

# Invasion genetics of marsh frogs (*Pelophylax ridibundus sensu lato*) in Switzerland

CHRISTOPHE DUFRESNES<sup>1,\*</sup>, JULIEN LEUENBERGER<sup>2,3</sup>, VALENTIN AMRHEIN<sup>4</sup>, CHRISTOPH BÜHLER<sup>5</sup>, JACQUES THIÉBAUD<sup>6</sup>, THIERRY BOHNENSTENGEL<sup>7</sup> and SYLVAIN DUBEY<sup>2,5</sup>

<sup>1</sup>Department of Animal & Plant Sciences, University of Sheffield, Alfred Denny Building, Western Bank, Sheffield S10 2TN, UK

<sup>2</sup>Department of Ecology & Evolution, University of Lausanne, Biophore Building, CH.1015 Lausanne, Switzerland

<sup>3</sup>Botanical Gardens and Museum of the Canton de Vaud, Avenue de Cour 14bis, CH-1007 Lausanne, Switzerland

<sup>4</sup>Zoological Institute, University of Basel, Vesalgasse 1, CH-4051 Basel, Switzerland

<sup>5</sup>Hintermann & Weber SA, Rue de l'Eglise-Catholique 9b, 1820 Montreux, Switzerland

<sup>6</sup>karch-GE (Swiss Coordination Center for the Conservation of Amphibians and Reptiles – Geneva Regional Branch), 1200 Geneva, Switzerland

<sup>7</sup>info fauna – CSCF & karch, Passage Maximilien-de-Meuron 6, CH-2000 Neuchâtel, Switzerland

Received 7 September 2017; revised 21 October 2017; accepted for publication 22 October 2017

The marsh frog (*Pelophylax ridibundus* s.l.) is the number one amphibian invader in Western Europe. In Switzerland, marsh frogs were introduced in the 1950–1960s and progressively colonized most of the northern parts of the country. We investigated this invasion using molecular tools. We mapped the cryptic presence of three monophyletic mitochondrial lineages (*P. ridibundus*, *Pelophylax kurtmuelleri*, and *Pelophylax* cf. *bedriagae* from southeastern Europe) consistent with registered importations by a local frog-leg industry. High nuclear diversity supports that invasive frogs probably originated from genetically rich import batches, and patterns of population differentiation confirm that multiple independent introduction sites were involved. Moreover, several lines of evidence suggest occasional hybridization with local hybridogenetic water frogs. This invasion emphasizes the issues of frequent amphibian releases and translocations at the international and regional scale for commercial and recreational purposes, and stresses the need for more adequate legislation, control, and information for the general public. Given the parallel invasion by exotic pool frogs (i.e. the Italian *Pelophylax bergeri* has replaced the local *Pelophylax lessonae*), the situation of water frogs in Switzerland is critical. The water frog complex provides an alarming symbol of the anthropogenic mark left on wildlife diversity and distributions.

**ADDITIONAL KEYWORDS:** alien species – biological invasions – conservation – hybridogenesis – introduction – water frogs.

## INTRODUCTION

The management of biological invasions can benefit from genetic surveys to characterize the nature, sources, and dynamics of invaders in space and time. Molecular tools are also necessary to understand whether native closely related taxa are threatened by hybridization

with invasive ones. This is particularly true for amphibians, where closely related species are often morphologically similar and thus impossible to monitor by biometric means (e.g. Dufresnes *et al.*, 2015, 2016).

Marsh frogs from the *Pelophylax ridibundus* species complex [i.e. *Pelophylax ridibundus sensu lato* (s.l.)] are the most problematic amphibians in Western Europe (Holsbeek & Jooris, 2010; Holsbeek *et al.*, 2008). This complex involves at least seven species, including *P.*

\*Corresponding author. E-mail: C.Dufresnes@sheffield.ac.uk

*ridibundus sensu stricto* (s.s.), *Pelophylax kurtmuel-leri*, *Pelophylax cerigensis*, *Pelophylax* cf. *bedriagae*, *Pelophylax cypriensis*, *Pelophylax cretensis*, and *Pelophylax epeiroticus*. Marsh frogs were introduced in the mid-20th century from Eastern Europe by the frog-leg industry and have since invaded most of France, Belgium, the Netherlands, Germany, and Switzerland, as well as parts of Britain. These large frogs are causing various ecological damage, such as competition with and predation on smaller amphibians (Roth *et al.*, 2016). Moreover, they can threaten the indigenous *Pelophylax* L-E hybridogenetic system, *P. lessonae* (LL)/*P. esculentus* (R'L), by quickly replacing the native *P. lessonae* (L) and *P. ridibundus* s.s. (R') genomes through hybridization (Supporting Information, Fig. S1). In this system, the hybridogenetic hybrid *P. esculentus* (R'L) eliminates its L genome before meiosis and contribute gametes only with its R' genome (Vorburger & Reyer, 2003). Given that it is transmitted clonally, the R' genome has accumulated deleterious mutations, and R'R' offspring from *P. esculentus* × *P. esculentus* crosses are not viable (Vorburger *et al.*, 2009). However, crosses with introduced marsh frogs (RR), yield marsh frog offspring with viable R'R combinations (Leuenberger *et al.*, 2014; Supporting Information, Fig. S1). Although the invasions are often attributed to the nominal species *P. ridibundus* s.s., genetic studies unravelled the cryptic presence of other taxa, namely the Balkan (*P. kurtmuel-leri*) and Levant water frogs (*P. cf. bedriagae*) (Holsbeek *et al.*, 2010; Dubey *et al.*, 2014; Dufresnes *et al.*, 2017a), sometimes leading to complex patterns of water frog assemblages and distributions, and potentially inducing new hybridogenetic systems (Dufresnes *et al.*, 2017a).

