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Evolutionary history of *Ichthyosaura alpestris* (Caudata, Salamandridae) inferred from the combined analysis of nuclear and mitochondrial markers

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ABSTRACT

Widespread species with morphologically and ecologically differentiated populations are key to understand speciation because they allow investigating the different stages of the continuous process of population divergence. The alpine newt, *Ichthyosaura alpestris*, with a range that covers a large part of Central Europe as well as isolated regions in all three European Mediterranean peninsulas, and with strong ecological and life-history differences among populations, is an excellent system for such studies. We sampled individuals across most of the range of the species, and analyzed mitochondrial (1442 bp) and nuclear (two nuclear genes ~1554 bp- and 35 allozyme loci) markers to produce a time-calibrated phylogeny and reconstruct the historical biogeography of the species. Phylogenetic analyses of mtDNA data produced a fully resolved topology, with an endemic, Balkan clade (Vlasina) which is sister to a clade comprising an eastern and a western group. Within the former, one clade (subspecies *I. a. veluchiensis*) is sister to a clade containing subspecies *I. a. montenegrina* and *I. a. serdara* as well as samples from southern Romania, Bosnia-Herzegovina, Serbia and Bulgaria (subspecies *I. a. reiseri* and part of *I. a. alpestris*). Within the western group, populations from the Italian peninsula (subspecies *I. a. apuana* and *I. a. inexpectata*) are sister to a clade containing samples from the Iberian Peninsula (subspecies *I. a. cyreni*) and the remainder of the samples from subspecies *I. a. alpestris* (populations from Hungary, Austria, Poland, France, Germany and the larger part of Romania). Results of *BEAST analyses on a combined mtDNA and nDNA dataset consistently recovered with high statistical support four lineages with unresolved inter-relationships: (1) subspecies *I. a. veluchiensis*; (2) subspecies *I. a. apuana* + *I. a. inexpectata*; (3) subspecies *I. a. cyreni* + part of subspecies *I. a. alpestris* (the westernmost populations, plus most Romanian populations); and (4) the remaining populations, including subspecies *I. a. serdara*, *I. a. reiseri* and *I. a. montenegrina* and part of subspecies *I. a. alpestris*, plus samples from Vlasina. Our time estimates are consistent with ages based on the fossil record and suggest a widespread distribution for the *I. alpestris* ancestor, with the split of the major eastern and western lineages during the Miocene, in the Tortonian. Our study provides a solid, comprehensive background on the evolutionary history of the species based on the most complete combined (mtDNA + nDNA + allozymes) dataset to date. The combination of the historical perspective provided by coalescent-based analyses of mitochondrial and nuclear DNA variation with individual-based multilocus assignment methods based on multiple nuclear markers (allozymes) also allowed identification of instances of discordance across markers that highlight the complexity and dynamism of past and ongoing evolutionary processes in the species.

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1. Introduction

Widespread species with morphologically and ecologically differentiated populations are key to understand the process of speciation because they allow investigation of the early stages of population divergence, eventually leading to species formation. The upsurge of molecular data in the study of such species has frequently revealed unanticipated high levels of genetic differentiation and geographic structuring, even in species that are morphologically uniform (e.g., Calvo et al., 2009; Oliver et al., 2009; Recuero et al., 2012; Vences et al., 2013). Despite the complexity of genetic and demographic processes underlying cryptic speciation (e.g., DeVitt, 2006; Pereira and Wake, 2009), much progress is being made towards the integration of these data sources, leading to a better understanding of the mechanisms that have generated current diversity patterns. In any case, to avoid erroneous interpretations in the reconstruction of evolutionary scenarios from molecular data it is paramount to analyze and compare multiple markers with 'vertical' inheritance, different evolutionary rates, and demographic and coalescence histories (Hudson and Turelli, 2003).

The newts of the western Palearctic constitute a good system to study the evolutionary forces shaping patterns of diversity at regional and continental scales. With c. 22 species in 6 genera distributed across Eurasia (AmphibiaWeb, 2014) the group is diversified and widespread, with some species having large ranges while others are restricted and local. In some lineages hybridization and admixture is fairly common, allowing the study of reproductive interactions. The group is thought to be of Paleocene or Eocene origin (Steinfartz et al., 2007; Zhang et al., 2008) and for several species fossils have been identified from the late Oligocene – early Miocene (Marjanović and Laurin, 2013; Martín and Sanchiz, 2013). The western Palearctic newts, thus, offer good prospects to study diversification and speciation at different spatial and temporal scales (Weisrock et al., 2006).

We here focus on the alpine newt, *Ichthyosaura alpestris* (Laurenti, 1768), the only extant representative of the genus *Ichthyosaura,* with a range that covers a large part of Central Europe and the Balkans (Roček et al., 2003), as well as isolated regions in all three European Mediterranean peninsulas (Recuero and Martínez-Solano, 2002; Andreone and Tripepi, 2006; Sotiropoulos et al., 2008). The species' fossil record goes back to the Pliocene of Slovakia (Martín and Sanchiz, 2013), suggesting a long-term presence in Central Europe. Congeneric fossils, furthermore, date back to the early Miocene (Martín and Sanchiz, 2013). *Ichthyosaura alpestris* shows extensive morphological and ecological variation across its range, which has resulted in the description of numerous subspecies (Roček et al., 2003), although the relevance of some of them is under debate (Breuil and Guillaume, 1985; Arano and Arntzen, 1987; Herrero and Arano, 1987; Zuiderwijk, 1997; Sotiropoulos et al., 2001, 2007; Dubois and Raffaëlli, 2009; Speybroeck et al., 2010; Lužnik et al., 2011). Most authors recognize from six to ten subspecies, which have been defined on the basis of body proportions and coloration patterns or on differences in life-history traits. Remarkably, paedomorphosis is common in this species, although apparently it is restricted to southern populations (especially in Italy and the Balkans), with a variable frequency, affecting most individuals in some populations (Denoël et al., 2001). Such morphological and ecological diversity, geographically structured over a wide distribution (Andreone, 1990; Denoël, 1996), makes *I. alpestris* a promising system for studying processes underlying diversification and early stages of speciation.

A mitochondrial DNA (mtDNA) based phylogeography for *I. alpestris* (Sotiropoulos et al., 2007) resolved five clades with the following distributions: (1) Southwestern (Iberian peninsula), western and central Europe (Clade C), (2) Apennine Peninsula (Clade B), (3) central Balkans (Clade E), (4) the southernmost Balkan peninsula (Clade D), and (5) the easternmost populations including the remainder of the Balkans (Clade A). These clades showed deep diversification and several instances of discordance with the current morphology-based taxonomy. Discrepancy also existed with the patterns recovered with nuclear markers (Arano and Arntzen, 1987; Sotiropoulos et al., 2007). Therefore, additional data are required to clarify the evolutionary history of the species and revise the taxonomy of the group. We here integrate new molecular data from the mitochondrial and nuclear genomes in a geographically comprehensive sample with a reanalysis of previously published allozyme data with the following goals: (1) to produce a fully-resolved, time-calibrated phylogeny; (2) to reconstruct the historical biogeography of the species; and (3) to identify major evolutionary lineages and relate them to current taxonomy in light of the multilocus-based historical analysis.

2. Materials and methods

2.1. Sampling

We obtained genomic DNA from one to six individuals of 42 populations of *I. alpestris*, totaling 131 individuals, encompassing a variety of subspecies distributed along the entire range of the species (Table 1 and Fig. 1). Our efforts to obtain samples from *I. a. piperiana* (Kapetanovo jezero and Manito jezero, Montenegro), and from the extinct *I. a. lacusnigri* (Črno jezero, Slovenia) failed. However, previous studies based on mtDNA do not support these populations as representing independent lineages (Sotiropoulos et al., 2007; Smith et al., 2008; Lužnik et al., 2011, but see comments in Denoël, 1996). Other described subspecies with restricted ranges (see for instance Dely, 1960) are not currently recognized. We obtained molecular data from individuals of *Ommatotriton ophryticus* ($n = 3$), *Lissotriton boscai* ($n = 2$) and *L. italicus* ($n = 2$) to be used as outgroups. Tissues for this study were obtained from a variety of sources, including recent field collections and donations of several researchers and institutions (see acknowledgments). A large proportion of the samples were previously used by Arano (1988) and Arano and Arntzen (1987) for allozyme analyses, and by Herrero and Arano (1987) for cytogenetic studies.

