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Cryptic invasion of Italian pool frogs (*Pelophylax bergeri*) across Western Europe unraveled by multilocus phylogeography

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Abstract

Human introductions of exotic amphibians can have catastrophic effects on native species. However, they usually remain unnoticed without genetic tools when species are difficult to distinguish morphologically. In Western Europe, pool frogs (*Pelophylax* sp.) make a worrisome case: recent genetic data showed the presence of Italian (*Pelophylax bergeri*) mtDNA haplotypes within the range of the threatened European *Pelophylax lessonae*, two morphologically similar taxa. Here we conduct a multilocus phylogeographic and population genetic survey of European and Italian pool frogs (combining present and historic samples), to investigate the origin(s) and consequences of potential introductions. Results are unequivocal: we show that the alien *P. bergeri* have extensively invaded France and north-Alpine Switzerland, and have also deeply introgressed with *P. lessonae*, which has led to the complete replacement of most populations. Alien specimens have probably been translocated multiple times from Central Italy at least prior to the 1960s. Based on our dense sampling, only two areas, north and south of the Alps still host native pool frogs in Switzerland, the Joux Valley near the French border and the canton of Ticino, respectively. Importantly, we show that these last *P. lessonae* populations are remnants of a private genetic diversity specific to Western Europe, which vanished during the *P. bergeri*'s invasion. Our study emphasizes the risk of genetic pollution during invasion by human-introduced taxa and brings alarming concern regarding uncontrolled amphibian translocations. Moreover, it demonstrates the necessity for genetic surveys to detect and monitor these invasions, especially where species determination is problematic.

Keywords Europe · Human introduction · Introgression · Invasion · Ranid frogs · *Pelophylax bergeri* · *Pelophylax lessonae*

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Introduction

Biological invasions constitute a major threat to biodiversity. Human introductions of exotic species may rapidly induce fundamental changes in ecosystem dynamics, with detrimental effects such as interspecific competition, predation and transmission of diseases (Pimentel et al. 2001). Alien species may also compromise the genetic integrity of related indigenous taxa, through introgressive hybridization (i.e. “genetic pollution”; Allendorf et al. 2001; Randi 2008; Laikre et al. 2010), leading to the formation of hybrid swarms. Genetic and ecological factors may further interact to promote the invasion of exotic genes, and thus the rapid replacement of the native genome (Fitzpatrick et al. 2010). This can also be accelerated when mate choice is asymmetric favoring the invasive species (While et al. 2015). Together, these factors can precipitate local declines and ultimately the extinction of native taxa.

Several cases of invasion involving introgressive hybridization have been documented in mammals (e.g. wolf, Verardi et al. 2006; Randi et al. 2014), birds (e.g. partridges, Negro et al. 2001; Barilani et al. 2007), fishes (e.g. trout, Madeira et al. 2005; Allen et al. 2016) and plants (e.g. poplar, Vanden Broeck et al. 2005). Invasive species are also an important conservation concern for amphibians and have contributed to their decline worldwide (e.g. Johnson et al. 2010; Dufresnes et al. 2016a). In Europe, human translocations of amphibians are extremely common (Smith and Sutherland 2014), increasing their potential to compromise native populations, genetically and/or ecologically (e.g. Johnson et al. 2010; Austin et al. 2011). Yet, these introductions usually go unnoticed. A major issue is that, in many amphibian systems, closely-related species are often morphologically indistinguishable, preventing the detection and efficient monitoring of introductions without genetic tools (Shaffer et al. 2015). In Europe, amphibian introductions have led to the genetic replacement of native taxa in several instances, sometimes in an invasive manner. In crested newts, the Italian lineage *Triturus carnifex* have established expanding alien populations in England, the Netherlands, Germany and Switzerland, where it has regionally replaced the threatened indigenous *T. cristatus* (Meilink et al. 2015; Dufresnes et al. 2016a). In Switzerland, a protected population of locally endangered tree frog *Hyla arborea* turned out to be deeply introgressed by exotic Italian *H. intermedia* genes, as a consequence of illegal releases (Dufresnes et al. 2015).

European water frogs (*Pelophylax* sp.) are iconic examples of problematic amphibian translocations. This group forms a large species complex with multiple, widespread, parapatric and sympatric lineages distributed throughout the Western Palearctic (Lymberakis et al. 2007). The best-known case of *Pelophylax* introduction is the marsh frog (*P. ridibundus*), which involves multiple lineages of closely-related taxa (including also *P. bedriagae*, Holsbeek et al. 2008, 2010; Dubey et al. 2014; *P. kurtmulleri*, Ficetola and Scali 2010; Dubey et al. 2014). Importantly, these invasive species are replacing the native pool frog *P. lessonae*, via competition for food and breeding resources, predation, and by eliminating its genome through hybridogenesis (Vorburger and Reyer 2003, detailed below). Furthermore, *Pelophylax* translocations were also reported between pool frog taxa (*P. lessonae* and relatives), raising further conservation concerns. In Umbria, the Albanian lineage *P. shqipericus* was detected within the range of the Italian/Corsican endemic *P. bergeri* (Domeneghetti et al. 2013). In Belgium, a specimen was genetically identified as the

Iberian pool frog *P. perezii* (Holsbeek et al. 2010). It is also known that *P. bergeri* was introduced to Sardinia and the United Kingdom, although it is unclear whether populations survived in the latter case (Andreone et al. 2009). Recently, Dubey et al. (2014) found alien mitochondrial haplotypes of *P. bergeri* in several Swiss populations, where only *P. lessonae* is naturally present. Given that these two lineages (considered to be subspecies by some authors, Canestrelli and Nascetti 2008) are hardly distinguishable in the field (Wycheley et al. 2002; Nöllert and Nöllert 2003), such findings suggest that the taxonomic identity of Western-European pool frogs (supposedly *P. lessonae*) may have been compromised by multiple cryptic introductions, which have remained undetected to date.

Documenting the extent of these introductions has important conservation implications. *Pelophylax lessonae* is widely distributed throughout Northern Italy and Eastern, Central and Western Europe, but is declining due to habitat loss, pollution, and the impact of the invasive marsh frogs. Yet, its conservation status is unresolved in many regions because of the difficulty identifying this species among other sympatric *Pelophylax* frogs (*P. ridibundus* and *P. esculentus*) by morphometrics. Conservation actions would clearly depend on the nature of populations, that is whether they are native, alien, or introgressed, which is a controversial issue (Allendorf et al. 2001; Jackiw et al. 2015; Fitzpatrick et al. 2015). Molecular surveys are therefore required, firstly to clarify the status of pool frog populations, and secondly to shed light on the processes associated with the *P. bergeri* invasion by discriminating between genetic pollution through introgressive hybridization and replacement without interbreeding.