In Switzerland, the historical progression of marsh frogs has been relatively well documented (Grossenbacher, 1988 and references therein; Meyer *et al.*, 2009; Fig. 1). The first reference specimens were collected in 1950 in western Switzerland around the Lemanic basin. Animals probably escaped from the import companies located in the area (e.g. Aigle, Vallorbe). In the following years, several populations were discovered around Lake Geneva and Lake Neuchâtel. The first records of marsh frogs in the eastern parts of the country were reported a decade later (1967 in the canton of Zurich, ZH), and more isolated stations were subsequently identified in additional cantons (Basel, BL; Graubünden, GR; St-Gallen, SG). All of these probably stemmed from releases of imported animals. As a symbolic anecdote, large populations established near Basel and Zurich international airports, where confiscated batches of imported frogs were even released directly on several occasions. In the 1980s and 1990s, they progressively replaced the native *P. esculentus* and *P. lessonae* s.l. in western Switzerland and rapidly propagated eastwards. Populations from eastern Switzerland remained

isolated for decades, but observations have increased in recent years. Marsh frogs now occupy most of the Swiss Plateau and adjacent valleys up to ~1000m a.s.l.

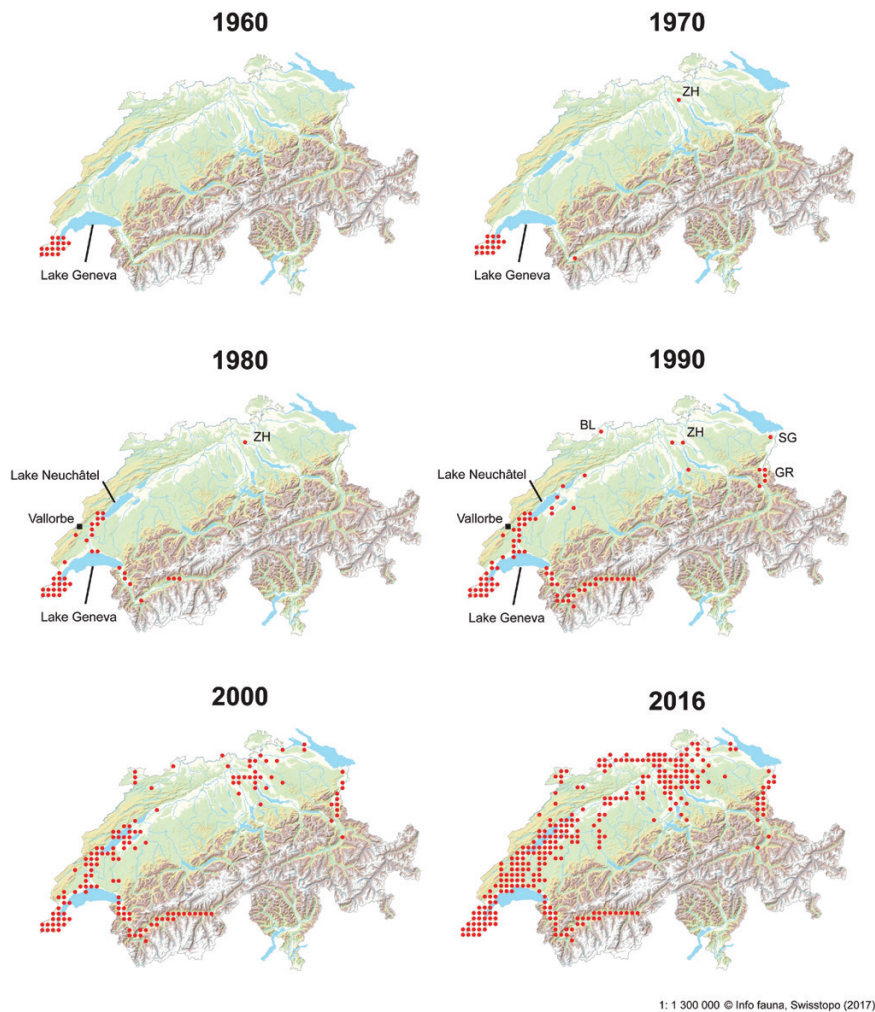
In a recent survey, Dubey *et al.* (2014) identified three mitochondrial lineages of invasive marsh frogs in western Switzerland, namely *P. ridibundus*, *P. kurtmuelleri*, and *P. cf. bedriagae*, based on a few populations. The water frog issue in Switzerland becomes even more complex given that the other hybridogenetic partners, the pool frogs (*P. lessonae* s.l.), are also represented by several hybridizing taxa (*P. lessonae* s.s. and *P. bergeri*; Dubey *et al.*, 2014; Dufresnes *et al.*, 2017b), thus potentially leading to multiple cross-species combinations of marsh frogs, pool frogs, and their hybridogenetic hybrids. As we recently did for pool frogs (Dufresnes *et al.*, 2017b), characterization of the distribution and structure of invasive marsh frog lineages and understanding how they interact with the local gene pool (in this case the native R' hemiclinal genome of autochthonous *P. esculentus*) are necessary to clarify this confusing situation.