2.2. mtDNA and nDNA extraction, amplification and sequencing

Whole genomic DNA was extracted from small amounts of frozen or ethanol-preserved tissues using proteinase-K and phenol–chloroform (Sambrook et al., 1989). We sequenced two mtDNA fragments for the full sample and two nuclear gene fragments for a subset including representatives of all major mtDNA lineages, yielding alignments of 562 base pairs (bp) of the large subunit ribosomal mtDNA gene (16S); 689 bp of the ND4 (nicotinamide adenine dinucleotide dehydrogenase subunit 4) gene, plus 191 bp corresponding to the adjacent tRNAs; 840 bp of intron 4 of the growth hormone gene (GH); and 714 bp of intron 11 of the platelet-derived growth factor receptor chain alpha gene (PDGFR). Amplification was done via the polymerase chain reaction (PCR) (Saiki et al., 1988), using the primer pairs "16Sar-16Sbr" (Palumbi et al., 1991) for 16S, "ND4-Leu" (Arévalo et al., 1994) for ND4 plus adjacent tRNAs, and PDGFR_F-PDGFR_R and GH_F-GH_R (Nadachowska and Babik, 2009) for PDGFR and GH respectively. PCR reactions were run in a total volume of 25 μl as described in Recuero et al. (2010), with annealing temperatures of 50 °C (16S), 55 °C (PDGFR, GH) and 56 °C (ND4). Double strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then re-suspended in 22 μl of H_2O. Sequencing reactions were performed using both strands with standard conditions and sequenced on an ABI PRISM 3700 DNA sequencer.

Table 1
Ichthyosaura alpestris - details of samples used in this study, including subspecific assignment, locality name and country, code in Fig. 1, latitude and longitude, sample ID, correspondence to mtDNA clades delineated in Sotiropoulos et al.'s 2007 study and GenBank Accession Numbers of newly generated sequences (GB).

Taxa	Locality	Loc. ID	Latitude	Longitude	Country	Sample ID	Clade in Sotiropoulos et al. (2007)	GB (16S)	GB (ND4)	GB (GH)	GB (PDGFR)
I. a. alpestris	Grazay (Mayenne)	8	48°17′41″N	0°28′44″W	France	MAB305	C2 + C3	KM253291	KM253511	–	–
I. a. alpestris	Grazay (Mayenne)	8			France	MAB306	C2 + C3	KM253292	KM253512	KM253420 KM253421	–
I. a. alpestris	Grazay (Mayenne)	8			France	MAB363	C2 + C3	KM253337	KM253557	KM253458 KM253459	–
I. a. alpestris	Grazay (Mayenne)	8			France	MAB364	C2 + C3	KM253338	KM253558	–	–
I. a. alpestris	Grazay (Mayenne)	8			France	MAB365	C2 + C3	KM253339	KM253559	–	–
I. a. alpestris	Kottenforst, Bonn	9	50°40′00″N	7°05′00″E	Germany	MAB348	C2 + C3	KM253334	KM253554	KM253454 KM253455	KM253680 KM253681
I. a. alpestris	Kottenforst, Bonn	9			Germany	MAB349	C2 + C3	KM253335	KM253555	KM253456 KM253457	KM253682 KM253683
I. a. alpestris	Kottenforst, Bonn	9			Germany	MAB350	C2 + C3	KM253336	KM253556	–	–
I. a. alpestris	Arnoldstein	10	46°32′60″N	13°43′00″E	Austria	MAB307	C2 + C3	KM253293	KM253513	KM253422 KM253423	KM253640 KM253641
I. a. alpestris	Arnoldstein	10			Austria	MAB308	C2 + C3	KM253294	KM253514	KM253424 KM253425	KM253642 KM253643
I. a. alpestris	Ajka	11	47°06′00″N	17°34′00″E	Hungary	MAB309	C2 + C3	KM253295	KM253515	KM253426 KM253427	KM253644 KM253645
I. a. alpestris	Ajka	11			Hungary	MAB310	C2 + C3	KM253296	KM253516	–	–
I. a. alpestris	Ajka	11			Hungary	MAB311	C2 + C3	KM253297	KM253517	–	–
I. a. alpestris	Ajka	11			Hungary	MAB312	C2 + C3	KM253298	KM253518	–	–
I. a. alpestris	Ajka	11			Hungary	MAB313	C2 + C3	KM253299	KM253519	–	–
I. a. alpestris	Inwald	12	49°52′00″N	19°24′00″E	Poland	MAB374	C2 + C3	KM253367	KM253587	–	–
I. a. alpestris	Inwald	12			Poland	MAB375	C2 + C3	KM253368	KM253588	–	–
I. a. alpestris	Inwald	12			Poland	MAB376	C2 + C3	KM253369	KM253589	–	–
I. a. alpestris	Inwald	12			Poland	MAB377	C2 + C3	KM253370	KM253590	–	–
I. a. alpestris	Chyszowki, near Limanowa	13	49°42′00″N	20°25′00″E	Poland	MAB378	C2 + C3	KM253371	KM253591	–	–
I. a. alpestris	Chyszowki, near Limanowa	13			Poland	MAB379	C2 + C3	KM253372	KM253592	–	–
I. a. alpestris	Chyszowki, near Limanowa	13			Poland	MAB380	C2 + C3	KM253373	KM253593	KM253462 KM253463	KM253686 KM253687
I. a. alpestris	Chyszowki, near Limanowa	13			Poland	MAB381	C2 + C3	–	–	KM253464 KM253465	KM253688 KM253689
I. a. alpestris	Chyszowki, near Limanowa	13			Poland	MAB382	C2 + C3	KM253374	KM253594	–	–
I. a. alpestris	Chyszowki, near Limanowa	13			Poland	MAB383	C2 + C3	KM253375	KM253595	–	–
I. a. alpestris	Văratec (Neamţ Dept.)	14	47°08′25″N	26°16′17″E	Romania	MAB412	C2 + C3	KM253396	KM253616	KM253476 KM253477	KM253700 KM253701
I. a. alpestris	Văratec (Neamţ Dept.)	14			Romania	MAB413	C2 + C3	KM253397	KM253617	KM253478 KM253479	KM253702 KM253703
I. a. alpestris	Văratec (Neamţ Dept.)	14			Romania	MAB414	C2 + C3	KM253398	KM253618	KM253480 KM253481	KM253704 KM253705
I. a. alpestris	Văratec (Neamţ Dept.)	14			Romania	MAB415	C2 + C3	KM253399	KM253619	–	–
I. a. alpestris	Văratec (Neamţ Dept.)	14			Romania	MAB416	C2 + C3	KM253400	KM253620	–	–
I. a. alpestris	Stanisinci near Krusevac	29	43°32′00″N	20°54′00″E	Serbia	MAB319	E2	KM253305	KM253525	–	KM253652 KM253653
I. a. alpestris	Stanisinci near Krusevac	29			Serbia	MAB320	E2	KM253306	KM253526	–	KM253654 KM253655
I. a. alpestris	Stanisinci near Krusevac	29			Serbia	MAB321	E2	KM253307	KM253527	KM253432 KM253433	KM253656 KM253657
I. a. alpestris	Stanisinci near Krusevac	29			Serbia	MAB322	E2	KM253308	KM253528	–	–
I. a. alpestris	Stanisinci near Krusevac	29			Serbia	MAB323	E2	KM253309	KM253529	–	–
I. a. alpestris	Smolyan	30	41°34′00″N	24°43′00″E	Bulgaria	MAB314	E2	KM253300	KM253520	KM253428 KM253429	KM253646 KM253647
I. a. alpestris	Smolyan	30			Bulgaria	MAB315	E2	KM253301	KM253521	KM253430	KM253648

(*continued on next page*)

Table 1 (*continued*)

