

Genetic characteristics of an introduced population of *Bombina bombina* (Linnaeus, 1761) (Amphibia: Bombinatoridae) in Moselle, France

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Abstract. The fire-bellied toad *Bombina bombina* has recently been introduced in Moselle, north-eastern France, in an area where the yellow-bellied toad *Bombina variegata* occurs naturally. Both species hybridize in a wide area throughout Europe where their distribution overlaps. Therefore, there is a risk of introgression regarding the *Bombina variegata* population in north-eastern France. In order to assess the status of the introduced population of *Bombina bombina* and its origin, we investigated its genetic characteristics and structure using both mitochondrial (cytochrome b) and nuclear DNA (microsatellites markers). The results demonstrated a lack of introgression in the *Bombina variegata* population. Though experiencing a bottleneck effect, the introduced *Bombina bombina* population displays a high genetic diversity. If a propensity for expansion is found within the introduced population of *Bombina bombina*, it could be considered as a potential invasive species in France, and thus threaten the native species.

Keywords. Invasive species, population genetics, conservation, cytochrome *b*, microsatellites.

INTRODUCTION

Introduction of allochthonous species in natural habitats represents one of the aggravating factors of the current loss of biodiversity (Kats and Ferrer, 2003). Such introductions may induce numerous effects on native species, such as ecological competition, over predation, transmission and dispersal of pathogens, and genetic introgression (Mooney and Cleland, 2001; Strayer et al., 2006). In the context of conservation biology, it is important to understand the factors which enabled introduced species to adapt to their new environment, in order to define prevention, monitoring, and management plans for these species (Strayer et al., 2006).

Amphibians are currently in the focus for conservation biologists as they represent the most endangered

group of vertebrates worldwide (Stuart et al., 2004; Stuart et al., 2008). Invasive alien species are a major threat to amphibians (Kats and Ferrer, 2003). Among them, other amphibian species can have an impact on native ones. For example, they can be an important source of dispersal of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), a pathogen which affects many amphibian species around the globe and causes their decline (Ficetola et al., 2008; Fisher and Garner, 2007; Garner et al., 2006). Another possible threat linked with introduced species is genetic introgression, which can, in some cases, lead to local extinctions (Arntzen and Thorpe, 1999; Dufresnes et al., 2016; Rhymer and Simberloff, 1996).

In France, at least six species of amphibians have been introduced: *Triturus carnifex*, *Discoglossus pictus*, *Lithobates catesbeianus*, *Xenopus laevis*, *Pelophylax*

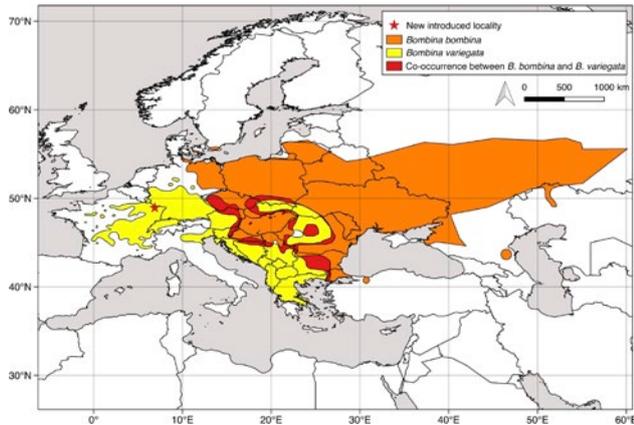


Fig. 1. Global distribution of *Bombina bombina* and *B. variegata* and location of the newly introduced population in France. Map sources: Natureearth/IUCN.

ridibundus, and *P. bedriagae* (Duguet and Melki, 2003; Lescure and de Massary, 2012). In 2009, an introduced population of the fire-bellied toad *Bombina bombina* has been discovered in the eastern part of the Moselle department, close to the Sarre valley (Vacher and Pichenot, 2012) (Fig. 1). In this region, the yellow-bellied toad *Bombina variegata* occurs naturally (Lescure et al., 2011; Lescure and de Massary, 2012; Thiriet and Vacher, 2010). This last species is considered as endangered in France and listed as ‘Vulnerable’ on the national red list published by the French Committee of the IUCN (IUCN France et al., 2015). The occurrence of introduced *Bombina bombina* close to natural populations of *B. variegata* raises conservation concern as *B. bombina* could lead to the decline of *B. variegata* through introgression. Indeed, hybridization has already been demonstrated where both species are in contact in their native range (Gollmann et al., 1988; Szymura, 1976; Yanchukov et al., 2006). In order to characterize the status of this introduced population, and its possible interaction with the native *Bombina variegata*, we assessed if hybridization already occurred between the native and the introduced species using genetic markers as well as the putative origin of the introduced individuals.