Here, we conduct a multi-locus phylogeographic study to characterize the introduction(s) of *P. bergeri* within the range of *P. lessonae*. Combining nuclear and mitochondrial markers with a widespread sampling, particularly dense in putatively introduced areas (Western Europe, particularly Switzerland), we aimed to (1) identify exotic *P. bergeri* from native *P. lessonae* populations, (2) infer the history of *P. bergeri*'s presence, and (3) understand the genetic impact on *P. lessonae*. For the latter, we specifically tested whether *P. bergeri*'s introduction resulted in (a) competitive exclusion of *P. lessonae* (without hybridization and introgression) in which case we expected *P. bergeri* individuals without admixture in introduced populations; or (b) introgressive hybridization, in which case we expect signs of admixture between the two genomes. In addition, we aimed to evaluate the reliability of morphological characters to distinguish pool frogs from other green frogs on the field.

Methods

Model system, DNA sampling and morphological species identification

The pool frog *P. lessonae* forms a hybridogenetic complex with the invasive marsh frog (*P. ridibundus* sp.) and the edible frog (*P. esculentus*). Briefly, *P. esculentus* is the first-generation hybrid between *P. lessonae* and *P. ridibundus* and secondary crosses between *P. esculentus* and *P. ridibundus* only produce *P. ridibundus* offspring (the *P. lessonae* germ line of edible frogs is eliminated). This two-step process thus contributes to the progressive replacement of the native pool frog genome in the field. The same mechanisms apply to the Italian lineages: crosses between Italian pool frogs (*P. bergeri*) and marsh frogs produce

Italian edible frogs (*P. hispanicus*), although marsh frogs are not yet present in Italy (Nöllert and Nöllert 2003). Importantly, the sympatric pool, edible and marsh frogs are difficult to determine on the field and identification requires confirmation by diagnostic genetic markers.

For our study, we included a total of 700 water frogs from across the native ranges of *P. bergeri* and *P. lessonae*, with a particular focus in Eastern France and Switzerland (Fig. 1), where *P. bergeri* was putatively introduced (Dubey et al. 2014). In Switzerland, the sampling densely covers all the main biogeographic regions of the country (Swiss Plateau, Jura Mountains, Alpine Valleys, Ticino) and represents most of the range of these declining frogs there. A total of 114 samples originate from museum collections (Paris, MNHP; Lausanne, MCZL; Geneva, MNGE; Vienna, NMW; SD personal collection) and 572 were collected on the field between 2013 and 2015. Sampling primarily focused on pool frogs (*P. lessonae* and *bergeri*) but edible and marsh frogs were also collected in places where pool frogs were rare or absent (such as the area of Geneva in western Switzerland), since these can be informative for the mitochondrial history of pool frogs. We also included published mitochondrial data of an additional 14 samples (see below). Museum samples date back from the 1960s to the early 2000s. Finally, one individual of *P. perezi* was collected to be used as an outgroup in the phylogenetic analyses. Species determination, based on the shape of the metatarsal tubercle, as well as relative body sizes (Nöllert and Nöllert 2003), was attempted in 255 instances. Samples consisted of ethanol-preserved tissues (museum samples) or non-invasive buccal swabs (live field-captured individuals), and DNA was extracted using the Qiagen Biosprint Robotic Workstation. File S1 provides detailed sampling information.

Genetic species identification

To unambiguously distinguish pool frogs (*P. bergeri/lessonae*) from marsh (*P. ridibundus* and relatives) and edible (*P. esculentus*) frogs, most individuals (n = 678 out of 686) were genotyped at a diagnostic microsatellite locus with species-specific alleles (*Rrid059A*, Leuenberger et al. 2014). This locus was amplified in multiplex along with other microsatellites; details are provided in the corresponding section below.

Mitochondrial sequencing

We inferred the mitochondrial lineage by sequencing a short fragment of *cytochrome-b* (114 bp) in 626 water frog samples, complemented by 14 published mitochondrial types of *P. bergeri/lessonae* (Beebe et al. 2005; Hofman et al. 2012; Komaki et al. 2015; Dubey et al. 2014). Because edible frogs are the primary offspring of pool frogs, and further transmit their genes to marsh frogs, the mitochondrial DNA of *P. lessonae* or *P. bergeri* (our taxa under focus) can be carried by *P. esculentus* and *P. ridibundus* species complex as well. Therefore, all water frog species were potentially informative and mitotyped. To this end, we used the newly developed primers *cytb_F2* (AGA CRT GAA ACA TTG GTG TA) and *cytb_R2* (CAG ATT CAT TGG ACT AG) with amplification conditions as follow: 3' at 94 °C (initial denaturation), 35 cycles consisting of 45'' at 94 °C (denaturation), 45'' at 48 °C (annealing), 1'30'' at 72 °C (elongation), followed by 7' at 72 °C (final elongation). PCRs were conducted in 25ul reaction volumes, containing 1.9 Qiagen PCR buffer, 0.2 mM of MgCl₂, 0.2 mM of

each primer, 0.5 mM of dNTPs, 0.25 units of Qiagen Taq and 2.5 µl of template DNA (10–100 ng).

For a subset of 361 individuals carrying the mitochondrial lineages of interest (*P. lessonae/bergeri*), we sequenced a longer fragment of *cyt-b* (974 bp) for phylogenetic analyses, complemented by four published sequences (Hofman et al. 2012). For amplification, we used the published primers *cytb_60F* and *cytb_60R* (Hofman et al. 2012), the same PCR reaction mix as above, and the following thermal conditions: 3' at 94 °C (initial denaturation), 35 cycles consisting of 60'' at 94 °C (denaturation), 60'' at 50 °C (annealing), 2' at 72 °C (elongation), followed by 7' at 72 °C (final elongation).

