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# Ecogeographic variation of body size in the spectacled salamanders (*Salamandrina*): influence of genetic structure and local factors

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## ABSTRACT

**Aim** The patterns and causes of ecogeographical body size variation in ectotherms remain controversial. In amphibians, recent genetic studies are leading to the discovery of many cryptic species. We analysed the relationships between body size and climate for a salamander (*Salamandrina*) that was recently separated into two sibling species, to evaluate how ignoring inter-specific and intraspecific genetic structure may affect the conclusions of ecogeographical studies. We also considered the potential effects of factors acting at a local scale.

**Location** Thirty-four populations covering the whole range of *Salamandrina*, which is endemic to peninsular Italy.

**Methods** We pooled original data and data from the literature to obtain information on the snout–vent length (SVL) of 3850 *Salamandrina* females; we obtained high-resolution climatic data from the sampled localities. We used an information-theoretic approach to evaluate the roles of climate, genetic features (mitochondrial haplogroup identity) and characteristics of aquatic oviposition sites. We repeated our analyses three times: in the first analysis we ignored genetic data on intraspecific and interspecific variation; in the second one we considered the recently discovered differences between the two sibling species; in the third one we included information on intraspecific genetic structure within *Salamandrina perspicillata* (for *Salamandrina terdigitata* the sample size was too small to perform intraspecific analyses).

**Results** If genetic information was ignored, our analysis suggested the existence of a relationship between SVL and climatic variables, with populations of large body size in areas with high precipitation and high thermal range. If species identity was included in the analysis, the role of climatic features was much weaker. When intraspecific genetic differences were also considered, no climatic feature had an effect. In all analyses, local factors were important and explained a large proportion of the variation; populations spawning in still water had a larger body size.

**Main conclusions** An imperfect knowledge of species boundaries, or overlooking the intraspecific genetic variation can strongly affect the results of analyses of body size variation. Furthermore, local factors can be more important than the large-scale parameters traditionally considered, particularly in species with a small range.

## Keywords

Amphibians, Bergmann's rule, body size, ecogeographical variation, haplotype, Italy, local factors, mitochondrial DNA, Salamandridae, *Salamandrina*.

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## INTRODUCTION

The relationship between climate and body size, known as Bergmann's rule, is one of the best-known ecogeographical generalizations. Bergmann's rule states that, within species or among closely related species, body size is greater in cold climates and, consequently, at high latitudes (Bergmann, 1847; Mayr, 1956; see also Watt *et al.*, 2010). Several mechanisms have been suggested to explain ecogeographical variation in body size (see Blackburn *et al.*, 1999; Litzgus *et al.*, 2004; and references therein for discussions). For endotherms, the hypothesis most often proposed suggests that large body size reduces the surface-to-volume ratio and may help thermoregulation in cold environments (Mayr, 1956). However, Bergmann's rule as a direct response to thermal environment has been questioned by several authors, who have argued that factors other than environmental temperature (e.g. sexual selection, predation, competition, type of food, age at sexual maturity, moisture, precipitation, species richness) may impose a stronger selection on body size (e.g. James, 1970; Calder, 1984; Butler & King, 2004; Yom-Tov & Geffen, 2006; Meiri *et al.*, 2007; Gaston *et al.*, 2008). Bergmann's rule was originally formulated for endothermic vertebrates (Bergmann, 1847; Watt *et al.*, 2010). More than one century after its original definition, it has been proposed that the same ecogeographical pattern may also be present in ectothermic vertebrates (Ray, 1960; Millien *et al.*, 2006); however, the applicability of Bergmann's rule to ectotherms remains controversial, as there is no simple mechanism explaining how large body size can improve the thermal physiology of individuals living in cold environments. Proposed hypotheses explaining ecogeographical variation in ectothermic vertebrates include heat balance, water availability, starvation resistance and length of the growing season (see Ray, 1960; Lindsey, 1966; Ashton & Feldman, 2003; Olalla-Tárraga & Rodríguez, 2007; Cvetković *et al.*, 2009; Ficetola *et al.*, 2010). Furthermore, patterns consistent with Bergmann's rule have been found in only some of the ectotherms analysed (Ashton, 2002a; Ashton & Feldman, 2003; Millien *et al.*, 2006; Olalla-Tárraga *et al.*, 2006; Adams & Church, 2008). For example, in amphibians some studies have observed a positive relationship between body size and elevation/latitude, at both the intraspecific and the interspecific level (Ashton, 2002a; de Queiroz & Ashton, 2004; Olalla-Tárraga & Rodríguez, 2007; Adams & Church, 2008), but a recent meta-analysis suggested that the negative relationship between population-level body size and temperature is lacking in most amphibians (Adams & Church, 2008). Nevertheless, in many cases the body size of amphibians covaries significantly with temperature and water availability, and the pattern of variation is often dependent on the life history features of the target taxa (Olalla-Tárraga & Rodríguez, 2007; Ficetola *et al.*, 2010).

Bergmann (see translations in James, 1970; Watt *et al.*, 2010) originally stated that the rule is observable mainly among closely related species, and the effects of the rule in

subspecies are not very apparent. Subsequently, Rensch (1938) and Mayr (1942, 1956) revised the original formulation of Bergmann's rule, suggesting that patterns of body size can be explained as adaptations of populations within species (also named neo-Bergmann's rule or James's rule; James, 1970; see also Blackburn *et al.*, 1999; Wilson, 2009). In practice, relationships between body size and temperature have been observed at multiple taxonomic levels: among communities living in different climates (Olson *et al.*, 2009), among species within lineages (Wilson, 2009), and among populations within species (e.g. Ashton, 2002a,b; Angilletta *et al.*, 2004a; Meiri *et al.*, 2007; Ficetola *et al.*, 2010). At both the intraspecific and interspecific levels, phylogenetic relationships among populations or among species can have profound confounding effects on body size (Pincheira-Donoso *et al.*, 2008), and should not be overlooked (Angilletta *et al.*, 2004b; Gaston *et al.*, 2008; Pincheira-Donoso *et al.*, 2008). This poses a further challenge to the study of ecogeographical variation of body size (Gaston *et al.*, 2008), as we have information on intraspecific phylogenetic relationships for only a subset of species.

