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SHORT COMMUNICATION

Introgressive hybridization of threatened European tree frogs (*Hyla arborea*) by introduced *H. intermedia* in Western Switzerland

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Abstract Hybridization by introduced taxa is a major threat to native species. Characterizing human introductions is thus one of the missions of conservation geneticists. Here we survey a declining population of the regionally endangered European tree frog (*Hyla arborea*) in the Grangettes natural reserve (Rhone valley, Western Switzerland), where previous evidence indicated human introduction of the Italian taxon *Hyla intermedia*. We combined fast-evolving mitochondrial and nuclear markers and an extended sampling to conduct population genetic analyses of the Grangettes and putative source areas. We show that the Grangettes population is a hybrid swarm, with all individuals featuring recent nuclear admixture and mitochondrial DNA of introduced *H. intermedia*, most likely of proximate south Alpine origin. In contrast, *H. arborea* and *H. intermedia* hardly introgress in their natural parapatric ranges, consistent with an advanced reproductive isolation. From our results, even distantly-related species can endanger the genetic integrity of local taxa in the context of introductions. Potential hybrid incompatibilities may thus account for the strong decline of this population, despite important conservation efforts. Although their hybrid nature makes them a priori unworthy of any protection, we propose specific measures to recover local *H. arborea* gene pool and preserve tree frogs in the Grangettes, the last population remaining from this heavily impacted part of the Alps.

Keywords Conservation · Exotic species · Human introduction · Hybridization · *Hyla*

Introduction

Hybridization by human-introduced taxa is a major threat to native species (Rhymer and Simberloff 1996; Allendorf et al. 2001). Lower viability and fertility of hybrids can diminish the overall fitness of indigenous populations, and genetic introgression may admix and eventually replace their gene pools. In both cases, it raises important management questions (Rhymer and Simberloff 1996; Allendorf et al. 2001). Identifying exotic and hybrid *versus* native populations is thus fundamental to prioritize protection plans. Such task often requires genetic tools, particularly where little or no reliable phenotypic differences exist between species (e.g. Dubey et al. 2014).

Many European amphibians had their distributions drawn by Quaternary glaciations, with the Alpine arc isolating lineages inhabiting Peninsular Italy from those ranging in north-western Europe (Dufresnes and Perrin 2015). Nevertheless, multiple human introductions of southern taxa, north of the Alps, have been reported, often with detrimental effects on indigenous species (e.g. crested newts, smooth newts; Meyer et al. 2009; water frogs; Dubey et al. 2014). In this context, European tree frogs present an interesting case: the Italian species, *Hyla intermedia*, naturally occurs in Peninsular Italy and Swiss

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Christophe Dufresnes and Sylvain Dubey have equally contributed to this work.

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Ticino (Canestrelli et al. 2007; Stöck et al. 2012; Fig. 1a), but its mtDNA haplotype was unexpectedly sampled north of the Alps in an isolated western Swiss population (Dubey et al. 2006). This pattern suggests a recent human introduction, as it is situated within the natural range of *Hyla arborea* (Dubey et al. 2006; Fig. 1a).

Although formerly distributed widely throughout Switzerland, tree frogs are now heavily threatened by land use and habitat fragmentation, and considered endangered by national authorities (Schmidt and Zumbach 2005). This decline was particularly pronounced in the Alpine valleys of western Switzerland (east of Lake Geneva), where tree frogs were already extinct by 1980 (Grossenbacher 1988) expect for one isolated population (Grangettes natural reserve), which persists in the area and thus has a potentially high conservation value. Its present situation is yet very precarious: whereas hundreds of breeders were recorded by 1990, the population rapidly declined despite substantial conservation efforts (i.e. biotope refection and creation; JM Fivat pers. com.). However, whether and how the Grangettes tree frogs are worth protecting is bound to their taxonomic and genetic nature, i.e. to what extent was the native *H. arborea* replaced by introduced *H. intermedia* and what are the genetic consequences. To address these questions and inform managing authorities, we conducted a population genetics survey of the Grangettes and putative source populations, using fast-evolving nuclear and mitochondrial markers.

Methods

DNA sampling, marker genotyping and sequencing

Tree frogs were captured from the Grangettes reserve at two different time points (2002, $n = 11$ and 2011, $n = 12$), as well as from two other populations of the Swiss plateau confirmed as native *H. arborea* (Dubey et al. 2009; Dufresnes et al. 2013; $n = 63$) and from six proximate populations of *H. intermedia* distributed south of the Alps ($n = 55$), for a total of 141 individuals (Table 1). DNA was sampled using non-invasive buccal swabs (adult frogs, Broquet et al. 2007) or from ethanol-fixed tadpoles, and extracted with the Qiagen Biosprint robotic workstation.