Sequencing of nuclear introns

We amplified and sequenced intronic portions of the nuclear genes *Cellular myelocytomatosis* (*Cmyc*, 207 bp) and *Serum Albumin* (973 bp) in 66 and 110 pool frogs respectively, which represent well the species' ranges (both native and introduced). For *Cmyc*, we used newly-developed primers (*Myel_F1*: CAG TGA ATG ACA GCA TTT CCA G; *Myel_R3*: GTC AAA GCC TTC AAA GAC CAT TG) and the following PCR thermal conditions: 3' at 94 °C (initial denaturation), 35 cycles consisting of 45'' at 94 °C (denaturation), 45'' at 54 °C (annealing), 60'' at 72 °C (elongation), followed by 7' at 72 °C (final elongation). For *Serum Albumin*, we used the published primers *ex1_F5* and *ex2_R2* (Plötner et al. 2009) and the following PCR thermal conditions: 3' at 94 °C (initial denaturation), 35 cycles consisting of 60'' at 94 °C (denaturation), 60'' at 52 °C (annealing), 1'45'' at 72 °C (elongation), followed by 7' at 72 °C (final elongation). For both markers, reactions volumes were the same as for *cyt-b* amplification (see above).

Both nuclear markers were directly sequenced and haplotypes of heterozygote individuals (i.e. featuring double peaks) were manually reconstructed for analyses. This was possible given the low number of polymorphic sites and haplotypes (see “Results” section) and that all variants were sampled in homozygotes individuals. Ambiguities remained for eight heterozygous individuals at *Cmyc*, which were discarded in subsequent analyses.

Microsatellite genotyping

Nine microsatellite loci were genotyped in 356 individuals confirmed as *P. bergeri/lessonae*. Amplifications were performed in two multiplex PCR including 4 (Mix A) and 5 loci (Mix B) respectively. File S2 provides the list of markers, their origins, and primer concentrations in PCR multiplexes. Reaction mixes (10 µl) included 3 µl of Qiagen Multiplex PCR Master Mix (MPMM), 3 µl of template DNA, and the different primers. PCR thermal conditions were as follow: 3' at 94 °C (initial denaturation), 35 (recent samples) or 40 (museum samples) cycles consisting of 30⁰⁰ at 94 °C (denaturation), 1⁰30⁰⁰ at 58 °C (Mix A) or 56 °C (Mix B) (annealing), 60⁰⁰ at 72 °C (elongation), followed by 7⁰ at 72 °C (final elongation). PCR products were diluted three (Mix A) or two times (Mix B) and ran to an ABI3130 Genetic Analyzer. Peaks were scored with Genemapper 4.0 (ABI).

Phylogenetic and population genetic analyses

We built a maximum-likelihood phylogenetic tree of mitochondrial *cyt-b* haplotypes with PhyML (Guindon and Gascuel 2003), choosing a GTR + G model of sequence evolution

(MrAIC, Nylander 2004). Sequences of *P. bedriagae*, *P. ridibundus*, *P. kurtmuelleri* (obtained from Swiss marsh frogs, see “Results” section), as well as *P. perezi* (this study) and *P. cretensis* (GenBank KM677928) were included as outgroups. Branch support was tested by 1000 bootstrap replicates. For each sequence dataset (mitochondrial *cyt-b*, nuclear *Cmyc* and *Serum albumin*), statistical networks of *P. lessonae/bergeri* haplotypes were built using TCS (Clement et al. 2000), considering indel polymorphism. Given the low polymorphism and incomplete lineage sorting of intronic sequences, no phylogenetic inference was performed for these.

We analyzed microsatellite variation through a Principal Component Analysis (PCA) on individual genotypes with the *ade4* and *adeget* R packages (Jombart 2008). In this analysis, we also simulated F1 hybrids between *P. bergeri* and *P. lessonae*, using genotypes from native pure populations (*P. bergeri*: pop. 1–15; *P. lessonae*: pop. 16–49, 63, 116–121; see “Results” section).

Because of the private diversity found in Western Europe and the limited ability of some nuclear markers/alleles to diagnose species, the genetic nature of populations from the Swiss Plateau and Eastern France was not straightforward at first glance (see “Results” section). Therefore, we used Approximate Bayesian Computation (ABC) to test whether these populations result from admixture between native *P. lessonae* and introduced *P. bergeri* (scenario “hybrids”); are recently derived from other European *P. lessonae* populations (scenario “pure *lessonae*”); or are recently derived from *P. bergeri* (scenario “pure *bergeri*”). Briefly, the ABC approach consists of: (1) generating datasets under the different scenarios with a coalescent simulator; (2) computing population genetic summary statistics from the simulated data; and (3) comparing simulated versus observed summary statistics to infer which scenario is closer to the empirical data. The analysis was performed with DIYABC 2.1.0 (Cornuet et al. 2014), following the detailed software’s manual. This approach is powerful when distinguishing between scenarios with or without admixture, based on datasets such as ours (Sousa et al. 2012). Each scenario involved three groups of individuals, corresponding to *P. lessonae* gene pool (i.e. loc. 63 and 116–121, representing the last *P. lessonae* populations of Western Europe, see “Results” section), to *P. bergeri* (i.e. loc. 1–15, representing most of the species’ diversity), and to ambiguous Swiss/French populations (i.e. loc. 55–62, 64–114). Figure 2 provides a graphic representation of the three scenarios and the parameters involved. The three types of genetic markers were included in the analysis: mitochondrial sequences (*cyt-b*), nuclear sequences (introns) and microsatellite loci. We considered the following 13 summary statistics (File S3), informative to distinguish between the three scenarios: diversity within the Swiss/French sample (heterozygosity and number of alleles for microsatellites, number of pairwise differences for sequences), genetic distances between Swiss/French versus *P. lessonae* and Swiss/French versus *P. bergeri* (F_{st} for microsatellites, W mean of pairwise differences for sequences) and admixture indices in the Swiss/French sample (with *P. lessonae* and *P. bergeri* set as parental populations). Prior distributions of parameters were optimized by several rounds of 300,000–500,000 simulations. For final analyses, we generated 10,000,000 simulations with

the optimal prior distributions. To evaluate which scenario best explains the data, we calculated two estimates of the posterior probabilities of each scenario: the emphdirect estimate (considering the best 500 simulations) and the logistic regression estimate (considering the best 10% of simulations).

Results

Species identification

Of the 678 barcoded specimens, 266 were assigned to

P. esculentus, 53 to marsh frog lineages (48 *P. ridibundus*, 4 *P. bedriagae*, 1 *P. kurtmuelleri*) and 359 to *P. lessonae/bergeri*. Morphological identification was relatively reliable: only 34 out of the 255 samples determined were misidentified (13%). Twenty-three pool frogs and one marsh frog were confused with edible frogs, five edible frogs were confused with pool frogs, one pool frog and four edible frogs were confused with marsh frogs. Most misidentification (31 out of 34) involved ethanol-preserved museum specimen, and almost no field samples were misidentified (3 out of 355 < 1%).

