A new population of European cave salamanders (genus *Hydromantes*) from west-central France: relict or introduction?

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INTRODUCTION

European cave salamanders, all belonging to the genus *Hydromantes*, are the only members of the family Plethodontidae living in Europe and are furthermore characterised by a unique disjunct distribution, with three American species living in California and eight European ones occurring in France and Italy, including Sardinia (Wake, 2013). This odd range is likely the result of Europe colonisation by *Hydromantes* from North America through the Beringia land bridge (Shen et al., 2015) and it is probably a relict of a past widest distribution, as witnessed also by fossil records from Slovakia (Venczel & Sanchiz, 2005) and by the recent discovery of the only known Asian plethodontid salamander, *Karsenia coreana* (Min et al., 2005).

Hydromantes salamanders miss an aquatic larval stage, having direct-developing larvae, and lack lungs, so needing moist and fresh retreats warranting suitable conditions for their skin-mediated respiration. They are therefore characterised by poor dispersal ability, subtroglophile habits and a mainly nocturnal activity, making hard the finding of new populations without a great sampling effort. Accordingly, several new sites inhabited by *H. strinatii* have been recently discovered in France, showing a wider and more continuous distribution than previously thought, despite this was a widely studied species (Renet et al., 2012). Due to their cryptic nature, the discovery of a new population of H*ydromantes* in France, in March 2015, would be relatively unsurprising unless this finding has some peculiar features that makes it worth of in depth studies. The site is located in west-central France in the Vienne County, at Angles-sur-l'Anglin, more than five hundreds kilometres outside the known range of the French Cave Salamander *H. strinatii* (Fig.1), whose range goes from north-western Italy to south-eastern France (Lanza et al., 2005). This is a limestone area of the Poitou-Charentes region hosting many caves, providing suitable natural habitats for European cave salamanders and so suggesting the finding of a possible relict population. On the other hand, it is known that in the past decades some experiments of translocation/introduction were made throughout the mainland part of European *Hydromantes* range. These experiments were only seldom publicised in the scientific literature, indicating that further unknown introductions may have occurred. Therefore, we decided to investigate the genetic structure of the population from Angles-sur-l'Anglin to discriminate between the hypotheses of having found a new relict in a suitable area or, more likely, an introduced population and, in this last case, to assess from which species and area the founders come from.
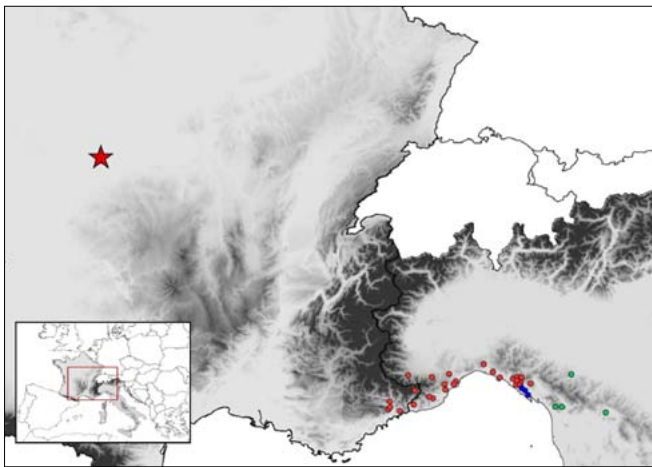
METHODS

