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Phylotranscriptomic evidence for pervasive ancient hybridization among Old World salamanders

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ABSTRACT

Hybridization can leave genealogical signatures in an organism's genome, originating from the parental lineages and persisting over time. This potentially confounds phylogenetic inference methods that aim to represent evolution as a strictly bifurcating tree. We apply a phylotranscriptomic approach to study the evolutionary history of, and test for inter-lineage introgression in the Salamandridae, a Holarctic salamanders group of interest in studies of toxicity and aposematism, courtship behavior, and molecular evolution. Although the relationships between the 21 currently recognized salamandrid genera have been the subject of numerous molecular phylogenetic studies, some branches have remained controversial and sometimes affected by discordances between mitochondrial vs. nuclear trees. To resolve the phylogeny of this family, and understand the source of mito-nuclear discordance, we generated new transcriptomic (RNAseq) data for 20 salamandrids and used these along with published data, including 28 mitochondrial genomes, to obtain a comprehensive nuclear and mitochondrial perspective on salamandrid evolution. Our final phylotranscriptomic data set included 5455 gene alignments for 40 species representing 17 of the 21 salamandrid genera. Using concatenation and species-tree phylogenetic methods, we find (1) *Salamandrina* sister to the clade of the "True Salamanders" (consisting of *Chioglossa*, *Mertensiella*, *Lyciasalamandra,* and *Salamandra*), (2) *Ichthyosaura* sister to the Near Eastern genera *Neurergus* and *Ommatotriton*, (3) *Triturus* sister to *Lissotriton*, and (4) *Cynops* paraphyletic with respect to *Paramesotriton* and *Pachytriton*. Combining introgression tests and phylogenetic networks, we find evidence for introgression among taxa within the clades of "Modern Asian Newts" and "Modern European Newts". However, we could not unambiguously identify the number, position, and direction of introgressive events. Combining evidence from nuclear gene analysis with the observed mito-nuclear phylogenetic discordances, we hypothesize a scenario with hybridization and mitochondrial capture among ancestral lineages of (1) *Lissotriton* into

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Ichthyosaura and (2) *Triturus* into *Calotriton*, plus introgression of nuclear genes from *Triturus* into *Lissotriton*. Furthermore, both mitochondrial capture and nuclear introgression may have occurred among lineages assigned to *Cynops*. More comprehensive genomic data will, in the future, allow testing this against alternative scenarios involving hybridization with other, extinct lineages of newts.

1. Introduction

Phylogenetic relationships are typically represented by bifurcating evolutionary trees. In their simplest form, phylogenies represent the split of population-level lineages that diverge and remain independent. However, in many cases a bifurcating tree oversimplifies evolutionary history by neglecting that lineages can often maintain gene flow throughout the divergence process. In some cases, hybrid populations can follow independent evolutionary trajectories (e.g. after allopolyploidization; Evans 2008) and result in new hybrid species. Hybridization can also leave genomic signatures through introgression, where part of the genetic material of one of the lineages is assimilated by the other (Arnold 1997, Mallet 2005). The amount of introgressed genomic material and its persistence through time, depend on many factors, including selection. The importance of these processes has been widely investigated in plants (Soltis & Soltis 2009) where the possibility of new species arising through hybridization was already discussed by Linnaeus (Baack & Rieseberg 2007). Mallet (2005) estimated that 25% of plant species are involved in hybridization, mostly between young and closely related species, but sometimes between more distant lineages (Whitney et al. 2010). By contrast, interspecific hybridization and introgression have been traditionally disregarded by zoologists (Mallet 2005). The development of genomic methods has provided new tools to identify hybrids and understand the role of introgressive hybridization in lineage diversification (Baack & Rieseberg 2007; Payseur and Rieseberg, 2016; Irisarri et al. 2018; Palkopoulou et al. 2018). Many studies have reported inter-species hybridization, emphasizing that hybridization in natural populations might be much more common than usually thought (Mallet et al. 2016) and that gene flow between distinct taxa should be considered an important process in shaping genomic diversity.

Genetic exchange between distinct species may confound phylogenetic inference methods that do not explicitly account for gene flow. Depending on loci sampling and the sorting of introgressed alleles, traditional phylogenetic approaches may yield phylogenetic relationships representative of the majority of the genome (Leaché et al. 2013). However, gene tree incongruence produced by introgression can be confounded with that of incomplete lineage sorting (ILS, Degnan & Rosenberg 2009). Hence, distinguishing their respective contribution remains a major challenge. In addition, ancient introgression can involve now-extinct species and thus be more difficult to detect. While the application of phylogenetic inference methods that account for ILS is now common, primarily in the framework of the Multi-Species Coalescent (MSC), introgression has been widely ignored in large scale phylogenetic studies (Eckert & Carstens 2008). The extension of the MSC into the Multi-Species Network Coalescent (Degnan 2018) allowed the development of models accounting for both ILS and introgression as sources of variation among gene trees. Although phylogenetic network estimation under this model remains a statistical challenge, particularly with large data sets, several implementations are already available based on maximum parsimony, maximum likelihood or Bayesian approaches. Alternative methods to detect ancient hybridization events include Patterson's D statistic (also named "ABBA/BABA" introgression tests), which is based on alignment site patterns (Green et al. 2010; Durand et al. 2011). Alongside this, the development of high throughput sequencing techniques make it possible to sample hundreds, or even thousands, of independent loci in non-model organisms for molecular analysis. This provides an unprecedented opportunity to study the variation of gene genealogies at the genome scale and gain insight into potential past hybridization events. As a result, phylogenomic studies have increasingly reported introgression in a wide range of taxa (Folk et al. 2018).

In this study, we apply a phylotranscriptomic approach to investigate the evolutionary history, and test for inter-lineage introgression, in a diverse clade of salamanders distributed in Europe, Northern Africa, Asia and North America, classified in the family Salamandridae (Sparreboom 2014). The currently recognized 118 salamandrid species and 21 genera (Amphibiaweb 2019) comprise aquatic and semiaquatic newts with sexual dimorphism and complex nuptial displays during their aquatic phase (Arnold 1977), as well as semi- and fully terrestrial salamanders with complex life histories and reproductive modes (Buckley 2012, Lourenço et al. 2019) involving specialized courtship pheromones (Van Bocxlaer et al. 2016). Equally intriguing are the potential anti-predator defensive systems, including biosynthesis of steroidal alkaloids (Lüddecke et al. 2018), accumulation of and resistance to tetrodotoxins (Geffeney et al. 2002, Hanifin & Gilly 2014), and putatively aposematic warning coloration in several taxa. Studying the evolution of these traits (Veith et al. 1998; Steinfartz et al. 2006; Wiens et al. 2011; Kieren et al. 2018) requires a well-resolved phylogeny.

Molecular phylogenies, mainly based on mitochondrial genes, have provided numerous surprising insights into salamandrid evolution (Titus and Larson, 1995; Veith et al. 1998; Weisrock et al. 2006; Steinfartz et al. 2006; Zhang et al. 2008; Veith et al. 2018), such as the non-monophyly of the "Modern European Newts" in the former genus *Triturus*, now separated into *Triturus, Ichthyosaura, Lissotriton*, and *Ommatotriton*, despite striking similarities in their aquatic-phase courtship and crest ornamentation (Steinfartz et al. 2006). Similarly, the former genus *Euproctus* of European mountain stream newts found on the islands of Corsica and Sardinia and the Pyrenees of the European mainland turned out to be non-monophyletic, resulting in the recognition of the genus *Calotriton* for the Pyrenean species (Carranza & Amat, 2005). Neither Asian nor European salamandrids formed reciprocally monophyletic groups, suggesting a complex evolutionary history stemming from multiple vicariant and dispersal events (Kieren et al. 2018). However, despite high support for many branches of the salamandrid tree, several other have remained poorly supported or discordant among different studies (reviewed by Veith et al. 2018). A recent tree inferred from a small set of nuclear genes (Veith et al. 2018) revealed discordances with mitochondrial trees regarding the position of some European (*Calotriton*, *Lissotriton, Ichthyosaura*) and Asian (*Cynops*) newts. Although these results may have been biased due to the limited amount of nuclear DNA data included, evolutionary processes such as ILS (McKay & Zink 2010) or introgression (of either nuclear genes or the mitochondrial genome, Wallis et al. 2017) could also underlie these discordances. Indeed, hybridization has been described in the wild in several salamandrid genera, including *Lissotriton* (Babik et al. 2003), *Lyciasalamandra* (Johannesen et al. 2006), *Triturus* (Arntzen et al. 2014), and *Pleurodeles* (Escoriza et al. 2016). Additionally, hybridization between genera has been reported under experimental conditions, including *Ichthyosaura, Lissotriton, Ommatotriton*, and *Triturus* (e.g. Pariser 1932; Mancino et al. 1978; Macgregor et al. 1990), and between *Pleurodeles* and *Tylototriton* (Ferrier & Beetschen 1973). Thus, the use of a genome-wide sampling of nuclear loci and methods that account for introgression may be necessary to resolve the disputed nodes in the salamandrid tree.

