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Hybridization of two ancient salamander lineages: molecular evidence for endemic spectacled salamanders on the Apennine peninsula

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Abstract

Current studies indicate that both processes and mechanisms of natural hybridization go far beyond the formation and maintenance of hybrid zones between species. These studies demonstrate that the evolutionary consequences of hybridization can include extinction or extirpation of lineages but may also favor the formation of new hybrid species in an ecological context. The unambiguous identification of occurrences of hybridization in natural populations is a crucial first step in addressing questions related to natural hybridization in both evolutionary and ecological terms. Here, we provide the first molecular evidence of extensive natural hybridization between two ancient sister species of spectacled salamanders – *Salamandrina perspicillata* in northern and *Salamandrina terdigitata* in southern Italy. Parental lineages diverged at least 10 million years ago during the Lower Pliocene and represent the most ancient split between any congeneric amphibian species endemic to the Italian peninsula. Analysis of the mitochondrial cytochrome b (Cyt *b*), nuclear-encoded recombination-activating protein (RAG-1) and propriomelanocortin (POMC) genes of more than 250 individuals from populations of both species and from the contact zone, show clear evidence of ongoing hybridization. Whereas 20% of the individuals from the contact population showed no signs of hybridization for the applied markers, the remainder (80%) were identified as first generation hybrids and backcrossed individuals. Our results suggest that hybridization between these two ancient lineages produces viable and fertile offspring, highlighting the need for research on possible mechanisms that prevent the intermixing and hybridization of parental species on a broader geographical scale.

Introduction

The intermixing of individuals from genetically, and most often morphologically, differentiated lineages in nature is considered to be part of the broader phenomenon of natural hybridization and has been reported to varying degrees across all groups of sexually reproducing organisms (Ridley, 2004; Mallet, 2005; Strickberger, 2008). In animals, hybridizing individuals may originate from distinct species or even from populations or lineages from within a single species. As a result of sexual intermixing, the respective parental genomes come into contact, and gene pools become intermixed. Current research addresses processes and consequences of hybridization that go far beyond the formation of classical hybrid zones and how reproductive isolation of parental species or lineages is maintained (e.g. by reinforcement or decreased hybrid fitness). It is now clear that the evolutionary consequences of hybridization may also include the extinction or local extirpation of one or both lineages; consequently, the formation of hybrid phenotypes with high fitness may represent new challenges for conservation (Fitzpatrick & Shaffer, 2007). Hybrid speciation in an ecological context, however, has been shown to overcome what is generally assumed to be the destructive force of hybridization. The formation of new genetic combinations between closely related species enables the new hybrid species to occupy open niches and become successfully established in new habitats (Seehausen, 2004; Nolte *et al*., 2005; Nolte & Tautz, 2010).

Hybridization is common among amphibian species (see Vences & Wake, 2007 for examples). The most widely studied amphibian systems in this respect are the hybrid zones between the yellow-bellied toads *Bombina variegata* and fire-bellied toads *Bombina bombina* (Szymura & Barton, 1991; MacCallum *et al*., 1998) and the hybridogenetic water frog complex (Plötner, 2005) for anurans. Among urodelans, well-studied systems include the partial hybridization of *Ensatina* salamander species of the formerly recognized ring-species complex (Moritz, Schneider & Wake, 1992; Alexandrino *et al*., 2005) and the contact zone between

crested newts *Triturus cristatus* and marbled newts *Triturus marmoratus* in western France (Arntzen & Wallis, 1991; Arntzen *et al*., 2009).

In this report, we present an analysis of a new case of hybridization in terrestrial salamanders resulting from contact between two ancient lineages of spectacled salamanders endemic to the Apennine peninsula. Spectacled salamanders are terrestrial with an aquatic larval stage spent in slow-flowing brooks or small, lentic water bodies (Angelini, Antonelli & Utzeri, 2008). After metamorphosis, their life cycle becomes terrestrial, and only adult females return to streams and ponds for the deposition of their eggs. Currently, little is known about their biology because active adults are rarely encountered above ground outside their breeding season. In addition, males and females are difficult to distinguish because of the lack of sexually dimorphic characters, though a method has recently been described for sexing these salamanders in the field (Romano, Bruni & Paoletti, 2009*a*). With an estimated phylogenetic age of *c*. 95 million years (Steinfartz *et al*., 2007; Zhang *et al*., 2008), the genus *Salamandrina* represents the most basal clade within the family Salamandridae, which also contains two well-supported monophyletic species assemblages of so-called true salamanders and newts (Steinfartz *et al*., 2007; Zhang *et al*., 2008). Until 2005, *Salamandrina* was considered to be a monotypic genus with *Salamandrina terdigitata* as the sole representative species. Mitochondrial sequence analysis, however, revealed a deep phylogenetic divergence between northern and southern populations of *Salamandrina* (Mattoccia, Romano & Sbordoni, 2005; Nascetti, Zangari & Canestrelli, 2005) that took place between 4.7 and 16.4 million years ago (depending on the calibration scenario used, see Mattoccia *et al*., 2005). Nuclear-encoded allozyme loci further corroborated the strong genetic differentiation between the lineages and confirmed the existence of two distinct species of *Salamandrina* (Canestrelli, Zangari & Nascetti, 2006*b*), *Salamandrina perspicillata*, distributed in northern Italy from Liguria to Campania, and *S. terdigitata*, which is distributed in southern Italy from Campania to Calabria (reviewed in Romano *et al*., 2009*b*). The Volturno River drainage has been identified as a potential contact zone between lineages of both species (Canestrelli *et al*., 2006*b*). Using image analysis of the ventral coloration patterns, which are unique and are retained throughout the lives of adults, of more than 300 individuals, Angelini *et al*. (2010*b*) showed that the coloration is discriminative and allows for correct assignment of nearly 99% of individuals to their respective species.

