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Evolution of the melanistic colour in the Alpine salamander *Salamandra atra* as revealed by a new subspecies from the Venetian Prealps

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ABSTRACT

In order to reconstruct the evolution of the body colour within the *Salamandra atra* group, a phylogenetic analysis of the mitochondrial DNA D-loop sequence was performed on seven populations of this group, including all three chromatically different forms and all known nominal subspecies. Also included were four outgroup species. Three major mitochondrial lineages were found within *S. atra*: (i) the invariably yellow-patched *S. a. auro-rae* lineage from the Altopiano dei Sette Comuni (Venetian Prealps); (ii) a genetically distinct lineage represented by a population from the Pasubio massif (Venetian Prealps), in which the yellow patches were either limited or completely lacking; (iii) a lineage comprising all fully melanistic populations from the Alps to the Dinarides, which were assigned to *S. a. atra* and also included the nominal *S. a. prenjensis*. Different phylogenetical reconstruction methods and statistical tests supported the hypothesis that the lineage of *S. a. auro-rae* was the sister group to both remaining lineages of *S. atra*. Within these latter lineages, *S. a. pasubiensis* emerged as the sister group to all populations of the *S. a. atra* lineage. This phylogenetic reconstruction suggests that the yellow-patched colour is the ancestral condition in *S. atra* and the evolutionary transition to fully melanistic colour occurred probably through an intermediate reduction in the extension of the patches. Therefore, the fully melanistic colour of *S. lanzai*, which is probably not strictly related to *S. atra*, has an independent origin. The population from the Pasubio massif is here described as *Salamandra atra pasubiensis* n. subsp.

KEY WORDS: *Salamandra atra* - Molecular phylogeny - Colour evolution - Melanism - Taxonomy - *Salamandra atra pasubiensis* - Venetian Prealps.

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INTRODUCTION

The urodelan genus *Salamandra* represents a monophyletic group widely distributed in the western Palearctic. It shows a complex pattern of evolutionary differentiation with respect to morphological, physiological and biological traits, probably fostered by the geographic and climatic changes that have occurred during the last few millions of years (Veith, 1994; Steinfartz *et al.*, 2000; Weitere *et al.*, 2004).

In this group, the post-metamorphic colour of the integument is generally characterised by yellow patches determined by epidermal xanthophores on dermal iridophores; these patches are interspersed on a uniformly black surface, where only epidermal and dermal melanophores are present (Klewen, 1988; Pederzoli & Trevisan, 1990; Trevisan *et al.*, 1991; Pederzoli *et al.*, 2003). Different lineages, however, differ in their integument colour, particularly in the pattern, extension, and apparent hue of the patches (Eiselt, 1958; Veith, 1994).

Two fully melanistic lineages are known, namely *Salamandra lanzai* Nascetti, Andreone, Capula & Bullini, 1988 and *Salamandra atra atra* Laurenti, 1768, in which the xanthophore-iridophore complexes do not develop at all and thus the body surface is invariably and uniformly black. Recent molecular phylogenetic analyses suggest that these two lineages are not sister clades within *Salamandra* and therefore full melanisation probably evolved independently in the two cases (Veith, 1996; Veith *et al.*, 1998; Steinfartz *et al.*, 2000).

Salamandra lanzai is today represented by only a few populations limited to a restricted area in the Cottian Alps (Grossenbacher, 1997a); all known populations are fully melanistic and are genetically highly homogeneous (Steinfartz *et al.*, 2000; Ribéron *et al.*, 2002). Conversely, *S. atra* is widely distributed from the Alps to the Dinarides (Fig. 1) and shows a remarkable inter-population variation in chromatic traits associated with a phylogeographic genetic structure (Bonato & Grossenbacher, 2000; Ribéron *et al.*, 2001, 2004). Therefore, this latter lineage represents a challenging system for exploring the evolution of body colour.

Salamandra atra has long been considered a monotypic and fully melanistic species. Indeed the southernmost populations in the Balkans have been described as a distinct subspecies, *S. a. prenjensis* Mikšić, 1969, but the validity of this taxon is not supported by morphological or serological data (Joger, 1986; Klewen, 1988). More recently, a chromatically and genetically differentiated form was discovered in the Altopiano dei Sette Comuni (Venetian Prealps), at the southern edge of the Alpine range of the species, allopatric with respect to *S. a. atra* [Trevisan, 1982, *Abstract* in *Boll. Zool.*, 49 (suppl.): 184; Trevisan, 1983; Trevisan *et al.*, 1982, Trevisan *et al.*, 1984, *Abstract* in *Boll. Zool.*, 51 (suppl.): 107; Joger, 1986; Pederzoli *et al.*, 2001]. This form, which was described as a separate subspecies *S. a. auro-rae* Trevisan, 1982, is characterised by a dorsal

pattern of largely coalescent yellow patches. Observation of more than two hundred individuals strongly suggested that these patches consistently occur in all individuals of *S. a. aurorae* (Grossenbacher, 1994; Bonato & Fracasso, 2000). Although molecular analyses revealed that *S. a. aurorae* is genetically distinct from *S. a. atra*, the phyletic position of the former within *S. atra* still remains uncertain (see Steinfartz *et al.*, 2000, and Ribéron *et al.*, 2001, 2004).