Using a phylotranscriptomic pipeline involving extensive quality controls (Irisarri et al. 2017; Simion et al. 2017), as well as new analyses of mitochondrial sequences, we here provide rigorous tests of the topologies proposed by previous studies. We particularly focus on testing the conflicting inter-generic relationships recovered by mitochondrial

genomes (Zhang et al. 2008) *versus* nuclear genes (Veith et al. 2018). We investigate the presence of introgression among salamandrid lineages using phylogenetic network reconstruction and introgression tests based on gene trees topologies. By combining these different markers and approaches, we aim to provide a more comprehensive understanding of the evolutionary history of the Salamandridae.

2. Methods

2.1. Phylogenetic analysis based on mitochondrial genomes

In order to get an alternative view of the mitochondrial phylogeny of the Salamandridae, and assess the potential effect of the employed substitution model on the inferred topology, we analyzed available data under the CAT-GTR model. By accounting for site-specific frequency profiles, CAT-GTR can outperform site-homogeneous substitution models (Lartillot & Philippe 2004), particularly in the case of non-recombining fragments.

Protein sequences of 13 mitochondrial genes were recovered from GenBank for 30 salamandrid species (Table S1) and aligned using mafft (Katoh & Standley 2013). Phylogenetic inference was then performed using PhyloBayes (Lartillot & Philippe 2004, 2006; Lartillot et al. 2007), with 1500 cycles and a burnin of 500, and the consensus tree was recovered using the command *bpcomp*. To further assess the branches' support, 100 bootstrap replicates were generated with seqboot (http://evolution.genetics.washington.edu/phylip/doc/seqboot.html) and analyzed in PhyloBayes, with the same settings as above.

2.2. Transcriptome sequencing, data set filtering and assembly

We performed RNA sequencing (RNAseq) of representative salamandrid taxa on a range of Illumina platforms (as specified in Table S2) and at varying sequencing depths, partly in the context of other projects (Stuglik & Babik 2016; Maex et al. 2018; Wielstra et al. 2019). Specifically for this project, we sequenced eight transcriptomes from 100 mg of tissue per specimen, consisting of combined or separate skin, muscle, or liver samples preserved in RNAlater and frozen at −80 °C. RNA extraction was performed using standard trizol protocols (for a detailed protocol see Supplementary Methods). After paired-end 150 bp sequencing, Illumina reads were quality-trimmed and filtered using Trimmomatic v. 0.32 (Bolger et al. 2014) with default settings and later filtered for rRNA sequences with SortMeRNA (Kopylova et al. 2012). Filtered reads were used for *de novo* transcriptome assembly using Trinity v. 2.1.0 (Grabherr et al. 2011) following published protocols (Haas et al. 2013). The same assembly approach was used for various RNAseq data sets downloaded as raw reads from the NCBI Sequence Read Archive (SRA, Table S2). All new RNAseq data were submitted to the NCBI Sequence Read Archive under Bioproject PRJNA607429 (see Table S2 for SRA accession numbers of particular transcriptomes). Assemblies and alignments are available from Figshare under DOI https://doi.org/10.6084/m9.figshare.11778672.

As a basis to extract orthologous genes from the transcriptomes, we employed an alignment covering all jawed vertebrate classes assembled by Irisarri et al. (2017). In brief, the authors of this study inferred putative orthologs from 21 reference proteomes representing most major clades of jawed vertebrates, and enriched them with genome and transcriptome data from 79 additional taxa, using the software "42" (D. Baurain, https://metacpan.org/release/Bio-MUST-Apps-FortyTwo). This software adds sequence data to existing Multi-Species Alignments (MSA) and controls for their orthology using strict three-way reciprocal best BLAST hit tests. These tests rely on a set of reference taxa available in the MSAs (*query_orgs*) and as complete proteomes (*ref_orgs*). A first BLAST search is performed between *query_orgs* and *ref_orgs*, producing a database of best hits (*query_best_hits*). A second BLAST search uses *query_orgs* to search the new transcriptomes to be added (*org*) and identify homologs. Finally, the identified homologs are BLASTed against the *ref_orgs*. Homologs are considered orthologs if the best hit with each of the reference proteomes is among the sequences in the *query_best_hit* list built earlier. The identified orthologs are subsequently added to the original MSAs, and alignment and redundancy filtering steps are performed.

The original data set of Irisarri et al. (2017) included five salamandrids (*Salamandra salamandra*, *Pleurodeles waltl*, *Notophthalmus viridescens*, *Cynops pyrrhogaster,* and *Calotriton asper*). For the present study, we followed the same procedure and used "42" to enrich the original data set with 34 additional salamandrid transcriptomes. To remove possible remaining paralogs and contaminant sequences from the resulting MSAs, we followed the pipeline described in Irisarri et al. (2017). Briefly, (1) putative contaminations were first identified as significant BLAST hits against a custom database of proteomes containing a large diversity of eukaryotic species; (2) in cases where several homologous transcripts per taxon were present in single gene alignments, redundant (i.e. >95% of length overlap) or highly divergent sequences were removed; (3) putative cross-contaminations and paralogs were identified by comparing patristic distances between sequences in gene trees vs in a concatenation tree (inferred with RAxML under a GTR model; Stamatakis 2014), a method known as Branch-Length Correlation (BLC; Simion et al. 2020); (4) gene alignments containing deep paralogs were split using newly-inferred gene trees (as above) and dividing the sequences into two clades of paralogous sequences separated by a long branch that maximized the taxonomic diversity in the two sub-alignments. These steps were done at the amino acid level and included all species in the original data set. Subsequently, salamandrids and several outgroups were extracted and the original nucleotide sequences were retrieved using leel (D. Baurain, https://metacpan.org/release/Bio-MUST-Apps-FortyTwo) for further analyses. As a means to control for potential biases in this orthology assessment pipeline — which could be seen as conservative as it selected genes identified as orthologs across jawed vertebrates — we also generated a set of orthologs using OrthoFinder (Emms & Kelly 2019, see Supplementary Methods for more details). The resulting set of genes yielded phylogenetic trees and networks congruent with those obtained using the markers identified with 42 (Fig. S1).

For eight species, RNAseq data were available for two individuals each. In preliminary analyses, these conspecific samples were kept separate to investigate possible contamination or hybridization events that would confound phylogenetic inference. First, we selected the individuals so that none of the conspecifics were sampled from potential hybrid zones. Next, we assembled a preliminary concatenated alignment using ScaFos v. 1.25 (Roure et al. 2007) without merging conspecifics and by building chimeras within individuals when several transcripts were available for the same gene. A maximum likelihood (ML) phylogenetic tree was inferred using RAxML (under a GTR + Γ substitution model) with 50 rapid bootstrap replicates. This confirmed that conspecific individuals consistently formed monophyletic groups and were separated from each other by short branches (Fig. S2). To maximize the amount of data for these species, we assembled a final data set by merging conspecifics using ScaFos, with the same settings as above. The final data set contained 31 taxa and 5455 gene alignments (a total of 9,546,906 aligned bp). To control for the effect of low-coverage taxa on phylogenetic reconstructions, we assembled a second data set after omitting two species (*Pachytriton brevipes* and *Ommatotriton ophryticus*) that were present in < 50% of gene alignments. Sequences from these two species were present in only 9% and 29% of the alignments, respectively, either because the respective data were recovered from a gut metatranscriptome with only a limited number of host reads (in the case of *Pachytriton*), or due to sequencing failure (*Ommatotriton*).

2.3. Phylotranscriptomic analyses

We inferred an ML tree from the concatenated matrix with IQ-TREE v. 1.6.8 (Nguyen et al. 2015; Chernomor et al. 2016) using the best-

fitting substitution models and gene-partitions selected with BIC in ModelFinder (Kalyaanamoorthy et al. 2017), as implemented in IQ-TREE. The branches' support was assessed using the SH-like approximate likelihood ratio test (aLRT) with 1000 pseudoreplicates. To further assess branch support with a more stringent criterion and to characterize the amount of data required to stabilize each branch in the tree, we performed a gene jackknifing analysis (Delsuc et al. 2008; Irisarri et al. 2017). For this, we randomly sampled gene alignments without replacement and produced sets of 100 concatenated matrices of increasing lengths of approximately 10 Kbp; 50 Kbp; 100 Kbp; 500 Kbp; 1000 Kbp and 5000 Kbp. For the 100 pseudoreplicates of each of these lengths, ML trees were inferred using RAxML's rapid hill-climbing algorithm (GTR + Γ). For each matrix length, we calculated jackknife support values as the number of times a given bipartition was recovered among the 100 ML trees. These analyses were performed on both the full data set as well as the data set without the two low-coverage species.

To account for the effect of ILS on phylogenetic inference, we also inferred a tree from the full data set using ASTRAL-II (Mirarab & Warnow 2015), a summary-tree method that is statistically consistent with the MSC. Gene trees were inferred using PhyloBayes under a CAT-GTR model, with 1100 cycles and a burnin of 100. Branch support of the ASTRAL tree was assessed using local posterior probabilities and quartet scores (i.e. the proportion of the most common quartet in the gene trees pool supporting a given branch; Sayyari and Mirarab, 2016).