Taxa	Locality	Loc. ID	Latitude	Longitude	Country	Sample ID	Clade in Sotiropoulos et al. (2007)	GB (16S)	GB (ND4)	GB (GH)	GB (PDGFR)
										KM253431	KM253649
I. a. alpestris	Smolyan	30			Bulgaria	MAB316	E2	KM253302	KM253522	–	KM253650
											KM253651
I. a. alpestris	Smolyan	30			Bulgaria	MAB317	E2	KM253303	KM253523	–	–
I. a. alpestris	Smolyan	30			Bulgaria	MAB318	E2	KM253304	KM253524	–	–
I. a. alpestris	Tăul Secat, Retezat National Park, (Hunedoara Dept.)	34	45°21′44″N	22°49′47″E	Romania	MAB404	E2	KM253383	KM253603	–	–
I. a. alpestris	Tăul Secat, Retezat National Park, (Hunedoara Dept.)	34			Romania	MAB405	E2	KM253384	KM253604	–	–
I. a. alpestris	Tăul Secat, Retezat National Park, (Hunedoara Dept.)	34			Romania	MAB406	E2	KM253385	KM253605	–	–
I. a. alpestris	Valea Mare (Retezat National Park, Hunedoara Dept.)	35	45°23′57″N	22°46′13″E	Romania	MAB399	E2	KM253391	KM253611	KM253470	KM253694
										KM253471	KM253695
I. a. alpestris	Valea Mare (Retezat National Park, Hunedoara Dept.)	35			Romania	MAB400	E2	KM253392	KM253612	KM253472	KM253696
										KM253473	KM253697
I. a. alpestris	Valea Mare (Retezat National Park, Hunedoara Dept.)	35			Romania	MAB401	E2	KM253393	KM253613	KM253474	KM253698
										KM253475	KM253699
I. a. alpestris	Valea Mare (Retezat National Park, Hunedoara Dept.)	35			Romania	MAB402	E2	KM253394	KM253614	–	–
I. a. alpestris	Valea Mare (Retezat National Park, Hunedoara Dept.)	35			Romania	MAB403	E2	KM253395	KM253615	–	–
I. a. alpestris	Cumpănița (Argeș Dept.)	36	45°26′03″N	24°36′22″E	Romania	MAB409	E2	KM253376	KM253596	–	–
I. a. alpestris	Cumpănița (Argeș Dept.)	36			Romania	MAB410	E2	KM253377	KM253597	–	–
I. a. alpestris	Cumpănița (Argeș Dept.)	36			Romania	MAB411	E2	KM253378	KM253598	–	–
I. a. alpestris	Sinaia (Prahova Dept.)	37	45°22′54″N	25°32′42″E	Romania	MAB388	E2	KM253379	KM253599	KM253466	KM253690
										KM253467	KM253691
I. a. alpestris	Sinaia (Prahova Dept.)	37			Romania	MAB389	E2	KM253380	KM253600	–	–
I. a. alpestris	Sinaia (Prahova Dept.)	37			Romania	MAB390	E2	KM253381	KM253601	KM253468	KM253692
										KM253469	KM253693
I. a. alpestris	Sinaia (Prahova Dept.)	37			Romania	MAB392	E2	KM253382	KM253602	–	–
I. a. alpestris	Timișu de Sus (Brașov Dept.)	38	45°31′43″N	25°34′32″E	Romania	MAB394	E2	KM253387	KM253607	–	–
I. a. alpestris	Timișu de Sus (Brașov Dept.)	38			Romania	MAB395	E2	KM253388	KM253608	–	–
I. a. alpestris	Timișu de Sus (Brașov Dept.)	38			Romania	MAB396	E2	KM253389	KM253609	–	–
I. a. alpestris	Timișu de Sus (Brașov Dept.)	38			Romania	MAB397	E2	KM253390	KM253610	–	–
I. a. alpestris	Teleajen Valley (Prahova Dept.)	39	45°16′49″N	26°02′36″E	Romania	MAB393	E2	KM253386	KM253606	–	–
I. a. alpestris	Zli Do	41	42°25′00″N	22°27′00″E	Serbia	Ma7	E2	KM253402	KM253622	–	–
I. a. alpestris	Zli Do	41			Serbia	Ma8	E2	KM253403	KM253623	–	–
I. a. alpestris	Zli Do	41			Serbia	Ma9	E2	KM253404	KM253624	KM253496	KM253722
										KM253497	KM253723
I. a. alpestris	Zli Do	41			Serbia	Ma10	E2	KM253405	KM253625	KM253498	KM253724
										KM253499	KM253725
I. a. alpestris	Zli Do	41			Serbia	Ma11	E2	KM253406	KM253626	KM253500	KM253726
										KM253501	KM253727
I. a. alpestris	Zli Do	41			Serbia	Ma12	E2	KM253407	KM253627	–	–
I. a. alpestris	Vlasina Lake	42	42°44′00″N	22°19′00″E	Serbia	Ma1	A	KM253409	KM253629	–	–
I. a. alpestris	Vlasina Lake	42			Serbia	Ma2	A	–	–	KM253490	KM253716
										KM253491	KM253717
I. a. alpestris	Vlasina Lake	42			Serbia	Ma3	A	KM253410	KM253630	KM253492	KM253718
										KM253493	KM253719
I. a. alpestris	Vlasina Lake	42			Serbia	Ma4	A	KM253411	KM253631	KM253494	KM253720
										KM253495	KM253721
I. a. alpestris	Vlasina Lake	42			Serbia	Ma5	A	KM253412	KM253632	–	–
I. a. alpestris	Vlasina Lake	42			Serbia	Ma6	A	KM253413	KM253633	–	–
I. a. apuana	S. Benedetto Belbo (Cueno - 1988)	15	44°29′29″N	8°03′32″E	Italy	MAB426	B	KM253346	KM253566	–	–
I. a. apuana	S. Bovo/ Castino (Cueno - 1988)	16	44°38′10″N	8°09′06″E	Italy	MAB427	B	KM253347	KM253567	–	–
I. a. apuana	Camporgiano (Lucca - 1989)	17	44°09′33″N	10°20′00″E	Italy	MAB433	B	KM253356	KM253576	–	–
I. a. apuana	Cardoso (Toscana)	18	44°01′00″N	10°29′00″E	Italy	MAB369	B	KM253362	KM253582	–	–
I. a. apuana	Cardoso (Toscana)	18			Italy	MAB370	B	KM253363	KM253583	–	–
I. a. apuana	Cardoso (Toscana)	18			Italy	MAB371	B	KM253364	KM253584	KM253460	KM253684
										KM253461	KM253685

