Contributions to Zoology, 84 (2) 85-113 (2015)

Vertebral number is highly evolvable in salamanders and newts (family Salamandridae) and variably associated with climatic parameters

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Key words: ancestral character states, body shape, climatic niche, comparative method, phylogeny, vertebral column

Abstract

In vertebrates, the relative proportion of the number of trunk and caudal vertebrae is an important determinant of body shape. While among amphibians frogs and toads show low variation in vertebrae numbers, in salamanders the numbers of trunk and caudal vertebrae vary widely, giving rise to phenotypes in the range from short-bodied and long-tailed to long-bodied and short-tailed. We analysed vertebral numbers in the family Salamandridae in a phylogenetic context and calculated the relationship between vertebral changes and changes in climate and other environmental parameters. A significant association was found between morphological change with precipitation and temperature. However, annual precipitation affected the two main groups of salamandrid salamanders differently, with trunk elongation in the terrestrial 'true salamanders' and tail elongation in the more aquatic 'newts'. A - male biased - sexual dimorphism was only observed in *Lissotriton vulgaris vulgaris* in the number of trunk vertebrae and in *Ommatotriton ophryticus* and *Lissotriton* species for the number of caudal vertebrae. Our data indicated that the number of trunk and caudal vertebrae are highly evolvable traits with frequent evolutionary reversals. In some groups (*e.g. Cynops, Lyciasalamandra, Neurergus* and the *Laotriton-Pachytriton-Paramesotriton* clade) the number of trunk vertebrae is stable, while in many groups it is subject to change (*e.g. Tylototriton*). This latter, species-rich genus appears to be an excellent group to further test effects of the environment on body shape.

Contents

Introduction

A major goal of evolutionary biology is to understand the origin of morphological diversity in the living world. Some morphological features are easily understood in an adaptive context, such as a cryptic versus aposematic colouration and the visibility to potential mates and predators, sturdiness of the skull and fossoriality, or limb length and running speed (*e.g.* Rettenmeyer, 1970; Gans, 1975; Garland and Janis, 1993; Galis, 1996). However, morphological diversification appears often constrained within lineages. One example concerns the conserved body plans that define the three orders of Amphibia, namely the tailless and compact built frogs and toads (order Anura), the legless, eel-shaped caecilians (order Gymnophiona) and the elongated, legged and tailed salamanders (order Urodela). The short trunk and the absence of a tail in frogs and toads may be an adaptation to digging backwards (Hillenius, 1976) or jumping (Handrigan and Wassersug, 2007). The latter authors further argue that the conservation of the short trunk and the absence of discrete caudal vertebrae in anurans are due to a limited modifiability of the developmental programs involved, which includes activity of the conserved *Hox* genes. The conservation of the limbless caecilian body plan (Nussbaum, 1977; Wake, 1980; Renous and Gasc, 1989) can at least in part be explained by a strong evolutionary constraint on the re-evolution of limbs and digits in amniotes. Mutations for such re-evolution are almost invariably accompanied by deleterious pleiotropic effects, which drastically lower fitness (*e.g.* Lande, 1978; Galis *et al.*, 2010). The body plan of urodeles is also conserved, yet they display considerable variation across families and genera (Litvinchuk and Borkin, 2003; Buckley *et al.*, 2013). In the well-studied group of *Triturus* newts, the number of trunk vertebrae appears to be associated with the length of the annual aquatic period (Arntzen, 2003; Ivanović and Arntzen, 2014). The series of five *Triturus* morphotypes varies from short trunks with 12 vertebrae in species that spend two months in the aquatic environment annually, to elongated trunks with 16 or 17 vertebrae in a species that spends six months in the water annually. This pattern suggests that environmental factors and species-specific ecological preferences may affect the evolutionary changes in the number of trunk vertebrae in salamandrid salamanders. The array of body shapes in salamanders as a function of the number of trunk and caudal vertebrae is illustrated in Fig. 1 for the European and Near-eastern species. The overall pattern of variation, with change along the horizontal axis and along the vertical axis (and not along the diagonal), suggests that the trunk and tail evolve largely independently.

A recent accumulation of morphological data (in particular Lanza *et al.*, 2010), global geo-spatial environmental information and species range maps (Hijmans *et al.*, 2005; IUCN, 2012), and the availability of molecular data for phylogeny building (in particular Babik *et al.*, 2005; Weisrock *et al.*, 2006; Zhang *et al.*, 2008; Wielstra and Arntzen, 2011; Wielstra *et al.,* 2014) forms the basis for the current analysis of body shape variation in the Salamandridae to which more than two-third of European urodele species belong. Our aims are to explore the evolution of body-shape in salamandrid salamanders (family Salamandridae) and to identify potentially important selective environmental

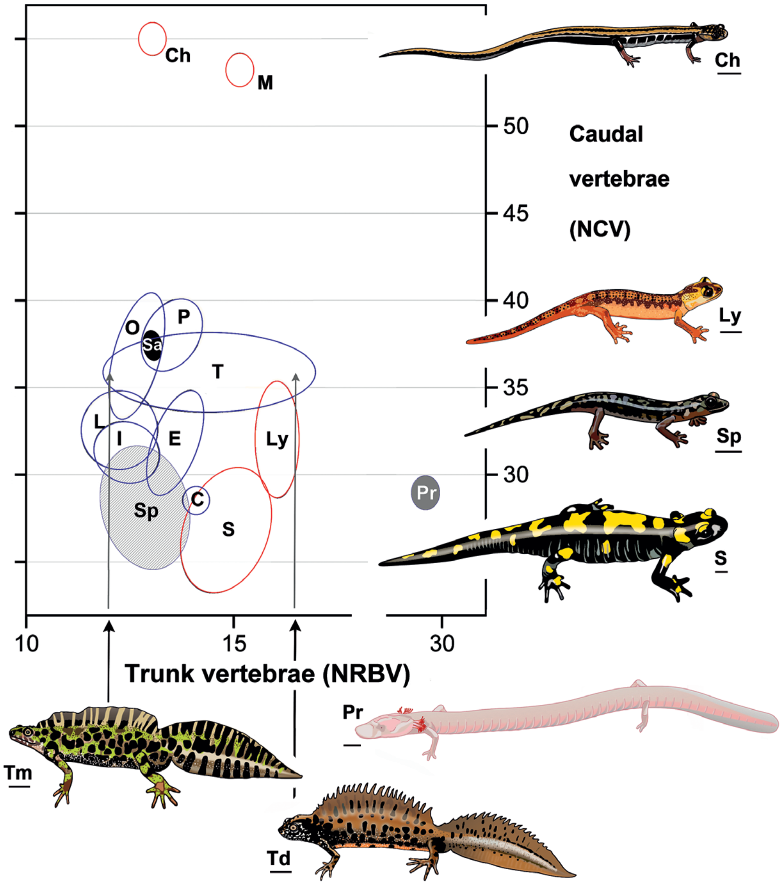

Fig. 1. Schematic representation of the numbers of trunk vertebrae (NRBV, horizontal axis) and caudal vertebrae (NCV, vertical axis) in European representatives of the salamander families Plethodontidae (Sp, light shading), Proteidae (Pr, grey shading) and Salamandridae with the subfamilies Salamandrininae (Sa, in black), Pleurodelinae (or 'newts'- in blue) and Salamandrinae or 'true salamanders' (in red). Data are summarized by ellipses for the genera *Calotriton* (C), *Chioglossa* (Ch), *Euproctus* (E), *Ichthyosaura* (I), *Lissotriton* (L), *Lyciasalamandra* (Ly), *Mertensiella* (M), *Ommatotriton* (O), *Pleurodeles* (P), *Proteus* (Pr), *Salamandrina* (Sa), *Salamandra* (S), *Speleomantes* (Sp) and *Triturus* (T). The extremes are *Chioglossa* with a short body and a long tail, *Speleomantes* with a short body and a short tail and *Proteus* with a long body and short tail, indicating that numbers of trunk and caudal vertebrae may evolve independently. Species highlighted for variation in number of trunk vertebrae, facing left, are *Triturus marmoratus* (Tm) and *T. dobrogicus* (Td) representing the *Triturus* morphoseries. Species highlighted for variation in the number of caudal vertebrae, facing right, are *Salamandra infraimmaculata* (S), *Lyciasalamandra antalaya* (Ly) and *Chioglossa lusitanica* (Ch). Non-salamandrid European urodeles shown for comparisons are *Speleomantes flavus* (Sp) that lives in and around dry caves and the obligatorily aquatic cave salamander *Proteus anguinus* (Pr). Drawings are not to scale (size bars underneath species codes indicate 1 cm).

factors by correlating morphological change with climate change in the context of a phylogeny.

Material and methods

Morphological data

Data on the number of trunk vertebrae (NRBV) were i) from X-rayed specimens selected from the collection of the Naturalis Biodiversity Center, Leiden. Salamanders were X-rayed on a Faxitron 43855C/D with an exposure of 20-40s at 3 mA and 70kV; ii) idem, material we borrowed from the Museum of Comparative Zoology, Harvard University, the Museum of Vertebrate Zoology, University of California, Berkeley and the Staatlichen Naturhistorischen Museums, Braunschweig; iii) from cleared and stained specimens from the herpetological collection of the Institute for Biological Research 'Siniša Stanković' (IBISS), Belgrade and iv) data we gathered earlier and were published by Lanza *et al.* (2010). We scored NRBV in 3439 specimens and the number of caudal vertebrae (NCV) in 1653 specimens. The database was supplemented with records from the literature (Appendix I). For sample sizes per taxon and author names for Salamandridae species see Appendix I.

The NRBV counts excluded the cervical vertebra (atlas) and the sacrum, as in Lanza *et al.* (2010). In some older publications the counting method could not be ascertained and decisions were made on the basis of consistency of results with other published and our own data. In the case of transitional trunk/sacral vertebra (incomplete homeotic transformation of trunk vertebra into sacral vertebra which produce an asymmetric pelvic articulation), the number of the vertebrae with ribs attached to both sides of the vertebra was counted (*e.g.* Appendix III H, see also J). The number of specimens with a transitional trunk/sacral homeotic transformation was determined for the samples of X-rayed and cleared and stained specimens. When counting NCV we excluded the sacrum and did not discriminate between caudosacral and caudal vertebrae. When reading from photos, we included all apical elements that were radiographically detectable (see Appendix III and *e.g.* Fig. 24 in Clergue-Gazeau, 1999 and Fig. 1 in Karakasiliotis *et al.,* 2013).

The intra(sub)specific variation in NRBV is low, with a standard deviation (SD, average weighted for sample size) of 0.53 (data from Lanza *et al.*, 2010). Because SD is markedly lower than unity, NRBV is represented in modal values. In *Lissotriton vulgaris vulgaris*. *Paramesotriton labiatus* and *Triturus dobrogicus* NRBV has two about equally frequent character states; we used the higher values since they appear to represent genuine cases of trunk elongation. Sexual dimorphism (SexDim) was calculated as ($\text{value}_{\text{males}}/(\text{value}_{\text{males}} + \text{value}_{\text{females}})$). We tested for sexual dimorphism in NRBV with the *G*-test for independence with one degree of freedom for taxa with a sample size ≥ 5 in both sexes.

The number of caudal vertebrae varied markedly, within and across taxa. Given that the average SD is in excess of unity (SD=2.6; data from Lanza *et al.*, 2010), NCV effectively represents a continuum due to which we worked with average group values. Groups considered had a minimum sample size of n=8 and included the genera *Cynops* and *Lyciasalamandra* and several subspecies of *Icthyosaura alpestris* and *Lissotriton vulgaris*. NCV SexDim (as above) was coded as significantly male biased, significantly female biased and the intermediate 'neutral' class as determined with Student's *t*-tests, be it that a female bias did not show in our data.

Trunk elongation by an increase in the number of vertebrae is common in salamanders whereas body elongation through the lengthening of vertebrae without a change in the number, is only known for *Pseudoeurycea lineola* (Cope, 1865) (Wake, 1991; Parra-Olea and Wake, 2001). Accordingly, vertebral lengthening was not taken into account as a source of variation and, for ease of presentation, we will speak of increase and decrease in the number of trunk and caudal vertebrate as equivalent to the increase / decrease of trunk size and tail size. We ignored the addition of caudal vertebrae and lengthening of the tail throughout life (Noble, 1931; Arntzen, 1994; Babcock and Blais, 2001; Vaglia *et al.*, 2012) because with few exceptions the specimens used in our analyses were adults. We also ignored the possible effects of developmental temperature on meristic characters (Orska and Imiołek, 1962; Jockush, 1997).

Phylogeny

A phylogeny of most salamandrid species was established from molecular data as detailed in Appendix II. Taxa for which vertebrae counts were absent or insufficient were excluded from the phylogenetic analysis, by *a posteriori* tree pruning. Accordingly, the number of taxa in the phylogenetic tree was 81 for NRBV and 40 for NCV. The phylogenetic trees are fossil calibrated so that estimates on the timing of character state changes can be made.

Environmental data

Mean and standard deviation of 19 'BioClimatic' variables on temperature and precipitation (Bio01 - Bio19) as well as altitude were extracted over the documented range of the (sub)species in ArcGIS 10.0 (ESRI, 2011; data from Hijmans *et al.*, 2005 and IUCN, 2012). From altitude we derived 'slope' using the ArcGIS Spatial Analyst extension. Environmental data for occasional single records (point data representing populations) were extracted over a circular area with 25 km radius. Missing data points (<0.4%) were restricted to Bio03, Bio14 and Bio17 and were filled in by the averages for that variable (Appendix IV). Following the UPGMA clustering of Pearson's correlation coefficient (*r*), six groups of parameters were recovered at the level of $r < 0.5$ (Appendix V), from which we selected one variable per group namely Bio01, Bio07, Bio09, Bio12 and Slope, under the following rationale. 'Annual mean temperature' (Bio01) and 'annual precipitation' (Bio12) represent the most general climate parameters, which are frequently used in amphibian research (Kozak and Wiens, 2012 and references therein). In *Triturus* newts NRBV appears to be associated with the length of the annual aquatic period (Arntzen, 2003). The climate parameter best expressing desiccation of the ponds that *Triturus* species use for reproduction is taken to be the 'mean temperature of driest quarter' (Bio09). Alternatively, the length of the aquatic period may be represented by the parameter 'slope' under the rationale that the seasonal presence of large bodies of standing water is more common in flat areas. From the UPGMA clusters not yet represented we included 'precipitation of coldest quarter' (Bio19) and from the remaining cluster we choose the most general parameter, which is 'temperature annual range' (Bio07) (Appen-

Table. 1. Overview on the statistical analyses of morphological evolution in salamandrid salamanders. Abbreviations are: NRBV - number of trunk vertebrae, NCV - number of caudal vertebrae, SexDim - sexual dimorphism and PGLS - phylogenetic generalized least squares.