In amphibians, the actual levels of intraspecific and interspecific diversity are not fully appreciated. Recent analyses suggest that the currently described amphibian species represent half or less than half of the actual species richness (Vieites *et al.*, 2009). Even in the best-known areas, the use of genetic markers is leading to the discovery of cryptic species; many of these newly described species are within already described taxa, for which deep substructuring was previously unrecognized (e.g. Dubois *et al.*, 2005, and references therein). Taxonomic uncertainties can have important consequences for the analysis of ecogeographical variation. For instance, an analysis of ecogeographical variation of body size in a taxon that overlooks the fact that the taxon is actually composed of multiple cryptic species might lead to misleading conclusions. However, the effects of ignoring phylogenetic relationships, and of confusing intraspecific and interspecific variation, remain little explored (Gaston *et al.*, 2008).

In this study, we investigated the relationships between body size and environmental variables in the Italian endemic salamanders *Salamandrina*. This genus was traditionally believed to be composed of a single, monotypic species (e.g. Zuffi, 1999). However, recent genetic studies, using both mitochondrial (mt) and nuclear (n) DNA markers, revealed that *Salamandrina* is actually composed of two parapatric cryptic species, *Salamandrina perspicillata* and *Salamandrina terdigitata*. The genetic divergence is among the highest exhibited among congeneric species within the salamandrid family, and is the result of an ancient cladogenetic event (4.5–10 Ma; Mattocchia *et al.*, 2005; Nascetti *et al.*, 2005; Canestrelli *et al.*, 2006; Hauswaldt *et al.*, 2008). This long-term isolation and genetic drift caused population divergence at the genetic species-level uncoupled from comparable change in phenotypic diversity (Winker, 2009). Strong genetic divergence unaccompanied by morphological divergence is frequent in amphibians (Wake *et al.*, 1978; Highton, 2000; Maletzky *et al.*,

2008), but cryptic species are evenly distributed among animal taxa and biogeographical regions (Pfenninger & Schwenk, 2007), and may result from both recent and old speciations (Bickford *et al.*, 2007; Elmer *et al.*, 2007). As for many other cryptic species, recent analyses show that the two *Salamandrina* species can be identified also on the basis of morphology (Romano *et al.*, 2009a; Angelini *et al.*, 2010), and thus in the future the two species could be referred to as pseudo-cryptic or pseudo-sibling species (see Sáez & Lozano, 2005).

The aims of this study were twofold. First, we investigated whether climatic or local factors can explain ecogeographical variation of body size in *Salamandrina*. Second, we evaluated how ignoring intraspecific and interspecific phylogenetic structure can affect the results of the analysis of body size variation. We performed our analyses at three levels. In the first analysis we assumed that *Salamandrina* is a single species, and we ignored any genetic data on the existence of the cryptic species. This 'naïve' analysis corresponds to the only one that could be performed before the availability of genetic data (i.e. before 2005); indeed, this is the only analysis that can be performed for most species (Gaston *et al.*, 2008). In the second analysis, we considered the differences between the two sibling species. Finally, in the third analysis, we also included information on intraspecific genetic structure. Comparing the results of these three analyses allows us to evaluate how inter- and intraspecific variation can affect body size variation and bias the results of ecogeographical studies. As factors other than climate can affect body size (e.g. Meiri *et al.*, 2007), we also considered local factors (i.e. types of aquatic oviposition sites), because survival probabilities could differ among populations breeding in running waters and those spawning in still waters (Angelini *et al.*, 2008), and therefore local factors may influence age structure, which in turn is related to body size in salamanders (Bovero *et al.*, 2006). Several mechanisms involving multiple climatic variables have been proposed to explain ecogeographical variation in the body size of amphibians (Ficetola *et al.*, 2010; see section 'Climatic variables and hypotheses'). For this reason, in our analyses we considered multiple environmental variables, integrated within an information-theoretic framework.

## MATERIALS AND METHODS

### Study species

The Italian endemic genus *Salamandrina* (Fitzinger, 1826) has been separated recently, on the basis of genetic evidence, into two species (Mattocchia *et al.*, 2005; Nascetti *et al.*, 2005; Canestrelli *et al.*, 2006): the northern spectacled salamander, *S. perspicillata* (Savi, 1821), and the southern spectacled salamander, *S. terdigitata* (Bonnaterre, 1789). Detailed information on the distribution of both species, inferred from genetic evidence, and their morphological differences, is available in Romano *et al.* (2009a). Phylogeographic analysis of three mitochondrial genes encoding the 12S and 16S ribosomal RNA and cytochrome *b* (1324 bp) of both species

showed that *Salamandrina* haplotypes cluster into a number of haplogroups. Three and two mitochondrial haplogroups were found in *S. perspicillata* and in *S. terdigitata*, respectively (Mattocchia *et al.*, 2005; Romano, 2007). In this study, each population was attributed to the corresponding haplogroup, taking into consideration the genetic data available. For populations for which no first-hand data on mtDNA sequences were available, the haplogroup was inferred by matching the population location and the known genetic profile of nearby populations, following Mattocchia *et al.* (2005), Romano (2007) and Romano *et al.* (2009a). In *Salamandrina*, haplogroup distribution has a strong geographic structure, and in most cases localities without first-hand genetic data were in regions where only one haplogroup was present. If in a given population more than one haplogroup occurred (three populations, see Table 1), we excluded this population from the analyses considering the haplogroup of the populations.

### Data collection

As a biometric parameter representative of body size, we preferred to use the snout-vent length (SVL) over the total length (i.e. SVL plus tail length). SVL is more reliable because the tail in salamanders can be partially missing or regenerated. We used both original and published literature data (Table 1). Owing to different methods of SVL measurement (cf. Bruce *et al.*, 2000), we used only the data obtained by measuring SVL from the tip of the snout to the anterior margin of the cloacal slit, which is considered to be the standard approach (e.g. Heyer *et al.*, 1994). We considered populations with at least nine measured individuals, and did not consider studies pooling individuals from multiple sites.

