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# An extended mtDNA phylogeography for the alpine newt illuminates the provenance of introduced populations

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**Abstract.** Many herpetofauna species have been introduced outside of their native range. MtDNA barcoding is regularly used to determine the provenance of such populations. The alpine newt has been introduced across the Netherlands, the United Kingdom and Ireland. However, geographical mtDNA structure across the natural range of the alpine newt is still incompletely understood and certain regions are severely undersampled. We collect mtDNA sequence data of over seven hundred individuals, from both the native and the introduced range. The main new insights from our extended mtDNA phylogeography are that 1) haplotypes from Spain do not form a reciprocally monophyletic clade, but are nested inside the mtDNA clade that covers western and eastern Europe; and 2) haplotypes from the northwest Balkans form a monophyletic clade together with those from the Southern Carpathians and Apuseni Mountains. We also home in on the regions where the distinct mtDNA clades meet in nature. We show that four out of the seven distinct mtDNA clades that comprise the alpine newt are implicated in the introductions in the Netherlands, United Kingdom and Ireland. In several introduced localities,

two distinct mtDNA clades co-occur. As these mtDNA clades presumably represent cryptic species, we urge that the extent of genetic admixture between them is assessed from genome-wide nuclear DNA markers. We mobilized a large number of citizen scientists in this project to support the collection of DNA samples by skin swabbing and underscore the effectiveness of this sampling technique for mtDNA barcoding.

*Keywords*: citizen science, *Ichthyosaura alpestris*, invasive species, mtDNA barcoding, skin swabbing.

## Introduction

Invasive species are species that have established outside of their natural distribution and have a negative environmental impact at the expense of native biodiversity (Simberloff, 2013; Bellard et al., 2016; Pyšek et al., 2020; Diagne et al., 2021). Therefore, mapping and monitoring of invasive species is crucial when contemplating conservation action, and genetic tools play an important role in this process (Allendorf et al., 2010). MtDNA typically displays strong geographical genetic structuring, allowing closely related species as well as intraspecific lineages to be distinguished (Avise, 2000).

For most European amphibians, comprehensive mtDNA phylogeographies have already been published (e.g., Babik et al., 2005; Fijarczyk et al., 2011; Vences et al., 2013; Wielstra et al., 2013; Dufresnes et al., 2020; Ambu et al., 2023). MtDNA barcoding involves the comparison of sequences for a particular mtDNA marker across many individuals that belong to e.g., different species or structured populations (Hebert et al., 2003; Mir et al., 2021). Matching mtDNA barcodes from introduced amphibian populations with published phylogeographic datasets can be used to determine from where within the natural distribution range these populations originate.

Sample collection for a project on mtDNA barcoding of invasive amphibian species lends itself well to 'citizen science' initiatives, where members of the public team up with professional scientists to collect data (Devictor et al., 2010; Bonney et al., 2016). A network of citizen scientists would allow comprehensive sampling to be achieved efficiently – particularly when the focal species is present in the volunteers' private garden ponds. For the purpose of citizen science, any DNA sample collection methodology needs to be simple and easy to follow, minimize the impact on animals and involve as little bureaucracy as possible.

Collecting amphibian tissue requires training and ethical approval and can be controversial with the public (Zemanova, 2019). While taking tail tips of salamanders has been proven not to negatively affect survival (Arntzen et al., 1999; Polich et al., 2013), amputating toes, as often conducted on frogs, may be harmful (McCarthy and Parris, 2004; Funk et al., 2005). Swabbing is considered a 'minimally invasive' alternative to tissue sampling (Broquet et al., 2007; Ringler, 2018; Ambu and Dufresnes, 2023). While one could argue about what would constitute 'minimally invasive', swabbing is in practice not considered an animal experiment in most instances, and is therefore exempt from approval by an animal ethics committee. Accordingly, the required permissions to take swabs from animals in their natural environment are relatively easy to obtain for a large group of people, particularly if they are already involved in monitoring schemes (i.e., with terrain access and permissions/licenses to handle animals already in place).

While buccal swabbing is a suitable source of DNA, it requires physically opening the mouth of the animal (Pidancier et al., 2003; Poschadel and Möller, 2004; Broquet et al., 2007). Buccal swabbing could risk injury if the person taking the sample is unexperienced and/or the mouth of the animal being sampled is small relative to the swab (Poschadel and Möller, 2004; Müller et al., 2013; Ringler, 2018). Skin swabbing has been used as an alternative for buccal swabbing for over a decade now (Prunier et al., 2012; Ward et al., 2019). While skin swabbing performs poorly in relatively dry-skinned

