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Skin swabbing as a new efficient DNA sampling technique in amphibians, and 14 new microsatellite markers in the alpine newt (*Ichthyosaura alpestris*)

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Abstract

This study introduces a novel DNA sampling method in amphibians using skin swabs. We assessed the relevancy of skin swabs relevancy for genetic studies by amplifying a set of 17 microsatellite markers in the alpine newt *Ichthyosaura alpestris*, including 14 new polymorphic loci, and a set of 11 microsatellite markers in *Hyla arborea*, from DNA collected with buccal swabs (the standard swab method), dorsal skin swabs and ventral skin swabs. We tested for quality and quantity of collected DNA with each method by comparing electrophoresis migration patterns. The consistency between genotypes obtained from skin swabs and buccal swabs was assessed. Dorsal swabs performed better than ventral swabs in both species, possibly due to differences in skin structure. Skin swabbing proved to be a useful alternative to buccal swabbing for small or vulnerable animals: by drastically limiting handling, this method may improve the trade-off between the scientific value of collected data, individual welfare and species conservation. In addition, the 14 new polymorphic microsatellites for the alpine newt will increase the power of genetic studies in this species. In four populations from France (n = 19–25), the number of alleles per locus varied from 2 to 16 and expected heterozygosities ranged from 0.04 to 0.91. Presence of null alleles was detected in two markers and two pairs displayed gametic disequilibrium. No locus appeared to be sex-linked.

Keywords: 454 sequencing, buccal swabs, DNA sampling, *Hyla arborea*, *Ichthyosaura alpestris*, skin swabs

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Introduction

Although widely distributed in most ecosystems, amphibians are the vertebrate group with the highest proportion of species threatened with extinction (Beebee & Griffiths 2005). Among the identified threats to amphibian populations, habitat fragmentation is considered crucial, impacting both demographic processes and genetic diversity (Cushman 2006). DNA-based methods using neutral genetic markers such as microsatellites offer an efficient way to assess the effects of fragmentation on biological processes and long-term population persistence by allowing the estimation of effective population sizes and gene flow (Holderegger & Wagner 2008; Balkenhol *et al.* 2009; Storfer *et al.* 2010). To increase inferential power, genetic approaches involve both the sampling of many loci across the genome (Selkoe & Toonen 2006), and the sampling of many individuals (Manel *et al.* 2003). In the first case, conservation biologists can rely on new sequencing technologies for identifying and developing new polymorphic microsatellite loci (Perry & Rowe 2011). In the latter case, because genetic sampling may have significant impacts on animals, field studies should be designed according to trade-offs between scientific value of collected data and individual or species welfare (Parris *et al.* 2010). Researchers should use the least destructive and least invasive methods, especially when sampling endangered, vulnerable, or declining amphibian species (Pidancier *et al.* 2003).

There are different methods for genetic sampling in amphibians, each one impacting the animals' welfare in different ways. The toe clipping method (McGuigan *et al.* 1998), which is classically used for individual marking, provides valuable amounts of DNA. This

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method is rather controversial (Funk *et al.* 2005) because it may reduce individual survival, especially in climbing species such as tree frogs (McCarthy & Parris 2004). The tail tipping method (Snell & Evans 2006) is usually applied to DNA sampling in anuran tadpoles, but may be used in urodeles at any life-stage (tail- or crest-clipping); however, a proportion of sampled animals may die as a result of stress during sampling, or later because of infection or reduced mobility (Parris *et al.* 2010). Collecting the entire tadpoles may have little impact on species welfare, but it obviously leads to the death of sampled individuals and implies being able to identify species (Parris *et al.* 2010). Buccal swabbing is an alternative method of genetic sampling that can provide enough DNA for microsatellite genotyping for a range of amphibian species (Pidancier *et al.* 2003; Broquet *et al.* 2007). However, collecting buccal cells with cotton swabs requires levering open the upper and lower jaw with a sterile spatula. Collecting buccal cells and opening the jaw may lead to an amount of bleeding (Pidancier *et al.* 2003). Some species, such as *Hyla arborea*, are easily handled as they tend to keep their jaws opened during sampling, whereas species such as *Triturus cristatus* usually try to keep their mouth closed, and can be easily injured with either rigid tape or cotton swabs. A sampling technique currently used to detect chytrids (*Batrachochytrium dendrobatidis*), a cutaneous pathogenic fungus associated with amphibian mass mortalities and population declines worldwide (Berger *et al.* 1998), relies on sloughing skin removal with skin swabs (Kriger *et al.* 2006; Soto-Azat *et al.* 2009). Although skin swabs can provide fungal DNA, this sampling technique was never used, to our knowledge, to study DNA from the sampled individuals themselves. Nevertheless, skin swabs may minimize stressful handling of animals and may thus overcome ethical issues linked to other DNA sampling methods.