MATERIALS AND METHODS

Sampling design and laboratory methods

We collected 61 DNA samples from individuals morphologically assigned to *Bombina bombina* from three localities in Moselle (6.87°E, 48.92°N) and 64 samples from individuals morphologically assigned to *Bombina variegata* from four neighbouring localities in Moselle and Bas-Rhin in 2011 and

2012. DNA samples were collected through buccal swabbing (Beebee, 2008; Pidancier et al., 2003). The two westernmost *Bombina bombina* localities were 1 km distant from each other, and the third was 5 km south-east from the others.

DNA was extracted from the buccal swabs using the QIAGEN DNeasy Blood & Tissue kit (QIAGEN). As we suspected that all *Bombina bombina* individuals would originate from the same locality, we amplified by PCR a fragment of 1200 bp of the mitochondrial cytochrome *b* (*cytb*) using L16245 and H17444 primers (Hofman and Szymura, 2007) from only five out of the 61 samples. Amplifications were performed following Hofman and Szymura (2007) and sequencing were performed by Macrogen (Amsterdam, the Netherlands). The new sequences were deposited in GenBank (Table 1).

Ten microsatellite loci specifically developed for the fire-bellied toad (BobomF2, Bobom5F, Bobom9H, Bobom1A, BobomF22, Bobom10F, Bobom8A, BobomD2, BobomB13 and Bobom11D) were amplified by PCR for all 125 samples, following the PCR conditions suggested by Hauswaldt et al. (2007) and Stuckas and Tiedemann (2006). Forward dyed primers were used in order to analyse them with an automatic sequencer (AB3130xl Applied Biosystem). Allele lengths were then read with the software PEAK SCANNER v.1.0 (Applied Biosystem).

Data analysis

We used *cytb* sequences from GenBank to confirm taxonomic assignment of our samples. The *cytb* sequences obtained were first aligned automatically using the software MAFFT (Kato and Standley, 2013), and then the alignment was checked in MEGA (Tamura et al., 2011). We subsequently grouped our sequences with other *Bombina bombina cytb* sequences published in a previous study on the phylogeography of the species (Fijarczyk et al., 2011). After inferring the best sequence evolutionary model in PartitionFinder v.1.1.1 (Lanfear et al., 2012), using a BIC approach, we constructed a phylogenetic tree with a Maximum Likelihood method in RAXML v.8 (Stamatakis, 2014) under the GTR+G model. We used one sequence of *Bombina variegata* as an outgroup to root our tree. After visualizing the position of our samples in the tree, we subsequently selected the sequences that were the closest to the samples from Moselle, and constructed a haplotype network using the software Hapview (Salzburger et al., 2011).

Each microsatellite locus was first examined for null allele occurrence with MICRO-CHECKER v.2.2.3 (Van Oosterhout et al., 2004) for each population. Loci showing a high probability ($P > 0.05$) of null alleles were discarded from the dataset. For each retained locus, we estimated allele frequency, allelic richness (A_R), observed and expected heterozygosity (H_O , H_E), and intrapopulation structuration (F_{IS}) with the packages *adegenet* (Jombart, 2008) and *hierfstat* (Goudet, 2005) implemented in R (R Development Core Team, 2016). Moreover, Hardy-Weinberg equilibrium was tested for each locus with allele randomizations (1000 permutations per test) with the package *pegas* (Paradis, 2010) implemented in R. In addition, we evaluated the number of genetic clusters (K) using a Bayesian clustering approach implemented in the software STRUCTURE v.2.3.3 (Pritchard et