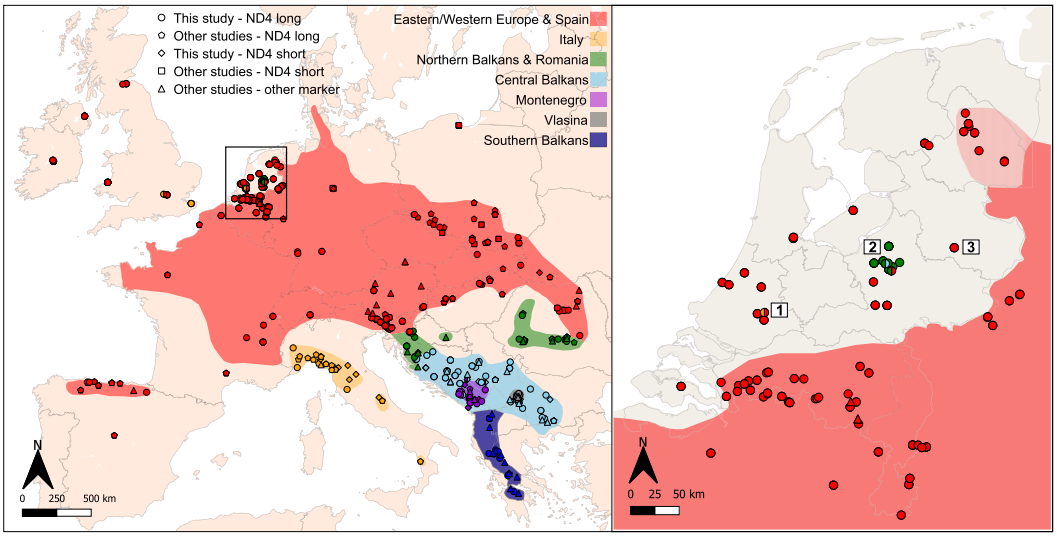


**Figure 1.** Distribution map for the alpine newt (*Ichthyosaura alpestris*). Background colour reflects the rough natural distribution of the mtDNA clades and symbols reflect sampled localities. The inset shows the Netherlands in more detail (lighter shading reflects uncertainty about the status of populations in the northeast of the country). Numbers refer to localities/regions mentioned in the text: 1 = Oudeland, 2 = Veluwe and 3 = Holterberg. Sampling information in supplementary table S1.

frog species (Ringler, 2018), it has been shown to work well in species with a relatively moist skin, such as newts (Prunier et al., 2012). Skin swabbing is arguably simpler and quicker than buccal swabbing and less impactful on the sampled animals, making it particularly suitable in citizen science projects.

The alpine newt (*Ichthyosaura alpestris*) occurs throughout most of Europe (fig. 1). The taxon harbours multiple highly distinct mtDNA clades (Sotiropoulos et al., 2007; Recuero et al., 2014) and is suspected to encompass multiple 'cryptic species' (Raffaëlli, 2018; Speybroeck et al., 2020; Frost, 2021). Unfortunately, previous studies have used different mtDNA markers, which hampers the combination of datasets (Sotiropoulos et al., 2007; Recuero et al., 2014; Chiocchio et al., 2017). Furthermore, despite multiple studies, sampling gaps remain. Most mtDNA clades meet in the Balkans. However, the distribution of these lineages and the extent of comingling between them are not yet well understood. Finally, the postglacially colonized range, such as Eastern and Western Europe, is genetically relatively homogeneous and has been poorly sampled.

Alpine newts are popular pets and have regularly been introduced outside of their native range (Sillero et al., 2014). MtDNA barcoding has been applied to determine the origin of several incidental introductions. Italian alpine newts have been introduced on the other side of the globe, in New Zealand (Arntzen et al., 2016). Less extreme are the introduction of alpine newts from the Cantabrian Mountains of northern Spain into central Spain (Palomar et al., 2017), Cantabrian and Italian alpine newts into Catalonia (Fibla et al., 2015), and alpine newts from unspecified but distinct sources in Eastern/Western Europe into northern Poland (Jakóbik et al., 2019) and southern France (Arntzen et al., 2016). The alpine newt has been introduced on a massive scale in The Netherlands (Creemers and van Delft, 2009; https://www.ravon.nl/Soorten/Soortinformatie/alpenwatersalamander) as well as the United Kingdom and Ireland (Meehan, 2013; Allain and Lynn, 2021). The provenance of these introductions is currently unclear.

The marked geographical genetic structure in mtDNA across the vast alpine newt range (Sotiropoulos et al., 2007; Recuero et al., 2014) facilitates determining the provenance of introduced alpine newt populations. However, the situation would benefit from the survey of a single mtDNA from individuals, throughout the natural distribution range. To this end, we employ mtDNA barcoding of a single mtDNA gene with the aim to: 1) expand the alpine newt mtDNA phylogeography and 2) determine the geographical origin of introduced alpine newt populations. Additionally, we 3) test the effectiveness of buccal versus skin swabs for mtDNA barcoding in alpine newts.

## Materials and methods

*Sampling and DNA extraction*

We obtained new mtDNA data for 456 individuals from 234 localities (fig. 1, supplementary table S1). For 247 individuals samples consisted of skin swabs. The outer surface of the newt was swabbed (40 times in total, 10 times on the dorsal, ventral and each lateral side) with Copan 155C Rayon swabs. Swabs were stored dry and moved to −20°C as soon as feasible. For 18 individuals, buccal swabs were also taken, i.e., alongside skin swabs, to compare DNA yield (measured with a DropSense96 machine) and PCR performance (i.e., if a sequence could be produced). We also obtained tissue samples, either freshly collected or taken from museum specimens (Džukić et al., 2015). DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). Samples from Belgium and Slovenia were provided as DNA extract.

