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[1]*CIBIO/InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Instituto de Ciências Agrárias de Vairão, Universidade do Porto, Vairão Portugal;* [2]*Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke Belgium;* [3]*Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, Oviedo Spain;* [4]*Unidad Mixta de Investigación en Biodiversidad (UMIB), CSIC-Universidad de Oviedo-Principado de Asturias, Mieres, Spain*

Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes

Wouter Beukema[1,2], Alfredo G. Nicieza[3,4], André Lourenço[1] and Guillermo Velo-Antón[1]

Abstract

The existence of two or more distinctly coloured phenotypes among individuals of an interbreeding population is known as colour polymorphism. In amphibians, this phenomenon is pervasive among anurans, but rare or absent among salamanders and caecilians, respectively. Here, we examine whether various distinct phenotypes of *Salamandra salamandra* in North Spain, used as a basis to describe the subspecies *S. s. bernardezi* and *S. s. alfredschmidti*, indeed warrant separate taxonomic status or that these co-occur and belong to a single taxon. Based on a sample of 1147 individuals from 27 local populations, six phenotype classes were designated. Although two phenotypes that are attributable to *S. s. alfredschmidti* show some degree of geographical restriction, these co-occur with those representing typical *S. s. bernardezi*. A fifth phenotype class could not be unambiguously attributed to either subspecies due to an overlap in previously suggested diagnostic characteristics. Mitochondrial (cytochrome b) and nuclear (β-fibrinogen) DNA analyses revealed *S. s. alfredschmidti* to be nested within several subclades of *S. s. bernardezi*, without displaying unique lineages. Furthermore, no significant divergence was recovered by means of niche overlap analyses. As a result, we revoke the subspecies status of *S. s. alfredschmidti*, which should be regarded as a junior synonym of *S. s. bernardezi*. The current findings confirm the existence of colour polymorphism in *S. salamandra* and the family Salamandridae, which provides exciting possibilities for future research.

Key words: Colour polymorphism – microgeographical variation – niche – taxonomy – mtDNA – nuDNA

Introduction

Colour polymorphism describes the presence of two or more distinct phenotypes in a single interbreeding population, of which the rarest is too frequent to simply represent the result of recurrent mutation (Huxley 1955). Because divergent phenotypes are often readily observed and registered in their natural environment, polymorphic species have often been used as models to study the fundamental processes that affect genetic variation. The mechanisms that maintain colour polymorphism are, however, complex, and comprise both intrinsic and extrinsic factors (e.g. Hoffman and Blouin 2000; Bond 2007; Noonan and Comeault 2009; Richards-Zawacki et al. 2012). For instance, phenotypic maintenance in the guppy *Poecilia reticulata* (Peters 1859), a polymorphic model species, is influenced by at least apostatic predation, sexual selection, sensory bias and disruptive correlational selection (Gray and McKinnon 2007 and references therein). Whereas genetic colour polymorphism is ubiquitous among birds, habitat diversity, mate choice and behaviour act as strong determinants for the relative abundance of different phenotypes within populations (Roulin 2004; Roulin et al. 2004). In general, prolonged maintenance of several phenotypes within a single species might result in incipient speciation and eventually the evolution of reproductive isolation (Gray and McKinnon 2007; Fisher-Reid et al. 2013).

In amphibians, colour polymorphism is generally expressed by differences in background and eye colour, as well as dorsal patterns (e.g. Hoffman and Blouin 2000; McKnight and Nelson 2007). Anuran species are frequently polymorphic due to which frogs have often been used as models to study colour polymorphism (Hoffman and Blouin 2000; Rudh and Qvarnström 2013). Contrarily to anurans, few salamanders and none of the caecilian species have been described to exhibit colour polymorphism (Wells 2007; Wollenberg and Measey 2009; Petranka 2010), although a large body of literature is available regarding the maintenance of several phenotypes in at least one salamander species, *Plethodon cinereus* (Green 1818) (e.g. Highton 1959; Fitzpatrick et al. 2009; Fisher-Reid et al. 2013; Venesky et al. 2015). As all other polymorphic salamander species have proven to be plethodontids (García-París et al. 2000; Petranka 2010), the phenomenon is considered confined to the family Plethodontidae (but see e.g. Wu et al. 2010). Indeed, previous discoveries of multiple 'morphs' or 'variants' within non-plethodontid salamander species have consistently been followed by taxonomic revisions, based on subsequent evidence which revealed distinct evolutionary histories and/or allopatric occurrence of such 'morphs' (e.g. Nussbaum et al. 1995; Carranza and Wade 2004; Nishikawa et al. 2013). Nevertheless, colour polymorphism seems to occur in populations of *Salamandra salamandra* (Linnaeus 1758), a member of the Salamandridae, although only anecdotal descriptions are available concerning the presence of this phenomenon (Eiselt 1958; Malkmus 1991; Barrio and Fonoll 1997; Pasmans and Keller 2000).

Salamandra salamandra comprises approximately 13 subspecies distributed across most of Europe, although intraspecific differentiation is most pronounced in the Iberian Peninsula (Thiesmeier and Grossenbacher 2004). In this region, Pleistocene climate oscillations coupled with the Iberian physiographic heterogeneity drove cyclic patterns of range contractions and expansions, during which allopatric divergence took place in glacial refugia (Steinfartz et al. 2000; García-París et al. 2003). These allopatric events likely resulted in the distinct phenotypes observed across the Iberian Peninsula, which led to the description of at least 10 subspecies in this area (Montori and Herrero 2004; Thiesmeier and Grossenbacher 2004). As such, *S. salamandra* is highly polytypic, although colour pattern

Corresponding authors: Wouter Beukema (wouter.beukema@gmail.com), Guillermo Velo-Antón (guillermo.velo@gmail.com)
Contributing authors: Alfredo G. Nicieza (agnicz@gmail.com), André Lourenço (andrelourenco300@gmail.com)

variation is generally continuous at local geographical scales (Boulenger 1911) due to which cases of polymorphism have not been explicitly acknowledged (but see e.g. Eiselt 1958; Malkmus 1991). Nevertheless, syntopy of up to four diagnosable phenotypes varying in background colour and presence/absence of colour pattern has been reported from several populations of *Salamandra salamandra bernardezi* in northern Spain (Villanueva 1993; Barrio and Fonoll 1997; Pasmans et al. 2004; Beukema 2006). It remains ambiguous whether the observed variation comprises colour polymorphism (Pasmans and Keller 2000), or actually indicates the presence of multiple taxa (Köhler and Steinfartz 2006). Indeed, despite the presence of typical *S. s. bernardezi* individuals (*sensu* Wolterstorff 1928), *Salamandra salamandra alfredschmidti* was described based on differences in their colour patterns and mitochondrial D-loop sequences in respect to a highly restricted sample of other *S. salamandra* subspecies (Köhler and Steinfartz 2006). Currently, *S. s. alfredschmidti* is considered to occupy a small enclave (Tendi and Marea valleys, central Asturias, northern Spain) within the distribution of *S. s. bernardezi*.

