Ann. Zool. Fennici 42: 57–68 ISSN 0003-455X
Helsinki 23 February 2005

Molecular characterisation of the endangered *Salamandra salamandra almanzoris* (Caudata, Salamandridae)

Iñigo Martínez-Solano*, Marina Alcobendas, David Buckley & Mario García-París

*Museo Nacional de Ciencias Naturales, CSIC, c/José Gutiérrez Abascal, 2, ES-28006 Madrid, Spain (*corresponding author's e-mail: mcnim548@mncn.csic.es)*

Received 16 Aug. 2004, revised version received 31 Aug. 2004, accepted 1 Sep. 2004

Martínez-Solano, I., Alcobendas, M., Buckley, D. & García-París, M. 2005: Molecular characterisation of the endangered *Salamandra salamandra almanzoris* (Caudata, Salamandridae). — *Ann. Zool. Fennici* 42: 57–68.

Salamandra salamandra is a polymorphic species with seven endemic subspecies in the Iberian Peninsula. Assignment of populations to subspecies in many areas is controversial because of discrepancies between morphological or molecular datasets. One potential intermixing area includes the Sistema Central Mountains, inhabited by *S. s. almanzoris*, a subspecies with a restricted distribution. In order to delimit the distribution area of *S. s. almanzoris*, we obtained sequences of the cytochrome-*b* gene in 84 individuals representing 26 populations covering the potential range of this subspecies and scored variation at 33 nuclear loci in ten of these populations through allozyme electrophoresis. *S. s. almanzoris* is fragmented into three disjoint groups of populations in the Sierras of Gredos, Guadarrama and San Vicente. The observation of (i) discordance between mtDNA and allozymes; (ii) coexistence of divergent mtDNA haplotypes in two populations; and (iii) discordance between external morphology and mtDNA haplotypes in different populations suggests recent events of secondary contact between *S. s. almanzoris* and other lineages within *S. salamandra* in the lowlands of the Sistema Central. We discuss the implications of these findings for the management of *S. s. almanzoris* populations in central Spain.

Introduction

In recent times, global and local extinctions of species and populations of amphibians have often been documented (Alford & Richards 1999, Houlahan *et al*. 2001). The introduction of alloctonous faunas, the increase in UVB radiation, emerging infectious diseases and habitat degradation and fragmentation due to anthropogenic activity are, among others, plausible causes for some of the reported extinctions. However, apart from the urgent need for studies that identify the causes of the amphibian decline, a parallel effort is needed to delimit what has to be conserved (Moritz 1994).

According to Moritz (2002), the main goal of conservation biology is the protection of biological diversity as well as the processes that generate and sustain it. Since resources to achieve this goal are limited, there is an ongoing debate on the

criteria that should be used to prioritise taxa that merit conservation (Moritz 1994, Cracraft 1997, Crandall *et al*. 2000). In particular, the delimitation and ranking of units that merit conservation below the specific status (subspecies, Evolutionary Significant Units — ESUs — and Management Units — MUs — *see* for example Crandall *et al*. 2000, Fraser & Bernatchez 2001, and Moritz 2002 for reviews on this topic) is complicated on both theoretical and practical grounds. The proper delimitation of ESUs and MUs is a crucial part in designing strategies for conservation and might be of interest to complement species and subspecies categories when applying IUCN's criteria of endangerment (IUCN 2001). This is especially true in the present context of an increasing demand for applying these categories at regional levels, accompanied by an awakening of the need to conserve not only special individuals, populations or species, but also the evolutionary processes that have generated their peculiarities (Crandall *et al*. 2000, Stockwell *et al*. 2003).

Many amphibian populations have recently declined or disappeared in the Iberian Peninsula (Bosch *et al*. 2001, Martínez-Solano *et al*. 2003a, 2003b). As an example, the fire salamander, *Salamandra salamandra*, has strongly declined in central Spain in recent times and most populations are considered to be in serious risk of local extinction in the short term, both those inhabiting low altitude, non-protected areas (Buckley & Alcobendas 2002), and those living in protected, pristine areas (Martínez-Solano *et al*. 2003a, 2003b).

Salamandra salamandra is a polymorphic species that has been divided into 13 subspecies, diagnosed by characters of external morphology, mostly patterns of body coloration (Thorn & Raffaëlli 2001). The highest diversification of *S. salamandra* took place in the Iberian Peninsula, where up to nine subspecies are currently recognized, seven out of them being endemic to this geographic area (Salvador & García-París 2001). However, recent molecular studies focused in the Iberian Peninsula identified only five distinct genetic lineages on the basis of a nested cladistic analysis of partial sequences of mitochondrial genes (García-París *et al*. 2003). These authors found discordances between groupings based on external morphology (i.e., the criteria currently used to recognize subspecies), nuclear DNA and mitochondrial DNA (mtDNA) data along two contact zones in northern Spain. These discordances are apparently the result of hybridization after secondary contact of previously isolated lineages. The disagreement between taxonomic units (subspecies) and evolutionary units (mitochondrial lineages) together with the need for a clear management policy raises the question of how to define and delimit the units for conservation in *S. salamandra*. To address this issue, in this paper we focus in a geographic area where declining of populations of the fire salamander have been reported (Martínez-Solano *et al*. 2003b), and where two different subspecies and two differentiated mitochondrial lineages co-occur: the Sistema Central mountain range in the central Iberian Peninsula.