All individuals were genotyped for 22 microsatellite markers cross-amplifying in both *H. arborea* and *H. intermedia* (Dufresnes et al. 2014a) and potentially covering most of the 12 *Hyla* chromosomes (16 markers mapped to at least 12 unlinked genomic regions, including eight linkage groups; Dufresnes et al. 2014b). Protocols followed Dufresnes et al. (2014a, b). Included markers are listed in Table S1.

Fig. 1 **a** Natural distribution ranges of *H. arborea* (*green*), *H. intermedia* (*blue*) and location of our study area (*red square*); **b** Bayesian clustering of individuals microsatellite genotypes; **c** principal component analysis (PCA) of microsatellite genotypes. *Bar plots* show assignment coefficients to *H. arborea* (*green*) or *H. intermedia* (*blue*) groups, averaged per population on the map. Pie charts are proportional to sample size; the PCA first two axes explain 19.2 and 5.6 % of the total variance, respectively. (Color figure online) ▶

To get further insights on how *H. intermedia* was introduced, we sequenced ∼900 bp of the mitochondrial *cytochrome-b* (methods: Dufresnes et al. 2013) in a total of 50 frogs from the Grangettes and proximate *H. intermedia* populations, complemented by seven published sequences (FJ226852-FJ226853, JF318122- JF318125, JX192589).