Response variable	Number of taxa in molecular phylogeny	Method of ancestral state reconstruction	Steps	Gains	Losses	Testing with stepwise multiple regression
NRBV	81	parsimony	29	21	8	logistic
NRBV_SexDim	81	parsimony	1	1	0	none
NCV	40	PGLS	136.6	57.7	78.9	linear
NCV_SexDim	24	parsimony	3	2	1	none

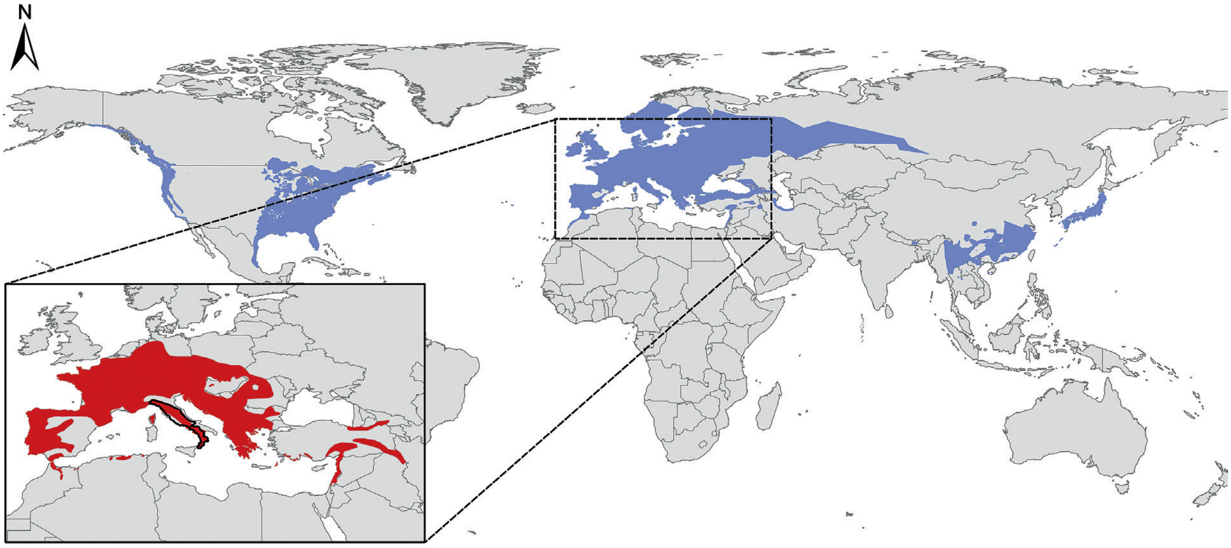

Fig. 2. Global distribution of the family Salamandridae with the 'newts', subfamily Pleurodelinae, in blue, the 'true salamanders' subfamily Salamandrinae in red (inset) and a basal lineage of the subfamily Salamandrininae, genus *Salamandrina*, in Italy (black outline).

dix IV). In an additional unguided analysis, we performed principal component analysis based on the correlation matrix (PCA), to reduce the number of explanatory parameters from the 15 not yet considered 'Bio-Climatic' variables to the number of components with eigenvalues in excess of unity. Selected variables were interpreted in a temporal/phylogenetic framework as described for the continuously distributed morphological variables.

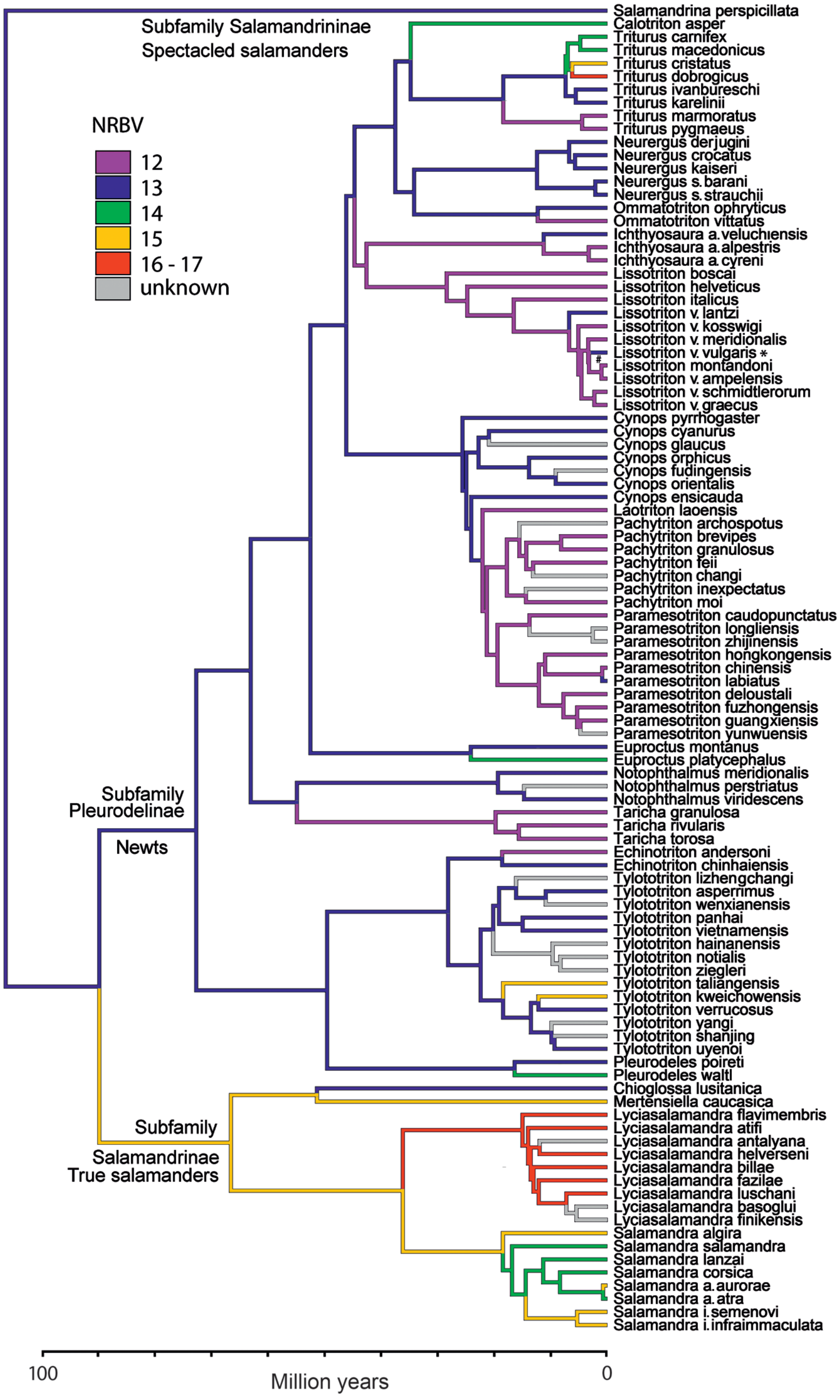

Fig. 3. Calibrated phylogenetic hypothesis for the family Salamandridae constructed from full mtDNA sequences (43 taxa) and sequences for the ND1 - ND2 mitochondrial genes (58 taxa). NRBV character states are allocated on the basis of 'AccTran' optimization and shown in colour as explained in legend. For details see Appendices I and II. For a representation that emphasizes the direction and possible ambiguity of NRBV character state change see Appendix VII. The taxon marked with an asterisk (*) shows a significant sexually dimorphism and the octothorpe symbol (#) indicates the branches where change is inferred to have taken place under the parsimony criterion. The phylogenetic position of *Lissotriton montandoni* is uncertain since this species' original mtDNA has throughout its range been replaced by that of *L. vulgaris*, with which it hybridizes (Babik *et al.*, 2005; Zieliński *et al.*, 2013).

Analyses of character state evolution

The number of trunk vertebrae is characterized by a punctuated continuous character state distribution, with modal values for NRBV in the range of 12-17. In the absence of a likelihood-based model to accommodate this transformation series, we applied the parsimony criterion. The program PAUP* (Swofford, 2003) was used to allocate NRBV and SexDim gains and losses over the molecular phylogenetic tree, under 'Acctran' as well as 'Deltran' optimization. Identical results for these methods were interpreted as 'unambiguous' and accordingly the inferred morphological change was linked to a single branch. When the results for both methods were different, this was seen as 'ambiguous' and morphological change was linked to more than one branch (actually, two branches).

For the continuously distributed variables (NCV and the environmental parameters) we explored the data for fit to the Brownian motion (BM) versus Ornstein-Uhlenbeck model (OU) of character state change. We compared the ln-likelihood values of the 'fitContinuousBM' versus 'fitContinuousOU' model fitting routine of the R (R Core Team, 2012) package Geiger (a program running in R; Harmon *et al.*, 2008). For NCV the Brownian motion model was selected and ancestral character states were estimated with the PGLS (phylogenetic generalized least squares) approach in Compare 4.6b software (http://compare.bio.indiana.edu; see also Martins and Hansen, 1997) in the subroutine 'ancestor reconstruction'. For all environmental parameters OU was the preferred model. The associated constraint alpha was noted for subsequent use in ancestral character state reconstruction. Alpha quantifies the strength of the tendency to move towards the central position of the character state change in the OU model. Alpha values were < 0.1 in all but three of the analyses. Ancestral character states were estimated with PGLS under the exponential model and alpha values as determined above.

Statistical evaluation

Statistical analyses were carried out with SPSS 21 (SPSS, 2013). Comparative phylogenetic analyses with NRBV and SexDim gave rise to a binary distribution of inferred gains and inferred losses (i.e., the increase or decrease of vertebrae numbers or sexual dimorphism as seen from the root of the tree). These data were analysed by stepwise logistic regression, in which 'ambiguous' state changes were allocated to all branches where they might have occurred (as in Appendix VII), and then down-weighted as to keep the total number of observations in the analysis unaffected. A second analysis had all branch allocations smaller than unity excluded.

Analysis of NCV yielded the inferred gain versus loss as a continuous variable, alongside with the standard error and associated level of statistical significance. Statistical analyses were performed by means of stepwise multiple regression, for which we considered i) all branches, and ii) all branches on which the morphological change was equal to or in excess of unity.

In stepwise regression analyses, the environmental variables were standardized to an average of zero and standard deviation of unity, to increase the comparability of their effects. Following Garland *et al.* (1992) all regressions with independent contrasts were forced through the origin. The fit of the descriptive models is expressed by the 'Area Under the Curve in Receiver Operating Characteristic' plots (ROC_AUC, logistic regression) and by Spearman's correlation coefficient (r_s, linear regression). For a summary of analytical procedures see Table 1. The groups analysed were the Salamandridae, the 'true salamanders' and the 'newts'; for the global distribution of these groups see Fig. 2.

Results

The phylogenetic analysis yielded three well-supported major groups (Fig. 3), comprising i) the subfamily Salamandrininae composed of the genus *Salamandrina*, with an independent history of 106.5 Ma (Million years before present), ii) the subfamily Salamandrinae comprising the 'true salamanders' with a most recent common ancestor (MRCA) at 66.7 Ma and iii) the subfamily Pleurodelinae comprising the 'newts' with a MRCA at 70.5 Ma. The Bayesian posterior probabilities associated with branches in the phylogeny of the Salamandridae are in excess of 0.99, with few exceptions (see Appendix II). The data on NRBV and NCV across the family Salamandridae are summarized in Appendix I. The frequency of transitional trunk-sacral vertebrae was recorded for 11 newt species and varied between 1% - 9% (genus *Ichthyosaura* 3.0%, n=93; genus *Lissotriton* 3.8%, n=277; genus *Triturus* 5.1%, n=1261). For the illustration of cleared and stained specimens and X-ray photography see Appendix III. The environmental data extracted from the BioClim data base are presented in Appendix IV.

The character NRBV required 29 steps distributed

Table 2. Parameter selection and model fit for the association of evolutionary morphological change and environmental variables in various groups of salamandrid salamanders. Panels A and B - results of stepwise logistic regression analysis with the change in modal number of trunk vertebrae as the dependent variable. Panels C and D – results for stepwise linear regression analysis with the change in number of caudal vertebrae as the dependent variable. The analyses were performed considering all changes (panels A and C) and all changes that were in excess of unity (panels B and D). Independent variables are Bio01 - annual mean temperature, Bio07 - temperature annual range, Bio09 - mean temperature of driest quarter, Bio12 - annual precipitation, Bio19 - precipitation of coldest quarter and Slope. Note that data were standardized to a mean of zero and standard deviation of unity prior to regressions. The analysis was repeated with 15 environmental parameters summarized in four axes of a principal component analysis (PCA).

	Selected environmental variables							15 Variables summarized				
	Bio01	Bio07	Bio09	Bio12	Bio19	Slope	Model fit #	PCA1	PCA2	PCA3	PCA4	Model fit
A - Trunk vertebrae - all change												
Salamandridae		-1.685				2.269	0.895			-2.045		0.813
True salamanders							no model					no model
Newts			1.014				0.719			-2.157		0.786
idem - *Triturus* excluded			1.291				0.714			-3.217		0.817
B - Trunk vertebrae - change ≥ unity												
Salamandridae				3.452		3.008	0.906			-1.742		0.750
True salamanders				95.57			1					no model
Newts			1.097				0.818			-1.887		0.709
idem - *Triturus* excluded	2.241				1.910		0.938			-2.723		0.719
C - Tail vertebrae - all change												
Salamandridae				-0.876			0.349	-3.002	-2.021		-1.524	0.423
True salamanders							no model					no model
Newts				-1.106			0.637	-2.860	-2.248		-1.341	0.648
idem - *Triturus* excluded				-1.206			0.688	-2.864	-2.495		-1.272	0.692
D - Tail vertebrae - change ≥ unity												
Salamandridae				-1.048			0.397					no model
True salamanders							no model					no model
Newts				-1.277			0.711	-2.621	-2.524			0.651
idem - *Triturus* excluded				-1.288			0.720	-2.552	-2.640			0.678

Model fit is expressed by ROC_AUC values for panels A and B and by Spearman's correlation coefficient for panels C and D.

