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RESEARCH ARTICLE

Inconsistent estimates of hybridization frequency in newts revealed by SNPs and microsatellites

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Abstract
Hybridization between the European smooth and palmate newts has recurrently been mentioned in the literature. The only two studies that attempted to quantify the frequency of hybridization and gene admixture between these two species came to strikingly opposite conclusions. According to Arntzen et al. (1998, 42 allozymes), hybrids are rare in nature and introgression negligible, while according to Johanet et al. (2011, 6 microsatellites), introgressive hybridization is significant and widespread across the shared distribution range. To clarify this question, we implemented high-throughput SNP genotyping with diagnostic biallelic SNPs on 965 specimens sampled across Europe. Our results are in line with Arntzen et al., since only two F1 hybrids were identified in two distinct French localities, and no further hybrid generations or backcrosses. Moreover, reanalysis of 78 of the samples previously studied by Johanet et al. (2011) using our SNPs panel could not reproduce their results, suggesting that microsatellite-based inference overestimated the hybridization frequency between these two species. Since we did not detect methodological issues with the analyses of Johanet et al., our results suggest that SNP approaches outperform microsatellite-based assessments of hybridization frequency, and that conclusions previously published on this topic with a small number of microsatellite loci should be taken with caution, and ideally be repeated with an increased genomic coverage.

Keywords Broad sympatry · Introgression · *Lissotriton vulgaris* · *Lissotriton helveticus* · SEQUENOME

Introduction

Introgressive hybridization is a process of major interest for evolutionary biology. By blurring the lines between species, interspecific gene flow reveals the gradual nature of reproductive isolation and its possible reversibility, complicates species delimitation, and more generally calls into question the centuries-old categorical conception we may have of species (Taylor et al. 2006; Harrison and Larson 2014; Barraclough and Humphreys 2015; Moran et al. 2021; Kim et al. 2022). The transfer, *via* hybridization and backcrossing, of novel alleles from one species into the gene pool of another species also constitutes a non-negligible source of genetic diversity, which may positively or negatively impact

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the adaptive capacities of species (Barton 2001, Pfenning et al. 2016, Seabra et al. 2019, Steensels et al. 2021, Wacker et al. 2021). Importantly, anthropogenic hybridization is increasingly common and its impact on conservation issues has been the topic of much debate (Chan et al. 2019, Mc Farelane & Pembertone 2019, Hirashiki et al. 2021).

Hybridization in animals has for a long time been considered as an anecdotal phenomenon, but in recent years, a growing body of evidence has demonstrated that a broad range of animal species experience it during their history (Mayr 1963; Mallet 2005; Taylor and Larson 2019; Adavoudi and Pilot 2022). In amphibians, introgressive hybridization has been documented in numerous anurans (e.g. Dufresnes et al. 2021), possibly as a result of their frequently external fertilization reducing the efficiency of pre-zygotic reproductive barriers. In Eurasian Urodela (with internal fertilization), several well documented examples have been reported in the genera *Triturus* (Jehle et al. 2001, Arntzen et al. 2009, 2021, Cogălniceanu et al. 2020) and *Lissotriton*, where *L. vulgaris* and *L. montandoni* exhibit some dramatic genomic consequences of past introgression (Babik et al. 2003, 2005; Babik and Rafiński 2004; Gherghel et al. 2012; Zieliński et al. 2013, 2014, 2016; Pabijan et al. 2017; Niedzicka et al. 2017, 2020; Dudek et al. 2019). Prior to these works, several sources (Griffiths 1987; Arntzen et al. 1998; Beebee et al. 1999, Schlüpmann et al. 1999) have also mentioned hybridization between the smooth and palmate newts, *Lissotriton vulgaris* and *L. helveticus*, two distantly related and non-sister species within the genus that diverged from each other relatively early (the divergence is imprecisely estimated between ~15 Mya and 24 Mya (Rage and Bailon 2005; Steinfartz et al. 2007; Böhme 2010; Arntzen et al. 2015). The first work aiming at quantifying the extent of hybridization between these two species was based on multivariate analyses of 16 morphological traits and electrophoretic analyses of 42 protein loci (Arntzen et al. 1998). Although this work irrefutably demonstrated natural hybridization between *L. vulgaris* and *L. helveticus*, it concluded that this phenomenon was rare (only one F1 hybrid identified from a large sample (> 5,000) of larvae, recently metamorphosed newts and adults) and no introgression was detected between the species (cf. Figure 3 of Arntzen et al.). More recently, and in striking contrast to this first work, Johanet et al. (2011) used mitochondrial and microsatellite markers on ~1,300 individuals from 37 sites across Europe and concluded that introgression was instead relatively widespread in the area of sympatry, with a frequency of hybridization of 1.7% and significant levels of introgression detected at most sites (73%) shared by both species. To determine the extent of hybridization and introgression between *L. vulgaris* and *L. helveticus* and to elucidate the causes of the discrepancy between the former studies, we reinvestigated the frequency of hybridization between the two species, using extended geographical sampling and, for the first time, high-throughput SNP genotyping. Most of the samples used in our study have never been analyzed before, but we also included in our SNP genotyping some of the samples genotyped for microsatellites and analyzed by Johanet et al. (2011) to make sure that our results are comparable with this previous study (see "Sampling" below). We also reanalyzed Johanet et al.'s (2011) microsatellite dataset with the same settings as our SNP datasets to provide fully comparable results.