2.4. Phylogenetic network inference and introgression tests

The presence of reticulations among salamandrid lineages was tested using two different approaches. First, we inferred phylogenetic networks from the gene trees previously used for the ASTRAL analysis. According to the mito-nuclear discordances and quartet scores from the nuclear transcriptomic data (results below), putative hybridization events were located within the "modern newts" group. Arguably, the discordances could also have been caused by introgression events involving genera outside of the "modern newts" (e.g. between *Calotriton* and *Euproctus*, or even a more distant genus). However, this hypothesis was not supported in preliminary introgression tests (Table S3), and is very unlikely given the present distribution and ecology of the taxa. Therefore, to reduce the computational burden of the analyses, we pruned gene trees to keep only the "modern newts", as well as *Pleurodeles* as an outgroup. As we focused on hybridization events between genera prior to their diversification, the following species (those with the highest coverage) were selected as representatives of their genus when several where available: *Triturus marmoratus*, *Lissotriton montandoni* and *Ommatotriton nesterovi*. This decision introduced new limitations to our analyses, as more recent intergeneric introgression events involving unsampled taxa could influence the results, but was necessary to reduce computational burden and provide manageably interpretable results. Since the monophyly of *Cynops* was not recovered in the concatenation analyses (c.f. results below), both species of this genus were used in these analyses.

Network inferences were first performed using PhyloNet v. 3.7.1 (Than et al. 2008; Wen et al. 2018) with the maximum partial-likelihood algorithm (InferNetwork_MPL command, Yu and Nakhleh, 2015). To determine the best-fitting number of reticulations, we ran PhyloNet assuming maximum numbers of reticulations ranging from 0 to 10, with 100 independent runs performed for each value. For each of these 11 analyses, the five models with the highest likelihood were kept, resulting in a total of 55 models that were ranked using the Akaike Information Criterion (AIC) as in Yu et al. (2014). Finally, we retained the 10 best models and visualized each using Dendroscope 3 (Huson & Scornavacca 2012). As a way to confirm PhyloNet results, phylogenetic networks were also inferred with SNaQ (implemented in the PhyloNetworks package, Solís-Lemus et al. 2017). Quartet concordance factors (CFs) were calculated from the previously used gene trees (*countquartetsintrees* command). The CFs were then used as input data to perform 11 network inferences (*snaq!* command) with the ASTRAL topology as starting tree and the maximum number of reticulations (hmax) set from 0 to 10. For each hmax value, 100 runs were performed. The five best runs per hmax were selected, and their pseudo-deviance ("Loglik", derived from the negative log-likelihood) plotted as a function of the inferred number of reticulations. The best-fitting number of reticulations was determined as the last one inducing a sharp decrease of the network Loglik (Solís-Lemus & Ané 2016).

Another popular approach to detect introgression is Patterson's D statistic, also known as the "ABBA/BABA" test (Green et al. 2010; Durand et al. 2011). In brief, this method tests for introgression in rooted asymmetric four-taxa trees (outgroup,(H1,(H2,H3))) using unlinked bi-allelic markers (usually SNPs). By comparing the proportion of site patterns, this test aims to reveal the presence of introgression between H1 and H2 (excess of ABBA pattern) or between H1 and H3 (excess of BABA), distinguishing it from random processes (i.e. ILS, which is generating similar proportions of ABBA and BABA patterns). Applying the original version of this test to our transcriptomic data would require sampling a single SNP per gene alignment to meet the non-linkage requirement. Since we are working with coding regions with low variability, this would highly reduce the data's informativeness and reduce the statistical power of the test. To overcome this problem, we used gene trees as markers for introgression tests rather than SNPs, in a way similar to Węcek et al. (2016) and Barlow et al. (2018). Under ILS alone, discordant branching patterns (equivalent to ABBA and BABA patterns in SNPs) should be present in four-taxa gene trees in even proportions, while introgression would generate excess of one topology (assuming the inferred gene trees are correct). To test that, we applied the following procedure on the gene trees used for the ASTRAL analysis : (1) we selected gene trees containing all three focal taxa (noted H1, H2 and H3) and an outgroup, and collapsed the nodes with a Posterior Probability (PP) < 70 to control for phylogenetic uncertainty; (2) we pruned gene trees to keep the four relevant taxa and then discarded those with polytomies (i.e. nodes with PP < 70 collapsed earlier); (3) we rooted the four-taxa trees using the outgroup; (4) we counted the number of occurrences of the two discordant patterns (respectively (H2, (H1, H3)) and (H3, (H1, H2))); and (5) we used these counts to calculate a statistic similar to Patterson's D, as follows $\frac{(N(H2(H1,H3)))-N(H3,(H1,H2)))}{(N(H2(H1,H3)))+N(H3,(H1,H2)))}$. A significant departure from expectations under ILS was assessed using 1000 bootstrap replicates. A graphical summary of the entire test is available in Fig. S5. This test was implemented in a custom R function (available at https://github.com/rancilhac/Introgression-tests-from-gene-trees) based on the ape package (Paradis et al. 2004). As in the network analyses, we focused on the two clades of "modern newts", using *Pleurodeles* as an outgroup. In both the "Modern Asian Newts" clade and the "Modern European Newts" clade, the test was performed for every four-taxa combination as described above, with the results of the IQ-TREE analysis used as a reference topology.

3. Results

3.1. New mitogenome analyses recover Salamandrina sister to the "True Salamander" clade

Analysis of the mitogenomic data set yielded a topology that was overall similar to that of Zhang et al. (2008) (Fig. 1a), recovering the main clades reconstructed in previous studies. One notable difference to previous studies (Veith et al. 2018; Zhang et al. 2008) was the position of *Salamandrina*, which we resolve as sister to the "True Salamanders" (i.e. *Salamandra, Lyciasalamandra,* and *Chioglossa*), although with low bootstrap support (BS = 0.6). It is also worth noting that the monophyly of *Cynops* and the position of *Ichthyosaura* as sister lineage to *Lissotriton* both received low support (BS = 0.42 and 0.53, respectively). All remaining branches received BS values > 0.9, except for the intrageneric relationships of both *Triturus* and *Salamandra*.

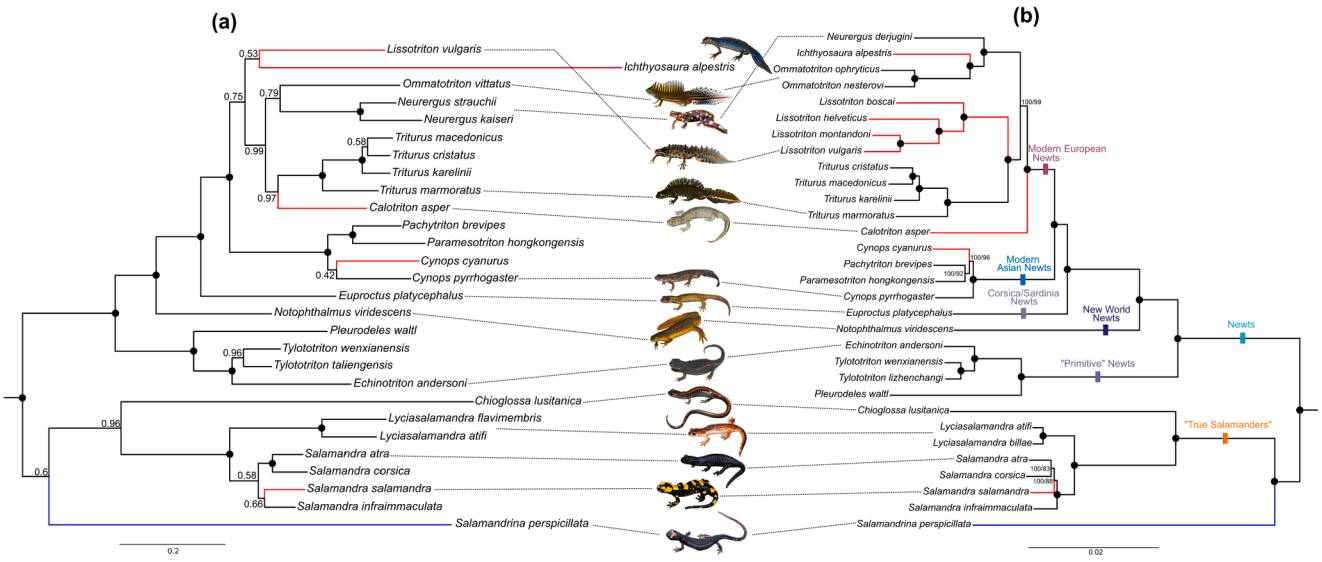

Fig. 1. (a) Phylogenetic tree inferred from amino acid sequences of 13 mitochondrial genes of 28 salamandrid representatives under a CAT-GTR model. The numbers at the nodes are Bootstrap Supports (black dots represent BS = 1.0). (b) Maximum likelihood tree of phylotranscriptomic relationships among the Salamandridae, inferred with IQ-TREE, using a by-genes partitioned analysis with best-fitting models and partitions on DNA sequences of 5455 genes. Numbers at the nodes represent the SH-like approximate likelihood ratio test support and the gene jackknifing proportion for 500 Kbp sampled, respectively, in percent (shown only when < 100%; all other nodes received 100% support in both analyses, represented by a black dot). Clade names follow Weisrock et al. (2006), Steinfartz et al. (2006), and Zhang et al. (2008). The red branches denote the taxa whose positions differ between the two topologies; the blue branches show the new position of *Salamandrina*. Both trees were rooted with sequences of *Ambystoma laterale* and either *Necturus beyurus* (mitochondrial tree) or *Andrias davidianus* (nuclear tree) were included as a hierarchical outgroup (both removed from the graphs to improve graphical resolution within Salamandridae). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Phylotranscriptomic analyses confirm mito-nuclear discordances