Two subspecies of *S. salamandra* were described from the Sistema Central: *S. s. almanzoris* Müller & Hellmich, 1935 and *S. s. bejarae* (Wolterstorff, 1934). The subspecies *S. s. almanzoris* is considered as "Vulnerable" (VU A2ce+B1ab) in the *Spanish Red Book of Amphibians and Reptiles* (Buckley & Alcobendas 2002). According to the original descriptions, individuals of *S. s. almanzoris* are characterized by their smaller adult size and by a characteristic pattern of coloration, including lower proportion and size of yellow spots and absence of red spots near the cephalic region (Wolterstorff 1934, Müller & Hellmich 1935). But more recent studies point out that variability in proportion and size of yellow spots in individuals from the type locality of *S. s. almanzoris* is larger than previously anticipated, making it difficult to distinguish them from individuals of *S. s. bejarae* (Brändle & Rödel 1994), and leaving the presence/absence of red pigmentation as the main diagnostic morphological character distinguishing the subspecies. This criterion was widely used before (since Salvador 1974) and will be also followed in this paper.

Following a nested cladistic analysis of mtDNA data (García-París *et al*. 2003), populations of *S. salamandra* along the Sistema Central cluster into two distinct mitochondrial lineages. One lineage includes populations geographically restricted to separate areas in high altitudes along the Sistema Central and that were assigned

to *S. s. almanzoris*. The first mtDNA lineage also includes the populations of the subspecies *S. s. crespoi* and *S. s. morenica*, present in the south of the Iberian Peninsula. The second lineage includes all the surrounding populations of salamanders in the Sistema Central range, traditionally ascribed to the subspecies *S. s. bejarae*. *S. s. bejarae* presents a wider distribution area in the central Iberian Peninsula. This second mtDNA lineage also includes the populations from the subspecies *S. s. gallaica*, *S. s. fastuosa* and *S. s. terrestris*.

According to the original description, *S. s. almanzoris* was initially thought to inhabit exclusively high altitude ponds in the Sierra de Gredos. However, re-evaluation of characters related to external morphology (Salvador 1974, Bosch & López-Bueis 1994) and molecular evidence (Alcobendas *et al*. 1994, García-París *et al*. 2003) suggests its presence in some other localities along the Sistema Central. In this context, the characterization of genetic diversity at the population level constitutes a necessary tool for their management (Moritz 1994). Our aim in the present study is to delineate the distribution area of *S. s. almanzoris* with the help of molecular markers (allozymes, mtDNA) and external morphology and to identify potential areas of contact and hybridization between these two groups in order to provide information for the adequate management of salamander populations in central Spain.

Materials and methods

Study area

The Iberian Sistema Central is the largest mountain range in central Iberia, with a maximum elevation of 2592 m above sea level (Almanzor, Sierra de Gredos). From west to east, it includes the following mountain ranges or "Sierras": Estrela, in Portugal, and Gata, Béjar, Francia, Gredos, Guadarrama, Somosierra and Ayllón in Spain. To the south of its main axis there are also some scattered mountains, like the Sierras of San Vicente and La Higuera. Salamanders are distributed throughout this region, with the exception of the easternmost part (Sierra de Ayllón), where no records are available (Buckley & Alcobendas 2002). Further south of the Sistema Central, populations of *S. salamandra* are scattered and restricted to other mountains as in the Sierra de Guadalupe and Montes de Toledo.

External morphology

We verified the presence/absence of red spots in all the individuals included in the study, considering this character as the main diagnostic morphological character distinguishing *S. s. bejarae* and *S. s. almanzoris*.

Protein electrophoresis

We scored allelic variants in 33 protein systems for 105 individuals from ten populations along the Sistema Central (Table 1). Allozyme data for two of the populations sampled (Peñalara (ID = 19) and Circo de Gredos (ID = 14)) were available from a previous study (Alcobendas *et al*. 1994). Protocols for protein electrophoresis were the same used in those studies. We calculated allele frequencies and estimates of genetic distance between populations with Biosys-1 (Swofford & Selander 1981). We generated UPGMA dendrograms grouping the populations studied according to Nei's genetic distance ($DNei_{72}$, Nei 1972) and Nei's unbiased genetic distance ($DNei_{78}$, Nei 1978).

DNA extraction and amplification

We sampled 84 individuals from 26 populations along the Sistema Central (Fig. 1 and Table 1), including 11 individuals representing ten populations studied by García-París *et al*. (2003), whose sequences were available in GenBank (for accession numbers, *see* Table 1). We extracted DNA from small amounts of tissue (muscle from toe tips of adults or tail tips of larvae in most cases) using a standard proteinase K – phenol chloroform protocol (Sambrook *et al*. 1989). We amplified 356 bp corresponding to part of the cytochrome-*b* gene via polymerase chain reaction (PCR) using the primers cytb2 and

MVZ15 (Kocher *et al*. 1989, Moritz *et al*. 1992), in order to allow comparison with a previous study (García-París *et al*. 2003). PCR reactions consisted of 40 cycles with a denaturing temperature of 94 °C (1 min), annealing at 50 °C (1 min) and extension at 72 °C (1 min). PCR reactions were performed in a total volume of 25 μl, including one unit of Taq polymerase (Biotools), 2.5 μM of each primer, 0.4 mM of dNTPs, 1.5 mM of $MgCl_2$ and 67 mM of a reaction buffer (Tris-HCl, pH = 8.3, Biotools). The amplified fragments were sequenced from both ends with the BigDye Deoxy Terminator cycle-sequencing kit (Perkin Elmer Biosystems) in an automated DNA sequencer (ABI PRISM 3100) following the manufacturer's instructions.

Table 1. Sampling localities for the populations studied. Population ID as in Fig. 1. *N* = sample size. Asterisks indicate sequences that were available from a previous study (García-París *et al*. 2003).