A few years ago, a new peculiar population of *S. atra* was discovered on the Pasubio massif (Venetian Prealps), about 20 km southwest of the Altopiano dei Sette Comuni where *S. a. aurorae* can be found. Although no more than twenty salamanders have been observed to date, individuals of this population are usually yellow-patched, but the total extent of the patches is significantly reduced with respect to *S. a. aurorae*, and some individuals are even completely black (Bonato, 2000; Bonato & Grossenbacher, 2000). The phyletic position of this population is unknown.

In order to investigate the evolution of the body colour within the *S. atra* group, we performed a molecular phylogenetic analysis for all three chromatically differentiated lineages within *S. atra*. Unlike previous phylogenetic analyses of this group, the inclusion of the peculiar population from the Pasubio massif appeared crucial for unravelling the evolution of the body colour within *S. atra*. We used the mitochondrial DNA D-loop, as this marker has already proven to be useful in revealing genetic differentiation at intraspecific level within different *Salamandra* species (Steinfartz *et al.*, 2000). In particular, we evaluated whether the yellow-patched colour of the southern Prealpine lineages is plesiomorphic or apomorphic within *S. atra*, and traced back the evolution of full melanisation in the *S. a. atra* lineage. We also assessed the phyletic position and the taxonomic status of the peculiar population from the Pasubio massif.

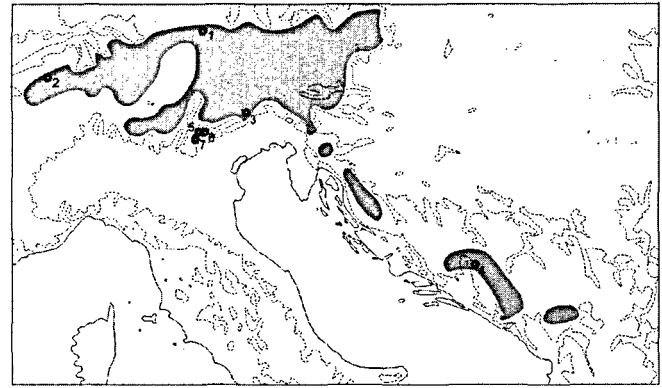


Fig. 1 - Geographic distribution of *S. atra* and sampled populations. Approximate range (shaded area) is mainly derived from Grossenbacher (1994, 1997b) and updated with subsequently published records (Krizmanić, 1997; Bonato & Grossenbacher, 2000). Sampled populations (circles) are coded according to Table I. Contour lines indicating a 500 m altitude are represented by dashed lines.

MATERIALS AND METHODS

Phylogenetic analysis

The complete mtDNA D-loop sequence was analysed for 20 individuals of *S. atra*, representing the three chromatically differentiated lineages within this species. The fully melanistic *S. a. atra* (including *S. a. prenjensis*) was represented by seven individuals from four different localities, the invariably yellow-patched *S. a. aurorae* was represented by six individuals from two different localities, and the population from the Pasubio massif, characterised by reduced patches, was represented by seven individuals (Table I, Fig. 1). Also individual representatives of all other European *Salamandra* species were analysed, i.e., *S. salamandra*, *S. lanzai*, and *S. corsica*. *Lyciasalamandra luschani* (formerly under *Mertensiella* or *Salamandra*; see Weisrock *et al.*, 2001; Garcia-Paris *et al.*, 2003; Veith & Steinfartz, 2004) was used for outgroup comparison,

TABLE I - Sampled populations, number of individuals and GenBank accession numbers for the mitochondrial D-loop sequences.

Code	Locality (country)	Taxon	n	GenBank no.
1	Mittenwald, near Garmisch (Germany) *	<i>S. a. atra</i>	3	AY628436
2	Berner Oberland (Switzerland) *	<i>S. a. atra</i>	2	AY628437
3	Tambre, near Belluno (Italy) *	<i>S. a. atra</i>	1	AY628438
4	Prenj-mountains (Bosnia) *	<i>S. a. prenjensis</i>	1	AY628439
5	Bosco del Dosso, Altopiano dei Sette Comuni (Italy) *	<i>S. a. aurorae</i>	3	AY628440
6	Val di Nos, Altopiano dei Sette Comuni (Italy)	<i>S. a. aurorae</i>	3	AY628441
7	Val Fontana d'Oro, Pasubio massif (Italy)	<i>S. a. pasubiensis</i> n. subsp.	7	AY628442
8	Monviso (Italy) *	<i>S. lanzai</i>	1	AY628443
9	Oviedo (Spain) *	<i>S. salamandra</i>	1	AY628444
10	Col de Bavella, Corsica (France) *	<i>S. corsica</i>	1	AF448816
11	Türbelinaz (Turkey) *	<i>L. luschani</i>	1	AY628445

*, sequences from Steinfartz *et al.* (2000).

as it represents the sister group of the entire *Salamandra* lineage (Titus & Larson 1995; Veith *et al.*, 1998; Weisrock *et al.*, 2001).

