata.* TYS: (i) the northernmost limit of this species falls into the municipality of Brallo di Pregola, Lombardia region (Bonini et al. 2004); (ii) the southernmost limit of *S. perspicillata* is in the municipality of Caserta, Campania (roughly at 41°06'20"N; i.e. site 40, previously unrecorded, and here reported); (iii) the westernmost limit is near Bolzaneto, Liguria region, province of Genoa (Barbieri 1994; Barbieri & Pellegrini 2006), although Bedriaga (1897) reported records also for Cogoleto, which is about 25 km further to the west (this fact was erroneously attributed to Vandoni (1914) by Barbieri & Pellegrini (2006)). ADS: (i) the northern limit for this species on the Adriatic side is in the municipality of Casalecchio di Reno, province of Bologna, Emilia Romagna, roughly at 44°28'40"N, which is the site 3 reported here; (ii) the southern limit is in the Municipality of San Bartolomeo in Galdo, province of Benevento, Campania (roughly at 41°25'00"N; i.e. a site previously unrecorded and here reported as site 21); (iii) the easternmost limit of *S. perspicillata* is in the municipality of S. Marco La Catola, province of Foggia, Apulia, roughly at 15°02'40"E; i.e. a site previously unrecorded and here reported as site 20.

*Range of Salamandrina terdigitata.* TYS: on the Tyrrhenian side, (i) the northernmost limit of this species is near Morcone, province of Benevento, Campania region (roughly at 41°19'00"N, which is the newly recorded site 42), where both species live in syntopy; (ii) the southernmost limit of *S. terdigitata* is in the municipality of Palizzi, province of Reggio Calabria, Calabria, roughly at 37°58'00"N (cf. distribution map in Barbieri & Pellegrini 2006); (iii) the westernmost population of this species occurs in the newly recorded site 41, which is very close to Cusano Mutri, province of Benevento, Campania (roughly at 14°32'30"E); (iv) the eastern limit is near Pietrapaola, province of Cosenza,

Calabria, roughly at 16°48'03"E (cf. distribution map in Barbieri & Pellegrini 2006).

#### *Comparative study of the dorsal pattern and morphometry*

CART analysis performed on our sample (Figures 3 and 4) showed that the red color on the dorsal and lateral sides of the tail (TAC) was the most important variable for predicting species, and the second variable was the total length (TOTL), while the less important variables were the spectacled proportion (SP) and the ratio TL/SVL. CART analysis split the data into two groups, using the TAC value of 0.47. In this first split, 95.56% of the salamanders with a TAC value smaller than this were correctly classified as *S. perspicillata*, and 81.33% of the salamanders with a TAC value larger than 0.47 were correctly classified as *S. terdigitata*. Considering also the terminal split, the correct classification rate was 98.91% for *S. perspicillata* and 89.33% for *S. terdigitata*. The overall correct classification rate was 94.61% ( $n=167$ ).

Some individuals exhibit a particular dorsal color pattern (see Lanza & Canestrelli 2002 and references therein), although no differences in coloration between populations were reported by Vanni (1980). This lack of chromatic variation was probably due to the examination of only one preserved southern specimen. On the contrary, our results indicate that the dorsal pattern can be useful, though not sufficient, for discriminating between species. Most of the specimens of both species were correctly classified by the CART analysis, which shows that the two species differ in their dorsal color pattern (tail coloration and median dorsal line). Our data indicate that *S. terdigitata* is more red painted (more extended red coloration on the tail) than *S. perspicillata*, and a reddish dorsal line was more often present in *S. terdigitata* than in the other species. This tendency could be related to data reported by Costa et al. (2005), where a southern population had red ventral coloration, which was more extended in comparison with four central Italian populations.

The mean size of *S. terdigitata* was  $2.94 \pm 0.38$  cm (range 2.07–3.70 cm) and  $7.18 \pm 0.89$  cm (range 4.76–9.76 cm), as estimated from the SVL and the TOTL, respectively (mean  $\pm$  SD;  $n=75$  for both variables), while the mean size of *S. perspicillata* was  $3.39 \pm 0.31$  cm (range 2.60–4.20 cm) and  $8.56 \pm 1.00$  cm (range 6.15–11.51 cm) for SVL and TOTL, respectively ( $n=132$ ). Welch's test clearly showed significant differences in SVL and TOTL between species ( $t=-8.796$ , d.f.: 130,  $p<0.001$  for SVL and  $t=-10.276$ , d.f.: 169,  $p<0.001$  for TOTL).