Here, we combine comprehensive phenotypic data, mitochondrial and nuclear DNA analyses and niche overlap tests to explore whether *S. s. alfredschmidti* and *S. s. bernardezi* indeed show geographical and evolutionary divergence or whether both taxa conform a single taxon characterized by colour polymorphism. These analyses are coupled with a comprehensive geographical coverage of both *S. s. alfredschmidti* and *S. s. bernardezi* distributions. We ask specifically whether (1) both subspecies are well diagnosable from each other and show geographical separation, (2) genetic divergence can be identified between them and (3) niche divergence might underlie diversification.

Materials and Methods

A total of 95 locations, mostly across the northern Iberian Peninsula, were visited in order to either collect distribution data of different phenotypes, to gather tissue samples for genetic analyses or to assemble a data set of geographical occurrence records to calibrate niches of *S. s. alfredschmidti* and *S. s. bernardezi*. Accordingly, phenotypic data were gathered at 27 of these 95 sites, tissue samples of several *S. salamandra* subspecies were collected at 34 of the 95 locations (Table 1, Fig. 1a, b), and geographical coordinates for 85 of the 95 sites (the remaining 10 coordinates fell within the same grid cells as already selected coordinates) were used for niche calibration. For a comprehensive overview summarizing all these data, see Fig. 1 and Table 1 and S1.

Distribution assessment and phenotype delimitation

Dorsal photographs were taken to record background colour and pattern of all encountered salamanders from 27 sites spread across central and eastern Asturias, comprising the entire known distribution of *S. s. alfredschmidti* and the eastern half of the *S. s. bernardezi* distribution (Fig. 1c). Despite the fact that four phenotype groups were proposed for *S. salamandra* in this region by Barrio and Fonoll (1997) and Pasmans and Keller (2000), we erected six phenotype groups to deal with all variation documented by dorsal photographs (Table 2; Fig. 2). As phenotype group 6 was erected to contain individuals not assignable to any other category, we did not provide a graphical example of this group in Fig. 2 (see also below). For subsequent genetic and niche overlap analyses, phenotype groups 1 and 2 were regarded to represent typical *S. s. bernardezi* following Wolterstorff (1928). As individuals from phenotype group 3 could be attributed to *S. s. bernardezi* as well as *S. s. alfredschmidti* due to overlapping diagnostic characteristics (Wolterstorff 1928; Köhler and Steinfartz 2006), we regarded this group as an intermediate class which was not ascribed to either subspecies. Phenotype groups 4 and 5 were classified as *S. s. alfredschmidti* following Köhler and Steinfartz (2006). Phenotype group 6 was erected to include individuals that were not attributable to any other of the phenotype groups.

DNA extraction and amplification

Tissue samples of the subspecies *S. s. alfredschmidti*/*S. s. bernardezi* (40), *S. s. bejarae*/*S. s. gallaica* (9) and *S. s. longirostris* (1) were collected in the field at 34 sites across Spain and Portugal (Table 1, S1). The latter two were used in the analyses as outgroups. As no specimens were collected, reference material in the form of tissue samples was deposited in the personal collection of GVA. Individuals belonging to phenotype groups 1 and 2 were classified as *S. s. bernardezi* in all genetic analyses, while those belonging to groups 4 and 5 were ascribed to *S. s. alfredschmidti*. Phenotypic assignment of these individuals was made based on their colour patterns. Genomic DNA was extracted from fresh tissue samples using Genomic DNA Tissue Kit (EasySpin), following the protocol of the manufacturer. Quantity and quality of DNA extract products were assessed on a 0.8% agarose gel. A section of ca. 1400 bp of the mitochondrial genome including the complete *cytochrome b gene b (cyt b*) and a fragment of ca. 700 base pairs of the intron of the nuclear gene β-fibrinogen (β*Fib*) were amplified and sequenced for each sample. The *cyt b* fragment was amplified using primers Glu14100L (forward, 5′ GAA AAA CCA AYG TTG TAT TCA ACT ATA A 3′) and Pro15500H (reverse, 5′ AGA ATT YTG GCT TTG GGT GCCA 3′) (Zhang et al. 2008), while the β*Fib* gene was amplified using BFIB_F (forward, 5′ TGG GAC TGG CAG TTG TTT AG 3′) and BFIB_R (reverse, 5′ TGA TTC ACG AGT TTG TTG CTC 3′) (Pereira et al. unpublished). The alignment of *cyt b* was trimmed to avoid missing data and resulted in a final alignment of ca. 1100 bp. Each polymerase chain reaction (PCR) had a total volume of 10–11 μl: 5 μl of MyTaqTM HS Mix 2X (Bioline), 3 μl of distilled H_2O, 0.5 μl of each primer from a primer solution of 10 μM and 1–2 μl of DNA extract (~50 ng/μl). A negative control was employed to identify possible contaminations. For *cyt b* gene, cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of 40 s at 94°C, 40 s of annealing at 51°C, 72°C for 2 min 30 s, ending with a final extension of 5 min at 72°C. PCR conditions for β*Fib* gene were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s of primer annealing at 59°C, elongation at 72°C for 45 s, finishing with a final extension of 5 min at 72°C. PCR product quality and quantity was assessed by visual inspection in a 2% agarose gel. Sequencing of PCR products was outsourced to Macrogen Inc. (Amsterdam, Netherlands) and Beckman Coulter Inc. (Grenoble, France). The same primers used in PCR were employed for sequencing, except for *cyt b*, where instead of Pro15500H we used an internal forward primer (available upon request). All the obtained chromatograms were verified, aligned and corrected by eye using GENEIOUS PRO v4.8.5 (http://www.geneious.com/).