Materials and methods

Sampling . — Currently, *Lissotriton vulgaris* has a broad Eurasian distribution, whereas *L. helveticus* is restricted to the western part of Europe (Wielstra et al. 2018; Sillero et al. 2014). Both species occur in sympatry across a wide area including Great Britain, the north of France, Switzerland, the Benelux countries and the west of Germany (Fig. 1). Designed to take into account their respective distribution, our sampling includes a total of 965 individuals from 59 localities (=ponds): 29 samples from 11 allopatric localities for *L. helveticus* (in Spain and southern part France), 23 samples from 8 allopatric localities for *L. vulgaris* (in Norway, Sweden, Poland, Romania and Hungary), and 913 samples of both species from 40 sympatric localities (in Great Britain and northern France), of which 829 are from 32 localities where both species co-occur in syntopy (see Fig. 1; Table 1). All these samples were collected during the reproductive phase (aquatic phase) and therefore correspond to viable adult individuals. For comparative purpose, we also included 78 syntopic individuals that were previously analyzed using microsatellites by Johanet et al. (2011), including those that showed the most substantial rates of introgression (see Table 1 for the populations origin of these 78 samples and Sup. Table 3 for the whole list of these 78 samples with their microsatellites and SNP Structure membership coefficients). These 78 samples were chosen on the basis of their Structure membership coefficients and positions in the PCA as reported by Johanet et al. (2011, see their Fig. 2 for the PCA) to include most samples showing clear signs of introgression in Johanet et al. (2011). The original microsatellites genotypes from Johanet et al. (2011) were also retrieved (Supplementary material S5).

Single-nucleotide polymorphism (SNP). — DNA extractions were done on 96 well plates with the DNeasy blood and tissues kit (QUIAGEN). DNA extracts were diluted to obtain a target concentration of 10-15ng/µL. To identify a set of candidate diagnostic SNPs, we used a total of 1380 nuclear ORF contigs/alignments from transcriptomic data