Tissue samples were obtained from live animals in the field. Following clipping off of a single toe, the individuals were immediately released at the capture location. Total genomic DNA was extracted using the SDS-proteinase K/Phenol-Chloroform extraction method. Published primer combinations (L-Pro-ML 5'GGCACCCAAGGCCAAAATTCT 3' and H-12S1-ML 5'CAAGGCCAGGACCAAACCTTTA 3'; Steinfartz *et al.*, 2000) were used to amplify the whole mitochondrial D-loop. PCR was performed in a 50- μ l scale under the following conditions (in final concentration): forward and reverse primer each 0.2 μ M, DNTPs each 0.2 mM, 1 \times reaction buffer, MgCl₂ 1.5 mM, 1 Unit Taq polymerase, 50-200 ng genomic DNA. The PCR reaction profile was the following: a single denaturation step at 95 °C for 2 min followed by 34 cycles; each cycle 95 °C for 25 s, 55 °C for 15 s, and 72 °C for 1 min; a single extension step at 72 °C for 10 min was run after the 34 cycles. Amplified mtDNA was purified using ultrafree-filters (Millipore, Bedford, MA, USA) and afterwards processed for symmetric cycle sequencing using the Big-Dye Ready-Reaction kit (Applied Biosystems, Foster City, USA).

Sequencing products were analysed on an ABI™ 377 (Perkin Elmer) using the Sequence Navigator software (Perkin Elmer). Sequences were aligned with the program CLUSTAL W (Higgins & Sharp, 1993) and subsequently adjusted by eye. Insertions and deletions were excluded.

Phylogeny was inferred through three different methods, i.e., Maximum Likelihood analysis (ML), Neighbour Joining algorithm (NJ) and Maximum Parsimony criterion (MP). ML was performed using PUZZLE (Strimmer & von Haeseler, 1996), whereas NJ and MP were performed using PAUP* 4.0 Beta version (Swofford, 1998). Taking into account variation among sites, we estimated the distribution parameter Γ from eight different rate categories from the dataset and corrected the sequences under the ML and NJ methods by the HKY-85_F substitution model (Hasegawa *et al.*, 1985). Robustness of branches was statistically estimated by 10,000 quartet puzzling replications for the ML reconstruction and by 10,000 bootstrap replications for NJ and MP reconstructions.

Analysis of colour evolution

The evolution of the body colour within the *S. atra* group was reconstructed through both Acctran and Deltran optimisation of the following two characters: (a) extension of the patches (patches present and relatively large in all individuals, vs. reduced and even absent in some individuals); (b) presence of the patches (patches present at least in some individuals, vs. lacking in all individuals).

We also applied the Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999) using the program PAUP* 4.0 to produce maximum likelihood estimates on different tree topologies, suggesting opposite evolutionary changes in the body colour within *S. atra*. Settings for the Shimodaira-Hasegawa test were the following: *L. luschni* was taken as the outgroup; ti/tv was estimated via ML; base frequencies, proportion of invariable sites, gamma distribution rates (from four rate categories) and the shape parameter alpha were estimated from the dataset. Ten thousands bootstrap replicates under the REL (Resampling Estimated Log-Likelihood) technique were performed to obtain statistical confidence intervals.

By using the reconstruction option in PAUP* 4.0 also the relative probability was estimated for character states which join the *S. atra* population from the Pasubio massif and *S. a. atra* into one group, while *S. a. aurorae* represents the sister group to this group, therefore supporting the hypothesis of a derived full melanisation within *S. atra*.

Morphology

The morphological description of the new subspecies was based on morphometric characters conventionally used for *Salamandra* taxa (e.g., Trevisan, 1983; Nascetti *et al.*, 1988; Krizmanić, 1997). Unambiguous definitions are here provided for some characters (see Table II): snout-vent length (from snout tip to anterior end of cloacal slit); head length (from snout to gular fold); head height (height measured at the level of jaw commissures); upper jaw length [from snout to jaw commissures; corresponding to 'head length' in Trevisan (1983)]; nostril-eye (from nostril to anterior eyelid commissure); eye diameter (from anterior to posterior eyelid commissure); forelimb length (from anterior

TABLE II - Morphometric characters of holotype and paratypes of *S. a. pasubiensis* n. subsp. Unambiguous definitions for some measures are provided under Materials and Methods.