Example: the logistic regression equation (1/(1+exp(-1.685*Bio07+2.269*Slope))) describes the probability of trunk elongation (versus shortening) over the evolutionary tree of the Salamandridae from experienced environmental change. Note that in logistic equations the sign of a variable is inverse to its effect.

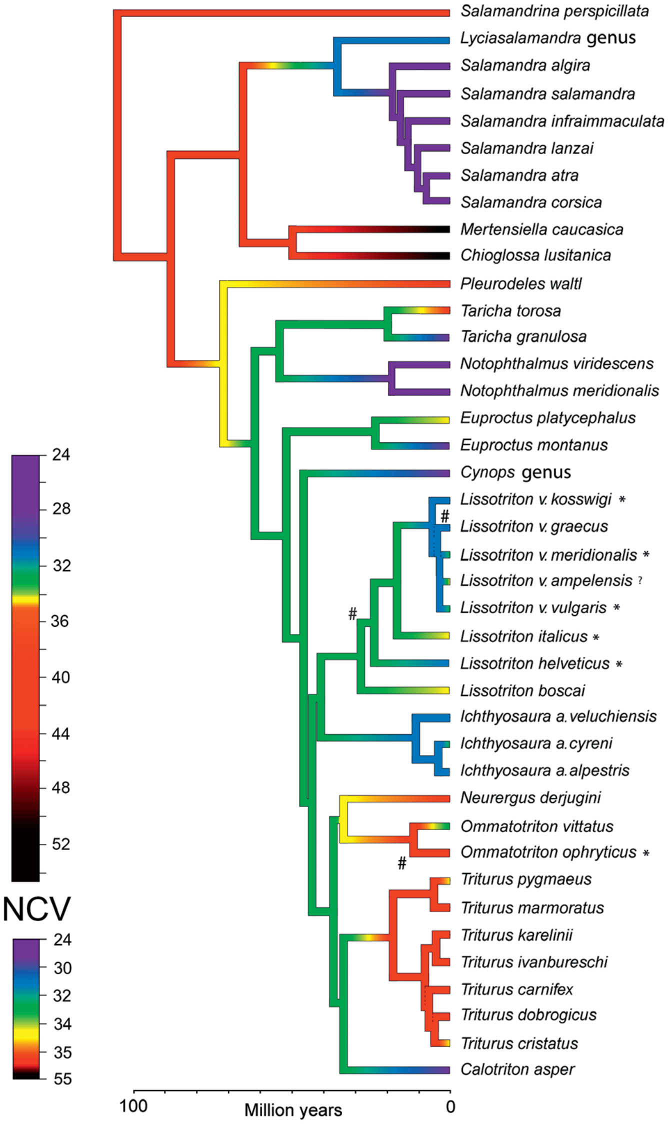

Fig. 4. Calibrated phylogenetic hypothesis for the family Salamandridae with colour coding indicating the average number of caudal vertebrae plotted along the branches (NCV, see text for details). Note that the colour scale is not linear. Taxa marked with an asterisk (*) show significant sexually dimorphism and the octothorpe (#) indicate the branches where change is inferred to have taken place under the parsimony criterion.

over 161 branches of the phylogenetic tree, of which 21 were inferred to be gains and 8 were inferred to be losses (Fig. 3, Appendix VII). This is significantly different from a 50:50 distribution (*G*-test for goodness of fit, G=6.04, P<0.05). The number of ambiguous steps was four in the true salamanders and two in the newts. Under stepwise logistic regression an increase in NRBV was associated with an increase in temperature annual range (Bio07), a decrease in annual precipitation (Bio12) and a decrease in Slope (Table 2AB).

Analysing the true salamanders and the newts separately yielded contrasting results. In the true salamanders we observed a negative relationship with annual precipitation (Bio12, Table 2B). In the newts we observed a negative relationship with the mean temperature of driest quarter (Bio09). The analysis was repeated excluding the genus *Triturus*, on the rationale that knowledge on this group motivated the parameter selection. This yielded either the same parameter selection (Table 2A) or a different model with Bio01 and Bio19 (Table 2B).

For NCV we observed change over all 79 branches of the phylogenetic tree and 37 changes that were in excess of unity. The total amounts of gains and losses were about the same (Table 1, Fig. 4) and not associated with the distribution of gains versus losses in NRBV

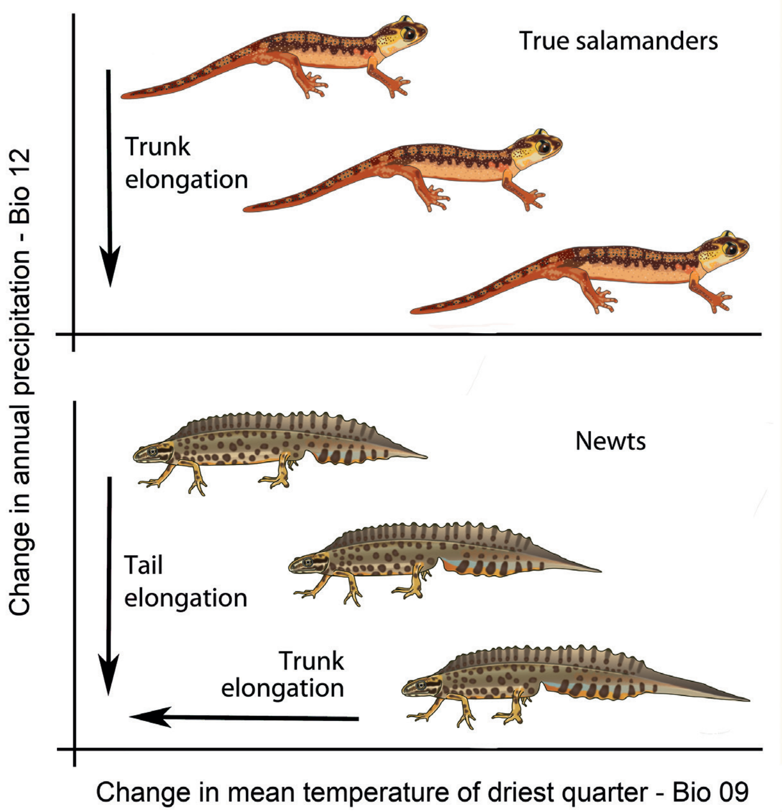

Fig. 5. Schematic representation of the results, in which vertebrae number is interpreted as a size vector. Arrows indicate elongation of the trunk and elongation of the tail as associated with change in the mean temperature of the driest quarter (Bio09, horizontal axis) and with change in annual precipitation (Bio12, vertical axis). Note that the true salamanders (upper panel) and newts (lower panel, *Triturus* excluded), respond differently to the same climatic parameters. The cartoon images with exaggerated body proportions are drawn after the true salamander *Lyciasalamandra antalyana* and the newt *Lissotriton vulgaris*.

(*t*-test, *t*=-0.68, not significant). No models were uncovered with stepwise linear regression for the true salamanders (Table 2CD). When the newts were analysed separately, a negative relationship with the parameter 'annual precipitation' (Bio12) was uncovered, with or without the genus *Triturus*. The results with analyses on the basis of just those branches carrying morphological change ≥ unity were similar. For a pictorial summary of the results see Fig. 5.

We also investigated the 15 non-chosen environmental parameters (Appendix V) as summarized by four PCA axes (Appendix IV C). The first, second, third and fourth axis explain 40.0%, 27.4%, 12.1% and 9.6% of the total variance, respectively. High loadings (>0.8) on the first axis are by Bio06, Bio11, Bio13 and Bio16 and this axis can be summarized as representing 'cold and wet' environmental conditions. High loadings on the second axis are by Bio02 and Bio17 and this axis can perhaps be summarized as 'generally favorable to amphibians' on account of even temperatures (Bio02) and precipitation in periods that it is most needed (Bio17, precipitation of driest quarter). Moderate high loadings (0.6<loading<0.8) on the third axis are by Bio03 and Bio04 and this axis can be summarized as 'temperature fluctuating over the year'. The fourth axis has a moderately high loading of 0.724 by the parameter Altitude. With this approach no models were retrieved for the true salamanders (Table 2). For the newts an increase in NRBV was positively related to PCA3, whereas changes in NCV were positively related to PCA1, PCA2 and sometimes PCA4. However, model fit with 15 variables summarized over four PCA axes was mostly lower than model fit with the pre-selected variables. Moreover, the PCA axes are not straightforward to interpret.

Significant sexual dimorphism in the number of trunk vertebrae was observed only in *Lissotriton v. vulgaris* (P < 0.0001), with a modal score of NRBV=13 in males and NRBV=12 in females, but also we also encountered populations of this subspecies in which sexual dimorphism was absent (results not shown). A significant sexual dimorphism in the number of caudal vertebrae, with males having higher numbers than females, was observed in six groups representing two lineages (the genus *Lissotriton*, *Ommatotriton ophryticus*) and involving three evolutionary events (two gains and one loss, Fig. 4). Because the number of evolutionary events is low, we refrained from testing for environmental signal possibly underlying the evolution of sexual dimorphism.

Discussion

Body shape and climate

Our study shows that climatic factors are significantly associated with numbers of trunk- and caudal vertebrae in the Salamandridae, which is in agreement with the notion that axial pattering in salamanders is to an important extent shaped by the environment (Jockusch, 1997; Blankers *et al.*, 2012). In addition, a significant result in our study is that one environmental parameter (namely the reduction in annual precipitation) affects the two main clades differently, i.e. leading to trunk elongation in the 'true salamanders' and tail elongation in the 'newts' (Fig. 5). Hence, different selection factors, reflecting different climatic niches, appear to be important for the groups of true salamanders and newts. This contrasting response is perhaps not surprising, given that the two groups have long, separate evolutionary histories and differ considerably in development and many life history traits. The most striking difference is the amount of time that they spend in the water or on land, with salamanders being largely terrestrial and newts more aquatic. Additionally, large eggs and embryo- and larval development within oviducts are the rule among the true salamanders, while newts are characterized by small eggs that develop in the surrounding aquatic environment (Thorn, 1969). Prior studies which explored causes underlying axial pattering in salamanders have proposed intrinsic (phylogenetic) as well as extrinsic (environmental) factors to explain the observed variation in body plan (*e.g.* Wake, 1966; Veith *et al.*, 1992; Jockusch, 1997).

The relationship between trunk and tail length and environmental factors is necessarily complex, since both true salamanders and newts have different ways of locomotion during larval and adult stages and adults have terrestrial and aquatic locomotory modes. Adding to the complexity is the wide array of habitat and climatic variables that are encountered by the species included in our study and more detailed studies are necessary to further interpret our results. Nonetheless, some of the conspicuous differences between true salamanders and newts may give us clues about the differences observed. In true salamanders, trunk elongation may be related to an increased importance of fossoriality in dry circumstances (also proposed for plethodontid salamanders by Jockusch, 1997), as surficial activity strongly depends on precipitation, on which many species of the true salamanders rely for foraging and mating (Duellman and Trueb, 1994). Arguably, when surface activity is restricted due to unsuitable climatic conditions, populations may adopt a more worm-like appearance that facilitates burrowing and the use of small holes and crevices. Indeed, a similar association between fossoriality and axial elongation has been found in lizards (Gans, 1975; Roscito and Rodrigues, 2013). The association between evolution of body form and environmental factors is well known in squamates (Wiens and Sinluff, 2001; Grizante *et al.*, 2012; Roscito and Rodrigues, 2013), which are, among tetrapods, the group with the most variable vertebrae number (Ward and Mehta, 2014). However, for salamanders, a consensus is lacking. An empirical study on a large number of plethodontid salamander species found no clear relationship between body shape and microhabitat use except for a few lineages (Blankers *et al.*, 2012). It seems that the relationship between change in morphology and ecological factors (microhabitat use or climatic parameters) in tailed amphibians is lineage-specific, but further study is necessary to find out to what extent ecological adaptations or lineage-specific constraints are responsible.

In newts, which are dependent on aquatic sites for their reproduction and spend a substantial part of their annual cycle in the water for mating and feeding, a lower mean temperature in the driest quarter (related to the increase in number of trunk vertebrae), and amount of annual precipitation (negatively related to number of caudal vertebrae) may relate to a larger and longer availability of lentic water as a result of low evaporation, which would allow for a longer aquatic phase, with an evolutionary morphological response opposite to that of the true salamanders (Fig. 5).

Locomotor behaviour is among factors that affect morphological evolution and could largely differ among groups and could be phylogenetically constrained (Blomberg *et al.*, 2003). For example, the obligate aquatic urodele *Proteus anguinus* Laurenti, 1768 - not a salamandrid - has exceptional body proportions with a long trunk and short tail (Fig. 1). This species has an anguilliform swimming mode that is drastically different from the stiff body with movement by tail propulsion in newts such as *Triturus* species (Gvoždik and Van Damme, 2006). For visual documentation see *e.g.* http://www.arkive.org/cave-salamander/proteus-anguinus/video-00.html on *Proteus anguinus* and, at the same address, smooth-newt/triturus-vulgaris/video-06b.html for *Lissotriton vulgaris*. To complicate the interpretation further, in species of the semi-aquatic New World brook salamanders of the genus *Eurycea* the subterranean forms show a *shortening* of the trunk relative to their surface exploiting counterparts (Bendik *et al.*, 2013).