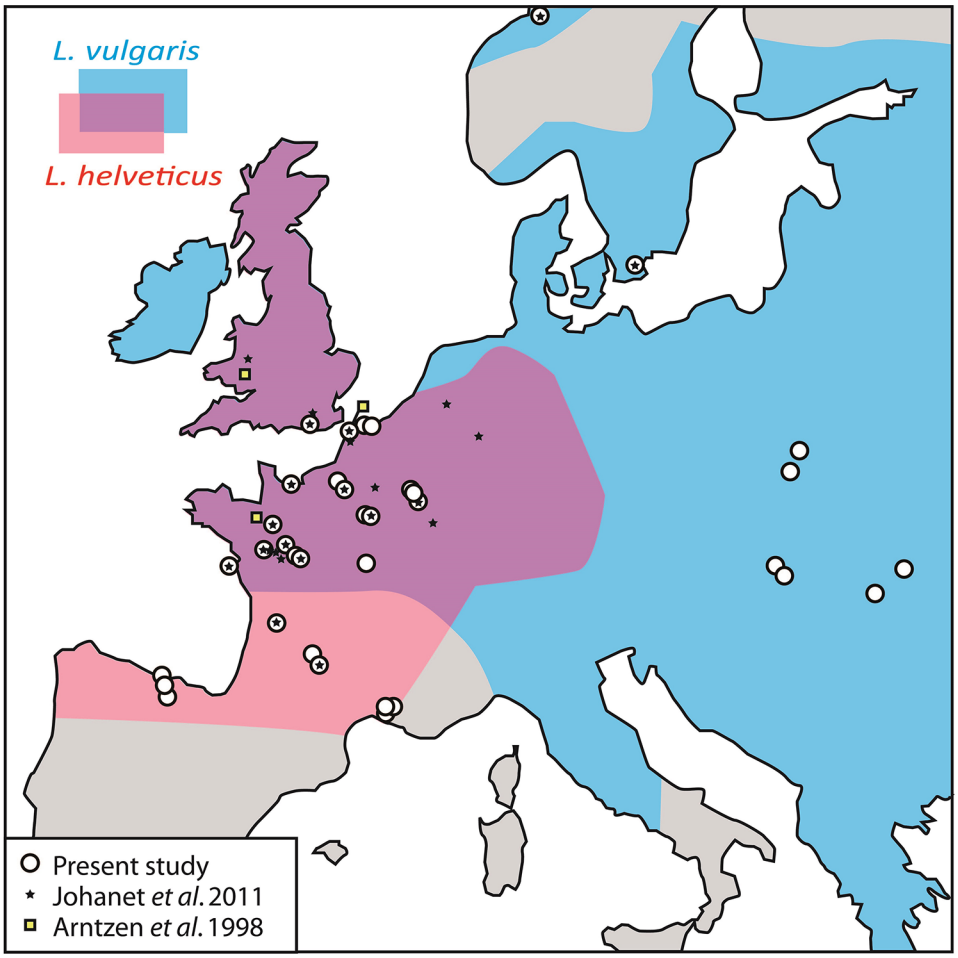

Fig. 1 Population sampling. The red and blue areas indicate the approximate distribution of *Lissotriton helveticus* and *L. vulgaris*, respectively (sympatric distribution range represented in purple). Each white dot represents one or several sampled ponds from the same area (cf. Table 1 for details)

for *L. vulgaris* and *L. montandoni* (Stuglik and Babik 2016). Each of these 1380 alignments encompassed the same 13 individuals: six *L. vulgaris* from Romania and Poland, six *L. montandoni* from Romania and Poland and one *L. helveticus* from France (cf. Supplementary Table 1).

For population-level genotyping, we selected the MassARRAY System approach (Sequenom). The MassARRAY assay consists of an initial locus-specific PCR reaction, followed by single base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Using MALDI-TOF mass spectrometry, the distinct mass of the extended primer identifies the SNP allele. This technology can genotype a SNP only if it presents biallelic variability and conserved upstream and downstream sequences of about 100 bp each (cf. Gabriel and Ziaugra 2004 and Gabriel et al. (2009) for more details about the Sequenom MassARRAY technology). Among the 1380 alignments examined, only 127 presented sequences meeting the above mentioned specifications and were used to develop 127 multiplexable candidate probes that were preliminarily tested on 20 *L. vulgaris* and 20 *L. helveticus* from different allopatric localities, in Poland (Krakow), Hungary (Pilisz mount and Budapest), Romania (Apuseni), Norway and Sweden for *L. vulgaris*, and Spain (Escobedo, Fresnedo and Poza de la Sal) and the southern part of France (Dordogne, Cazevielle, Ferrières-les-Verries, Notre-Dame-de-Londres and Aumelas) for *L. helveticus*. This screening phase enabled the extraction of 39 SNPs presenting a low amount of missing data and unambiguously distinguishing *L. vulgaris* from *L. helveticus* (cf. Supplementary Table 2 for the sequences of these probes). The selected alignments were used to design a 39-plex array to genotype the 1035 samples. The genotyping was subcontracted to the Genome Transcriptome facility at the Center for Functional Genomics in Bordeaux (CGFB), France.

Population structure. — Sample allocation and admixture level based on the 39 SNPs dataset were assessed with STRUCTURE v2.2 (Pritchard et al. 2000; Falush et al. 2003) under models assuming two populations (K = 2) as STRUCTURE has been shown to be less sensitive to the proportion of hybrids included in the sample than NEWHYBRIDS (another widely used population genetic programs to address questions related to genetic structure,

Table 1 Geographic sampling of *Lissotriton helveticus* and *L. vulgaris*. For each locality, the number of samples (N, assignment to a given species based on the present results) and the overall frequencies of the diagnostic alleles for each species (*F(h)* and *F(v)*, respectively) are indicated (only *F(h)* frequencies are indicated for the two hybrid specimens). Abbreviations : Hu. Hungary, No. Norway, Po. Poland, Ro, Romania, Sw. Sweden, Fr, France, Sp. Spain, UK. United Kingdom; F1 represents putative F1 hybrids. Localities including samples already analysed by Johanet et al. (2011) are indicated by an asterisk