The RNAseq data matrix included 31 salamandrid and two outgroup species, representing all salamandrid genera except for *Mertensiella*, *Taricha*, *Liangshantriton,* and *Laotriton*. Our alignment consisted of 5455 genes of a length ranging from 300 to 15,846 bp each, and a total of 9,546,906 bp, including 2,887,639 (30.25%) variable and 1,498,535 (15.70%) parsimony-informative sites. IQ-TREE produced a topology where all branches received 100% SH-like aLRT support (Fig. 1b). The exclusion of the two taxa with the highest proportion of missing data (91% and 71%) did not change the topology or branch support values (Fig. S3). Species-tree inference performed with ASTRAL recovered the same topology (Fig. S4), with every branch having local posterior probabilities of 1.0. This topology recovered the monophyly of the main mitochondrial clades, and confirms the new position of *Salamandrina* as sister to the "True Salamanders." However, the nuclear tree also confirmed the mito-nuclear discordances among the "Modern European Newt" clade suggested by Veith et al. (2018). The nuclear tree placed (1) *Ichthyosaura* nested within a group formed by *Neurergus* and *Ommatotriton*, (2) *Lissotriton* as the sister lineage to *Triturus,* and (3) *Calotriton* as sister lineage to the remainder of the group. In contrast, the mitochondrial tree placed *Ichthyosaura* sister to *Lissotriton* and *Calotriton* sister to *Triturus*. Within the "Modern Asian Newts", *Cynops cyanurus* formed a clade with *Paramesotriton* and *Pachytriton* in the nuclear tree, resulting in the paraphyly of the genus *Cynops*, while the mitochondrial tree recovered it as monophyletic. The relationships within the genus *Salamandra* also differed between the two trees, with *S. infraimmaculata* alternatively placed sister to *S. salamandra,* or to all the other species.

Gene jackknifing analyses further confirmed a well resolved RNAseq-derived topology, with most branches receiving high support even with relatively little data. With only 10 Kbp sampled (~6 genes), the average jackknife proportion among all the branches was 84%, and it increased to > 95% with 100 Kbp (~57 genes; Fig. 2a). However, a few nodes required more data to resolve, as shown in Fig. 2b-f. This included nodes within the genus *Salamandra*, the deep nodes of the "Modern European Newts", and the nodes of the "Modern Asian Newts." Interestingly, those nodes were also supported by rather low quartet proportions in the ASTRAL tree, although PP = 1.0 (Fig. S4), emphasizing high variation among gene-tree topologies.

3.3. Phylogenetic networks and introgression tests suggest pervasive introgression in modern newts

We inferred a total of 55 phylogenetic models that recovered 0–6 reticulations through 11 PhyloNet analyses, with maximum numbers of reticulations allowed ranging from 0 to 10. AIC scores supported models with at least one reticulation as substantially more likely than those with none (Fig. 3a). The best model was identified with 5 reticulations (Fig. 3b); however, AIC values did not clearly favor a specific number of reticulations. Therefore, we decided to also consider 9 sub-optimal networks (Table 1, Fig. S7) with 3–6 reticulations. While the limit to 10 networks is arbitrary, we selected it as a compromise to capture variation within and among runs of PhyloNet, while narrowing our sample to sufficiently few models with low AIC.

The best ranking network (Fig. 3b) identified both similarities and differences with the concatenated and coalescent reconstructions. In all, *Neurergus, Ichthyosaura,* and *Ommatotriton* formed a monophyletic group, and the two *Cynops* species were paraphyletic in regard to *Paramesotriton*. However, in the best ranking network (Fig. 3b), *Triturus* was sister to the *Neurergus, Ichthyosaura,* and *Ommatotriton* clade. *Calotriton* was placed as sister to the aforementioned taxa, and *Lissotriton* was sister to all the "Modern European Newts." *Paramesotriton* and the two species of *Cynops* also formed a monophyletic group, with relationships similar to the bifurcating trees. Across the nine sub-optimal networks, the topology was variable, caused by unstable positions of *Calotriton* and *Lissotriton*. The most common alternative reconstruction for these taxa (4 out of 10 networks, Fig. S6) placed *Calotriton* as a sister lineage to all other taxa, while *Lissotriton* was placed as the sister lineage to the *Paramesotriton* + *Cynops* clade.

The best ranking network featured five reticulations. One of them

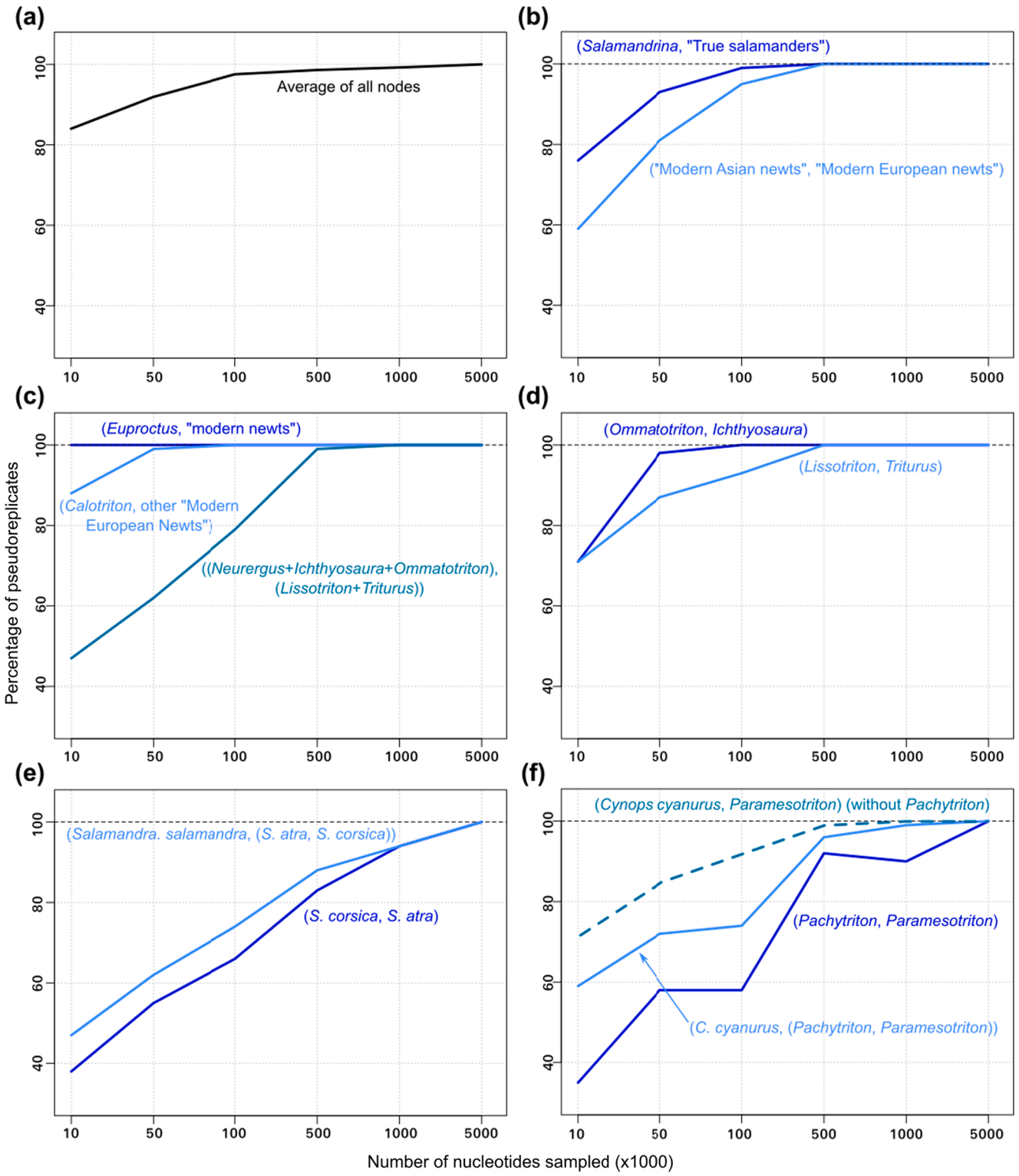

Fig. 2. Proportion (%) of gene jackknifing replicates supporting selected nodes of the maximum likelihood phylotranscriptomic tree (Fig. 1b), as a function of the number of nucleotides sampled: (a) average support for all the nodes of the tree and (b-f) support for specific nodes discussed in the text.

linked the ancestor of all ingroup taxa to the ancestor of the "Modern European Newts," while three others involved a putative extinct lineage sister to *Ommatotriton*. However, three of the hybridization branches had inheritance values that deviated strongly from the expected 0.50, with one as low as 0.002. Furthermore, the number (from 3 to 6) and position of the reticulations were variable across the nine sub-optimal networks (Fig. 3b, S6). Nonetheless, a reticulation event that reconstructed *Lissotriton* as the descendant of a hybridization event between *Triturus* and an extinct lineage was present in the 10 considered networks and had inheritance probabilities between 0.4 and 0.6.