Although, a rather narrow contact zone for these species has been described and mitochondrial haplotypes of both species were found in neighboring populations in Campania (Romano *et al*., 2009*b*), clear genetic evidence for hybridization between the two species is still lacking. On the basis of both mitochondrial and nuclear genes, we provide the first molecular evidence of ongoing natural hybridization between the two species.

Materials and methods

Sampling for molecular analysis and amplification of gene segments

To ensure unambiguous identification and classification of hybridization between *S. perspicillata* and *S. terdigitata*, a total of 113 individual samples were collected from six locations within the presumed range of *S. perspicillata*, and 83 individual samples were collected from four localities within the presumed range of *S. terdigitata* (see Table 1 and Fig. 1). Fifty-five individuals were sampled from the Morcone population, which is considered to be part of the contact area between both species. Tail tips of adults and larvae, as well as eggs, were used to extract total genomic DNA. We amplified and analyzed one mitochondrial gene and two nuclear gene segments for the presence of hybrid individuals. A 682 bp segment of the mitochondrial cytochrome *b* gene (Cyt *b*) was amplified using the published primers MVZ15 and MVZ16 (Moritz *et al*., 1992), corresponding to bp 14 211–14 892 of the entire mtDNA genome of *S. perspicillata* (EU880332; incorrectly referred to as *S. terdigitata* by the authors Zhang *et al*., 2008).

Table 1 List of sampling sites with their abbreviations and geographic coordinates as well as the number of samples analyzed for each marker

	Locality name (abbreviation)	Geographical coordinates (latitude: longitude)	Cyt *b* (682 bp)	POMC (382 bp)	RAG-1 (627 bp)
Salamandrina perspicillata	Rio Riazzu (RR)	44.683; 9.033	18	17	16
	Bologna (BC)	44.467; 11.283	15	9	9
	Mattere (MT)	42.717; 13.5	18	9	10
	Terni (ER)	42.517; 12.633	21	12	10
	Valle Ota (VO)	41.550; 13.050	16	16	10
	Casine Mainarde (CM)	41.617; 13.95	16	17	19
	Total number of individuals		**104**	**80**	**74**
S. perspicillata × *S. terdigitata*	Morcone (MR)	41.35; 14.683	**55**	**55**	**55**
Salamandrina terdigitata	Torrente Cerasuolo (ST)	40.783; 14.917	24	14	14
	Vallone Cupo (VC)	40.333; 15.35	18	17	15
	Foresta (FO)	39.983; 16.4	21	9	14
	Fiumara Samo (FS)	38.067; 16	19	14	11
	Total number of individuals		**82**	**54**	**54**

Total number of individuals for each species/group are in bold.