I. a. apuana	Cardoso (Toscana)	18			Italy	MAB372	B	KM253365	KM253585	–	–
I. a. apuana	Cardoso (Toscana)	18			Italy	MAB373	B	KM253366	KM253586	–	–
I. a. apuana	Lago della Bega (Lucca - 1989)	19	44°13′00″N	10°14′00″E	Italy	MAB431	B	KM253355	KM253575	–	–
I. a. apuana	Mont-Megna (Ferriere:Emilia-Romagna 1200 m)	20	44°37′00″N	9°32′00″E	Italy	MAB421	B	KM253348	KM253568	–	KM253706
											KM253707
I. a. apuana	Mont-Megna (Ferriere:Emilia-Romagna 1200 m)	20			Italy	MAB422	B	KM253349	KM253569	KM253482	KM253708
										KM253483	KM253709
I. a. apuana	Mont-Megna (Ferriere:Emilia-Romagna 1200 m)	20			Italy	MAB423	B	KM253350	KM253570	KM253484	KM253710
										KM253485	KM253711
I. a. apuana	Mont-Megna (Ferriere:Emilia-Romagna 1200 m)	20			Italy	MAB424	B	KM253351	KM253571	–	–
I. a. apuana	Negruzzo (Pavia - 1989)	21	44°42′59″N	9°13′03″E	Italy	MAB437	B	KM253359	KM253579	–	–
I. a. apuana	Parana (Mulazzo-Tuscany 600 m)	22	44°16′57″N	9°51′38.94″E	Italy	MAB418	B	KM253357	KM253577	–	–
I. a. apuana	Parana (Mulazzo-Tuscany 600 m)	22			Italy	MAB420	B	KM253358	KM253578	–	–
I. a. apuana	Rossiglione (Genova - 1988)	23	44°33′32″N	8°40′27″E	Italy	MAB430	B	KM253353	KM253573	–	–
I. a. apuana	S. Giulia (Genova - 1989)	24	44°18′13″N	9°22′23″E	Italy	MAB429	B	KM253352	KM253572	–	–
I. a. apuana	Lago di Val Noce (Genova - 1989)	25	44°29′00″N	9°02′00″E	Italy	MAB441	B	KM253354	KM253574	–	–
I. a. apuana	Serravezza	26	44°00′00″N	10°14′00″E	Italy	MAB351	B	KM253401	KM253621	–	–
I. a. apuana	Monti della Laga (Rieti - 1987)	27	42°32′08″N	13°31′57″E	Italy	MAB432	B	KM253360	KM253580	KM253486	KM253712
										KM253487	KM253713
I. a. apuana	Monti della Laga (Rieti - 1989)	27			Italy	MAB439	B	KM253361	KM253581	KM253488	KM253714
										KM253489	KM253715
I. a. cyreni	Peñalara	1	40°50′51″N	3°56′55″W	Spain	MAB366	C1	KM253340	KM253560	–	–
I. a. cyreni	Peñalara	1			Spain	MAB367	C1	KM253341	KM253561	–	–
I. a. cyreni	Peñalara	1			Spain	MAB347	C1	KM253333	KM253553	–	–
I. a. cyreni	Lago del Valle	2	43°02′36″N	6°08′18″W	Spain	MAB329	C1	KM253315	KM253535	KM253438	KM253662
										KM253439	KM253663
I. a. cyreni	Lago del Valle	2			Spain	MAB330	C1	KM253316	KM253536	KM253440	KM253664
										KM253441	KM253665
I. a. cyreni	Lago del Valle	2			Spain	MAB331	C1	KM253317	KM253537	–	–
I. a. cyreni	Pola de Siero	3	43°23′35″N	5°39′43″W	Spain	MAB341	C1	KM253327	KM253547	–	–
I. a. cyreni	Pola de Siero	3			Spain	MAB342	C1	KM253328	KM253548	–	–
I. a. cyreni	Pola de Siero	3			Spain	MAB343	C1	KM253329	KM253549	–	–
I. a. cyreni	Pola de Siero	3			Spain	MAB344	C1	KM253330	KM253550	–	–
I. a. cyreni	Pola de Siero	3			Spain	MAB345	C1	KM253331	KM253551	–	–
I. a. cyreni	El Fito-Colunga, Asturias	4	43°26′58″N	5°14′33″W	Spain	MAB386	C1	KM253344	KM253564	–	–
I. a. cyreni	El Fito-Colunga, Asturias	4			Spain	MAB387	C1	KM253345	KM253565	–	–
I. a. cyreni	Lago Ercina	5	43°16′11″N	4°58′51″W	Spain	MAB324	C1	KM253310	KM253530	KM253434	KM253658
										KM253435	KM253659
I. a. cyreni	Lago Ercina	5			Spain	MAB325	C1	KM253311	KM253531	KM253436	KM253660
										KM253437	KM253661
I. a. cyreni	Lago Ercina	5			Spain	MAB326	C1	KM253312	KM253532	–	–
I. a. cyreni	Lago Ercina	5			Spain	MAB327	C1	KM253313	KM253533	–	–
I. a. cyreni	Lago Ercina	5			Spain	MAB328	C1	KM253314	KM253534	–	–
I. a. cyreni	Santillana del Mar	6	43°23′20″N	4°06′33″W	Spain	MAB332	C1	KM253318	KM253538	KM253442	KM253666
										KM253443	KM253667
I. a. cyreni	Santillana del Mar	6			Spain	MAB333	C1	KM253319	KM253539	KM253444	KM253668
										KM253445	KM253669
I. a. cyreni	Santillana del Mar	6			Spain	MAB334	C1	KM253320	KM253540	–	–
I. a. cyreni	Santillana del Mar	6			Spain	MAB335	C1	KM253321	KM253541	–	–
I. a. cyreni	Santillana del Mar	6			Spain	MAB336	C1	KM253322	KM253542	–	–
I. a. cyreni	Fresnedo, Cantabria	7	43°21′55″N	3°34′20″W	Spain	MAB384	C1	KM253342	KM253562	–	–
I. a. cyreni	Fresnedo, Cantabria	7			Spain	MAB385	C1	KM253343	KM253563	–	–
I. a. inexpectata	Lago Trifoglietti, Montalto Uffugo, Cosenza	40	39°32′55″N	16°01′20″E	Italy	Ma13	B	KM253408	KM253628	KM253502	KM253728
										KM253503	KM253729
I. a. inexpectata	Lago Trifoglietti, Montalto Uffugo, Cosenza	40			Italy	Ma14	B	–	–	KM253504	KM253730
										KM253505	KM253731
I. a. montenegrina	Bukumirsko Jezero	32	42°36′17″N	19°33′24″E	Montenegro	MAB346	E1	KM253332	KM253552	–	KM253678
											KM253679
I. a. reiseri	Prokosko Jezero	28	43°57′28″N	17°45′18″E	Bosnia-Herzegovina	MAB337	E2	KM253323	KM253543	KM253446	KM253670

(*continued on next page*)

Table 1 (*continued*)

Taxa	Locality		Loc. ID	Latitude	Longitude	Country	Sample ID	Clade in Sotiropoulos et al. (2007)	GB (16S)	GB (ND4)	GB (GH)	GB (PDGFR)
											KM253447	KM253671
I. a. reiseri	Prokosko Jezero		28			Bosnia-Herzegovina	MAB338	E2	KM253324	KM2535544	KM253448	KM253672
											KM253449	KM253673
I. a. reiseri	Prokosko Jezero		28			Bosnia-Herzegovina	MAB339	E2	KM253325	KM2535545	KM253450	KM253674
											KM253451	KM253675
I. a. serdara	Zminicko Jezero		31	43°06′04″N	19°14′47″E	Montenegro	MAB340	E1	KM253326	KM2535546	KM253452	KM253676
											KM253453	KM253677
I. a. veluchiensis	Karpenisi		33	38°55′00″N	21°48′00″E	Greece	MAB300	D	KM253286	KM2535506	KM253414	KM253634
											KM253415	KM253635
I. a. veluchiensis	Karpenisi		33			Greece	MAB301	D	KM253287	KM2535507	–	–
I. a. veluchiensis	Karpenisi		33			Greece	MAB302	D	KM253288	KM2535508	–	–
I. a. veluchiensis	Karpenisi		33			Greece	MAB303	D	KM253289	KM2535509	KM253416	KM253636
											KM253417	KM253637
I. a. veluchiensis	Karpenisi		33			Greece	MAB304	D	KM253290	KM2535510	KM253418	KM253638
											KM253419	KM253639

All sequences were compiled using Sequencher v4.0 (Gene Codes Corp.). For nuclear sequences we tested for recombination using Phi tests as implemented in the software SplitsTree v4 (Huson and Bryant, 2006), and two alleles per individual were phased using SeqPHASE (Flot, 2010) and PHASE 2.1.1 (Stephens et al., 2001), under default settings. Alignments were produced with MAFFT v7 (Katoh and Standley, 2013) and refined manually.

2.3. Phylogenetics analyses and divergence time estimation

We used MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012) to infer phylogenetic relationships in the mtDNA dataset, including all *I. alpestris* samples and the outgroups. PartitionFinder v.1.1 (Lanfear et al., 2012) was used to select the optimal partitioning strategy and the substitution models for each partition. Accordingly, three independent partitions were defined, corresponding to 1st positions in the ND4 sequences (substitution model: HKY + I), 2nd positions in ND4 (TN93 + G), and 3rd positions + tRNAs + 16S sequences (HKY + I + G). Analyses were run for 20 million generations, sampling every 2000 generations. Convergence was assessed through examination of the standard deviation of split frequencies, which was well below recommended thresholds. A consensus phylogram was computed after discarding trees reconstructed during the default burn-in period. For convenience, and given the overall complementarity of our results with those of Sotiropoulos et al. (2007), we refer to their clade denominations to present our results (see also Tables 1 and 2 and Fig. 1).

Based on the major *I. alpestris* clades identified in the previous analysis (see Results), we selected one sample per clade to prepare a reduced dataset including one representative per clade as well as several outgroups with the purpose of estimating divergence times across major clades in *I. alpestris*. Additional sequences of representatives of other genera in the Salamandridae were downloaded from GenBank, including *Lissotriton vulgaris* (Accession: EU880339), *Neurergus kaiseri* (EU880320), *Calotriton asper* (EU880307), *Triturus pygmaeus* (NC015796), *T. marmoratus* (NC015795), *T. macedonicus* (NC015794), *T. ivanbureschi* (NC015792), *T. dobrogicus* (NC015791), *T. carnifex* (NC015788), and *T. cristatus* (NC015790). We used BEAST 1.8.0 (Drummond and Rambaut, 2007) to analyze this reduced mtDNA dataset and estimate divergence times across clades. The dataset was analyzed as three partitions, corresponding to ND4, tRNAs and 16S. Optimal nucleotide substitution models (or the best fitting option available in BEAST) were selected with jModeltest 2.1.1 based on the Bayesian Information Criterion (Darriba et al., 2012). For ND4, we used the GTR + G model, whereas for tRNAs and 16S we selected the HKY + G model. We used a Yule tree prior (Yule, 1924; Gernhard, 2008) and a single strict clock model for the three partitions. The molecular clock was calibrated setting a prior for the time for the most common ancestor (TMRCA) of the *Triturus* clade with a lognormal distribution with an offset at 23.8 millions of years (mya), according to the oldest known *Triturus* fossil (Wiens et al., 2011). We set a log(mean) of 1.0 and a log(stdev) of 1.0, determining a 95% quantile range between 24 and 38 mya. Additionally, we restricted the maximum age of the root of the tree by specifying a prior for the treeModel.rootHeight with a normal distribution (mean: 40 mya, st. dev.: 6) producing a wide range of dates (30–50 mya) encompassing estimates from previously published phylogenies of Salamandridae using different calibration points (Zhang et al., 2008; Wiens et al., 2011). We enforced the monophyly of several clades, in accordance with the results of these studies: the genera *Lissotriton* and *Triturus,* the ingroup (*I. alpestris*) and *Lissotriton + Ichthyosaura.* Substitution rates of the different partitions were estimated during the analysis, using uninformative priors. Analyses were run for 50 million generations, sampling every 5000 generations. Traces were visually inspected in Tracer 1.6 (Rambaut and Drummond, 2007) to ensure effective sample