al., 2000). First, we conducted the analysis for the three putative populations of *B. bombina*, and then for *B. bombina* and *B. variegata* populations together. We performed ten independent runs for each K, and tested between 1 and 3 for *B. bombina* alone, and up to seven clusters for *B. bombina* and *B. variegata* grouped together, according to the number of localities we sampled. Each replicate was run for 400,000 iterations following a burn-in period of 200,000. As the three sampled populations of *B. bombina* were supposedly closely related, we used the admixture model with allele frequencies that were correlated among populations (Falush et al., 2003). In order to identify the most likely value of K, the logarithmic probability of the data [Ln P(D)] was estimated for each simulation. Additionally, the value of ΔK , representing the second order of change, was estimated (Evanno et al., 2005). Finally, we tested if a bottleneck effect (significant heterozygosity excess) was detected within the population of *Bombina bombina* with the software BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1996; Piry et al., 1999), using a Wilcoxon signed-rank test. Such an effect is expected after a strong reduction in population size (Hedrick et al., 1986), such as in recent introduced populations.

RESULTS

BLAST and haplotypes

The BLAST results showed that the five samples matched with *Bombina bombina cytb* sequences deposited in GenBank. Maximum Likelihood inference suggests that the samples from Lorraine are nested within a clade that originates from Austria and Czech Republic (Fig. 2). The haplotypes found in Moselle cluster within haplogroup B3-1 (Fijarczyk et al., 2011), that contains specimens from southern Europe. More precisely, one individual of Moselle is identical to haplotype B14 (Fig. 3), that includes specimens from Czech Republic, Slovakia, Austria, Croatia, Serbia, Hungary, and Ukraine (Fijarczyk et al., 2011), and the four other haplotypes retrieved from the specimens from Moselle only differ from B14 by three to eight substitutions (Fig. 3).

Genetic variation and diversity

We detected an excess of homozygosity, thus the probable presence of null alleles for BobomF2, BobomF22, and BobomD2 within *B. bombina* populations only. Therefore, these markers were discarded from the subsequent analyses for *B. bombina*, which were consequently conducted with seven microsatellites markers (Bobom5F, Bobom9H, Bobom1A, Bobom10F, Bobom8A, BobomB13, and Bobom11D).

The number of alleles in *B. bombina* of Moselle varies from three (Bobom5F and Bobom1A) to ten (Bobom9H)