*PCR and sequencing*

For 411 individuals we amplified a 897 bp fragment of the ND4 (NADH dehydrogenase subunit 4) mtDNA gene and adjacent tRNA. We adjusted the 'universal' ND4 primers (ND4 and Leu) from Arévalo et al. (1994) to exactly match the sequence in the *Ichthyosaura alpestris* mitogenome published by Zhang et al. (2008), using Geneious Prime 2021.1.1. This resulted in the primers ND4-Ich (5′-CACCTCTGACTTCCAAAAGCCCACGTA GAGGC-3′) and Leu-Ich (5′-CATAACTTTTACTTGGAG TTGCACCA-3′). For 45 individuals for which we could not obtain a usable sequence, presumably due to DNA degradation, internal primers were designed in Geneious Prime to amplify a stretch of 241 bp, namely primer pairs ND4-Ich-i299vlasiF (5′-TGCTTCTAGCCCGCGGAACC-3′) and Leu-Ich-i559vlasiR (5′-ATGGGTTTATCAGGCGA AGA-3′) for the distinct Vlasina mtDNA clade or ND4-Ich-i299F (5′-TACTCTTAGCCCGCGGAGTM-3′) and Leu-Ich-i559R (5′-ATGGGTTTATCGGGYGAAGG-3′) for the remaining mtDNA lineages.

PCRs were performed in 12 $\mu$l reactions, containing 0.06 $\mu$l of both forward and reverse primer (0.05 $\mu$M end concentration of each primer), 7.2 $\mu$l QIAGEN multiplex PCR master mix, 3.68 $\mu$l purified water and 1 $\mu$l of DNA extract. PCR conditions were: a hot start for 15 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C, and extension at 72°C of ten minutes. Sanger sequencing was outsourced to BaseClear B.V. and sequences were edited and trimmed in Geneious Prime.

*Compilation of mtDNA ND4 reference database*

ND4 sequences were available from previous studies from across the natural range of *I. alpestris* (Pabijan and Babik, 2006; Recuero et al., 2014; Vörös et al., 2021). To be able to compare between studies, we focussed on a shared stretch of 651 bp and we trimmed our sequences accordingly. ND4 sequences of sufficient length were available from several introduced populations as well (Recuero et al., 2014; Arntzen et al., 2016). This database of 651 bp ND4 haplotypes provides a comprehensive overview of genetic variation across the natural range and allows us to link introduced populations to the part of the natural range that they originate from. We introduce a new naming system for these 651 bp ND4 haplotypes (supplementary tables S1 and S2).

Our 241 bp ND4 data, together with additional studies that focussed on either a different fragment of ND4 (i.e., not our target 651 bp) (Jakóbik et al., 2019) or different mtDNA markers (Sotiropoulos et al., 2007; Šunje et al., 2021), were incorporated to help further delineate the geographical distribution of mtDNA clades (supplementary table S1). The colour scheme we use to highlight the different mtDNA clades follows Recuero et al. (2014) and Arntzen et al. (2016).

*Genetic analyses*

The Haplotype Collapser function in FaBox (Villesen, 2007) was used to determine if the mtDNA haplotypes of the newly sequenced individuals match any of the previously identified 651 bp ND4 haplotypes in our database. To determine the phylogenetic position of newly identified haplotypes we conducted Bayesian phylogenetic inference with MrBayes v.3.2.6. (Ronquist et al., 2012). The IQ-TREE web server functionality ModelFinder (Trifinopoulos et al., 2016; Kalyaanamoorthy et al., 2017) was used to approximate the most appropriate model of sequence evolution for each codon position (HKY + G, GTR + G and GTR + I for position 1, 2 and 3, respectively). The northern crested newt *Triturus cristatus* (haplotype Tcri01, Genbank Code: GU982383, taken from Wielstra et al., 2013) was used as an outgroup. We ran two runs of four-chains for five million generations with a sampling frequency of 0.001 and a heating parameter of 0.2 in MrBayes and used a 25% burnin. Tracer v1.7 (Rambaut et al., 2018) was used to confirm that runs converged and that ESS was above the standard thresh-

old of 200. A median spanning haplotype network was constructed in PopArt 1.7 under default settings (Leigh and Bryant, 2015). We calculated nucleotide diversity ($\pi$) across the natural distribution range in DNAsp v6 (Rozas et al., 2017), at the level of the country and for each mtDNA clade separately (if more than one occurred in the same country).

## Results

Our database of 651 bp ND4 sequences contains 411 newly sequenced and 185 previously published individuals (from 273 localities). This dataset encompasses 100 haplotypes, including 62 not previously identified (figs. 1-3, supplementary tables S1 and S2). These haplotypes are used to conduct phylogenetic reconstruction and to tie introduced populations to the natural distribution range (see below). For another 45 individuals (covering 36 localities) an internal 241 bp ND4 fragment is available. Furthermore, another 80 individuals (covering 62 localities) are included for which either a relatively short stretch of ND4 or a different mtDNA marker are available from previous studies. These data are only used to assign individuals to mtDNA clade to help delineate the distribution of mtDNA clades (fig. 1, supplementary table S1).