In concordance with PhyloNet, SNaQ recovered a strong signal for hybridization within the "modern newts", as adding one reticulation to the model significantly improved the log-likelihood (mean Loglik of 248.31 vs 45.32 for respectively zero and one reticulation; Fig. 3c).

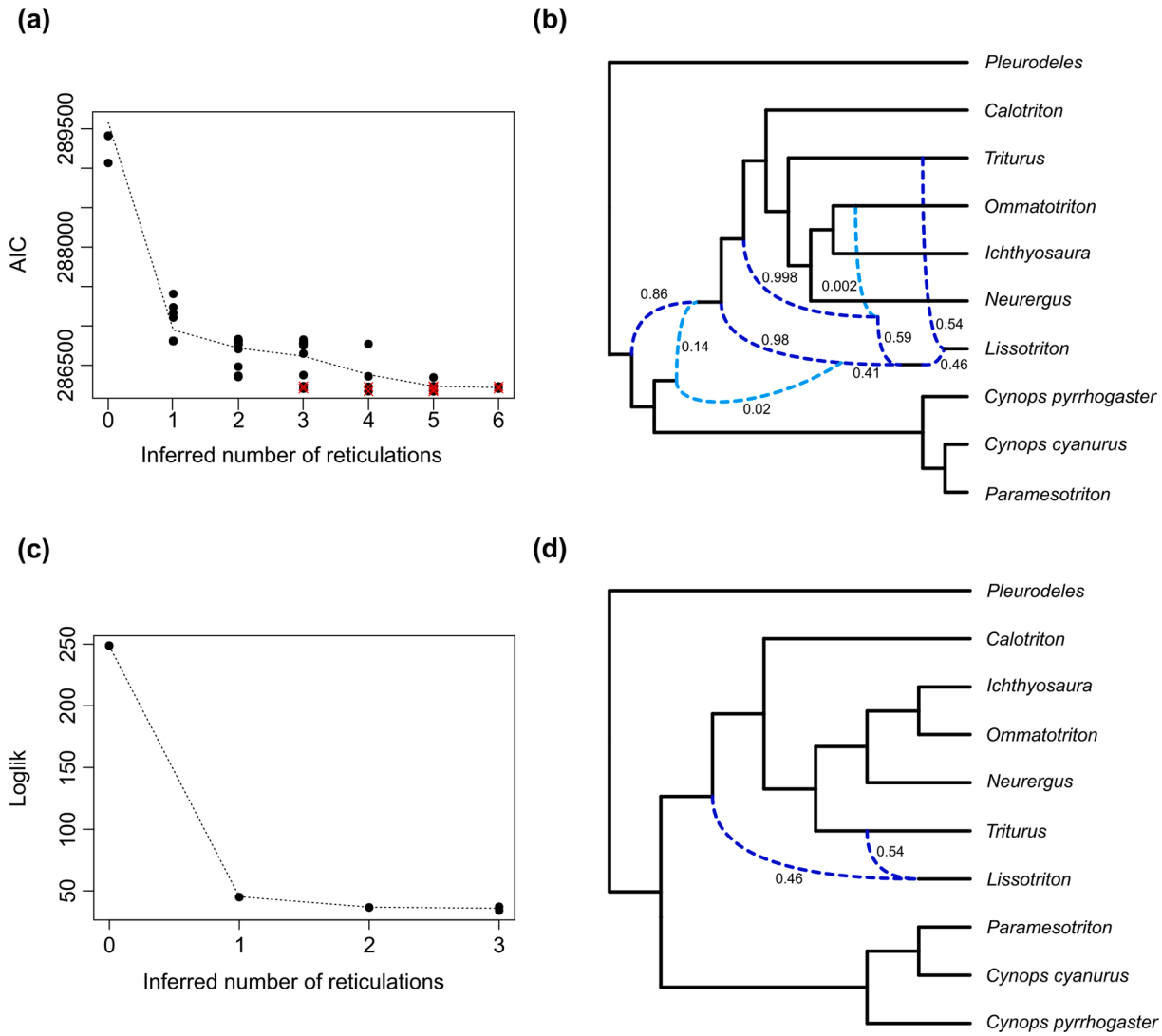

Fig. 3. Results of the phylogenetic networks inference for the "modern newts". (a) Akaike Information Criterion (AIC) values of the 55 phylogenetic networks inferred with PhyloNet, as a function of the number of inferred reticulations. The line shows the average AIC for each number of reticulations, and the red crosses identify the 10 best models, considered for further investigation. (b) The best network according to AIC. (c) Loglik of the 55 phylogenetic networks inferred with SNaQ as a function of the inferred number of reticulations. The line shows the average Loglik. (d) Best SNaQ network identified using the Loglik. Blue dashed lines in (b) and (d) show hybridization branches (light blue = inheritance probability < 0.20). Numbers adjacent to hybridization branches denote the inheritance probabilities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Details of the 10 best networks inferred with PhyloNet, ranked according to their Akaike Information Criterion (AIC). The last column indicates which figure illustrates the relationships corresponding to the respective network.

Maximum number of reticulations	Inferred number of reticulations	Ln(L)	AIC	Figure
7	5	−143047.0	286170.0	3b
10	4	−143051.9	286171.7	S5a
10	5	−143058.9	286193.8	S5b
7	3	−143072.1	286204.3	S5c
10	5	−143060.3	286204.5	S5d
8	4	−143080.0	286228.0	S5e
8	5	−143076.5	286229.0	S5f
7	6	−143074.2	286232.4	S5g
8	5	−143082.6	286235.1	S5h
8	3	−143087.8	286235.7	S5i

When allowing up to 10 reticulations in the networks, only two (hmax = 2) or three (hmax = 3–10) were recovered, but these additional reticulations did not significantly improve the Loglik (ranging from 34.93 to 37.76; Fig. 3c). Thus, we consider a single reticulation to be best fitting our data. Similarly to PhyloNet's best ranking network, this reticulation recovers *Lissotriton* as the descendant of a hybridization event between *Triturus* and an extinct lineage branching at the root of the "Modern European Newts" (Fig. 3d). The inheritance values are very close to those recovered by PhyloNet: 0.54 for the edge linking to *Triturus* and 0.46 for the edge linking to the root of the "Modern European Newts", respectively.

Within the "Modern European Newts" clade we performed introgression tests on a total of 20 four-taxa combinations, of which 11 yielded a significant signal of introgression (Fig. 4). Taxa combinations that did not yield significant signals for introgression are summarized in Fig. S3. The test including *Triturus*, *Lissotriton,* and *Calotriton* yielded significant signal of introgression between *Triturus* and *Calotriton* (Fig. 4a). Tests including *Calotriton*, *Lissotriton,* and either *Ommatotriton*, *Ichthyosaura* or *Neurergus* all yielded significant introgression between *Calotriton* and these latter three taxa (Fig. 4b). The same result was produced when using *Triturus* instead of *Calotriton* (Fig. 4c). However, tests including *Calotriton, Triturus* and either *Ommatotriton*, *Ichthyosaura*