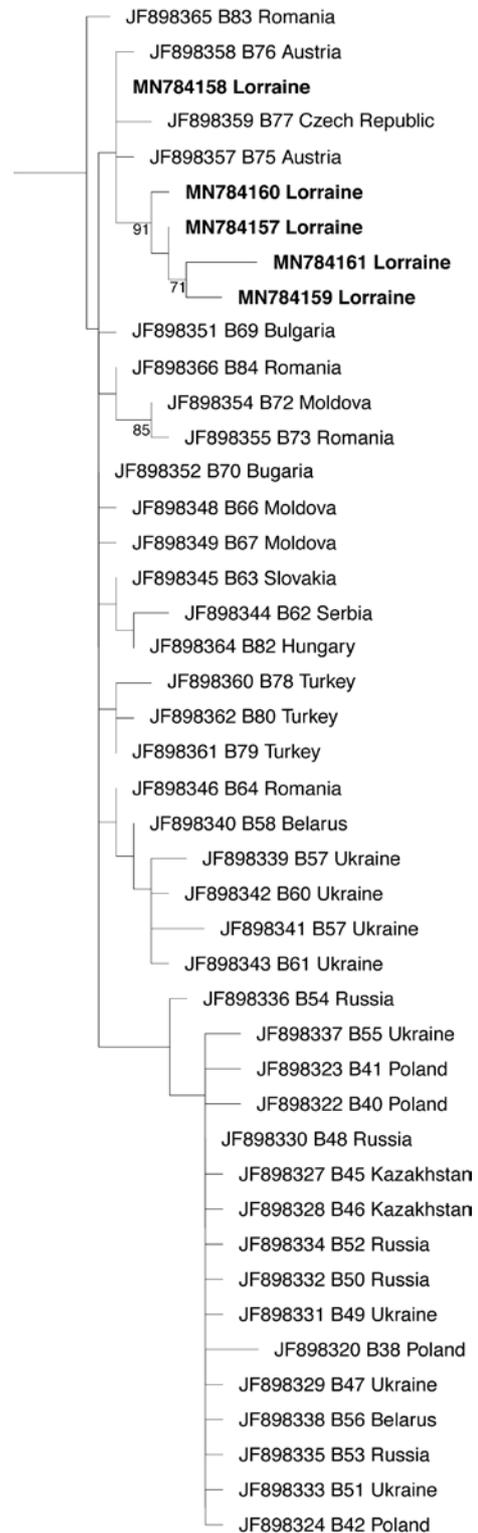


Fig. 2. Best Maximum Likelihood inference obtained from RAxML using ~1000 bp of *cytb* mtDNA of *Bombina bombina*. Bootstrap values above 70 are given at each node. The tree is rooted on *Bombina variegata* (not shown). The GenBank accession numbers are provided, new sequences are indicated in bold.

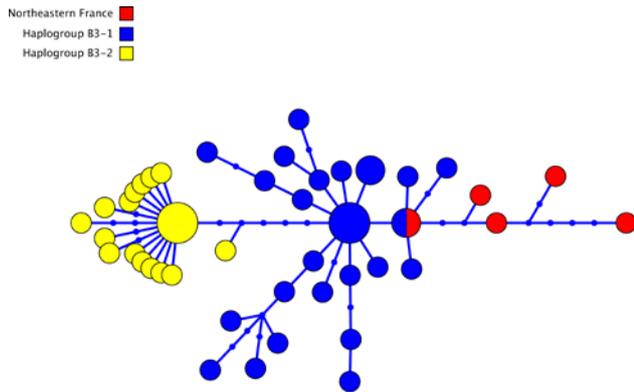


Fig. 3. Haplotype network based on a fragment of the mitochondrial *cytb* in *Bombina bombina*. The haplogroups were defined in Fijarczyk et al. (2011). The five new haplotypes from the introduced specimens in Moselle (this study) are represented in red. Each line represents a single mutation, and the size of the circles represents the frequency of a haplotype.

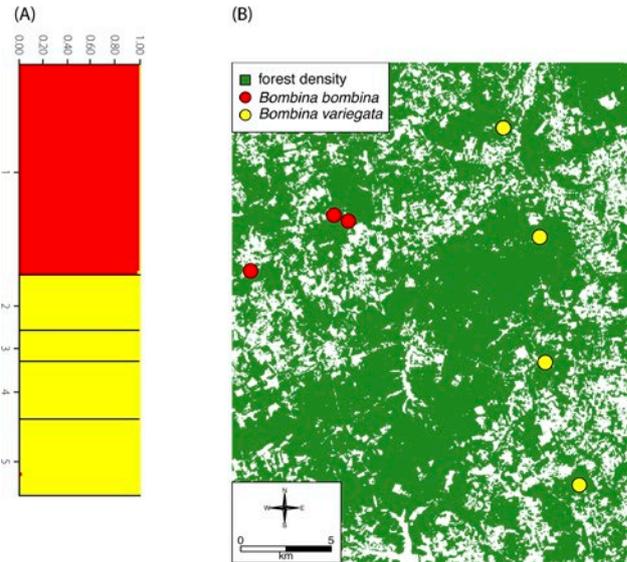


Fig. 4. (A) Clusters from retrieved in the STRUCTURE analysis from seven microsatellite markers; (B) Distribution in space of the two nuclear DNA clusters. The geographic coordinates are not provided on purpose. Map source: Global Forest Watch.

Table 1. GenBank accession numbers and additional information of the locality of the newly introduced *Bombina bombina* in France (in bold) and the samples from GenBank used in the genetic analysis.