The relationship between the number of caudal vertebrae and environmental variables is less robust than that found for the number of trunk vertebrae and more study is required to find out which factors affect tail length. It is noteworthy though that the extremely long tails such as found in *e.g. Chioglossa lusitanica*, a species living in very conditions (Arntzen, 1981, 1994) contrast sharply with the short tails of the *Salamandra* species, especially those living in arid conditions (Eiselt, 1958) (Fig. 1). At the level of the analyses, we attribute the paucity of significant results to the NRBV changes that could not be linked to a single lineage (four out of eight in the true salamanders versus two out of 21 in the newts, see Appendix VII). Another reason may be the scarcity of data for the number of caudal vertebrae and the limited taxon sampling especially in the true salamanders (see also Ficetola *et al.*, 2013). Indeed, large sample sizes are required to deal with the substantial intraspecific variation in NCV. To circumvent the tedious analyses based upon X-ray imagery, we propose to use tail length relative to snout – vent length as a proxy parameter, provided that centrae-length along the primary body axis is more or less uniform (for extensive data in a variety of urodeles see *e.g.* Wake, 1966 and Worthington and Wake, 1972). A few X-rays per species would be sufficient to test the assumptions coming with body measurements as proxies to vertebrae counts.

Significant sexual dimorphism in the number of trunk vertebrae was only observed in *Lissotriton v. vulgaris*, but not in all populations, allowing for a study of geographical variation in a search for the underlying causes. Sexual dimorphism in the number of caudal vertebrae was restricted to the two newt genera *Lissotriton* and *Ommatotriton*. As it happens these are the groups in which males are larger than females in overall size, suggesting that sexual selection drives the dimorphism (Wiens *et al.*, 2011; Ficetola *et al.*, 2013). For a discussion of sexual dimorphism in the context of Rensch's rule see Colleoni *et al.* (2014).

Evolvability

The data here presented indicate a high level of variation in axial pattering in the family Salamandridae, with similar high rates of change over the evolutionary trajectories in true salamanders and in newts. We observed more gains than losses in trunk vertebrae and more or less equal numbers of gains and losses in tail vertebrae. The relatively high frequency of transitional trunk-sacral vertebrae that we observed is in support of the evolvability of the number of trunk vertebrae. It further suggests that evolutionary changes of the trunk count in salamandrid salamanders are due to homeotic changes in the vertebral identity at the trunk-sacral boundary. Transitional vertebrae represent incomplete homeotic transformations (*e.g.* Varela-Lasheras *et al.*, 2011). Although it is often thought that changes of vertebral count do not necessarily require homeotic transformations and can be solely the result of increases or decreases in the number of vertebrae of a certain region, this is not true, except for vertebrae in the tail region which is the part of the vertebral column formed last. Homeotic transformations are unavoidably involved, because of the sequential head-to-tail generation of the embryonal segments from which the vertebrae develop (somites) and the patterning of these segments under the influence of head-to-tail signaling gradients (for a detailed discussion, see ten Broek *et al.*, 2012). Initial mutations for homeotic transformations usually lead to incomplete transformations. Hence, evolutionary changes of vertebrae counts are only expected if sufficient individuals with transitional vertebrae can survive and reproduce (Galis *et al.*, 2014). The absence of a correlation among changes in number of trunk and caudal vertebrae is not surprising, given that the formation of trunk and caudal vertebrae in vertebrates is decoupled at early development, suggesting that these two parts of the axial skeleton represent independent evolutionary modules and can evolve independently in response to selective pressures (Polly *et al.*, 2001; Ward and Brainerd, 2007; Woltering, 2012).

While the numbers of trunk and caudal vertebrae are variable across urodeles (Jockusch, 1997; Litvinchuk and Borkin, 2003; present study), the number of cervical and sacral vertebrae is highly conserved (one cervical and one sacral vertebra - Duellman and Trueb, 1994). A similar strong constraint has been found for the number of cervical vertebrae in mammals where conservation appears to be explained by the high interactivity of developmental processes at the early developmental stage, during which the number of cervical vertebrae is determined, such that a change is invariably associated with other, usually deleterious changes (Galis, 1999; Varela-Lasheras *et al.*, 2011; ten Broek *et al.*, 2012).

The conservation in the number of cervical and sacral vertebra is in strong contrast with the high intraspecific variation in the number of trunk vertebrae in the Salamandridae and in some other urodeles, such as *Salamandrella keyserlingii* Dybowski, 1870 (Litvinchuk and Borkin, 2003) and the ambystomatid salamanders (Worthington, 1974; Peabody and Brodie, 1975). However, in some lineages such as the genus *Bolitiglossa,* numerous species have a fixed number of 14 trunk vertebrae, regardless of the length of the trunk (Jockusch, 1997; Parra-Olea and Wake, 2001). 'Eco-Evo-Devo' studies are required to find out why the number of trunk vertebrae is conserved in *Bolitoglossa* and highly evolvable in most other urodele taxa. Our data on a selection of salamanders and newts also indicate that the number of trunk and caudal vertebrae are highly evolvable traits with frequent changes and reversals, affecting both sexes equally. In a few groups however (*e.g. Cynops, Lyciasalamandra, Neurergus*, and the *Laotriton-Pachytriton-Paramesotriton* clade) the number of trunk vertebrae is stable, but in most groups it is variable (*e.g. Tylototriton*). With 18 species (Frost, 2014; Sparreboom, 2014) of which only seven were included in the present study, the latter genus appears an excellent natural group to further investigate effects of the environment on body shape.

Conclusions

We found significant associations between vertebral column changes and climatic conditions in salamandrid salamanders, with a positive relationship between annual precipitation and axial elongation, manifested through trunk elongation in the terrestrial 'true salamanders' and by tail elongation in the more aquatic 'newts'. Furthermore, we observed a positive relationship between trunk elongation and mean temperature of the driest quarter in 'true salamanders'. Our results support the notion that the selective factors that are important for body shape evolution along environmental gradients differ between the two clades - true salamanders and newts - examined in our study. In addition, we found a high rate of gains and losses of both trunk and caudal vertebrae, indicating significant evolvability of these traits.

Acknowledgements

We thank John J. Wiens for advice, Dirk van de Marel and Rob Langelaan for X-raying collection material, Esther Dondorp for collection management, Maja Slijepčević for counting vertebrae on cleared and stained specimens from the IBISS collection and Bas Blankevoort for species drawings (copyright Blankevoort © Naturalis Biodiversity Center – see also Appendix VI). We thank J. Rosado, J. Hanken and J.B. Losos (Museum of Comparative Zoology, Harvard University), C. Spencer and D.B. Wake (Museum of Vertebrate Zoology, University of California, Berkeley) and U. Joger (Staatlichen Naturhistorischen Museums, Braunschweig) for the loan of specimens under their care. AI acknowledges financial support from the Serbian Ministry of Education and Science (grant no. 173043), grants from SyntheSys (NL-TAF 1245 and 3082) and an NCB Naturalis Temminck fellowship.

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Received: 31 Januari 2014
Revised and accepted: 19 November 2014

Editor: M. Brazeau

Online Supplementary Information

S1. ND1 and ND2 sequence data underlying thev phylogeny of the Salamandridae (Fig. 3, Appendix I) in NEXUS format. The full mitogenomic data can be found at the TreeBase repository under the numbers S9945 and S11081.

Appendix I

Overview of molecular and morphological data.

Taxon	Phylogeny		Morphology[**]			
	Full mtDNA	ND1, ND2[*]	Modal NRBV	Mean NCV	References[***]	Comments
Calotriton arnoldi Carranza and Amat, 2005	no	none	14		1	
Calotriton asper (Dugès, 1852)	yes	both	14 (37)	28.4 (35)		
Chioglossa lusitanica Bocage, 1864	yes	both	13 (24)	54.9 (12)		
Cynops cyanurus Liu, Hu and Yang, 1962	yes	both	13 (2)	27.3 (15)	2, 3	
Cynops ensicauda (Hallowell, 1861)	yes	both	13 (6)		3	
Cynops fudingensis Wu, Wang, Jiang and Hanken, 2010	no	ND2			4	
Cynops glaucus Yuan, Jiang, Ding, Zhang and Che, 2013	no	ND2			5	
Cynops orientalis (David, 1873)	yes	both	13 (8)		2, 3	
Cynops orphicus Risch, 1983	yes	both	13		3	
Cynops pyrrhogaster (Boie, 1826)	yes	both	13		3	
Cynops wolterstorffi (Boulenger, 1905)	no	none	13 (1)			
Echinotriton andersoni (Boulenger, 1892)	yes	both	12		6	
Echinotriton chinhaiensis (Chang, 1932)	yes	both	13		7	
Euproctus montanus (Savi, 1838)	yes	ND1	13 (37)	29.3 (35)		
Euproctus platycephalus (Gravenhorst, 1829)	yes	both	14 (37)	34.0 (33)		
Ichthyosaura alpestris alpestris (Laurenti, 1768)	yes	both	12 (240)	30.4 (138)		
Ichthyosaura alpestris cyreni (Wolterstorff, 1932)	no	both	12 (84)	32.4 (30)		
Ichthyosaura alpestris veluchiensis (Wolterstorff, 1935)	no	ND1	13 (56)	30.0 (9)	8	
Laotriton laoensis (Stuart and Papenfuss, 2002)	yes	both	12		9, 10	as *Paramesotriton laoensis*
Lissotriton boscai (Lataste, 1879)	no	both	12 (42)	34.2 (34)	11	
Lissotriton helveticus (Razoumowsky, 1789)	no	ND2	12 (74)	31.5 (45)	11	
Lissotriton italicus (Peracca, 1898)	no	ND2	12 (111)	34.0 (100)	11	
Lissotriton montandoni (Boulenger, 1880)	no	both	12 (68)		11	
Lissotriton vulgaris ampelensis (Fuhn and Freytag, 1952)	no	ND2	12 (34)	34.1 (8)	11, 12	lineage J of Babik *et al.* (2005)
Lissotriton vulgaris graecus (Wolterstorff, 1906)	no	ND2	12 (14)	31.8 (11)	11, 12	lineage D of Babik *et al.* (2005)
Lissotriton vulgaris kosswigi (Freytag, 1955)	no	ND2	12 (68)	30.6 (31)	12, 31	
Lissotriton vulgaris lantzi (Wolterstorff, 1914)	no	both	13		11	
Lissotriton vulgaris meridionalis (Boulenger, 1882)	no	ND2	12 (59)	32.7 (59)	11, 12	lineage H of Babik *et al.* (2005)
Lissotriton vulgaris schmidtlerorum (Raxworthy, 1988)	no	ND2	12 (101)		11, 12	lineage E of Babik *et al.* (2005)
Lissotriton vulgaris vulgaris (Linnaeus, 1758) - males	yes	both	12 (106)	31.7 (128)	11	
Lissotriton vulgaris vulgaris (Linnaeus, 1758) - females	yes	both	13 (141)			
Lyciasalamandra antalyana (Basoglu and Baran, 1976)	no	both		30.5 (19)		
Lyciasalamandra atifi (Basoglu, 1967)	yes	both	16 (6)			
Lyciasalamandra basoglui (Baran and Atatür, 1980)	no	both				
Lyciasalamandra billae (Franzen and Klewen, 1987)	no	both	16 (5)			
Lyciasalamandra fazilae (Basoglu and Atatür, 1974)	no	both	16 (2)			
Lyciasalamandra finikensis (Basoglu and Atatür, 1975)	no	both				
Lyciasalamandra flavimembris (Mutz and Steinfartz, 1995)	yes	both	16 (2)			

Cont. Overview of molecular and morphological data.

	Phylogeny		Morphology**			
Taxon	Full mtDNA	ND1, ND2*	Modal NRBV	Mean NCV	References***	Comments
Lyciasalamandra helverseni (Pieper, 1963)	no	both	16 (1)	30.5 (19)		
Lyciasalamandra luschani (Steindachner, 1891)	no	both	16 (6)			
Mertensiella caucasica (Waga, 1876)	yes	both	15 (21)	53.4 (14)		
Neurergus crocatus Cope, 1862	no	both	13 (4)			
Neurergus derjugini (Nesterov, 1916)	no	both	13 (16)	35.7 (15)		as *N. microspilotus*
Neurergus kaiseri Schmidt, 1952	yes	both	13 (1)			
Neurergus strauchii barani Öz, 1994	no	both	13 (3)			
Neurergus strauchii strauchii (Steindachner, 1887)	yes	both	13 (13)			
Notophthalmus meridionalis (Cope, 1880)	yes	both	13 (10)	27.6 (9)		
Notophthalmus perstriatus (Bishop, 1941)	no	both				
Notophthalmus viridescens (Rafinesque, 1820)	yes	both	13 23)	27.9 (29)		
Ommatotriton ophryticus nesterovi (Litvinchuk, Zuiderwijk, Borkin and Rosanov, 2005)	no	none	12 (47)	40.1 (25)		
Ommatotriton ophryticus ophryticus (Berthold, 1846)	no	both	13 (33)		13	
Ommatotriton vittatus cilicensis (Wolterstorff, 1906)	no	none	12 (20)			
Ommatotriton vittattus vittatus (Gray, 1835)	yes	both	12 (30)	33.0 (36)	13	
Pachytriton archospotus Shen, Shen and Mo, 2008	no	ND2			14	
Pachytriton brevipes (Sauvage, 1876)	yes	both	12 (5)		2, 3	
Pachytriton changi Nishikawa, Matsui and Jiang, 2012	no	ND2			19	
Pachytriton feii Nishikawa, Jiang and Matsui, 2011	no	ND2	12		15, 16	
Pachytriton granulosus Chang, 1933	yes	both	12		2, 3, 9, 17	as *P. labiatus* (see Nishikawa *et al.*, 2011b and Frost, 2014).
Pachytriton inexpectatus Nishikawa, Jiang, Matsui and Mo, 2011	no	ND2			18	
Pachytriton moi Nishikawa, Jiang and Matsui, 2011	no	ND2	12		16, 19	
Paramesotriton caudopunctatus (Liu and Hu, 1973)	yes	both	12 (1)		2, 3	
Paramesotriton chinensis (Gray, 1859)	no	both	12 (1)		2, 3	
Paramesotriton deloustali (Bourret, 1934)	yes	both	12 (5)		3	
Paramesotriton fuzhongensis Wen, 1989	no	both	12		3, 20	
Paramesotriton guangxiensis (Huang, Tang and Tang, 1983)	no	both	12		2, 3	
Paramesotriton hongkongensis (Myers and Leviton, 1962)	yes	both	12		2, 3	
Paramesotriton labiatus (Unterstein, 1930)	no	ND2	13		9, 16, 21	as *P. ermizhao*; as *P. chinensis*, locality Mt. Yao, Dayao Shan, Guangxi Province, China
Paramesotriton longliensis Li, Tian, Gu and Xiong, 2008	no	ND2			22	
Paramesotriton yunwuensis Wu, Jiang and Hanken, 2010	no	ND2			23	
Paramesotriton zhijinensis Li, Tian and Gu, 2008	no	ND2			22	
Pleurodeles nebulosus (Guichenot, 1850)	no	none	13		24	
Pleurodeles poireti (Gervais, 1835)	yes	both	13 (7)		24	
Pleurodeles waltl Michahelles, 1830	yes	both	14 (21)	38.3 (20)	24, 32	
Salamandra algira Bedriaga, 1883	no	ND2	15 (12)	27.4 (8)		
Salamandra atra atra Laurenti, 1768	no	both	14 (32)	25.2 (36)		
Salamandra atra aurorae Trevisan, 1982	no	both	15 (10)			