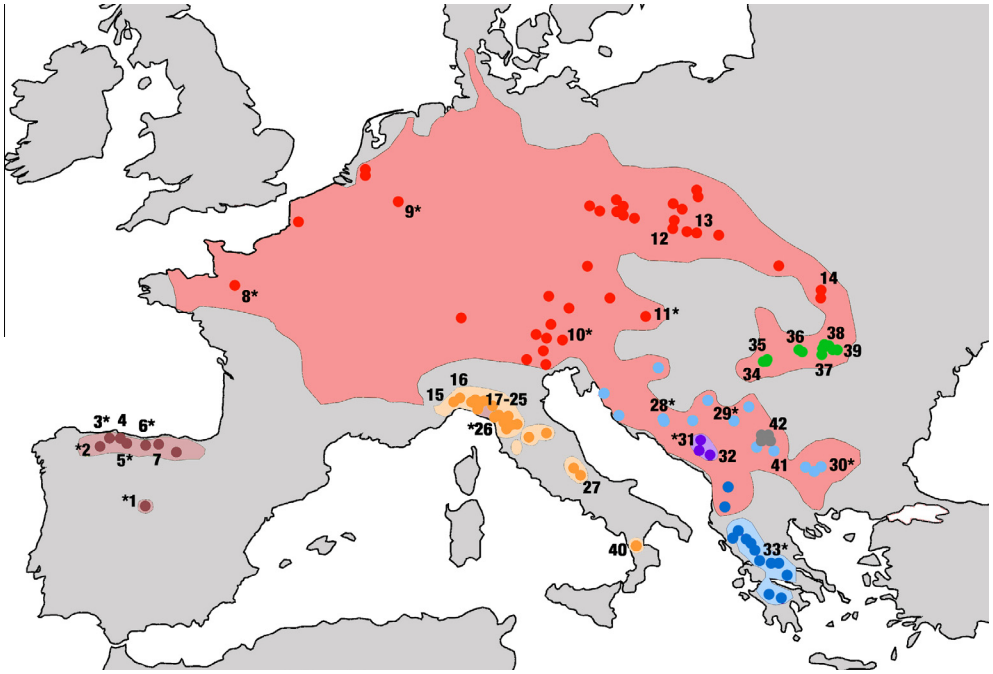

Fig. 1. Distribution of *Ichthyosaura alpestris* (color shading) and sampling. Colored areas indicate putative ranges of subspecies: brown: *I. a. cyreni*; red: *I. a. alpestris*; orange: *I. a. apuana* and *I. a. inexpectata*; purple: *I. a. montenegrina* (includes *I. a. serdara* and *I. a. piperiana*, see Discussion) and blue: *I. a. veluchiensis*. Colored dots indicate mtDNA-based lineages based on the present (numbered localities, see Table 1 for details) and previous studies (see references and Table 2). Asterisks represent populations analyzed for both sequence and allozyme data. Colored dots with no associated code indicate populations included in previous molecular studies (Pabijan and Babik, 2006; Sotiropoulos et al., 2007; Pabijan et al., 2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sizes (ESSs) of parameters were >200, as recommended by the authors, and convergence of parameter estimates across runs.

2.4. Nuclear data: haplotype networks and the multilocus coalescent

We built haplotype networks for both nDNA markers using the median-joining algorithm in the Network version 4.5.1.0 software (Bandelt et al., 1999). We used as input the maximum clade credibility trees generated for each gene with BEAST under a Birth and Death tree prior and the strict clock model, using HKY + G (for GH) and GTR + G (for PDGFR) as substitution models (selected by jModeltest) and no constraints regarding TMRCAs. Run lengths were 10 million generations, sampling parameters and topologies every 1000 generations. The maximum clade credibility consensus trees were computed after discarding the first 25% topologies as "burn-in", and were subsequently modified to collapse unsupported branches before using them for the network construction.

Table 2
Summary of the results in Sotiropoulos et al. (2007) and the present study, with correspondence across major clades and subclades and described subspecies.

Sotiropoulos et al. (2007) (mt DNA)					Present study			
Lineage	Clade	Subclade	Geographical location	Subspecies	Clade	Nuclear data (*Beast analyses)	Allozymes	Combined
Vlasina	A		SE Serbia	*alpestris*	A			
Western	B		Italy	*apuana, inexpectata*	B	B	B	B (*apuana, inexpectata*)
Western	C	C1	Spain	*cyreni*	C1	C1 + C2 + C3 + pE2**	C1	C (*cyreni, alpestris*)
		C2	NE Italy	*alpestris*	C2		C2	
		C3	Central Europe, N Romania	*alpestris*	C3		C3	
Eastern (Southern)	D	D1	Balkans	*alpestris**	D	D	D	D (*veluchiensis*)
		D2	Balkans	*veluchiensis*				
		D3	Balkans	*veluchiensis*				
		D4	Balkans	*veluchiensis*				
Eastern (Northern)	E	E1	Balkans	*serdara, piperiana, montenegrina*	E1	A + E1 + pE2	E1	A + E (*reiseri, montenegrina*)
		E2	Central and northern Balkans, south Carpathians	*alpestris, reiseri*	E2		E2a	
							E2b	

* Morphological data (vertebral count numbers) indicate these populations can be assigned to *Ichthyosaura alpestris veluchiensis*, in accordance with molecular data (Arntzen et al., in press).

** pE2 refers to the fact that subclade E2 is divided in two clusters.

Relationships between major lineages in *I. alpestris* were investigated through application of the multispecies coalescent implemented in *BEAST (Drummond and Rambaut, 2007; Heled and Drummond, 2010). The analyses included three separate partitions (the two nuclear genes and the mitochondrial dataset), with substitution models selected by jModeltest (or the closest option available in BEAST: mtDNA: TN93 + G; GH: HKY + G; PDGFR: HKY + G). We used a Yule tree prior with the "piecewise: constant" option and strict clock models for each partition, with mtDNA fixed at 1.0 substitutions/site per million years and the relative rates of the two nuclear partitions estimated during the analyses, which were run for 100 million generations, sampling every 10,000, yielding high ESSs (>200) for all parameters.

Since *BEAST does not take into account the possibility of gene flow across lineages, we investigated the possible violation of this assumption with the posterior predictive checking approach implemented in JMLv1.02 (Joly et al., 2009; Joly, 2012). JML takes as input the posterior distribution of species trees from *BEAST and simulates replicate datasets under the coalescent with no migration. Minimum genetic distances between species across datasets were calculated to generate a posterior predictive distribution which was used to compare with empirical (observed) values.

2.5. Allozymes

We studied 98 alpine newt individuals from 16 populations across a large part of the species range (Fig. 1, Table 1). Genetic profiles were established for 35 nuclear gene loci by protein electrophoresis, following published protocols (Arntzen, 2001). We included six individuals of *Lissotriton boscai*, ten *L. italicus*, two *Neurergus strauchii* and five *Ommatotriton ophryticus* as outgroups for the analyses. Data were missing for six loci in the outgroups and in one sample of *I. alpestris*.

We estimated the number of distinct gene pools using the software BAPS v5.3 (Corander et al., 2009). BAPS makes no a priori assumptions about k and can be used to appoint individuals to as many distinct ancestral gene pools as there are individuals present in the input dataset. BAPS was run on the complete allozyme dataset, assuming from 1 to 16 groups (the number of *I. alpestris* populations sampled). Here, k was evaluated over the $2 \leqslant k \leqslant 16$ range (the number of *I. alpestris* populations sampled), under BAPS default settings.

