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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

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definition of reliable flanking positions and the remaining parameters at default settings. Heterozygous positions were included using degenerate base codes (IUPAC ambiguity codes) in the concatenated analyses, and separated into haplotypes (see below) for the species-tree analyses. The final matrix comprised 9666 bp of 35 terminals (see Table SM2). It included all known species and most subspecies of the genus *Salamandra*, and representatives of *Lyciasalamandra*, the sister genus of *Salamandra* (Weisrock et al., 2006; Zhang et al., 2008), as outgroup.

We used PartitionFinder 1.0.1 software (Lanfear et al., 2012) to infer the best-fitting model of molecular evolution and partition scheme applying to our combined dataset in the phylogenetic reconstructions. The best-fitting partition/substitution model scheme, as determined by the AICc (Table SM3), was implemented in a Bayesian inference (BI) phylogenetic analysis of the concatenated DNA sequences with MrBayes 3.2 (Ronquist et al., 2012). Results of two independent runs of 20 million generations, each comprising four Markov Chains (three heated and one cold), were sampled every 1000 generations. Chain mixing and stationarity were assessed by examining the standard deviation of split frequencies and by plotting–lnL per generation using Tracer 1.5 software (Rambaut and Drummond, 2007). Results were combined to obtain a majority-rule consensus tree and the respective posterior probabilities of nodes, after discarding 25% of the generations as burn-in. The same procedure was applied independently for the nuclear and the mitochondrial DNA datasets, with specific optimal models and partitions determined using PartitionFinder.

The concatenated mitochondrial DNA and nuclear DNA (mtDNA + nucDNA) dataset was furthermore submitted to a bootstrap analysis under the maximum likelihood (ML) and maximum parsimony (MP) optimality criteria. We conducted MP bootstrap analyses with 2000 pseudoreplicates in PAUP* v4.10 (Swofford, 2002), using a tree-bisection-reconnection (TBR) branch-swapping algorithm and 20 random-addition sequence replicates. Of the 9666 characters in the final alignment, 8507 were constant, and 895 were parsimony-informative. All characters were equally weighted and gaps were treated as missing data. We performed ML bootstrap analyses in RaxML v7.2.6 (Stamatakis, 2006) using 500 pseudoreplicates and estimating free parameters under a GTRGAMMA model applied to the 25 optimal partitions previously determined.

Using the combined information from all genetic markers, we inferred a species tree for the genus *Salamandra* using the multispecies coalescent approach implemented in *BEAST v.1.7.4 (Heled and Drummond, 2010). Prior to this analysis, alignments of nuclear genes were pruned to exclude stretches with missing data at the beginning and the end of some sequences. Subsequently, the haplotypes of nuclear markers showing more than one heterozygous position in the alignments were inferred using the phasing algorithm implemented in DNASp 5 (Librado and Rozas, 2009) with 10,000 replications. Parameters for the *BEAST run were specified using BEAUti 1.7 (Drummond et al., 2012). Alignments of mitochondrial DNA sequences were considered a single locus, as all mitochondrial genes are located on the same molecule and are linked with each other. As coalescent species-tree analyses do not require specifying an outgroup, we performed separate analyses with and without outgroup. For the former we grouped the samples of the genus *Lyciasalamandra* (outgroup) into a single artificial species and arranged the species of *Salamandra* as ingroup samples. All ingroup species were represented by haplotypes derived from 3 to 12 individuals. Substitution models were estimated for each alignment with jModelTest (Posada, 2008) (see Table SM5). A Yule prior was chosen for the species tree, and an uncorrelated lognormal clock was specified for the mitochondrial fragment, while strict clocks were set for the nuclear genes. To calibrate the molecular clock, we used an average mitochondrial substitution rate of 1%/site/million years (my) as a fixed parameter (corresponding to 0.01 substitutions per site per million years) following Hauswaldt et al. (2014) and, in a second run, a lower rate of 0.5%/site/my (see Section 4). Substitution rates for the nuclear genes were co-estimated. Additional analyses were run without specifying a substitution rate but instead using published divergence time estimates as calibration. Zhang et al. (2008), in two separate analyses, estimated the *Lyciasalamandra*/*Salamandra* split at either 27.7 mya (with confidence intervals of 16.1–39.9 mya), or as 43.4 mya (37.2–50.2 mya). The first of these estimates was implemented in our analysis as uniform prior with hard boundaries of 16–40 mya. A similar calibration with the second estimate (uniform prior: 37–50 mya) was not possible because starting likelihood values would reach minus infinity, preventing the MCMC to start; therefore, in this case we used a normal prior with 95% of the distribution between 37–50 mya and no hard boundaries.