When analysing ecogeographical variation, sexes should be considered separately, because they may respond differently to climatic variation (for example for the relationships between maternal investment and climate; Blanckenhorn *et al.*, 2006; Ficetola *et al.*, 2010) and, consequently, analyses performed on unsexed populations could be biased by different sex ratios, in particular in the presence of sexual size dimorphism (Romano *et al.*, 2009b). In *Salamandrina*, though, a method to sex spectacled salamanders has become available only recently (Romano *et al.*, 2009b), and sexes have been considered indistinguishable by external morphology for a long time (e.g. Zuffi, 1999; Angelini *et al.*, 2007). However, only females are found in water bodies during the oviposition phase (Lanza, 1983; Zuffi, 1999). Therefore, we used only data obtained from salamanders sampled in oviposition water bodies and that can be thus considered adult females.

By pooling original and literature data, we obtained SVL from 3850 individuals representing 34 populations of *Salamandrina* (Table 1). The study populations covered the whole geographical and almost the whole elevational range of both species (Fig. 1). Twenty-six populations were of *S. perspicillata*, seven populations were of *S. terdigitata* and one population was in the area where haplotypes of both species were found in the same population (see Romano *et al.*, 2009a). Geographic

**Table 1** Details of the sampling localities in Italy and populations of *Salamandrina perspicillata* (SP) and *Salamandrina terdigitata* (ST) considered [size (snout–vent length, SVL) is expressed in mm]. A given *S. perspicillata* haplogroup was attributed to the corresponding population taking into consideration the available genetic data (analysed), or taking into consideration genetic data available for nearby populations (inferred) (see text). If more than one haplogroup occurred in a given population, the most common is reported in bold. Haplogroups of *S. terdigitata* are not reported because they were not used in the analyses owing to the small sample size available for this species (see text). The distribution of populations is shown in Fig. 1. Locality names are provided together with the official code for Italian provinces.

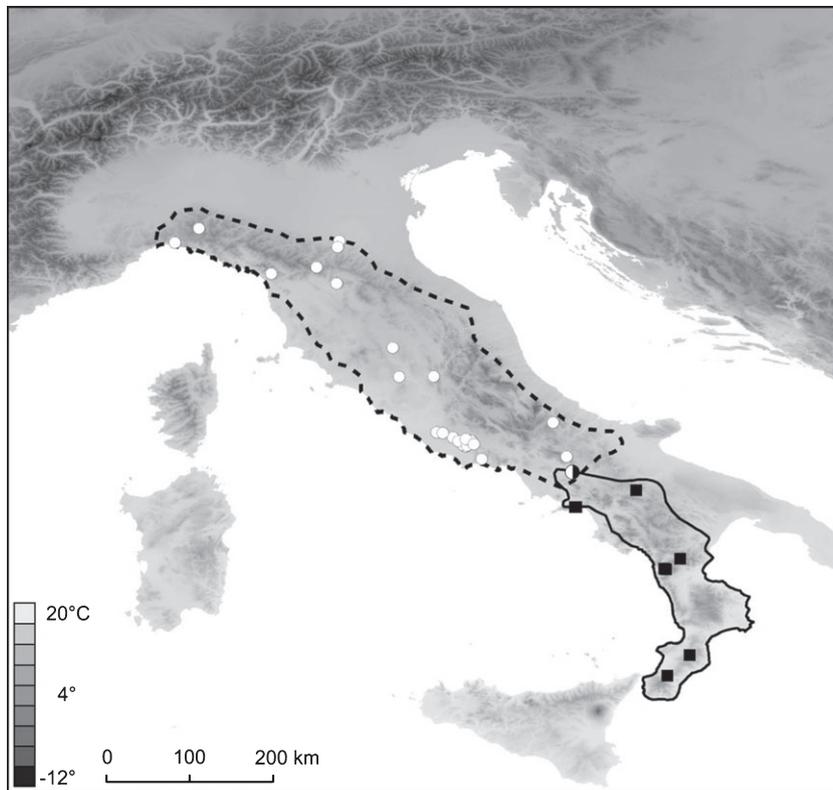
Species	Locality	Altitude (m a.s.l.)	Aquatic habitat type	<i>n</i>	SVL (mm)		Haplogroup	
	Locality, Province				Mean ± SE	References	Analysed	Inferred
SP	Carpineto R., RM	908	Drinking-trough	839	38.5 ± 0.1	Angelini <i>et al.</i> (2008)		1
SP	Sezze, LT	910	Spring	148	41.7 ± 0.1	Angelini <i>et al.</i> (2008)		1
SP	Sezze, LT	680	Spring	147	42.1 ± 0.2	Angelini <i>et al.</i> (2008)		1
SP	Bassiano, LT	935	Stream	559	36.7 ± 0.1	Angelini <i>et al.</i> (2008)		1
SP	Morolo, FR	816	Drinking-trough	277	42.0 ± 0.2	Angelini <i>et al.</i> (2008)	1	
SP	Roccassima, LT	400	Residual pool	24	45.3 ± 0.2	G. Forcina & A. Romano (unpublished data)	2 + 3	
SP	Monte S. Biagio, LT	368	Drinking-trough	107	38.5 ± 0.2	This study	1	
SP	Norma, LT	600	Stream	59	36.1 ± 0.3	This study	1	
SP	Maenza, LA	1047	Drinking-trough	150	39.7 ± 0.2	Romano & Mattoccia (2005)	1	
SP	Morolo, FR	1005	Drinking-trough	30	40.8 ± 0.5	This study	1	
SP	Gorga, RM	707	Drinking-trough	15	39.3 ± 0.5	This study	1 + 2	
SP	Patrica, FR	875	Drinking-trough	11	38.5 ± 0.7	This study	2	
SP	Viterbo, VT	240	Stream	192	37.0 ± 0.3	Angelini <i>et al.</i> (2008)		3
SP	Rocca di Papa, RM	500	Residual pool	11	40.5 ± 0.5	Angelini <i>et al.</i> (2006)		2
SP	Rocca di Papa, RM	810	Tunnel-stream	50	38.8 ± 0.3	Angelini <i>et al.</i> (2006)		2
SP	Guardiaregia, CB	1100	Stream	40	34.9 ± 0.5	This study	2	
SP	Rosello, CH	825	Stream	12	32.7 ± 0.8	M. Pellegrini (unpublished data)	2	
SP	Terni, TE	200	Pond	207	33.6 ± 0.2	Angelini <i>et al.</i> , 2008	2	
SP	Piegara, PE	375	Stream	75	37.3 ± 0.4	Spilinga <i>et al.</i> , 2006	2	
SP	Stazzema, LU	300	Stream	20	35.2 ± 0.8	G. Forcina & A. Romano (unpublished data)	3	
SP	Sambuca Pistoiese, PT	480	Stream	33	38.7 ± 0.3	Romano <i>et al.</i> (2009b)	3	
SP	Sesto F., FI	470	Stream	26	36.8 ± 0.8	S. Vanni (unpublished data)	3	
SP	Casalecchio di R., BO	150	Stream	229	35.5 ± 0.1	Dall'Alpi & Sazzini, 2007	3	
SP	Sasso Marconi, BO	250	Stream	298	41.7 ± 0.1	Dall'Alpi & Sazzini, 2007	3	
SP	Ottone, PC	825	Stream	43	36.6 NA	F. Barbieri & M.A.L. Zuffi (unpublished data)		3
SP	Genoa, GE	425	Stream	22	38.2 NA	F. Barbieri & M.A.L. Zuffi (unpublished data)		3
SP + ST	Morcone, BN	750	Stream	20	32.2 ± 0.3	S. Marta & A. Romano (unpublished data)		
ST	Vietri sul Mare, SA	400	Stream	9	31.9 ± 0.6	M. Mattoccia & S. Marta (unpublished data)		
ST	Rionero in V., PZ	350	Stream	37	33.0 ± 0.4	S. Marta & A. Romano (unpublished data)		
ST	Viggianello, PZ	1280	Stream	13	34.0 NA	E. Sperone (unpublished data)		
ST	Orsomarso, CS	278	Stream	15	36.2 NA	E. Sperone (unpublished data)		
ST	Orsomarso, CS	350	Stream	108	34.7 NA	E. Sperone (unpublished data)		
ST	Serra S. Bruno, VV	850	Stream	13	31.8 ± 0.7	This study		
ST	Oppido M., RC	500	Stream	11	30.9 ± 0.8	This study		