Salamandra corsica Savi, 1838	no	both	14 (43)	24.2 (40)		
Salamandra infraimmaculata infraimmaculata (Martens, 1885)	no	both	15 (19)	28.3 (19)	25	
Salamandra infraimmaculata semenovi Nesterov, 1916	no	both	15 (2)			
Salamandra lanzai Nascetti, Andreone, Capula and Bullini, 1988	no	both	14 (54)	23.8 (52)		
Salamandra salamandra (Linnaeus, 1758)	yes	both	14 (215)	26.5 (201)		
Salamandrina perspicillata (Savi, 1821)	yes	both	13 (29)	38.0 (25)	9, 17	as *S. terdigitata*
Salamandrina terdigitata (Bonnaterre, 1789)	no	none	13 (24)			
Taricha granulosa (Skilton, 1849)	yes	both	12 (7)	28.4 (10)		
Taricha rivularis (Twitty, 1935)	yes	both	12 (5)			
Taricha sierrae (Twitty, 1942)	no	none	12 (9)			
Taricha torosa (Rathke, 1833)	no	both	12 (12)	35.9 (10)		
Triturus carnifex (Laurenti, 1768)	yes	both	14 (250)	36.5 (139)		
Triturus cristatus (Laurenti, 1768)	yes	both	15 (288)	34.7 (71)		
Triturus dobrogicus (Kiritzescu, 1903)	yes	both	16 - 17 (166)	35.2 (36)		
Triturus ivanbureschi Arntzen and Wielstra, 2013	yes	both	13 (171)	35.5 (44)		
Triturus karelinii (Strauch, 1870)	yes	both	13 (199)	36.9 (38)		
Triturus macedonicus (Karaman, 1922)	yes	both	14 (82)			
Triturus marmoratus (Latreille, 1800)	yes	both	12 (104)	37.0 (24)		
Triturus pygmaeus (Wolterstorff, 1905)	yes	both	12 (60)	34.8 (10)		
Tylototriton asperrimus Unterstein, 1930	yes	both	13		26	
Tylototriton hainanensis Fei, Ye and Yang, 1984	no	both				
Tylototriton kweichowensis Fang and Chang, 1932	no	both	15		26	NRBV=14 in Yu and Zhao (2007)
Tylototriton lizhengchangi Hou, Zhang, Jiang, Li, 2012	no	ND2			27	
Tylototriton notialis Stuart, Phimmachak, Sivongxay and Robichaud, 2010	no	both			28	
Tylototriton panhai Nishikawa, Khonsue, Pomchote and Matsui, 2013	no	ND2	13		29	
Tylototriton shanjing Nussbaum, Brodie and Yang, 1995	no	both				
Tylototriton taliangensis Liu, 1950	no	both	15 (1)		26	
Tylototriton uyenoi Nishikawa, Khonsue, Pomchote and Matsui, 2013	no	ND2	13		29	
Tylototriton verrucosus Anderson, 1871	no	both	13 (12)		26	
Tylototriton vietnamensis Böhme, Schöttler, Nguyen and Köhler, 2005	no	both	13		28, 30	
Tylototriton wenxianensis Fei, Ye and Yang, 1984	yes	both				
Tylototriton yangi Hou, Zhang, Zhou, Li and Lu, 2012	no	ND2			29	
Tylototriton ziegleri Nishikawa, Matsui and Nguyen, 2013	no	ND2			27	

* If multiple sequences were available the choice was for i) Weisrock *et al.* (2006), ii) the nominotypical subspecies, iii) the longest sequence and iv) random. For GenBank accession numbers see Appendix II.

** For NCV the mean is given only for taxa with sample size ≥ 10 and utilized in Fig. 4. Data in boxed cells are pooled over taxa. In brackets, sample size per taxon pooled from the following sources: Naturalis Biodiversity Center, Leiden; Museum of Comparative Zoology, Harvard University; Museum of Vertebrate Zoology, University of California, Berkeley; Staatlichen Naturhistorischen Museums, Braunschweig; herpetological collection of the Institute for Biological Research "Siniša Stanković", Belgrade and published by Lanza *et al.* (2010).

***References in addition to Lanza *et al.* (2010): 1 - Carranza and Amat (2005), 2 - Zhao *et al.* (1988), 3 - Chan *et al.* (2001), 4 - Wu *et al.* (2010a), 5 - Yuan *et al.* (2013), 6 - Nussbaum and Brodie (1982), 7 - Chang (1932), 8 - Vukov *et al.* (2011), 9 - Weisrock *et al.* (2006), 10 - Stuart and Papenfuss (2002), 11 - Skorinov and Litvinchuk (2012), 12 - Babik *et al.* (2005), 13 - Litvinchuk *et al.* (2005), 14 - Wu *et al.* (2010b), 15 - Wu *et al.* (2012a), 16 - Nishikawa *et al.* (2011a), 17 - Zhang *et al.* (2008), 18 - Wu *et al.* (2013), 19 - Wu *et al.* (2012b), 20 - Wen (1989), 21 - Wu *et al.* (2009), 22 - Wang *et al.*, unpublished data, 23 - Wu *et al.* (2010c), 24 - Carranza and Wade (2004), 25 - Veith *et al.* (1992), 26 - Liu (1950), 27 - Nishikawa *et al.* (2013a), 28 - Stuart *et al.* (2010), 29 - Nishikawa *et al.* (2013b), 30 - Böhme *et al.* (2005), 31 - Nadachowska and Babik (2009) and 32 - Karakasiliotis *et al.* (2013).

Appendix II

Molecular phylogeny of the family Salamandridae. To obtain a dated phylogeny for the genus *Triturus* we reconstructed the phylogeny of the entire family of the Salamandridae from full mitochondrial DNA (mtDNA) data under reference to seven fossil calibration points. The mtDNA data consisted of 11 sequences for eight *Triturus* species, 35 sequences for another 35 salamandrid species and four outgroup species (Zajc & Arntzen, 1999; Zhang *et al.*, 2008; Wielstra & Arntzen, 2011). Following Steinfartz *et al.* (2007) and Wiens *et al.* (2011) we used as fossil calibration points the oldest taxon that can be confidently assigned to a given clade and then took the minimum age of the stratum to which that fossil is assigned: 1 - minimum age for the Salamandridae of 55 million years before present (Ma), based on the oldest known salamandrid fossil, the newt-like *Koalliella genzeli* Herre, 1950 dated to 65-55 Ma (Estes, 1981). The prior has a 95% highest probability density (HPD) interval ranging from 54.4-74.3 Ma (median 57.7); 2 - minimum age for the clade consisting of *Tylototriton, Echinotriton*, and *Pleurodeles* of 44 Ma (HPD median 46.0, range 44.3-58.3), based on *Chelotriton weigelti* (Herre, 1935) (Milner, 2000) from the middle Eocene (Mammal Paleogene 13; Haubold and Thomae 1990) dated at 44 Ma (Mertz *et al.,* 2000); 3 - the clade of *Notophthalmus* and *Taricha*, minimum age 22 Ma (HPD median 24.0, range 22.3-36.3) based on *Taricha oligocenica* (van Frank, 1955) from the upper Oligocene and dated to 22 Ma (Estes, 1981); 4 - the crown group *Triturus* is at least 23.8 Ma (median HPD 26.0, range 24.3-38.3), given fossils apparently related to *Triturus marmoratus* from the Lower Miocene (Estes 1981) and dated to 24.2-23.8 Ma (Böhme, 2003); 5 - the clade of *Cynops, Laotriton, Pachytriton,* and *Paramesotriton* that is at least 15 Ma (median HPD 17.0, range 15.3-29.3), based on a fossil (*Procynops miocenicus* Young, 1965) (Estes, 1981), similar to *Cynops orientalis* from the Shanwang series (18-15 Ma; Qiu and Qiu, 1995); 6 - the clade of *Salamandra* and *Lyciasalamandra* is at least 33.9 Ma (median HPD 36.7, range 34.4-53.3), based on the fossil *Salamandra sansaniensis* Lartet, 1851 from the Eocene (33.9-55.8 Ma; Milner, 2000); 7 - the clade of *Chioglossa* and *Mertensiella* is at least 23.03 Ma (median HPD *25.0, range 23.3-37.3)*, based on *Chioglossa meini* Estes & Hoffstetter, 1976 and *Mertensiella mera* Hadrova, 1986 from the early Miocene (Ivanov, 2008).

We performed a combined phylogeny and divergence-time estimation using the Bayesian uncorrelated lognormal approach (Drummond *et al.,* 2006) implemented in BEAST version 1.5.4 (Drummond and Rambaut, 2007). Analyses were conducted with separate partitions for different codon positions in 13 genes and yet other partitions for 12S, 16S and the combined RNA's (with rate parameters, rate heterogeneity and base frequencies unlinked across partitions, but clock and tree models linked), estimated base frequencies, with trees generated using a Yule speciation process and with nucleotide substitution models suggested by MrModelTest (Nylander, 2004). Temporal calibration points were treated as priors on clade ages, with a lognormal distribution (following Drummond *et al.,* 2006), a standard deviation of one Ma (a standard but arbitrary number) and an offset equal to the estimated minimum age of the clade (allowing the calibration points to function as constraints on the minimum ages of clades). A mean of 5 Ma was used for most clades, creating a 95% highest probability density distribution (HPD) spanning from roughly 1 Ma older than the minimum age of the fossil to roughly 15 Ma older, with a median roughly 3 Ma older than the fossil calibration point. This allows the fossil calibration point to constrain the minimum age of the clade, and allows for the possibility that the clade could be substantially older than the oldest known fossil. For two clades with greater uncertainty in the ages of the fossils (calibration points 1 and 6), we used a mean of 10 Ma (such that the 95% HPD extends to roughly 30 Ma older than the fossil calibration point). We ran three replicate analyses of 100 million and two of 200 million generations in BEAST. All five runs yielded effective sample sizes (ESS) > 200 for likelihood and ages of all clades as shown with Tracer, v. 1.5 (A. Rambaut and A.J. Drummond, available from http://beast.bio.ed.ac.uk/Tracer). Trees were combined using LogCombiner, after excluding the initial 90% (90-180 million generations) of each run as burn-in.

Secondly, we gathered ND1 / ND2 mtDNA sequence data from Genbank with the final consultation on 1 January 2014 , for altogether 101 salamandrid taxa (90 species and 11 subspecies), including the full mitogenomic data used above. Alignment of the protein-coding sequences was straightforward and done by eye, assisted by MacClade (Madisson & Madisson, 2000). Data unavailable for one or the other gene fragment amounted to 12.7%. The sequence data file is presented in the Online Supplementary Information. We analysed these data with the full mtDNA tree as a backbone constraint in PAUP* (Swofford, 2003), under likelihood settings determined by jModelTest 2.1.3 under default settings (Darriba *et al.*, 2012). The tree was dated on the basis of 42 nodes for which the 95% confidence estimates (CI) were available from the full mtDNA phylogeny, with r8s v. 1.8 software (by M. Sanderson, made available at http://loco.biosci.arizona.edu/r8s/, The subroutine used was NPRS with Powell optimization with constraints set over the 95% CI). The resulting phylogeny for the family Salamandridae is described below. It is similar to that based on nuclear data (which had limited taxon sampling (Wiens, 2007)), indicating that the results based on mtDNA are not widely misleading (cf. Rubinoff and Holland, 2005). The erroneous reconstruction of a phylogeny *e.g.* due to incomplete lineage sorting or hybridization and introgression events, is likely to be most pronounced at the more recent branches of a tree. A case in point is *Lissotriton montandoni* in which the species' original mtDNA has throughout its range been replaced by that of the congeneric species *L. vulgaris,* with which it hybridizes (Babik *et al.*, 2005; Zieliński *et al.*, 2013).