Measurements (mm)	Holotype ZMF26139	Paratype ZMF26140	Paratype ZMF26141
Total length	87	91*	62
Snout-vent length	47.2	63.8	34.6
Head length	11.7	15.5	9.6
Head width	10.0	13.5	7.6
Head height	4.6	6.2	3.4
Upper jaw length	7.6	10.0	5.8
Internarial distance	3.6	4.9	2.9
Nostril-eye	2.9	4.3	2.2
Eye diameter	2.4	2.9	2.0
Parotoid length (left/right)	6.4/6.1	8.9/7.6	4.3/4.1
Parotoid maximum width (left/right)	3.1/3.1	4.7/4.5	2.0/1.7
Axilla-groin	24.2	33.4	18.7
Forelimb length	14.1	17.7	9.9
Hindlimb length	16.4	21.5	11.4
Hand length	6.3	9.3	4.5
Foot length	8.5	11.4	5.3
Tail length	35.7	22.3*	24.5

*, the tail of paratype ZMF26140 is mutilated.

attachment of forelimb to tip of finger III); hindlimb length (from anterior attachment of hindlimb to tip of finger IV); hand length (from inner border of hand to tip of finger III); foot length (from inner border of foot to tip of finger IV); tail length (from anterior end of cloacal slit to tail tip). All measurements were made with a calliper on dead specimens preserved in 70% alcohol. Total length was taken to the nearest mm, whereas all other measures were taken to the nearest 0.1 mm.

The number of vertebrae was estimated for each individual on the basis of radiographs performed by B. Lanza (Lanza B., Arntzen J. W., Gentile E., in prep.).

RESULTS

Phylogeny

In a comparison of 24 mitochondrial D-loop sequences, 590 bp out of 719 bp were constant. Genetic variation within the three chromatically differentiated forms of *S. atra* was very low, ranging from identical haplotypes to haplotypes that differed only by a single base pair. All individuals from the Pasubio massif shared a unique diagnostic haplotype and all *S. a. aurorae* individuals from both the sampled localities in the Altopiano dei Sette Comuni shared a different diagnostic haplotype.

Mean base frequencies were estimated at $\pi_A = 0.309$, $\pi_C = 0.223$, $\pi_G = 0.138$ and $\pi_T = 0.330$, resulting in a clear deviation from equal base frequencies. The distribution parameter Γ was estimated at 0.30, thus indicating strong variation among sites. Molecular distances, corrected by the HKY-85 $_{\Gamma=0.30}$ substitution model, for inter-specific comparisons ranged between 4.8% (*S. corsica*/*S. atra*) and 20.9% (*L. luschani*/*S. atra*), whereas those among populations within *S. atra* ranged between 0.6% and 1.5%.

All three phylogenetic reconstructions (MP, NJ, ML) resulted in the same tree topology (Fig. 2). They all strongly support the existence of three genetically distinct lineages within the *S. atra* group, corresponding to the three chromatically differentiated forms, with the following relationships: the largely patched *S. a. aurorae* emerges as a distinct lineage which separated early from the other extant ones; the poorly patched population from the Pasubio massif is clearly differentiated and is the sister group of a lineage including all remaining populations; the latter lineage comprises all fully melanistic populations represented by *S. a. atra*, also including the nominal *S. a. prenjenensis*. *Salamandra corsica* emerges as the sister group of *S. atra*, whereas a sister relationship between *S. atra* and *S. lanzai* can be reliably excluded, although the phyletic position of the latter species within the *Salamandra* group is not resolved.

Evolution of body colour

Both Acctran and Deltran optimisations of the characters describing the body colour within the *S. atra* group agreed in suggesting that (i) the consistent presence of large patches is the ancestral state within this group, (ii) a reduction of the extension of the patches occurred af-