Sampling site (population field number)		*Lissotriton helveticus*		*Lissotriton vulgaris*		F1		Coordinates
		N	*F(h)*	N	*F(v)*	N	*F(h)*	
	Allopatric localities (*L. vulgaris* only)							
Hu.	Budapest hill (S27-13)	–	–	3	*1*	–	–	47.5764°N 8.8686°E
	Pilisz mount (S24-13)	–	–	3	*0.996*	–	–	47.7294°N 9.0068°E
No.	Trondheim (Tpon-Nor)	–	–	3	*0.991*	–	–	63.9442°N 0.3552°E
Po.	Gartatowice.(S10-12)	–	–	4	*1*	–	–	50.5742°N 0.6239°E
	Krakow (S11-12)	–	–	2	*1*	–	–	50.0397°N 9.9176°E
Ro.	Coma, Apuseni mountains (S29-13)	–	–	3	*1*	–	–	46.2831°N 3.1215°E
	Strâmba, Bistrita district (S30-13)	–	–	3	*1*	–	–	47.2475°N 4.6533°E
Sw.	Sjöbo (TponSwe)	–	–	2	*1*	–	–	55.6729°N 3.7927°E
	total			*23*	*0.998*			
	Allopatric site (*L. helveticus* only)							
Fr.	Aumelas (T08-12).	1	*1*	–	–	–	–	43.5743°N 3.6442°E
	Causses du Quercy (PalQue)*	2	*1*	–	–	–	–	44.2539°N 1.9702°E
	Cazevieille (T01-12).	2	*1*	–	–	–	–	43.7597°N 3.7772°E
	Dordogne, Montouleix (S37-07*, PalGir)	5	*0.995*	–	–	–	–	45.6450°N 0.5885°E
	Ferrières-les-Verreries (T02-12)	6	*0.991*	–	–	–	–	43.8654°N 3.7828°E
	Ile d'Yeu (palYeu)	2	*1*	–	–	–	–	46.6955°N 2.3391°W
	ND-de-Londres (T04-12)	2	*0.987*	–	–	–	–	43.8339°N 3.7460°E
Sp.	Escobedo (S38-07)	3	*0.996*	–	–	–	–	43.3889°N 3.8853°W
	Fresnedo.(S36-07)	4	*0.993*	–	–	–	–	43.3652°N 4.1589°W
	Poza de la Sal (T31-13)	2	*0.987*	–	–	–	–	42.6686°N 3.5288°W
	total	*29*	*0.994*					
	Sympatric site without evidence of syntopy							
Fr.	Ambleteuse, #330 (PalAm)*	4	*1*	0	–	0	–	50.8086°N 1.6473°E
	Bois de l'Epinay, Bl.-Gohier (pal38)*	4	*1*	0	–	0	–	47.4111°N 0.3977°W
	Ile St Aubin, Angers (pon44)*	0	–	4	*1*	0	–	47.5123°N 0.5356°W
	Grande Synthe, Prédembourg (S3-12)	0	–	22	*1*	0	–	51.0200°N 2.2811°E
	Orée du bois, Ahuillé (palOr)*	4	*1*	0	–	0	–	48.7743°N 2.6588°E
	Ozoir -la-Ferrière (T10-12)	16	*0.998*	0	–	0	–	48.7732°N 2.6561°E
	Rocan, Briquenai (PalAr)*	1	*1*	0	–	0	–	49.3926°N 4.8432°E
	Zuydcoote (S2-12)	0	–	29	*1*	0	–	51.0633°N 2.4867°E
	total	*29*	*0.999*	*55*	*1*	*0*		
	Sympatric site with evidence of syntopy							
Fr.	Bazenville (palBaz)*	3	*1*	1	*1*	0	–	49.2835°N 0.5820°W
	Boire des Ecouilles, Liré (pal33)*	5	*0.990*	6	*0.998*	0	–	47.3601°N 1.1380°W
	Bois Boureau (S35-11, S56-14, pal41)*	51	*0.990*	10	*1*	0	–	47.4142°N 0.5428°W
	Notre Dame, Sucy-en-Brie (BDN3, 6)	3	*1*	2	*0.993*	0	–	48.7591°N 2.6038°E

Table 1 (continued)

Sampling site (population field number)		*Lissotriton helveticus*		*Lissotriton vulgaris*		F1		Coordinates
		N	*F(h)*	N	*F(v)*	N	*F(h)*	
	Briollay, Nord (S9-12)	7	*0.994*	31	*1*	0	–	47.5707°N 0.5205°W
	Briollay, Sud (S05-12)	0		24	*0.999*	0	–	47.5636°N 0.5255°W
	Ecordal I (T35-14)	29	*0.997*	0		0	–	49.5291°N 4.6107°E
	Ecordal II (T36-14)	10	*0.999*	41	*1*	1	*0.502*	49.5257°N 4.5888°E
	Faisseault (T39-14)	27	*1*	3	*1*	0	–	49.5988°N 4.4802°E
	Launois sur Vence (T38-14)	5	*1*	32	*1*	0	–	49.6854°N 4.5361°E
	Lorraine, Liverdun (P.Lor)*	4	*1*	1	*0.987*	0	–	48.7462°N 6.0402°E
	Poix Terron (T37-14)	23	*1*	20	*1*	0	–	49.6460°N 4.6557°E
	Puisaye I (T40-14)	24	*0.996*	0		0	–	47.6271°N 3.1879°E
	Puisaye II (T41-14)	30	*0.994*	29	*1*	0	–	47.6379°N 3.1546°E
	Puisaye III (T42-14)	29	*0.995*	0		0	–	47.6382°N 3.2514°E
	Puisaye IV (T43-14)	30	*0.996*	1	*1*	0	–	47.6560°N 3.1984°E
	Puisaye V (T44-14)	30	*0.997*	25	*1*	0	–	47.6720°N 3.1820°E
	Roissy-en-Brie (T9-12)	23	*0.994*	18	*1*	1	*0.502*	48.7923°N 2.6812°E
	Denée (S57-14)	30	*0.993*	10	*1*	0	–	47.3957°N 0.6199°W
	St Jean de la Croix II (S58)	26	*0.996*	21	*1*	0	–	47.4063°N 0.5945°W
	Signy l'abbaye I (T32-14)	21	*1*	1	*1*	0	–	49.7103°N 4.3794°E
	Signy l'abbaye II (T33-14)	22	*1*	1	*1*	0	–	49.7214°N 4.3559°E
	Signy l'abbaye III (T34-14)	27	*1*	1	*1*	0	–	49.7343°N 4.3892°E
	St Germer de Fly I (S22-13)	13	*0.993*	4	*1*	0	–	49.4379°N 1.8087°E
	St Germer de Fly, II (S20-13)	3	*0.996*	8	*1*	0	–	49.4383°N 1.8069°E
	St Germer de Fly, III (S21-13)	8	*0.988*	1	*1*	0	–	49.4377°N 1.8181°E
	St Germer de Fly, IV (S19-13)	8	*0.992*	5	*1*	0	–	49.4375°N 1.8125°E
	St Germer de Fly, V (PalBa)*	23	*0.994*	0	*1*	0	–	49.4399°N 1.8195°E
	Varennes-sur-Loire I (pal43)*	4	*0.997*	3	*1*	0	–	47.2555°N 0.0110°E
	Varennes-sur-Loire II (pal42)*	3	*0.987*	4	*1*	0	–	47.2241°N 0.0732°E
UK	Offham Marshes (palOff)*	1	*1*	2	*1*	0	–	50.9167°N 0.0833°E
	total	*522*	*0.996*	*305*	*1*	*2*		