3. Results

3.1. Phylogenetic analyses and divergence time estimation

Analysis of molecular evolution of the ND4 sequences showed that they had a typical "mitochondrial" behavior (Zhang and Hewitt, 1996; Parra-Olea, 2002). Most variable sites were in the third codon position as is typical for protein coding regions, and the reading frame was conserved. The number of amino acid changes across sequences was low, suggesting that random base changes, as would be expected for non-functional nuclear copies, were not present. According to Phi tests, there was no significant evidence of recombination in the PDGFR and GH sequences.

The mtDNA alignment included 1442 positions, of which 475 were variable (287 in the ingroup). The 129 sequences of the ingroup were collapsed into 56 unique haplotypes, with frequencies ranging from 1 (32 haplotypes) to 13 (one haplotype found in samples from the Iberian Peninsula – *cyreni*, clade C1-). The observed uncorrected genetic distances were similar to those reported by Sotiropoulos et al. (2007), with pairwise genetic distances ranging from 0% (*veluchiensis*, clade D) to 0.8% (*apuanus*) within clades; and from 1.3% (*cyreni* vs. clades C2 + C3) to >10% ("Vlasina" lineage *vs.* rest of clades) between clades.

MrBayes and BEAST analyses produced fully resolved topologies consistent with the results of Sotiropoulos et al. (2007) but with higher resolution within the western lineage, in which their sampling was sparse (Figs. 2 and 3). Clade A ("Vlasina") is sister to all other lineages within *I. alpestris*, and there are two major lineages corresponding to the eastern (D + E) and western (B + C) lineages. Within the eastern linage, clade D (*I. a. veluchiensis*) is sister to a clade containing the subspecies *I. a. montenegrina* and *I. a. serdara* (clade E1) and samples from southern Romania, Bosnia-Herzegovina, Serbia and Bulgaria (subspecies *I. a. reiseri* and the eastern part of *I. a. alpestris*, clade E2). Clades E1 and E2 are sister groups. Within the western lineage, populations from the Italian peninsula (subspecies *apuana* and *inexpectata*, clade B) are sister to a clade containing samples from the Iberian Peninsula (subspecies *I. a. cyreni*, clade C1) and the remainder of the samples from subspecies *I. a. alpestris* (clades C2 + C3, populations from Hungary, Austria, Poland, France, Germany and the larger part of Romania). Median and 95% HPD (Highest Posterior Density) intervals, that is, those that include 95% of the posterior probability function for a given parameter (in parentheses) for the estimated TMRCAs for *I. alpestris* lineages (in million years) were as follows (Fig. 3): all *I. alpestris* lineages (clades A-E): 19.6 (15.9–23.7), eastern-western lineages (clades B-E): 11.2 (9.1–13.6), eastern lineage (clades D-E): 7.3 (5.6–9.3), western lineage (clades B-C): 9.2 (7.1–11.3), western *I. a. alpestris* (clades C2 + C3): 1.0 (0.5–1.7), *I. a. apuana* (clade B): 2.1 (1.3–3.1), western *I. a. alpestris* + *I. a. cyreni* (clade C): 1.6 (1.0–2.4).

3.2. Nuclear data: haplotype networks and the multilocus coalescent

Haplotype networks based on the nuclear DNA data are shown in Fig. 4. In the GH network, most lineages had non-overlapping sets of haplotypes (clades B, C1, C2 + C3, D, E1 and E2), although samples from the "Vlasina" lineage (clade A of Sotiropoulos et al., 2007) grouped with clade E2. Samples of southern Italy (subspecies *I. a. inexpectata*) shared haplotypes with other populations in the Apennine Peninsula (subspecies *I. a. apuana*). On the other hand, the PDGFR network showed several instances of shared haplotypes across clades, including those more closely related to each other (clades C1, C2 and C3) as well as other cases involving well-differentiated lineages (C1 + C2 + C3 with E2 and B). In general, populations from Romania presented the highest levels of allele admixture, including heterozygous individuals with haplotypes of both eastern and western lineages.

Results of *BEAST analyses consistently identified with high support four lineages with unresolved inter-relationships: (1) subspecies *I. a. veluchiensis* (clade D); (2) subspecies *I. a. apuana* + *I. a. inexpectata* (clade B); (3) subspecies *I. a. cyreni* (clade C1) + part of subspecies *I. a. alpestris* (the westernmost populations, corresponding to clades C2 + C3, plus most Romanian populations -part of clade E2); and (4) the remaining populations, including subspecies *I. a. serdara, I. a. reiseri* and *I. a. montenegrina* and part of subspecies *I. a. alpestris* (clades E1 and part of clade E2), plus samples from Vlasina (Table 2). Thus, there is discordance regarding the phylogenetic position of Romanian populations included in mitochondrial clade E2, which are part of the major Eastern clade based on mtDNA but cluster with Western *I. a. alpestris* (clade C) in the multispecies coalescent. However, according to JML results, this is consistent with a strictly bifurcating species tree with no gene flow across lineages, and therefore these results may reflect incomplete lineage sorting rather than hybridization.

3.3. Allozymes

Based on the allozyme dataset, BAPS resolved eight groups including the following populations: (1) Spain (Cantabria and

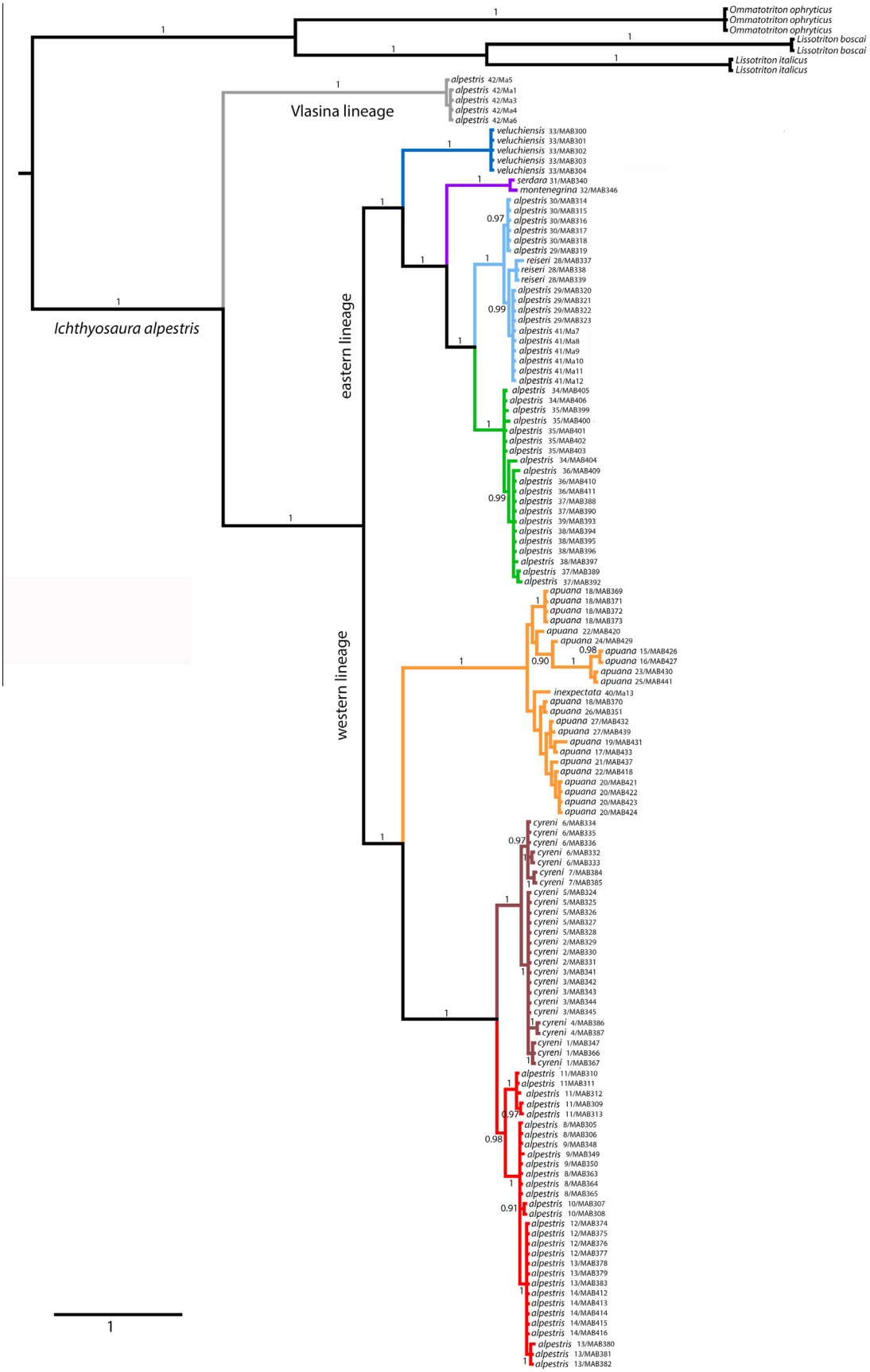

Fig. 2. Bayesian consensus phylogram of mtDNA data, showing major clades in *Ichthyosaura alpestris* and their respective support values (posterior probabilities, only values $\geqslant 0.9$ are shown). Colors represent clades as in Fig. 1; sample and locality codes are as in Table 1. Scale is number of substitutions per site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