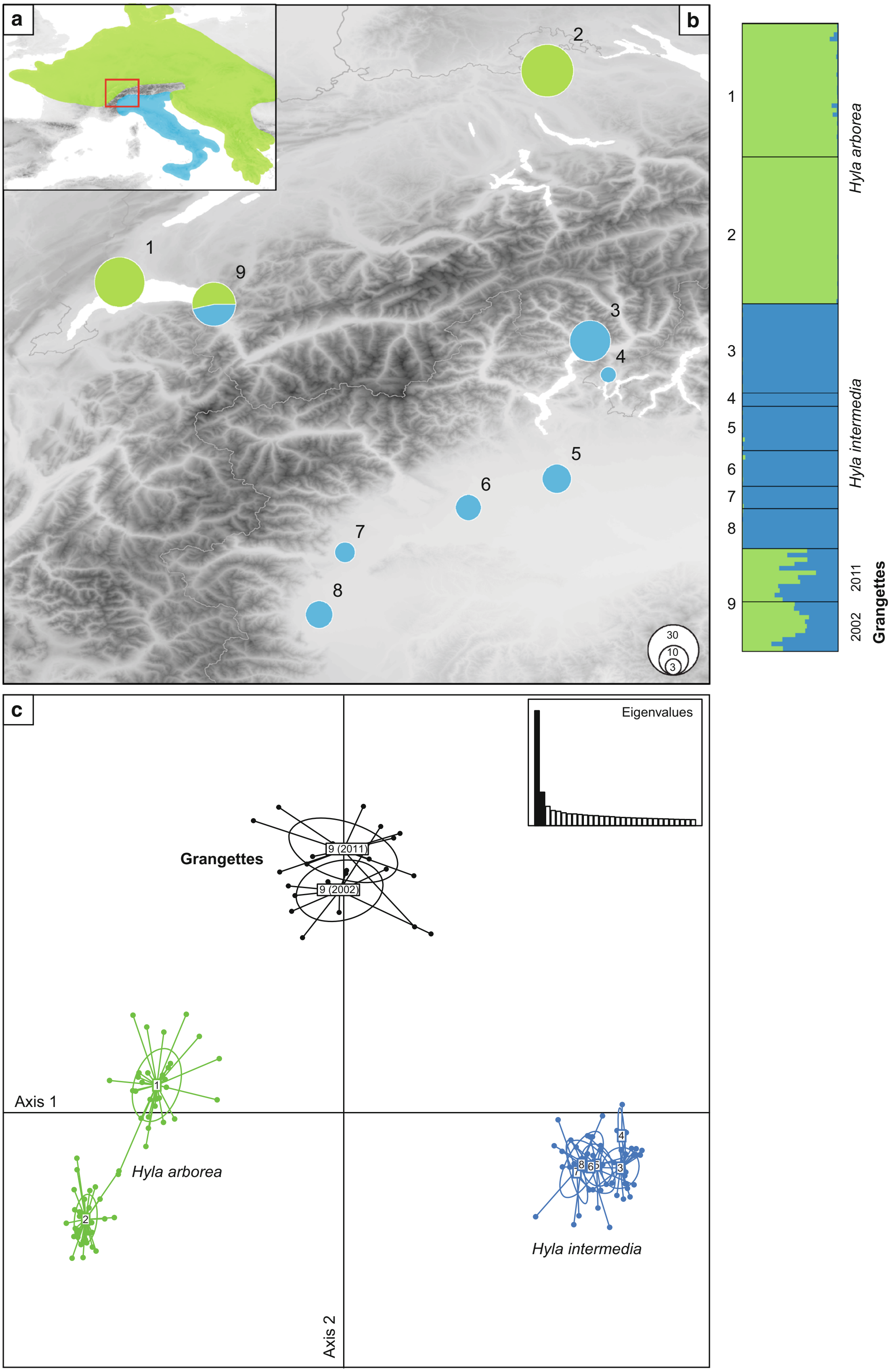
Population genetic analyses

Three sets of analyses were performed on our nuclear dataset. First, we conducted Bayesian clustering of microsatellite genotypes into groups with STRUCTURE (Pritchard et al. 2000), using the admixture model without prior on sample origin, and tested from one to 10 groups (K) with 10 replicate runs per K. Each run consisted of 100,000 iterations following a burn-in of 10,000. The most likely number of groups was determined by the Evanno method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). Replicates were combined using CLUMPP (Jakobsson and Rosenberg 2007) and graphical displays of posterior point estimates of the admixture proportions (barplots) were obtained with DISTRUCT (Rosenberg 2004). Second, we decomposed the genetic variation of microsatellite genotypes by a Principal Component Analysis (PCA, *adegenet* R package, Jombart 2008). Third, we computed pairwise F_{st} between populations where $n \geq 8$, as well as allele frequencies for each species and the Grangettes population in FSTAT (Goudet 1995).

Maximum-likelihood phylogenetic reconstruction of the sampled mtDNA haplotypes was performed with PHYML (Guindon and Gascuel 2003) using a HKY + I model of sequence evolution (MrAIC, Nylander 2004) and 1000 bootstrap replicates. The southern clade of *H. intermedia* (∼9 % of *cyt-b* divergence, Canestrelli et al. 2007) was used as outgroup.

Results

Seventeen *cyt-b* haplotypes were sampled in *H. intermedia* populations, with little association to geography (Fig. 2). Since at least 2002, a single *H. intermedia* haplotype (H16)


a
b
1
2
3
4
5
6
7
8
9
30
10
3
Hyla arborea
Hyla intermedia
2011
2002
Grangettes
c
Eigenvalues
9 (2011)
9 (2002)
Grangettes
Axis 1
Axis 2
Hyla arborea
Hyla intermedia

was fixed in all individuals from the Grangettes, elsewhere found in only one locality (Swiss Ticino, loc. 3). However, this haplotype is closely related (singleton) to haplotypes H09-H15, segregating across all populations (Fig. 1).

Bayesian clustering of microsatellite genotypes unambiguously recovered two groups (Δ-K = 1045.6), corresponding to *arborea* and *intermedia* gene pools, and to which all individuals from putatively pure populations were expectedly assigned (Fig. 1b). In contrast, all Grangettes frogs show intermediate probabilities of assignment, indicating introgressive hybridization. A similar result also stems from the PCA: samples from the Grangettes feature intermediate scores on the first axis (Fig. 1c), which mainly accounts for interspecific differences (19.2 % of the total variance). The PCA highlighted genetic homogeneity within native *H. intermedia* populations, but some differentiation between *H. arborea* from western and eastern Switzerland (Fig. 1c), which was also depicted by pairwise F_{st} (Table 1). Interestingly, the second PCA axis summarized genetic variance that was specific to the Grangettes population (5.6 % of the total variance). No private alleles were found for all but two loci in this population ($\sim$3 % of the total number of alleles), which only possesses subsets of *H. arborea* and *H. intermedia* diversity (Table S1), in further support of its recent hybrid nature. Our two different time samples were not significantly differentiated, but the 2011 samples featured higher F_{st} with all pure populations compared to 2002 samples (Table 1).

Discussion

Corroborating previous studies (Dubey et al. 2006, based on $\sim$300 bp of mtDNA sequences), all Grangettes tree frogs possess *H. intermedia* mtDNA. However, their nuclear genome is a mixture of *H. arborea* and *H. intermedia*, in support of a recent introduction of *H. intermedia* followed by introgression with local *H. arborea*. The fixation of a single *cyt-b* haplotype since at least 2002 points to a unique source; but because south-Alpine *H. intermedia* populations show little genetic differentiation, its precise origin remains unclear. The genomic portion of *H. arborea* from the Grangettes tree frogs is closely-related to proximate western Swiss individuals (loc. 1), as one would expect: the most westerly *H. arborea* populations (including western Switzerland) were shown to be genetically homogeneous, but isolated by distance from more eastern populations (including eastern Switzerland; Dufresnes et al. 2013).