In the species-tree analyses using a mutation rate as calibration MCMCs were run for 5×10^8 generations, sampling every 50,000 trees; while in the analyses using a root age as calibration the MCMCs were run for 1×10^9 generations, sampling every 100,000 trees. Results of the MCMC run were inspected for convergence and effective sample sizes (ESS) using Tracer 1.5 software (Rambaut and Drummond, 2007). Resulting trees were summarized with a burn-in of 20% in TreeAnotator 1.7.4 (Drummond et al., 2012). Analyses were run until ESS values were higher than 200,000 with unimodal posterior distributions.

Haplotype networks were built to visualize variation in all nuclear markers (details in Supplementary Materials). To analyze whether nuclear haplotype sharing among *Salamandra* species might be the result of hybridization, incomplete lineage sorting or PCR contamination, we analyzed DNA sequences of 24 specimens of *S. atra* and 21 of *S. salamandra* from an area of sympatry near Wolfenschiessen, central Switzerland, for one mtDNA marker (*12S*) and three nucDNA markers (*POMC*, *RAG2* and *PDGFRA*).

2.3. Alkaloid analysis from skin glands

Secretions were collected from wild-caught specimens by gently squeezing the parotoid glands and collecting the fluid with a small piece of laboratory-grade filter paper (Schleicher &-Schuell GmbH, Germany). The filter paper was preserved in a 2 ml glass vial with a Teflon-lined lid, filled with ca. 200 µl dichloromethane. In some cases, the fluid was directly sprayed from the gland into the glass vial without using filter paper.

The secretions were directly used for combined gas chromatography/mass spectrometry (GC/MS) analysis. Alkaloids were identified by GC/MS on an Agilent 7890A GC system fitted with a HP-5MS-fused silica capillary column (30 m, 0.25 mm i.d., 0.25 µm film; J&W Scientific, USA), connected to an Agilent 5975C inert mass detector. The following conditions were used: inlet pressure: 77.1 kPa, 23.3 ml He min^{-1}; injection volume: 1 µl; transfer line: 300 °C; electron energy: 70 eV; GC program: 5 min at 50 °C, then increasing with 10 °C min^{-1} to 320 °C, operated in splitless mode (60 s valve time). Known alkaloids (samandarine, samandarone, and *O*-acetyl-samandarine) were identified by comparison of mass spectra and gas chromatographic retention times with known data (Habermehl and Spiteller, 1967).

3. Results

Bayesian Inference analysis of the concatenated mtDNA + nucDNA dataset (9666 bp, after exclusion of hypervariable regions as suggested by Gblocks) produced a highly supported tree (Fig. 1A). Most nodes depicting interspecific relationships received Bayesian posterior probabilities (BPP) of $\geqslant 0.99$, and most of them were concordantly recovered by BI, ML and MP methods. A