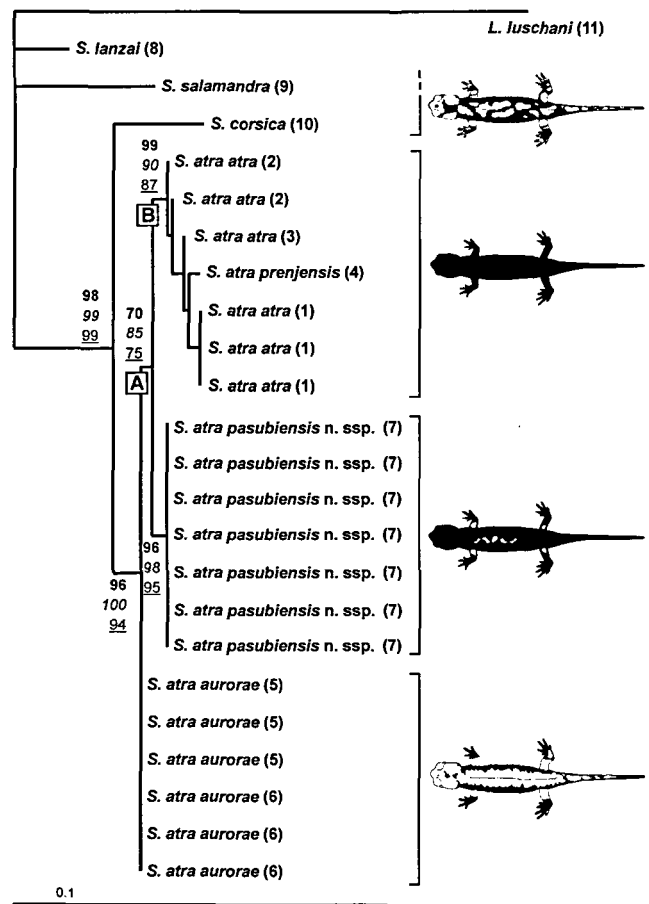


Fig. 2 - Phylogenetic tree for the *S. atra* group based on mitochondrial D-loop sequences, as inferred by the ML analysis (NJ and MP produced the same topology). Numbers at branches indicate puzzle values for ML reconstruction (bold) and bootstrap values for NJ (italic) and MP (underlined) reconstructions. Population codes are given in brackets (see Table I). Letters mark two hypothetical steps towards complete melanisation: A, reduction in the mean extension of patches; B, invariable absence of patches.

ter the emergence of the *S. a. aurorae* lineage and before the emergence of the lineage from the Pasubio massif, (iii) a complete melanisation occurred after the emergence of the latter lineage and at the basis of the clade including all populations of *S. a. atra* and *S. a. prenjenensis* (Fig. 2).

The same reconstruction was also obtained after extending the character optimisation to all *Salamandra* lineages represented in our analysis and considering all the alternative topologies for the unresolved basal trichotomy among *S. lanzai*, *S. salamandra* and *S. corsica* + *S. atra* (Fig. 2). Only the Deltran optimisation under the hypothesis that *S. lanzai* is the sister group of all other *Salamandra* lineages supported the alternative hypothesis that a fully melanistic colour is ancestral within the *S. atra* group, and that patches emerged independently in the lineage from the Pasubio massif and in *S. a. aurorae*. However, this hypothesis appears unlikely because the same option suggested that a fully melanistic colour is

the ancestral condition in the whole *Salamandra* clade and that patches appeared independently in four lineages, i.e., in *S. salamandra*, in *S. corsica*, in *S. atra* from the Pasubio massif, and in *S. a. aurorae*.

The tree topology [*L. luscbani* (*S. lanzai* (*S. corsica* (*S. a. aurorae* (*S. a. pasubiensis* n. subsp. + *S. a. atra*)))]), which suggests a plesiomorphic condition for the patched pattern and a derived condition for the completely melanistic colour in *S. atra*, had the highest likelihood of all possible tree reconstructions under ML (-ln L = 1623.34). The likelihood of the alternative tree [*L. luscbani* (*S. corsica* (*S. lanzai* (*S. a. atra* (*S. a. pasubiensis* n. subsp. + *S. a. aurorae*)))]), which suggests the opposite evolutionary change from a completely melanistic colour to the patched pattern was significantly lower (-ln L = 1634.30, P = 0.045) compared to the best tree.

The grouping of the population from the Pasubio massif and *S. a. atra* was supported by two character changes. A transition from G (present in *S. corsica* and *S. a. aurorae*) to A (present in *S. atra* from the Pasubio massif and *S. a. atra*) had a relative probability of 0.86. A transversion from C (present in *S. corsica* and *S. a. aurorae*) to A (present in *S. atra* from the Pasubio massif and *S. a. atra*) had a relative probability of 0.96. No synapomorphic character was found for a grouping of *S. a. aurorae* with the population from the Pasubio massif or with *S. a. atra*.

DISCUSSION

Phylogeny

The analysis of the mitochondrial D-loop sequences revealed that the three chromatic forms recognisable within the *S. atra* group correspond to three well-differentiated phyletic lineages. The analysis also produced a well supported hypothesis on the phylogenetic relationships among these three major lineages.

According to our phylogeny and on the basis of the molecular clock calibration provided by Steinfartz *et al.* (2000), *S. a. aurorae* separated from the other *S. atra* lineages about 1 million years ago and the population from the Pasubio massif separated from *S. a. atra* more recently, but most probably before the last glacial period. Therefore, the current geographic distribution of the three major lineages within *S. atra* was most probably shaped by the Pleistocene climatic oscillations: in particular, after the last retreat of the ice sheets, *S. atra aurorae* and the population from the Pasubio massif remained isolated in different Prealpine mountainous areas, whereas the *S. a. atra* lineage successfully recolonised most of the Alps and the Dinarides. This recent expansion would also explain the low degree of genetic differentiation within *S. a. atra* observed by us and other Authors (Ribéron, 1999; Ribéron *et al.*, 2001, 2004).

