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New distribution and genetic data extend the ranges of the spectacled salamanders, genus *Salamandrina*, in the Apulia region (South Italy)

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Abstract. Additional data on the distribution of the genus *Salamandrina* in the Apulia region (southern Italy) are provided. Based on fieldwork carried out from May to August 2011 in two new localities, Volturara Appula (Foggia province) and Spinazzola (Barletta province), the presence of *Salamandrina* species was recorded. Results from the genetic analyses of the 12S rRNA gene fragment from six individuals demonstrated that *S. perspicillata* occurs in Volturara Appula while *S. terdigitata* in the Spinazzola locality. The latter species is reported for the first time for the Apulia region. These new distribution data represent considerable range extensions for the *Salamandrina* species, indicating that more surveys are needed to complement the existing knowledge on their distribution as well as of the herpetofauna from the Apulia region. The conservation implications of our findings are also discussed.

Keywords. *Salamandrina perspicillata, Salamandrina terdigitata*, distribution, 12S rRNA, genetic diagnosis, Apulia, Italy.

INTRODUCTION

The genus *Salamandrina,* endemic to the Italian Peninsula, includes two vicariant species, the northern spectacled salamander *S. perspicillata* (Savi, 1821) and the southern spectacled salamander *S. terdigitata* (Bonnaterre, 1789). While the genetic distinction of the two species is obvious both at the mitochondrial (Nascetti et al., 2005; Mattoccia et al., 2005) and at the nuclear level (Nascetti et al., 2005; Canestrelli et al., 2006), no morphological traits allow a clear distinction between the two species (Angelini et al., 2007). Although some differences in size and coloration would allow a tentative distinction between adults of *S. terdigitata* and *S. perspicillata* (Romano et al., 2009), it is not possible to identify neither young individuals nor larvae based exclusively on their morphology.

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As a consequences of the difficulty of identifying the two species on the base of their morphology, the knowledge of their distribution ranges is currently still coarse in areas where the two species' ranges meet. Genetic data from the previously referenced published studies indicated that *S. terdigitata* occurs in the southern Italian regions of Calabria, Basilicata and southern Campania, while *S. perspicillata* is distributed from the northern districts of Campania and Apulia regions to the northern Apennines (Piemonte region). The sympatry between *S. terdigitata* and *S. perspicillata* has been recorded in a small area of the Campania region for which recent genetic studies suggested the occurrence of hybridization between the two species (Hauswaldt et al., 2011; Mattoccia et al., 2011). Another candidate area of sympatry between the two species could be in the northern part of the Apulia region, which is quite close to the north-eastern limit of the *S. terdigitata*'s range, but where only one record of *S. perspicillata* is known (Romano et al., 2009).

With the aim of filling the gap of knowledge on the distribution of the genus *Salamandrina* in the Apulia region, we carried out an extensive field survey in two different districts at the border between the Apulia region and the regions Molise, Campania and Basilicata. The results of the genetic analyses carried out for the identification of the specimens found provided new distribution data for the genus *Salamandrina* in the Apulia region.

MATERIALS AND METHODS

Extensive field surveys were carried out in two geographical districts of the Apulia region: the Dauno Sub-Apennine, at the border with the Campania region, and the north-western Murgia, at the border with the Basilicata region. Using IGM and orthophotos maps numerous springs and streams were identified and then inspected in areas potentially suitable for the *Salamandrina* species. Surveys were carried out between May and August at fortnightly intervals. We surveyed both larvae, by inspecting water bodies by means of nets, and adults, which were actively sought in the wooded areas adjacent to water.

The urodele larvae found were collected, photographed, assigned to the genus *Salamandrina* using the identification keys provided by Lanza et al. (2007) and immediately released on site. All the equipment was disinfected after each sampling session, following the requirements of the Conservation Commission of the Societas Herpetologica Italica (available at http://www-3.unipv.it/webshi).