Several processes may lead to this contrasted pattern between mitochondrial and nuclear markers in the Grangettes. First, a female-biased introduction would increase the chance of fixing the maternally transmitted mtDNA of *H. intermedia*, while *H. arborea* haplotypes may have been lost by drift. The Grangettes population has been completely isolated and maintained low population size for the last 25 years (<30 breeding males on average, JM Fivat pers. com.), preventing inflocks of *H. arborea*, and enhancing genetic drift. This may account for the lesser differentiation from native populations of our older compared to recent samples, for which drift had acted for longer. Second, such pattern may also arise from asymmetric hybrid incompatibilities i.e. offspring of female *H. intermedia* being fitter than offspring of female *H. arborea*. These two species diverged since the Mio-Pliocene period ($\sim$5 Mya, Stöck et al. 2012) and hardly hybridize at their secondary contact zone in northeastern Italy (Verardi et al. 2009), suggesting advanced reproductive isolation and thus probable hybrid dysfunctions (see also Dufresnes et al. 2015). These may stem from genetic and/or ecological post-

Table 1 Origin, sample size, and nuclear genetic differentiation (pairwise F_{st}) of the tree frog populations included in this study

Species	Locality	Lat.	Long.	n	*H. arborea*		*H. intermedia*				Grangettes (**9**)	
					1	2	3	5	6	8	2011	2002
H. arborea	**1**–CH, Lavigny	46.50	6.42	30	–	0.18*	0.49*	0.46*	0.45*	0.46*	0.30*	0.25*
	2–CH, Flaach	47.58	8.60	33		–	0.59*	0.57*	0.57*	0.57*	0.47*	0.43*
H. intermedia	**3**–CH, Piazzogna	46.14	8.82	20			–	0.10*	0.07*	0.14*	0.40*	0.36*
	4–IT, Lugano	46.03	8.91	3								
	5–IT, Cameri	45.50	8.65	10				–	0.07	0.02	0.37*	0.32*
	6–IT, Santhià	45.35	8.20	8					–	0.05	0.36*	0.32*
	7–IT, Druento	45.12	7.57	5								
	8–IT, Palot	44.81	7.44	9						–	0.36*	0.32*
Grangettes	**9**–CH, Grangettes (2011)	46.39	6.48	12							–	0.03
	9–CH, Grangettes (2002)	46.39	6.48	11								–

F_{st} was only calculated when $n \geq 8$ (* $p < 0.05$)