Previous phylogenetic analyses of the *S. atra* group (Ribéron, 1999; Steinfartz *et al.*, 2000; Ribéron *et al.*, 2001, 2004) did not include the peculiar population

from the Pasubio massif and were therefore not suitable for exploring the evolution of body colour within *S. atra*. All these analyses are consistent in suggesting an early separation of the *S. atra aurorae* lineage within the *S. atra* group but a phylogenetic tree based on the mitochondrial cytochrome b (Ribéron, 1999; Ribéron *et al.*, 2001) and a similarity tree based on the AFLP technique (Ribéron *et al.*, 2004) differ from our phylogeny by suggesting a close relationship between *S. a. aurorae* and some northern Dalmatian populations. However, both the latter analyses used samples from Dalmatian localities different to ours, which might be essential for the outcome. Moreover, mitochondrial (Cyt b gene; Ribéron *et al.*, 2001) and nuclear genes (AFLP tree; Ribéron *et al.*, 2004) seem to suggest significantly different phylogenetic hypotheses.

Taxonomy

The three major lineages within the *S. atra* group correspond to populations or population clusters which are different in colour features and allopatrically distributed and therefore they can be treated as different subspecies. The populations from the Altopiano dei Sette Comuni (Venetian Prealps), composed of invariantly largely yellow-patched individuals, are currently recognised as the subspecies *S. a. aurorae* Trevisan, 1982. A population from the Pasubio massif (Venetian Prealps), with individuals either completely black or with limited yellow patches, is here described as *S. a. pasubiensis* n. subsp. (see Appendix and Fig. 3). A cluster including all other known populations of *S. atra* throughout the whole range, invariably composed of completely black individuals, has to be assigned to the subspecies *S. a. atra* Laurenti, 1768, also including the nominal subspecies *S. a. prenjensis* Mikšić, 1969. The validity of the latter taxon is neither confirmed by our phylogeny nor by previous serological and morphological investigations (Joger, 1986; Klewen, 1988).

The classification of the population from the Pasubio massif as a new subspecies is not only justified by our molecular analysis, but it also allows a phylogenetically consistent and practically useful partition of the *S. atra* group. Conversely, assigning this population either to *S. a. aurorae* or to *S. a. atra* would be unsatisfactory: in the first case, *S. a. aurorae* would become paraphyletic and thus invalid following a cladistic approach; in the second case, the diagnostic features of *S. a. atra* should be extended to include also yellow-patched individuals and thus the most significant distinctive feature with respect to *S. a. aurorae* would not pertain.

Evolution of body colour

Our phylogenetic hypothesis provided a consistent framework for reconstructing the evolution of the body colour in the *S. atra* group (Fig. 2). Under the most parsimonious hypothesis, the ancestral populations of *S. atra* were composed of consistently patched individu-

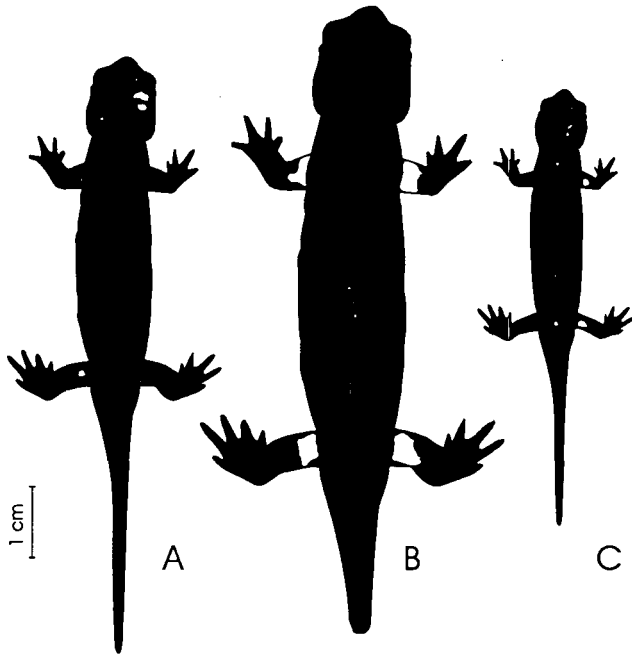


Fig. 3 - *Salamandra a. pasubiensis* n. subsp. A, holotype ZMF26139, female, from Pasubio Massif (Val Fontana d'Oro), 1750 m a.s.l. B, paratype ZMF 26140, female (tail mutilated), from Pasubio Massif (Val Fontana d'Oro), 1520 m a.s.l. C, paratype ZMF26141, juvenile, sex unknown, from Pasubio Massif (Val Fontana d'Oro), 1750 m a.s.l.