In this study, we investigated Swiss marsh (*P. ridibundus* s.l.) and edible (*P. esculentus*) frogs in a dense population genetic framework. Combining mitochondrial and nuclear markers on contemporary and historical samples, we aimed at: (1) mapping the occurrence of the different lineages reported; (2) inferring how the introduction events shaped the genetic diversity and structure of populations; and (3) assessing whether the native marsh frog hemiclones of *P. esculentus* have been replaced during the invasion.

## MATERIAL AND METHODS

### DNA SAMPLING

Tissue samples were obtained from 371 adult marsh (*P. ridibundus* s.l.) or edible frogs (*P. esculentus*) captured from natural populations throughout Switzerland and the neighbouring French Alsace and Jura ( $N = 347$ , buccal swabs and toe clips), as well as from historical museum specimens from the MZL (Musée cantonal de Zoologie, Lausanne, 1961–1967;  $N = 15$ , ethanol preserved) and the MHNG (Muséum d'Histoire Naturelle de la ville de Genève, Geneva, 1978–2002;  $N = 9$ , ethanol preserved). DNA was extracted using the Qiagen Biosprint robotic workstation or the Qiagen blood and tissue extraction kit. Identification as marsh/edible frogs was done morphologically by the shape of the metatarsal tubercle (Nöllert & Nöllert, 2003) and could be verified by diagnostic microsatellite loci in many of the samples ( $N = 239$ ; see section below, 'Microsatellite genotyping and population genetic analyses'). Only animals informative for this study were included, i.e. those carrying marsh frog mitochondrial DNA and/or confirmed as marsh or edible frogs. Sampling details are provided in the Supporting Information (Table S1 and Fig. S2).



**Figure 1.** Records of marsh frogs in Switzerland. Red dots show presence data over 5 km × 5 km quadrats. The first verified records date back to 1950. Note that monitoring effort was higher in the recent (1990s–2010s) compared with the earlier decades (1960s–1980s). Letters refer to the cantons mentioned in the main text. Source: © Info fauna, Swisstopo (2017).

#### MITOCHONDRIAL SEQUENCING AND PHYLOGENETIC ANALYSES

We barcoded the mitotypes of 351 individuals using a short fragment of *cytochrome-b* (113 bp), allowing us to distinguish between all Western Palearctic lineages (methods: Dufresnes *et al.*, 2017b). In addition, we sequenced a larger fragment of *cyt-b* (974 bp) in a subset of 64 samples for phylogenetic analyses (methods: Dufresnes *et al.*, 2017b), representative of the different taxa identified and the geographical range.

We combined our sequences with the reference datasets of Dufresnes *et al.* (2017a), including one with full sequence coverage (974 bp) for phylogenetic discrimination of taxa, and one with lower sequence coverage (515 bp) but more reference sequences from eastern species to infer the phylogeographic origins of our samples (mostly based on Lymberakis *et al.*, 2007). We performed maximum likelihood phylogenetic analyses

of haplotypes with PhyML (Guidon & Gascuel, 2003), using GTR+G+I models of sequence evolution (inferred with jModelTest; Darriba *et al.*, 2012) and assessing bootstrap support with 1000 replicates.

#### MICROSATELLITE GENOTYPING AND POPULATION GENETIC ANALYSES

We genotyped eight polymorphic microsatellite markers with R/R' and L alleles diagnostic of the marsh (*P. ridibundus* s.l.) and pool frog (*P. lessonae* s.l.) genomes, respectively (*Rica5*, *Rica1b5*, *Rica1b6*, *Re1Caga10*, *ReGa1a23*, *Rrid013A*, *Rrid059A*, and *Rica18*) (Dufresnes *et al.*, 2017b and references therein). This allowed us to ascertain identification of our marsh and edible frog samples, the latter featuring R'L or RL hybrid genotypes. Loci were amplified in multiplex PCRs for 237 samples, diluted, and run on an ABI3130 genetic analyser, as described by



Dufresnes *et al.* (2017b). A total of 45 and 192 marsh and edible frogs were identified, respectively. The R or R' haplotypes of edible frogs were phased directly from the data, which is straightforward because of the species diagnosticity of alleles in Switzerland (for a similar approach, see Leuenberger *et al.*, 2014) and the availability of pure RR (marsh frog data from this study) and LL genotypes (pool frog data from Dufresnes *et al.*, 2017b) for comparison.

We explored the genetic structure of marsh frog genotypes (diploid RR or RR' *P. ridibundus s.l.* individuals and haploid R or R' hemiclones from *P. esculentus*) by principal component analyses (PCA; *ade4* and *ade4genet* packages in R). Moreover, we computed population-based statistics of population differentiation (pairwise  $F_{st}$ ) and genetic diversity (expected heterozygosity,  $H_e$ ; allelic richness,  $A_r$ ) in FSTAT (Goudet, 1995), grouping nearby localities into regions (Table 1; only when  $N \geq 4$ ). We tested the association between genetic differentiation and geographical distance by Mantel tests, with 10 000 permutations.