Our extended mtDNA phylogeography mostly confirms the distinct mtDNA clades identified in previous studies and the relationships among them (Sotiropoulos et al., 2007; Recuero et al., 2014) – for consistency between studies we stick to the previous division into mtDNA clades as much as possible. However, haplotypes from Spain are not reciprocally monophyletic from, but are nested within, a clade containing haplotypes from across Eastern and Western Europe.

While we do not improve much upon the known distribution of the mtDNA clades here referred to as the Italy, Southern Balkans and

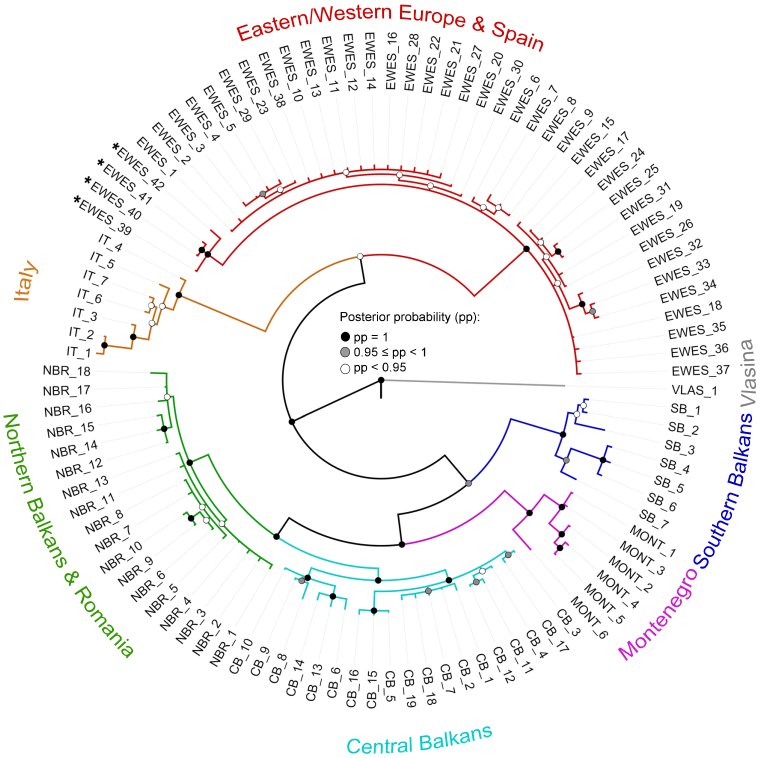


**Figure 2.** Majority rule consensus Bayesian phylogeny for the alpine newt (*Ichthyosaura alpestris*) based on 651 bp of the mtDNA gene ND4. The outgroup is not shown. Spanish haplotypes are highlighted with an asterisk. Haplotype details can be found in supplementary table S1 and a more detailed version of this phylogeny is available in supplementary fig. S1.

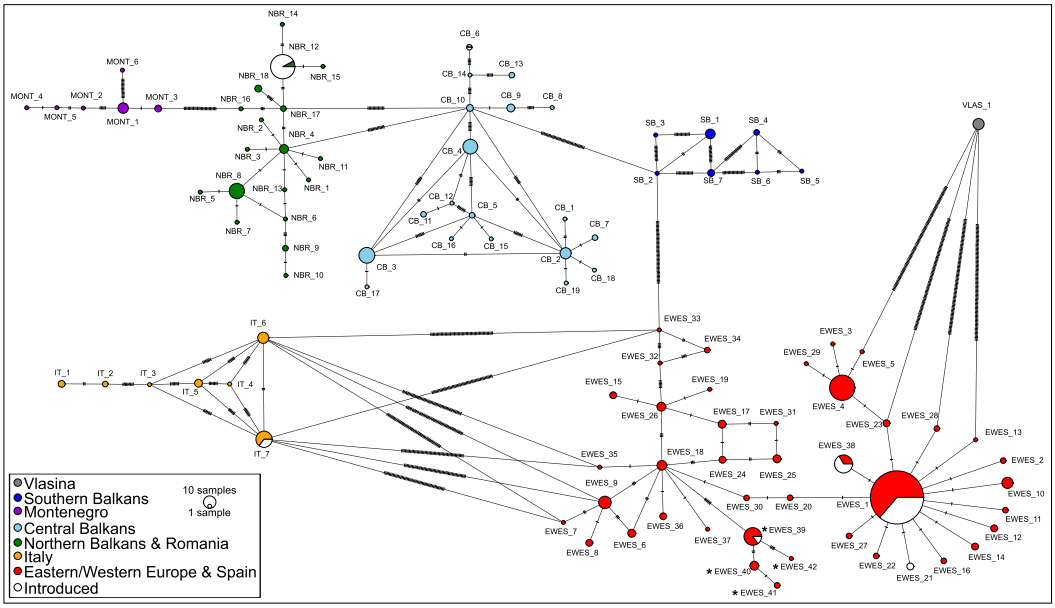


**Figure 3.** Haplotype network for the alpine newt (*Ichthyosaura alpestris*) based on 651 bp of the mtDNA gene ND4. Pie size reflects haplotype frequency and bars reflect the number of substitutions. White pie slices represent introduced localities. Spanish haplotypes are highlighted with an asterisk. Haplotype details can be found in supplementary table S1.