The *Salamandrina* individuals sampled were assigned either to *S. perspicillata* or to *S. terdigitata* based on genetic diagnoses. Total genomic DNA was extracted from tail tips from six specimens (four individuals from Volturara Appula and two from Spinazzola), following the standard saline method (Sambrook et al., 1989). A fragment of the mitochondrial 12S rRNA gene was amplified by PCR using primers and conditions as in Salvi et al. (2011). PCR products were purified and sequenced by an external service (Macrogen Korea). Multiple 12S rRNA sequences alignment was performed by Clustal W (Thompson et al., 1994) including ten sequences from *S. perspicillata* and *S. terdigitata* and one from *Lissotriton vulgaris* downloaded from Genbank (accession numbers reported in Fig. 3). A Maximum Likelihood (ML) phylogenetic analysis was carried out in Mega 5 (Tamura et al., 2011) employing the Tamura's 3-parameter model of evolution (Tamura, 1992) selected by Mega 5 under the Bayesian Information Criterion. Node support (BP) was calculated over 1000 bootstraps replicates.

RESULTS

Field Surveys

The presence of *Salamandrina* larvae was found at two sites about 100 km apart (Fig. 1). In both cases, animal at a larval stage were found. Adults were not encountered neither in aquatic nor in terrestrial environments. The first site where the animals were found is located in the Sub-Apennine, in the municipality of Volturara Appula (Foggia province, FG) about 7 km from the border with Campania region. Spectacled salamanders larvae (developmental stage 42-43; Harrison, 1969) were found in an artificial reservoir fed by a natural perennial spring located about 800 m asl, within a mixed mesophilous forest. The second site is located in the territory of Spinazzola (Barletta province, AT). At this

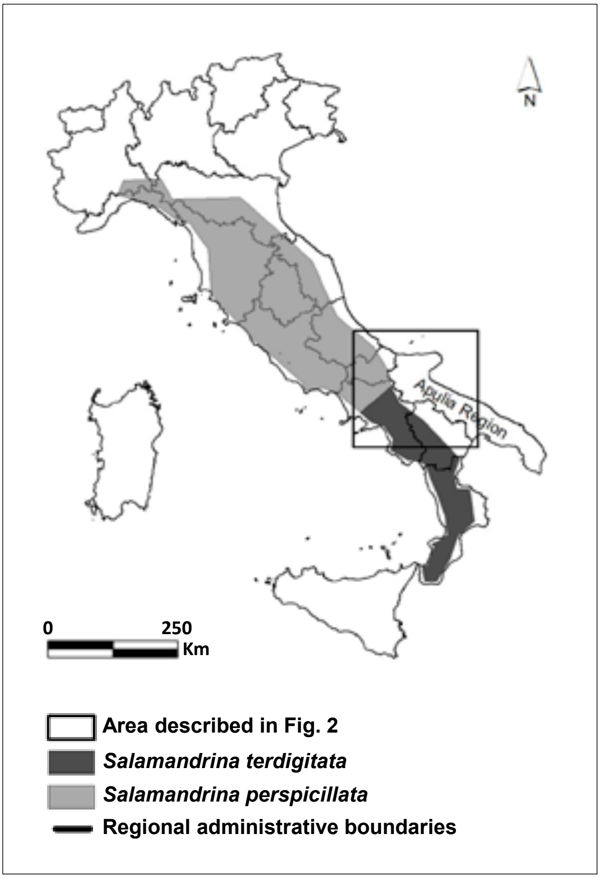

Fig. 1. Distribution ranges of the spectacled salamanders, genus *Salamandrina*: light grey, range of the northern spectacled salamander *S. perspicillata*; dark grey, range of the southern spectacled salamander *S. terdigitata*. The boxed area is described in Fig. 2.