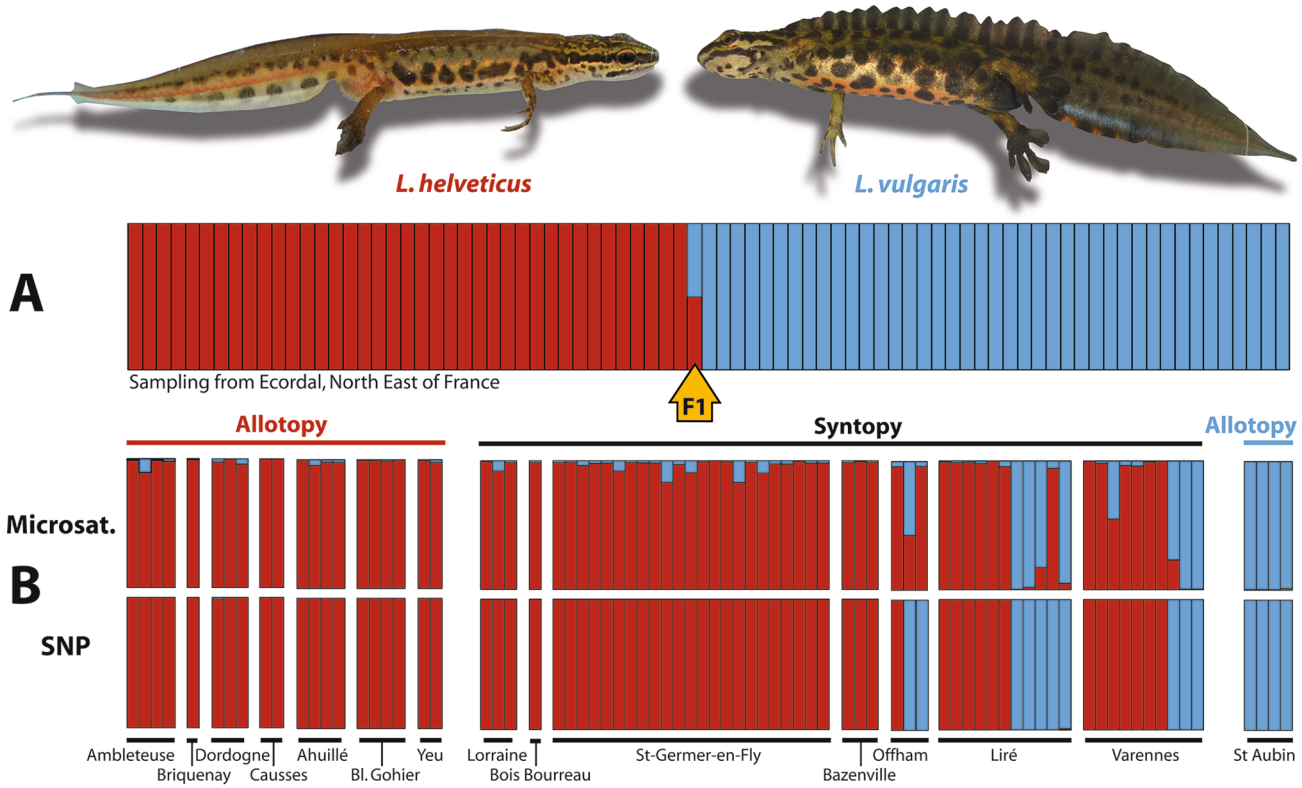

Fig. 2 Overview of the genetic structure using STRUCTURE (with K = 2). **(A)** All the samples genotyped with SNPs are unambiguously inferred as pure representatives of one species or another, except for two putative F1 hybrid specimens with membership coefficients of 0.50 to both species. Only results from Ecordal (NE France), where one of the two hybrids was detected, are illustrated (cf. Table 1 for overall results). **(B)** Two STRUCTURE analyses based on the same set of samples from various localities illustrating the differences between microsatellite and SNP genotyping: the structure inferred from the microsatellite dataset suggests significant introgressive hybridization between both species (above, raw data from Johanet et al. 2011), whereas on the contrary, the structure inferred from the newly generated SNP dataset does not show any signs of admixture between species (below). The photos represent anesthetized males of each species in nuptial coloration

admixture, and hybridization, Anderson and Thompson 2002) (Vähä and Primmer 2006). We estimated posterior distributions based on 3 million MCMC generations of which 50% were discarded as burnin. We used a model that considers the possibility of mixed population ancestry and of correlated allele frequencies among populations due to migration or shared ancestry (Falush et al. 2003), with an alpha value of 1/k (0.5) (Wang 2017). For comparison, we repeated the same analyses using the microsatellite dataset generated by Johanet et al. (2011, six loci: Tv3ca9, Th09, Tv3Ca19, Th14, Th27, Thca14), to compare membership coefficients of the 78 specimens for which both SNP and microsatellite data were generated. The microsatellites genotype data were obtained from the authors of Johanet et al. (2011) and microsatellite genotyping was not repeated for our study. We used the same options for the microsatellite dataset STRUCTURE analyses as for the SNPs. Probability intervals (90% probability intervals) for the membership coefficients were obtained with STRUCTURE by turning on the ANCESDIST option. To assess the power of the microsatellites dataset to reliably estimate ancestry in Smooth and Palmate Newts, we used the software Hybridlabs (Nielsen et al. 2006) to generate simulated genotypes of various ancestry classes. Because many individuals in Johanet et al. (2011) had missing data, we generated simulated genotypes with all six loci and with only four loci (th09, Tv3Ca19, th14 and thca14) to match the loci present in the real specimens with missing data and 90% probability intervals that did not overlap 0/1. With the six loci dataset we simulated 1000 simulated genotypes of each class: pure *helveticus*, pure *vulgaris*, F1 hybrids, backcross to *helveticus* and backcross to *vulgaris*. With the four loci dataset only pure genotypes of each species were simulated (1000 individuals of each class). Parental populations used to simulate genotypes of the various classes were made of 1392 *L. helveticus* and 220 *L. vulgaris* selected from the microsatellites dataset and having a probability of membership to their own cluster of 0.99 or more. Simulated genotypes were analyzed in the exact same way as real genotypes.