Nine individuals were sampled in August 2015 in a natural cavity located on a Natura 2000 area ("Vallée de l'Anglin" – FR5400535) from Angles-sur-l'Anglin (AsA). Permissions were issued by order of the prefect [2015-DRCLAJ/BUPPE-173], according to French law. Tissue samples were obtained cutting the tail tip from each individual and total DNA was isolated using the CTAB protocol slightly modified from Doyle and Doyle (1987). Two mitochondrial regions comprised within the genes cytochrome-b (cytb) and NADH dehydrogenase subunit 2 (ND2) were amplified using primers and PCR conditions as reported in Cimmaruta et al. (2015). PCR products were purified and sequenced by Macrogen Inc. (www.macrogen.com). After visual electropherograms checking, the sequences were deposited in GenBank (KX347904 and KX347905 for Cytb; KX347911and KX347912 for ND2). To assign the obtained sequences to a species of *Hydromantes*, they were compared to representative sequences of the three mainland species: six *H. strinatii* sequences were recovered from Cimmaruta et al. (2015) while 4 specimens of *H. italicus* (from Florence, Lucca and Reggio Emilia) and 2 of *H. ambrosii ambrosii* (from the environs of La Spezia) were specifically sequenced for cytb and ND2 (KX347898-KX347903 and KX347906-KX347910 respectively). These sequences were used to build a maximum likelihood (ML) tree in RAxML GUI v.1.3 (Silvestro and Michalak, 2011). We set a GTRGAMMA model with partitions by gene, one thousand bootstrap replicates and ten independent runs. A median-joining network was then built on a concatenated alignment consisting of the whole *H. strinatii* mitochondrial dataset from Cimmaruta et al. (2015) and the nine sequences from AsA, using Network v.4.6.1.2 (http://www.fluxus-engineering.com) under Greedy FHP criterion (Bandelt et al., 1999; Foulds et al., 1976).
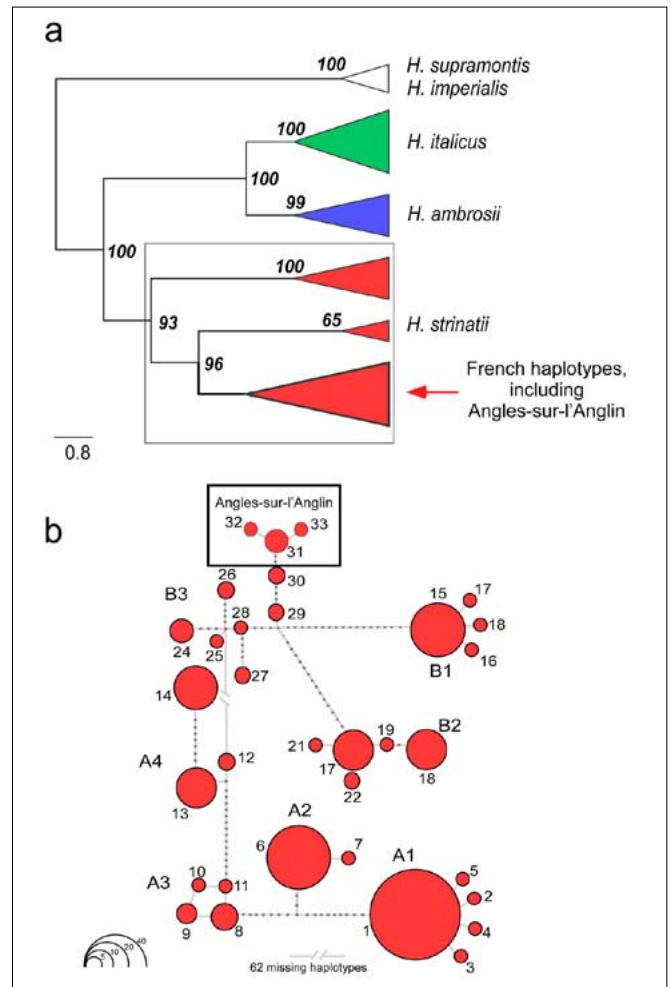
RESULTS AND DISCUSSION

The fragments obtained from the nine individuals from AsA were 638 bp long for cytb and 676 bp for ND2, providing a final concatenated alignment of 1314 bp. The 9 specimens from AsA provided three haplotypes (named 31, 32, 33), which differed for one or two mutations from each other.