In short, *S. terdigitata* is significantly smaller than *S. perspicillata*. The distributions of the two species, the one in Southern Italy and the other in Northern Italy, could suggest a latitudinal trend for body size. The ecogeographic rule according to which animals from cooler climates tend to be larger than congeners from warmer climates was formulated for homeotherms in origin (Bergmann 1847), although a wealth of research has also described such a pattern (or lack thereof) in poikilotherm vertebrates and in various invertebrate taxa (see Meiri & Dayan 2003 and references therein). The application of this rule is very controversial in amphibians (e.g. Adams & Church 2008 and references therein), however, and we think that the hypothesis that differences in body size between *S. perspicillata* and *S. terdigitata* are related to Bergmann's rule cannot be tested, considering the distribution of the samples we used for morphometric comparisons. The sampling locations vary little, both in latitude (less than 6°) and altitude (less than 800 m). Although latitude, altitude and climate are likely to be broadly correlated, this correlation may be weaker when assessed on a finer scale (cf. Blackburn et al. 1999). Furthermore, many sites we sampled in southern Italy were cooler habitats (i.e. beech woods at high elevation) than others in central and northern Italy (which were in a Mediterranean climate with Mediterranean bush vegetation). All of this enables a test of the intra- and interspecific association (with temperature, latitude or altitude), which can provide reliable results only when latitude or altitude vary considerably (Imre et al. 2005; Adams & Church 2008; see also Romano & Mattoccia 2005 for a brief discussion on Bergmann's rule applied on *Salamandrina perspicillata*).

In *Salamandrina*, the sexes are not unambiguously distinguishable by external morphology (Brizzi et al. 1989; Zuffi 1999), although only females can be found in water bodies during the oviposition phase (Lanza 1983). However, the males in a given population are shorter than the females (Vanni 1980). Consequently we pooled and analyzed the morphometric data of the salamanders that were collected exclusively on the ground because they were of unknown sex. Morphometric data on *S. perspicillata* females is widely available (e.g. Della Rocca et al. 2005; Romano & Mattoccia 2005; Spilinga et al. 2006; Angelini et al. 2008), while comparative data on *Salamandrina terdigitata* females are still lacking. In our sampling we also found 49 confirmed females of *S. terdigitata* (i.e. captured in water bodies during spawning activity). Their mean size was  $3.24 \pm 0.27$  cm (range 2.39–3.70 cm) and  $8.11 \pm 0.51$  cm (range 6.99–9.76 cm), as estimated from SVL and TOTL, respectively (mean  $\pm$  SD;



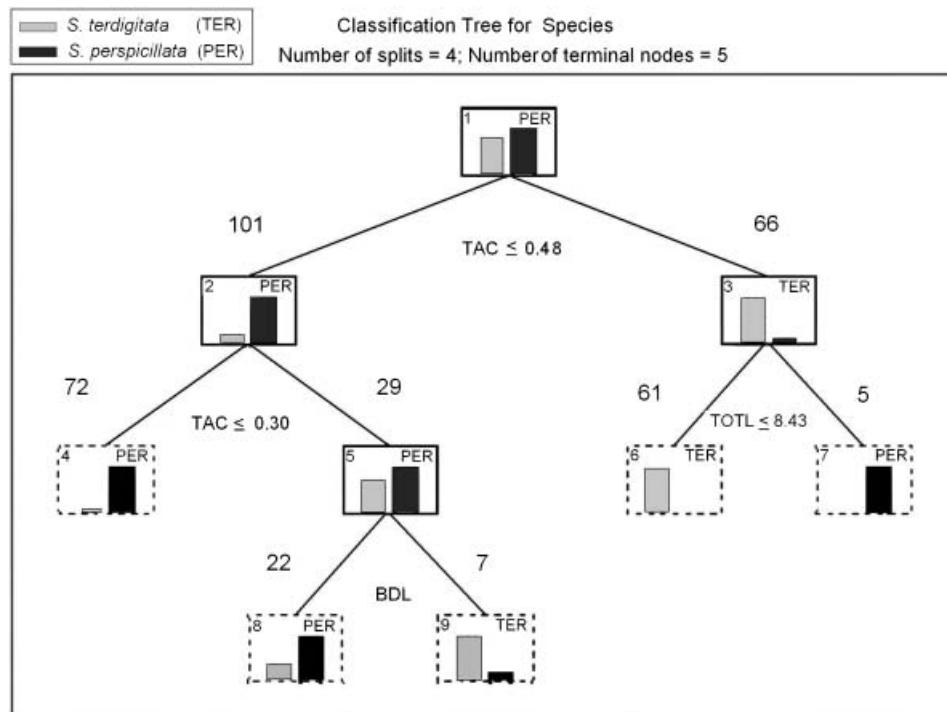


Figure 3. Classification tree CART-style obtained for the *Salamandrina perspicillata* and *S. terdigitata* using dorsal color pattern and morphometric variables. The tree was based on five variables: (i) the presence or absence of a reddish body dorsal line (BDL); (ii) the ratio between the extension of red on tail and the total tail length (TAC); (iii) the ratio between the light area of the spectacles and the head surface including both spectacles and the black snout area in front of spectacles (SP); (iv) the total length (TOTL); and (v) the ratio TL/SVL (where TL is the tail length and SVL is the snout-vent length). Root and intermediate nodes include the variable and ratio value used to create the split. Samples containing less than splitting value are classified on the left, and samples containing more than this value are classified on the right. Numbers of cases sent to child nodes are reported above nodes. Numbers of cases in each node are reported within or above corresponding bars. Labels within each node denote predicted classes. Terminal nodes are outlined with dotted lines, while the remaining decision nodes or split nodes are outlined with solid lines.