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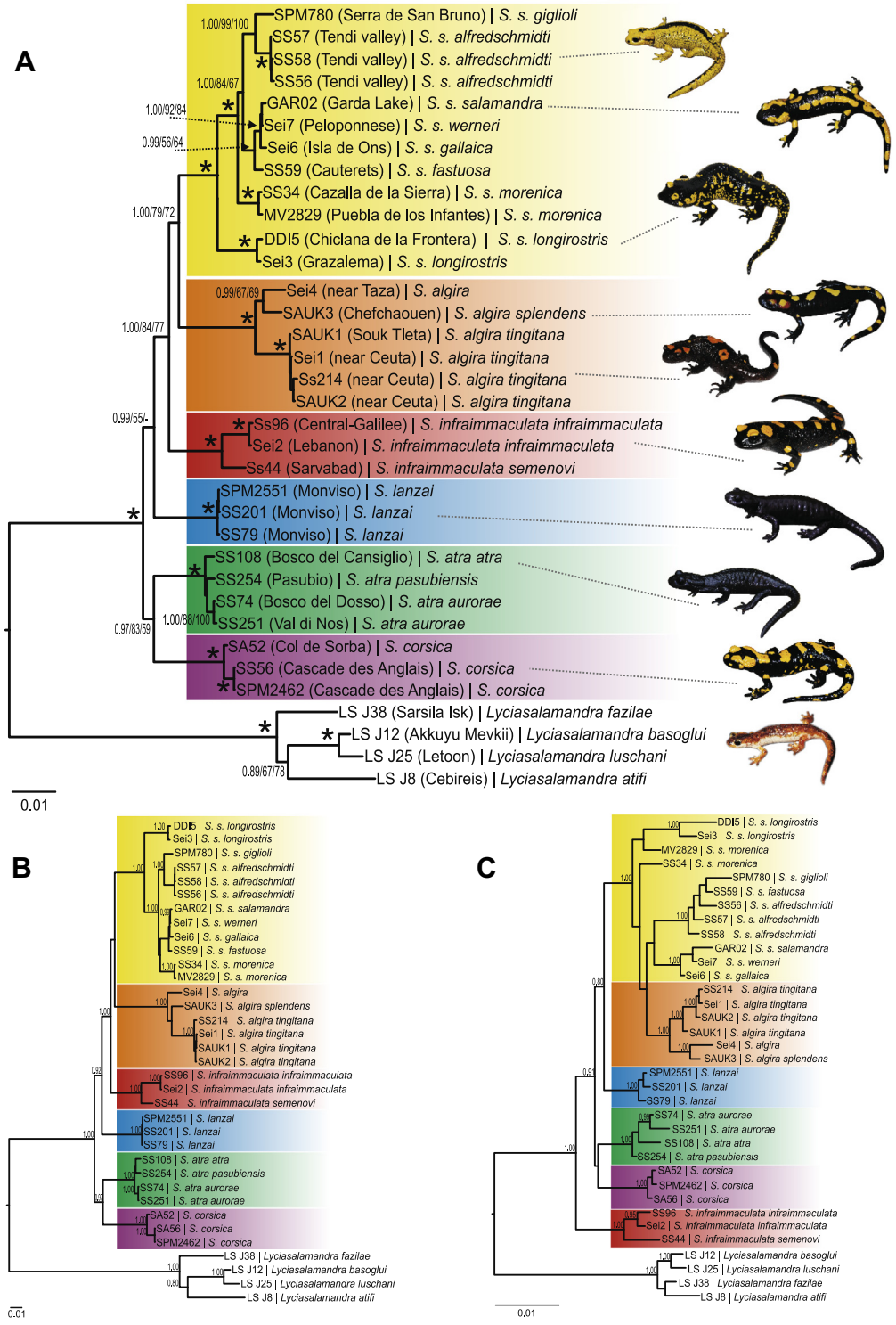

Fig. 1. Phylogenetic relationships of the *Salamandra* species inferred by Bayesian analyses of concatenated DNA sequences: (A) using all the genes, nuclear and mitochondrial combined, (B) using only the mitochondrial data, and (C) using only the nuclear data. Values at branches are Bayesian posterior probabilities ⩾0.9, followed in (A) by bootstrap values from MP and ML analyses. An asterisk indicates maximum support values (1.0/100) from all analyses. Samples of *Lyciasalamandra* were used as outgroup.

moderately supported clade of *S. atra* and *S. corsica* (BPP = 0.97) formed the sister group of a clade containing all other species. Within the latter clade, the deepest node separates *S. lanzai* (weakly supported) from the three remaining species, followed by separation of *S. infraimmaculata* from a common ancestor of *S. algira* and *S. salamandra*.

The tree based on the concatenated mitochondrial DNA sequences only (Fig. 1B) agrees with the combined mtDNA + nucDNA tree for the most part. All taxa were supported equally well and species were reciprocally monophyletic. The *S. atra*/*S. corsica* clade received moderate support (BPP = 0.97), like in the combined analysis, but the clade grouping *S. algira*/*S. salamandra* did not receive significant support by the mitochondrial data alone.

For the phylogenetic tree reconstructed solely on the concatenated nucDNA genes (Fig. 1C), support values for most of the basal nodes were relatively low. Here, *S. infraimmaculata* was placed as

the sister taxon of a clade containing all other species, and within the *S. algira*/*S. salamandra* clade, *S. algira* was nested within a paraphyletic *S. salamandra*. Extensive haplotype sharing was observed for most of the nuclear genes, even among the phylogenetically most distant species (see haplotype networks in Supplementary Materials; Figs. SM1–13). Some genes, such as *RHOD*, *H3*, *NCX1* or *POMC*, were particularly invariable, with numerous species sharing one predominant haplotype, while for a few other genes (*RAG2*, *PDGFRA*, *TTN*) most species had private haplotypes. The 21 individuals of *S. salamandra* and the 24 individuals of *S. atra* from a contact zone in central Switzerland were all phenotypically assignable to either species without ambiguity. The haplotypes determined for mtDNA (12S) and two highly variable nucDNA markers (*PDGFRA*, *RAG2*) confirmed the phenotypic species assignment, and accordingly no signs of hybridization were observed. For *POMC*, the haplotype sharing as observed among other *Salamandra* species was also confirmed for these populations. Of two haplotypes detected, one was found exclusively in *S. atra* while the other was found in all *S. salamandra* and in four *S. atra* (Fig. SM14) of which three were heterozygotes.