Phylogenetic analyses

Phylogenetic relationships were analysed using Bayesian analyses conducted in BEAST v1.7.5 (Drummond et al. 2012). JMODELTEST v.2.1.4 (Darriba et al. 2012) was used to test for the best fitting model of nucleotide substitution, under Bayesian information criteria correction (BIC; HKY+G). A lognormal relaxed clock and a coalescence constant size model were used as tree priors. Markov chain Monte Carlo (MCMC) analyses were run in three independent runs of 100 million generations, with a sampling frequency of 1000 generations and discarding 25% trees as burn-in. Parameter convergence was verified by examining the effective sample sizes (ESSs) using TRACER v1.6 and used the remaining trees to obtain the subsequent maximum clade credibility summary tree with posterior probabilities for each node using TREEANNOTATOR v1.7.5 (distributed with the BEAST package). Phylogenetic relationships at nuclear β*Fib* were analysed using a haplotype network. Heterozygous sequences within the nuclear β*Fib* fragment were phased using the PHASE algorithm as implemented in DNASP 5 (Librado and Rozas 2009). Phase probabilities parameter was set at 0.7, and all other settings were set by default. TCS v.1.21 (Clement et al. 2000) was used to construct the haplotype network and applying default settings (probability of parsimony cut-off: 95%).

Niche overlap

Bioclimatic data at a 30′ resolution consisting of 19 temperature- and precipitation-related parameters (Hijmans et al. 2005) were downloaded from the WORLDCLIM database (www.worldclim.org). These parameters were

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Table 1. *Salamandra salamandra* tissue samples used in this study, including subspecies identity, locality data, geographical coordinates, voucher codes and GenBank accession numbers for each locus sequenced. See Fig. 1a, b for a graphical overview of the visited locations.

Subspecies	Locality	Lat	Long	Voucher	*cyt b*	β*Fib*
S. s. bernardezi	Villaviciosa	43.44	−5.49	GVA3794	KT799695	KT799741, KT799742
S. s. bernardezi	Pesanca	43.26	−5.33	GVA4601	KT799711	ND
S. s. bernardezi	Mañangas	43.39	−4.80	GVA4675	KT799712	KT799761, KT799762
S. s. bernardezi	Pimiango	43.39	−4.53	GVA4687	KT799713	KT799763, KT799764
S. s. bernardezi	Buferrera	43.27	−4.98	GVA4692	KT799714	KT799765, KT799766
S. s. bernardezi	FaRío	43.43	−5.57	GVA4707	KT799716	ND
S. s. bernardezi	Tuiza	43.02	−5.91	GVA3532	KT799686	KT799733, KT799734
S. s. bernardezi	Somiedo	43.09	−6.20	GVA1921	KT799684	ND
S. s. bernardezi	Somiedo	43.15	−5.98	GVA3626	KT799688	ND
S. s. bernardezi	Mondoñedo	43.39	−7.30	GVA3673	KT799689	KT799735, KT799736
S. s. bernardezi	Soto	43.54	−6.06	GVA3711	KT799690	ND
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3755	KT799693	ND
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3770	KT799694	KT799739, KT799740
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3954	KT799701	KT799747, KT799748
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3719	KT799691	KT799737, KT799738
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3724	KT799692	ND
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3976	KT799702	ND
S. s. bernardezi	Oviedo	43.35	−5.84	GVA3992	KT799703	KT799749, KT799750
S. s. bernardezi	Salas	43.40	−6.20	GVA3836	KT799699	ND
S. s. bernardezi	Serra do Xistral	43.42	−7.52	GVA3873	KT799700	ND
S. s. bernardezi	La Cuevona de Cuevas	43.43	−5.07	GVA5102	KT799728	ND
S. s. bernardezi	Restriello	43.29	−6.18	GVA4988	KT799726	ND
S. s. bernardezi	N Borinés	43.40	−5.31	GVA4971	KT799696	ND
S. s. bernardezi/S. s. alfredschmidti	Púron	43.37	−4.69	GVA4053	KT799708	KT799759, KT799760
S. s. bernardezi/S. s. alfredschmidti	Llerandi	43.31	−5.21	GVA4697	KT799715	KT799767, KT799768
S. s. bernardezi/S. s. alfredschmidti	Sueve	43.44	−5.19	GVA4722	KT799717	KT799769, KT799770
S. s. bernardezi/S. s. alfredschmidti	Río Tendi 3	43.20	−5.15	GVA3546	KT799687	ND
S. s. bernardezi/S. s. alfredschmidti	Caldevilla	43.34	-5.23	GVA3816	KT799697	ND
S. s. bernardezi/S. s. alfredschmidti	Caldevilla	43.34	−5.23	GVA3815	KT799696	KT799743, KT799744
S. s. bernardezi/S. s. alfredschmidti	La Roza	43.30	−5.24	GVA3822	KT799698	KT799745, KT799746
S. s. bernardezi/S. s. alfredschmidti	Río Marea 3	43.32	−5.39	GVA4944	KT799718	ND
S. s. bernardezi/S. s. alfredschmidti	Río Marea 4	43.30	−5.40	GVA4947	KT799719	ND
S. s. bernardezi/S. s. alfredschmidti	Río Marea 5	43.29	−5.42	GVA4950	KT799720	KT799771, KT799772
S. s. bernardezi/S. s. alfredschmidti	Río Marea 5	43.29	−5.42	GVA4951	KT799721	KT799773, KT799774
S. s. bernardezi/S. s. alfredschmidti	Río Marea 6	43.27	−5.41	GVA4958	KT799722	ND
S. s. bernardezi/S. s. alfredschmidti	Río Marea 6	43.27	−5.41	GVA4959	KT799723	ND
S. s. bernardezi/S. s. alfredschmidti	Río Marea 6	43.27	−5.41	GVA4968	KT799724	ND
S. s. bernardezi/S. s. alfredschmidti	Río Marea 6	43.27	−5.41	GVA4969	KT799725	KT799775, KT799776
S. s. bernardezi/S. s. alfredschmidti	Nava	43.34	−5.48	GVA4051	KT799706	KT799755, KT799756
S. s. bernardezi/S. s. alfredschmidti	Nava	43.34	−5.48	GVA4052	KT799707	KT799757, KT799758
S. s. gallaica/S. s. bejarae	Boñar	42.86	−5.29	GVA4114	KT799709	ND
S. s. gallaica/S. s. bejarae	Abadim	41.56	−7.98	GVA1848	KT799681	ND
S. s. gallaica/S. s. bejarae	Cubillos del Sil	42.59	−6.56	GVA5001	KT799727	ND
S. s. gallaica/S. s. bejarae	Mindelo	41.32	−8.72	GVA1863	KT799682	KT799729, KT799730
S. s. gallaica/S. s. bejarae	Mirandela	41.52	−7.18	GVA4003	KT799705	KT799753, KT799754
S. s. gallaica/S. s. bejarae	Muxía	43.10	−9.12	GVA1009	KT799680	ND
S. s. gallaica/S. s. bejarae	Viana do Castelo	41.71	−8.81	GVA1891	KT799683	ND
S. s. gallaica/S. s. bejarae	Sedano	42.68	−3.73	GVA4248	KT799710	ND
S. s. gallaica/S. s. bejarae	Nuez	41.77	−6.51	GVA4000	KT799704	KT799751, KT799752
S. s. longirostris	San Pablo de Buceite	36.49	−5.40	GVA1948	KT799685	KT799731, KT799732