Lat. latitude, *Long.* longitude, *CH* Switzerland, *IT* Italy

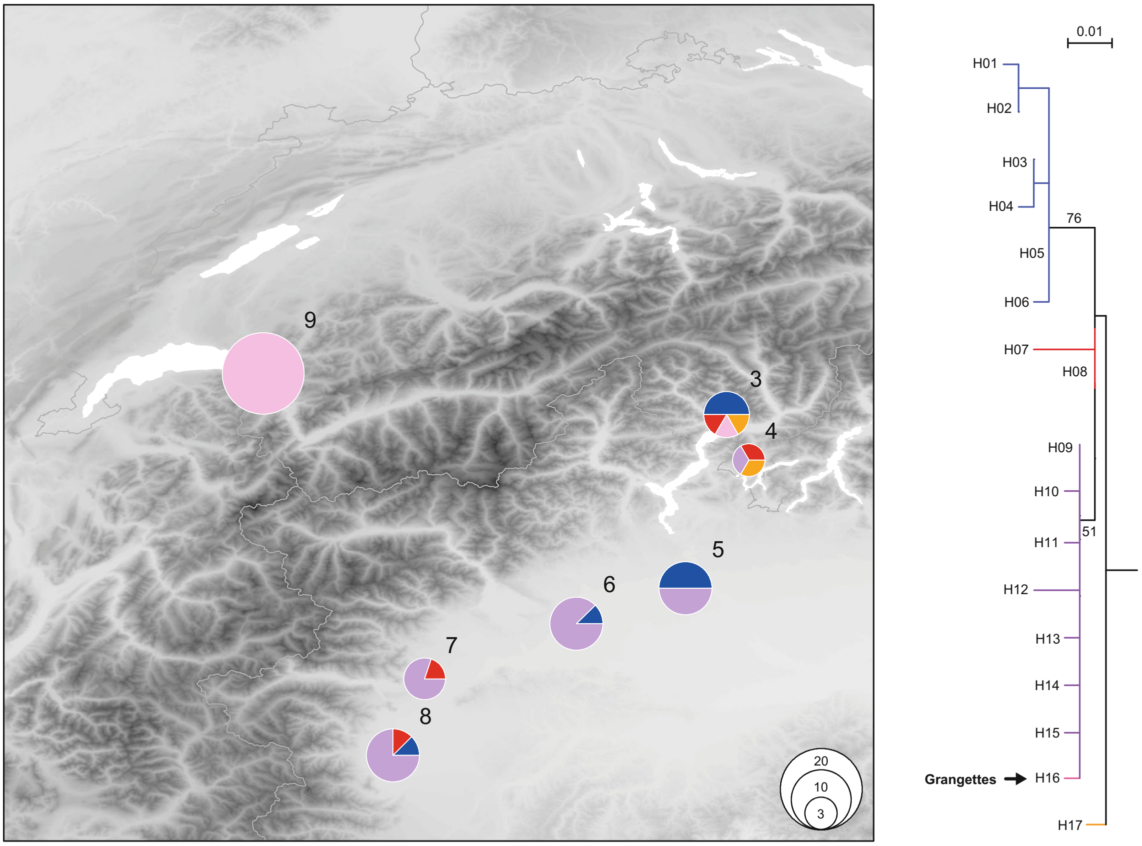


Fig. 2 Phylogenetic relationships of *H. intermedia cytochrome-b* haplotypes and their geographic distribution. *Colors* highlight closely-related alleles for visualization. Only one haplotype (H16, shown in *pink*) was sampled in all Grangettes individuals from both time points. Pie charts are proportional to sample size. (Color figure online)

zygotic incompatibilities, reducing the fitness of individuals in this population. In natural contact zones, admixture is most likely prevented by pre-zygotic barriers and reinforcement (e.g. assortative mating through advertisement calls, Höbel and Gerhardt 2003). These processes may be less efficient in the small and isolated Grangettes population: the lack of opportunity for mate choice in small populations are expected to weaken pre-zygotic isolation, particularly as the calls of these two species only show slight differences, at least in allopatric populations (Schneider 2004). Thus, from our results, its long-term decline despite important conservation efforts, might reflect incompatibilities stemming from hybridization. Experimental crosses aiming at measuring hybrid fitness may help testing this hypothesis and understand the underlying causes.

From a conservation point of view, whether to include anthropogenic hybrid populations in protection plans is controversial (Kraus 1995; Allendorf et al. 2001). Hybridization is a source of diversity but introgressive hybridization due to human disturbances (linked to the introduction of non-native species) can compromise the integrity of natural species to the point of causing population extinctions. To help prioritize conservation actions, Allendorf et al. (2001) proposed a classification depending on the level of introgression, in which the Grangettes population falls under “Type 5: widespread introgression”, a category considered of little conservation value. Nevertheless, our findings stress the need for specific management planning. Here, we propose to purge the exotic *H. intermedia* genome by frequent translocation of *H. arborea* individuals (e.g. clutches) from large, closely-related indigenous populations (e.g. loc. 1). Alternatively, the current hybrid population could be exterminated and fully replaced by native *H. arborea* captured nearby. The most appropriate action shall depend on the mechanisms allowing interbreeding: purging through regular translocations would only be efficient if pre-zygotic isolation is weak and hybrid incompatibilities are limited; full replacement would be more suitable otherwise. Restoring the native gene pool should eliminate putative incompatibilities, which in turn may help overriding the decline of this population. Nevertheless, the current isolation of the Grangettes (>40 km away from the closest occupied sites) might still remain an issue for its long-term persistence. Future measures should also enhance habitat connectivity at the regional scale (which had proven efficient for *Hyla*, Angelone and Holderegger 2009), in order to preserve the last Alpine population of tree frogs from western Switzerland.

Our analyses provide strong evidence for the introgressive hybridization of a valuable European tree frog population by an introduced exotic species. This result emphasizes that even distantly-related species can endanger the genetic integrity of local taxa in the context of introductions. Future research documenting individual hybrid fitness should help understanding the mechanism(s) of reproductive isolation involved, and especially how it relates to the decline of this protected population. These findings should inform efficient and appropriate management planning.

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Compliance to ethical standards

Conflict of interest The authors declare no conflict of interest. Our study was approved by the Service de la Consommation et des Affaires Vétérinaires du Canton de Vaud (Epalinges, Switzerland; authorization No.°1798) and our research was carried out in compliance with the Convention on Biological Diversity (CBD) and Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES); DNA of adult frogs was sampled using non-invasive techniques and tadpole collection was done without impact on populations.

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