No	Location	Accession GenBank	Reference	No	Location	Accession GenBank	Reference
1	Moselle, France	MN784157	This study	27	Serbia	JF898344	Fijarczyk et al., 2011
2	Moselle, France	MN784158	This study	28	Romania	JF898355	Fijarczyk et al., 2011
3	Moselle, France	MN784159	This study	29	Moldova	JF898354	Fijarczyk et al., 2011
4	Moselle, France	MN784160	This study	30	Ukraine	JF898339	Fijarczyk et al., 2011
5	Moselle, France	MN784161	This study	31	Romania	JF898365	Fijarczyk et al., 2011
6	Hungary	JF898363	Fijarczyk et al., 2011	32	Czech Republic	JF898359	Fijarczyk et al., 2011
7	Austria	JF898357	Fijarczyk et al., 2011	33	Ukraine	JF898341	Fijarczyk et al., 2011
8	Romania	JF898366	Fijarczyk et al., 2011	34	Belarus	JF898338	Fijarczyk et al., 2011
9	Bulgaria	JF898352	Fijarczyk et al., 2011	35	Russia	JF898330	Fijarczyk et al., 2011
10	Hungary	JF898356	Fijarczyk et al., 2011	36	Poland	JF898321	Fijarczyk et al., 2011
11	Romania	JF898353	Fijarczyk et al., 2011	37	Poland	JF898326	Fijarczyk et al., 2011
12	Ukraine	JF898347	Fijarczyk et al., 2011	38	Slovakia	JF898325	Fijarczyk et al., 2011
13	Bulgaria	JF898351	Fijarczyk et al., 2011	39	Russia	JF898334	Fijarczyk et al., 2011
14	Bulgaria	JF898350	Fijarczyk et al., 2011	40	Ukraine	JF898333	Fijarczyk et al., 2011
15	Moldova	JF898348	Fijarczyk et al., 2011	41	Russia	JF898332	Fijarczyk et al., 2011
16	Hungary	JF898364	Fijarczyk et al., 2011	42	Ukraine	JF898329	Fijarczyk et al., 2011
17	Slovakia	JF898345	Fijarczyk et al., 2011	43	Kazakhstan	JF898328	Fijarczyk et al., 2011
18	Romania	JF898346	Fijarczyk et al., 2011	44	Poland	JF898324	Fijarczyk et al., 2011
19	Belarus	JF898340	Fijarczyk et al., 2011	45	Kazakhstan	JF898327	Fijarczyk et al., 2011
20	Turkey	JF898362	Fijarczyk et al., 2011	46	Ukraine	JF898337	Fijarczyk et al., 2011
21	Turkey	JF898361	Fijarczyk et al., 2011	47	Russia	JF898335	Fijarczyk et al., 2011
22	Ukraine	JF898343	Fijarczyk et al., 2011	48	Ukraine	JF898331	Fijarczyk et al., 2011
23	Ukraine	JF898342	Fijarczyk et al., 2011	49	Poland	JF898322	Fijarczyk et al., 2011
24	Turkey	JF898360	Fijarczyk et al., 2011	50	Russia	JF898336	Fijarczyk et al., 2011
25	Moldova	JF898349	Fijarczyk et al., 2011	51	Poland	JF898320	Fijarczyk et al., 2011
26	Austria	JF898358	Fijarczyk et al., 2011	52	Poland	JF898323	Fijarczyk et al., 2011

Table 2. Microsatellite loci used for the genetic analyses of 61 individuals of *Bombina bombina* introduced in Moselle, north-eastern France. The estimations were conducted with the *adegenet* and *hierfstat* packages implemented in R. bp: base pairs; A_R : allelic richness; H_O : observed heterozygosity; H_E : expected heterozygosity; F_{IS} : intrapopulation structure index.

Microsatellite	Length (bp)	Alleles number	A_R	H_O	H_E	F_{IS}
Bobom5F	126-146	3	3	0.54	0.64	0.09
Bobom9H	115-203	10	7.6	0.83	0.86	0.00
Bobom1A	341-353	3	2.95	0.57	0.54	-0.04
Bobom10F	207-223	4	3.97	0.75	0.73	-0.02
Bobom8A	275-315	5	3.77	0.61	0.63	-0.01
BobomB13	117-147	4	3.21	0.63	0.66	0.12
Bobom 11D	268-296	6	5.12	0.82	0.78	-0.05

(Table 1). The mean A_R was 4.23 (calculated from 61 diploid individuals). The mean H_E value was 0.65, and the mean H_O value was 0.66. There was no significant difference between the overall values of H_O and H_E (Bartlett's K-squared = 0.035, df = 1, P = 0.8). The overall F_{IS} value was 0.01 and ranged from -0.05 for Bobom 11D to 0.09 for Bobom 5F (Table 1).