This article presents an alternative genetic sampling method for amphibians, which meets ethical expectations when studying these vulnerable species. We assessed the efficiency of skin swabs compared with buccal swabs to collect genetic data, in a urodele (*Ichthyosaura alpestris*) and an anuran (*H. arborea*). This work is also an opportunity to present a supplementary set of polymorphic microsatellite loci in the alpine newt *I. alpestris*, a widespread species in France sharing many ecological characteristics with amphibians such as *Lissotriton vulgaris* or the endangered *T. cristatus* (Emaresi *et al.* 2011). We detail the conditions for successful multiplex polymerase chain reaction (PCR) amplifications of the new markers in addition to three markers developed by Garner *et al.* (2003), and present results of cross-species amplifications in two other urodele species: *Lissotriton helveticus* and *T. cristatus*.

Material and methods

Study area and DNA sampling

All samples were collected in France during the breeding season. To develop microsatellite markers, muscle and liver samples were collected from a single *I. alpestris* male found moribund on a road in 2009 and stored in 95% ethanol (code GL in Table 1).

All other individuals were trapped in breeding ponds in 2009 or 2010 and sampled using buccal and/or skin swabs. Buccal sampling was performed by opening the animals' mouth with a sterile rigid spatula (Pidancier *et al.* 2003) and swabbing the surfaces of the buccal cavity with an ordinary cotton bud (Poschadel & Moller 2004).

Table 1 Sampling characteristics

Protocol	Region	Code	Latitude	Longitude	Species	*N*	Sample type
DMM	Rhône-Alpes	GL	45°25′13.3″N	5°24′56.0″E	*I. alpestris*	1	T
EGV	Ile-de-France	CN	48°42′02.7″N	2°43′10.2″E	*I. alpestris*	22	B
EGV	Ile-de-France	CW	48°41′12.3″N	2°42′24.1″E	*I. alpestris*	19	B
EGV	Bourgogne	X43	46°52′07.3″N	5°11′47.8″E	*I. alpestris*	25	B
EGV	Franche-Comté	Z23	46°54′25.1″N	5°27′31.5″E	*I. alpestris*	24	B
SSE	Rhône-Alpes	F	45°46′47.7″N	4°52′04.5″E	*I. alpestris*	23	B, D, V
SSE	Rhône-Alpes	I	45°44′18.0″N	5°21′06.3″E	*H. arborea*	4	B, D
CSA	Bourgogne		**47°39′41.0″N**	**3°02′00.0″E**	*T. cristatus*	11	B
CSA	Bourgogne		**46°46′24.0″N**	**5°29′23.0″E**	*L. helveticus*	12	B

DMM, development of microsatellite markers; EGV, estimation of genetic variability; SSE, skin sampling efficiency; CSA, cross-species amplification; T, tissues; B, buccal swabs; D, dorsal swabs; V, ventral swabs.
For the CSA protocol, geographical coordinates (in bold) correspond to the northwestern and southeastern corners of the corresponding sampling area.

The skin swab technique was inspired by Kriger *et al.* (2006) and performed by firmly running an ordinary cotton bud three times over the dorsal (dorsal skin swab) or ventral surface (ventral skin swab).

Skin swab sampling efficiency was assessed using 23 alpine newts (*I. alpestris*), captured in 2010 in site F, and four European tree frogs (*H. arborea*), captured in 2010 in site I (Table 1). Each individual was sampled using buccal swabs and dorsal skin swabs. Eight alpine newts and the four tree frogs were also sampled using ventral skin swabs. In frogs, the abdomen was too dry to allow a sample, and swabs were not considered for DNA extraction. Individuals were immediately released after the sampling, except four alpine newts and the four European tree frogs that were kept in observation for a month. All swabs were kept at ambient temperature (around 15–20 °C) in a hermetic container with silica gel providing total desiccation.