Mitochondrial variation

Among the 626 mitotyped individuals, 575 carried the pool frog *cyt-b* haplotypes of interest (*P. lessonae/bergeri*): 339 were sequenced directly from pool frogs, 223 were carried by edible frogs, five by marsh frogs and eight from non-barcoded museum specimen. In total, 589 individuals with *P. lessonae/bergeri* mtDNA were included (575 new + 14 published sequences).

The mitochondrial *cyt-b* unambiguously discriminates between the monophyletic *P. bergeri* and *P. lessonae* (6.0% of divergence, File S4; 121 polymorphic sites, including 85 parsimony-informative). *Pelodytes lessonae* forms a homogeneous clade with closely-related haplotypes ($g = 0.00447$ for 27 haplotypes). Mitochondrial diversity was higher for *P. bergeri* ($g = 0.00940$ for 23 haplotypes), and we could distinguish three phylogeographic clades: (1) Sicily (BER22–23, loc. 1), southern mainland Italy (BER01–05, loc. 2–6) and the rest of the ranges (BER06–21). Haplotype networks and distributions of lineages are displayed in Fig. 1.

Alien *P. bergeri* haplotypes were present (and often fixed) within the range of *P. lessonae* in almost all French and Swiss populations north of the Alps. In this area, eight different *P. bergeri* haplotypes were identified. BER21 was the most abundant (209 out of all 232 alien *P. bergeri* mitotypes). Other haplotypes were restricted to French populations (BER12–13 in loc. 53 and 58), the Eastern coast of Lake Geneva (BER09–11 in loc. 72–74), the Jura mountains (BER18 in loc. 85–87 and 92), as well as Central (BER17 in loc. 99) and Eastern Switzerland (BER20 in loc. 109). Several localities host the mitotypes of both species (Eastern France: loc. 57, 61; Western Switzerland: loc. 74–75 and 77; Swiss Plateau: 98–99, 102 and 108). In Switzerland, despite intensive sampling effort, we found only two populations free of alien *P. bergeri*'s mtDNA north of the Alps: the isolated Joux (loc. 63) and upper Rhone valleys (loc. 79); although, the latter became extinct 15 years ago and only

one historical sample was sequenced. In addition, south-Alpine Swiss populations from Ticino (loc. 116–121) only host the native *P. lessonae*'s mitotypes. Alien *P. bergeri*'s haplotypes were also sequenced in historical French and Swiss samples, supporting the presence of this species at least since the 1970–1980s in France (loc. 55–56 and 60) and since the 1960s in Western Switzerland (loc. 74–75, 77–78 and 93).

Nuclear sequence variation

The two slower-evolving nuclear introns feature lower polymorphism (over all samples, *Cmyc*: $g = 0.01078$ for 5 haplotypes; *Serum Albumin*: $g = 0.01289$ for nine haplotypes) and unresolved phylogenies. Yet, both markers featured species-specific alleles, whose distributions were concordant with mtDNA (Fig. 3). *Cmyc*'s haplotypes CM01–04 and CM05 are associated with *P. lessonae* and *P. bergeri*, respectively; haplotypes CM03–04 were found exclusively in the Western Alps (N-Italy, loc. 16, 18; Ticino, loc. 116–119; Joux, loc. 63). For *Serum Albumin*, five closely-related alleles are associated to *P. bergeri* (SA01–04, SA09), four of which being restricted to southern and insular populations (SA01–02, SA04, SA09; loc. 4–14); haplotype SA06 was fixed throughout most *P. lessonae*'s range except in the Western Alps (Ticino and Western Switzerland) where distinct haplotypes also segregate (SA05, SA07–08). For both markers, typical *P. bergeri*'s haplotypes (CM05 and SA03) were present in high frequencies across *P. lessonae*'s French and Swiss ranges (Fig. 3).

Interestingly, 21 out of 138 Swiss individuals sequenced featured cyto-nuclear discordances (15%), i.e. carrying the mtDNA haplotype of one lineage, but intronic alleles associated to the other. This was detected in loc. 62–64, 69, 85–87, 89, 92, 95, 99 and 119, and was clearly asymmetric: 17 of these 21 samples carried *P. bergeri* mitotypes while retaining nuclear alleles typical of *P. lessonae*, whereas 4 samples featured the reverse pattern.

Microsatellite variation

Microsatellite loci discriminate between the two lineages, but the signal was mixed with high inter-specific variation within both species, particularly *P. lessonae* (Fig. 4). The PCA using individual genotypes distinguished *P. bergeri* (axes 1 and 3; red), Central/Eastern European *P. lessonae* (axis 2; light green), the Joux Valley (axes 2 and 3; blue), as well as Fig. 3 Haplotypes networks and geographic distributions of haplogroups from the nuclear introns *Cmyc* and *Serum Albumin*. Alleles are colored according to which species they are associated with. Zoomed frames containing the main introduced ranges (Switzerland) show the proportion of each haplogroup per population, with pie size proportional to sample size individuals from N-Italy/Ticino (axis 1; dark green) and France/Swiss Plateau (axis 1; black). Southern *P. bergeri* populations (loc. 1–7), represented by only a few individuals, also differ. Simulated *P. bergeri*/*P. lessonae* hybrids (yellow) did not group with any observed populations based on axis 1, but many hybrids shared genetic variation with individuals sampled in introduced ranges, based on axes 2 and 3.

ABC analyses

The admixture scenario received the highest posterior probabilities of likelihood (direct estimate, $PP = 0.87$; logistic regression estimate: $PP = 0.92$; Fig. 2). In contrast, the alternative scenarios that French/Swiss populations are derived from pure *P. bergeri* or remained pure *P. lessonae* were much rarer in the best simulations (direct estimates: $PP = 0.13$ and 0.01 respectively; logistic regression estimate: $PP = 0.06$ and 0.02 respectively). Confidence intervals of posterior probabilities do not overlap between the admixture and alternative scenarios (Fig. 2).