Results

Samples with three or more missing SNP loci (70 samples) were discarded from the dataset, leading to a final biallelic SNP data-set for 965 usable individual newts, with only 5.3% of missing data (4,256 undetermined SNP genotypes out of a total of 80,730). From the 39 selected SNPs, two showed shared alleles at significant frequencies: SNP 10,797 and SNP 10,913. For both loci, the "vulgaris" allele was found in *Lissotriton helveticus* in low frequencies that were similar between syntopic (3.3% for SNP 10,797 and 13.2% for SNP 10,913) and allopatric populations (4.0% and 20.0%, respectively); these two loci are thus not fully diagnostic. In spite of this, the SNPs allowed specific discrimination of every sample, including newly collected sample as well as those previously studied by Johanet et al. (2011), and no significant sign of introgression between species was detected, i.e., species-specific alleles of one species were not found in the other. Only two samples collected in two syntopic localities in France revealed an F1-hybrid genotype: one (sample T9-12-LH8f-2, from Roissy-en-Brie) is heterozygous (*vh*) for 37 SNP loci (homozygous for the two remaining SNP loci, one of *vv* type and one *hh* type) and a second sample (T36-14-24-1, from Ecordal) is heterozygous for all the 38 SNP loci available (genotype at one locus missing). No other signs of hybridization or introgression were detected (Fig. 2A; Table 1).

In contrast with the SNP-based results, the sample allocation and admixture level presently inferred from the microsatellite dataset previously generated by Johanet et al. (2011) suggest a substantial admixture of genes from the other species (i.e., ≥ 0.1) for 11 of the 78 samples presently reanalyzed with SNPs, in accordance with the results of the original publication (See Fig. 2B and Supplementary Table 3 for a comparison, for the same 78 samples, of the admixture levels inferred from microsatellites and SNP, respectively). Note however that, for all these 78 samples, the 90% probability intervals include 0/1, even when the assignation is 0.45/0.55, so none of these samples is "significantly" admixed when it comes to assessing their ancestry with Structure (Sup Table 4). Only six *helveticus* out of 1923 samples of both species genotyped with microsatellites by Johanet et al. (2011) have 90% probability intervals that do not overlap 0/1 in our reanalysis and their assignation to *helveticus* ranges from 0.14 to 0.34 (Sup Material 5).