NA, data not available.

coordinates were directly measured by GPS (GARMIN eTrex H-Sensitivity Personal Navigator) or indirectly estimated from the 'Portale Cartografico Nazionale' (<http://www.pcn.minambiente.it/PCN/>) on the basis of reported localities or after clarifications provided by the authors.

### Climatic variables and hypotheses

We used WorldClim (Hijmans *et al.*, 2005) at a resolution of 30 arcseconds to obtain a series of climatic features describing the thermal environment of populations, as well as water



**Figure 1** Distribution of the sampled populations of *Salamandrina perspicillata* (white circles) and *Salamandrina terdigitata* (black squares) in Italy. The ranges of species are delimited by a dashed line and by a black line, respectively. The black and white circle represents the location of the population in which *S. terdigitata* and *S. perspicillata* are syntopic (Romano *et al.*, 2009a). The background map shows the average annual temperatures; darker colours indicate colder temperatures. Species ranges were redrawn on the basis of maps from the Global Amphibian Assessment (<http://www.iucnredlist.org/amphibians>) and from Romano *et al.* (2009a).

availability, corresponding to the multiple hypotheses proposed to explain the relationship between body size and climate (see Introduction; reviewed in Ficetola *et al.*, 2010). Climatic features considered were: annual thermal range (difference between maximum temperature of July and minimum temperature of January) (relationship proposed by the endurance hypothesis; Ashton, 2002a); mean annual temperature (heat balance hypothesis; Olalla-Tárraga *et al.*, 2006; Ficetola *et al.*, 2010); and summed annual precipitation (water availability hypothesis; Ashton, 2002a; Olalla-Tárraga *et al.*, 2006). Furthermore, we calculated the number of months with average temperature  $\geq 8^\circ\text{C}$  (months  $\geq 8^\circ$ ), as a proxy for the length of the activity period, as *Salamandrina* start activity when temperature rises above  $8^\circ\text{C}$  (Utzeri *et al.*, 2004) (seasonality hypotheses; Olalla-Tárraga *et al.*, 2006; Ficetola *et al.*, 2010). Analyses performed using a different activity threshold (e.g.  $9^\circ\text{C}$ ) yielded identical results. Months  $\geq 8^\circ$  was strongly correlated with annual temperature (Pearson's correlation,  $r = 0.96$ ,  $n = 34$ ,  $P < 0.001$ ); however, these two variables were never included in the same candidate model (see below) and therefore this correlation did not bias our results. The other pairwise correlations between climatic variables were not significant (all  $P > 0.2$ ).