Discussion

Our study allowed us to document a cryptic invasion of *P. bergeri* north of the Alps within *P. lessonae*'s range. Importantly, we could distinguish between the two major processes driving this invasion: replacement by competitive exclusion only, or by introgressive hybridization. A cryptic invasion of *P. bergeri* north of the Alps

Our phylogeographic survey revealed that alien mitochondrial haplotypes of the Italian pool frog *P. bergeri* are widespread north of the Alps. *Pelophylax bergeri* mtDNA is almost fixed throughout the entire Swiss Plateau (as recently suspected in some populations, Dubey et al. 2014), as well as most sampled French populations. In parallel, intronic alleles typical of *P. bergeri* also occur in high frequencies throughout this part of the range. In contrast, northern and eastern-European populations only displayed mitochondrial haplotypes and nuclear alleles associated to *P. lessonae*. Altogether, these findings support that the exotic *P. bergeri* mitochondrial and nuclear genomes are widely present across Western Europe north of the Alps, where it has replaced the native *P. lessonae*. From our sampling and analyses, only a handful of populations were spared by this invasion, notably the Joux valley in Switzerland. It is worth noting that in Joux, some individuals featured intronic *Serum Albumin*'s haplotype SA03 (associated to *P. bergeri*). However it is unclear whether this may stem from ancestral polymorphism rather than recent introgression, since this *P. lessonae* population appears otherwise free of *P. bergeri* alleles, features private diversity (see below), and this marker shows little species differentiation despite complex variation. Similar hypotheses may explain why one specimen from Ticino carried *Cmyc*'s *P. bergeri* allele (CM05). Yet, natural introgression cannot be excluded there, given the proximity with *P. bergeri*'s native range. From our data, Ticino, along with the rest of the Po valley south of the Alps, do not seem affected by human-driven *P. bergeri*'s introductions, at least not yet. The fact that Joux and Ticino are isolated from the Swiss plateau by mountain ranges, might explain why they have, so far, been spared by the invasion.

This invasion is likely from the result of multiple introduction events, followed by a widespread and rapid expansion: we sampled nine different exotic mtDNA haplotypes in France and Switzerland, with a single one (BER21) widespread throughout introduced ranges. The origin is likely to be central Italy; southern peninsular and insular (Sicily) populations belong to different mtDNA clades, as previously shown for Sicily (Canestrelli and Nascetti 2008). Since the introduced and native ranges of *P. bergeri* are highly isolated, with the Po Plain (inhabited by *P. lessonae*) and the Alps acting as strong dispersal barriers,

these introductions clearly result from human voluntary (e.g. as garden ornaments) or involuntary (e.g. along trade traffic) translocations. These species are absent from south-eastern France (see e.g. the detailed study by Pagano et al. 2001), precluding a natural colonization from Western Europe. The mosaic geography of introgression patterns north of the Alps also argues against a natural contact following post-glacial expansion. Given the widespread presence of *P. bergeri* and that all our museum samples (dating back to the 1960s) carried *P. bergeri*'s mtDNA, the first translocations are probably quite ancient (i.e. at least the first part of the twentieth century). In contrast to marsh frogs (*P. ridibundus* sp.) which were recorded early on (since the 1950s, Grossenbacher 1988), the alien presence of *P. bergeri* in Switzerland was never suspected before the genetic era (Dubey et al. 2014) and no information regarding its history of occurrence, nor the reasons of translocations, is available.

Invasion driven by introgressive hybridization

Our results support introgressive hybridization of *P. lessonae* by *P. bergeri*, rather than competitive exclusion only. Most individuals from introduced ranges possess *P. bergeri* nuclear and mitochondrial alleles, but some feature cyto-nuclear discordances,

i.e. carrying the mtDNA haplotype of one lineage, but intronic alleles associated to the other. In addition, the PCA on microsatellite genotypes places Western-European populations apart from most native *P. bergeri* and *P. lessonae*. Instead, these populations feature intermediate scores on the second axis, which mainly discriminates between the two species (Fig. 4); accordingly, these scores strongly overlap with the simulated *P. lessonae*/*P. bergeri* hybrid genotypes. Finally, combining all data, ABC simulations clearly suggest that the admixture scenario best explained the origin of these populations. Note however that our understanding and confidence of the admixture history in Western-Europe is limited by several inherent features of the study system. Given the high relatedness between the species, our nuclear markers show limited power for distinguishing between *P. lessonae* and *P. bergeri*. In addition, this signal is partially mixed with the strong intraspecific polymorphism found within *P. lessonae*. Admixture analyses are sensitive to the reference populations used: here the original diversity of the invaded *P. lessonae* populations, which appears endemic to this part of the range (see below), has been almost completely wiped out. Thus we could only include the two pure W-European

P. lessonae populations as reference in ABC simulations. Finally, the basic coalescent model of DIYABC may not well reflect the evolutionary dynamics of water frogs, particularly hybridogenetic aspects. Screening for species-specific SNPs using Next Generation Sequencing (NGS) approaches, as well as implementing more realistic coalescent models involving hybridogenesis (e.g. Quilodran et al. 2014) may help to get more robust inferences. From a taxonomic point of view, both pool and edible frogs inhabiting invaded ranges pose a designation issue. This is especially true in the latter; as they arise from

P. lessonae/*bergeri* hybrids, they correspond to neither *P. esculentus* nor *P. hispanicus*. Given the extent of *P. bergeri* presence, it is likely that it has been taking over *P. lessonae* and that the current situation is not stable, but rather progresses towards the complete replacement of the native gene pool.

Since they diverged relatively recently [mid-Pleistocene, estimated at *1.3 Mya by Canestrelli and Nascetti (2008)], hybridization between European and Italian pool frogs is not surprising as these two lineages may not have had time to accumulate reproductive isolation. In anuran amphibians, species formed at the Plio-Pleistocene era (3 My) still strongly admix in natural hybrid zones (Dufresnes et al. 2014, 2016b) and reproductive isolation is only detected between older divergences (C3 My, Colliard et al. 2010; Dufresnes et al. 2015). The extensive mixing of *P. lessonae* and *bergeri* is in line with weak or absent reproductive isolation. The asymmetric cyto- nuclear discordances detected in Switzerland (biased towards *P. bergeri* mtDNA) may therefore reflect differential sex-biased dispersal between the two species (i.e. better dispersal capability of invasive female *P. bergeri* than native female *P. lessonae*), rather than differential sex-specific hybrid fitness. Allopatric *P. bergeri* and *P. lessonae* seem to have distinct mating calls (Günther and Plötner 1994; Sinsch and Schneider 1996), but different dialects are also common within species in amphibians, including *P. lessonae* (Wyherley et al. 2002) and seem insufficient to promote pre-zygotic isolation in our case. The case of European pool frogs is remarkably analogous to other vertebrate systems, notably wall lizards (*Podarcis muralis*), where native German gene pools were rapidly assimilated by strong introgression from introduced Italian lineages (Schulte et al. 2012). Similarly, the extensive introgression observed parallels the situation in crested newts (*Triturus cristatus* by introduced Italian *T. carnifex*; Meilink et al. 2015; Dufresnes et al. 2016a); blueback herrings and alewife (where human disturbances triggered introgression, Hasselman et al. 2014); it is only prevented by strict hybrid control programs to limit the invasion of ruddy ducks genes into endangered white-headed duck populations in Spain (Muñoz-Fuentes et al. 2007, 2013). Without proper control (e.g. Muñoz-Fuentes et al. 2013), introduction by invasive congeners can thus rapidly promote the creation of hybrid swarms and threaten the genetic integrity of native taxa.

