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Nuclear and mitochondrial multilocus phylogeny and survey of alkaloid content in true salamanders of the genus *Salamandra* (Salamandridae)

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ABSTRACT

The genus *Salamandra* represents a clade of six species of Palearctic salamanders of either contrasted black–yellow, or uniformly black coloration, known to contain steroidal alkaloid toxins in high concentrations in their skin secretions. This study reconstructs the phylogeny of the genus *Salamandra* based on DNA sequences of segments of 10 mitochondrial and 13 nuclear genes from 31 individual samples representing all *Salamandra* species and most of the commonly recognized subspecies. The concatenated analysis of the complete dataset produced a fully resolved tree with most nodes strongly supported, suggesting that a clade composed of the Alpine salamander (*S. atra*) and the Corsican fire salamander (*S. corsica*) is the sister taxon to a clade containing the remaining species, among which *S. algira* and *S. salamandra* are sister species. Separate analyses of mitochondrial and nuclear data partitions disagreed regarding basal nodes and in the position of the root but concordantly recovered the S. *atra*/*S. corsica* as well as the *S. salamandra*/*S. algira* relationship. A species-tree analysis suggested almost simultaneous temporal splits between these pairs of species, which we hypothesize was caused by vicariance events after the Messinian salinity crisis (from late Miocene to early Pliocene). A survey of toxins with combined gas chromatography/mass spectroscopy confirmed the presence of samandarine and/or samandarone steroidal alkaloids in all species of *Salamandra* as well as in representatives of their sister group, *Lyciasalamandra*. Samandarone was also detected in lower concentrations in other salamandrids including *Calotriton*, *Euproctus*, *Lissotriton*, and *Triturus*, suggesting that the presence and possible biosynthesis of this alkaloid is plesiomorphic within the Salamandridae.

1. Introduction

True salamanders of the genus *Salamandra*, largely distributed over the Western Palearctic, are an intriguing group of amphibians exhibiting a huge variation in coloration patterns and reproductive modes. As presently understood (Thiesmeier, 2004; Speybroeck et al., 2010), *Salamandra* includes six distinct species. Of these, four share a contrasted black-yellow coloration: the widespread European *S. salamandra* (including *S. s. longirostris* which some authors consider as a distinct seventh species; Frost, 2013), covering major parts of southern and middle Europe with numerous subspecies; *S. algira,* with a fragmented distribution of various subspecies in northern Africa; *S. corsica,* endemic to the island of Corsica; *S. infraimmaculata*, with three recognized subspecies, distributed in the Near and Middle East. Additionally, two mainly uniformly black species are adapted to higher-elevation habitats in the Alps: the widespread *Salamandra atra* with two completely black subspecies (*S. a. atra*, *S. a. prenjensis*) and two partially black and yellow colored subspecies (*S. a. aurorae* and *S. a. pasubiensis*) as well as the monotypic *S. lanzai* restricted to a small area in the Cottian Alps bordering France and Italy. Melanistic populations also occur in species having typically a black/yellow pattern, such as *S. algira* and *S. salamandra* (see Seidel et al., 2012 for a graphical overview).

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Although it has never been thoroughly tested, it is commonly assumed that the contrasting color pattern of fire salamanders serves as an aposematic signal for potential predators, given that species of *Salamandra* are toxic (Schöpf, 1961). The major toxic compounds in the skin secretions of *S. salamandra* and *S. atra* are steroidal alkaloids called samandarines, of which nine derivatives have so far been identified (e.g. Schöpf, 1961; Habermehl and Spiteller, 1967; Daly et al., 2005). Comparatively little recent work has been done on these compounds, and their presence has neither yet been assessed in other species of *Salamandra*, nor in other representatives of the Salamandridae.

Additionally, true salamanders show varying degrees of viviparity, thus providing an excellent model for the study of reproductive modes and development. As summarized by Buckley et al. (2007), several taxa of *Salamandra* are ovoviviparous, with 20–60 larvae growing within the female on yolk nutrition only and being released to different kinds of water bodies (a reproductive mode called larviparity sensu Greven, 2003). Some other taxa can bear 1–15 fully metamorphosed juveniles. In these cases, different types of nutritional modes have been reported, such as maternal nutrition through unfertilized eggs, intrauterine cannibalism, and secretion of nutritious material in the uterus as seen e.g. in *S. atra* (Wake, 1993; Greven and Guex, 1994; Greven, 2003; Buckley et al., 2007). Larviparity occurs in *S. algira*, *S. corsica*, *S. infraimmaculata*, and most populations of *S. salamandra*, while release of metamorphosed juveniles (or pueriparity sensu Greven, 2003) occurs in *S. atra*, *S. lanzai*, some populations and subspecies of *S. salamandra* (e.g. *S. s. bernardezi*/*alfredschmidti*) and in *S. algira tingitana*, as well as in the sister genus *Lyciasalamandra*.