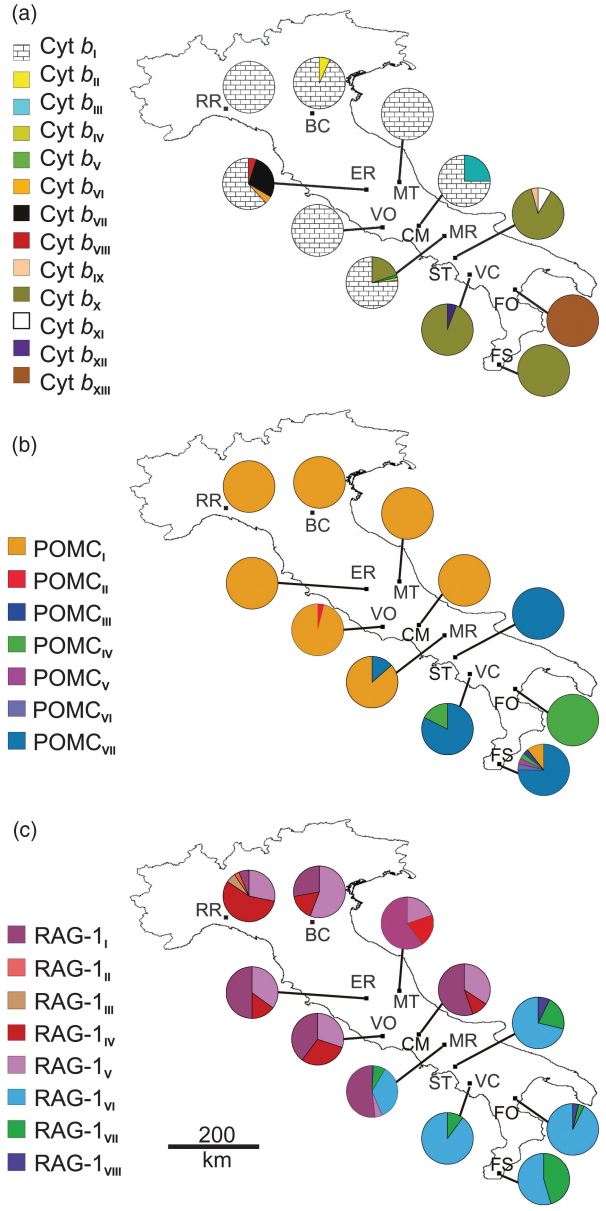

Figure 1 Sample collection sites of *Salamandrina perspicillata* (RR, BC, ER, MT, VO, CM), *S. terdigitata* (ST, VC, FO, FS) and for the hybrid population Morcone (MR). Haplotype distributions, as well as their respective frequencies (pie diagrams proportional to the frequencies of haplotypes) are provided separately for (a) Cyt *b*, (b) POMC and (c) RAG-1. See Table 1 for detailed information on abbreviated sampling sites.

The nuclear gene segments obtained encoded a 382 bp segment of the propriomelanocortin (POMC) gene and a 627 bp segment of the nuclear-encoded recombination-activating protein (RAG-1) gene. For amplification of PCR products, we used primers POMC_Sal-F (5′-AAGTTCGGCAGAAGG AACAGCACC-3′) and POMC_SAL-R (5′-GTCTGACTCC GCTCAGGGGTCATG-3′), corresponding to bp 204-585 of *Cynops pyrrhogaster* (AB572298; Hasunuma *et al.*, 2010), and RAG1-SAL-F2 (5′-ACAGGATATGATGAGAAGCTYGT GAG-3′) and RAG1-SAL-R1 (5′-CGTGGGCTAGTGTCT TGTGGAAATAG-3′), corresponding to bp 721–1347 of *Lyciasalamandra luschani* (AY323753; Hoegg *et al.*, 2004). Whereas the primers for POMC were designed based on sequences obtained with POMC-DRV-F + R (Vieites, Min & Wake, 2007), primers for RAG-1 were designed based on sequence information for *Salamandrina* obtained with other amphibian primers that are used in our laboratory. PCR amplifications were performed and amplicons cleaned and sequenced as described in Hauswaldt *et al.* (2010). Annealing temperatures for amplifications of gene segments were 50.7 °C for the Cyt *b* gene, 57 °C for the POMC and 54 °C for the RAG-1 gene.

Sequence analysis

All sequences were aligned and edited using the CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA) and collapsed into haplotypes using FABOX 1.35 (Villesen, 2007). Nucleotide composition, the ratio of transitional to transversional distances per site (R), averaged over all sequence pairs using the Kimura 2-parameter model, and uncorrected *p*-distances within and among sequences were calculated using MEGA 4 (Tamura *et al.*, 2007). To reconstruct haplotypes for the nuclear sequences, we used PHASE 2.1 (Stephens & Donelly, 2003) as implemented in DNASP v. 5 (Librado & Rozas, 2009). We accepted haplotype reconstructions with Bayesian posterior probabilities >95% for the nuclear markers. Genetic variability within populations was estimated in terms of haplotype diversity (h), nucleotide diversity (π) and the average number of pairwise nucleotide differences (k) using DNASP. Observed heterozygosity was calculated as the ratio of heterozygotes to the total number of individuals sampled. We used TCS 1.21 (Clement, Posada & Crandall, 2000) to generate haplotype networks for all three of the applied markers.

The patterns of genetic differentiation among populations were estimated by pairwise F_{ST} values using ARLEQUIN 3.5 (Excoffier & Lischer, 2010) and tested for statistical significance on the basis of 10 000 permutations. The haplotypes of all genes were deposited in GenBank (HQ915029–HQ915638).