Furthermore, differences in life-history traits, such as dispersal capacity, ecological preferences, and/or reproductive and survival success, may have contributed to the rapid invasion of *P. bergeri* over *P. lessonae*. The presence of a single alien mitochondrial haplotype in most Swiss and French pool frogs, implying a recent *P. bergeri* expansion, would be in line with this hypothesis. *Pelophylax lessonae* is known to be specialized in sunny shallow swamps and ponds with vegetation, but unlike marsh frogs, it tends to avoid disturbed (e.g. urban) water bodies (Meyer et al. 2009). It is unknown whether *P. bergeri* has more generalist habits, which could explain its success in this heavily impacted part of Europe. To our knowledge, the fitness and ecological niches of these two closely-related species have never been compared, but could be assessed by ecological surveys and common garden experiments.

The case of pool frogs raises serious concerns on cryptic human introductions of potentially invasive species. In groups like amphibians, the difficulty to distinguish closely-related taxa (and even more their hybrids) makes the use of genetic tools essential for biological invasion surveys (Allendorf et al. 2010; Rius et al. 2015). Given the alarming rates of amphibian translocations (Fisher and Garner 2007; Smith and Sutherland 2014), similar situations may likely occur in other species systems. Genetic screening therefore has a vital role to play in active conservation where hybridization contributes to an invasion (Shaffer et al. 2015). However, the proper management decisions regarding these

introgressed populations remains complex since their conservation value may be decided upon their level of admixture (Fitzpatrick et al. 2015). Moreover, consensual decisions to “protect” versus “eradicate” requires understanding between conservationists, scientists and legislators, especially with regard to the genetic results (Fitzpatrick et al. 2015).

Implications for conservation

Our study has significant applied outcomes for the conservation of pool frogs. First, we show that the metatarsal tubercle criteria (Nöllert and Nöllert 2003) can be used with a high confidence to distinguish them from other water frogs. Pool frogs are declining over much of their range (IUCN 2016), but population monitoring is often challenging due to the difficulty of their identification. As a consequence, they are listed as “Data Deficient” (DD) in many regional red lists, and lack adequate conservation plans. Second, we identified the main remaining indigenous *P. lessonae* populations of Switzerland, namely the Joux Valley in the West and the Canton of Ticino south of the Alps. These populations are thus of national importance and should be prioritized for protection. More isolated populations, notably in mountain areas (pool frogs occur as high as up to 1200 m in Switzerland) may still carry the native diversity and should thus be screened with genetic markers. Third, these remnant *P. lessonae* populations are valuable at the European scale as well because they are genetically distinct from other biogeographic parts of its range. This is well-illustrated by private intronic alleles (*Cmyc*’s CM03–04, *Serum Albumin*’s SA05, SA07–08) and microsatellite genotypes (disruptive PCA clustering). Such endemic diversity may result from strong genetic drift and isolation by distance following post-glacial expansions (Excoffier et al. 2009), as found in European tree frogs (Dufresnes et al. 2013). Alternatively it may point to independent Holo-Pleistocene refugia during recent cold episodes (e.g. Last Glacial Maximum, 21,000 years ago; Younger Dryas, 11,000 years ago) in N-Italy/Ticino and north of the Alps (Joux). The latter would be a particularly important finding: northern continental areas are considered unsuitable to most terrestrial vertebrates during glacial periods, especially for ectothermic species like amphibians, which rather survived the glaciations in Mediterranean temperate regions (Dufresnes and Perrin 2015). This relic diversity is now on the verge of extinction due to the invasion by *P. bergeri*, which adds to the multiple threats already faced by *P. lessonae* in this part of the Palearctic. More globally, our analyses demonstrated significant nuclear structure throughout *P. lessonae*’s range, in accordance with a previous study based on RADP markers (Snell et al. 2005), which should be taken into account when developing global conservation plans (Thomassen et al. 2011).

Conclusion

In conclusion, we have uncovered a cryptic, yet extensive invasion of the Italian pool frog (*P. bergeri*) across the Western range of the European pool frog (*P. lessonae*). This invasion, which probably started more than half a century ago, involves genetic introgression by the exotic *P. bergeri*, which have almost completely replaced all sampled French and Swiss populations. Importantly, our dense phylogeographic framework allowed us to pinpoint the last remaining

refugia of endemic *P. lessonae* diversity in Switzerland, which should be considered by managing authorities.

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Wycherley J, Doran S, Beebee TJC (2002) Frog calls echo microsatellite phylogeography in the European pool frog (*Rana lessonae*). J Zool 258:479–484