Simulated genotypes revealed a pattern similar to the empirical samples. With the six loci dataset, nine simulated "pure *helveticus*" samples out of 1000 have admixed estimations > 0.10 (probability of *helveticus* assignation < 0.9), with a maximum *vulgaris* ancestry of 0.17, and four out of 1000 have 90% probability intervals that doe not overlap 0 /1 (with *vulgaris* ancestry estimates from 9 to 16%). Among the 1000 pure *vulgaris* simulated genotypes, 14 have admixed estimation > 0.10, with a maximum *helveticus* ancestry $= 19\%$, and two have 90% probability intervals that doe not overlap 0 /1 (with *helveticus* ancestry of 11% and 16%). Simulated F1 were all identified as admixed but their ancestry estimates varied widely, from 0.18/0.82 to 0.76/0.24 (*vulgaris*/*helveticus* membership, respectively), and 200 simulated F1 genotypes have their probability of membership to the majority cluster > 0.6. Simulated backcrosses were all assigned to the species with the highest ancestry with a high probability and did not differ visibly from pure genotypes (Sup. Material S5).

Counterintuitively, STRUCTURE performance with the 4-loci dataset was better for simulated *helveticus* samples: out of 1000 simulated genotypes, none had a probability of assignation below 0.9 (minimum: 0.93) and none had a 90% probability interval that did not overlap 0/1. Pure simulated *vulgaris* genotypes were less reliably identified by STRUCTURE: 11 were recovered as admixed over 10%, with a maximum value of 0.342 assignation probability to *helveticus*, but again none had a 90% probability interval that did not overlap with 0/1.

Discussion

These results confirm the conclusions of Arntzen et al. (1998): *Lissotriton helveticus* and *L. vulgaris* can hybridize in nature but this phenomenon remains rare, as we found only two F1 individuals out of a total of 829 adults from syntopic populations. Such hybrids are likely sterile as we detected no signs of introgression between both species. In addition, SNP analyses of 78 of the samples previously analysed by Johanet et al. (2011), including samples identified as admixed individuals by microsatellite genotypes, detected no sign of hybridization either, suggesting that detection of hybridization and introgression with microsatellites produced biased results that overestimated the frequency of hybridization between these two species (Fig. 2B). The lack of introgression is consistent with the maintenance of a broad sympatric zone and supports the hypothesis that convergence of certain color traits (dorsum, tail) observed in sympatry would primarily be the result of plastic or adaptive responses to environmental variables and not introgression (de Solan et al. 2022). This result also echoes the findings of Drillon et al. (2019) who found hardly any hybridization and no introgression between *Hyla* tree frog species that diverged around 20 Mya. Yet, the possibility of introgressive hybridization can persist for a very long time in newts, as exemplified by *Triturus cristatus* and *T. marmoratus* that hybridize frequently and can still exchange genes in spite of their divergence estimated at around 24 Mya (Wielstra et

al. 2011, Arntzen et al. 2021, comparable with the timing of divergence between *L. vulgaris* and *L. helveticus* presented in introduction).

An alternative explanation would be that the SNP genotyping underestimated hybridization and introgression, whereas the microsatellites returned the correct pattern of admixture. Our SNP loci have been selected to be (near) perfectly diagnostic (fixed alternative alleles in both species) and might therefore over-represent genomic regions highly differentiated between species, and thus, indirectly, genes involved in barriers to gene flow (which, by counter-selection, would be less likely to introgress in the long term in the other species). On the contrary, microsatellites have been designed and selected independently of their level of divergence between species and should thus not overrepresent genomic regions that resist introgression. This hypothesis could be valid if the microsatellite results were used to detect long-term (historical) interspecific gene flow, as this would indeed be reduced in genomic regions involving barrier loci. However, it does not apply to the detection of recent admixture (F1 hybrids and backcrosses), in which case the SNP results are expected to be more robust than the microsatellite results due to a larger number of loci and unambiguous allelic assignment in the former. Thus, this hypothesis does not explain the low frequency of F1s and the total absence of F2 or backcrosses in the SNP dataset, in contradiction with the previous study based on microsatellites who inferred the presence of recently admixed individuals (Johanet et al. 2011).