Vlasina mtDNA clades, we clarify the distributions of the other mtDNA clades (fig. 1). Haplotypes from the Southern Carpathians and the Apuseni Mountains in Romania and the northwest Balkans (northern Croatia and adjacent Slovenia and Bosnia and Herzegovina) compose a monophyletic clade that we refer to as the Northern Balkans & Romania mtDNA clade (figs 1 and 2, supplementary fig. S1). The Central Balkans mtDNA clade stretches from Bulgaria (and just a bit of Greece), through Serbia to Bosnia and Herzegovina, and slightly extends into Montenegro and Croatia (fig. 1). The Montenegro mtDNA clade is mainly found in Montenegro but slightly protrudes into neighbouring Bosnia and Herzegovina, Serbia and Albania (fig. 1). We show that the Eastern/Western Europe & Spain mtDNA clade extends as far south as the Southern Carpathians and the Slovenian/Croatian border (fig. 1). Most genetic diversity in this mtDNA clade is found at the southern fringe of its distribution, just north of the Balkan Peninsula (fig. 4, supplementary table S3).

Most introduced localities in the Netherlands contain one particular haplotype from the Eastern/Western Europe & Spain mtDNA clade, namely EWES_1, with the exception of introduced locality Holterberg, where we find EWES_21 (figs 1 and 3, supplementary table S1). Additional haplotypes from distinct mtDNA clades are present in several introduced localities, sometimes in syntopy (fig. 1). Namely, introduced locality Oudeland features haplotype IT_7 (Italy mtDNA clade) and haplotype EWES_1. In the Veluwe region, haplotype NBR_12 (Northern Balkans & Romania mtDNA clade) is widespread, while haplotype CB_6 (Central Balkans mtDNA clade) is restricted to a single locality (close to NBR_12). We also find the EWES_1 haplotype in two localities in this region; in one it is the only haplotype identified, in the other it co-occurs with NBR_12. In both Ireland and Northern Ireland, the only haplotype found is EWES_38 of the Eastern/Western Europe & Spain mtDNA clade. Scotland and Wales host haplotype EWES_1. In one locality from England we find EWES_1