clipped in ARCGIS 10.1 using a rectangular area comprising the distribution of both *S. s. alfredschmidti* and *S. s. bernardezi*, ranging between N 43.96–42.69 to W −8.40 – −4.44 (Fig. 1b). In order to calibrate niches, these parameters were combined with a data set of georeferenced occurrences gathered during field visits between 2004 and 2014, which were supplemented with literature sources and additional personal observations. Ten populations with the presence of individuals attributable to phenotype groups 4 and 5 (Fig. 1c, Table S1) were used in combination with two literature records (Villanueva 1993; Pasmans and Keller 2000), one personal record (David Buckley) and 17 personal records of GVA and AN to construct a final database of 28 *S. s. alfredschmidti* occurrences (Table S1). For *S. s. bernardezi*, occurrence data for 17 populations without the presence of individuals from phenotype groups 4 and 5 were gathered (Fig. 1c), in addition to 40 personal observations of GVA, AN and WB resulting in a total of 57 occurrences (Table S1). All records used for niche calibration were characterized by a resolution of at least 30′. Niches were calibrated and overlap was measured on a 2D representation of environmental space in R 2.14.1, represented by the first two axes of a principal component analysis (PCA) using the PCA-env ordination approach presented by Broennimann et al. (2012). The PCA was used to summarize information contained in the climatic parameters. Environmental space thereby consists of a grid of $r \times r$ cells, with a standard resolution of 100. Based on occurrence records, smoothed occurrence densities were constructed for each entity using a Gaussian kernel (standard bandwidth) density function applied to all cells. PCA-env selects orthogonal and linear combinations of environmental parameters explaining as much variance as possible, which were subsequently summarized into the first two components. Hence, different positions of species' smoothed occurrence densities represent dissimilar occupation of environmental space. PCA-env is calibrated on the entire environmental space of the study area rather than using only climatic values corresponding to occurrence records. Subsequently, actual niche overlap was calculated based on overlap of the occurrence densities using the *D* metric of Schoener (1968), which varies between 0 (no overlap) and 1 (complete overlap).

We used two tests to measure the degree of niche overlap between *S. s. alfredschmidti* and *S. s. bernardezi* (Warren et al. 2008). First, the

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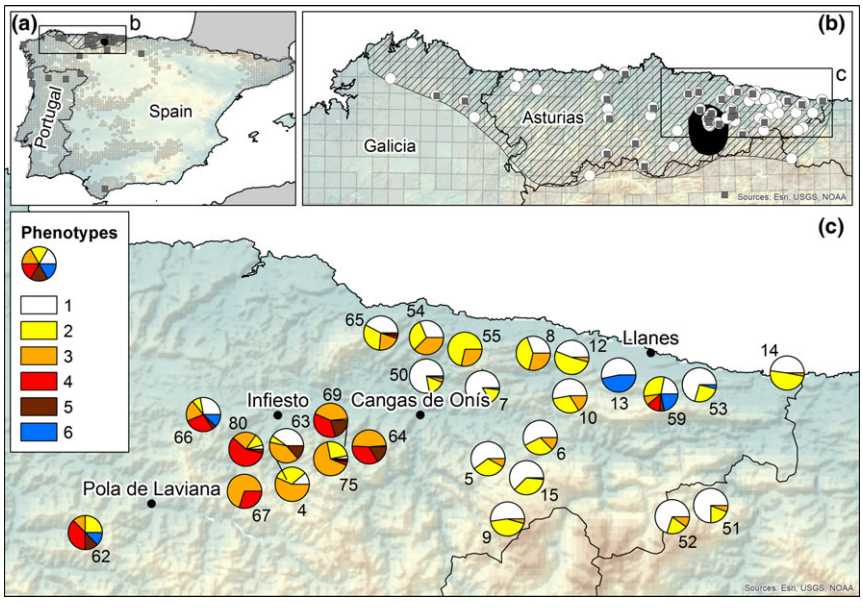

Fig. 1. (a) Range of *Salamandra salamandra* on the Iberian Peninsula (light grey grid) including the distribution of *S. s. bernardezi* (hatched) and hitherto assumed occurrence of *S. s. alfredschmidti* in black; localities at which tissue samples were collected are indicated by dark grey squares. (b) Detail comprising the ranges of the aforementioned taxa, tissue sample locations and occurrences (white dots) used for niche modelling. (c) Distribution of phenotype groups in central and eastern Asturias. Population numbers correspond to those in Table S1.