## RESULTS AND DISCUSSION

Our genetic survey clarifies the ongoing invasion of marsh frogs in Switzerland and adjacent areas. We show that import batches involved a mixture of distinct lineages that are likely to have expanded through multiple human-driven stepping-stone introduction events, resulting in the present population structure and high genetic diversity. Moreover, we found evidence of hybridization with local water frogs. These two aspects are discussed in turn, with emphasis on the conservation issues posed by the marsh frog invasions in Western Europe, and particularly in Switzerland.

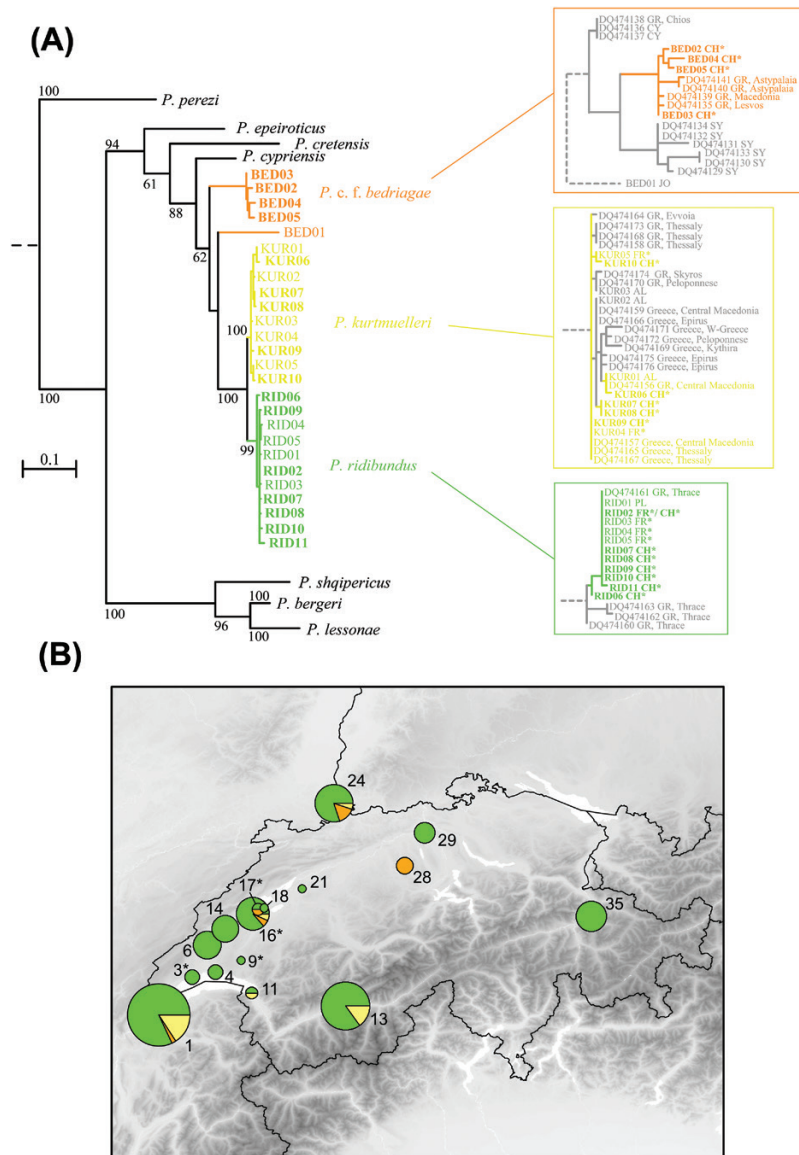
### ALIEN LINEAGES AND GENETIC NATURE OF MARSH FROG INVADERS

We mapped the occurrence of three foreign mitochondrial lineages of marsh frog throughout Switzerland (Fig. 2), extending the previous results of Dubey *et al.* (2014): the European marsh frog (*P. ridibundus s.s.*, seven *cyt-b* haplotypes), the Balkan water frog (*P. kurtmuelleri*, five haplotypes), and the Levant water frog (*P. cf. bedriagae*, western lineage, four haplotypes). Whereas multiple haplotypes suggest many introduction events, these particular lineages point to a single geographical region: southeastern Europe, and more specifically, Thrace/Greek Macedonia, where all three co-occur and harbour a similar genetic diversity (Lymberakis *et al.*, 2007; Fig. 2). Accordingly, the largest company for commercial frog importation in Switzerland, based in Vallorbe (close to our location 14), informed us that their supplies originate from Thrace in European Turkey, and that escapes were common with the equipment used a few decades ago (Fivaz SA, personal communication). Historical specimens from the 1970s–2000s confirmed the early presence of at least *P. kurtmuelleri* and *P. ridibundus s.s.* mitochondrial DNA. The introduced populations thus appear to be a mixture of all three lineages, although largely dominated by *P. ridibundus s.s.* (Fig. 2). Interestingly, except for the widespread RID02, none of our Swiss haplotypes is shared with another similarly surveyed invasive range in France (Dufresnes *et al.*, 2017a; Fig. 2). The nuclear signatures of our Swiss samples are also different (Supporting Information, Fig. S3). Our results thus emphasize that marsh frogs in Western Europe progressed through repeated, independent releases, rather than by natural dispersal from a few initial sites. In Switzerland, historical reports (Grossenbacher, 1988 and references therein)