### Statistical analyses

We used Moran's  $I$  to evaluate whether spatial variation of body size among populations was spatially autocorrelated. We used the  $t$ -test to compare the climatic features of the localities

of the two species, and Levene's test for homogeneity of variance to evaluate whether the range of variation of climatic variables encountered by populations is different between the two species, or between *S. perspicillata* and the whole *Salamandrina* genus (i.e. *S. perspicillata* + *S. terdigitata*). Subsequently, we used generalized linear models (GLMs) to evaluate the factors that may explain spatial variation of SVL. As potential predictors we considered: (1) types of breeding site (stream/still water not stream); (2) the four climatic factors; (3) genetic information defining species identity or the mitochondrial haplogroup observed in the population. To identify the best models describing the relationship between SVL and the potential predictors, we used an information-theoretic approach (Burnham & Anderson, 2002) following the procedure detailed in Denoël *et al.* (2009). First, we built GLMs including all possible combinations of 1–3 environmental variables. We limited our models to a maximum of three independent variables because of the limited sample size. We also tested for interactions, but we did not find that interactions significantly improved the fitness of our models and therefore interactions are not included in our candidate models. For each model, we calculated Akaike's information criterion (AIC) and  $\Delta\text{AIC}$ , the difference in AIC values between the candidate model and the model with the lowest AIC (i.e. the best model). We did not correct AIC for small sample size because analyses suggest that such correction does not improve model selection (Richards, 2005; Raffalovich *et al.*, 2008). However, we repeated the analyses using the correction for small sample size (Burnham & Anderson, 2002)

and obtained equivalent results to the reported ones (results not shown). AIC may select overly complex models (Richards, 2008). Therefore, we considered a complex model as a candidate model only if it had a  $\Delta$ AIC lower than the  $\Delta$ AIC of all its simpler nested models (Richards, 2008). Subsequently, we calculated the AIC weight  $w$  of all candidate models;  $w$  corresponds to the likelihood that a given model is the best among the candidate models, given the data (Burnham & Anderson, 2002). We used standard variance partitioning to evaluate the percentage of variation explained by the independent variables in the best AIC models (Cushman & McGarigal, 2002).

To evaluate the relative roles of climatic features and interspecific and intraspecific genetic differences, we repeated this analysis three times. First, we considered all 34 populations, without taking into account species identity. In this analysis, we considered as potential predictors only breeding-site type and climatic features. This therefore corresponds to a naïve analysis, performed without knowledge of the existence of two divergent cryptic taxa. Second, we included species identity determined using molecular data as a further potential predictor. This analysis therefore evaluated body size differences among *S. terdigitata* and *S. perspicillata*, and the intraspecific variation within the latter. We excluded from this analysis one population in which *S. terdigitata* and *S. perspicillata* are syntopic (Romano, 2007; Romano *et al.*, 2009a; see Fig. 1 and Table 1). Finally, to evaluate the role of intraspecific genetic diversity, we considered the haplogroup observed in populations as a further potential predictor. We performed this analysis for *S. perspicillata* only, because of the small sample size available for *S. terdigitata*; we did not include in this analysis two populations in which more than one haplogroup occurred (total: 24 populations considered). To maximize statistical power, this analysis was performed using both observed and inferred haplogroups (see above). An analysis excluding those populations for which no first-hand genetic data were available (see Table 1) yielded identical results.

The residuals of our best models were not spatially autocorrelated, and therefore it is unlikely that spatial autocorrelation biases our results. However, to evaluate whether spatial autocorrelation may affect our models, we also built error-spatial autoregressive models (error-SAR) (Beale *et al.*, 2010). Error-SARs are autoregressive models incorporating spatial error; simulation and comparative studies have shown that error-SARs are among the best-performing models when dealing with spatial autocorrelation (Kissling & Carl, 2008; Beale *et al.*, 2010). For most models, error-SARs had non-significant values of the spatial autoregressive coefficient  $\rho$  and higher AIC than ordinary least square models (i.e. GLMs). This suggests that error-SARs are unnecessary, and that the results of GLMs are reliable and not biased by autocorrelation. The results of error-SARs were nearly identical to those of GLMs.

We built correlograms in SAM 3.0 (Rangel *et al.*, 2010); we performed all other analyses under the R environment

(R Development Core Team, 2007) using the package SPDEP to build error-SARs.