The species tree (Fig. 2; calculated with outgroup) shows relatively weakly supported relationships among *Salamandra* species. The unrooted topology is identical to that of the concatenated analysis (Fig. 1A) but the root is recovered at the branch separating *S. lanzai* from the remaining species. Highest node probability is recovered for the sister-species relationship between *S. algira* and *S. salamandra* (0.66), followed by the sister-species relationship between *S. corsica* and *S. atra* (0.44). The most recent common ancestor (MRCA) of all *Salamandra* species is at 4.4 mya (95% HPD: 5.6–3.4 mya) and the splits of *S. algira*/*S. salamandra* and *S. atra*/*S.corsica* are both close to 3 mya. The species-tree analysis without outgroup (Fig. SM17) suggested similar divergence times, except for a younger *S. atra*/*S. corsica* split at 2.3 mya (Fig. SM17).

A species tree with an alternative mitochondrial substitution rate of 0.5%/site/my (see Section 4) yielded ages between 5 and 6 mya for the splits of *S. algira*/*S. salamandra* and *S. atra*/*S.corsica*, and 8 mya for the earliest divergence within *Salamandra*, respectively. Species trees calibrated with priors for the *Lyciasalamandra*/*Salamandra* divergence (alternatively, 16–40 or 37–50 mya) rather than using substitution rates yielded ages around either 3.5 or 7.5 mya for the splits of *S. algira*/*S. salamandra* and *S. atra*/*S.corsica*, and either 5 or 10 mya for the earliest divergence within *Salamandra*. In either case, confidence intervals overlapped with the values obtained in most of the other analyses.

Regarding the phylogeny at the subspecies-level entities, the combined tree, as well as the mtDNA and nucDNA trees (Fig. 1A–C), provided several concordant insights. Within *S. salamandra*, mtDNA and nucDNA combined data placed the subspecies *longirostris* from the Betic (Penibetic) mountain chains in southern Spain, and the equally southern Iberian *morenica*, as the sister groups of all other samples of the species. Furthermore, both sets of markers placed a sample from the southern Italian region Serra San Bruno (subspecies *gigliolii*) together with the subspecies *alfredschmidti* from northern Spain as a clade. Subspecies of *S. atra*, *S. infraimmaculata*, and *S. algira* included in the analysis all showed substantial amounts of divergence and were monophyletic in the mtDNA tree, and samples of the same subspecies were also placed in the same clades in the nucDNA tree.

The GC/MS analysis of the toxin samples revealed mass spectra typical for *O*-acetylsamandarine in moderate to high concentrations in *S. atra, S. corsica*, *S. infraimmaculata*, *S. lanzai*, and *S. salamandra*, as well as in *Lyciasalamandra* (Table 1). Samandarone was observed at high concentrations in *S. algira, S. atra, S. corsica. S. infraimmaculata*, *S. lanzai*, *S. salamandra* (including numerous subspecies), and *Lyciasalamandra*, and in lower concentrations in *Calotriton*, *Euproctus*, *Lissotriton*, and *Triturus*. Samandarine was detected in high concentrations in *S. atra* and *S. lanzai*, and in moderate amounts in a few other *Salamandra* samples. Numerous other undetermined alkaloid compounds were observed in *Salamandra* and *Lyciasalamandra*, but not in the other salamandrids except trace amounts in *T. cristatus* (Supplementary Figs. SM15 and SM16).

4. Discussion

4.1. Phylogeny of Salamandra species

This study provides the first comprehensive multilocus genetic analysis comprising all species of the genus *Salamandra* and combining mtDNA and nucDNA sequence data. The combined concatenated dataset suggests a highly supported phylogenetic topology in disagreement with the hypothesis of Veith et al. (1998). The (unrooted) topology recovered largely agrees with that of Steinfartz et al. (2000) based on the mitochondrial control region, and fully agrees with that of Weisrock et al. (2006). This and the congruence of mitochondrial and combined concatenated trees suggests that the results of the combined analysis have mainly been driven by the mitochondrial data. However, despite