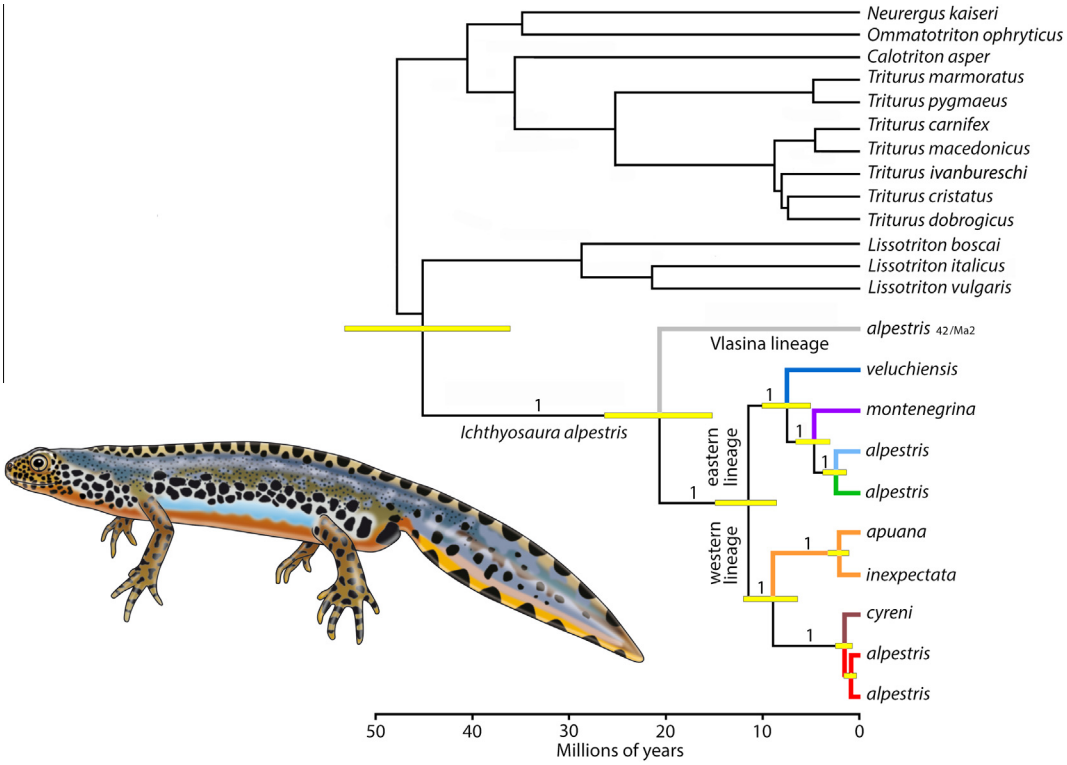

Fig. 3. Maximum clade credibility tree based on the reduced *Ichthyosaura alpestris* mtDNA dataset recovered by BEAST, showing time estimates for major clades within *I. alpestris* (colors as in Figs. 1 and 2). Node bars (yellow) represent 95% HPD intervals for node ages. Also shown are posterior probabilities for each node in the ingroup (only values $\geqslant$0.9 are shown). The scale is in millions of years. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Guadarrama, subspecies *I. a. cyreni*), (2) western Europe (Germany and France in Western *I. a. alpestris*), (3) central Europe (Austria and Hungary in Western *I. a. alpestris*), (4) three central Balkan populations (Eastern *I. a. alpestris*), (5) Bulgaria (Eastern *I. a. alpestris*), (6) northern Italy (subspecies *I. a. apuana*), (7) Bukumirsko Jezero in eastern Montenegro (subspecies *I. a. montenegrina*) and (8) Greece (subspecies *I. a. veluchiensis*) (Table 2).

4. Discussion

The evolutionary history of *Ichthyosaura alpestris*, as revealed by molecular phylogenetic approaches, is old and complex. Sotiropoulos et al. (2007) identified several divergent mitochondrial lineages, estimating their major splits from the Miocene-Pliocene boundary. The phylogenetic hypotheses based on mtDNA from our study recovered the same lineages and phylogenetic structure. However our time estimates, based on a different external calibration point, are sensibly older (especially for deeper splits). Our new proposal fits well with ages proposed based on the fossil record (Marjanović and Laurin, 2013) but conflict at least in part with the biogeographic scenario proposed by Sotiropoulos et al. (2007). According to the latter study, the evolutionary history of *I. alpestris* dates back to the Upper Miocene, when the ancestor of *I. alpestris* was either restricted to the Balkan region, from where it expanded westward right before the start of the Pliocene, or it was already widespread along most of the continent during the Miocene. Our results are consistent with this "widespread" hypothesis, with the split of the major eastern and western lineages during the Tortonian. Palaeogeological reconstructions indicate that during this period, oscillations in the Paratethys Sea level would have isolated the Alps region from the Balkan territories (Popov et al., 2004), setting the scenario for vicariant processes. Additionally, paleoclimatic inferences indicate that temperatures in Europe during the Miocene and the Pliocene were significantly higher than during the Quaternary, with a shift during the Tortonian that intensified a latitudinal temperature gradient (Fauquette et al., 2007). In view of the current range of *I. alpestris*, it seems likely that higher temperatures promoted the isolation of population groups in different mountain ranges and latitudes, reinforcing the process of parapatric differentiation during the Miocene and the Pliocene. Similarly old diversification processes are not uncommon in amphibians, including other salamandrids (Zhang et al., 2008; Wiens et al., 2011). The eastern populations of *Lissotriton vulgaris*, for instance, harbour several lineages that pre-date the Pleistocene (Babik et al., 2005). Lineages of Miocene origin are also found in *L. boscai* (Martínez-Solano et al., 2006), while species in the *Triturus cristatus* species group seem to have radiated also during the Tortonian (Arntzen et al., 2007).

The ranges of the different lineages appear to have shifted during the Pleistocene glaciations, which complicates inferring their evolutionary history. For example, the populations currently inhabiting the Iberian Peninsula most likely originated after a southwards range expansion of the species during the lower/middle Pleistocene, where it has survived since then. Two clades corresponding to western and eastern Cantabrian population groups were recovered in the analyses (Fig. 2). The population in Central Spain (Peñalara) is very similar to the rest of Iberian samples and clusters with the western clade, supporting the hypothesis of a recent introduction from a source population in this region (Arano et al., 1991). Shifts in the distribution ranges of most lineages (if not all) must have been common during the alternation of glacial cycles, including both local extinctions and recolonizations. These shifts may have also caused admixture following