Pop. ID	Locality	*N* (mtDNA)	*N* (Allozymes)	GenBank accession numbers	Haplotypes
1	Spain: Cáceres: Guadalupe	1	8–10	AY514638	II
2	Spain: Cáceres: Cabezuela del Valle	1	6–10	AY196249*	III
3	Spain: Cáceres: Hervás	1		AY196250*	III
4	Spain: Ávila: Mijares	11	8–11	AY196252-53*; AY514639-42; AY722844-48	I, V
5	Spain: Ávila: Casillas	2		AY514643-44	I
6	Spain: Ávila: El Tiemblo	4		AY514645-46; AY722849-50	I
7	Spain: Ávila: Cebreros	2		AY514647-48	I
8	Spain: Ávila: La Adrada	2		AY514649-50	I
9	Spain: Madrid: Robledo de Chavela	1		AY514651	I
10	Spain: Madrid: Pelayos de la Presa	5		AY514652-53; AY722851-53	I
11	Spain: Madrid: Valdemaqueda	3		AY514654-56	I
12	Spain: Madrid: Zarzalejo	10		AY514657-58; AY722854-61	I, V
13	Spain: Toledo: San Pablo de los Montes	1	3–5	AY196251*	I
14	Spain: Ávila: Circo de Gredos	2	6–16	AY196255*; AY722862	V
15	Spain: Ávila: Cinco Lagunas	3	5	AY196254*; AY722863-64	V
16	Spain: Ávila: El Hornillo	5	9–10	AY196256*; AY722865-68	V
17	Spain: Madrid: Guadarrama	3		AY514659-60; AY722869	V
18	Spain: Madrid: El Escorial	3		AY514661-62; AY722870	V
19	Spain: Madrid: Peñalara	2	10	AY196257*; AY722871	V
20	Spain: Madrid: Los Pájaros	2	7	AY196258*; AY722872	V
21	Spain: Madrid: Miraflores de la Sierra	5		AY514663-64; AY722873-75	V
22	Spain: Madrid: Navafría	5		AY514665-66; AY722876-78	V
23	Spain: Madrid: Cenicientos	1		AY514667	V
24	Spain: Toledo: Pelahustán	1		AY514668	V
25	Spain: Toledo: El Real de San Vicente	7	9–10	AY514669-72; AY722879-81	V, VI
26	Portugal: Serra da Estrela: Manteigas	1		AY196246*	IV

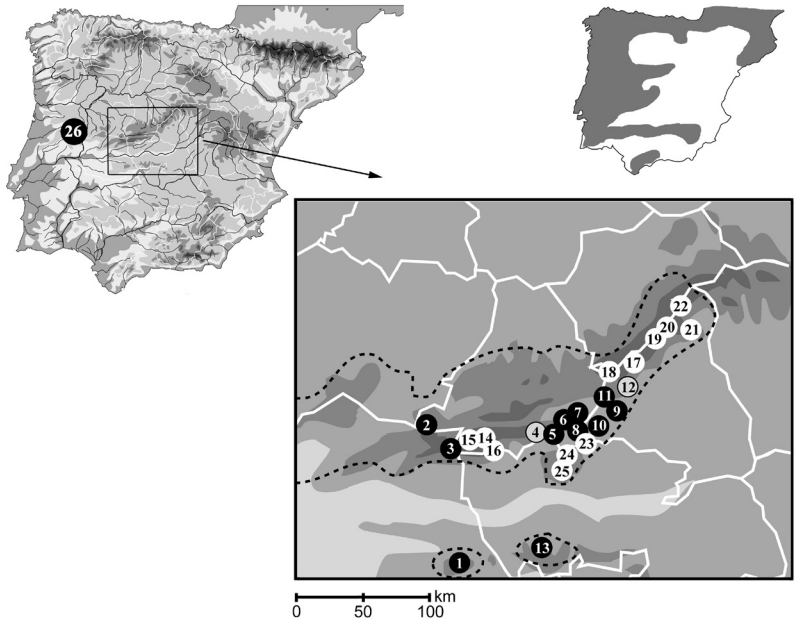

Fig. 1. Sampling localities. Top right-hand-side corner: map of the Iberian Peninsula showing the distribution of *S. salamandra* (shaded). Sampling localities: white dots indicate mtDNA haplotypes of *S. s. almanzoris*; black dots indicate mtDNA haplotypes of *S. s. bejarae*. The dashed line delimits the distribution of *S. salamandra* in the highlighted area. We found discordances between mtDNA and allozymes and/or coexistence of haplotypes assigned to both subspecies at the populations of Mijares (4) and Zarzalejo (12), respectively.

Sequence alignment and analyses

We compiled and aligned all sequences using Sequence Navigator™ version 1.0.1 (Applied Biosystems). Data were analyzed with the software ModelTest 3.0.6 (Posada & Crandall 1998) in order to determine the substitution model that best fits our data and the transitions/transversions ratio (ti/tv). We also calculated the pairwise genetic distances among the haplotypes present in different populations by means of the software PAUP*4.0b10 (Swofford 2002). In order to visualize the relationships among the haplotypes, a median joining haplotype network (Bandelt *et al*. 1999) was constructed with the software Network 4101 (available on the web at: http://www.fluxus-engineering.com).

Results

External morphology

None of the salamanders studied here along the Sistema Central from Cabezuela del Valle (Fig. 1 and Table 1, pop. ID = 2) to Navafría (ID = 22), near the easternmost record for the species along the Sistema Central, had the red spots characterising *S. s. bejarae*. However, red spots were present in the populations of Guadalupe (ID = 1) and San Pablo de los Montes (Montes de Toledo, ID = 13; Fig. 1).