Genetic structure of populations

The analysis conducted with STRUCTURE did not reveal any population differentiation between the three sampling sites of *Bombina bombina* in Moselle. In the analysis conducted with all the samples of *B. bombina* and *B. variegata*, both species formed two well-differentiated clusters, indicating a complete lack of introgression (Fig. 4).

Bottleneck

The BOTTLENECK analysis revealed a bottleneck effect within the *Bombina bombina* population of Moselle. Indeed, the Wilcoxon test showed an excess of heterozygotes compared to the expected equilibrium heterozygosity under both the SMM and TPM models (Wilcoxon test: P = 0.007).

DISCUSSION

Globally, the genetic diversity observed in this introduced population of *B. bombina* is rather high, since the mean H_E value of 0.65 for seven loci is similar to the ones

found in 11 natural populations of *Bombina bombina* that occur in the core of the range of the species in Germany and that averages 0.70 [0.59-0.78] for six loci (Dolgener et al., 2012). This could be explained by the introduction of numerous individuals, maybe through different episodes. As the biggest population was observed in a series of lakes that are used for fish farming, it is highly probable that the presence of *Bombina bombina* in this area resulted from the transport of tadpoles caught together with young fishes from one or several close localities in Central Europe. Such cases of translocations have been observed in Brandenburg and in Saxony (Berger, 1996; Dolgener et al., 2012), so it is very likely that the occurrence of *B. bombina* in Moselle might result from a similar event. As tadpoles are small organisms, it is possible that hundreds, or maybe thousands of them have been introduced, therefore maintaining a high genetic diversity. Still it was expected to detect a bottleneck effect within this population as it is the case with recently introduced populations (Lee, 2002; Puillandre et al., 2008). In comparison with other amphibian species, the fire-bellied toad does not have a high fertility rate, with around 300-400 eggs per female per year (Gollmann et al., 2011). Therefore, the high diversity and the high number of alleles observed in some markers indicate that introduction of only a few founder individuals, as observed in other introduction of amphibians in France such as the bullfrog *Lithobates catesbeianus* (Ficetola et al., 2008), is unlikely. We could think that even though the primary source of the *B. bombina* population in Moselle resulted from numerous tadpoles, they still represent a small fraction of a broader population located in the core area of distribution and that though a bottleneck effect could be detected, it was not sufficient enough to affect the genetic diversity of this population.

The discovery of *Bombina bombina* in Lorraine is recent, certainly dating back to 2009 (Vacher and Pichenot, 2012). Right now, its distribution is geographically restricted and does not directly overlap with that of *B. variegata* (Fig. 4). As it can hybridize with *B. variegata* in the wild, a monitoring of *B. bombina* in the area should be set up to track the population dynamics, its dispersal behaviour, the evolution of its distribution in the area, and to determine possible concurrence with *B. variegata*. However, both species seem to display contrasted ecological preferences in their native habitat: *B. bombina* is known to prefer ponds or swamps for reproduction compared to *B. variegata* that favours puddles (Barandun and Reyer, 1997; Gollmann B. et al., 2011; Gollmann G. et al., 2011; Kruuk and Gilchrist, 1997). Consequently, we might expect that ecological competition should be limited. Moreover, *B. bombina* shows

higher site fidelity, suggesting lower dispersal capacities (Gollmann et al., 2011). Still, *B. bombina* seems to display a broader ecological tolerance by colonizing puddles in areas where ponds are scarce and where both species occur and hybridize (MacCallum et al., 1998). Therefore, a close attention to habitat components in the landscape (i.e., density of ponds and small lakes) should be integrated in a monitoring protocol to track the dynamics of this introduced population. The removal of a species at an early stage is normally the best method to avoid future competition with native species. We can consequently recommend to monitor the competition between both species of *Bombina* in the area, and perhaps also conduct some actions to reduce or remove this introduced population. Additionally, it would be necessary to scan for *Bd* and maybe other pathogens within this introduced population, as they can represent a further threat on native amphibian populations that occur in the area such as the European tree frog *Hyla arborea* or the common frog *Rana temporaria* (Ohst et al., 2013).

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