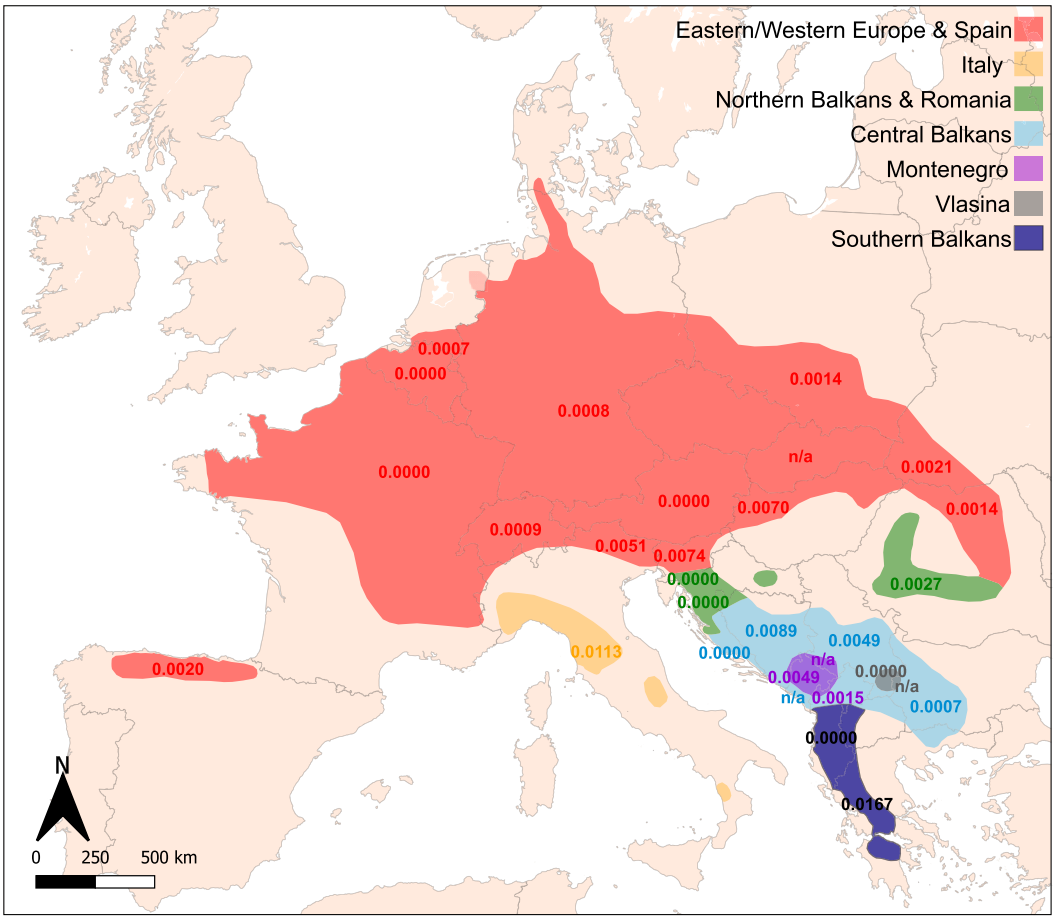


**Figure 4.** Nucleotide diversity ($\pi$) across the natural distribution range of the alpine newt (*Ichthyosaura alpestris*) for the main mtDNA clades and at country-level, based on 651 bp of the mtDNA gene ND4. N/a means $\pi$ could not be established because only a single individual was available. Background colour reflects the rough natural distribution of the mtDNA clades. Further details can be found in supplementary table S3.

again, another features haplotype IT_7 of the Italy mtDNA clade, and a third contains both EWES_1 and IT_7.

We have obtained sequence data for 247 swab samples. Another 32 swabs (c. 11%) did not produce a sequence. For the 18 individuals that have been both buccal- and skin-swabbed we obtain sequences from both approaches for 16 individuals (for one individual the buccal swab failed and for another individual the skin swab failed). There is accordingly no difference in performance between buccal and skin-swabbing (Chi-squared test, $p > 0.05$, $df = 1$, $n = 36$). The two sequences per individual based on buccal and skin swabs are identical. Even though success rate in terms of obtaining a sequence is similar for buccal and skin swabs, DNA yield is consistently lower for skin swabs (supplementary table S4).

## Discussion

### *New insights from extended mtDNA phylogeography*

While the alpine newt has received acute phylogeographic interest, the sampling schemes and markers employed in previous studies differ,

and sampling gaps remain (Sotiropoulos et al., 2007; Recuero et al., 2014; Chiocchio et al., 2017; Šunje et al., 2021; Vörös et al., 2021). Our range-wide mtDNA phylogeography, based on a single marker, allows us to clarify the distribution of the main mtDNA clades and locate (potential) contact zones between them.

We find Spanish haplotypes nested within a clade that also contains haplotypes from Eastern and Western Europe. We suggests this pattern reflects our greatly increased sampling, rather than low phylogenetic resolution. We recommend a genomic study should test the phylogenetic position of Spanish alpine newts.

While, outside of Spain, the Eastern/Western Europe & Spain mtDNA clade is genetically homogeneous across most of its range, considerable genetic variation is found at its southern fringe, just north of the Balkan Peninsula, in the north-east of Italy, Slovenia and Hungary (fig. 3, supplementary table S3). Haplotype EWES_1 occurs most frequently and is widely distributed, including in the Italian eastern Alps, adjacent to the region that harbours most of the genetic diversity for the Eastern/Western Europe & Spain mtDNA clade. This pattern suggests that the eastern Alps were the point of origin for the postglacial recolonization of western and central Europe (Sotiropoulos et al., 2007).

Another new insight concerns to which mtDNA clade the haplotypes from the northwest Balkans (northern Croatia and adjacent Slovenia and Bosnia and Herzegovina) should be allocated (fig. 1). In an attempt to integrate data from a previous study (Sotiropoulos et al., 2007), concerning a different, relatively low-resolution mtDNA marker, these localities were misinterpreted as belonging to the Central Balkans mtDNA clade in Recuero et al. (2014). Rather, the haplotypes are part of an mtDNA clade that is further distributed in the Southern Carpathians and Apuseni Mountains in Romania (figs 1 and 2). Currently, these two range sections are allopatric (Sillero et al., 2014), being separated by hundreds of kilometres of unsuitable lowland habitat. This disjunct distribution pattern suggests that alpine newts had a more extensive range in the past (presumably during glacial cycles), in line with montane relictual distribution patches observed in other parts of the range (Pabijan et al., 2009; Chiocchio et al., 2017; Vörös et al., 2021). A phylogenomic study should further explore the phylogenetic position of these geographical populations.

Our denser sampling allows us to delineate the distributions of the distinct mtDNA clades with more accuracy and informs upon where they (potentially) meet. In particular, we pinpoint the transition between the Central Balkans and Northern Balkans & Romania mtDNA clade, in the north-west of Bosnia and Herzegovina and just across the border in Croatia (fig. 1) (see also Šunje et al., 2021). Syntopy of the Central Balkans and Montenegro mtDNA clade is observed at the crossover between Serbia, Bosnia and Herzegovina and Montenegro. While we show that the Vlasina mtDNA clade extends slightly into Bulgaria (fig. 1), further sampling is required to determine if it is geographically isolated from the Central Balkans mtDNA clade. Additional sampling in Kosovo is required to assess whether the Southern Balkans mtDNA clade comes into contact with the Montenegro and Central Balkans mtDNA clades. We show that the Eastern/Western Europe & Spain mtDNA clade is distributed as far south as the Southern Carpathians and the border between Slovenia and Croatia, in close proximity to the Northern Balkans & Romania mtDNA clade.