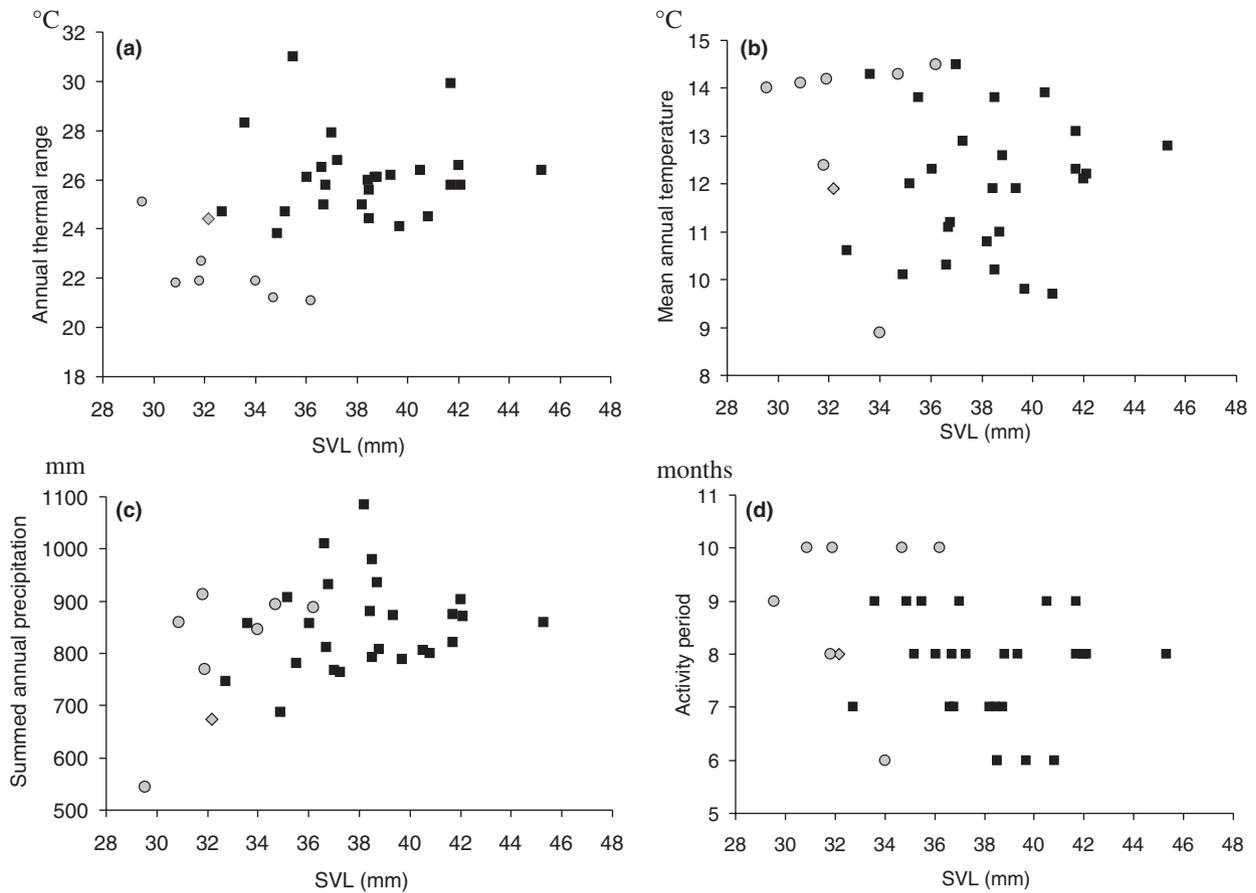
## RESULTS

Spatial autocorrelation of SVL was positive and significant at distances up to 100 km. Above 100 km, spatial autocorrelation was either negative or non-significant (see Fig. S1 in Supporting Information).

*Salamandrina perspicillata* and *S. terdigitata* are exposed to slightly different climatic features (Fig. 2). Populations of *S. perspicillata* live in areas with a larger thermal range ( $t$ -test:  $t_{31} = 5.68$ ,  $P < 0.001$ ) and shorter activity period ( $t_{31} = 2.72$ ,  $P = 0.011$ , Fig. 2a,d). Differences in mean annual temperature and precipitation were not significant (temperature:  $t_{31} = 1.84$ ,  $P = 0.075$ ; precipitation:  $t_{31} = 0.91$ ,  $P = 0.37$ ); for these variables, there was strong overlap between the two species (Fig. 2b,c). However, the range of variation of climatic variables was similar between the two species (Levene's test,  $P > 0.2$  in all comparisons). For all climatic variables, *S. perspicillata* populations encountered most of the variation encountered by the whole genus (thermal range: 73% of the variation of the whole genus; annual temperature: 85%; precipitation: 73%; activity period: 75%) (Fig. 2). Although the range of variation experienced by *S. perspicillata* was slightly lower than that for the whole genus, differences in variance were small and not significant (Levene's test,  $P > 0.2$  in all comparisons).

The GLMs performed by pooling all populations together suggested the existence of a relationship between SVL and climatic variables. For this analysis, the best models indicated that large SVL was associated with populations laying eggs in still waters and living in areas with high precipitation and high thermal range (Table 2a). Simpler models had lower support (Table 2a). The best model, including wetland type, precipitation and thermal range, explained a substantial proportion of the variation in body size ( $R^2 = 0.57$ ). In variance partitioning, type of oviposition site was the variable with the largest independent contribution (39% of variation accounted for); the independent contribution of the two climatic variables (precipitation and thermal range) accounted for 18% of the variance. Thirteen per cent of the variation was explained by the joint effect of aquatic site type and climatic variables.

However, when we included species identity in our analysis, the role of climatic features was much weaker. The best model indicated that the largest SVLs are found in populations of *S. perspicillata* spawning in still waters, living in areas with high annual precipitation (Fig. 2a; Table 2b); this model explained 60% of the variation. In variance partitioning, species identity was the variable with the largest independent contribution (17% of variation accounted for). Type of oviposition site accounted for 15% of the variance; precipitation was the variable with the lowest independent contribution (3.5% of variance accounted for). Twenty-five per cent of the variation was explained by the joint effect of multiple variables.



**Figure 2** The relationship between mean body size (snout–vent length, SVL in mm) of 34 populations of *Salamandrina* and climatic features describing the thermal environment [(a) annual thermal range and (b) mean annual temperature], (c) water availability (summed annual precipitation) and (d) length of the activity period. Black squares, *Salamandrina perspicillata*; grey circles, *Salamandrina terdigitata*; grey diamond, population in which haplogroups of both species are syntopic.

A simpler model, not taking into account the effect of precipitation, had slightly lower support. Models not considering the differences between species or oviposition site type showed very low support (Table 2b).

When we considered also intraspecific genetic differences (haplogroups) within *S. perspicillata*, the best model included oviposition site type and haplogroup identity only (Table 2c). This model explained 39% of the variation within *S. perspicillata* (Table 2c). In variance partitioning, aquatic site type was the variable with the largest independent contribution (16% of variation accounted for). Variation among haplogroups within species (Fig. S2b) accounted for 13% of the variation; the joint effect of aquatic site type and haplogroup accounted for 11% of the variance. A model with slightly lower support did not include intraspecific genetic diversity (Table 2c). Climatic variables were not included in any of the candidate models. Within *S. perspicillata*, there was no correlation between average SVL of populations and climatic variables (Pearson’s correlations: precipitation:  $r = 0.16$ ,  $P = 44$ ; thermal range:  $r = 0.02$ ,  $P = 0.91$ ; temperature:  $r = 0.06$ ,  $P = 0.78$ ; months  $\geq 8^\circ$ ,  $r = 0.08$ ,  $P = 0.70$ ,  $n = 26$  in all correlations).