For the estimation of microsatellite genetic variability in natural populations, we sampled 90 individuals in four distinct breeding ponds, located from 2 to 286 km apart, with 19–25 alpine newts at each site (codes CN, CW, X43 and Z23 in Table 1). Each individual was morphologically sexed, sampled using buccal swabs and immediately released. We also tested microsatellite markers for cross-species amplification using 23 individuals of two other urodele species: *L. helveticus* (n = 12) and *T. cristatus* (n = 11). The 23 individuals were randomly collected across Bourgogne (France) during an independent fieldwork campaign (Table 1). They were sampled using buccal swabs and immediately released.

Development of microsatellite markers

Genomic DNA was extracted from tissue samples collected at GL site (Table 1), following Nurnberger *et al.* (2003). To identify alpine newt specific microsatellites, genomic DNA was sequenced using 454 FLX pyrosequencing technology (Abdelkrim *et al.* 2009) at the Savannah River Ecology Laboratory (University of Georgia). Raw DNA sequences were cleaned of remnant vector and screened for appropriate microsatellite motifs using the program MSATCOMMANDER (Faircloth 2008). Among 1015 fragments containing microsatellites, 61 were used to design primer pairs with PRIMER3 version 0.4.0 (Rozen & Skaletsky 2000). Only 14 pairs of primers amplified easy-to-score and polymorphic microsatellites (Table 2).

Microsatellite genotyping

DNA extractions from swabs were performed using a Qiagen DNeasy Tissue Kit (Qiagen), following the manufacturer's instructions. DNA was eluted in a final volume of 100 μL of buffer BE (Qiagen). DNA concentrations were estimated using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc) and each sample was treated by dilution or evaporation with a Speed-Vac apparatus (Concentrator plus; Eppendorf) to reach 20 ng/μL.

For *I. alpestris*, the 14 developed markers were studied together with *Ta1Ca1*, *Ta2Caga3* and *Ta1Caga4* microsatellite loci (Garner *et al.* 2003). Among the seven primer pairs developed by Garner *et al.* (2003), only those three microsatellite loci worked well in our experimental conditions. The 17 primer pairs were used to constitute four sets of 4–5 markers for multiplex PCR amplification (Table 2). PCR was conducted on PTC-100 Thermal Cycler (MJ Research, Inc.) using Type-it™ Microsatellite PCR Kit (Qiagen) in a 10 μL volume containing 5 μL Qiagen multiplex PCR Master Mix, 3 μL of water, 1 μL of primer mix (2 μM of both forward and reverse primers) and 1 μL of genomic DNA at 20 ng/μL. Cycling conditions were the following: 95 °C for 5 min; 32 cycles of denaturation at 95 °C (30 s), annealing at 60 °C (90 s) and extension at 72 °C (30 s); lastly, 60 °C for 30 min. The forward of each primer pair was labelled with a fluorescent dye: FAM or HEX (Sigma Aldrich), NED or PET (Applied Biosystems). Cross-species amplifications in *L. helveticus* and *T. cristatus* were attempted using the PCR conditions optimized for *I. alpestris*.

In *H. arborea*, PCR amplifications were performed with two sets of 5 and 6 primer pairs (set 1: Ha-B5R3, Ha-A119, WHA1-2, Ha-D3R3, Ha-A11, Ha-A127; set 2: D106, H116, Ha-D115, A136, Ha-A130; (Arens *et al.* 2000; Berset-Brandli *et al.* 2008a, b) at the following cycling conditions: 95 °C for 15 min; 30 cycles of denaturation at 94 °C (30 s), annealing at 59 °C (90 s) and extension at 72 °C (30 s); lastly, 60 °C for 30 min.

The PCR products were diluted 50 times and discriminated using capillary electrophoresis on a 3730xl DNA Analyser (Applied Biosystems). For each locus, alleles were scored with GENEMARKER 1.95 (SoftGenetics), using GS600 LIZ size standard (Applied Biosystems). Ambiguous genotypes were amplified and sized a second time.