Results

Genetic analyses

None of the supposed Cyt *b* sequences contained a stop codon, and the mean nucleotide frequencies were $T = 32.1$, $A = 29.0$, $C = 22.7$, $G = 14.2$, indicating the typical bias against guanine on the light strand of mitochondrial DNA (Kocher *et al.*, 1989). In addition, the average ratio of transitional to transversional distances per site (R) determined using the Kimura 2-parameter model was 3.27, strongly supporting a mitochondrial origin of obtained sequences (Zhang & Hewitt, 1996). Of the 682 bp obtained, 109 sites were variable, and 103 of these were parsimony informative. The mean uncorrected *p*-distances within

Table 2 Genetic diversity measures of species and populations of *Salamandrina perspicillata* (*S. per.*) and *Salamandrina terdigitata* (*S. ter.*)

	Cyt *b*						POMC							RAG-1						
Site	N	H	Ht	*h*	*k*	*p*	N	H	Ht	*h*	*k*	*p*	H_O	N	H	Ht	*h*	k	p	H_O
RR	18	1	I	–	–	–	17	1	I	–	–	–	–	16	5	I, II, III, IV, V	0.615	3.23	0.515	0.437
BC	15	2	I, II	0.133	0.133	0.02	9	1	I	–	–	–	–	9	3	I, IV, V	0.621	3.954	0.631	0.778
MT	18	1	I	–	–	–	9	1	I	–	–	–	–	10	3	I, IV, V	0.589	3.537	0.564	0.6
ER	21	4	I, VI, VII, VIII	0.557	0.6570	0.096	12	1	I	–	–	–	–	10	3	I, IV, V	0.637	4.032	0.643	0.6
VO	16	1	I	–	–	–	13	2	I, II	0.077	0.077	0.020	0.077	10	3	I, IV, V	0.695	4.168	0.666	0.7
CM	16	2	I, III	0.4	0.4	0.006	17	1	I	–	–	–	–	19	3	I, IV, V	0.582	3.76	0.00.6	0.526
S. per.	104	6	I, II, III, VI, VII, VIII	0.232	0.259	0.038	77	2	I, II	0.013	0.013	0.003	0.013	74	5	I, II, III, IV, V	0.675	4.05	0.647	0.581
MR	55	4	I, IV, V, X	0.383	32.339	4.742	55	2	I, VII	0.238	1.188	0.311	0.273	55	5	I, V, VI, VII, VIII	0.604	2.726	0.435	0.636
ST	24	3	IX, X, XI	0.236	0.243	0.036	14	1	VII	–	–	–	–	14	3	VI VII, VIII	0.455	0.772	0.123	0.286
VC	18	2	X, XII	0.111	0.111	0.016	17	2	IV, VII	0.299	0.599	0.157	0.235	15	2	VI, VII	0.186	0.372	0.059	0.2
FO	21	1	XIII	–	–	–	9	1	IV	–	–	–	–	14	3	VI, VII, VIII	0.14	0.209	0.033	0.071
FS	19	1	X	–	–	–	14	6	I, III, IV, V, VI, VII	0.437	1.415	0.371	0.286	11	2	VI, VII	0.519	1.039	0.166	0.545
S. ter.	82	5	IX, X, XI, XII, XIII	0.456	1.64	0.24	54	6	I, III, IV, V, VI, VII	0.441	1.026	0.269	0.148	54	3	VI, VII, VIII	0.349	0.643	0.013	0.258

Number of haplotypes (H), haplotypes (Ht), haplotype diversity (*h*), average number of nucleotide differences (*k*), nucleotide diversity per site ($p \times 10^{-2}$), and observed heterozygosity for the nuclear markers (H_O). See Table 1 for other abbreviations.

each species (excluding individuals from the Morcone population) were 0.0004 for *S. perspicillata*, 0.0024 for *S. terdigitata* and 0.145 for between-species comparisons. Across all populations analyzed, 13 distinct haplotypes were found that were not shared between the two species. Six haplotypes (Cyt b_{I}, Cyt b_{II}, Cyt b_{III}, Cyt b_{VI}, Cyt b_{VII} and Cyt b_{VIII}) were restricted to populations of *S. perspicillata*, whereas five haplotypes (Cyt b_{IX}, Cyt b_{X}, Cyt b_{XI}, Cyt b_{XII} and Cyt b_{XIII}) were diagnostic for *S. terdigitata*. Two haplotypes, Cyt b_{IV} and Cyt b_{V}, were present only in the Morcone population (for a detailed description of the haplotype occurrence per population, see Table 2 and Fig. 1). Whereas Cyt b_{I} was the most common haplotype found in *S. perspicillata*, present in every population and even fixed in three populations (RR, MT and VC; see Table 2 and Fig. 1a), the most common haplotype for *S. terdigitata* was Cyt b_{X}, which was fixed in the Fiumara Samo (FS) population. Salamanders from Foresta were fixed for haplotype Cyt b_{XIII}, which differs by four inferred mutational steps from the common Cyt b_{X} haplotype.