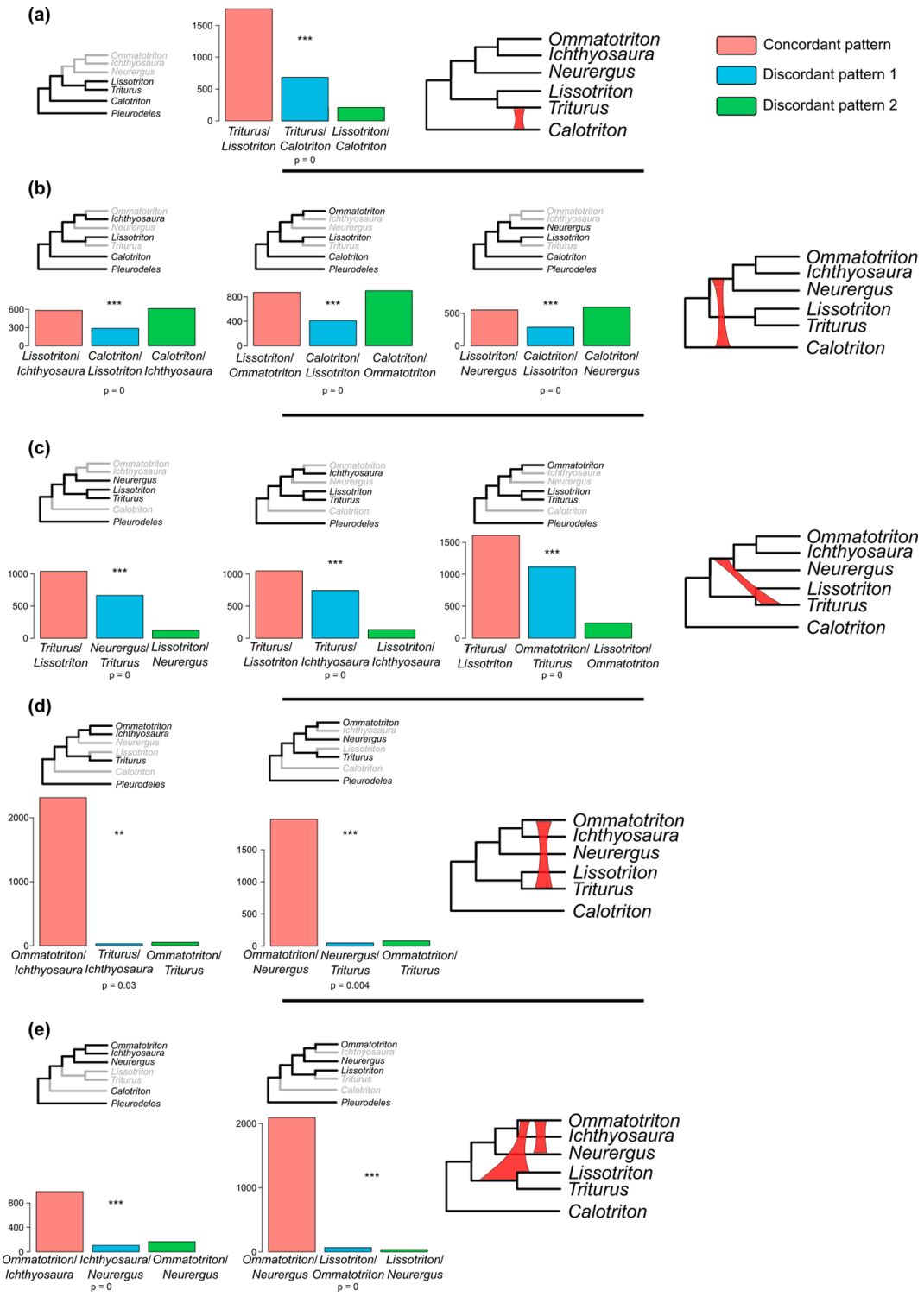

Fig. 4. Summary of the significant introgression tests within the "Modern European Newts." Each tree in the left part shows the subsampling used, and the barplots represent the counts of the three alternative patterns within the gene trees. The trees on the right are schematic representations of the gene flow events inferred from the different tests (their positions along the branches and widths are arbitrary). **: $0.05 > p > 0.01$; ***: $p < 0.01$.

or *Neurergus,* did not detect introgression (Fig. S6). Similarly, testing *Calotriton* against each possible taxon pair of the *Ommatotriton-Ichthyosaura-Neurergus* group did not detect introgression (Fig. S6). In contrast, *Triturus* consistently showed introgression with *Ommatotriton*, relative to both *Neurergus* and *Ichthyosaura* (Fig. 4d). Finally, both *Lissotriton* and *Neurergus* yielded significant signals of introgression with *Ommatotriton*, relative to *Neurergus* and *Ichthyosaura*, respectively (Fig. 4e).

For "Modern Asian Newts," only four four-taxa combinations could be tested, three of which resulted in significant signals for introgression (Fig. 5). These tests were performed on only 139 to 2072 gene trees, due to the low coverage in the *Pachytriton brevipes* transcriptome. Introgression tests between the two *Cynops* species and *Paramesotriton* and *Pachytriton*, gave significant positive signals (Fig. 4a), as did tests between *C. pyrrhogaster* and either *Pachytriton* or *Paramesotriton* (Fig. 4b).

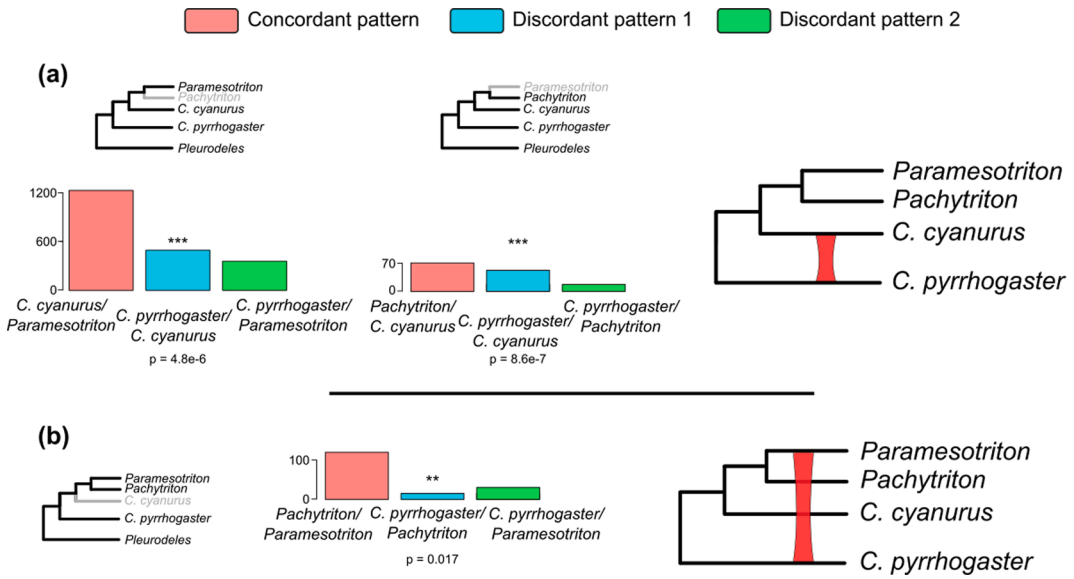

Fig. 5. Summary of the significant introgression tests within the "Modern Asian Newts." Each tree in the left part shows the subsampling used, and the barplots represent the counts of the three alternative patterns within the gene trees. The trees on the right are schematic representations of the gene flow events inferred from the different tests (their positions along the branches and widths are arbitrary). ** : $0.05 > p > 0.01$; *** : $p < 0.01$.

In contrast, *C. cyanurus* did not show significant introgression with any of these two taxa (Fig. S6).

4. Discussion

4.1. Phylotranscriptomic data resolve salamandrid phylogeny and confirm mito-nuclear discordances

Our analyses of both mitochondrial sequences and nuclear phylotranscriptomic data provide the most comprehensive phylogenomic assessment of the evolutionary relationships of salamandrids to date. While phylogenetic discordances were confirmed between mitochondrial and nuclear genomes, both genomes yielded overall similar topologies, with many relationships concordant with those inferred in previous studies. The family was divided into two major clades, the "True Salamanders" and the "Newts." Most of the previously defined major groups within these clades were also confirmed by our data. This was particularly relevant for the newts, in which the Asian and European taxa did not form reciprocally monophyletic groups, confirming their complex biogeographical history (Zhang et al. 2008; Kieren et al. 2018). However, some other relationships recovered by our phylotranscriptomic analysis differed from previous topologies. Perhaps most significantly, *Salamandrina*, a taxon most often inferred as the sister lineage to all the other salamandrids (Zhang et al. 2008; Veith et al. 2018), formed a monophyletic group with the "True Salamanders" in both the mitochondrial and the nuclear transcriptomic trees. While this position received low support in the mitochondrial tree (BS = 0.6), it was fully supported in the nuclear IQ-TREE and ASTRAL analyses, and by more than 90% of the short gene-jackknifing replicates with $\geq$ 50 Kbp (Fig. 2b). This suggests that the previously recovered position of *Salamandrina* was the result of an artifact in phylogenetic reconstruction, possibly due to its long branch. Concordantly, Hime et al. (2020) also recovered *Salamandrina* as sister to the "True Salamanders" in a recent phylogenomic analysis of all amphibian families. Both the low quartet score of this branch (q = 0.51) in the ASTRAL analysis and the short internal branch preceding the ancestor of *Salamandrina* + "True Salamanders" could be interpreted as suggesting the presence of ILS, which would cause difficulties for phylogenetic inference (Degnan & Rosenberg 2006). However, this low quartet score could also be the result of gene tree errors, as such deep and short branches can be difficult to recover (Parks & Goldman 2014).