Protein electrophoresis

Seven out of the 33 loci were monomorphic (Aat-1, Ak-1, Ak-2, Fum-1, Gld-1, Gpd-1, and Ldh-1). Two groups of populations were well differentiated, one including the populations of Cabezuela del Valle (ID = 2), Guadalupe (ID = 1) and San Pablo de los Montes (Montes de Toledo, ID = 13), and the other comprising the remaining seven populations (Fig. 2). There were no fixed allelic differences between the two groups of populations, although the locus Alb-1 presented a diagnostic allele (B) and the allele A of the locus Hkm-1 was fixed in one group and occurred in low frequencies in the other group (Table 2). The population of Guadalupe (ID = 1) had private alleles in the following loci: Alb-1, Ck-1, Est-h, Icd-1, Lap-1 and Ldh-2. Most of them were present in low frequencies (0.06%–0.95%). Other populations, like Mijares (ID = 4), Cabezuela del Valle (ID = 2), Circo de Gredos (ID = 14), El Real de San Vicente (ID = 25), San Pablo de los Montes (Montes de Toledo, ID = 13) or Los Pájaros (ID = 20) had also private alleles.

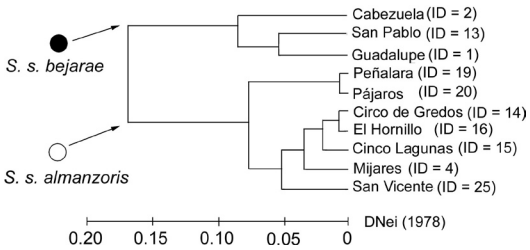

Fig. 2. Dendrogram of the populations of *S. salamandra* studied based on Nei's (1978) unbiased genetic distances, showing the two main groups identified, referred to as *S. s. bejarae* (black spot) and *S. s. almanzoris* (white spot). Population IDs as in Fig. 1 and Table 1. Cophenetic correlation coefficient = 0.910.

Average $DNei_{72}$ was 0.080 for populations within the first group (0.006–0.092), 0.049 within the second (0.005–0.102), and 0.178 between both groups (0.112–0.254; Table 3).

mtDNA

We found six haplotypes among the 84 specimens analyzed (Fig. 3 and Table 4). There were nine variable positions defining haplotypes, four involving mutations in the first positions (in all cases involving amino acid replacements) and

Table 2. Allele frequencies for 26 polymorphic loci in 10 populations of *S. salamandra*. Population ID as in Table 1. *N* = Sample size.

Locus/Population	1	2	4	13	14	15	16	19	20	25
ALB-1										
N	9	7	10	3	16	5	10	10	7	10
A	0.11	0	0	0	0	0	0	0	0	0
B	0.77	0.14	0	1	0	0	0	0	0	0
C	0.05	0.28	1	0	0.81	1	1	1	1	1
D	0.05	0.57	0	0	0.18	0	0	0	0	0
ALB-2										
N	8	6	9	5	16	5	10	10	7	10
A	0	0.50	0	0	0	0	0	0.30	0	0
B	1	0.33	1	1	1	1	1	0.70	1	1
C	0	0.16	0	0	0	0	0	0	0	0
AAT-2										
N	9	10	10	5	16	5	10	10	7	10
A	0	0.25	0	0	0.15	0.10	0	0.80	0.92	0
B	0	0	0	0	0.06	0	0	0	0	0
C	0.50	0.45	1	0.70	0.78	0.90	1	0.20	0.07	1
D	0	0.20	0	0.30	0	0	0	0	0	0
E	0.50	0.10	0	0	0	0	0	0	0	0
ACO-1										
N	10	6	10	5	6	5	10	10	7	10
A	0	0	0	0	0	0	0	0	0	0.05
B	1	0.91	1	1	1	1	1	1	1	0.95
C	0	0.08	0	0	0	0	0	0	0	0
CK-01										
N	8	10	10	5	15	5	10	10	7	10
A	0.93	1	1	1	1	1	1	1	1	1
B	0.06	0	0	0	0	0	0	0	0	0
EST-h										
N	9	10	10	5	16	5	10	10	7	10
A	0.11	0	0	0	0	0	0	0	0	0
B	0.88	1	1	1	1	0.80	1	1	1	0
C	0	0	0	0	0	0.20	0	0	0	1
EST-m										
N	9	10	10	5	16	5	10	10	7	10
A	0	0	0	0	0.15	0.10	0.10	0	0	0.05
B	0.77	1	1	0.80	0.84	0.90	0.90	1	1	0.95
C	0.22	0	0	0.20	0	0	0	0	0	0

continued

Table 2. Continued.