For POMC, 382 bp were analyzed from 80 individuals of *S. perspicillata*, 54 individuals of *S. terdigitata* and all 55 individuals from Morcone. Overall, six polymorphic sites were encountered, of which five were parsimony informative. Only a single individual of *S. perspicillata* from Valle Ota was heterozygous at a single position (haplotype $\mathrm{POMC_{II}}$), whereas all remaining individuals analyzed were homozygous. Ranging from exhibiting a single heterozygous site to five heterozygous sites per individual, eight individuals were found to be heterozygous in *S. terdigitata*. Overall, seven distinct haplotypes for the POMC gene were determined unambiguously with PHASE (Fig. 2). Five POMC haplotypes were restricted to populations of *S. terdigitata* (see Table 2 and Fig. 1b). No diagnostic haplotypes were found for *S. perspicillata*: all but one individual of *S. perspicillata* were fixed for haplotype $\mathrm{POMC_I}$, but this haplotype was also found in *S. terdigitata* in the FS population, present in one homozygous and in one heterozygous individual.

We analyzed 627 bp of the RAG-1 gene from 74 individuals of *S. perspicillata*, 54 individuals of *S. terdigitata* and all 55 individuals from Morcone. Overall, we detected 12 polymorphic sites within this gene segment, of which eleven were parsimony informative. Most of the individuals of *S. perspicillata* harbored at least one and at most seven heterozygous positions. In contrast, only a quarter of the individuals of *S. terdigitata* exhibited one or two heterozygous positions, whereas the remainder had none. Altogether, eight distinct haplotypes were unambiguously identified with PHASE (Fig. 2). Five haplotypes were diagnostic for *S. perspicillata*, and three haplotypes were specific for *S. terdigitata* (Table 2). We were not able to detect any haplotype that was shared between populations of the two species.

The average pairwise population differentiation was greater for *S. terdigitata* than for *S. perspicillata* across all markers (Cyt *b*: $F_{\mathrm{ST}}\mathrm{per} = 0.12 \pm 0.101$, $F_{\mathrm{ST}}\mathrm{ter} = 0.49 \pm 0.533$; POMC: $F_{\mathrm{ST}}\mathrm{per} = 0.00 \pm 0.007$, $F_{\mathrm{ST}}\mathrm{ter} = 0.45 \pm 0.398$ and RAG-1: $F_{\mathrm{ST}}\mathrm{per} = 0.07 \pm 0.099$, $F_{\mathrm{ST}}\mathrm{ter} = 0.13 \pm 0.139$; see also Tables 3 and 4).

Evidence for hybridization in the Morcone population

Among the 55 individuals analyzed for the three gene segments, several combinations of mitochondrial haplotypes and nuclear genotypes clearly represented molecular evidence of hybridization between *S. perspicillata* and *S. terdigitata* (see Table 5). With respect to mitochondrial sequences, haplotype Cyt b_{I}, which is diagnostic for *S. perspicillata*, was found in 44 individuals (80%), and haplotype Cyt b_{X}, which is diagnostic for *S. terdigitata*, was found in the remaining 11 individuals (i.e. 20%). Haplotypes Cyt b_{IV} and Cyt b_{V} were found only in this population, but in just one individual each. Hybridization of individuals was assigned as follows (see Table 5): 10 individuals (18%) showed no sign of hybridization and were potentially pure *S. perspicillata*; one individual (0.02%) was potentially a