Our analyses confirmed most of the previously detected mito-nuclear incongruencies. First, the genus *Cynops* was paraphyletic in our phylotranscriptomic trees. The position of *C. cyanurus* as sister to *Pachytriton* and *Paramesotriton* was strongly supported in our trees, but 500 Kbp were necessary to recover this relationship with high support (Fig. 2f). When repeating this analysis after removing *Pachytriton brevipes*, which has a very low gene coverage, the support for a *C. cyanurus* + *Paramesotriton* clade increased substantially (from 74% to 90% at 100 Kbp). The monophyly of *Cynops* has been consistently supported by complete mitogenome analyses (Kieren et al. 2018; Zhang et al. 2008), and our mitochondrial analysis also supported it, although with only low support (BS = 0.43). A previous study focusing on *Cynops* phylogeny also found this genus to be paraphyletic with respect to *Paramesotriton* and *Pachytriton* (Tominaga et al. 2013), although this was based on one mitochondrial gene. While some authors have already placed *C. cyanurus* and the other Chinese species of *Cynops* into a different genus, *Hypselotriton* (Dubois & Raffaëlli 2009), a more extensive taxon sampling would be necessary for reliable conclusions on the evolutionary relationships and taxonomy of these newts.

Other important mito-nuclear incongruencies affect the clade of "Modern European Newts." In the mitochondrial topologies, including the one inferred in the present study, *Calotriton* was sister to *Triturus*, and *Lissotriton* was placed as sister to *Ichthyosaura* (albeit poorly supported). In the phylotranscriptomic tree, however, *Triturus* and *Lissotriton* were sister clades, while *Ichthyosaura* was nested within a clade also containing *Neurergus* and *Ommatotriton*, and *Calotriton* was sister to all these five taxa. These results are partially concordant with the four-nuclear-gene phylogeny of Veith et al. (2018), with the only difference being that the phylotranscriptomic tree placed *Ommatotriton* as sister to *Ichthyosaura*, rather than to *Neurergus*. All relevant branches received full SH-like aLRT support in the ML analysis of the phylotranscriptomic data, as well as maximum local posterior probabilities in the ASTRAL tree. While some of the bipartitions received low support from the shorter jackknife pseudo-replicates when few positions were sampled (Fig. 2 c-d), they all stabilized at full (100%) support with $\geq$ 500 Kbp. Interestingly, most of these contentious branches received a rather low quartet score in the ASTRAL analysis (Fig. S4), suggesting a relatively high level of variation among gene genealogies.

One possible explanation for the observed discordances between the

phylogenetic signal of mitochondrial and nuclear markers could be ILS affecting the nuclear markers. In this scenario, the mitochondrial genome could be a more reliable marker than nuclear genes due to its smaller effective population size and faster sorting (Harrison 1989), thus making it less sensitive to ILS. However, the concordance between the concatenation and ASTRAL analyses of the nuclear markers suggested ILS has a limited impact on the phylogenetic signal, as the latter approach is theoretically able to recover the correct topology even under high levels of ILS (Mirarab & Warnow 2015). This is concordant with previous studies where concatenation and ASTRAL approaches were found to converge to very similar topologies in the presence of ILS, when using large amounts of molecular data (e.g. Irisarri et al. 2018).

4.2. Inter-species gene flow as a source of incongruence?

A second possible explanation for the observed mito-nuclear incongruences is gene flow between lineages (i.e. introgression of nuclear genes and/or mitochondrial capture). In salamandrids, we hypothesize that ancient introgressive hybridization between the ancestors of different genera explains some of the variations in nuclear genes trees, as well as the differences between mitochondrial and nuclear phylotranscriptomic topologies in both the "Modern European Newts" and "Modern Asian Newts." When performing phylogenetic network analyses using two different algorithms on a subset of taxa including all of the "modern newts", introducing reticulations in the models substantially increased their likelihoods, supporting the presence of introgression within this group. However, the exact number of reticulations, as well as their position, were more difficult to assess. In that respect, it has been shown that sequence and gene tree data are often not informative enough to distinguish between complex networks with several reticulations occurring along the same branch (Pardi and Scornavacca, 2015), which might be the cause of the uncertainties in our results. On the one hand, the best PhyloNet network, according to AIC values, had five reticulations. We also considered nine other sub-optimal models for comparison, which displayed between three and six reticulations. On the other hand, SNaQ strongly supported a model with a single reticulation, and only recovered up to three reticulations. Determining whether the PhyloNet or SNaQ networks are closer to the actual evolutionary history is not straightforward. Considering the results displayed in Fig. 3a, one could hypothesize that PhyloNet artificially increases the likelihood when adding reticulations, regardless of the true hybridization events. This result was partially supported by the very low inheritance probabilities of some hybridization branches (Yu et al. 2012), suggesting that at least some of the recovered reticulations could be over-fitted. However, SNaQ might oversimplify the model by only allowing a single hybridization event per branch (Solís-Lemus & Ané 2016). While this restriction enables a better statistical distinction between candidate models, it might not reflect the actual complexity of the network.

When comparing the 10 selected PhyloNet models, the topology and the number and location of hybridization branches were quite variable. However, it was possible to identify some consistent features. Regarding the topology, two groups had unambiguous relationships. First, among the "Modern European Newts," a monophyletic group comprising *Triturus*, *Neurergus*, *Ichthyosaura*, and *Ommatotriton* (branching hierarchically in that order; hereafter referred to as the "TNOI" group) was present in all 10 networks. The monophyly of this group relative to *Lissotriton* was also consistent with our introgression tests, albeit the latter do not allow to further clarify the relationships within the "TNOI" group. Among the "Modern Asian Newts," the relationships were similar to those recovered in the phylotranscriptomic tree, with the genus *Cynops* resolved as paraphyletic with respect to *Paramesotriton* (*Pachytriton* was excluded from this analysis because of its low coverage). Thus, the uncertainty in the network topologies can be traced to two unstable taxa, *Calotriton* and *Lissotriton*. The number and position of reticulations in the different networks was also variable. All recovered hybridization branches were relatively deep, and thus involved extinct populations. One possibility to explain such a pattern could be the presence of strong genetic structure within the ancestral populations of the extant species, which could leave a signal very similar to gene flow (Slatkin & Pollack 2008). Although models that are theoretically able to differentiate between gene flow and structure in ancestral populations have been developed (Theunert & Slatkin 2017), they are not yet scalable to data sets with high numbers of both loci and taxa, such as ours. A wrong phylogenetic placement of *Calotriton* and *Lissotriton* could also artificially inflate the signal for gene flow, although PhyloNet is theoretically able to address that issue by allowing topological rearrangements. SNaQ's best network supports a topology similar to that of the concatenation and ASTRAL trees, but a closer examination of the sub-optimal networks (i.e., with two or three reticulations) also revealed some variation in the position of *Lissotriton* and *Calotriton*. Because of these inconsistencies, the network analyses do not allow us to unambiguously determine the number and positions of the reticulations in our tree. However, by comparing the results of our different analyses, including the mitochondrial-nuclear discordances, we could identify common patterns supporting several introgression events, discussed in detail thereafter. We summarized them in a hypothetical evolutionary scenario for the "modern newts", represented in Fig. 6.

Although most reticulations were poorly supported, it is interesting to note that all considered PhyloNet networks recovered *Lissotriton* as originating from a hybridization event between *Triturus* and an unsampled lineage. This hybridization event was further supported by the inheritance probabilities between 0.4 and 0.6, suggesting a relatively equal genetic contribution of both parental populations (Yu et al. 2012). This result is also confirmed by SNaQ, as the preferred network recovers the same reticulation with near-identical inheritance probabilities, and matches the discordance between the mitochondrial and nuclear trees for these two genera. This supports a scenario (Fig. 6) in which *Lissotriton* inherited part of its genetic material (including its mitochondrial genome) from a lineage that diverged early within the "Modern European Newts," with subsequent introgressive genetic contribution from *Triturus*. Although introgression tests did not allow for direct investigation of introgression between these two genera, a high proportion of the nuclear genes supported the monophyly of the "TNOI" group, which is consistent with this hypothesis. However, it is important to note that such pattern could also arise if *Lissotriton* were the sister group of *Triturus,* with subsequent introgression of nuclear genes from a more distant, unsampled lineage into *Lissotriton* (Fig. 6), although such a hypothesis contradicts the network results. *Lissotriton* species are widespread ecological generalists, often occurring in sympatry with *Triturus* species, and the two genera share an overall similar reproductive behavior. Given that experimental hybridization between *Triturus* and *Lissotriton* (and *Lissotriton* and *Ichthyosaura*) can result in viable offspring (Pariser 1932; Mancino et al. 1978), it is reasonable to assume that their respective ancestors could have successfully hybridized in the wild. Some PhyloNet networks suggest that ancestral populations of *Lissotriton* have exchanged genes with other lineages. However, as discussed above, inconsistent reticulations could result from artifacts and should rather be taken cautiously in the absence of further evidence.