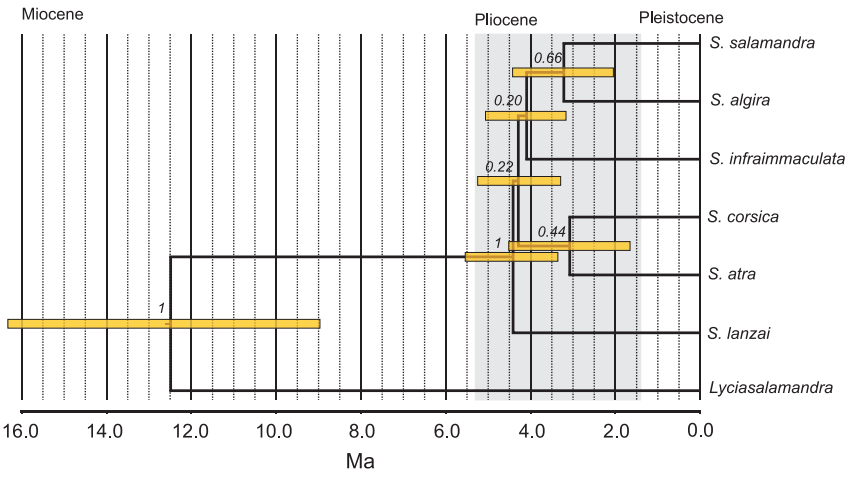

Fig. 2. Species tree of the genus *Salamandra* with *Lyciasalamandra* as outgroup, inferred from nuclear and mitochondrial DNA sequence data using *BEAST software and time-calibrated using a prior of 1%/site/mya for mtDNA. The posterior probabilities are shown above nodes. Bars at nodes represent the 95% highest posterior density of the node age.

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Table 1
Steroidal alkaloids (samandarine, *O*-acetyl-samandarine, samandarone) and undetermined alkaloids (some of which are structurally related to samandarine and samandarone) observed in *Salamandra* and other salamandrids. Symbols indicate relative amount as determined by GC/MS analysis (xxx high (peak height 30–100% of that of major component of the extract, which was either an alkaloid or cholesterol), xx moderate (3–30%), x low (less than 3%)). Cholesterol occurs in moderate to major amounts in every sample. Samples were taken in the wild except for some captive specimens, which are marked as such.

Species	Locality	Samandarine	Samandarone	*O*-Acetyl-samandarine	Unidentified alkaloids
Salamandra atra	Wolffenschiessen, Switzerland	xxx	xxx	xxx	xxx
S. atra	Wolffenschiessen, Switzerland	xxx	xxx	xxx	xx
S. atra	Wolffenschiessen, Switzerland	xxx	xxx	xxx	xx
S. lanzai	Monviso Massif, Italy	xxx	xxx	xxx	xxx
S. corsica	Vizzavona, France		xx	x	xxx
S. corsica	Niello, France		xxx	x	x
S. corsica	Badella, France		xxx	xx	xx
S. infraimmaculata	Central Galilee, Israel	x	xxx	xxx	xx
S. infraimmaculata	Tel Dan, Israel		xxx	x	x
S. algira splendens	Taza, Morocco		xxx		xx
S. algira splendens	Taza, Morocco		xxx		xxx
S. algira splendens	Morocco, central Rif (captive)		xxx	x	xx
S. algira tingitana	Sidi Mulay Abdesalam, Morocco (captive)		xxx	x	xxx
S. s. salamandra	Reitlingstal/Elm, Germany	x	xxx	xxx	xx
S. s. gallaica	Grixoa, Spain (captive)		xxx	xxx	x
S. s. crespoi	Portugal, Rio Mira (captive)		xxx	xx	x
S. s. crespoi	Portugal, Rio Mira (captive)		xxx	x	x
S. s. alfredschmidti	Tendi valley, Spain (captive)	xx	x	xxx	x
S. s. alfredschmidti	Tendi valley, Spain (captive)		xxx	xxx	xxx
S. s. morenica	Cazalla de la Sierra, Spain (captive)		xx	xxx	x
S. s. longirostris	Medina, Spain (captive)		xxx	xxx	
S. s. longirostris	Montecoche, Spain (captive)		xxx	xxx	
S. s. longirostris	Alcala de los Gazules, Spain		xxx	x	x
Lyciasalamandra facilae	Unknown locality, captive		xxx	xx	xx
Lyciasalamandra billae	Unknown locality, captive	x	xxx	x	x
Euproctus montanus	Badella, France		xx		
Triturus pygmaeus	Alcala de los Gazules, Spain		x		
Triturus cristatus	Elm, Germany		xx		x
Lissotriton boscai	Mera, Spain		x		
Calotriton asper	Respomuso, Spain		x		
Neurergus kaiseri	Unknown locality, captive				

this agreement in topology, the placement of the root of the *Salamandra* clade remains highly contentious. In Weisrock et al. (2006) it was recovered on the branch leading to *S. algira,* while in the concatenated analysis of our study it is placed on the branch leading to the *S. atra/S. corsica* clade (Fig. 3).