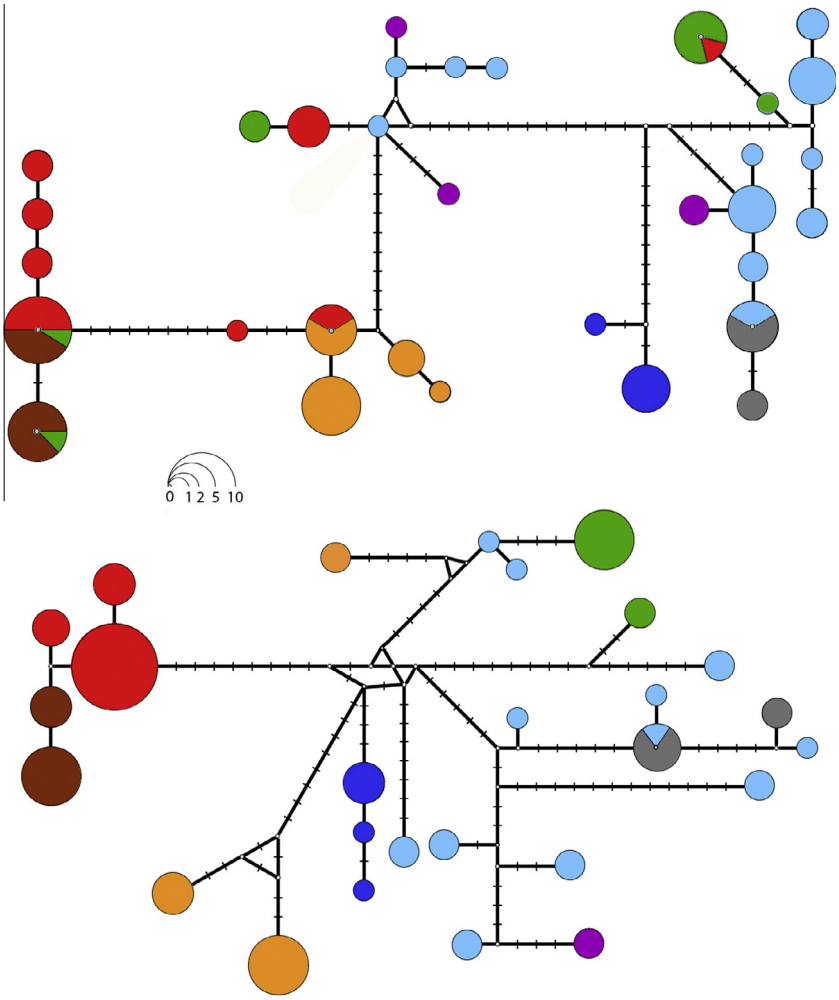

Fig. 4. Haplotype networks of nuclear sequences (top: PDGFR; bottom: GH), showing frequencies (proportional to size, see scale) and distribution across major mtDNA clades (in colors as in Figs. 1–3).

secondary contact, explaining discordance across loci in our dataset. For instance, samples from the "Vlasina" lineage share nuclear haplotypes with individuals from other clades in the Balkans (clade E2). Additionally, patterns of allele admixture in samples from Romania might be indicative of admixture in secondary contact zones, perhaps associated with Pleistocene cycles of range contractions-expansions, although the results from the JML analysis suggest they may equally well result from incomplete lineage sorting. High levels of genetic diversity in this region are in line with evidence supporting the existence of Carpathian glacial refugia in several taxa: brown bear (*Ursus arctos*) (Sommer and Benecke, 2004), bank vole (*Clethrionomys glareolus*) (Deffontaine et al., 2005), newts (*Lissotriton vulgaris* and *L. montandoni*) (Babik et al., 2005), frogs (*Bombina bombina* and *B. variegata*) (Vörös et al., 2006; Hofman et al., 2007), and trees (*Alnus*, *Betula*, *Picea*, *Pinus*, *Salix*, see Willis and van Andel, 2004, and references cited therein), in which the long-term persistence of large populations explains the current patterns of regional diversity (Schmitt and Varga, 2012).

The groups recovered in the allozyme analyses correspond, respectively, to: (1) *I. a. cyreni* – clade C1-; (2) *I. a. alpestris* – clade C3; (3) *I. a. alpestris* – clade C3; (4) *I. a. alpestris* + *I. a. serdara* + *I. a. reiseri* – clades E1 + E2; (5) *I. a. alpestris* – clade E2; (6) *I. a. apuana* – clade B; (7) *I. a. montenegrina* – clade E1; and (8) *I. a. veluchiensis* – clade D (Table 2). These results are largely congruent with analyses of mtDNA variation and current taxonomy, with a few interesting exceptions. Clade C3, for instance, is extremely homogeneous in mtDNA but consists of two subgroups in allozymes. This nuclear structure could result from the comparatively larger geographic distances between sampled populations in allozymes *vs.* mtDNA, but also from different demographic histories at the local scale and the existence of impermeable habitats reducing the amount of gene flow between populations. In line with this, Pabijan et al. (2005) and Pabijan and Babik (2006) reported significant levels of nuclear genetic differentiation between geographically close populations in Poland, even though these were of recent, Holocene origin and showed no divergence in mtDNA sequences. On the other hand, allozyme structure in clade E does not correspond to mtDNA results, with populations from the same mtDNA clade (E2) clustering in different allozyme-based groups, and *vice versa*. These discrepancies are found in the geographic region that harbours most genetic (four mtDNA lineages) and life-history variation (paedomorphic populations), an area that is characterized by a complex biogeographic history. The different patterns observed at allozymes and mtDNA may reflect past and/or ongoing events of isolation coupled with secondary contact and gene flow among well-differentiated mitochondrial lineages. Additionally, male-biased dispersal in *I. alpestris* (Joly and Grolet, 1996) can accentuate these discrepancies between mtDNA and nuclear markers. Finally, discordance might result from regional selective pressures acting over some of the analyzed loci. All these plausible and non-exclusive scenarios have to be further explored with *ad hoc* experimental and field studies.

The work of Sotiropoulos et al. (2007), including the surprising finding of a highly divergent lineage exclusive from the Vlasina lake, triggered some debate on the taxonomic status of subspecies within *I. alpestris* (Dubois and Raffaëlli, 2009; Speybroeck et al., 2010) to which our results can shed some light. Dubois and Raffaëlli (2009) proposed that all three main lineages identified by Sotiropoulos et al. (2007) (eastern, western and Vlasina), could deserve specific consideration but made no official proposal at the time, whereas Speybroeck et al. (2010) were more conservative, and expressed the opinion (that we share) that taxonomic changes should not be based solely on mtDNA data.

As seen from the nuclear data, the evolutionary history of the Vlasina population is not independent from the remainder of the Balkan populations, as the mtDNA data would seem to reveal. In our view, therefore, the Vlasina population does not deserve specific status. Our estimates place the split of the Vlasina mitochondrial lineage in the lower Miocene. Recently, Schoch and Rasser (2013) described a new *Ichthyosaura* species of similar age from the German Miocene, indicating a higher diversity of the genus in the past, which is consistent with our scenario (see also Marjanović and Laurin, 2013; and Martín and Sanchiz, 2013, about the fossil record of *Ichthyosaura*). Such "ghost" mitochondrial DNA lineages are not uncommon (Wilson and Bernatchez, 1998; Pinho et al., 2008) and are a good reason not to base taxonomic decisions on the results of single-marker studies. Based on our data and analyses, a minimum of four lineages can be delineated (see Table 2), corresponding respectively to: (1) subspecies *I. a. veluchiensis*; (2) subspecies *I. a. apuana* and *I. a. inexpectata*; (3) subspecies *I. a. alpestris* (Central European populations, which include the type locality "in Etschero monte", northern Alps, west of Mariazell, Austria - Laurenti, 1768) and *I. a. cyreni*; and (4) subspecies *I. a. alpestris* (populations in Eastern Europe and the Balkans) and *I. a. montenegrina*. While approximate ranges for these groups can be delineated based on ours and previous studies (Fig. 1, see also Canestrelli et al., 2006), finer scale studies are needed to address the possibility (or the extent) of gene flow across lineages, which is key to understand speciation processes.

5. Conclusions

Amongst the newts of the western Palearctic, *I. alpestris* represents an exceptional model for the study of evolutionary processes at very different geographical and temporal scales. First, it has a wide geographic distribution and its continued presence in three major glacial refugia is both well documented in the fossil record and also inferred from genetic data. Second, the species includes very old genetic lineages that have survived major geological and climatic events since the Miocene. Third, there is considerable variation in life-history traits associated to differing ecological settings, like the presence of paedomorphic populations (Denoël et al., 2001). Our study provides a solid, comprehensive background on the evolutionary history of the species based on the most complete combined (mtDNA + nucDNA + allozymes) dataset to date. The combination of the historical perspective provided by coalescent-based analyses of mtDNA variation with individual-based multilocus assignment methods based on multiple nuclear markers also allows identifying important discordances that highlight the complexity and dynamism of evolutionary processes in the species. For instance, processes of gene flow and admixture after secondary contact have been widely invoked to explain conflict between datasets in amphibians (García-París et al., 2003; Babik et al., 2005; Wielstra and Arntzen, 2012; Wielstra et al., 2013), but little is known about the actual demographic and ecological processes favouring or impeding gene flow in *I. alpestris*. In the same vein, the relative importance of demographic, historical and life-history factors in explaining contrasting patterns of population structure in Clade E is unclear. Future studies comparing fine-scale gene flow patterns across ecological gradients in genetically homogeneous (Central Europe) *vs.* genetically diverse (Balkans) regions might clarify the roles of ecological and historical factors in population divergence and speciation.

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