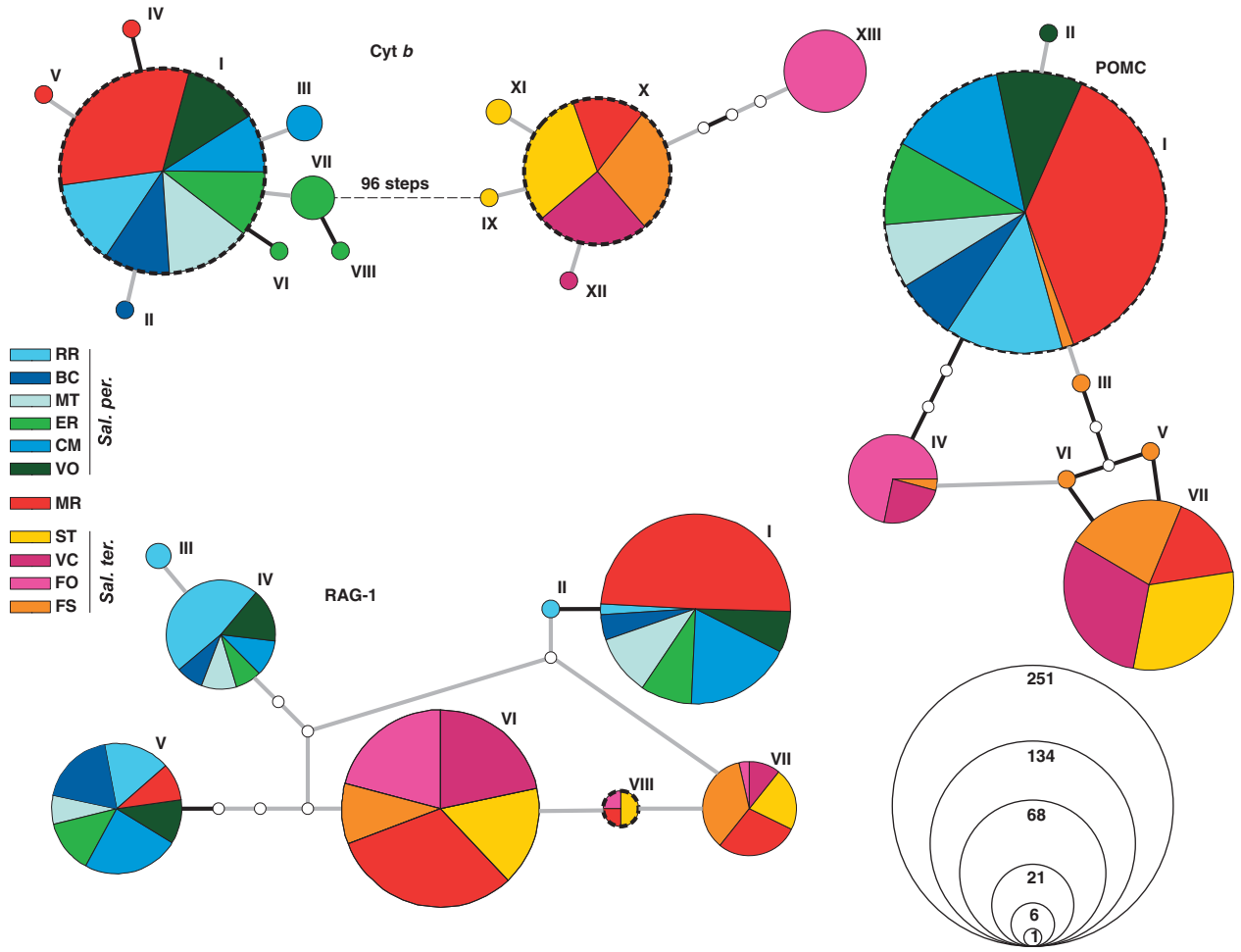

Figure 2 Haplotype networks of *Salamandrina perspicillata* and *Salamandrina terdigitata* for the Cyt *b*, POMC and RAG-1 genes. Connections between haplotypes equal one mutational step unless indicated otherwise. Gray connections indicate synonymous and black connections nonsynonymous mutations. Circle sizes represent number of copies found of each haplotype as indicated in the schematic diagram on the bottom right, for example a total of 251 copies were found of haplotype $POMC_I$. Note that per nuclear gene two haplotypes can be found. Pie slices indicate the frequency of a haplotype in a given population. Missing haplotypes are indicated by small open circles. A dotted line around a haplotype indicates its inferred ancestry.

Table 3 Pairwise F_{ST} estimates based on Cyt *b* for eleven populations of *Salamandrina*

	RR	BC	MT	ER	VO	CM	MR	ST	VC	FO
RR										
BC	0.013									
MT	0	0.013								
ER	**0.215**	**0.177**	**0.215**							
VO	0	0.004	0	**0.202**						
CM	**0.215**	0.154	**0.215**	**0.213**	0.200					
MR	0.110	0.101	0.110	**0.117**	0.104	0.106				
ST	**0.999**	**0.998**	**0.999**	**0.996**	**0.999**	**0.997**	**0.734**			
VC	**1**	**0.999**	**1**	**0.996**	**0.999**	**0.998**	**0.717**	0.014		
FO	**1**	**1**	**1**	**0.997**	**1**	**0.999**	**0.731**	**0.969**	**0.987**	
FS	**1**	**0.999**	**1**	**0.997**	**1**	**0.998**	**0.720**	0.016	0.003	**1**

Significant values ($P<0.05$) are in bold. Enboxed values represent within-species comparisons. See Table 1 for other abbreviations.