Locus/Population	1	2	4	13	14	15	16	19	20	25
GDH-1										
N	9	10	11	5	16	5	10	10	7	9
A	0.11	0	0.18	0	0	0	0	0	0	0
B	0.88	1	0.72	1	1	1	1	1	1	1
C	0	0	0.09	0	0	0	0	0	0	0
GPI-1										
N	10	10	11	5	16	5	10	10	7	10
A	0	0	0.09	0	0	0	0	0	0	0
B	1	1	0.90	1	1	1	1	1	1	1
HKH-1										
N	8	10	11	5	16	5	10	10	7	10
A	0	0	0.13	0.10	0	0	0	0.15	0	0.05
B	1	1	0.86	0.50	1	1	1	0.85	1	0.95
C	0	0	0	0.40	0	0	0	0	0	0
HKM-1										
N	10	10	10	5	16	5	10	10	7	10
A	1	1	0.10	1	0	0	0.05	0	0.07	0
B	0	0	0.90	0	1	1	0.95	1	0.92	1
ICD-1										
N	10	10	11	5	16	5	10	10	7	10
A	0	0.05	0.09	0	0	0	0	0.10	0.14	0
B	0.90	0.95	0.90	1	1	1	1	0.90	0.85	1
C	0.10	0	0	0	0	0	0	0	0	0
ICD-2										
N	8	10	11	5	16	5	10	10	7	10
A	0	0	0	0	0	0	0.95	0	0.14	0
B	1	1	1	1	1	1	0.05	1	0.85	1
LAP-1										
N	9	10	11	5	16	5	10	10	7	10
A	0.11	0	0	0	0	0	0	0	0	0
B	0.88	0.35	0	0.50	0.06	0	0	0	0	0
C	0	0.65	1	0.50	0.93	1	1	1	1	1
LDH-2										
N	10	10	11	4	11	5	10	10	7	10
A	0.95	0	0	0	0	0	0	0	0	0
B	0.05	0.70	0	1	0.09	0.20	0	0	0	0
C	0	0.30	0.09	0	0.04	0	0	0	0	0
D	0	0	0	0	0.04	0	0	0	0	0
E	0	0	0.90	0	0.81	0.80	1	1	1	1
MDH-1										
N	10	10	11	5	16	5	10	10	7	10
A	0	0	0	0	0	0	0	0	0	0.05
B	0.65	1	1	0.40	1	1	1	1	1	0.95
C	0.35	0	0	0.60	0	0	0	0	0	0
MDH-2										
N	10	10	11	5	16	5	10	10	7	10
A	1	0.95	0.95	1	1	1	0.95	1	1	0.90
B	0	0	0.04	0	0	0	0.05	0	0	0.10
C	0	0.05	0	0	0	0	0	0	0	0
ME-01										
N	10	10	11	5	16	5	10	10	7	10
A	1	1	0.90	1	1	1	1	1	1	1
B	0	0	0.09	0	0	0	0	0	0	0
MPI-1										
N	9	10	8	5	16	5	10	10	7	10
A	0	0	0.06	0	0	0	0	0	0	0
B	0.94	1	0.87	1	1	1	1	1	1	0.80
C	0.05	0	0.06	0	0	0	0	0	0	0.20

continued

Table 2. Continued.

Locus/Population	1	2	4	13	14	15	16	19	20	25
PEP-1										
N	10	10	10	5	16	5	10	10	7	10
A	0.45	1	0.05	0.20	0.68	1	0.75	1	1	0.95
B	0.55	0	0.95	0.80	0.31	0	0.25	0	0	0.05
PGD-1										
N	10	10	11	5	16	5	10	10	7	10
A	0.10	0	0	0	0.12	0	0	0	0	0
B	0.90	1	1	1	0.87	1	1	1	1	1
PGM-1										
N	10	10	11	5	14	5	9	10	7	10
A	0	0.10	0.18	0	0.03	0.70	0.11	0.30	0.21	0
B	1	0.80	0.31	1	0.03	0	0	0	0	0
C	0	0.10	0.50	0	0.89	0.30	0.88	0.70	0.78	1
D	0	0	0	0	0.03	0	0	0	0	0
PK-01										
N	10	10	11	5	16	5	10	10	7	10
A	0.05	0	0	0	0.06	0	0	0	0	0
B	0.95	1	1	1	0.93	1	1	1	1	1
SDH-1										
N	8	10	10	5	15	5	10	10	7	10
A	0	0	0	0	0	0	0	0	0.14	0
B	0	0	0.10	0	0	0	0.15	0.15	0.07	0
C	1	1	0.90	1	1	1	0.85	0.85	0.78	1
SOD-2										
N	10	10	11	5	16	5	10	10	7	10
A	0.90	0.65	1	1	1	1	1	1	1	1
B	0.10	0.35	0	0	0	0	0	0	0	0
XDH-1										
N	10	10	11	5	16	5	10	10	7	10
A	1	1	1	1	1	1	1	0.20	0	1
B	0	0	0	0	0	0	0	0.80	1	0

Table 3. Matrix of genetic distances between the populations studied based on electrophoretic data. Above diagonal, Nei's (1972) genetic distance; below diagonal, Nei's (1978) unbiased genetic distance.

Population	Cabezuela	San Pablo	Peñalara	Circo de Gredos	Mijares	Guadalupe	Cinco Lagunas	Pájaros	San Vicente	El Hornillo
Cabezuela		0.089	0.142	0.113	0.143	0.092	0.112	0.166	0.171	0.128
San Pablo	0.080		0.238	0.162	0.146	0.060	0.177	0.254	0.233	0.178
Peñalara	0.136	0.231		0.047	0.084	0.231	0.050	0.007	0.085	0.050
Circo de Gredos	0.107	0.156	0.044		0.027	0.162	0.020	0.062	0.041	0.005
Mijares	0.136	0.139	0.080	0.023		0.160	0.044	0.102	0.071	0.024
Guadalupe	0.084	0.051	0.225	0.157	0.154		0.178	0.242	0.227	0.178
Cinco Lagunas	0.105	0.170	0.046	0.016	0.039	0.172		0.069	0.040	0.017
Pájaros	0.160	0.247	0.003	0.058	0.098	0.237	0.064		0.101	0.065
San Vicente	0.166	0.228	0.082	0.039	0.068	0.222	0.037	0.098		0.036
El Hornillo	0.123	0.172	0.047	0.003	0.021	0.174	0.013	0.062	0.034	

five in third positions (in all cases involving synonymous substitutions). The choice of the mutation model was based on a hierarchical log likelihood test procedure implemented in ModelTest 3.0.6 (Posada & Crandall 1998), using 1% significance cut-off level, and Akaike information criterion. The mutation model suggested for the data set was K81uf, with ti/tv = 7:1 and