**Table 1.** Nuclear diversity estimates in Swiss marsh frogs and R/R haplotypes phased from *Pelophylax esculentus*

	Region	Locality	<i>N</i>	$H_e$	$A_r$
R/R haplotypes from <i>Pelophylax esculentus</i>	Jura (France)	2	4	0.21	1.15
	Lemanic Basin	3–11	24	0.23	1.23
	Lake Neuchatel	15–20	94	0.11	1.11
	Bern Pre-Alps	22–23	4	0.17	1.14
	Central Switzerland	25–32	38	0.12	1.12
	St-Gallen	33	7	0.18	1.14
	Graubünden	36–38	19	0.09	1.09
<i>Pelophylax ridibundus s.l.</i>	Geneva	1	17	0.59	1.59
	Lemanic Basin	7–12	4	0.58	1.64
	Lake Neuchatel	16–18	16	0.47	1.47
	Central Switzerland	28	4	0.57	1.58
	Graubünden	35	5	0.52	1.52

$A_r$ , allelic richness, scaled to one diploid individual and thus varying from one to two;  $H_e$ , expected heterozygosity; *N*, sample size.

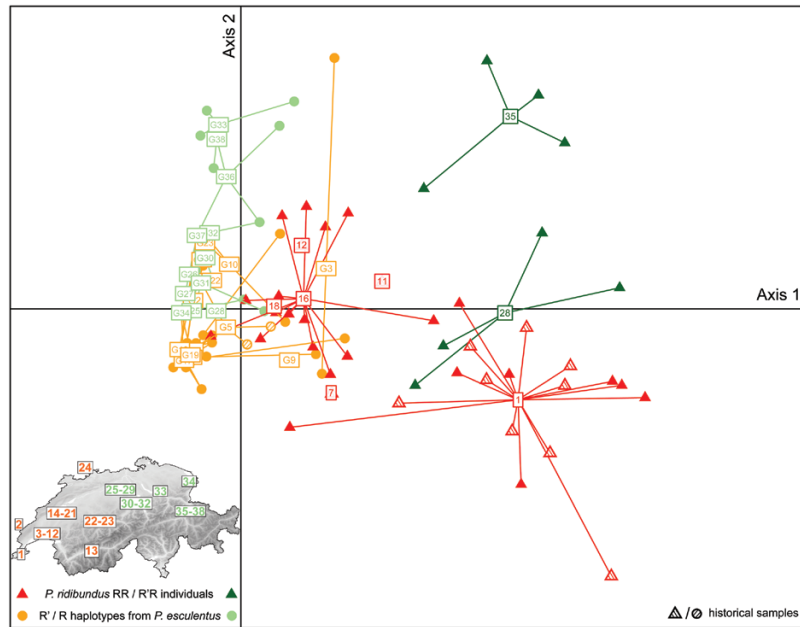


**Figure 2.** A, mitochondrial phylogeny of Western Palearctic marsh frogs. B, distribution of taxa introduced in Switzerland. The tree includes haplotypes analysed by Dufresnes *et al.* (2017a); taxa and haplotypes present in Switzerland are shown in colours and in bold, respectively. Bootstrap support is provided for major nodes. For each taxon identified in Switzerland, we provide details of the corresponding clade, including the phylogeographical data from Lymberakis *et al.* (2007), based on more sequences but lower sequence overlap (see Materials and methods). AL, Albania; CH, Switzerland; CY, Cyprus; FR, France; GR, Greece; JO, Jordan; PL, Poland; SY, Syria. On the map, the pie charts show the relative frequencies of each taxon, with pie size proportional to sample size. Locality codes correspond to Supporting Information, Table S1 and Figure S1.

also support this scenario, as do the complex geographical patterns of expansion, originally composed of several unconnected sites throughout the country (Fig. 1).

In parallel, we found evidence for high nuclear diversity (Table 1) and subtle structure among our marsh frog populations. The PCAs (Fig. 3; Supporting Information, Fig. S4) grouped individuals from the Lemanic Basin/Lake Neuchâtel (localities 7–18),

from the east (locality 35) and from Geneva/Central Switzerland (localities 1 and 28). Pairwise  $F_{st}$  also supported this pattern (Supporting Information, Table S2), with marsh frogs from eastern Switzerland being the most differentiated (average  $F_{st} = 0.22$ ), but with no clear isolation by distance ( $P = 0.08$ ). Individuals from across the canton of Geneva (pooled together as locality 1) did not show signs of nuclear



**Figure 3.** Principal component analysis (PCA) of individual genotypes from marsh frogs (triangles) and the marsh frog hemiclones of edible frogs (circles). The two first axes explain 7.6 and 4.3% of the total variance, respectively. Western (localities 1–24) and eastern Switzerland (localities 25–38) are shown in warm (orange/red) and cold (dark/light green) colours, respectively. The map shows their approximate locations. Historical samples are shown in dashed symbols (locality 1, 1978–2002; other localities, 1960s; see Supporting Information, Table S1 for details).

or mitochondrial structure. Expected heterozygosity and allelic richness were relatively high in all areas ( $H_e = 0.47$ – $0.59$ ;  $A_r = 1.52$ – $1.64$ , scaled to one individual; Table 1). Genetic homogeneity and low diversity are usually expected in the case of biological invasions, owing to founder effects and the strong drift associated with demographic expansions. In contrast, here the fine-scale structure and strong diversity among and within populations confirm that invasive frogs are likely to have originated from genetically rich import batches and expanded from multiple introduction sites. However, except near Geneva (locality 1), the widespread *P. ridibundus* s.s. shows uniform mitochondrial diversity, with a single haplotype (RID02) fixed across the country (Supporting Information, Table S1). This haplotype was probably the most common among released *P. ridibundus* s.s. individuals and soon became fixed in most populations. The Swiss situation is reminiscent of the marsh frog invasion in the UK, characterized by pronounced genetic structure and diversity levels comparable to their original ranges, despite rapid population expansions (Zeisset *et al.*, 2003). As in the UK, urban-centred dispersal by humans is thus likely to have promoted the marsh frog invasion within Switzerland.