als, their integument regularly developing xanthophore-iridophore complexes. This is also the case for most of the living lineages of the other *Salamandra* species, including all species which have been proposed as sister groups of *S. atra* either by our own or by other phylogenetic analyses (Veith, 1996; Veith *et al.*, 1998; Steinfartz *et al.*, 2000). This ancestral condition is also maintained in *S. a. aurorae*, which is currently a relic lineage with a narrow distribution. The fully melanistic colour of the widespread *S. a. atra*, in which only melanophores are expressed, evolved secondarily from this ancestral condition. Up to now, conversely, most Authors hypothesized that *S. a. aurorae* is a recently derived lineage within *S. atra* (Trevisan, 1983; Grossenbacher, 1994; Bonato, 2000; but see Lapini *et al.*, 1998), thus implying that its patched colour is apomorphic with respect to the fully melanistic condition.

The reduced pattern of patches observed in *S. a. pasubiensis* may be confidently considered the stable and definitive colour of those individuals. In *Salamandra*, the colour pattern of each individual is usually a stable character through the post-metamorphic life. In *S. salamandra*, in particular, the definitive pattern of chromatophores develops at metamorphosis, through a deep structural remodelling of the integument (Pederzoli *et al.*, 2003), and only slight changes may occur subsequently. This is also the case for the patched pattern of *S. a. aurorae*, as observed in both free living and captive-reared individuals (Klewen, 1988; Steinfartz, 1998; also personal observation). An extensive and

quite rapid change of the hue of all patches was actually documented in two captive individuals of *S. a. aurorae*, the yellow having become dark brown within a few months (Steinfartz, 1998), possibly because of the melanisation of the xanthophores (Ide, 1978; Pederzoli & Trevisan, 1990); however, even in this case both individuals maintained their original pattern of patches, although less distinctly.

In *S. a. pasubiensis*, xanthophore-iridophore complexes still develop in most (but not all) individuals. In any case, the total extension of the yellow patches is significantly lower than in *S. a. aurorae* (Bonato, 2000). Assuming that the peculiar population from the Pasubio massif represents an intermediate condition in the evolutionary transition towards full melanisation, as suggested by its phyletic position, we can hypothesize that this change occurred through an intermediate, step-wise reduction in the individual extension of the yellow patches rather than through the gradual substitution between two (or more) discrete colour morphs (largely patched vs. fully melanistic), temporarily coexisting in polymorphic populations. However, whether the evolutionary fixation of a fully melanistic colour resulted from natural selection or is just the result of drift (possibly associated with the Pleistocene climatic oscillations) is an open question.

Worthy of note is the fact that the *S. a. atra* lineage seems to have completely lost the ability to develop the ancestral xanthophore-iridophore complexes. All known populations of *S. a. atra* (including the nominal subspecies *S. a. prenjensis*) are invariably fully melanistic, and no yellow patches putatively due to the typical ancestral xanthophore-iridophore complexes have ever been observed in any individual. Individuals with brown patches are actually rarely found within otherwise uniformly black populations (reports reviewed by Klewen, 1988; also personal observation), but this exceptional colour is more probably due to the local lack of melanophores than to the presence of different chromatophores, as suggested by histological investigations (Klewen, 1988; Pederzoli & Trevisan, 1990; Trevisan *et al.*, 1991). The only record of 'yellow' patches in *S. a. atra* was from the ventral side of an individual (Freitag, 1955) and therefore not resembling the ancestral pattern.

Our phylogenetic analysis also supports the hypothesis of convergent evolution of complete melanism in two distinct lineages of *Salamandra*: both *S. lanzai* and *S. a. atra* have evolved from yellow-patched ancestors and acquired independently a fully melanistic habit. Both lineages seem to have also evolved other similar features convergently, such as a relatively small body size and an intrauterine completion of the larval development. In the other species of *Salamandra*, conversely, no melanistic populations or even polymorphic populations including melanistic morphs are known and, although the extension and patterns of patches vary strongly both within and among populations, single uniformly black individuals are only rarely found (e.g., Pinston & Craney, 1998; Cochet *et al.*, 1999; Rivera *et al.*, 2001).

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APPENDIX

Salamandra atra pasubiensis new subspecies

Diagnosis. A *S. atra* with body coloration variable among individuals of the same population, from uniformly black (like in *S. a. atra*) to yellow-patched (like in *S. a. aurorae*). Patches mainly straw-yellow but even brownish, only on dorsal surface, most often on proximal part of limbs (arms and thighs) but also on head and trunk. Pattern of patches variable among individuals. Patches much less extended than in *S. a. aurorae*.