Table 2. Background colour and pattern of the *Salamandra salamandra* phenotypic groups native to central and eastern Asturias, Spain

	Phenotype					
	1	2	3	4	5	6
Background colour	Yellow	Yellow	Yellow	Yellow/light brown	Brown	Yellow or black
Pattern	Broad and continuous dorsal and lateral black stripes	Thin and discontinuous dorsal and lateral black stripes	Either only dorsal black stripe or also with vestigial lateral stripes	Yellow or orange coloured head region and tiny irregular lighter flecks covering the body	Lateral and/or dorsal black stripes and occasionally lighter parotoids	Not corresponding to groups 1–5

identity or equivalency test assesses whether niches of two taxa are identical; the occurrences of both subspecies were pooled, two random sets of occurrences with the same original sample sizes were extracted, and the overlap scores were determined. This procedure was repeated 100 times in order to create a null distribution of overlap scores, which was compared to the actual overlap. When the actual overlap value falls beyond 95% of the simulated values, the hypothesis of niche identity is rejected. Second, the background or similarity was used to assess whether niches of the two subspecies are more similar than expected by chance based on the geographical regions (environmental background) in which they occur (as opposed to solely the actual occurrence points used in the first test). Again, 100 randomizations were created by placing the kernel density of occurrences at random within the background of entity A, which was compared to the background of entity B and vice versa. When the actual overlap value is significantly ($p < 0.05$) higher or lower than expected from the null distribution based on a two-tailed test, the null hypothesis that the two entities are not more similar to each other can be rejected.

Results

A total of 1147 individuals from 27 local populations (± 42 individuals per population) were assigned to the six phenotypic groups. The relative occurrence of these groups decreased gradually (Fig. 3), with individuals assigned to group 1 being the most common ($n = 466$) and those belonging to group 6 the fewest in number ($n = 22$). Individuals assigned to groups 1, 2 and 3 occur throughout the studied area, although the relative occurrence of the former two groups decreases greatly in the area formed by the mid-altitude valleys from the central Asturian Basin (between Peñamayor Mountains and the River Sella), while that of groups 4–5 increases (Fig. 1c). Individuals belonging to groups 4 and 5 were found in 10 of the 27 visited populations, ranging from Urbiés in the east, towards Sueve in the north and Purón in the west (Fig. 1c). All populations but one (Río Marea 2) shows co-occurrence of individuals from groups 1 and 2 and those from groups 4 and 5. The relatively high occurrence of group 6 in the area south of Llanes is associated with the presence of individuals characterized by highly restricted and occasionally irregular yellow markings.

Phylogenetic analyses

Bayesian analyses of mtDNA sequences showed *S. s. longirostris* as sister to a well-supported clade (BPP = 0.98) that includes all the remaining studied subspecies (Fig. 4). This main clade is divided into two subclades: a well-supported subclade that includes all *S. s. gallaica* and *S. s. bejarae* samples and a moderately supported clade comprising all *S. s. bernardezi* and *S. s. alfredschmidti* samples that is further

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Fig. 2. Phenotypes of *Salamandra salamandra* in northern Spain, in series of three. Photographs by WB unless stated otherwise. Phenotype 1: Moreda, central Asturias (a), Río Tendi, eastern Asturias (Mario Riedling, b), La Caridad, western Asturias (c). Phenotype 2: La Caridad, western Asturias (d), Purón, eastern Asturias (e and f). Phenotype 3: Río Marea (Philip Gerhardt, g), Urbiés, central Asturias (h), Río Infierno, central Asturias (i). Phenotype 4: Río Tendi, eastern Asturias (Sergé Bogaerts, j), Río Tendi, eastern Asturias (Frank Deschandol, k), Urbiés, central Asturias (l). Phenotype 5: Río Tendi, eastern Asturias (Frank Deschandol, m), Río Tendi, eastern Asturias (Sebastian Voitel, n), Río Tendi, eastern Asturias (Mario Riedling, o)

subdivided into five groups. Samples of individuals attributed to groups 4 and 5, and ascribed to *S. s. alfredschmidti* during analyses, are intermixed with *S. s. bernardezi* individuals of groups 1 and 2 in two of the five monophyletic sublineages instead of constituting a monophyletic clade (Fig. 4). Four haplogroups were identified in the nuclear haplotype network (Fig. 4). While *S. s. longirostris*, *S. s. bejarae* and *S. s. gallaica* group together, *S. s. bernardezi* and *S. s. alfredschmidti* are intermixed in two of the three remaining haplogroups (Fig. 4).

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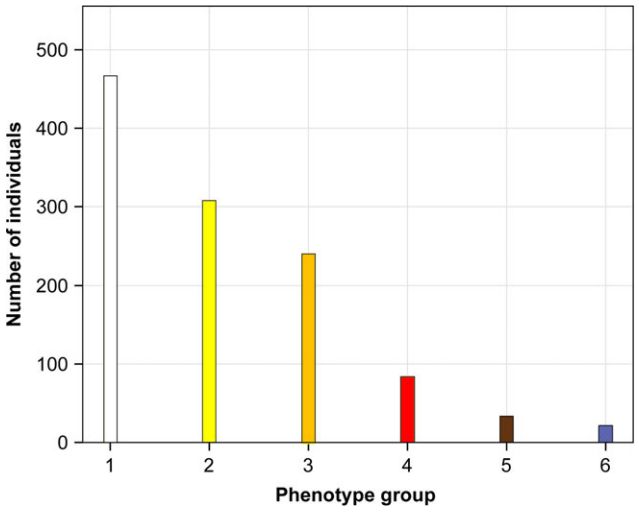

Fig. 3. Number of recorded *Salamandra salamandra* individuals belonging to each phenotypic group

Niche overlap

The niches of individuals from groups 1 and 2 (*S. s. bernardezi*) and that of groups 4 and 5 (*S. s. alfredschmidti*) occupy similar positions in environmental space (Fig. 5a, b, respectively). While the niche of *S. s. bernardezi* is clearly wider, the actual overlap with *S. s. alfredschmidti* was moderate (D = 0.25). The equivalency test showed that both niches were not identical as the actual overlap fell beyond the null distribution, leading to a p value of 1 (Fig. 5c). The actual overlap did fall within the null distributions created during the similarity tests, both when comparing the *S. s. bernardezi* niche to that of *S. s. alfredschmidti* and vice versa (Fig. 5d, e). In both cases, the actual overlap was therefore not found to be significantly higher or lower than expected in respect to these null distributions (Fig. 5d, e). Visual inspection of the niches in environmental space revealed that the niche of *S. s. alfredschmidti* represents a subset of the *S. s. bernardezi* niche.