nucleotide frequencies A: 0.3107, C: 0.2480, G: 0.1374, T: 0.3130. Two groups of haplotypes were apparent (Fig. 3). The first group of haplotypes (I–IV) included the populations of Manteigas (Serra da Estrela, ID = 26), Guadalupe (ID = 1), Cabezuela del Valle (ID = 2), Hervás (ID = 3), populations from transitional lowlands between the sierras of Gredos and Guadarrama (part of the samples from Mijares as well as all samples from Casillas, El Tiemblo, Cebreros, La Adrada, Robledo de Chavela, Pelayos de la Presa and Valdemaqueda, ID = 4–11), the sample from San Pablo de los Montes (Montes de Toledo, ID = 13) and one of the samples from Zarzalejo (ID = 12). The second group (haplotypes V–VI) comprised samples from the sierras of Gredos (Circo de Gredos, Cinco Lagunas, El Hornillo, ID = 14–16), Guadarrama (Guadarrama, El Escorial, Peñalara, Los Pájaros, Miraflores de la Sierra, Navafría, ID = 17–22), La Higuera (Cenicientos, Pelahustán, ID = 23–24) and San Vicente (El Real de San Vicente ID = 25). This second group included also two samples from Mijares as well as nine samples from Zarzalejo (Fig. 1). Mean K81uf corrected sequence divergence ranged from 0.56% to 1.13% among samples within the first group (haplotypes I–IV) and 0.28% between haplotypes V and VI (Table 4). Sequence divergence ranged from 1.41%–1.99% between both haplotype groups.

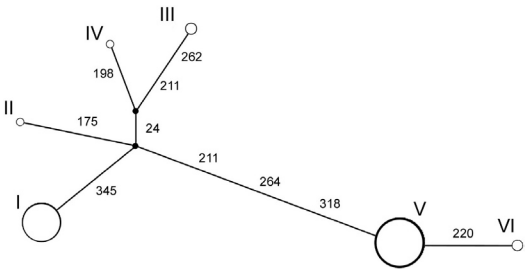

Fig. 3. Median-joining network connecting haplotypes (roman numerals) found in the populations studied. Positions defining haplotypes are mapped on to the network. The size of the ellipses representing haplotypes is proportional to their observed frequency.

Discussion

On the basis of congruence between data on external morphology, allozymes and mtDNA, two groups of populations of *S. salamandra* should be considered for management purposes in the Central Iberian Peninsula.

The populations of Circo de Gredos (ID = 14), Cinco Lagunas (ID = 15), Peñalara (ID = 19), Los Pájaros (ID = 20), El Real de San Vicente (ID = 25) and El Hornillo (ID = 16) form a first group of populations, defined by mtDNA haplotypes, allozyme allele frequencies and colour characteristic to *S. s. almanzoris*. Thus, from a taxonomic perspective, this first group can be readily assigned to *S. s. almanzoris* (Fig. 1). The distribution of *S. s. almanzoris* comprises three disjunct groups including the Sierras of Gredos (*terra typica* of this subspecies), Guadarrama and San Vicente. The latter population was not previously assigned to this subspecies; however, the congruence of both nuclear and mitochondrial markers, as well as observations on external morphology support its assignment to *S. s. almanzoris*. Data from allozymes support a closer relationship between these populations and those from the uplands of Gredos (Table 3).

A second group includes the populations of Guadalupe (ID = 1), San Pablo de los Montes (Montes de Toledo, ID = 13) and Cabezuela del Valle (ID = 2), although individuals from the latter population lack the red pigmentation in the cephalic region characteristic of *S. s. bejarae*. Populations from the second group are part of a mitochondrial lineage that is widespread in Iberia and western Europe (Steinfartz *et al.* 2000, García-París *et al.* 2003, this study). In the Iberian Peninsula, populations from this lineage occupy the southern slopes of the Pyrenees in Cataluña, Aragón and Navarra, the eastern and southern slopes of the Cantabrian mountains,

Table 4. Average (%) genetic distances (K81uf-corrected) between mtDNA haplotypes observed.

Haplotypes	I	II	III	IV	V	VI
I						
II	0.564					
III	1.133	1.133				
IV	0.848	0.848	0.848			
V	1.419	1.420	1.419	1.706		
VI	1.706	1.707	1.706	1.994	0.282	

northwestern Spain (Galicia), most of Portugal, including the Sierras of Sintra and São Mamede, the northern and southern Iberian tablelands ("mesetas") and part of the Sistema Central, including some isolated mountains south of this range (Guadalupe, Montes de Toledo). The lineage includes populations that are highly differentiated phenotypically and were previously assigned to four subspecies: *S. s. terrestris*, *S. s. fastuosa*, *S. s. gallaica* and *S. s. bejarae*. Disentangling the relationships among these populations and re-evaluating their taxonomic status will require further studies combining molecular (allozymes, microsatellites) and morphological data. For instance, *S. s. bejarae*, as currently considered, includes a set of populations very diverse from a phenotypic, ecological and evolutionary point of view.

Reciprocal monophyly and significant divergence at nuclear loci supports the qualification of both groups of populations as ESUs (*sensu* Moritz 1994, 2002). According to molecular clock estimates for allozymes (Veith 1992) and mtDNA data (Tan & Wake 1995), both population groups diverged 1.7–3.3 million years ago, during the Pliocene–Pleistocene. The combination of the results of García-París *et al*. (2003) together with ours suggest that the Sistema Central is an area of confluence among several differentiated intraspecific lineages within *S. salamandra*. On the contrary, other studies focused on amphibians and reptiles (*Rana iberica* and *Triturus boscai*; I. Martínez-Solano unpubl. data; *Lacerta schreiberi*, Godinho *et al*. 2003) suggest that the colonization of the Sistema Central in these species was a relatively recent event that progressed in a west-east direction. In those cases, the Sistema Central is inhabited by a single, genetically homogeneous lineage, whereas in *S. salamandra* several lineages with different evolutionary histories coexist along the Sistema Central.