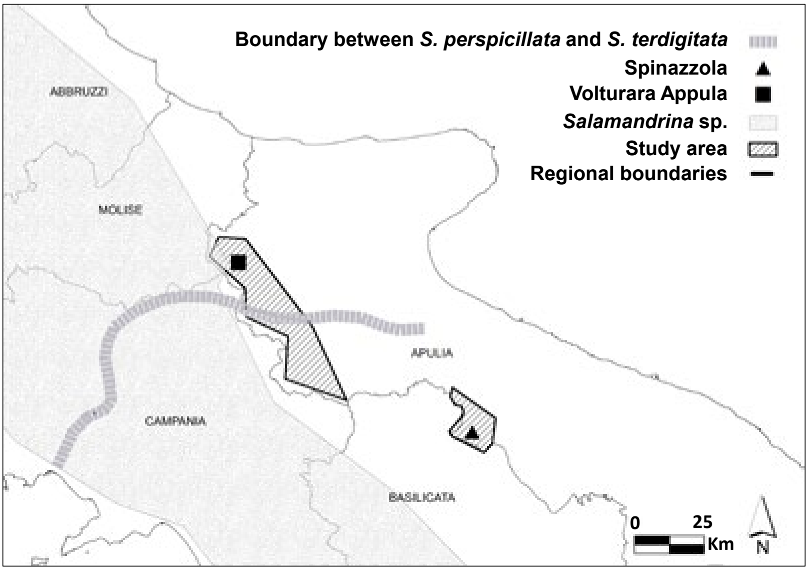

Fig. 2. Map of the study area. The new distribution sites of Volturara Appula and Spinazzola are indicated by square and triangle symbols respectively. The putative boundary area between *S. perspicillata* and *S. terdigitata* is defined according to Romano et al. (2009).

site, the presence of *Salamandrina* larvae (developmental stage 42-43; Harrison, 1969) was recorded in a perennial stream in a narrow valley with a stand of Turkey Oak (*Quercus cerris*) located at around 400 m asl. In this site larvae were found in water from early June through to the end of July, while during a survey on the 29th of August, no larvae were found, indicating that metamorphosis presumably takes place before the end of August. The temperature and pH of the water in the period investigated remained fairly stable, at respectively 15 ± 0.5 °C and 8.3 ± 0.2, respectively. Additionally, at both sites the presence of both larvae and adults of *Rana italica* was recorded.

Genetic Analyses

DNA sequences of the 12S rRNA gene fragment resulted in an alignment of 367 base pairs (bp) among which 58 sites were variable. Sequences from the six larvae of *Salamandrina* sampled (Genbank accession numbers HE610671-HE610676) collapsed in two haplotypes, one present in salamanders from Volturara Appula and the other one occurring in salamanders from Spinazzola. The sequence divergence (uncorrected *p*-distance) between these two haplotypes was equal to 3%. The Maximum Likelihood phylogenetic tree (Fig. 3) clearly showed that spectacled salamander from Volturara Appula clustered with *S. perspicillata*, while those from Spinazzola are included in the clade of *S. terdigitata* with high bootstrap support (BP ≥ 98).