**DISCUSSION**

In recent years, a growing number of studies have investigated the factors explaining body size variation in amphibians and in other ectotherms, at both the intraspecific and the interspecific level. In principle, analyses at these two levels can have different underlying mechanisms, and the intraspecific genetic structure representing the evolutionary history of populations can have important effects on body size variation (Angilletta *et al.*, 2004b). Despite the fact that genetic studies are rapidly improving our understanding of amphibian systematics, studies of intraspecific and interspecific genetic variation remain limited to a subset of species, mostly in temperate areas (Vences & Köhler, 2006). Our study showed that some body size differences exist between the two *Salamandrina* species (Fig. S2), while relationships within species were less clear, particularly when the species with the largest sample size was analysed in detail (*S. perspicillata*), or when intraspecific phylogenetic data, measured using presumably neutral markers, were taken into account (as in Table 2c). Observing interspecific differences in body size may or may not indicate an effect of climatic variables; the analysis of interspecific ecogeographical

**Table 2** Candidate models analysing the relationship between snout–vent length (SVL) of *Salamandrina perspicillata* and *Salamandrina terdigitata* populations and (a) climatic variables, (b) climatic variables and species identity, (c) climatic variables, species identity and information on mitochondrial haplogroups. All the models perform significantly better than the null model; for brevity, we list only models with  $w$  (AIC weight)  $\geq 0.01$ . For the variable oviposition site type (site type), (–) indicates smaller size in streams.

Rank	K	Candidate model	$\Delta$ AIC	$w$
(a) Models considering climatic variables only (34 populations, both species)				
1	3	Site type (–), Precipitation (+), Thermal range (+)	0.0	0.813
2	2	Site type (–), Thermal range (+)	4.0	0.109
3	2	Site type (–), Precipitation (+)	5.2	0.059
4	1	Site type (–)	7.7	0.018
(b) Models considering also interspecific differences (33 populations, both species)				
1	3	Species, Site type (–), Precipitation (+)	0.0	0.551
2	2	Site type (–), Species	0.6	0.369
3	3	Site type (–), Precipitation (+), Thermal range (+)	7.2	0.053
4	2	Site type (–), Thermal range (+)	7.5	0.013
(c) Models considering also differences among haplogroups (24 populations, <i>S. perspicillata</i> )				
1	4	Haplogroup, Site type (–)	0.0	0.574
2	1	Site type (–)	0.6	0.389
3	3	Haplogroup	3.6	0.088

K, number of parameters in the model;  $\Delta$ AIC, difference between the Akaike information criterion (AIC) value of each model and the AIC value of the best model.

variation requires the comparison of multiple species, taking into account phylogenetic structure (Pincheira-Donoso *et al.*, 2008). On the other hand, taking population as the replication unit allows analysis of intraspecific variation (e.g. Adams & Church, 2008; Ficetola *et al.*, 2010). Intraspecific and interspecific variation can originate through different processes occurring over very different time-scales; for instance, the evolutionary divergence between the study species dates back to the Pliocene (4.7–10 Ma; Mattoccia *et al.*, 2005; Nascetti *et al.*, 2005; Canestrelli *et al.*, 2006), while most populations of each species probably diverged after the Last Glacial Maximum (Romano, 2007; M. Mattoccia *et al.*, submitted). An analysis merging all populations would have confounded intraspecific and interspecific variation, providing conclusions that do not necessarily correspond to the actually ongoing processes. Pooling all populations together suggests a pattern of among-population variation (which might have been interpreted as intraspecific, perhaps recent, variation; Table 2a), while differences are likely to be caused by interspecific, perhaps more ancient, processes.

An imperfect knowledge of species boundaries, or overlooking the intraspecific genetic structure, can therefore affect the results of analyses of body size variation (see also Ashton,

2001). Studies on ecogeographical variation of body size have often observed weaker relationships when phylogenetic relationships are taken into account (Blackburn *et al.*, 1999; Angilletta *et al.*, 2004b). In our study case, the analysis of spatial autocorrelation showed that populations at distances up to 100 km have similar body size (Fig. S1), suggesting that factors occurring approximately at this geographical scale affect body size. However, we found a relationship between body size and climatic parameters only when genetic differences were not taken into account. This occurred because the northern species is larger (Romano *et al.*, 2009a; see Fig. S2), so merging the data across species produced a latitudinal trend. This difference cannot be explained on the basis of the standard version of Bergmann's rule, as northern and southern populations live in areas with similar temperature (Fig. 2). Furthermore, an analysis of European and North American salamanders suggests that larger species are often found in warmer/southern areas (Olalla-Tárraga & Rodríguez, 2007). The importance of climatic parameters was much lower when considering the distinction between the two sibling species, and no climatic parameter was important when genetic variation within *S. perspicillata* was accounted for (Table 2). These findings suggest that intraspecific and interspecific genetic structure is the major cause of body size variation occurring across a large spatial extent. *Salamandrina* has been considered to be a monotypic genus for a long time (Zuffi, 1999); it was only a few years ago that genetic evidence suggested the existence of two distinct species (Mattoccia *et al.*, 2005; Nascetti *et al.*, 2005; Canestrelli *et al.*, 2006). If analyses of ecogeographical body size variation has been carried out before 2005, all populations would have been pooled as belonging to the same species (i.e. a 'naïve analysis') and the results would have supported the existence of an intraspecific ecogeographical pattern of body size variation, related to thermal range and precipitation. However, when phylogenetic factors are considered (i.e. the existence of two species genetically distinct but morphologically similar), the importance of climatic variables in explaining size variation is reduced, and a large amount of the variation is indeed explained by species identity and by the intraspecific genetic structure.

It is possible that precipitation has a role with regard to body size, particularly for *S. terdigitata*, as populations with large body size were partially associated with areas having more precipitation (Fig. 2c, Table 2b). In many amphibians, activity is associated with wet periods (Zug *et al.*, 2001); therefore, high precipitation can be advantageous. For instance, salamanders can be more active in areas with more precipitation, and perhaps forage for prolonged periods. Unfortunately, the small geographic range and the limited sample size available hampered intraspecific analyses on *S. terdigitata*.