Holotype. Subadult, female, 87 mm long (91 mm alive), Pasubio Massif: Val Fontana d'Oro, UTM 32T 0671 5072, 1750 m a.s.l., L. Bonato leg. 19-V-2002, ZMF26139 (Fig. 3A).

Paratypes. Two specimens: (i) adult, female, 91 mm long (96 mm alive) (tail mutilated), Pasubio Massif: Val Fontana d'Oro, UTM 32T 0671 5071, 1520 m a.s.l., L. Bonato leg. 7-VII-2002, ZMF 26140 (Fig. 3B); (ii) juvenile, sex unknown, 62 mm long (64 mm alive), Pasubio Massif: Val Fontana d'Oro, UTM 32T 0671 5072, 1750 m a.s.l., L. Bonato leg. 29-VI-2002, ZMF26141 (Fig. 3C).

Type locality. Pasubio Massif (Venetian Prealps, North-East Italy): Val Fontana d'Oro, 1520-1750 m a.s.l.

Depository of holotype and both paratypes. Museo di Scienze Naturali, Sezione di Zoologia 'La Specola', Università di Firenze, Firenze (ZMF).

Etymology. From Pasubio massif (Venetian Prealps).

Description of holotype (measurements in Table II). Head 0.2 times as long as snout-vent length. Head oval from above, 1.2 times longer than wide; anterior margin rounded, maximum width at level of jaw commissure. Head depressed, 2.3 times wider than high; dorsal surface quite flat, with a median shallow concavity. Nostrils nearer to snout tip than to anterior commissure of eyelid, internarial distance 0.4 times the head width and 1.7 times the distance between nostril and eye. Eyes prominent, their horizontal diameter 0.2 times as long as head. Each parotoid gland swollen and pierced with ca. 15 pores; 0.5 times as long as head and 1.7 times longer than wide; width gradually increasing rearwards; external margins of glands almost parallel, internal margins slightly concave and converging rearwards. Upper jaw 1.2 times wider than long and 0.7 times as long as head; jaw commissure behind the posterior angle of eye. Gular fold straight, evident. Trunk slightly depressed, uniform in width; dorsal and ventral surfaces smooth, lateral surface with papillae and ca. 11 weak transversal grooves; two mid-longitudinal dorsal rows of ca. 15

glandular pores and two paramedian longitudinal rows of ca. 11 glandular pores. Forelimb and hindlimbs ca. 0.6 times as long as axilla-groin distance, the latter 1.1 times longer than the former. Hand with four fingers, phalangeal formula 1-2-3-2, digits in order of increasing length I-IV-II-III; foot with five fingers, phalangeal formula 1-2-3-3-2, digits in order of increasing length I-V-II-III-IV; all fingers stout and truncate at tip, without interdigital membrane. Tail sub-elliptical in section, laterally compressed, gradually tapering backwards, ending with a pointed tip; transversal grooves on lateral sides; two mid-longitudinal dorsal rows of glandular pores. Coloration (in life): uniformly black, slightly brownish on ventral surface of head and trunk; two straw-yellow blotches on the dorsal right side of head and one brown dot on the dorsal left side; a brown dot on the dorsal side of left thigh; no other blotches or dots. Number of vertebrae: 14 trunk vertebrae (atlas and sacral excluded), ca. 25 post-sacral vertebrae.

Description of paratype (i). Morphological traits corresponding to holotype except for the following points: internarial distance 1.3 times that between nostril and eye; tail mutilated, the tip rigidly bent ventrally. Coloration (in life): uniformly black, slightly brownish on ventral surface of head and trunk; two brown dots on dorsal side of trunk at about mid-length; a straw-yellow blotch, partially surrounded by a narrow brown contour, on the dorsal side of each arm and thigh. Number of vertebrae: 14 trunk vertebrae (atlas and sacral excluded).

Description of paratype (ii). Morphological traits corresponding to holotype except for the following points: head 0.3 times as long as snout-vent length; internarial distance 1.9 times that between nostril and eye; parotoid gland only slightly swollen, 0.4 times as long as head and 2.2 times longer than wide; trunk not evidently depressed; limbs and fingers more slender. Coloration (in life): uniformly black, slightly brownish on ventral surface of head and trunk; two straw-yellow dot and one brown dot on the dorsal right side of head, four tiny dots on the dorsal left side of head; a straw-yellow blotch on the dorsal side of the right arm and of the right thigh; two mid-longitudinal straw-yellow tiny blotches on the dorsal side of trunk, one just behind the neck, the other at the level of the groin; no other blotches or dots. Number of vertebrae: 15 trunk vertebrae (atlas and sacral excluded), ca. 24 post-sacral vertebrae.

Other studied specimens. Eighteen specimens, observed but not collected, from 52 to 130 mm long, both males and females, same locality as the holotype and paratypes, from 1520 m to 1760 m a.s.l.