Two clades depicting sister-species relationships need to be especially highlighted. On one hand, there is a clade including *S. algira* and *S. salamandra* that received maximum support from nucDNA (*S. algira* even being nested within *S. salamandra*) (Fig. 1C). This clade was also recovered, albeit without significant support, by mtDNA alone (Fig. 1B). On the other hand, the sister-group relationship of *S. atra* and *S. corsica* was strongly supported by mtDNA (BPP = 0.97), but recovered without significant statistical support by nucDNA. Neither of these two clades was strongly supported by both datasets. Yet, their recovery by analyses of two largely independent data sets, as well as by the combined analyses (concatenated and species tree) provides some confidence that they represent the true phylogenetic relationships between these species.

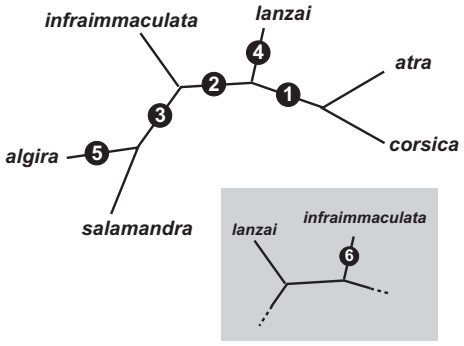

Fig. 3. Simplified unrooted tree of species in the genus *Salamandra* following analyses herein and Weisrock et al. (2006). The numbered black circles indicate alternative placements of the root, as suggested by different analyses: (1) mtDNA and concatenated mtDNA + nucDNA (Fig. 1A-B); (2) species tree with outgroup and root priors for age calibration (SI), (3) species tree without outgroup and substitution rate prior (SI); (4) species tree with outgroup and substitution rate prior (Fig. 2); (5) mtDNA analysis of Weisrock et al. (2006). The concatenated nucDNA analysis (Fig. 1C) is the only one suggesting (with low support) a different topology in which *S. infraimmaculata* and *S. lanzai* exchange their positions (gray box) and the root is placed (6) on the *S. infraimmaculata* branch.

On the contrary, despite inclusion of almost 10 kbp of mtDNA and nucDNA, the more basal nodes in the phylogeny and especially the placement of the root of the *Salamandra* clade still could not be unambiguously resolved. Whether *S. atra*/*S. corsica* constitutes the sister group of all other species of *Salamandra* (as suggested by the combined concatenated tree and the mtDNA tree; Fig. 1A–B), or this position corresponds to *S. infraimmaculata* (as suggested by the nucDNA tree; Fig. 1C), or by *S. lanzai* (as suggested by the species tree analysis in Fig. 2), or to yet other clades (Fig. 3; Figs. SM17–SM19) basically remains unsolved, as also indicated by the low node support in the species tree (Fig. 2).

The lack of unambiguous phylogenetic resolution within *Salamandra* despite the exceptionally large dataset is largely caused by the unexpectedly low genetic variation and the high degree of haplotype sharing in numerous of the nuclear gene segments analyzed. This phenomenon can be explained partly by the short average length of the DNA segments analyzed (ranging from 209 to 783 bp). As discussed by Huang et al. (2010), accurateness of species tree inference depends on multiple factors, among others on an adequate a-priori choice of markers, and the short gene segments used herein might simply confer too little information. Extensive interspecific haplotype sharing, however, was also observed in the nuclear marker *SACS* for which we analyzed a

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larger segment (682 bp). Haplotype sharing also characterized genes such as *RAG1* or *POMC* which, despite being rather evolutionarily conserved, had exclusive haplotypes in various closely related tropical and temperate amphibian species such as *Boophis* (Vences et al., 2010, 2012), *Discoglossus* (Pabijan et al., 2012) or *Salamandrina* (only *RAG1*: Hauswaldt et al., 2014).