$n=49$  for both variables). These data were not included either in the database used in the CART analyses or in the statistical size comparison between species. However, these data are very interesting because they are the first available morphometric data on *S. terdigitata* females. The mean size and the maximum length of the females (both SVL and TOTL) of the southern species are smaller than in the populations of *Salamandrina perspicillata* (cf. Vanni 1980; Romano & Mattocchia 2005; Angelini et al. 2006, 2008; Spilinga et al. 2006; Dell'Alpi & Sazzini 2007). Also, the first specimen of *Salamandrina terdigitata* measured by Lacépède (1788) was a tiny salamander, because it was 12 “lignes” long (SVL) and 28.5 “lignes” long (TOTL), that is 2.71 and 6.43 cm, respectively. The homogeneity of the biometrical characters between the northern and the southern populations previously reported by Vanni (1980) was due, as previously mentioned, to the lack of southern samples. Furthermore, statistically relevant differences in size between the geographically close central

Italian populations of *S. perspicillata* have previously been reported (Romano & Mattocchia 2005).

The average size of *S. perspicillata* here obtained (about 8.6 cm) agrees with the data reported by Zuffi (1999), i.e. average 8–9 cm, with the maximum size found in a female of 11.6 cm. However, recent data showed that in a restricted area in the Latium (i.e. the Lepini Mountains) particularly large salamanders occur. In fact, some females in the Lepini Mountains exceeded 13 cm in TOTL (Romano & Mattocchia 2005; Bovero et al. 2006). As a matter of caution, we did not include the morphometric data of specimens from this area in order to avoid bias due to these localized large-sized populations in the calculation of the mean size of *S. perspicillata*. However, this consideration strengthens the evidence for a great difference in size between the two species, even if analogous restricted areas inhabited by large-sized salamanders in southern Italy cannot be excluded.

The genetic differentiation between *S. perspicillata* and *S. terdigitata* is corroborated by differences in morphometry and coloration features. Morphometric

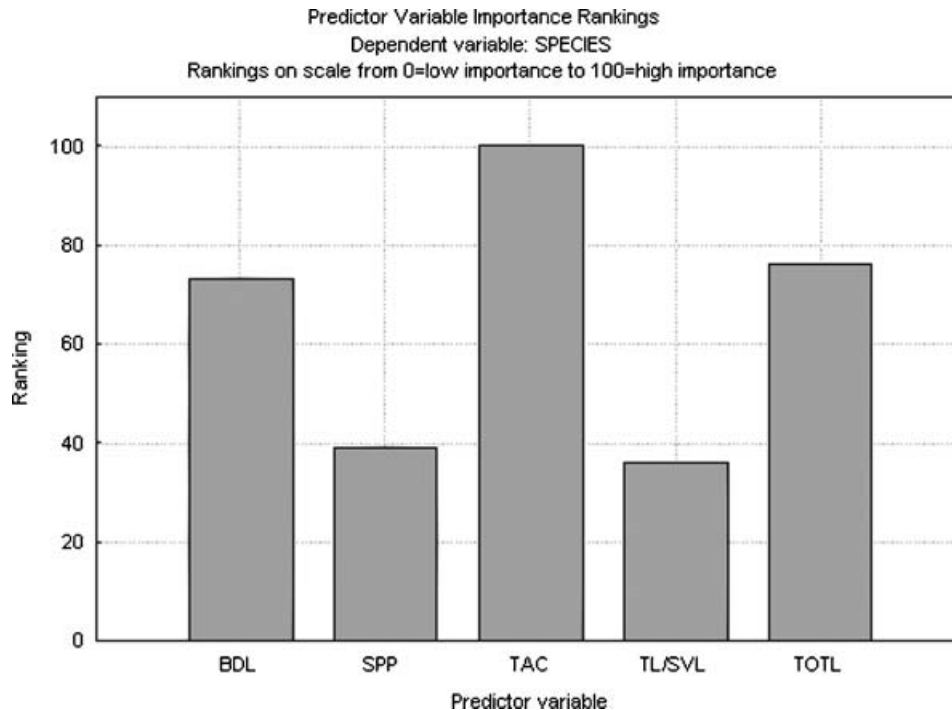


Figure 4. Potential importance, in accounting for responses on the dependent variable, of the five predictor variables used between *Salamandrina perspicillata* and *S. terdigitata* (see the text). The ratio between the extension of the red on the tail and the total tail length (TAC) turns out to be the most important predictor.

and chromatic data on both species were collected from different localities within the whole distribution range and, therefore, should be representative of the two species.