Table 4 Population pairwise F_{ST} estimates based on RAG-1 (below the diagonal) and POMC (above the diagonal)

	RR	BC	MT	ER	VO	CM	MR	ST	VC	FO	FS
RR	–	0	0	0	0.011	0	**0.076**	**1**	**0.936**	**0.845**	**1**
BC	**0.165**	–	0	0	−0.015	0	0.055	**1**	**0.917**	**0.799**	**1**
MT	**0.240**	**0.143**	–	0.000	−0.015	0	0.055	**1**	**0.917**	**0.799**	**1**
ER	**0.194**	0.026	−0.017	–	−0.003	0	0.065	**1**	**0.925**	**0.820**	**1**
VO	**0.094**	0.030	−0.003	−0.030	–	0.011	**0.068**	**0.993**	**0.921**	**0.818**	**0.985**
CM	**0.241**	0.064	−0.010	−0.036	0.001	–	**0.076**	**1**	**0.936**	**0.845**	**1**
MR	**0.375**	**0.275**	**0.093**	**0.119**	**0.150**	**0.115**	–	**0.796**	**0.751**	**0.663**	**0.685**
ST	**0.506**	**0.481**	**0.534**	**0.473**	**0.452**	**0.447**	**0.307**	–	**0.135**	**0.139**	**1**
VC	**0.554**	**0.552**	**0.611**	**0.550**	**0.526**	**0.509**	**0.369**	0.047	–	**0.044**	**0.772**
FO	**0.570**	**0.573**	**0.630**	**0.569**	**0.545**	**0.525**	**0.386**	**0.109**	−0.018	–	**0.613**
FS	**0.446**	**0.396**	**0.443**	**0.381**	**0.362**	**0.368**	**0.246**	0.053	**0.257**	**0.343**	–

Significant values ($P<0.05$) are in bold. Enboxed values represent within-species comparisons. See Table 1 for other abbreviations.

Table 5 Assignments of 55 salamanders from the hybrid population Morcone to the species and hybrid categories based on diagnostic haplotypes

Absolute number of individuals	Cyt-*b*		RAG-1		POMC	
	per	*ter*	*per*	*ter*	*ter*	Species/hybridization category
10	+		+ +			*Salamandrina perspicillata*
1		+		+ +		*Salamandrina terdigitata*
5	+		+	+	+	F1 with *S. perspicillata* mother
16	+		+	+		F1 with *S. perspicillata* mother
4		+	+	+	+	F1 with *S. terdigitata* mother
3		+	+	+		F1 with *S. terdigitata* mother
4	+		+ +		+	Unspecified background
2	+			+ +	+	Unspecified background
7	+			+ +		Unspecified background
3		+	+ +			Unspecified background

Cyt *b* is mono-allelic, whereas RAG-1 is di-allelic for an individual. As we found no diagnostic alleles of POMC for *S. perspicillata* and no individuals with two *S. terdigitata*-specific alleles were found, POMC is indicated only for *S. terdigitata* as mono-allelic state.

S. terdigitata specimen; 21 individuals may have been first generation hybrids with a *S. perspicillata* mother (38%); seven others (13%) may have been first generation hybrids with a *S. terdigitata* mother; 16 individuals (29%) had mitochondrial haplotypes specific to one species and nuclear genotypes with alleles specific to the other species. This category of unspecified genetic background (Table 5) indicates that first-generation hybrid individuals must have successfully backcrossed with one of the parental species.

Discussion

Along with the Iberian–North African and the Pontic–Mediterranean areas, the Apennine peninsula is regarded as one of the major southern glacial refugia in Europe (Taberlet *et al.*, 1998; Schmitt, 2007). For many taxa, such as beetles (genus *Tomicus*; Horn *et al.*, 2006), lizards (genus *Podarcis*; Podnar, Mayer & Tvrtkovic, 2005), anurans (genus *Bombina*; Canestrelli *et al.*, 2006*a*), salamanders (genus *Salamandra*; Steinfartz, Veith & Tautz, 2000) and pond turtles (genus *Emys*; Fritz *et al.*, 2005), populations in southern Italy are genetically more diverse and differentiated than northern populations. This pattern suggests that refugial populations of these species survived in southern Italy during periods of unfavorable climatic conditions and expanded northward during warmer interglacial periods.

Amphibian species of the Apennine peninsula have been intensively studied for their phylogeographic patterns. Three other endemic amphibian species with geographic ranges similar to that of *Salamandrina* can be found in Italy: *Hyla intermedia* (Lapini, 2007), *Rana italica* (Picariello, Guarino & Bernini, 2007) and *Bombina pachypus* (Guarino, Picariello & Venchi, 2007), although the latter may not be a separate species (see Hofman *et al.*, 2007). Among these taxa, very different phylogeographic patterns have been identified.