While it has been well-established that the alpine newt harbours multiple, highly distinct mtDNA clades (Sotiropoulos et al., 2007; Recuero et al., 2014), only limited nuclear DNA data have been studied so far (Recuero et al., 2014). In some instances, the distinct mtDNA clades are associated with distinct nuclear DNA alleles, suggesting they probably represent cryptic species. However, the highly distinct Vlasina mtDNA clade might concern a

'ghost lineage' that does not reflect a diverged nuclear DNA gene pool (Recuero et al., 2014). Mitochondrial surveys are known to be misleading in amphibian phylogeography and ensuing taxonomy (Dufresnes and Jablonski, 2022). Introgression of mtDNA across species boundaries is regularly observed (Toews and Brelsford, 2012).

Genome-scale data are becoming readily available for newts (Rancilhac et al., 2021) and have already been successfully applied for species delineation in related newt genera (Wielstra and Arntzen, 2016; Pabijan et al., 2017; Wielstra et al., 2018), also in combination with exploring potential ecological (Wielstra et al., 2012; van Riemsdijk et al., 2017) and morphological differentiation (Gerlach and Fahrbach, 2016; Üzüm et al., 2019). A phylogenomic study is required to clarify the evolutionary significance of the tremendous mitochondrial diversity in the alpine newt. In this respect, our improved mtDNA phylogeography helps to home in on the contact zones between the potential cryptic species hiding within the alpine newt (fig. 1).

*Provenance of introductions illuminated*

We focus on alpine newt introductions in the Netherlands as well as the United Kingdom and Ireland, where, compared to earlier studied incidental introductions (Arntzen et al., 2016; Palomar et al., 2017; Jakóbik et al., 2019), alpine newt introduction has occurred on a massive scale (Creemers and van Delft, 2009; Allain and Lynn, 2021). While these newly established populations are thriving and expanding, based on both the rapidness with which new localities have been discovered, and the geographical distances between them, tens if not hundreds of introduction and/or translocation events must have been involved.

In the Netherlands, the Veluwe region has been rapidly colonized since c. 1995 (Creemers and van Delft, 2009). Haplotypes from three distinct mtDNA clades are recovered in this area. One (EWES_1) is naturally widespread across Europe and also occurs natively in the Netherlands, while the other two are indigenous to the Balkan Peninsula (fig. 1). The most widespread of the two Balkan haplotypes (NBR_12) is also present in a locality from western Croatia. Another newt species that has been introduced in the Veluwe region, the Italian crested newt *Triturus carnifex*, could also be linked to a locality from western Croatia (less than thirty kilometres away) based on mtDNA barcoding (Meilink et al., 2015). Yet, we also find a haplotype (CB_6) in a single alpine newt that is naturally only known from a single locality in Bosnia and Herzegovina, c. 135 kilometres further eastwards. We suggest that these two distinct Balkan haplotypes may have a wider natural distribution and may occur in syntopy in nature, implying that the introduction could have involved a single Balkan stock. However, we acknowledge that the difference in frequency is striking. For example, two distinct mtDNA haplotypes also segregate in an introduced population of *Pelobates* toads in the Netherlands, but here haplotype frequency is considerably more equal (Koster et al., 2023). Nonetheless, the presence of an additional haplotype, natively distributed throughout Eastern and Western Europe, suggests that at least two stocks must have been involved.

In Oudeland we observe a haplotype that naturally occurs in Central Italy (IT_7). The same haplotype has also been found in the introduced population in New Zealand (Arntzen et al., 2016). However, this area (and practically all other introduced populations of the Netherlands) is also inhabited by newts that feature a widespread European and even Dutch native haplotype. There is one exception, Holterberg, where a distinct haplotype from the same Eastern/Western Europe & Spain mtDNA clade (EWES_21) is found, but because we did not sequence this haplotype across the natural range we cannot map its geographical origin.

We now have a situation in the Netherlands where genetically distinct mtDNA clades, presumably representing different cryptic species,

occur in syntopy. A similar situation has previously been observed in e.g., Spain (van Riemsdijk et al., 2018) and the Netherlands (Kuijt et al., 2023), where the mtDNA of two recently recognized species of *Ommatotriton* newts and *Hyla* treefrogs were found together in introduced populations. Such a situation may stem from the fact that cryptic species are typically mixed in the pet trade, particularly before they have been characterized as such in major taxonomic lists. According to current taxonomy, the alpine newt is regarded a single species, despite the presumed presence of cryptic species (Speybroeck et al., 2020). We urge for an evaluation of the extent of genetic admixture between the potential cryptic species within the introduced alpine newt populations based on many unlinked nuclear DNA markers.