ML analysis strongly supported the clusterisation of AsA haplotypes within the *H. strinatii* clade, where they grouped with the samples from the French Maritime Alps (Fig.2a). The same result was obtained by the outcome of the median-joining network reported in Fig.2b, where 30 haplotypes from the entire species range were included. The three haplotypes from AsA resulted directly connected to the haplogroup from Col de Tende (haplotypes 29 and 30), from which they are separated by 3 (haplotype 31) or 4 (haplotypes 32 and 33) mutational steps.

Figure. 1 Distribution map of the samples considered in this study. Green circles are *H. italicus*, blue circles are *H. ambrosii*, red circles are *H. strinatii*. The red star spots the location of the new population from Angles-sur-l'Anglin.

These data showed that the three haplotypes recovered from Angles-sur-l'Anglin tightly nested within *H. strinatii*, assigning to this species the newly discovered locality. In particular, the three haplotypes showed a greater affinity with *H. strinatii* populations from the French Maritime Alps in the Col de Tende area, as shown by the network analysis. The comparison between the high geographical distance and the low genetic differentiation between Angles-sur-l'Anglin and Col de Tende, indicates that the discovered population is the result of a human introduction, rather than a relict. In fact, such a geographical distance is far superior to that between populations from the two main lineages found within *H. strinatii*, living respectively in the western (French Maritime and Ligurian Alps) and eastern (eastern Liguria) parts of its range. These are indeed separated by an amount of 62 mutational steps over a geographic distance of tens of kilometres (Cimmaruta et al., 2015), while Angles-sur-l'Anglin and Col de Tende are hundreds of kilometres distant but show only 3 to 4 mutational steps from each other. The haplotypes shown by the nine individuals from Angles-sur-l'Anglin were however unique, never observed in other previously examined French sites, preventing

Figure. 2. a) Maximum likelihood tree built on concatenated sequences of cytb and ND2 of *H. italicus* (green), *H. ambrosii* (blue) and *H. strinatii* (red). Bootstrap values are showed on each node. The Sardinian species *H. supramontis* and *H. imperialis* represent the outgroups. **b)** Median-joining network based on concatenated sequences of cytb and ND2 including 194 sequences of *H. strinatii* from Cimmaruta et al. (2015) and the nine sequences from Angles-sur-l'Anglin of this study (squared). Haplogroups A1-4 and B1-3 are named as in Cimmaruta et al. (2015).

the identification of the exact source of the introduced population. This finding evidences the need of expanding the genetic survey of *H. strinatii*, with particular regard to the Maritime Alps, an area with a peculiar paleoclimatic history resulting in particularly high levels of biodiversity (Schönswetter et al., 2005) and in highly fragmented and genetically differentiated populations of *H. strinatii*, each one characterised by private haplotypes in this zone (Cimmaruta et al., 2015 and Fig. 2b).

The discovery of an introduced population is not surprising since other introductions of *Hydromantes* took place in the past, either within or outside the range of the genus and with different aims. For example, the parapatric species *H. strinatii* and *H. a. ambrosii* were put in syntopy in the southernmost part of *H. strinatii* range to demonstrate the role of competitive interactions in generating replacement patterns (Cimmaruta et al., 1999). An artificial syntopy between *H. italicus* and *H. a. ambrosii* was carried out outside the genus range, in Tuscany, to test for their

reproductive isolation (Forti et al., 2005; Cimmaruta et al., 2013). Other introductions took place between 1965-70 in a mine gallery of the French Pyrenees (in the Salat Valley, Ariege) by Durand and in north-eastern Italy (Gorizia) where *H. strinatii* specimens were released (Lanza, 2005). In both cases the populations survived and settled in the sites. These data show that *Hydromantes* introductions may easily result in local viable populations, provided that the site has subterranean suitable retreats, which is also the case of Angles-sur-l'Anglin where, after the discovery of the site in March 2015, up to 28 individuals were recovered simultaneously including some juveniles, so suggesting that the population is reproducing and has permanently settled in the site.

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