It thus seems more likely that the microsatellite analyses by Johanet et al. (2011) led to an overestimation of introgression. In general, SNPs have recurrently been shown to outperform microsatellites (e.g. Camacho-Sanchez et al. 2020, Bradbury et al. 2015, Hoffman et al. 2014, Lemopoulos et al. 2019, Sunde et al. 2020, Zimmerman et al. 2020, Szatmári et al. 2021). These works most often involved a far greater number of SNP loci than in the present study (i.e. hundreds or thousands of SNPs *versus* 39 SNPs) but they did not select diagnostic SNP loci, which would be trivial as diagnostic SNP provide unambiguous detection of F1 and backcrosses. More specifically, several recent studies comparing relative performance of SNPs and microsatellites for hybridization detection effectiveness have shown that many hybrids indicated by microsatellites are not validated by SNPs (Daïnou et al. 2017, Szatmári et al., 2021). Assessment of hybridization usually rests, as in the present case, on statistical estimation of the likelihood to observe a given multilocus genotype in a population (assignment methods such as STRUCTURE or NEWHYBRIDS) or comparisons of multivariate axes scores (Johanet at al. 2011). These assignment methods can produce unreliable results when a small number of microsatellites are used or when there is too much missing data (Grünwald et al. 2017; Hodel et al. 2017; Yi and Latch 2022). Analyses of datasets simulated under scenarios with different levels of genetic divergence and varying number of loci have shown that two popular model-based Bayesian methods for detecting hybridization (i.e. STRUCTURE and NEWHYBRIDS) require a significantly higher number of loci than commonly applied in microsatellite-based studies (Vähä and Primmer 2006). According to this study, efficient detection of F_1 hybrid individuals would require the use of 12 or 24 loci (for pairwise F_{ST} between hybridizing parental populations of 0.21 or 0.12, respectively), and separating backcrosses from pure individuals would require an even more significant genotyping effort (≥ 48 loci, even when divergence between parental populations are high). In addition to the low number of loci usually involved in microsatellites studies ($n = 6$ in Johanet et al. 2011), it should be noted that these markers are also notoriously difficult to use to assess hybridization because they are highly polymorphic (from 12 to 35 alleles per locus, with an average of 25 alleles in both species in Johanet et al. 2011) which, coupled with homoplasy, often result in a lack of diagnostic loci (Putman and Carbone 2014; Daïnou et al. 2017; Šarhanová et al. 2018). Additionally, the study by Johanet et al. (2011) was affected by a significant amount of microsatellite missing data (22.2% for the original dataset). Within the 1924 samples involved, 41.8% were missing at least one third of the data (i.e., 2 or 3 loci out of a total of six) and 11.4% were missing half of the data (i.e., 3 out of 6). However, some of the samples for which microsatellites suggested substantial admixture of genes from the other species (i.e., ≥ 0.1, $n = 11$) had no missing data, including two of them where the 90% probability interval did not overlap 0/1 (Sup. Material 5).

Taken together, these considerations prompt us to conclude that the most parsimonious explanation for the discrepancy between Johanet et al. (2011) on the one hand, and Arntzen et al. (1998) and the present study on the other hand, is the poor performance of NEWHYBRIDS (microsatellite analysis by Johanet et al. 2011) and STRUCTURE (reanalysis of the same dataset in the present study) to reliably assess ancestry using a small number of hypervariable microsatellite loci. This hypothesis is strengthened by the examination of the 90% probability intervals of the STRUCTURE membership coefficients (see Sup. Table S3 and S4) that all overlap 0 or 1 except for 6 individuals (in bold in Sup. Table S3). In particular, the four individuals that seem to have the strongest signs of admixture in Fig. 2B (one in population Offham, one in population Liré and two in population Varennes) all have 90% probability overlap that overlap 0 or 1. It thus seems that the microsatellite data simply did not have enough resolution to assess reliably the occurrence (or lack of) admixture in the previous study. This interpretation

is strengthened by the STRUCTURE analyses of simulated pure and F1 microsatellite genotypes that confirm that (i) STRUCTURE infers as admixed (admixture > 0.1) some pure genotypes, some of them with 90% probability intervals that do not overlap 0/1 and (ii) STRUCTURE assignation probabilities of simulated F1 hybrids have a wide range of values reaching as low as 20% of admixture. The "admixture" identified by Johanet et al. (2011) thus seem to result from a mixture of low frequency hybridization (as confirmed with the SNPs) and false signals of admixture resulting from the low power of the microsatellite dataset.

Over the past decades, microsatellite genotyping has been the approach favored by the research community to look for hybridization between non-model species. The present study suggests that many of these works may have overestimated the frequency of this phenomenon, as was the case here. The conclusions from these studies, and the subsequent works based on them, should therefore be carefully reconsidered in the light of their respective datasets coverage. By providing a broad coverage of loci, including diagnostic loci, genomic approaches (such as the SNP approach of the present study) would certainly represent preferable alternatives to microsatellite approaches to investigate introgressive hybridization, and should contribute to refining our understanding about the extent of this phenomenon in nature.

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Declarations

Conflict of interest The authors have no competing financial interests to declare.

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