Discussion

Through the combination of phenotypic, genetic and environmental data, we found that individuals of *S. s. alfredschmidti* and *S. s. bernardezi* are not significantly differentiated. In addition, we confirmed the usefulness of integrative analyses to tackle taxonomical issues (Haig and Winker 2010; Wielstra et al. 2012; Torstrom et al. 2014).

Phenotypic and subspecific diagnosability

Although we presented evidence on the occurrence of at least six diagnosable phenotypic groups in central and eastern Asturias (see also Barrio and Fonoll 1997; Pasmans and Keller 2000), we acknowledge that phenotypic attribution of individuals in some cases can be problematic; especially, groups 1–3 seem to display a continuum regarding the extent of their dorsal and lateral black stripes, which motivated Pasmans and Keller (2000) to treat these as subgroups of a single phenotypic class. It should be pointed out that the extent of dorsal and lateral stripes among individuals of *S. s. bernardezi* might change during ontogeny (Pasmans and Keller 2000; Bogaerts 2002), although this point has never been studied comprehensively (see also Beukema et al. 2009; Beukema 2011). In contrast, phenotypes 4 and 5 show clearly distinct colours and patterns due to the, respectively, lack of a striped pattern, occasionally in addition to a yellow or orange head region and small light flecks all over the body (phenotype 4) and a brown instead of yellow background coloration with dorsal and sometimes lateral black stripes as well as lighter parotoids (phenotype 5). Up to three highly distinct phenotypes therefore seem to occur within Asturian populations of *S. salamandra*, which in turn show a considerable variation.

While the differentiation between the vast majority of phenotypes analysed herein was unambiguous, an initial attempt to attribute these to distinct subspecies was far from being clear-cut as diagnostic traits between *S. s. alfredschmidti* and *S. s. bernardezi* overlap. Wolterstorff (1928) described *S. s. bernardezi* based on a sample of 22 individuals, which were different in their hue of yellow background colour, and the extent and demarcation of dorsal and lateral black stripes. In turn, Köhler and Steinfartz (2006) included individuals characterized by dirty to greyish-yellow coloration, absent lateral stripes and overall irregularly demarked stripes in the diagnosis of *S. s. alfredschmidti*, under the rationale that *S. s. bernardezi* showed sharply delimited dorsal and dorsolateral stripes. However, individuals displaying both sharply and irregularly demarcated black stripes, those lacking dorsolateral stripes and those displaying various hues of yellow background colour occur throughout the populations of *S. s. bernardezi* analysed herein (Fig. 1c). Moreover, although at least groups 4 and 5 do comprise phenotypes that are highly distinct from 'classical' *S. s. bernardezi*, these are nearly without exception intermixed with individuals of phenotype groups 1, 2 and 3 (Table S1; Pasmans et al. 2004). A geographical basis for the occurrence of *S. s. alfredschmidti* therefore seems to be lacking even when restricting this subspecies to the former two phenotype groups.

Polytypism versus polymorphism

Perhaps the most remarkable finding of the current study comprises the fact that individuals attributed to *S. s. alfredschmidti* solely on the basis of colour phenotypes do not represent a monophyletic unit. Rather, these were found to be interspersed within several subclades of *S. s. bernardezi* according to both mitochondrial and nuclear data. Similarly, niche divergence between these subspecies is absent as the niche of *S. s. bernardezi* completely overlaps that of *S. s. alfredschmidti* in environmental space, while their niche centres (assumed to correspond to the environmental optimum; Austin 1985) closely match. These data do not suggest that environmental variation currently maintains divergence, which is not unexpected as *S. salamandra* occurs continuously from sea level up to at least 2000 m in the Cantabrian Mountains (Martínez-Rica and Reiné-Viñales 1988). We therefore do not see sufficient grounds to acknowledge *S. s. alfredschmidti* as a separate subspecies (see also below), and regard the existence of several discrete phenotypes in *S. s. bernardezi* as a classic case of colour polymorphism. In other words, *S. salamandra* is both polytypic as the species encompasses a high number of well-diverged subspecies (Montori and Herrero 2004; Thiesmeier and Grossenbacher 2004), but shows geographically restricted colour polymorphism as well. To the best of our knowledge, this is the first explicitly confirmed case of this phenomenon in the family Salamandridae.

The occurrence of both polytypism and polymorphism in a single species is rare; however, these two phenomena are not mutually exclusive. Colour polymorphism is associated with accelerated speciation rates, due to which an initially polymorphic species can end up as polytypic when phenotypes diverge and receive taxonomic recognition (Gray and McKinnon 2007;