### Conclusions

*Salamandrina perspicillata* and *S. terdigitata* can be distinguished on the basis of mtDNA haplotypes and allozyme profiles (Mattocchia et al. 2005; Nascetti et al. 2005; Canestrelli et al. 2006) but they differ also in their body size and dorsal coloration. *Salamandrina terdigitata* has a smaller size and more extended red coloration on the tail, and it more often exhibits a median reddish dorsal line than does *S. perspicillata*. In other words, to attribute individuals to either of the two species, the morphometry and the dorsal pattern can be very useful, but it is not determinative. At the moment, the only way to definitively distinguish between the two species is on the basis of mtDNA haplotypes and allozyme profiles because any distinction on the basis of body size and dorsal coloration is only statistical and is not definitive for any given specimen.

Between the two species of *Salamandrina*, therefore, there is a deep genetic divergence that is not reflected in a comparable morphological differentiation. We should keep in mind, however, that analogous situa-

tions are widely known in other amphibians (e.g. Wake et al. 1978; Kraus & Petranka 1989; Nascetti et al. 1996; Glaw & Vences 2002; Maletzky et al. 2008). The significant increase in the numbers of samples included in the present study compared to those reported in previous studies (Mattocchia et al. 2005; Nascetti et al. 2005; Canestrelli et al. 2006) has made it possible to trace the range boundary between the ranges of the two species and to reveal the occurrence of a contact zone between them. The two related taxa show clearly parapatric distributions and the areas in which they are in contact seem, at least on the basis of the mtDNA results, to be very restricted.

Further studies using nuclear markers are needed to clarify whether the syntopic area we found – or perhaps even a wider area – also represents a hybrid zone. Some instructive cases have shown that the use of mtDNA markers as the sole genetic marker can lead to incomplete conclusions (e.g. Arntzen & Wallis 1999; Babik et al. 2003; Maletzky et al. 2008). The cline centers are often not coincident for all DNA markers (e.g. Babik et al. 2003; Sequiera et al. 2005), and the introgression of mtDNA and nuclear loci may differ remarkably in natural populations (e.g. Babik et al. 2003).

At present our sampling effort is focused on collecting as many samples from as large an area as possible including the contact zone between the two

species, and studies of these samples based on nuclear markers are in progress. The preliminary data (Mattoccia et al. in prep.) seem to indicate the existence of likely hybrids, but further research is required to quantify the extent of the phenomenon and the degree of introgression between the species.

From the beginning of the twenty-first century, after a long period of receiving scanty attention from herpetologists, the endemic Italian genus *Salamandrina* has become the subject of numerous studies relating to its ecology and ethology (Utzeri et al. 2005; Romano et al. 2008a,b; Romano & Ruggiero 2008), its reproductive biology (Della Rocca et al. 2005; Angelini et al. 2008) and its genetic structure (Mattoccia et al. 2005; Nascetti et al. 2005; Canestrelli et al. 2006). The interesting and unexpected results produced so far on the genetic characterization of the *Salamandrina* genus have important conservation implications which must account for the existence of the two distinct species of salamanders. Moreover they also make this genus an ideal candidate and a good model for evolutionary studies concerning hybrid zones.

### Acknowledgments

The Italian “Ministero dell’Ambiente e della Tutela del territorio e del Mare” issued capture permits (DPN/2D/2004/17393). For their contributions to this work, we are grateful to Emanuele Biggi, Francesco Chesi, Massimo Delfino, Tiziano Fattizzo, David Fiacchini, Giovanni Forcina, Stefano Mazzotti, Cristiano Spilinga, Sandro Tripepi, AISA-Isernia, LIPU Italia, WWF Italia, “Lago Piccolo di Monticchio” Natural Reserve, and Majella National Park. In particular, we are indebted to Marco Carafa, Pino Eremita, Antonio Iannibelli, Luigi and Mino Di Florio, Vincenzo Di Martino, Gianfranco Lionetti, Carlo Meo (“Casacalenda” LIPU reserve), Mario Pellegrini (“Abetina di Rosello” WWF reserve), Valeria Ramacciato, Edoardo Razzetti, Antonio Vito Romano, Marco Sazzini, Anton Bruno Smidth and Costantino Tedeschi for their invaluable help in the field. We are also grateful to Sebastiano Salvidio and two other anonymous referees for their helpful comments. We are indebted to Richard Sundahl for the linguistic revision of the paper.

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