In *B. pachypus*, maximal sequence divergence among Cyt *b* haplotypes was found to be only 1%, still providing genetic differentiation support for a glacial refugium in southern Italy (Calabria; Canestrelli *et al.*, 2006*a*). During the Pleistocene, this region was repeatedly split by two grabens, indicated by the Crati-Sibari (CS) and Catanzaro (CA) Plains, which functioned as barriers to dispersal and resulted in allopatric differentiation of populations between them. Postglacial admixture has added to the genetic

diversity found in the Calabrian populations of this species (Canestrelli *et al*., 2006*a*).

Sequence divergence between the two mitochondrial Cyt *b* lineages of *R. italica* was found to be 1.3%, with two distinct glacial refugia, one on the Aspromonte massif at the very tip of Calabria and the other ranging from the Volturno River to the Sila massif in central Calabria (Canestrelli, Cimmaruta & Nascetti, 2008).

A very different pattern was found for *H. intermedia*: three distinct glacial refugia were identified, including the Padanovenezian Plain, the region north of the CS Plain along the southern edge of the northern Apennines and the area south of the CS Plain. Divergence between the northern and the central and southern groups of Cyt *b* haplotypes was fairly high (9.7% uncorrected *p*-distance), resembling that found between several congeneric species of salamander (discussed in Canestrelli *et al*., 2006*b*); genetic divergence at the nuclear level (assayed by allozyme loci) was clearly below that of congeneric amphibian species ($D_{Nei} = 0.07$; Canestrelli, Cimmaruta & Nascetti, 2007).

Our data show a strong genetic differentiation at both the mitochondrial and nuclear levels between northern (*S. perspicillata*) and southern (*S. terdigitata*) spectacled salamanders. Fixed F_{ST} values for the mitochondrial marker (see Table 3) in combination with high F_{ST} values for nuclear genes (F_{ST} ranging from 0.5 to 1; see Table 4) are in accordance with previously observed average genetic distances based on allozyme loci ($D_{Nei} = 0.47$; Nascetti *et al*., 2005) and mitochondrial sequence divergence (Mattoccia *et al*., 2005; Nascetti *et al*., 2005). Further, the detailed phylogeographic analysis of our study now provides the first direct evidence of ongoing genetic hybridization between these two species in a contact population. Of the 55 individuals analyzed from the Morcone population, only 11 (20%) showed no sign of hybridization, whereas the majority (80%) were first-generation hybrids and backcrossed individuals (see Table 5 for details). These findings demonstrate that despite a long-lasting separation (at least several million years) that resulted in strong genetic differentiation, hybridization between two species can still result in the formation of viable and fertile offspring.

Classically, hybridization has been seen as an accidental phenomenon associated with destructive forces leading to maladapted phenotypes. For example, in the case of *Bombina*, hybrid individuals between *B. variegata* and *B. bombina* are only able to survive within a small tension zone between the distribution ranges of these species and are unable to compete with either parental genotype/phenotype in its native environment (Kruuk, Gilchrist & Barton, 1999). At the moment, it is unclear whether hybridization in *Salamandrina* has resulted in a stable hybrid zone or what the dimensions of such a zone would be. Intermixed mitochondrial haplotypes of both species have been found in another population (Cusano Mutri) that is in close geographic proximity to Morcone (Romano *et al*., 2009*b*). This may suggest that hybridization is not restricted to a single population and might be common where both species come into contact. If hybridization in the contact zone is common, however, it will be of great interest to identify the separation mechanisms (both pre- and post-zygotic) that prevent the intermixing of the species on a larger scale.

Because *Salamandrina* was, until recently, considered to be a single species, no one has yet attempted to identify possible ecological differentiations between the populations of the recently distinguished species. Whereas populations of *S. perspicillata* have been studied intensively for differences in breeding behavior and life-history traits (see Della Rocca, Vignoli & Bologna, 2005; Angelini *et al*., 2008; Angelini, Antonelli & Utzeri, 2010*a*), such detailed information is lacking for *S. terdigitata*. Such studies, however, are critical for investigations into whether niche separation in *Salamandrina* prevents the spread of hybridized individuals as in the case of *Bombina*, thus keeping both species genetically separate from one another. We therefore strongly suggest not only further analysis of the dimensions and fine-scale genetic structure of the potential hybrid zone between *S. perspicillata* and *S. terdigitata*, but also investigation the possible role of ecological adaptation in affecting the outcome and patterns of hybridization in this system.

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