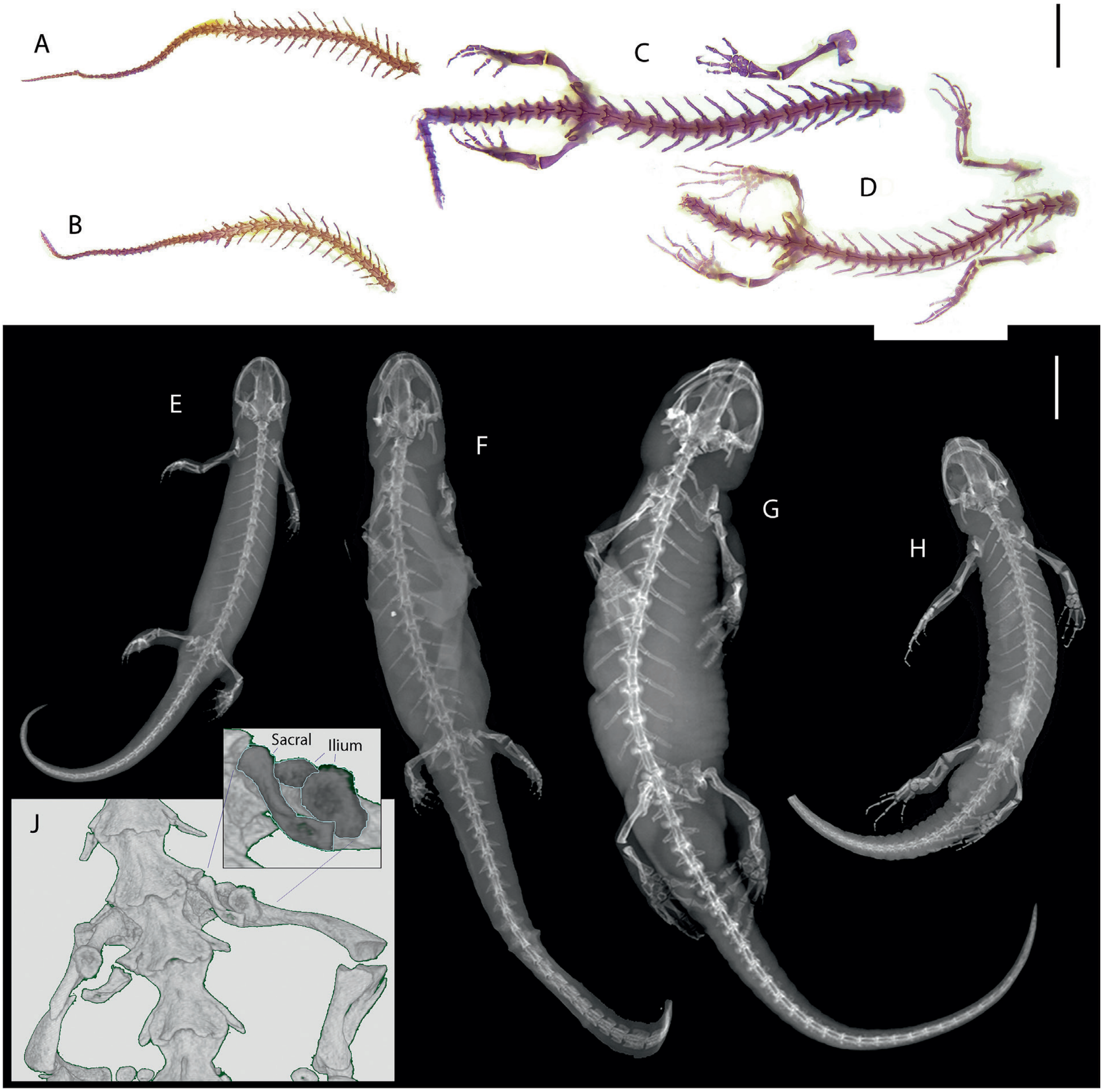
The time calibrated phylogeny of the Salamandridae that we accept as the basis of the analytical work is as follows:

“(((((((((*Calotriton_asper*:34.61,((((*Triturus_carnifex*:4.51,*Triturus_macedonicus*:4.51)node01{4.51,CI=3.22-5.59}:2.19,(*Triturus_cristatus*:5.90,*Triturus_dobrogicus*:5.90)node02{5.90,CI=6.07-9.17}:0.79)node03{6.70,CI=6.50-9.64}:0.40,(*Triturus_ivanbureschi*:5.27,*Triturus_karelinii*:5.27)node04{5.27,CI=5.64-8.67}:1.83)node05{7.10,CI=7.26-10.65}:11.06,(*Triturus_marmoratus*:4.14,*Triturus_pygmaeus*:4.14)node06{4.14,CI=3.83-6.20}:14.01)node07{18.16,CI=22.46-29.15}:16.45)node08{34.61,CI=33.80-41.85}:2.70,((((*Neurergus_crocatus*:5.42,*Neurergus_kaiseri*:5.42)node09{5.42}:1.02,*Neurergus_derjugini*:6.44)node10{6.44}:5.73,(*Neurergus_s_barani*:1.81,*Neurergus_s_strauchii*:1.81)node11{1.81}:10.37)node12{12.17,CI=12.17-17.31}:21.81,(*Ommatotriton_ophryticus*:12.05,*Ommatotriton_vittatus*:12.05)node13{12.05}:21.93)node14{33.98,CI=32.68-40.32}:3.33)node15{37.31,CI=35.91-46.08}:7.25,(((*Ichthyosaura_a_alpestris*:2.97,*Ichthyosaura_a_cyreni*:2.97)node16{2.97}:8.00,*Ichthyosaura_a_veluchiensis*[KC149522]:10.97)node17{10.97}:31.44,(*Lissotriton_boscai*:28.20,(*Lissotriton_helveticus*:24.54,(*Lissotriton_italicus*:16.30,((((((*Lissotriton_montandoni*:0.71,*Lissotriton_v_ampelensis*:0.71)node18{0.71}:2.24,*Lissotriton_v_vulgaris*:2.95)node19{2.95}:0.10,*Lissotriton_v_meridionalis*:3.05)node20{3.05}:1.21,(*Lissotriton_v_schmidtlerorum*:1.95,*Lissotriton_v_graecus*:1.95)node21{1.95}:2.31)node22{4.26}:0.55,*Lissotriton_v_kosswigi*[FJ588950]:4.81)node23{4.81}:1.64,*Lissotriton_v_lantzi*:6.45)node24{6.45}:9.85)node25{16.30}:8.24)node26{24.54}:3.66)node27{28.20}:14.21)node28{42.41,CI=37.48-47.45}:2.14)node29{44.56,CI=42.22-52.96}:1.49,((((*Cynops_cyanurus*:20.70,*Cynops_glaucus*[KC762304]:20.70)node30{20.70}:1.85,((*Cynops_fudingensis*[GU301787]:8.83,*Cynops_orientalis*:8.83)node31{8.83}:4.73,*Cynops_orphicus*:13.56)node32{13.56,CI=9.67-14.36}:8.99)node33{22.55,CI=19.68-26.62}:2.14,(*Cynops_ensicauda*:23.82,(*Laotriton_laoensis*[DQ517808]:21.82,(((*Pachytriton_archospotus*[GQ303628]:15.27,((*Pachytriton_brevipes*:7.86,*Pachytriton_granulosus*[DQ517798]:7.86)node34{7.86,CI=7.86-11.79}:6.22,(*Pachytriton_feii*[JX237735]:12.86,*Pachytriton_changi*[JX237745]:12.86)node35{12.86}:1.23)node36{14.08}:1.18)node37{15.27}:2.22,(*Pachytriton_inexpectatus*[JX907835]:14.11,*Pachytriton_moi*[JX237747]:14.11)node38{14.11}:3.38)node39{17.49,CI=17.49-24.59}:3.57,((*Paramesotriton_caudopunctatus*:13.50,(*Paramesotriton_longliensis*[FJ169608]:2.28,*Paramesotriton_zhijinensis*[FJ169609]:2.28)node40{2.28}:11.22)node41{13.50}:5.69,(((*Paramesotriton_chinensis*:0.59,*Paramesotriton_labiatus*[FJ744601]:0.59)node42{0.59}:10.13,*Paramesotriton_hongkongensis*:10.72)node43{10.72}:1.09,(*Paramesotriton_deloustali*:7.44,(*Paramesotriton_fuzhongensis*:5.02,(*Paramesotriton_guangxiensis*:4.43,*Paramesotriton_yunwuensis*[GU980577]:4.43)node44{4.43}:0.58)node45{5.02}:2.43)node46{7.44}:4.37)node47{11.81,CI=8.69-13.19}:7.38)node48{19.19,CI=16.70-23.61}:1.87)node49{21.06}:0.76)node50{21.82,CI=18.93-26.00}:2.00)node51{23.82,CI=23.82-32.16}:0.87)node52{24.69,CI=23.10-30.66}:0.67,*Cynops_pyrrhogaster*:25.36)node53{25.36,CI=18.25-25.36}:20.68)node54{46.04,CI=44.85-58.03}:6.36,(*Euproctus_montanus*:23.94,*Euproctus_platycephalus*:23.94)node55{23.94,CI=20.75-27.17}:28.47)node56{52.40,CI=48.83-63.25}:10.58,((*Notophthalmus_meridionalis*:19.08,(*Notophthalmus_perstriatus*:14.46,*Notophthalmus_viridescens:*14.46)node57{14.46}:4.61)node58{19.08,CI=14.50-20.82}:35.68,(*Taricha_granulosa*:19.50,(*Taricha_rivularis*:15.37,*Taricha_torosa*:15.37)node59{15.37}:4.13)node60{19.50,CI=13.63-19.50}:35.26)node61{54.76,CI=46.57-60.83}:8.22)node62{62.98,CI=55.08-71.33}:9.67,(((*Echinotriton_andersoni*:18.37,*Echinotriton_chinhaiensis*:18.37)node63{18.37,CI=12.85-18.37}:9.58,(((((*Tylototriton_asperrimus*:10.62,*Tylototriton_wenxianensis*:10.62)node64{10.62,CI=10.62-15.36}:5.25,*Tylototriton_lizhengchangi*[AB769532]:15.87)node65{15.87}:3.04,(*Tylototriton_panhai*[AB830735]:14.72,*Tylototriton_* *vietnamensis*[HM770088]:14.72)node66{14.72}:4.19)node67{18.91}:1.02,(*Tylototriton_hainanensis*:9.40,(*Tylototriton_notialis*[HM462063]:8.03,*Tylototriton_ziegleri*[AB769539]:8.03)node68{8.03}:1.38)node69{9.40}:10.53)node70{19.93}:2.22,(((*Tylototriton_kweichowensis*:11.88,*Tylototriton_verrucosus*:11.88)node71{11.88}:1.34,((*Tylototriton_shanjing*:9.05,*Tylototriton_uyenoi*[AB830729]:9.05)node72{9.05}:0.56,*Tylototriton_yangi*[AB830739]:9.61)node73{9.61}:3.61)node74{13.22}:4.94,*Tylototriton_taliangensis*:18.17)node75{18.17}:3.98)node76{22.15}:5.80)node77{27.95,CI=21.74-27.95}:21.48,(*Pleurodeles_poireti*:16.12,*Pleurodeles_waltl*:16.12)node78{16.12,CI=15.53-22.23}:33.31)node79{49.43,CI=40.89-49.43}:23.23)node80{72.66,CI=62.05-80.23}:17.22,((*Chioglossa_lusitanica*:51.22,*Mertensiella_caucasica*:51.22)node81{51.22,CI=43.43-55.64}:15.30,(((((*Lyciasalamandra_antalyana*:11.70,*Lyciasalamandra_helverseni*:11.70)node82{11.70}:1.57,((((*Lyciasalamandra_basoglui*:5.17,*Lyciasalamandra_finikensis*:5.17)node83{5.17}:1.81,*Lyciasalamandra_luschani*:6.97)node84{6.97}:4.90,*Lyciasalamandra_fazilae*:11.87)node85{11.87}:0.92,*Lyciasalamandra_billae*:12.79)node86{12.79}:0.48)node87{13.27}:0.48,*Lyciasalamandra_atifi*:13.75)node88{13.75}:0.99,*Lyciasalamandra_flavimembris*:14.73)node89{14.73,CI=11.43-16.29}:21.26,((((((*Salamandra_a_aurorae*:0.44,*Salamandra_a_atra*:0.44)node90{0.44}:7.66,*Salamandra_corsica:*8.10)node91{8.10}:2.97,*Salamandra_lanzai*:11.07)node92{11.07}:3.10,(*Salamandra_i_semenovi*:5.03,*Salamandra_i_infraimmaculata:*5.03)node93{5.03}:9.14)node94{14.17}:2.41,*Salamandra_salamandra*:16.58)node95{16.58}:1.65,*Salamandra_algira*:18.24)node96{18.24}:17.76)node97{36.00,CI=31.49-38.36}:30.53)node98{66.52,CI=57.87-76.24}:23.35)node99{89.88,CI=72.62-98.43}:16.65,*Salamandrina_perspicillata*[DQ517823]:106.53);"

All branches had posterior probabilities (pp) in excess of 0.99, with three exceptions, namely pp=0.83 for the branch leading to all taxa minus *Salamandra perspicellata*, pp = 0.58 for the branch leading to the genus *Cynops* and pp= 0.95 for the branch leading to *Laotriton laoensis* and the genus *Pachytriton*. Numbers following a colon are branch-lengths in Ma. Numbers in curly brackets are calibration points in Ma; if accompanied by the code 'CI' the three numbers refer to the estimate and to the 95% confidence interval of the estimate, respectively. GenBank accession numbers are provided in square brackets in cases of potential ambiguity (see also Appendix I). To draw the phylogeny as in Fig. 3 use *e.g.* TreeView (Page, 1996) after curly brackets have been replaced by straight brackets. To obtain phylogenetic trees suitable for character state analysis, taxa with no morphological data were trimmed from the data set with PhyUtility (Smith and Dunn, 2008).

Appendix III

Illustrative cleared and stained specimens (upper part) and X-ray imagery (lower part) with collection registration numbers and vertebrae counts as follows: A and B – *Ichthyosaura alpestris veluchiensis* IBISS 7108, NRBV = 13, NCV = 34 and IBISS 7109, NRBV = 13, NCV = 27; C and D - *Triturus karelinii* IBISS G22832, NRBV = 13, NCV = 16 with tail tip missing and G22825, NRBV = 13, NCV not available; E - *Ichthyosaura alpestris veluchiensis* ZMA.RENA.5308, NRBV = 13, NCV = 28; F - *Pachytriton brevipes* RMNH.RENA.5890, NRBV = 12, NCV = 23; G - *Taricha torosa* ZMA.RENA.5011, NRBV = 12, NCV = 31; H - *Triturus karelinii* RMNH.RENA.46931, NRBV = 13 (transitional - 13 left and 14 right) and NCV = 22 with tail tip missing. J – CT-scan of a transitional sacral vertebra in *T. dobrogicus* (ZMA.RENA.9120). The sacral rib is developed on the left side on the 18th vertebra and at the right side on the 17th vertebra. The iliac bones of the pelvic girdle are attached to the corresponding sacral ribs of 17th (see insert) and 18th vertebra. The size bar represents 1 cm.



Appendix IV

Database of environmental data Bio 01 to Bio 19, altitude and slope, derived from Hijmans *et al.* (2012). Species distribution maps largely follow IUCN (2012).