Figures

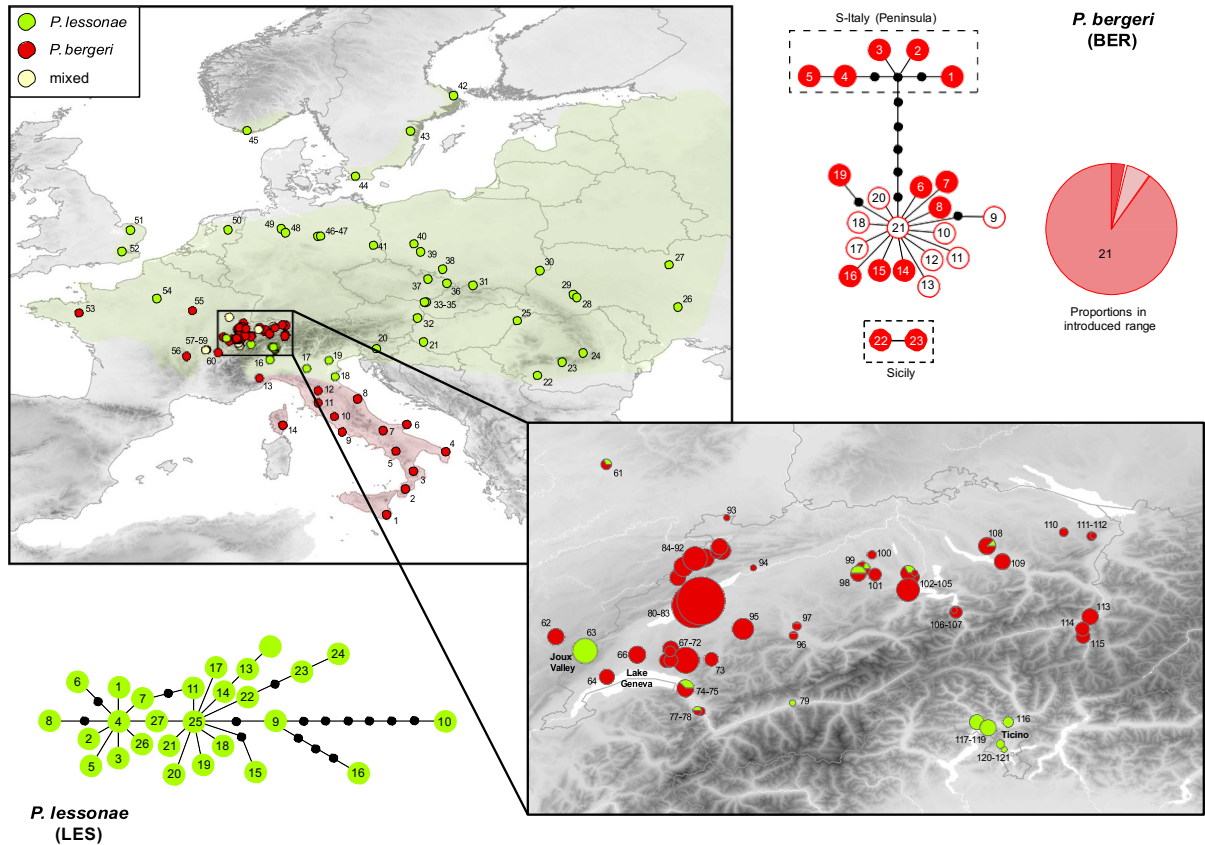


Fig. 1 Haplotype networks and geographic distributions of mitochondrial lineages. Native ranges of species are displayed by background colors (European map). *Pelophylax bergeri* haplotypes sampled in introduced ranges are represented by *hollow circles* on the network, and their proportions of occurrence are given by the pie chart. The *bottom-right* frame zooms in on the main introduced range (Switzerland), where circle diameters are proportional to sample size. In Switzerland, only populations from Ticino, the Joux valley, and perhaps deep in the Rhône Valley (only one individual sequenced) are free of *P. bergeri*'s mtDNA

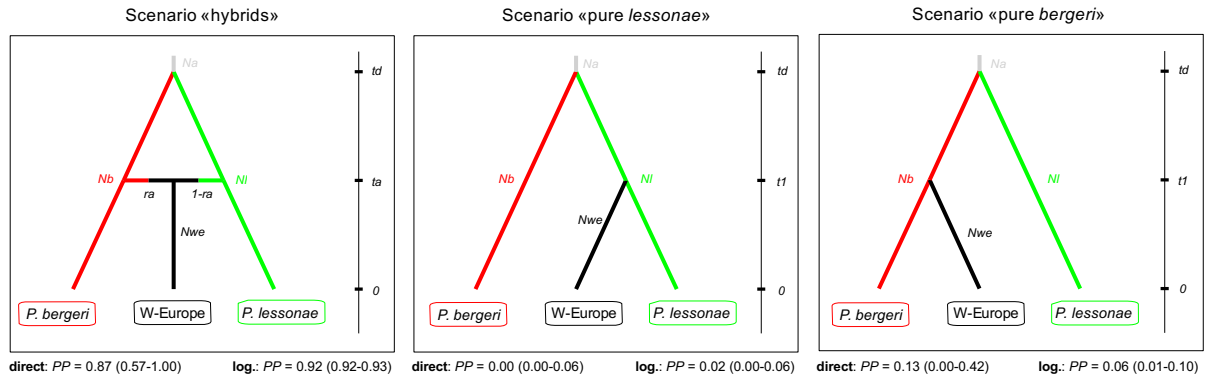


Fig. 2 Scenarios tested with Approximate Bayesian Computation (ABC) to infer the genetic nature of Western-European populations. Parameters of the coalescent models are shown. N_a , N_b , N_l and N_{we} : effective population sizes of the ancestral population, *P. bergeri*, *P. lessonae* and W-Europe, respectively; t_d , t_1 and t_a : divergence time of *P. bergeri/lessonae*, of W-European populations (for scenarios “pure *lessonae*” and “pure *bergeri*”) and admixture time (for scenario “hybrids”), respectively; ra : rate of admixture (for scenario “hybrids”). Posterior probabilities PP of each scenario (and their credible intervals) calculated from the direct and logistic estimates are given