With regard to the adaptive distinctness of these two groups of populations, data are not conclusive. Ecological data about *S. s. almanzoris* and *S. s. bejarae* are scarce (Salvador 1974, Lizana *et al*. 1986, Guerrero *et al*. 1990). According to these studies, there are some differences in life-history traits, such as period of activity and diet. Besides, there are differences in developmental traits. A small proportion of the larvae of *S. s. almanzoris* living in high altitude mountain ponds spend several years in the water before metamorphosis (Alcobendas & Castanet 2000). However, there is no evidence that the differences in phenotypic or ecological traits are heritable and adaptive, and they might also result from pure environmental effects (Weitere *et al*. 2004). Thus, the null hypothesis of ecological exchangeability of both taxa remains untested (Crandall *et al*. 2000). In any case, we believe that the lack of evidence for adaptive distinctness cannot be considered as an argument for the management of both taxa as a single unit.

Even so, the delimitation of ESUs is made difficult by the discrepancies between datasets. These discrepancies probably reflect events of secondary contact between well-differentiated lineages that took place recently along the Sistema Central. We found three types of discordances: (i) the discordance between mtDNA and allozyme markers in the population of Mijares (ID = 4); (ii) the coexistence of divergent mtDNA haplotypes in the populations of Mijares (ID = 4) and Zarzalejo (ID = 12); and (iii) the discordance between external morphology and mtDNA haplotypes in some of the populations studied. For example, most individuals from the river Tiétar valley (populations 5–8 as well as Pelayos de la Presa, ID = 10) are morphologically very similar to individuals from Sierra de Guadarrama (ID = 17–21) but, at the same time, their mtDNA corresponds with the lineage found in the populations of Guadalupe (ID = 1) and Montes de Toledo (ID = 13) (individuals characterized by the presence of red spots in the cephalic region) as well as in the populations of Cabezuela and Hervás (ID = 2–3). This suggests the existence of a large intermixing area along the transitional lowlands between the Sierras of Gredos (ID = 14–16) and Guadarrama (ID = 17–21). It has been argued that areas where secondary contact between historically isolated lineages has been documented should be preserved because they can be regarded as natural laboratories to study some aspects of the evolutionary process (Moritz 2002). Large intermixing areas between incompletely differentiated lineages might be commonplace due to the nature of the speciation process (*see* figures in García-París 1995 and Lushai *et al*. 2003 for

examples in toads and butterflies, respectively). In the case of geographically restricted lineages (such as *S. s. almanzoris*), this intermixing might lead to the extinction *via* hybridization of the more restricted lineage. Comparative studies involving diverse taxa will help in identifying these areas, which are of special relevance for the preservation of unique lineages as well as evolutionary patterns and processes.

Acknowledgements

We thank two anonymous referees for their valuable comments on the manuscript. The "Consejerías de Medio Ambiente" of Castilla-La Mancha, Castilla-León, Extremadura and Madrid (Spain) extended legal permits for the collection of samples. We also thank R. Esteban, C. Grande, A. Iraola, R. López, P. Merelo, E. Recuero, V. Rodríguez and I. Urbán for help during collecting trips and A. Salvador for providing us with relevant references. The work of IM-S was supported by a CAM-CSIC-MNCN predoctoral fellowship. Financing was partially provided by the project 07M/0090/2002 (Comunidad Autónoma de Madrid).

References

Alcobendas, M. & Castanet, J. 2000: Bone growth plasticity among populations of *Salamandra salamandra:* interactions between internal and external factors. — *Herpetologica* 56: 14–26.

Alcobendas, M., Dopazo, H. & Alberch, P. 1994: Genetic structure and differentiation in *Salamandra salamandra* populations from the northern Iberian Peninsula. — *Mertensiella* 4: 7–23.

Alford, R. A. & Richards, S. J. 1999: Global amphibian declines: a problem in applied ecology. — *Annu. Rev. Ecol. Sist.* 30: 133–165.

Bandelt, H. J., Forster, P. & Röhl, A. 1999: Median-joining networks for inferring intraspecific phylogenies. — *Mol. Biol. Evol.* 16: 37–48.

Bosch, J. & López-Bueis, I. 1994: Comparative study of the dorsal pattern in *Salamandra salamandra bejarae* (Wolterstorff, 1934) and *S. s. almanzoris* (Müller & Hellmich, 1935). — *Herp. J.* 4: 46–48.

Bosch, J., Martínez-Solano, I. & García-París, M. 2001: Evidence of a chytrid fungus infection in the decline of the midwife toad in protected areas of Central Spain (Anura: Discoglossidae). — *Biol. Cons.* 97: 331–337.

Brändle, M. & Rödel, M. O. 1994: Beiträge zur Kenntnis der iberischen Herpetofauna (Amphibia; Reptilia). — *Herpetozoa* 7: 19–27.

Buckley, D. & Alcobendas, M. 2002: *Salamandra salamandra*. — In: Pleguezuelos, J. M., Márquez, R. & Lizana, M. (eds.), *Atlas y Libro Rojo de los Anfibios y Reptiles de España*: 55–57. Dirección General de la Conservación de la Naturaleza — Asociación Herpetológica Española, Madrid, Spain.

Cracraft, J. 1997: Species concepts in systematics and conservation biology: an ornithological viewpoint. — In: Claridge, M. F., Dawah, H. A. & Wilson, M. R. (eds.), *Species: the units of biodiversity*: 325–339. Chapman & Hall, London.

Crandall, K. A., Bininda-Edmonds, O. R. P., Mace, G. M. & Wayne, R. K. 2000: Considering evolutionary processes in conservation biology. — *Trends Ecol. Evol.* 15: 290–295.