A - Environmental data

Taxon	Comments	Range (km^2)	Bio 01	Bio 02	Bio 03	Bio 04	Bio 05	Bio 06	Bio 07
Calotriton asper		43349	77.673	84.834	35.131	5430.721	210.772	-26.917	237.689
Chioglossa lusitanica		85023	127.696	86.315	40.100	4382.838	247.734	34.814	212.920
Cynops cyanurus		87981	156.699	106.361	45.438	4446.509	252.453	20.625	231.828
Cynops ensicauda		2565	215.724	55.833	29.274	4648.066	307.679	119.766	187.913
Cynops orientalis		294001	160.646	81.305	25.021	8371.431	318.931	0.310	318.621
Cynops orphicus		13131	191.560	75.390	31.467	5612.494	303.810	67.783	236.027
Cynops pyrrhogaster		411188	119.993	86.040	26.680	8144.233	282.823	-33.821	316.644
Echinotriton andersoni		2880	215.447	55.578	29.140	4653.980	307.480	119.689	187.791
Echinotriton chinhaiensis		67	157.970	65.448	22.075	7864.448	301.463	13.836	287.627
Euproctus montanus		12185	124.781	60.908	29.142	5131.382	239.629	34.616	205.013
Euproctus platycephalus		3095	133.051	71.230	30.508	5608.977	266.068	36.130	229.939
Ichthyosaura a. alpestris		2196898	82.848	86.358	32.287	6420.025	224.946	-39.534	264.480
Ichthyosaura a. cyreni		43193	108.514	86.843	39.379	4593.986	231.611	14.101	217.510
Ichthyosaura a. veluchiensis	Includes the northern Greek lineage which has also NRBV 13	35831	114.874	106.367	36.060	6417.029	279.136	-12.205	291.341
Laotriton laoensis		688	191.251	109.368	56.109	2353.856	268.100	74.983	193.118
Lissotriton boscai		345484	140.948	100.438	38.883	5363.559	289.894	33.246	256.648
Lissotriton helveticus		1909090	97.814	83.350	35.577	5362.989	225.506	-5.685	231.191
Lissotriton italicus		114355	139.469	78.079	31.540	5865.031	275.406	32.401	243.005
Lissotriton v. ampelensis	Babik *et al.* (2005)	36000	81.667	96.420	31.461	7354.181	235.853	-65.373	301.226
Lissotriton v. graecus	Babik *et al.* (2005)	2594	122.111	100.724	34.226	6642.641	281.910	-8.299	290.209
Lissotriton v. kosswigi	Babik *et al.* (2005), Schmidtler and Frantzen (2004)	39037	105.977	98.412	34.897	6443.883	255.506	-22.687	278.193
Lissotriton v. lantzi	Babik *et al.* (2005), Schmidtler and Frantzen (2004)	268674	72.338	98.188	31.618	7384.321	233.134	-73.203	306.337
Lissotriton v. meridionalis	Babik *et al.* (2005)	15206	121.504	85.230	30.691	6579.712	270.279	-2.892	273.171
Lissotriton v. schmidtlerorum	Babik *et al.* (2005), Schmidtler and Frantzen (2004)	72714	140.514	103.629	36.148	6514.206	296.355	14.343	282.011
Lissotriton v. vulgaris	Babik *et al.* (2005)	68656	77.699	92.564	28.716	8024.608	238.868	-78.031	316.899
Lyciasalamandra atifi		3086	142.513	105.406	36.051	6632.815	299.789	11.052	288.737
Lyciasalamandra billae		87	171.471	111.701	37.908	6449.667	331.253	40.149	291.103
Lyciasalamandra fazilae		1005	171.042	121.051	40.777	6263.764	332.784	39.278	293.506
Lyciasalamandra flavimembris		525	171.691	111.918	37.463	6633.960	337.608	42.851	294.756
Lyciasalamandra helverseni		518	178.774	62.757	30.116	5230.069	286.724	82.361	204.363
Lyciasalamandra luschani		1452	161.463	113.826	39.444	6176.335	319.330	33.762	285.567
Mertensiella caucasica		38173	82.017	103.464	33.734	6901.154	240.638	-60.902	301.540
Neurergus crocatus		19653	90.287	107.838	27.890	9635.701	293.128	-86.434	379.562
Neurergus kaiseri		227	193.749	157.656	38.454	8663.009	415.542	10.872	404.670
Neurergus derjugini		821	116.150	146.939	35.554	9029.581	340.676	-67.084	407.760
Neurergus s. barani		1867	117.438	105.292	27.544	9481.918	321.277	-54.268	375.545
Neurergus s. strauchii		24156	97.488	108.628	28.455	9262.644	303.844	-71.314	375.158
Notophthalmus meridionalis		117014	234.438	110.732	47.000	4428.673	342.670	106.754	235.916
Notophthalmus viridescens		5215453	107.078	119.850	32.663	8820.786	288.923	-81.618	370.541
Ommatotriton ophryticus	Litvinchuk *et al.* (2005)	412893	90.248	107.182	34.152	7108.960	255.373	-53.660	309.033
Ommatotriton vittatus	Litvinchuk *et al.* (2005)	77895	158.310	109.793	35.693	7079.256	320.313	15.436	304.877
Pachytriton brevipes		583823	179.934	82.106	28.493	7097.060	319.451	33.847	285.604
Pachytriton feii		427	131.433	71.792	23.269	8106.707	279.494	-22.670	302.164
Pachytriton granulosus		60998	156.028	77.356	23.873	8419.440	314.585	-2.780	317.365
Pachytriton moi		3257	155.402	79.507	27.986	7141.639	291.185	11.980	279.205
Paramesotriton caudopunctatus		48586	160.149	82.038	28.008	7328.505	303.324	14.939	288.386
Paramesotriton chinensis		163067	164.152	76.462	25.743	7522.468	309.882	17.401	292.481
Paramesotriton deloustali		29597	218.333	79.882	39.346	4459.567	309.583	108.759	200.824
Paramesotriton fuzhongensis		7486	183.010	81.919	29.469	6779.370	313.978	40.836	273.142

Bio 08	Bio 09	Bio 10	Bio 11	Bio 12	Bio 13	Bio 14	Bio 15	Bio 16	Bio 17	Bio 18	Bio 19	Altitude	Slope
77.002	82.797	149.617	12.153	1062.020	115.092	64.426	15.991	308.821	222.726	240.726	252.319	1281.094	8.486
82.313	184.236	185.833	74.198	1137.781	158.569	27.395	43.227	435.366	114.586	126.214	413.120	459.812	3.803
205.858	95.171	205.874	93.588	1002.517	207.422	12.181	85.863	575.773	40.688	572.820	40.719	1924.125	4.852
252.094	155.604	274.166	155.604	2452.615	333.030	126.421	31.005	834.299	409.558	716.872	409.876	119.891	2.757
232.324	73.980	265.264	49.837	1451.310	243.231	39.775	52.018	618.008	150.005	548.853	172.421	228.942	3.040
227.504	137.106	258.098	115.928	1596.153	259.067	32.233	57.466	677.007	119.574	615.034	163.915	478.242	4.848
203.108	40.647	224.889	17.000	1822.945	261.294	73.490	40.423	691.952	242.651	652.189	276.619	414.111	4.072
248.995	155.345	274.056	155.345	2448.342	331.625	126.144	30.579	831.821	409.766	711.006	410.049	121.745	2.790
256.925	82.731	256.925	56.612	1426.224	198.104	49.597	41.045	505.642	176.209	505.642	187.627	191.090	5.206
104.034	191.188	193.679	64.915	738.779	99.278	13.997	42.632	281.526	75.045	96.940	236.743	613.659	7.635
95.837	206.950	208.969	68.162	706.144	104.599	9.436	50.420	276.337	52.731	67.627	261.550	649.866	5.006
128.134	36.320	163.477	-1.071	836.176	98.011	49.155	23.041	273.722	158.331	252.846	183.558	518.326	2.913
76.284	167.642	169.604	52.758	964.548	118.605	45.534	25.127	319.732	167.384	177.473	278.651	719.797	5.741
54.749	197.634	198.327	35.077	871.093	131.032	22.766	48.282	356.114	85.571	86.298	333.449	918.986	7.433
210.278	158.355	213.612	156.205	1681.067	384.395	4.843	94.099	974.403	30.715	804.320	34.890	1401.971	6.780
88.744	211.064	212.476	75.625	767.874	106.225	12.780	48.919	297.844	63.464	67.474	284.065	487.210	2.522
97.163	95.393	166.729	29.603	855.795	94.447	50.259	18.037	263.198	167.223	202.315	227.116	307.532	1.863
111.332	210.656	216.320	68.735	722.548	96.602	25.673	36.263	267.941	93.675	103.541	228.384	425.829	3.757
156.711	-3.523	171.289	-17.283	698.617	104.945	35.706	38.783	277.976	111.749	267.060	121.871	632.747	3.690
74.651	200.343	207.412	38.051	740.694	104.703	25.350	39.187	283.017	89.244	96.664	254.577	646.420	5.231
57.598	180.384	186.509	21.885	748.590	95.823	39.553	27.931	256.045	123.777	141.803	237.940	735.657	4.922
107.189	4.130	165.109	-23.084	875.107	118.889	40.290	34.853	318.062	136.070	274.643	164.732	1192.721	6.505
119.534	126.489	205.708	37.528	878.193	107.012	43.817	24.907	286.935	154.564	187.705	199.667	394.558	3.524
70.531	222.528	224.780	59.743	712.587	126.101	13.914	59.960	325.434	54.113	55.373	313.775	290.145	3.098
166.640	-9.946	175.444	-31.029	665.638	93.382	32.225	37.210	258.707	103.036	252.026	111.438	378.016	1.625
59.013	226.753	227.236	59.013	874.702	179.433	5.605	81.520	484.150	26.230	30.886	484.150	787.544	8.810
92.471	254.713	255.126	92.471	880.172	192.598	3.736	90.092	525.287	18.080	20.494	525.287	317.793	9.559
94.617	252.853	252.962	94.617	989.979	237.476	3.363	94.081	592.134	21.022	21.317	592.134	216.719	4.325
92.093	258.802	259.095	91.739	999.375	230.425	3.625	91.189	586.722	22.413	22.819	586.650	291.152	5.400
122.840	245.923	246.295	115.118	712.757	170.037	0.869	94.838	416.998	4.573	11.290	344.521	262.797	6.523
85.506	241.204	241.534	85.506	829.236	194.100	4.000	93.414	504.216	20.200	21.967	504.216	471.125	7.575
91.033	100.085	167.428	-8.050	840.157	104.426	41.973	27.826	284.243	143.209	172.233	207.760	1270.011	8.613
21.324	209.289	209.540	-36.605	635.210	111.990	3.263	71.336	304.327	11.953	15.059	240.890	1871.327	8.681
82.595	303.599	303.599	82.264	426.595	89.194	0.000	88.317	222.203	0.053	0.062	222.176	943.687	9.955
17.787	230.597	231.055	-2.174	652.493	120.565	0.000	85.392	341.228	0.223	0.512	303.536	1784.358	9.760
19.147	236.831	236.857	-5.267	586.658	82.986	2.461	62.866	231.667	12.097	13.884	230.251	1443.330	7.510
22.559	215.260	215.275	-20.751	796.316	116.448	5.228	61.634	326.225	22.379	23.095	291.763	1642.133	6.434
271.766	192.679	283.672	171.986	886.750	175.231	21.550	61.971	399.418	88.891	330.192	106.762	83.162	0.614
162.075	46.422	215.966	-11.778	1102.969	124.059	61.693	21.475	343.447	207.806	309.721	242.439	239.491	0.866
83.468	133.712	178.423	-3.110	671.061	88.681	28.228	34.816	236.378	96.819	133.385	179.961	1144.411	5.466
66.948	245.351	246.668	66.312	758.092	145.932	4.188	76.462	391.375	20.008	23.687	388.762	657.142	3.943
222.417	106.795	265.840	84.418	1621.177	273.299	42.674	56.297	729.864	154.272	571.048	195.703	375.349	3.932
187.225	43.012	232.361	23.482	1794.157	302.496	48.115	51.014	758.883	177.501	678.824	208.621	765.726	8.531
218.754	70.877	261.132	44.624	1501.651	248.563	44.799	48.569	618.272	163.674	550.055	190.490	275.223	23.395
208.645	78.600	241.165	58.668	1612.209	288.724	47.540	56.781	735.181	170.576	603.971	183.417	806.724	7.566
218.090	61.301	249.517	60.654	1269.464	212.511	32.844	55.293	554.252	114.636	485.916	115.640	668.754	4.282
217.282	88.809	257.562	65.007	1643.061	265.473	43.950	50.295	668.790	164.212	581.151	202.856	397.759	4.908
266.758	162.412	267.962	156.043	1761.586	357.799	13.972	84.132	972.558	68.138	962.426	72.183	369.000	4.818
225.958	110.449	262.606	90.801	1552.530	289.038	45.605	60.078	722.674	146.882	576.456	180.704	454.425	4.427