The weakness of relationships between body size and climatic factors when taking into account genetic structure can have multiple explanations. First, size clines are strongest for species with large distribution ranges encompassing greater climatic variation (Meiri *et al.*, 2007). Despite our having

sampled the whole range of the genus *Salamandrina*, both the longitudinal and the latitudinal gradient had limited extent (Fig. 1; Table 1). The range of *Salamandrina* extends less than 7° in latitude, and the extent of occurrence (*sensu* IUCN, 2001) of *S. perspicillata* and *S. terdigitata* is about 75,000 km<sup>2</sup> (4° in latitude) and 35,000 km<sup>2</sup> (3° in latitude), respectively. Small geographic ranges are common in amphibians. For instance, the distribution ranges of 70% of anurans investigated by Cooper *et al.* (2008) were smaller than the range of *S. perspicillata*. As ecogeographical variation increases in species with large ranges (Meiri *et al.*, 2007), it is perhaps not surprising that many amphibians do not have a body size trend (Adams & Church, 2008). On the other hand, significant ecogeographical patterns have been found in several amphibians with range extent equal to or smaller than that in *S. perspicillata* (e.g. *Plethodon jordani*, *Desmognathus quadramaculatus*, *Rana sauteri* in de Queiroz & Ashton, 2004; *P. jordani*, *Plethodon aureolus*, *Plethodon metcalfi*, *Plethodon shermani*, *Plethodon teyahalee* in Adams & Church, 2008) and even in amphibians with very small geographic ranges (e.g. *Schistometopum thomense*, with a range extending less than 50 km; Measey & Van Dongen, 2006). Furthermore, in ectotherms, theory predicts patterns conforming to Bergmann's rule only for species that thermoregulate (Olalla-Tárraga & Rodríguez, 2007). Urodeles are reported to have limited thermoregulation ability (reviewed in Brattstrom, 1963; Hutchinson & Dupré, 1992; see also Olalla-Tárraga & Rodríguez, 2007), which might lead to less detectable trends.

Amphibians have indeterminate growth, and modification in longevity/mortality pattern is a further hypothesis to explain patterns of body size variation (Angilletta *et al.*, 2004b; Cvetković *et al.*, 2009). Adolph & Porter (1996) predicted that ectotherms in cold climates mature slowly but attain maturity later; therefore, their model predicts smaller size at a given age in cold climates, but invariant adult body size across climates (Adolph & Porter, 1996). Estimating age in amphibians is extremely complex and time-consuming, and we are aware of only one study integrating large-scale data on age structure within the analysis of amphibian ecogeographical variation (Cvetković *et al.*, 2009). An analysis of common toad (*Bufo bufo*) populations suggested that high-elevation/high-latitude populations can have greater longevity, but the large-scale variations in longevity determined by elevation/latitude are insufficient to determine patterns of body size variation (Cvetković *et al.*, 2009).

Our study suggests that the type of aquatic oviposition site may be a key determinant of body size through its effect on survival. Both species of *Salamandrina* spawn in a variety of aquatic sites (Angelini *et al.*, 2007). Breeding sites may be divided into two main categories: running water (i.e. streams and brooks) and still water (drinking troughs and small springs). Mediterranean streams and brooks are subject to unpredictable and frequent floods in early spring. *Salamandrina* are mainly terrestrial salamanders: females have a limited swimming ability, and can be easily drifted by streams and flooding. Capture–mark–recapture studies have confirmed

that populations breeding in running water suffer higher mortality than populations breeding in lentic water (Angelini *et al.*, 2008). Drifting of reproductive females is known to reduce longevity in natural populations of amphibians (e.g. Ogielska *et al.*, 1999; Tucker *et al.*, 2001; Cogalniceanu & Miaud, 2003; Wheeler & Welsh, 2008), while greater longevity is often observed in predictable and stable habitats (cf. Caetano & Leclair, 1996; Cogalniceanu & Miaud, 2003). Indeed, the populations of *S. perspicillata* with the largest females are from still-water bodies (Romano & Mattocchia, 2005; Angelini *et al.*, 2008; see also Table 1).

A few studies have reported that local factors may obscure large-scale variation trends in body size. For example, in the bear *Ursus arctos*, populations with large body size are found in areas with high food availability, and the effect of trophic resources can be more important than climatic parameters in explaining body size (Meiri *et al.*, 2007). Local features are key factors that are spatially linked to the patches where species live, do not operate along the standard geographical clines, but may be strong predictors of body size (e.g. Meiri *et al.*, 2007; Gaston *et al.*, 2008). Studies on ecogeographical variation usually focus on broad-scale environmental gradients (e.g. climatic features). Our analysis stresses the importance of integrating data collected at multiple spatial scales, as local factors can have overwhelming effects on key parameters such as mortality or growth rate, thus confounding the actual relationships (Ficetola *et al.*, 2007; Meiri *et al.*, 2007). Unfortunately, integrating local factors into ecogeographical studies may be challenging: analyses are often based on large-scale layers (e.g. climate, altitude), obtained through geographic information systems, that cannot capture the variety of microhabitats experienced by animals. Furthermore, multiple local features can affect mortality and growth, but it is difficult to predict *a priori* if a given local feature is important, because the same factor may influence different taxa differently.

Relationships between ecogeographical factors and intra-specific body size variation have intrigued biogeographers for decades. However, multiple factors can covary with the overall pattern of body size. Moreover, local factors can be more important than large-scale parameters, particularly in species with limited range size, such as the two *Salamandrina* species. Actually, most species have small ranges, and this should not be overlooked when searching for generality in ecogeographical patterns (Gaston *et al.*, 2008). Furthermore, our analysis showed that knowledge of genetic structure can be extremely important. It can be impossible to draw robust conclusions when genetic information is lacking, and conclusions can be particularly misleading in the presence of cryptic taxa.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Spatial autocorrelation of body size (snout–vent length, SVL) among populations of *Salamandrina* based on Moran's *I*.

**Figure S2** Body size differences between *Salamandrina terdigitata* and *Salamandrina perspicillata* and among the three haplogroups within *S. perspicillata*.

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## BIOSKETCHES

**A. Romano** is a post-doctoral researcher who uses multiple approaches to examine the ecology, distribution, biogeography and conservation of Italian herpetofauna, for which he is a Red-List assessor for the IUCN. In particular, his research focuses on endemic Italian amphibians.

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