The lack of reference genotypes from native *P. ridibundus* s.s., *P. kurtmuelleri*, and *P. cf. bedriagae* precludes any conclusions regarding the exact genetic

nature of the marsh frogs present in Switzerland. We could not assign our genotypes to the well-defined *P. kurtmuelleri* and *P. ridibundus* s.s. clusters invasive in southern France (Dufresnes *et al.*, 2017a; Supporting information, Fig. S3); individuals of different origins are likely to be involved. All three species frequently hybridize in their native parapatric ranges, resulting in long-lasting cytonuclear discordance (e.g. Kolenda *et al.*, 2017). They probably do so in introduced ranges, as previously suggested, e.g. *P. ridibundus* s.s.  $\times$  *P. kurtmuelleri* (Dufresnes *et al.*, 2017a) and *P. ridibundus* s.s.  $\times$  *P. cf. bedriagae* (Holsbeek *et al.*, 2010). Here, the sympatric occurrence of all three mitochondrial lineages, together with the strong individual genetic variation, probably stems from pre- and/or post-introduction admixture. Geographical patterns of nuclear differentiation, which do not mirror the distribution of mitochondrial lineages, also argue in favour of a hybrid swarm between these gene pools. If not limited to neutral loci, the resulting high diversity might actively contribute to the success of marsh frogs in Switzerland, by increasing their potential for local adaptation (e.g. Kolbe *et al.*, 2004).

Altogether, these findings emphasize the clear role of human societies in this invasion, as a consequence of both importation practices and human-driven translocations across the country. Weak legislative and executive actions on international commercial trade

favoured the introductions of genetically diverse alien water frogs for decades (e.g. Holsbeek *et al.*, 2008). At the regional level, frequent uncontrolled releases, usually for ornamental purposes, are a major driver (Dufresnes *et al.*, 2017a). Although the persons responsible are often wildlife enthusiasts ('it would be nice to have wild animals in our garden' or 'better release it than kill it'), their lack of knowledge has promoted the introductions of many amphibian and reptile alien taxa, particularly in Switzerland (Dufresnes *et al.*, 2015, 2016, 2017b; Dubey *et al.*, 2017). Despite being non-native, marsh frogs were historically considered as an enrichment of the Swiss herpetofauna (Escher, 1972).

#### GENETIC INTERACTIONS WITH 'NATIVE' WATER FROGS

Are invasive marsh frogs (RR) genetically replacing the native R' genome of *P. esculentus*? This taxon is most probably native to Switzerland, with records as old as the 19th century (Thierry Bohnenstengel, personal communication). In a local study, Leuenberger *et al.* (2014) found few R'R and RL individuals stemming from crosses between *P. esculentus* (R'L)  $\times$  *P. ridibundus s.l.* (RR) and *P. lessonae s.l.* (LL)  $\times$  *P. ridibundus s.l.* (RR), respectively (Supporting Information, Fig. S1). They concluded that preferences for different microhabitats limited hybridization and that all three species could coexist in the long term.

Here, two lines of evidence confirm that *P. ridibundus s.l.* occasionally hybridizes with other sympatric water frogs, especially in western Switzerland, and probably since the early presence of invaders. As emphasized by Leuenberger *et al.* (2014), they may hybridize with both edible frogs (yielding new R'R marsh frogs) and pool frogs (yielding new RL edible frogs). First, some *P. esculentus* harboured *P. ridibundus s.l.* mitotypes, possibly obtained via ♀ *P. ridibundus s.l.*  $\times$  ♂ *P. lessonae s.l.* F1 crosses (framed RL<sup>rid</sup> in Supporting Information, Fig. S1B). This, however, remains limited to few individuals (seven out of 101 *P. esculentus* coexisting with marsh frogs; Fig. 2). Reciprocally, one marsh frog individual carried mitochondrial DNA from a pool frog, potentially born from a ♀ *P. esculentus*  $\times$  ♂ *P. ridibundus s.l.* cross (framed R'R<sup>les</sup> or RR<sup>les</sup> in Supporting Information, Fig. S1B, C).

Second, part of the observed nuclear structure and diversity of invasive marsh frogs might result from hybridization with local *P. esculentus* (R'L). Marsh frogs from the Vaud canton (localities 7–18) have intermediate PCA scores between most *P. esculentus* hemiclones (supposedly R') and the other marsh frogs (supposedly RR; Fig. 3), and could thus represent R'R individuals (R'R<sup>les</sup> and R'R<sup>rid</sup> in Supporting Information, Fig. S1C). The R and R' genomes may admix through

recombination in these individuals, and subsequent backcrossing by invasive RR marsh frogs will ultimately dilute the R' gene pool. Reciprocally, few *P. esculentus* possess hemiclones that cluster with invasive marsh frogs (supposedly RR; Fig. 3), and may thus represent new RL *P. esculentus* (e.g. localities 3, 9, and 16–18; RL<sup>les</sup> and RL<sup>rid</sup> in Supporting Information, Fig. S1B). Importantly, if these RL individuals were to breed together, they should yield viable RR offspring (as the R genome is free of deleterious mutations), thus boosting the invasion of the R genome (RR<sup>les</sup> and RR<sup>rid</sup> in Supporting Information, Fig. S1B). From this interpretation of the data, the two kinds of hypothetical genotypes (RL and R'R) were already present among historical specimens collected in the 1960s, i.e. during the very first years of the invasions (localities 5, 7, and 11–12). Despite potential differences in microhabitats, all three taxa may thus coexist and hybridize in some populations, as they sometimes do in natural ranges (e.g. Herczeg *et al.*, 2017).