The analysis of samples from a contact zone of *S. atra* and *S. salamandra* (the only two species of the genus *Salamandra* occurring occasionally in syntopy), found neither signs of hybridization or introgression in mtDNA nor in the two nucDNA fragments that frequently (*RAG2*) or completely (*PDGFRA*) lacked haplotype sharing in the multispecies analysis. Hence, these markers provided no indication for hybridization, even in a contact zone of both species. In contrast to the situation in *Salamandrina* species, which show extensive hybridization in a contact zone (e.g. Hauswaldt et al., 2011), the extensive haplotype sharing among species of *Salamandra* is probably caused by incomplete lineage sorting, and might be related to the relatively young ages of most species in the genus (Fig. 2).

Results herein confirm the status of numerous taxa within *Salamandra,* but they also expose the need for revision in several cases although the limited population-level sampling in our analysis hampers final conclusions. The included individuals assigned to the subspecies *S. atra aurorae* and *S. a. pasubiensis* clustered separate from typical *S. atra* in the concatenated analysis of the combined dataset, and a similar situation applies to individuals belonging to other subspecies-level taxa (e.g., *S. algira tingitana*). The placement of *S. s. longirostris* as the sister group of all other *S. salamandra* lineages would be compatible with its interpretation as a distinct species (e.g., Frost, 2013); this view was not shared in a recent revision of the systematics of the European herpetofauna, however (e.g., Speybroeck et al., 2010).

The results of our study merit discussion from two biogeographic perspectives. First of all, two competing hypotheses can be postulated regarding the geographic origin of the genus. Based on nucDNA, the Eastern Mediterranean and Near Eastern *S. infraimmaculata* splits off at the most basal node of the genus. *Lyciasalamandra*, the sister group of *Salamandra*, is exclusively distributed in the Eastern Mediterranean region (Weisrock et al., 2001; Veith and Steinfartz, 2004). This could indicate that the MRCA of *Salamandra* also was distributed in this region, and that the genus subsequently expanded into central and western Europe and from there to North Africa. Alternatively, as suggested by the combined and mtDNA analyses, respectively, the position of the Corsican and Alpine species (namely *S. atra*, *S. corsica* and *S. lanzai*) placed paraphyletically spanning the base of the tree, suggests a possible origin of the MRCA of *Salamandra* in the mountainous and alpine areas of central Europe. This latter hypothesis would be supported by the fossil record, as most Oligocene–Miocene remains assignable to the extinct species *S. sansaniensis* (reviewed in Veith et al., 1998) which has been discovered in central Europe. The analysis of deep salamandrid relationships by Zhang et al. (2008) suggests an initial diversification of the family in Europe during the Cretaceous, but the fact that the sister group of the *Lyciasalamandra*/*Salamandra* clade comprises one European (*Chioglossa*) and one Eastern Mediterranean (*Mertensiella*) genus inhibits an easy inference of the ancestral range of these more inclusive clades and hence, of the MRCA of *Salamandra*.

The second biogeographic aspect concerns the sister-group relationships of *S. atra*/*S. corsica*, and *S. algira*/*S. salamandra*. Both of these clades occur on adjacent geographical areas separated by the Mediterranean Sea: *S. atra* is widespread in the Alps, while *S. corsica* occurs mainly in mountainous areas of Corsica. In the second species pair, *S. algira* occurs in northern Africa, and in *S. salamandra* the subspecies separated by the most basal nodes within *S. salamandra* are endemic to the southern Iberian Peninsula, i.e. *S. s. longirostris* and *S. s. morenica*. Furthermore, it is relevant that in the species-tree analysis, these two species pairs show very similar divergence times, suggesting that splitting might have been triggered by the same biogeographic/climatic event. Despite the uncertainty of the time estimates, it is appealing to hypothesize that these divergences were caused by vicariance after the desiccation of the Mediterranean at the end of the Messinian salinity crisis, at the Miocene–Pliocene boundary, approximately 5.33 mya (Duggen et al., 2003; Rouchy and Caruso, 2006). Obtaining a reliable estimate of molecular age to confirm or reject this hypothesis will be rather difficult to achieve. Solid ingroup calibrations would require a comprehensive cladistic analysis of fossils to verify whether fossils assigned to *S. sansaniensis* can indeed be assigned to the *Salamandra* clade or whether they rather correspond to ancestors of the *Salamandra* + *Lyciasalamandra* clade. Hauswaldt et al. (2014) used outgroup fossil dating to estimate a substitution rate of 1%/site/my for the Salamandridae, a rate that we also used for the age estimation herein (Fig. 2). The estimated divergence times in our preferred analysis (Fig. 2) are post-Messinian, although at least the maximum values of the 95% confidence intervals are close to the age of the Messinian salinity crisis (5.33 mya). However, the 1%/site/my substitution rate was originally calculated for the cytochrome *b* gene, and our mitochondrial data set includes several genes known to evolve more slowly (e.g., *12S* and *16S* after exclusion of hypervariable regions). Therefore it is likely that the evolutionary age of lineages within *Salamandra*, as shown in Fig. 2, is an underestimation. An exploratory analysis employing a mtDNA substitution rate of 0.5%/site/my indeed resulted in divergences of *S. atra*/*S. corsica*, and *S. algira*/*S. salamandra* between 5–6 mya, and a single-gene analysis using the 1%/site/my with only cytochrome *b* sequences (enforcing monophyly of the two sister species pairs) yielded divergence estimates of 5.9 and 7.5 mya. Also, in the two analyses calibrated with previous time estimates for the *Lyciasalamandra*/*Salamandra* split (Zhang et al., 2008), confidence intervals overlapped with the age of the Messinian salinity crisis (Figs. SM18 and SM19). However, we here refrain from presenting these data in more detail because only a more comprehensive analysis, which should include precise cladistic assignment of fossils and additional nuclear markers that are not showing extensive haplotype sharing, could clarify shallow ages within the Salamandridae with sufficient precision and reliability.