Cont. **A - Environmental data**

Taxon	Comments	Range (km^2)	Bio 01	Bio 02	Bio 03	Bio 04	Bio 05	Bio 06	Bio 07
Paramesotriton guangxiensis		565	209.391	75.669	35.712	4971.818	303.297	93.904	209.393
Paramesotriton hongkongensis		25974	223.743	70.977	32.435	5146.707	320.039	105.047	214.992
Paramesotriton labiatus		134874	178.751	80.397	28.216	7079.104	315.574	34.387	281.187
Pleurodeles poireti		2681	178.648	92.264	38.345	5145.231	308.518	70.866	237.653
Pleurodeles waltl		536648	150.244	109.364	38.662	5854.752	311.639	31.086	280.554
Salamandra a. atra		188564	43.808	82.631	30.573	6397.965	183.178	-80.823	264.001
Salamandra a. aurorae		129	89.787	86.669	29.551	6951.669	243.898	-43.378	287.276
Salamandra algira		29270	151.054	106.350	37.913	5757.217	307.177	30.697	276.480
Salamandra corsica		6923	110.140	56.406	27.211	5214.736	224.640	21.362	203.278
Salamandra i. infraimmaculata		90459	121.767	113.593	34.601	7455.241	296.989	-28.724	325.713
Salamandra i. semenovi		44534	100.862	111.429	29.018	9373.376	304.887	-71.801	376.688
Salamandra lanzai		1250	31.989	72.855	29.676	5841.851	162.560	-77.895	240.455
Salamandra salamandra		3592466	98.872	90.333	33.637	6258.459	243.733	-22.050	265.783
Salamandrina perspicillata		117141	121.730	83.270	31.384	6184.554	266.989	6.818	260.171
Taricha granulosa		651184	70.251	97.087	38.630	5245.136	211.730	-34.403	246.133
Taricha rivularis		21243	119.864	136.692	53.253	4228.157	269.530	13.531	255.998
Taricha torosa		71315	137.827	139.503	52.346	4572.156	288.261	20.253	268.008
Triturus carnifex		549616	105.766	85.937	30.890	6605.643	253.924	-19.391	273.315
Triturus cristatus		8571399	59.752	83.351	25.692	8758.810	231.596	-98.091	329.687
Triturus dobrogicus		456514	105.715	97.461	30.390	7788.760	270.598	-44.934	315.532
Triturus ivanbureschi	Wielstra *et al.* (2010)	46583	110.435	101.809	33.062	7176.147	273.783	-29.572	303.355
Triturus karelinii		76333	105.021	106.829	31.375	8062.161	284.305	-51.232	335.537
Triturus macedonicus		252541	106.572	99.467	33.390	6764.289	266.150	-26.996	293.145
Triturus marmoratus		773002	114.631	94.062	38.308	5295.555	250.613	8.253	242.361
Triturus pygmaeus		258462	157.372	106.991	38.470	5765.692	318.108	42.017	276.091
Tylototriton asperrimus		367495	175.161	79.771	28.714	7029.887	310.896	33.382	277.514
Tylototriton kweichowensis		44968	129.965	86.455	33.168	5980.856	254.073	-3.063	257.137
Tylototriton panhai		936	256.637	121.322	54.013	2141.555	357.322	134.811	222.511
Tylototriton taliangensis		35381	108.711	99.130	38.193	5439.404	223.535	-32.844	256.379
Tylototriton uyenoi		4003	233.268	120.046	50.667	2430.491	341.645	106.897	234.748
Tylototriton verrucosus		436372	201.583	114.574	49.039	3529.276	296.421	65.098	231.323
Tylototriton vietnamensis		36555	222.436	77.977	37.101	4737.562	317.464	110.102	207.362

B - brief description of the bioclimatic variables, from http://www.worldclim.org/bioclim.

Bio01 = annual mean temperature
Bio02 = mean diurnal range (mean of monthly (max temp - min temp))
Bio03 = isothermality (Bio2/Bio7) (* 100)
Bio04 = temperature seasonality (standard deviation *100)
Bio05 = max temperature of warmest month
Bio06 = min temperature of coldest month
Bio07 = temperature annual range (Bio5-Bio6)
Bio08 = mean temperature of wettest quarter
Bio09 = mean temperature of driest quarter
Bio10 = mean temperature of warmest quarter
Bio11 = mean temperature of coldest quarter
Bio12 = annual precipitation
Bio13 = precipitation of wettest month
Bio14 = precipitation of driest month
Bio15 = precipitation seasonality (coefficient of variation)
Bio16 = precipitation of wettest quarter
Bio17 = precipitation of driest quarter
Bio18 = precipitation of warmest quarter
Bio19 = precipitation of coldest quarter

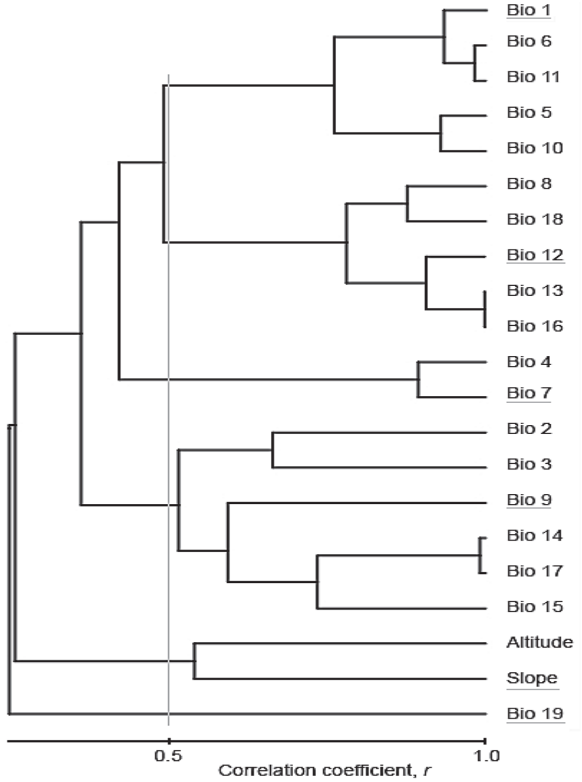
Bio 08	Bio 09	Bio 10	Bio 11	Bio 12	Bio 13	Bio 14	Bio 15	Bio 16	Bio 17	Bio 18	Bio 19	Altitude	Slope
264.051	155.781	264.051	139.995	1738.773	339.288	26.892	79.023	956.743	100.965	956.743	113.039	428.044	4.268
280.452	164.451	281.532	152.269	1730.463	308.671	26.116	72.982	867.192	95.523	864.485	107.493	53.094	1.517
221.553	102.259	263.539	83.032	1539.581	268.224	46.569	56.343	706.192	157.327	542.000	186.558	462.910	4.497
131.170	241.747	248.336	116.985	754.820	132.311	3.336	67.776	364.029	27.779	43.326	352.850	102.731	2.359
108.860	226.321	228.701	79.212	530.482	74.614	6.875	49.644	206.447	39.610	43.227	186.615	520.697	1.898
113.693	-22.192	124.309	-38.724	1187.587	143.887	64.996	27.920	410.302	209.374	399.479	221.279	1370.058	10.072
151.008	7.528	176.992	-1.031	826.441	94.945	37.945	29.087	265.323	123.189	262.150	124.913	842.299	11.885
92.467	226.098	228.907	81.264	738.541	120.501	3.985	63.332	336.149	29.265	39.141	322.342	765.326	6.120
89.111	177.895	180.497	49.821	785.399	102.543	17.005	39.909	291.349	86.283	106.639	247.128	895.826	9.354
51.162	212.690	213.923	23.588	672.642	120.264	4.432	67.950	323.989	20.318	30.088	316.351	1156.598	5.362
22.339	218.605	219.031	-20.209	697.224	119.631	2.425	73.857	331.790	9.761	11.151	278.344	1804.829	8.533
15.753	63.438	107.310	-39.550	1320.091	127.495	87.232	10.322	358.199	292.775	305.250	326.210	2010.569	12.393
116.488	89.174	178.429	18.247	821.469	99.734	42.327	27.112	275.919	142.478	206.160	205.817	523.201	3.205
99.553	185.188	202.109	45.199	843.576	108.638	37.782	27.672	291.448	142.043	147.244	221.499	544.223	4.596
25.254	129.406	139.435	7.625	1718.226	266.414	46.779	54.715	741.464	171.139	193.892	655.730	625.999	6.938
73.387	173.780	175.856	69.882	1331.618	254.315	2.773	85.071	700.955	21.294	35.341	685.022	401.012	4.433
86.681	194.942	198.623	83.911	635.139	127.937	1.541	87.719	342.847	9.391	16.027	336.524	548.076	4.435
125.383	92.168	190.077	21.271	915.501	111.613	46.043	27.404	309.372	156.020	233.162	200.812	533.805	4.197
154.204	-22.358	169.462	-54.482	646.911	82.152	32.766	28.871	224.330	109.020	215.032	130.467	199.584	0.829
189.015	22.445	201.514	1.208	606.622	78.760	34.513	26.143	207.283	109.692	198.205	122.510	137.173	0.673
103.015	132.216	200.703	16.980	669.002	92.010	29.966	34.351	244.256	100.189	134.731	202.902	579.277	3.224
112.279	123.946	206.485	0.647	533.740	75.874	20.473	41.023	200.049	71.019	115.715	129.954	904.059	3.457
75.314	162.256	192.463	19.369	883.554	112.370	40.622	29.086	305.501	136.537	148.048	267.735	751.842	5.331
85.266	158.637	183.417	47.958	807.610	94.969	40.045	23.134	259.757	143.057	153.775	235.081	424.958	1.912
103.715	232.980	234.570	87.359	586.922	82.666	4.451	54.782	234.845	32.184	34.432	222.876	392.738	1.856
234.436	87.624	259.957	79.541	1405.669	239.609	34.253	59.668	650.265	123.694	585.995	130.826	512.686	3.786
200.616	46.305	201.178	46.305	1002.634	188.963	13.987	77.565	529.080	44.539	528.703	44.539	1698.330	5.628
264.285	225.721	279.874	225.431	1217.789	240.895	3.368	79.237	607.664	27.815	404.315	30.751	389.122	3.033
173.556	33.717	173.972	33.717	1111.745	235.031	7.769	87.901	632.272	28.233	630.270	28.233	2308.301	10.151
241.365	212.060	259.603	197.365	1069.867	203.730	6.045	81.141	527.655	27.594	320.138	30.124	909.700	5.069
233.465	158.290	237.191	149.450	1685.401	355.556	9.356	86.254	957.848	44.440	819.536	49.370	1057.664	6.055
274.619	165.742	275.381	156.587	1633.707	330.138	14.516	83.983	908.145	68.822	903.445	74.196	261.585	2.963

C - loadings of 15 environmental variables to four PCA axes with eigenvalues larger than unity. High loadings (>0.8) are in boldface type and moderately high loadings (0.6<loading<0.8) are in italics.

Environmental variable	PCA1	PCA2	PCA3	PCA4
Bio02	-0.124	-**0.813**	-0.013	0.099
Bio03	0.366	-0.601	*-0.630*	-0.073
Bio04	-0.560	0.061	*0.781*	0.239
Bio05	0.589	-0.445	0.600	-0.149
Bio06	**0.897**	-0.057	-0.144	-0.340
Bio08	0.764	0.421	0.101	0.056
Bio10	0.775	-0.165	0.571	-0.128
Bio11	**0.925**	-0.159	-0.158	-0.255
Bio13	**0.846**	0.258	-0.021	0.386
Bio14	-0.058	**0.914**	-0.048	-0.085
Bio15	0.576	-0.680	0.096	0.341
Bio16	**0.834**	0.250	-0.047	0.414
Bio17	-0.026	**0.925**	-0.062	-0.107
Bio18	0.663	0.559	-0.038	0.405
Altitude	-0.394	-0.233	-0.227	*0.724*
Variance explained (%)	40.0	27.4	12.1	9.6

Appendix V

Clustering of 21 environmental variables on the basis of pairwise correlation coefficients (Pearson's product-moment correlations, *r*) with the UPGMA method. Note the presence of six clusters within which variables are correlated at $r > 0.5$. Selected variables are underlined.



Appendix VI

Drawn images of a variety of salamander species, as used in Figs 1 and 5. Artwork Bas Blankevoort © Naturalis Biodiversity Center.

Species depicted are: a. *Chioglossa lusitanica*, b. *Lyciasalamandra antalyana*, c. *Proteus anguinus*, d. *Salamandra infraimmaculata*, e. *Speleomantes flavus*, f. *Triturus marmoratus*, g. *Triturus dobrogicus* and h. *Lissotriton vulgaris*.

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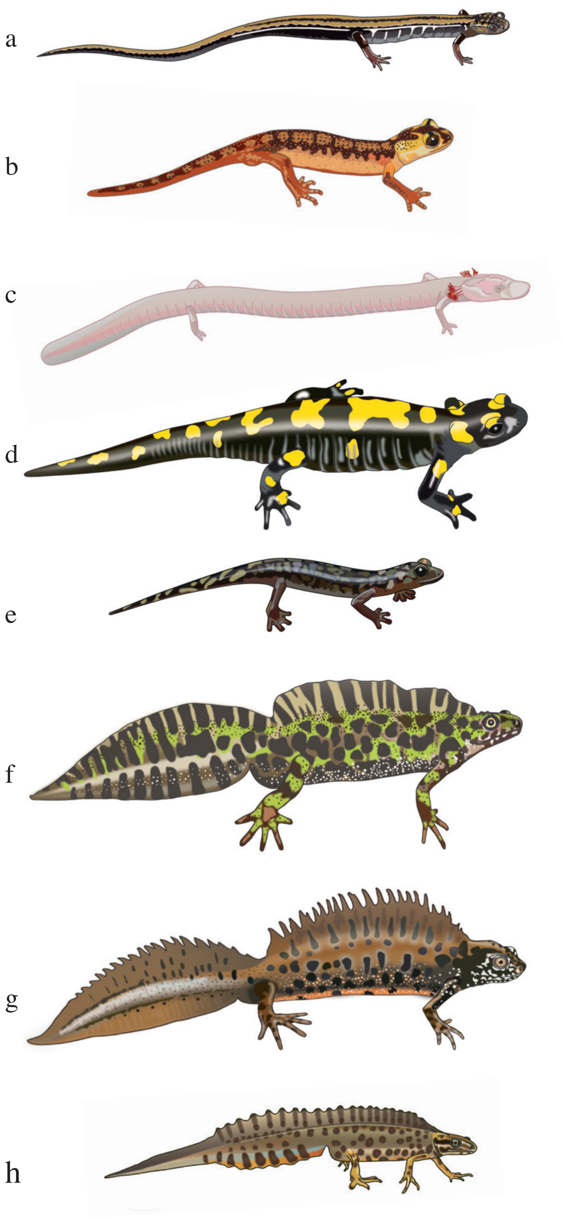

Appendix VII

Phylogenetic relationships for 81 salamandrid salamanders with NRBV documented. Branch lengths represent the amount of morphological change, as shown in legend. Right-headed arrows represent inferred gains and left-headed arrows represent inferred losses of vertebrae. Morphological change not unambiguously allocated to a single branch is shown in red.