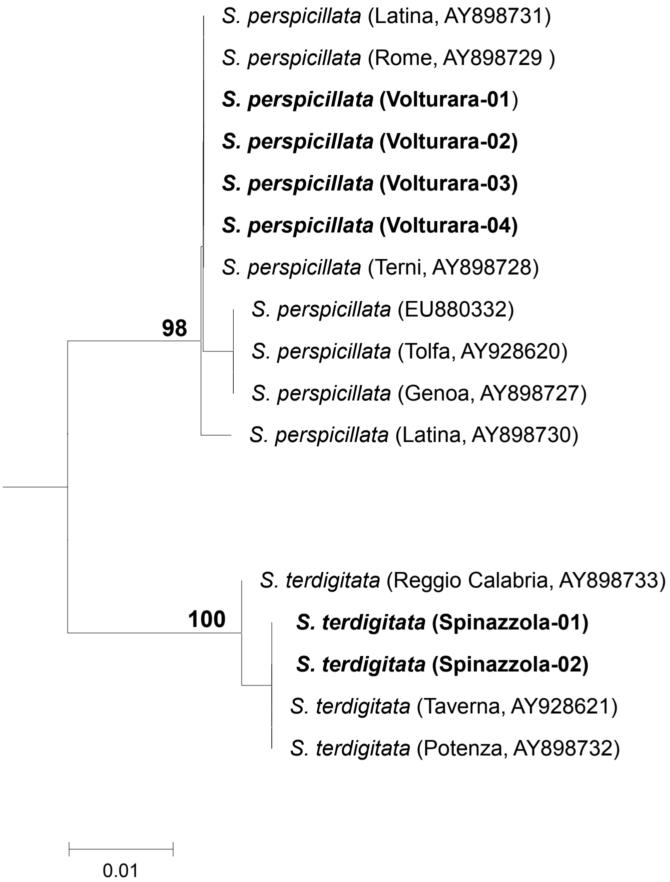

Fig. 3. Maximum Likelihood phylogenetic tree based on12S rRNA sequences depicting the relationships between *Salamandrina* haplotypes identified by previous studies (Nascetti et al., 2005; Mattoccia et al., 2005; Zhang et al., 2008) and those from the six individuals (in bold) sampled in the Volturara Appula and Spinazzola populations (Apulia region, South Italy). *Lissotriton vulgaris* (Genbank Accession number TVU04704) was used as outgroup (not shown). Bootstrap support values over 1000 replicates are indicated above the nodes.

DISCUSSION

This study provided two new distribution records for the *Salamandrina* genus in the Apulia region. Based on genetic diagnoses, the *Salamandrina* population breeding at the Volturara Appula was assigned to *S. perspicillata,* while the population breeding at the site of Spinazzola was identified as *S. terdigitata.*

Volturara Appula is adjacent to the only known location for *S. perspicillata* in Apulia, which is San Marco La Catola in Foggia province (Romano et al., 2009). On the other hand, the *S. terdigitata* population recorded in the site of Spinazzola represents the first sighting of this species for the Apulia region and is rather distant from any other known site where the genus *Salamandrina* has been recorded. The closest known sites of *S. terdigitata* are two localities in the Basilicata region about 45 km far from Spinazzola: one near Rionero in Vulture and the other near Accettura (Potenza province, PZ; Romano et al., 2009). The nearest record of *S. perspicillata* relative to Spinazzola is even further away of about 90 km (San Bartolomeo in Galdo, Benevento province, BN, Campania region; Romano et al., 2009).

Based on the results of this study, the range of both *S. perspicillata* and *S. terdigitata* was extended eastwards in the Apulia region. No evidence of sympatry between these two species was found based on our surveys. *S. perspicillata* is currently known to occur in two Apulian localities, while the site of Spinazzola represents the only known locality of *S. terdigitata* in the Apulia region. This finding is worthy of attention, since the Spinazzola site is discontinuous with any geographical and ecological areas potentially suitable for the species. Indeed, with the exception of a few regimented channels, which dry out for most of the year, the surroundings of the site are dominated by cereal and forage crops.

From the results of this preliminary study, the following conclusions can be drawn: i) it is plausible that the western border of the Apulia region is the south-eastern outpost for both species of the genus *Salamandrina*; ii) *S. perspicillata* and *S. terdigitata* have an allopatric distribution in their eastern range limit. Forthcoming studies should focus on the northwestern district of Apulia and on areas not surveyed in this study in order to conclusively validate the allopatry between these two species and to definitely define their eastern range limits.

Finally, knowledge of the fauna in the Apulia region, such as that reported herein, is an important first step to ensure that these areas - currently not subject to any kind of protection - may enter into a functional network of protected areas. Indeed, among other animals, many species of amphibians such as *Salamandrina*, *Triturus carnifex*, *Rana italica* and *Bombina pachypus,* which are identified by European Community lists as deserving conservation priority, have in the Apulia region the limit of their range.

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