Fraser, D. J. & Bernatchez, L. 2001: Adaptive evolutionary conservation: towards a unified concept for defining conservation units. — *Mol. Ecol.* 10: 2741–2752.

García-París, M. 1995: Variabilidad genética y distribución geográfica de *Alytes obstetricans almogavarii* en España. — *Rev. Esp. Herp.* 9: 133–138.

García-París, M., Alcobendas, M., Buckley, D. & Wake, D. B. 2003: Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. — *Evolution* 57: 129–143.

Godinho, R., Paulo, O. S., Ferrand, N., Luis, C., Rosa, H. D. & Crespo, E. G. 2003: Major patterns of population differentiation in the Iberian Schreiber's green lizard (*Lacerta schreiberi*) inferred from protein polymorphism. — *Herp. J.* 13: 35–42.

Guerrero, F., Pérez-Mellado, V., Gil, M. J. & Lizana, M. 1990: Food habits and trophic availability in the high mountain population of the spotted salamander from Spain (*Salamandra salamandra almanzoris*) (Caudata: Salamandridae). — *Fol. Zool.* 39: 341–353.

Houlahan, J. E., Findlay, C. S., Schmidt, B. R., Meyer, A. H. & Kuzmin, S. L. 2000: Quantitative evidence for global amphibian population declines. — *Nature* 404: 752–755.

IUCN 2001: *IUCN Red List Categories: Version 3.1*. — IUCN, Gland, Switzerland and Cambridge, United Kingdom.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. & Wilson, A. C. 1989: Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. — *Proc. Natl. Acad. Sci. USA* 86: 6196–6200.

Lizana, M., Ciudad Pizarro, M. J. & Pérez-Mellado, V. 1986: Uso de los recursos tróficos en una comunidad ibérica de anfibios. — *Rev. Esp. Herp.* 1: 209–271.

Lushai, G., Smith, D. A. S., Gordon, I. J., Goulson, D., Allen, J. A. & MacLean, N. 2003: Incomplete sexual isolation in sympatry between subspecies of the butterfly *Danaus chrysippus* (L.) and the creation of a hybrid zone. — *Heredity* 90: 236–246.

Martínez-Solano, I., Bosch, J. & García-París, M. 2003a: Demographic trends and community stability in a montane amphibian assemblage. — *Cons. Biol.* 17: 238–244.

Martínez-Solano, I., Barbadillo, L. J. & Lapeña, M. 2003b: Effect of introduced fish on amphibian species richness and densities at a montane assemblage in the Sierra de Neila (Spain). — *Herp. J.* 13: 167–173.

Moritz, C. 1994: Defining "Evolutionary Significant Units" for conservation. — *Trends Ecol. Evol.* 9: 373–375.

Moritz, C. 2002: Strategies to protect biological diversity and the evolutionary processes that sustain it. — *Syst. Biol.* 51: 238–254.

Moritz, C., Schneider, C. J. & Wake, D. B. 1992: Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. — *Syst. Biol.* 41: 273–291.

Müller, L. & Hellmich, W. 1935: Mitteilungen über die Herpetofauna der Iberischen Halbinsel. Über *Salamandra salamandra almanzoris* n. ssp. und *Bufo bufo gredosicola* n. ssp., zwei neue Amphibienrassen aus der Sierra de Gredos. — *Zool. Anz.* 112: 49–57.

Nei, M. 1972: Genetic distance between populations. — *Am. Nat.* 106: 283–292.

Nei, M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. — *Genetics* 89: 583–590.

Posada, D. & Crandall, K. A. 1998: MODELTEST: testing the model of DNA substitution. — *Bioinformatics* 14: 817–818.

Salvador, A. 1974: *Guía de los anfibios y reptiles españoles*. — ICONA, Madrid, Spain.

Salvador, A. & García-París, M. 2001: *Anfibios Españoles*. — Esfagnos, Talavera de la Reina, Spain.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989: *Molecular cloning*, 2nd ed. — Cold Spring Harbor Laboratory Press, New York, USA.

Steinfartz, S., Veith, M. & Tautz, D. 2000: Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of *Salamandra salamandra*. — *Mol. Ecol.* 9: 397–410.

Stockwell, C. A., Hendry, A. P. & Kinnison, M. T. 2003: Contemporary evolution meets conservation biology. — *Trends Ecol. Evol.* 18: 94–100.

Swofford, D. 2002: *PAUP*: Phylogenetic analysis using parsimony (*and other methods), Version 4*. — Sinauer Associates, Sunderland, Massachusetts, USA.

Swofford, D. L. & Selander, R. B. 1981: *Biosys-1, a computer program for the analysis of allelic variation in genetics*. — University of Illinois, USA.

Tan, A. M. & Wake, D. B. 1995: MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata: Salamandridae). — *Mol. Phyl. Evol.* 4: 383–394.

Thorn, R. & Raffaëlli, J. 2001: *Les salamandres de l'ancien monde*. — Boubée, Paris, France.

Veith, M. 1992: The fire salamander, *Salamandra salamandra* L., in central Europe: subspecies distribution and intergradation. — *Amph-Rept.* 13: 297–313.

Weitere, M., Tautz, D., Neumann, D. & Steinfartz, S. 2004: Adaptive divergence *vs.* environmental plasticity: tracing local genetic adaptation of metamorphosis traits in salamanders. — *Mol. Ecol.* 13: 1665–1677.

Wolterstorff, W. 1934: Über *Salamandra maculosa* Laur. (= *salamandra* L.) *molleri* und eine neue Form, subsp. *bejarae*. — *Blätter Aquar. Terrar. Kde* 45: 147–149.