The evolutionary history of *Calotriton* is more convoluted. The observed mito-nuclear discordances could be explained by mitochondrial introgression from *Triturus* into the *Calotriton* lineage, leading to their sister relationship in the mitochondrial phylogeny. From a biological perspective, their highly different mating behavior makes hybridization unlikely, although occasional occurrence in spatial proximity can be observed (e.g. of *T. marmoratus* with *C. arnoldi* and with *C. asper* in north-eastern Spain). However, it is important to keep in mind that the suspected introgression probably occurred millions of years ago, implying that both the distribution and behavior of the considered populations might have been very different. For example, Pleistocene fossils tentatively assigned to *Euproctus* (likely referring to *Calotriton*) have been found in the Spanish region of Asturias (Sanchìz 1977), an area far outside the current range of *Calotriton*, but

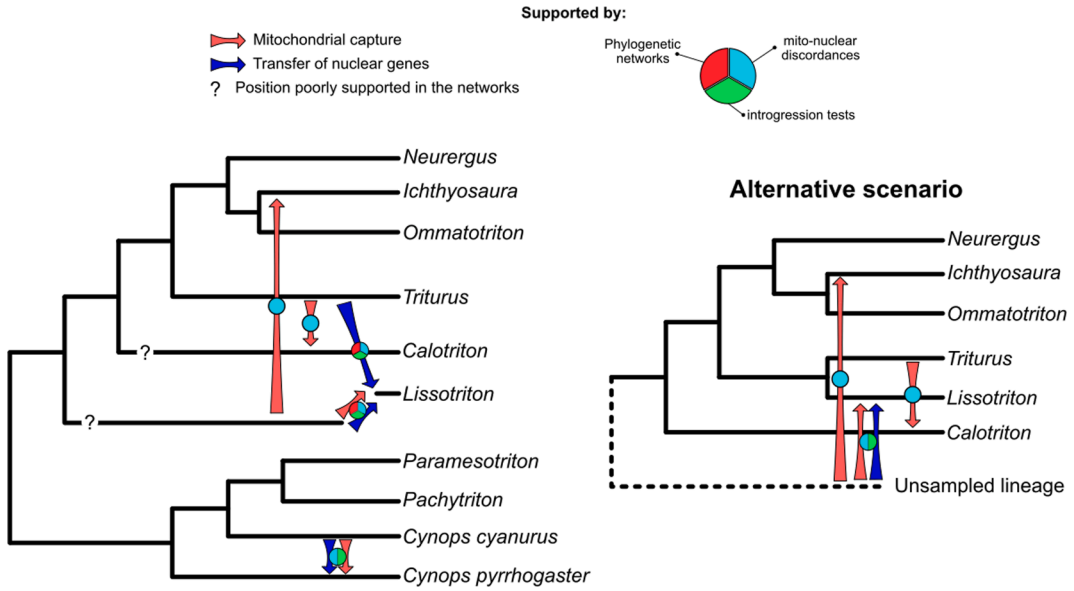

Fig. 6. Schematic representations of two hypothetical evolutionary scenarios for the "modern newts" involving several introgression events. The trees represent the splits between lineages, while the arrows represent directional introgressions (from donor to recipient lineage) of either mitochondrial genomes (red), nuclear genes (blue) or both. The position and direction of introgressions were estimated based on mito-nuclear discordances, phylogenetic networks and introgression tests. Circles on the arrows indicate the type of analyses supporting each hybridization event. The relative order of the introgression events is putative, and the branches lengths are arbitrary. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

climatically more suitable during glacial maxima, as revealed by modelling (Carranza and Amat 2005). This suggests that during Pliocene-Pleistocene times, the range of *Calotriton* may have been more broadly overlapping with that of *Triturus*. Introgression tests identified *Calotriton* as sister to the "TNOI" clade, relative to *Lissotriton,* but did not support significant introgression with *Triturus* when compared to either *Neurergus, Ichthyosaura* or *Ommatotriton*. This result suggests introgression at the root of this group, rather than more recently between *Calotriton* and *Triturus*, which might seem contradictory to the branching in the mitochondrial tree. However, it is possible that a mitochondrial capture occurred from the ancestral populations of *Triturus* to those of *Calotriton* (summarized in Fig. 6) without introgressed nuclear genes being retained (Toews & Brelsford 2012; Good et al. 2015; Bonnet et al. 2017).

Within the "Modern Asian Newts" only one network recovered a reticulation, linking *Paramesotriton* and *Cynops cyanurus*, with the two *Cynops* species forming a monophyletic group. When testing *C. cyanurus* against *Paramesotriton* and *Pachytriton,* no introgression could be detected, but introgression tests including *Pachytriton* may have been prone to stochastic error due to its low gene coverage. The introgression test performed without this taxon suggested introgression between *C. cyanurus* and *C. pyrrhogaster*. This result fits a scenario where the lineage represented by *C. pyrrhogaster* split early within the "Modern Asian Newts" and later hybridized with the lineage represented by *C. cyanurus*, resulting in both mitochondrial capture and introgression of nuclear genes from *C. pyrrhogaster* into *C. cyanurus* (Fig. 6). This hypothesis is also consistent with the mitochondrial topology. However, given the few tests that could be performed, the possibility that *Cynops* paraphyly is the result of introgression of nuclear genes from the *Paramesotriton* clade into *C. cyanurus* cannot be completely ruled out. As advocated earlier, further studies with a special focus on this group of Asian newts and a more comprehensive taxon sampling (particularly including more species of *Cynops*) will be needed to clarify these relationships.

It is surprising that we did not find evidence for nuclear introgression directly involving *Ichthyosaura* given that its placement differs between the mitochondrial and nuclear transcriptomic tree. However, as hypothesized for *Calotriton*, it is possible that *Ichthyosaura* captured a mitochondrial lineage similar to *Lissotriton* following an ancient hybridization event (both scenarios summarized in Fig. 6) without retaining any introgressed nuclear genes. *Ichthyosaura* displayed an extremely long branch in the mitochondrial tree, which could result from an accelerated substitution rate subsequent to mitochondrial replacement, caused by the introgressed mitochondrial genes adapting to the new genomic environment of mitochondrial-related nuclear genes (Sloan et al. 2017). However, it is also possible that *Ichthyosaura* has a higher mitochondrial substitution rate for another, unrelated reason, and this hindered accurate phylogenetic reconstruction.

To conclude, we confirmed that some aspects of the evolutionary history of the Salamandridae are difficult to solve, even with extensive genomic data sets. The deep nodes of the tree were uncontroversial in all analyses, and largely in agreement with previous studies. On the other hand, relationships within several groups are unclear, to say the least. Our results provide evidence for introgression between lineages at different phylogenetic scales, but we can only speculate about the exact number of reticulations and the branches along which they occurred. Further in-depth and focused analyses of the contentious clades, using a more complete species-level taxon sampling, could lead to greater resolution of relationships within this family. Moreover, the potential confounding effect of introgression in deep phylogenetic inference (e.g. regarding the placement of *Salamandrina*) remains to be clarified, as the power of our approaches decreased at these evolutionary scales. Ultimately, studying signals of introgression based on whole-genome sequences may be needed to fully understand the relationships among the Salamandridae, a goal still difficult to achieve at present due to very large genome sizes and high content of repetitive elements in salamandrid genomes.

4.3. *Gene flow in phylogenomic studies*

Consistent with many recent studies, our results identify the importance of considering introgression in phylogenetic studies. This is particularly relevant when analyzing large genomic data sets, which tend to give high, but potentially spurious, branch support while obscuring signals of discordance among markers. Even the gene-jackknifing method, which provides a more strict testing of

monophyly than bootstrapping, recovered with strong support some relationships that were most likely influenced by gene flow (e.g. the *Lissotriton/Triturus* clade; Fig. 2d). On the other hand, large phylogenomic data sets also offer an unprecedented opportunity to integrate introgression into the phylogenetic paradigm. Indeed, sampling numerous loci at a genomic scale allows in-depth insights into the various evolutionary histories supported by one genome, especially using long loci sequences, as in phylotranscriptomic approaches, which can yield well-resolved gene trees.

The main remaining challenge, to integrate the inference of reticulations into phylogenetic studies, is methodological, as the various methods available all have shortcomings. Inferring phylogenetic networks under the Multi-Species Network Coalescent might be the most promising approach, as it integrates explicit modeling of both ILS and introgression. However, such analyses are computationally intensive when including large numbers of taxa, and still have shortcomings, particularly regarding the identification of the optimal number of reticulations (Pardi and Scornavacca, 2015; Solís-Lemus & Ané 2016; Wen et al. 2016). As a result, most analyses using phylogenetic networks focus on a small number of reticulations, often only one, at shallow evolutionary scales (e.g. Yu et al. 2012). Thus, the applicability of phylogenetic networks to extensive phylogenomic data sets remains limited, but further theoretical developments might improve both the computational efficiency and the accuracy of the existing algorithms. Alternative approaches to detect introgression in phylogenetic studies have been used, including introgression tests (Green et al. 2010) or model testing based on simulations (Burbrink & Gehara 2018), but they all have limitations that might narrow their applicability to specific questions and data. However, even if these methods do not allow a full characterization of hybridization over species-tree or species-network history, we advocate for their use, as they can still yield very valuable insights into the presence of reticulations among the branches of a tree. Several very simple approaches, such as quartet sampling (Pease et al. 2018) or the comparison of mitochondrial and nuclear markers, can be used to define putative introgression events. Depending on the size of the data set, and the anticipated complexity of the evolutionary scenario, additional methods, as described above, can then be used to develop a more accurate picture of the evolutionary history.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106967.

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