4.2. Preliminary insights into the evolution of salamandrid alkaloids

Our data on alkaloid toxins suggest that these compounds are non-informative in elucidating phylogenetic relationships within *Salamandra* as they were universally distributed in all species of the genus. Until now, steroidal alkaloids, such as samandarine, samandarone and related compounds, had been known only from *S. salamandra* and *S. atra*. Presumably, samandarines are synthesized by salamanders from cholesterol, rather than sequestered from the diet, as are the alkaloids of poison frogs; however, this biosynthetic pathway has been suggested based on in vitro experiments only (see Habermehl and Haaf, 1968). The relative concentration of the toxins varies among individuals and seasons (Mebs and Pogoda, 2005). Several other salamandrids were known to contain tetrodotoxins, possibly synthesized by symbiotic bacteria (Daly et al., 2005), but so far had not been reported to secrete steroidal alkaloids. The broad screening of toxins in our study demonstrates that steroidal alkaloids are a prominent component of the skin secretions of all species of *Salamandra*. It also provides evidence for their high concentrations in the secretions of *Lyciasalamandra*. One of these alkaloid compounds, samandarone (but not samandarine and *O*-acetyl-samandarine) was furthermore found in lower concentration in various other genera of salamandrids. Based on our findings, we hypothesize that the biosynthetic pathway for these compounds evolved early in salamandrid evolution,

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and evolved into a major and highly concentrated toxic combination in the *Lyciasalamandra*/*Salamandra* clade. This demonstrates that knowledge of toxin composition of urodelans is incomplete and that surprises can be expected from in-depth analyses in progress. A wider screening of salamandrids other than *Salamandra* is needed to understand whether indeed samandarone is their only major alkaloid compound, or whether individual variations might occur. Our results also demonstrate that coloration (contrasting black-yellow vs. uniformly black) is not correlated with content and concentration of alkaloid toxins, which were present in comparable concentrations in all taxa studied (to the exception of samandarine that was found in high proportion only in *S. atra* and *S. lanzai*, the two mainly uniformly black species). It is probable that uniformly black color serves for thermoregulation in the alpine habitats of *S. a. atra* and *S. lanzai*. Given the toxicity of these salamanders, it is worth testing whether a uniform black color, which contrasts strongly with some types of substrate, could also serve an aposematic function.

Each of the alternative phylogenetic hypotheses resulting from the data herein suggest homoplasy in regard to color evolution, in agreement with previous analyses (Veith et al., 1998; Steinfartz et al., 2000). The fully black taxa (*S. a. atra* and *S. lanzai*) are not resolved as a clade, and the *S. atra* subspecies with the most yellow coloration (*S. atra aurorae*) is phylogenetically nested among melanic forms. Homoplasy is also suggested in the evolution of ovoviviparity versus viviparity, as the viviparous taxa included in the analysis (*S. atra, S. lanzai, S. salamandra alfredschmidti*, *S. algira tingitana*, and *Lyciasalamandra*) never formed a monophyletic group (note that *S. s. alfredschmidti* is very closely related to the equally viviparous *S. s. bernardezi*, not included in our study). Given the poor resolution provided by most nuclear gene fragments included, it is likely that only phylogenomic methods will be able to provide a robustly resolved phylogenetic tree for true salamanders. Such a phylogenomic tree will reveal the evolution of the many remarkable phenotypic characters that have attracted the attention of scientists for centuries.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.12.009.

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