In Ireland and Northern Ireland we find a single haplotype (EWES_38) that was previously reported from an introduced population in Larzac, southern France (Arntzen et al., 2016). This haplotype clearly belongs to the Eastern/Western Europe & Spain mtDNA clade. However, despite our increased sampling, which spans the previously hypothesized source in western Switzerland (Arntzen et al., 2016), we could not locate this haplotype in the natural distribution range. Given the apparently restricted geographical distribution of this haplotype in the wild, it is likely that the ancestors of these geographically distinct introduced populations derive from the same source. Note that Arntzen et al. (2016) identified a haplotype in two individuals from Larzac, France (GenBank Accession numbers KR107546 and KR107549) that is quite distinct from any of the haplotypes known throughout the natural range – also when considering the enhanced sampling of the current study – and appeared 'intermediate' between Spanish and Eastern/Western European haplotypes. We noticed that this haplotype is identical to multiple haplotypes found throughout Eastern and Western Europe for our 241 internal primer stretch, while carrying three fixed differences compared to Spanish haplotypes. Yet, in the remainder of the sequence, the opposite pattern is occasionally found. Therefore, we considered it likely that this haplotype represents a chimeric sequence and therefore opted to exclude it from our dataset.

Wales, Scotland and two out of three localities from England harbour a different haplotype (EWES_1) of the Eastern/Western Europe & Spain mtDNA clade; a haplotype which is widely distributed across the natural range of this mtDNA clade and is also the most common haplotype observed in the introduced localities in the Netherlands. A genome-scale dataset would be required to try and provide further insight on the provenance of populations bearing this naturally widespread haplotype.

In one of those two England localities where the Eastern/Western Europe & Spain mtDNA clade is present, we also find the same Italian mtDNA clade haplotype (IT_7) that has also been introduced in Oudeland in the Netherlands (see above) as well as in New Zealand (Arntzen et al., 2016). Again this raises a question about the extent of genetic admixture between two potential cryptic alpine newt species. The same Italian mtDNA clade haplotype is the sole haplotype present in the third locality from England we studied. Many more introduced alpine newt localities, in large part revealed by citizen science records, are known from England (Allain and Lynn, 2021) and Scotland (C. Cathrine, unpublished data); additional sampling is required to get a better understanding of the number of different geographical stocks involved and where these occur in syntopy.

Based on our extended sampling across the natural range, a good number of introduced alpine newt localities can now be connected to a native source population. Yet, the genetic homogeneity in temperate Europe makes it difficult to pinpoint the provenance of many others (a well-known limitation of the mtDNA barcoding of invasive species, see e.g., Vliegenthart et al., 2023). We can confirm that multiple distinct sources are involved. How did

these alpine newts get introduced? In most cases this is unknown. Alpine newts are common in the pet trade and release by owners seems the most plausible route (Arntzen et al., 2016), with an additional possible transmission route being 'hitchhiking' (perhaps as eggs) in aquatic vegetation (Schmeller et al., 2020). Ironically, alpine newts in Ratho, Scotland were introduced because they were mistakenly thought to be the native great crested newt (*T. cristatus*); the population is now expanding (C. Cathrine, unpubl. data).

A particular risk when closely related species or distinct populations of a single species are brought into contact with native counterparts is 'genetic pollution', where native gene copies are driven to extinction, because they are replaced by invasive ones (Rhymer and Simberloff, 1996; Huxel, 1999; Meilink et al., 2015; Todesco et al., 2016; Dufresnes et al., 2017). In this context, the introduction of highly distinct alpine newt mtDNA clades, presumably representing cryptic species, close to native populations, is of particular concern. Given the limitations associated with the use of a single DNA marker such as mtDNA, genome-scale data would be required to screen for potential genetic pollution (Wielstra et al., 2016).

*Swabbing by citizen scientists*

This project benefited from citizen science. Involving citizens in scientific projects helps to reduce the gap between science and society (Devictor et al., 2010; Bonney et al., 2016). Most of our samples from the Netherlands were collected by volunteers of NGO RAVON (Reptile, Amphibian and Fish Conservation Netherlands) to whom we sent swabs with detailed instructions on how to use these. Evidently, this was an efficient way to amass geographically dense sampling (fig. 1, supplementary table S1).

We show that the success rate of skin swabbing, in terms of obtaining an mtDNA barcode, is the same as for buccal swabbing. Skin swabs have a wider application than simple mtDNA barcoding. Both skin and buccal swabs have been used to obtain nuclear DNA data, including microsatellite (Broquet et al., 2007; Prunier et al., 2012) and KASP genotyping (Fahrbach et al., 2021; de Brouwer et al., 2023). Buccal swabs are also being applied in amphibians to obtain genome-scale datasets via RAD-sequencing (Ambu and Dufresnes, 2023) and we would recommend testing skin swabs for this purpose as well (while DNA concentrations of skin swabs are lower than those of buccal swabs, many should yield sufficient DNA for RAD-sequencing).

To the best of our knowledge, the stress associated with buccal and skin swabbing on amphibians has never been quantified. However, it is reasonable to assume that skin swabbing is relatively less stressful than buccal swabbing (Ringler, 2018). Our study illustrates the potential of the technique, particularly in projects on aquatic amphibians where conventional molecular techniques are applied and citizen scientists are involved.

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