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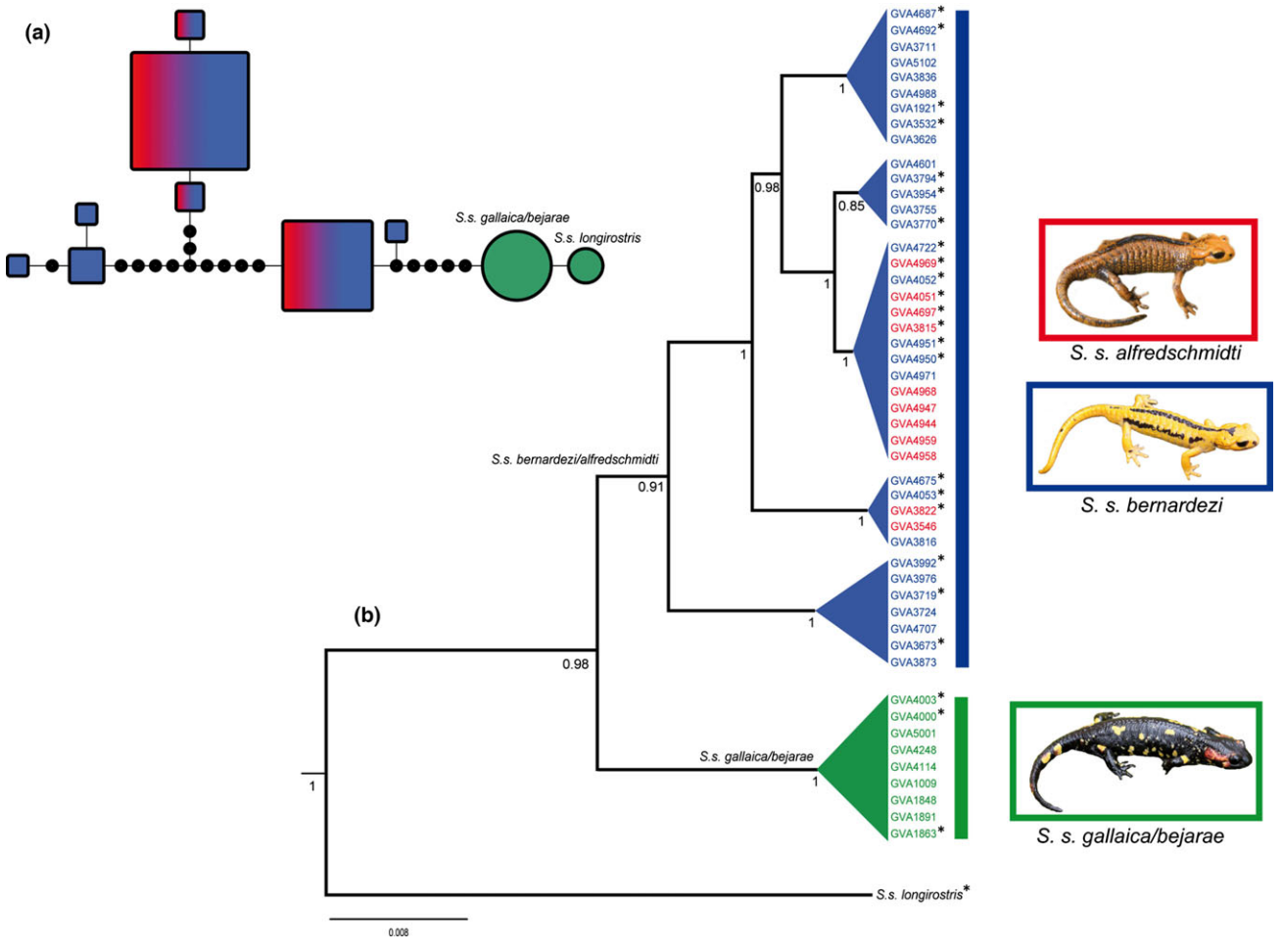

Fig. 4. Genetic relationships between *S. s. bernardezi* (blue) and *S. s. alfredschmidti* (red) and the outgroups used in this study (*S. s. gallaica/bejarae* in green, and *S. s. longirostris*) displayed by a β*Fib* haplotype network inferred by TCS under the 95% criterion showing four haplogroups, two of which show shared haplotypes between *S. s. bernardezi* and *S. s. alfredschmidti* (a). The size of each haplotype symbol is proportional to its frequency, and lines represent mutational steps separating observed haplotypes. Also shown is a Bayesian consensus phylogram based on mtDNA data cytochrome b. Posterior probability values are shown below each node (b). Colours are concordant with the nuclear haplotype network (a). Asterisks denote individuals sequenced for β*Fib*.

Hugall and Stuart-Fox 2012; Fisher-Reid et al. 2013). Among salamanders, polytypism is relatively more common than polymorphism (e.g. Petranka 2010). Occurrence of the former among at least several temperate salamander species can be explained by processes of isolation and subsequent recolonization during the Pleistocene glacial cycles, when currently recognized subspecies diverged in local (micro)refugia for varying amounts of time. Accordingly, species of the genera *Ensatina*, *Lissotriton* and *Salamandra* encompass large numbers of recently derived subspecies which form broad secondary contact zones (Steinfartz et al. 2000; García-París et al. 2003; Pereira and Wake 2009; Vences et al. 2014; Pabijan et al. 2015). Development of considerable morphological divergence during short periods of isolation (leading to subspecific recognition) is, however, exception rather than rule among salamanders, as most species remain morphologically conserved despite possessing high levels of intraspecific genetic structure (but see Wake et al. 1983; Arntzen et al. 2015). This situation seems to hold truth for *S. s. bernardezi*, which displays an overall conserved morphology despite its considerable genetic heterogeneity (current results; see also García-París et al. 2003; Velo-Antón et al. 2007). Nevertheless, colour polymorphism did also evolve in this subspecies, although the drivers that lead to this situation remain unknown. Specifically, we currently cannot infer whether colour polymorphism arose within populations independently or that the present situation is the result of complete intermixing between 'classical' *S. s. bernardezi* and individuals attributed to phenotypes 4 and 5. In case of the latter scenario, the area characterized by high occurrence of phenotype groups 4 and 5 located between Urbiés, Peñamayor and the River Sella (Fig. 1) might have functioned as a former microrefugium, after which individuals dispersed eastwards (García-París et al. 2003). As the overall lack of genetic divergence and occurrence of these phenotypes in several subclades of *S. s. bernardezi,* however, does not support such a hypothesis, we stress the need for increased sampling and more elaborate molecular analyses to shed light on the origin of colour polymorphism in *S. s. bernardezi*. It should additionally be noted that Boulenger (1911), Eiselt (1958) and Malkmus (1991) gave anecdotal descriptions regarding the presence of multiple phenotypes within populations of *S. s. gallaica* (e.g. through the presence of both striped and spotted dorsal patterns).

Maintenance of colour polymorphism

Colour polymorphism within salamander populations is known to have a genetic basis (Highton 1959), although at least assortative mating (Acord et al. 2013), apostatic predation (Fitzpatrick et al. 2009), the chytrid *Batrachochytrium dendrobatidis*