Despite this multifaceted biological interest in the genus *Salamandra*, no well-supported and complete phylogenetic hypothesis exists for this genus to this date. Numerous molecular phylogenetic studies have incorporated representatives of *Salamandra* and revealed the genus as part of the true salamanders, a clade of terrestrial genera within the Salamandridae. This terrestrial clade is the sister taxon to a clade of partly aquatic newt genera. Within the true salamanders, *Salamandra* is the sister taxon to *Lyciasalamandra*, and the clade of these two taxa is the sister taxon to a clade comprising *Chioglossa* and *Mertensiella* (Titus and Larson, 1995; Veith et al., 1998; Weisrock et al., 2001, 2006; Veith and Steinfartz, 2004; Frost et al., 2006; Steinfartz et al., 2007a; Zhang et al., 2008; Vieites et al., 2009). However, within *Salamandra*, conflicting topologies have been obtained based on different, mainly mitochondrial DNA sequence datasets: ((*infraimmaculata*, *atra*), (*algira*, (*lanzai*, (*salamandra*, *corsica*)))) according to Veith et al. (1998) based on a short stretch of the 16S rRNA gene; (*infraimmaculata*, *lanzai*, (*atra*, *corsica*), (*algira, salamandra*)) according to Steinfartz et al. (2000) based on mitochondrial control region sequences; and (*algira*, (*salamandra*, (*infraimmaculata*, (*lanzai*, (*atra*, *corsica*))))) according to Weisrock et al. (2006) based on 2700 bp of several mitochondrial genes. Additional molecular studies targeted specific aspects of the phylogeography and systematics of *Salamandra* species and subspecies (e.g. Joger and Steinfartz, 1994; García-París et al., 1998, 2003; Riberon et al., 2001; Martínez-Solano et al., 2005; Steinfartz et al., 2007b; Beukema et al., 2010; Reis et al., 2011; Velo-Antón et al., 2012). Yet, no comprehensive assessment of the phylogeny of this genus exists that also includes sequence data from nuclear genes.

The present study aims at a better understanding of the evolution of *Salamandra* by reconstructing phylogenetic relationships among all species and most subspecies of the genus, based on a comprehensive DNA sequence dataset of segments of 10 mitochondrial and 13 nuclear genes comprising almost 10 kbp. In addition, all species of the genus plus a set of other representatives of the Salamandridae were screened for skin alkaloids to reveal whether steroidal alkaloids are unique for the genus *Salamandra*, thus representing a derived character.

2. Materials and methods

2.1. Tissue sampling, DNA extraction, PCR and sequencing

Sampling was designed to include samples of all species of the genus *Salamandra*, as well as most subspecies. Tissue samples from toe clips of adults or fin clips of larvae were collected from a variety of specimens, either in the wild or from captive-bred specimens with known locality information of the parents. For some specimens, swabs (MW113, Medical Wire & Equipment Co.) were used to obtain buccal cells. All samples were preserved in 96% ethanol. Some of the tissue samples were identical with those used in Steinfartz et al. (2000) and Beukema et al. (2010). Total genomic DNA was extracted from tissue or swab samples using Proteinase K (10 mg/ml) digestion followed by a standard salt-extraction protocol (Bruford et al., 1992). Primers targeting four segments of mitochondrial DNA and 13 nuclear gene markers were employed in standard polymerase chain reactions (PCRs) for amplification. The selected markers include segments or entire sequences of the following mitochondrial markers: genes encoding 12S ribosomal RNA (*12S*), Cytochrome b (*COB*), NADH dehydrogenase 2 (*ND2*), tRNA-Trp, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, Cytochrome oxidase I (*COX1*), mitochondrial D-Loop gene (*DLOOP*); as well as the following nuclear markers: genes encoding brain-derived neurotrophic factor (*BDNF*), chemokine (C-X-C motif) receptor 4 (*CXCR4*), histone H3 (*H3*), leucine-rich repeat and WD repeat-containing protein (*KIAA1239*), sodium/calcium exchanger 1 (*NCX1*), propiomelanocortin (*POMC*), recombination activating genes 1 (*RAG1*) and 2 (*RAG2*), rhodopsin exon 1 (*RHOD*), sacsin (*SACS*), solute carrier family (*SLC*), titin (*TTN*), and platelet-derived growth factor receptor alpha intron 11 (*PDGFRA*). Polymerase chain reactions were performed in a final volume of 10 µl using 0.3 µM of each primer, 0.25 mM of dNTPs, 0.4 U GoTaq and 1.25 × Reaction Buffer (Promega). Primer characteristics, sources, and specific thermal cycling schemes are given in Supplementary Material Table SM1. For several markers, new primers were developed by first using a variety of universal primers, or primers established for other amphibians, to get one or a few *Salamandra* sequences for the respective gene, and subsequently use these sequences to design specific primers.

PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (SAP) or Antarctic Phosphatase (AP) according to the manufacturer's instructions (NEB). Purified PCR templates were directly sequenced using dye-labeled dideoxy terminator chemistry on an ABI 3130 automated DNA sequencer (Applied Biosystems). Chromatograms were checked and sequences manually corrected in CodonCode Aligner 3.5.6 (CodonCode Corporation). Newly obtained sequences were submitted to GenBank (accession numbers: KF645351-KF645999).

2.2. Phylogenetic analysis

Using the software MEGA 5 (Tamura et al., 2011), protein-coding sequences (*COB*, *ND2*, *COX1*, *BDNF*, *CXCR4*, *H3*, *KIAA1239*, *NCX1*, *POMC*, *RAG1*, *RAG2*, *RHOD*, *SACS*, *SLC*, *TTN*) were aligned by hand and translated into amino acids for authentication. Non-coding sequences (*12S*, *DLOOP*, tRNAs, *PDGFRA*) were aligned with the MUSCLE algorithm under default settings implemented in MEGA. Alignments of mitochondrial fragments that included insertions and deletions (*12S*, *DLOOP* and tRNAs) were processed with Gblocks 0.91b software (Castresana, 2000) to remove ambiguously aligned sections, with a less stringent 50% threshold for the