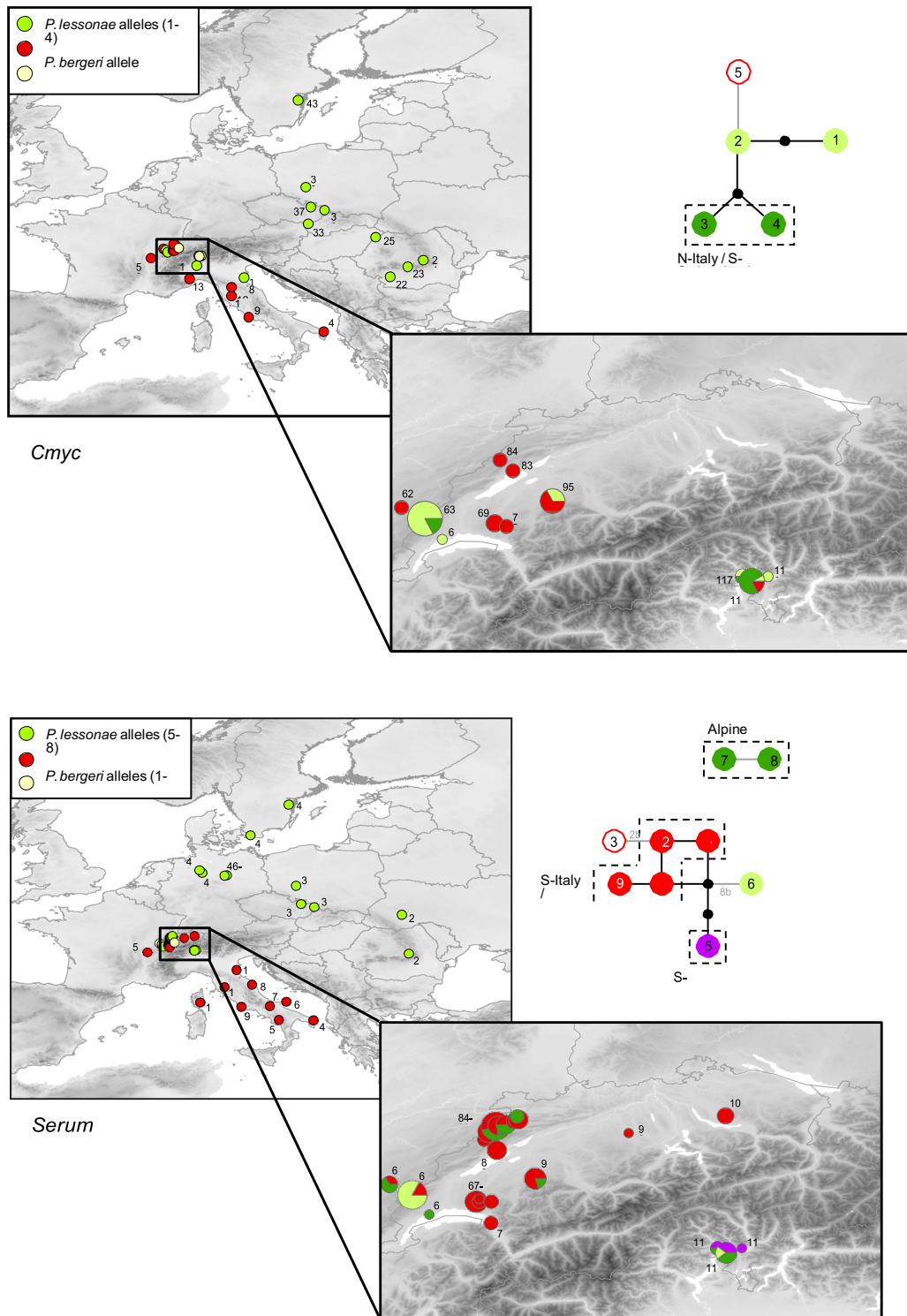


Fig. 3 Haplotypes networks and geographic distributions of haplogroups from the nuclear introns Cmyc and Serum Albumin. Alleles are colored according to which species they are associated with. Zoomed frames containing the main introduced ranges (Switzerland) show the proportion of each haplogroup per population, with pie size proportional to sample size

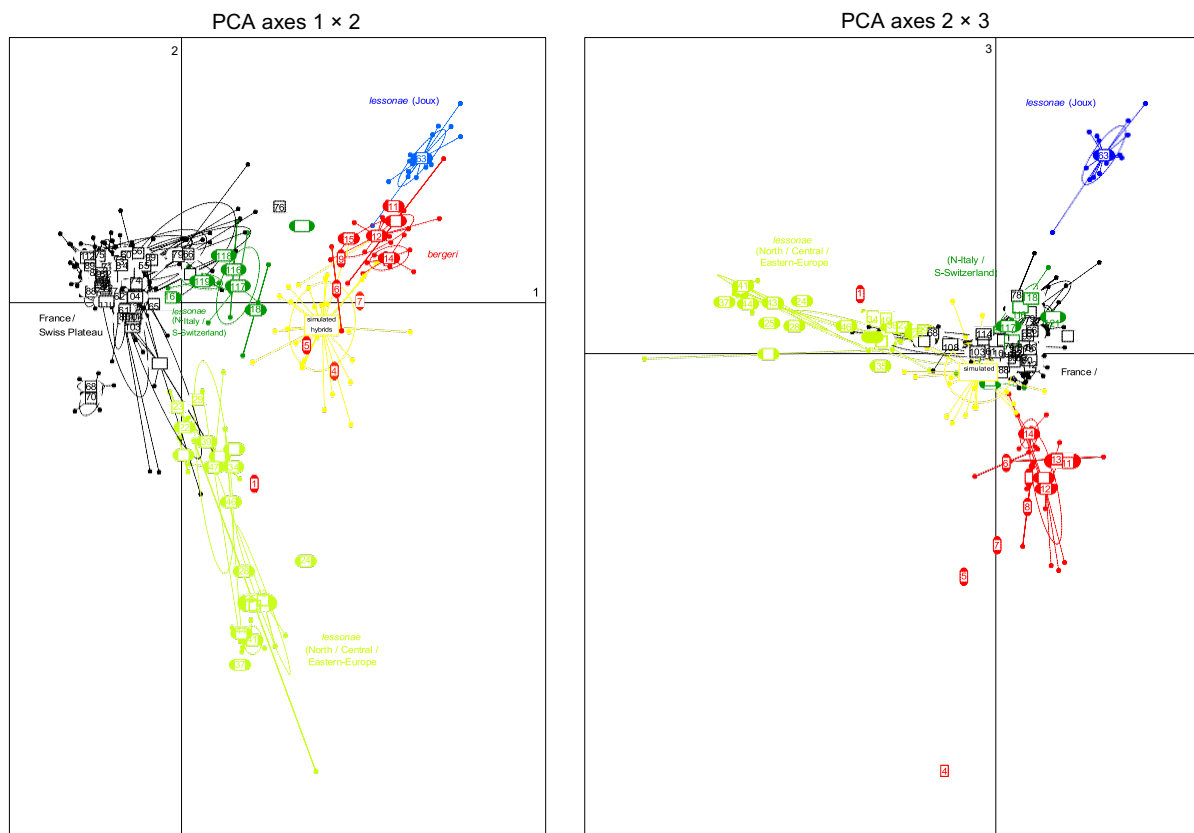


Fig. 4 PCA analysis of individual microsatellite genotypes. Dots represent individuals, linked to their populations (labels); colors show geographic origin. The three main axes explain 4.5, 3.5 and 2.8% of the total variance respectively. Simulated hybrids correspond to 30 samples generated by *adegenet* as F1 hybrids between pure *P. lessonae* and *P. bergeri*