Although such interactions may occur in the west, marsh frogs from eastern Switzerland remained differentiated from the hemiclones of *P. esculentus* (Fig. 3; localities 28–35). Accordingly, in western Switzerland, pairwise  $F_{st}$  between marsh frogs and marsh frog hemiclones phased from *P. esculentus* are much weaker (average  $F_{st} = 0.31$ ) than in the eastern ranges (average  $F_{st} = 0.62$ ; Supporting Information, Table S2). We did not find cytonuclear discordance in the latter either. The more recent spread of marsh frogs in eastern Switzerland may not yet have allowed opportunities for frequent hybridization with other water frogs.

Finally, we found little geographical structure among these *P. ridibundus s.l.* hemiclones across Switzerland. Weak east–west differentiation (Fig. 3; Supporting Information, Fig. S5) was not significantly explained by isolation by distance ( $P = 0.11$ ). Most of the genetic variance stands from outlier haplotypes potentially acquired from the exotic marsh frogs (Supporting Information, Fig. S5). Yet, the pairwise  $F_{st}$  between populations remains strong overall (up to 0.88; Supporting Information, Table S2), which is not surprising given the low effective size of non-recombining clonal genomes.

The native R' genome of autochthonous edible frogs is one of the last authentic entities among Swiss water frogs. The pool frog *P. lessonae* has been replaced by the Italian *P. bergeri* throughout most of the country (Dufresnes *et al.*, 2017b) and adjacent regions (including French Alsace, locality 24; S.D., C.B., and V.A., unpublished data). As a result of these multiple invasions, the situation for water frogs in Switzerland is thus extremely delicate. Conservation measures should prioritize the protection of the remaining wetlands where invaders have not yet been reported, by maintaining and/or restoring habitat quality and preventing alien



introductions. The latter might benefit from raising public awareness regarding the problem caused by translocations, even at the regional level. Given that marsh frogs are preferentially a lowland species, populations from the Alpine and Jura mountain ranges might represent the last strongholds of native *Pelophylax* diversity in the country.

## ACKNOWLEDGEMENTS

We greatly thank the Federal Office for the Environment (FOEN/OFEV/BAFU; Francis Cordillot), the state of Geneva (G. Dandliker), the foundation Lovioz, and Hintermann & Weber SA for funding and the karch for support, as well as M. L. Kieffer, H. Schmoker, C. Stickelberger, karch-GE (L. Barbu, S. de Chambrier, L. Merlier), the University of Zurich (Reyer group), the Museum of Natural History of the City of Geneva, and the Cantonal Museum of Zoology (Vaud) for tissue samples. We thank the relevant authorities for issuing collecting permits. We are also grateful to Lionel di Santo for his help in the laboratory, Johan Schuerch for field assistance, and Benedikt Schmidt for comments on the manuscript. We also thank three anonymous reviewers for their useful feedback. S.D. organized the study. J.L., V.A., C.B., J.T. and S.D. conducted fieldwork. S.D. supervised laboratory work. C.D. and T.B. conducted the analyses. C.D. drafted the manuscript, subsequently improved by all co-authors.