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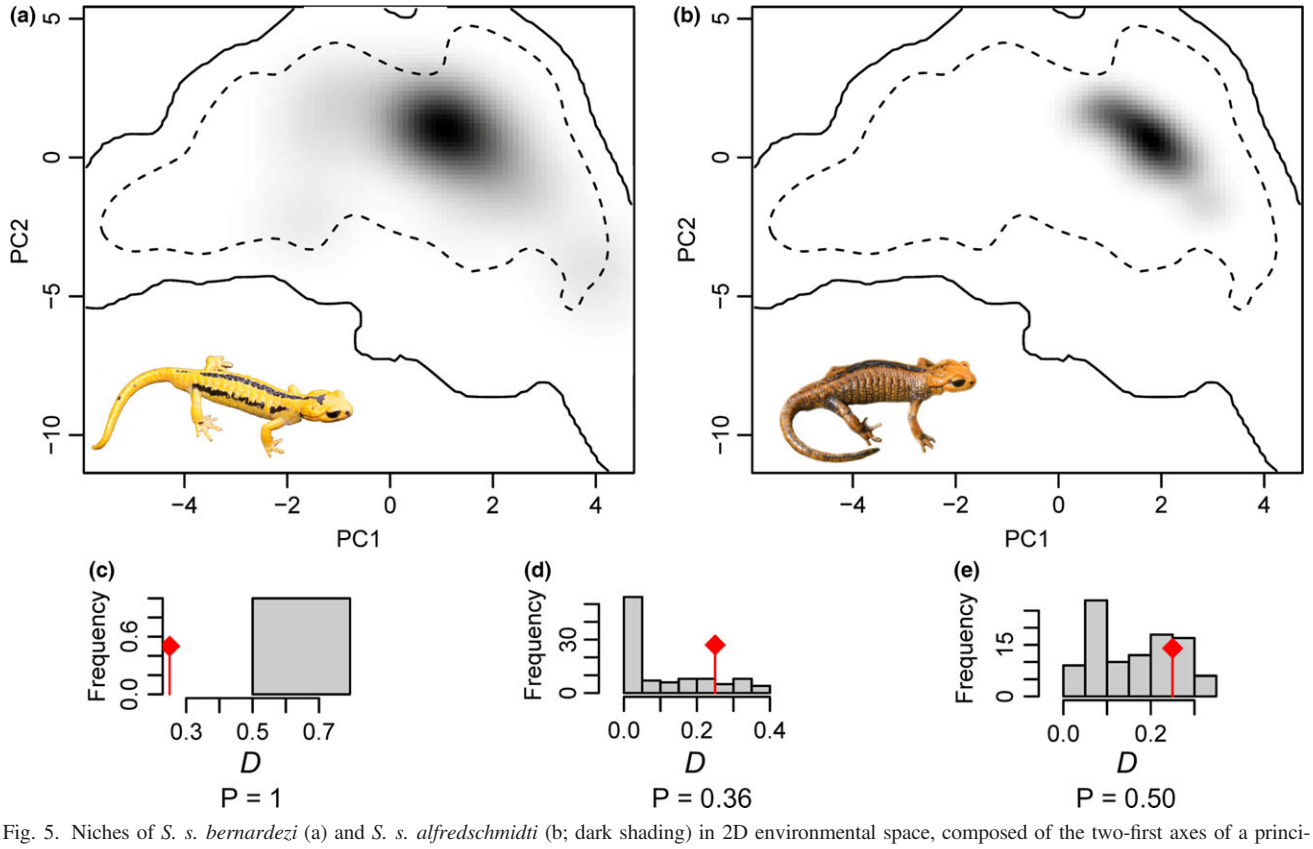

Fig. 5. Niches of *S. s. bernardezi* (a) and *S. s. alfredschmidti* (b; dark shading) in 2D environmental space, composed of the two-first axes of a principal component analysis summarizing information of bioclimatic parameters. The solid and dashed contour lines illustrate 100% and 50% of the background environment. Panels c–e show histograms displaying the null distributions consisting of 100 randomizations (grey bars) in respect to the actual niche overlap (D = 0.25; red arrow). From left to right, the histograms show tests of niche equivalency (c), niche similarity of *S. s. bernardezi* to *S. s. alfredschmidti* (d) and vice versa (e). Significance of the tests is shown below.

(Venesky et al. 2015) and possibly climate (Fisher-Reid et al. 2013) play significant roles in maintaining different phenotypes. Moreover, alleles coding for striped colour patterns are dominant over unstriped patterns among amphibians in general, making uniform phenotypes among polymorphic populations generally less abundant (O'Neill and Beard 2010). In *S. s. bernardezi*, the latter factor could perhaps explain the relatively low occurrence of phenotype group 4, although phenotype maintenance in this subspecies is undoubtedly much more complex. Populations of *S. s. bernardezi* are, in contrast to nearly all other populations of *S. salamandra*, characterized by pueriparous reproduction (Velo-Antón et al. 2015). As such, Pasmans and Keller (2000) suggested that the transition from larviparous to pueriparous reproduction might be associated with the decreased surface activity and the loss of aposematic colours, leading to a darker background colour or loss of pattern. Pueriparity has indeed been associated with the loss of yellow coloration in several *Salamandra* taxa, although it remains to be investigated whether these traits show correlational selection or whether these play a role in maintaining phenotypes of *S. s. bernardezi*. On the other hand, *S. s. bernardezi* is assumed to display pueriparity throughout most of its distribution, generally without showing a decrease in aposematic coloration. It seems likely that at least apostatic predation could have a significant effect in maintaining different phenotypes as well, due to the fact that largely yellow individuals starkly contrast with those of, for example, phenotype group 5. However, at this point, we can merely speculate on the necessarily complex factors that uphold colour polymorphism in this subspecies. Future experimental trials and increased field research are needed to assess the differences in survival between the herein established phenotypes.

Taxonomical implications

Populations of *S. s. alfredschmidti* do not represent a distinct geographical or genetic unit, as these are without exception interspersed (phenotypically, genetically, ecologically and geographically, in various degrees) by *S. s. bernardezi*. Recognizing *S. s. alfredschmidti* as subspecies renders *S. s. bernardezi* paraphyletic and impairs subspecific diagnosability. Consequently, we explicitly reject subspecies status for *S. s. alfredschmidti* and regard this taxon as a junior synonym of *S. s. bernardezi*. In this decision, we took the long-standing notion that subspecies should be erected for the sake of convenience into account (Mayr 1982; Fitzpatrick 2010). Intraspecific taxonomy of *S. salamandra* is highly confused, especially within the Iberian Peninsula (Eiselt 1958; Speybroeck et al. 2010), due to which there is an obvious need to move towards a comprehensive systematic revision. Taxonomic rearrangements like the current work are an essential part of this process and will hopefully provide a basis and shift focus towards eco-evolutionary studies aiming to explore the exciting high degree of phenotypic variation observed in *S. salamandra*.

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Supporting Information

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

Table S1. Overview of locations used for population and genetic analyses and niche modelling.

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