## REFERENCES

- Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModel-Test 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772.
- Dubey S, Leuenberger J, Perrin N. 2014. Multiple origins of invasive and 'native' water frogs (*Pelophylax* spp.) in Switzerland. *Biological Journal of the Linnean Society* **112**: 442–449.
- Dubey S, Ursenbacher S, Schuerch J, Golay J, Aubert P, Dufresnes C. 2017. A glitch in the *Natrix*: cryptic presence of alien grass snakes in Switzerland. *Herpetology Notes* **10**: 205–208.
- Dufresnes C, Dubey S, Ghali K, Canestrelli D, Perrin N. 2015. Introgressive hybridization of threatened European tree frogs (*Hyla arborea*) by introduced *H. intermedia* in Western Switzerland. *Conservation Genetics* **16**: 1507–1513.
- Dufresnes C, Pellet J, Bettinelli-Riccardi S, Thiébaud J, Perrin N, Fumagalli L. 2016. Massive genetic introgression in threatened northern crested newts (*Triturus cristatus*) by an invasive congener (*T. cristatus carnifex*) in Western Switzerland. *Conservation Genetics* **17**: 839–846.
- Dufresnes C, Denoël M, di Santo L, Dubey S. 2017a. Multiple uprising invasions of *Pelophylax* water frogs, potentially inducing a new hybridogenetic complex. *Scientific Reports* **7**: 6506.
- Dufresnes C, di Santo L, Leuenberger J, Schuerch J, Mazepa G, Grandjean N, Canestrelli D, Perrin N, Dubey S. 2017b. Cryptic invasion of Italian pool frogs (*Pelophylax bergeri*) across Western Europe unraveled by multilocus phylogeography. *Biological Invasions* **5**: 1407–1420.
- Dufresnes C, Leuenberger J, Amrhein V, Bühler C, Thiébaud J, Bohnenstengel T, Dubey S. 2017c. Data from: Invasion genetics of marsh frogs (*Pelophylax ridibundus sensu lato*) in Switzerland. *Dryad Digital Repository*. <https://doi.org/10.5061/dryad.4v57t>
- Escher K. 1972. Die amphibien des Kantons Zürich. *Vierteljahrsschrift Naturforschenden Gesellschaft in Zürich* **117**: 337–380.
- Goudet J. 1995. FSTAT (Version 1.2): a computer program to calculate F-Statistics. *Journal of Heredity* **86**: 485–486.
- Grossenbacher K. 1988. *Verbreitungsatlas der Amphibien der Schweiz*. Documenta faunistica helvetiae Nr. 7. Neuchâtel, Switzerland: Zentrum für die Kartografie der Fauna.
- Guidon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 692–704.
- Herczeg D, Vörös J, Christiansen DG, Benovics M, Mikulicek P. 2017. Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary. *Journal of Zoological Systematics and Evolutionary Research* **55**: 129–137.
- Holsbeek G, Jooris R. 2010. Potential impact of genome exclusion by alien species in the hybridogenetic water frogs (*Pelophylax esculentus* complex). *Biological Invasions* **12**: 1–13.
- Holsbeek G, Mergeay J, Hotz H, Plötner J, Volckaert FAM, De Meester L. 2008. A cryptic invasion within an invasion and widespread introgression in the European water frog complex: consequences of uncontrolled commercial trade and weak international legislation. *Molecular Ecology* **17**: 5023–5035.
- Holsbeek G, Mergeay J, Volckaert FAM, De Meester L. 2010. Genetic detection of multiple exotic water frog species in Belgium illustrates the need for monitoring and immediate action. *Biological Invasions* **12**: 1459–1463.
- Kolbe JJ, Glor RE, Rodríguez Schettino L, Lara AC, Larson A, Losos JB. 2004. Genetic variation increases during biological invasion by a Cuban lizard. *Nature* **431**: 177–181.
- Kolenda K, Pietras-Lebioda S, Hofman M, Ogińska M, Pabijan M. 2017. Preliminary genetic data suggest the occurrence of the Balkan water frog, *Pelophylax kurtmueleri*, in southwestern Poland. *Amphibia-Reptilia* **38**: 187–196.
- Leuenberger J, Gander A, Schimidt BR, Perrin N. 2014. Are invasive marsh frogs (*Pelophylax ridibundus*) replacing the native *P. lessonae*/*P. esculentus* hybridogenetic complex in Western Europe? Genetic evidence from a field study. *Conservation Genetics* **15**: 869–878.
- Lymberakis P, Poulakakis N, Manthou G, Tsigenopoulos CS, Magoulas A, Mylonas M. 2007. Mitochondrial phylogeography of *Rana* (*Pelophylax*) populations in the Eastern Mediterranean region. *Molecular Phylogenetics and Evolution* **44**: 115–125.
- Meyer A, Zumbach S, Schmidt B, Monney J. 2009. *Les amphibiens et les reptiles de Suisse*. Bern: Haupt Verlag.



- Nöllert A, Nöllert C. 2003. *Guide des amphibiens d'Europe*. Paris: Delachaux et Niestlé.
- Roth T, Bühler C, Amrhein V. 2016. Estimating effects of species interactions on populations of endangered species. *The American Naturalist* **187**: 457–467.
- Vorburger C, Reyer HU. 2003. A genetic mechanism of species replacement in European waterfrogs? *Conservation Genetics* **4**: 141–155.
- Vorburger C, Schmeller DS, Hotz H, Guex GD, Reyer HU. 2009. Masked damage: mutational load in hemiclinal water frogs. In: Schön I, Koen M, van Dijk P, eds. *Lost sex: the evolutionary biology of parthenogenesis*. Dordrecht, Netherlands: Springer, 433–446.
- Zeisset I, Beebee TJ. 2003. Population genetics of a successful invader: the marsh frog *Rana ridibunda* in Britain. *Molecular Ecology* **12**: 639–646.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Potential genotypes obtained by first generation hybridogenetic crosses between invasive marsh frogs (RR) with local pool (LL) and edible frogs (R'L), as well as their backcrosses.

**Figure S2:** Sampled localities.

**Figure S3.** Principal component analysis on marsh frog genotypes from Switzerland and southern France, where the taxonomy was assessed.

**Figure S4.** Principal component analysis on *P. ridibundus s.l.* microsatellite genotypes in Switzerland.

**Figure S5.** Principal component analysis on marsh frog haplotypes (R'/R) phased from *P. esculentus* in Switzerland and adjacent regions.

**Table S1.** Information on the water frog individuals included in this study.

**Table S2.** Pairwise *F*<sub>st</sub> among sample sets of marsh frogs (*P. ridibundus s.l.*) and R'/R germlines phased from *P. esculentus*.

## SHARED DATA

The new data associated with this article can be found on Dryad ([Dufresnes et al., 2017c](#)) and GenBank (MG575218-MG575231).