We checked the presence of null alleles in the four populations CW, CN, X43 and Z23 (Table 1) by analysing homozygote excess with MICROCHECKER (Van Oosterhout *et al.* 2004) and by calculating the number of individuals apparently homozygous for a null allele, that is which repeatedly failed to amplify any alleles at just one locus while all other loci amplified normally (Selkoe & Toonen 2006). We estimated the number of alleles per locus, observed and expected heterozygosity, and checked Hardy–Weinberg equilibrium and gametic disequilibrium with GENEPOP 4.0 (Rousset 2008). Tests for Hardy–Weinberg equilibrium (HWE) were conducted using the sequential Bonferroni correction to account for multiple-related tests (Rice 1989).

Table 2 Fourteen microsatellite loci from *Ichthyosaura alpestris* with PCR primers, repeat motifs, size ranges (bp: base pair) and accession number

Locus	Primer (5′–3′)	Repeat motif	Size range (bp)	Accession number
PCR multiplex set 1				
CopTa1	F: (FAM)-CATGAGGGTATGGGTGGTCAGC R: CCATTGCCCACAATGCAAACTG	(AC)10	85–93	JN048427
CopTa2	F: (HEX)-ACAGGAACTTACAGCCTACCC R: AGCAGTCCCTGGCTATTTAAG	(AC)10	88–96	JN048428
CopTa3	F: (NED)-AGTCACTTAAGGCCACAGGG R: CTAAAGCTCCTTCATGGGAGGC	(AC)11	75–127	JN048429
CopTa4	F: (PET)-TCTTCCTCCTAGACCCTTGTGG R: GACACAGTAGATTGGCAAGTGG	(AC)12	196–206	JN048430
PCR multiplex set 2 (completed with Ta1Caga4)				
CopTa5	F: (HEX)-CTGGCATGAGTGGCCTTGGTTC R: TGTCTGTTTGGGAAAGCACAG	(AC)10	48–67	JN048431
CopTa6	F: (FAM)-AACCTACAGATCACCCTTTCC R: CCCTCGTGTGCCTTGAGACCC	(AATC)11	70–87	JN048432
CopTa7	F: (FAM)-CGAGCGGAACTACAATTGAAAC R: CTCCCGCAGCACGGAGATACC	(AGAT)10	188–231	JN048433
PCR multiplex set 3				
CopTa8	F: (NED)-ACATAAGCTGAGCAAACCATGC R: GGTCTTACAGTTCGATTTACTGTGG	(AC)10	86–116	JN048434
CopTa9	F: (HEX)-ACACTACCAATTTCTGAACGC R: TGAAGTGGAAGTTACATCGGG	(AGAT)14	189–262	JN048435
CopTa10	F: (FAM)-GCACGAGCGGAACTCCTTCTG R: TTCCCACAGCTTACCTGAGCAC	(AGAT)16	185–295	JN048436
CopTa11	F: (PET)-GACTGTCAGAGAACCACTTACC R: GTGTGTGTAGAACTGCCTCC	(ACTC)15	172–223	JN048437
PCR multiplex set 4 (completed with Ta1Ca1 and Ta2Caga3)				
CopTa12	F: (HEX)-CTTTGCATGGGAAACAAAGGCG R: CCCTTGCAAACAGTGTATAGG	(AC)12	68–72	JN048438
CopTa13	F: (HEX)-GGGACACAGGAAATGAGACAGGC R: GAACCATTAAGCGTGTCCCTGC	(AG)11	192–204	JN048439
CopTa14	F: (FAM)-GTGGGATGTATGTTGGATTTCAC R: TAAGAGGGCTTCAGGGACAGTGG	(AC)10	199–205	JN048440

Skin sampling efficiency

The inconsistency between genotypes obtained from different swabbing methods was assessed by calculating e_1, the ratio between the number of single-locus genotypes obtained from ventral (e_{1v}) or dorsal (e_{1d}) swabs including at least one allelic difference with genotypes obtained from buccal swabs and the total number of single-locus genotypes. This index is analogous to e_1, the mean error rate per locus (Pompanon *et al*. 2005) using the genotypes obtained from buccal swabs as reference genotypes. It was calculated for each individual collected in populations F and I (Table 1) and averaged over all individuals in each species. We used the free software R (R Development Core Team 2011) to test for the correlation between e_1 (pooling e_{1d} and e_{1v}) and the concentrations of DNA in newt skin swab samples, using the Kendall rank correlation test. We also used a Wilcoxon rank sum test to compare error rates when using dorsal and ventral samples. To test for DNA quality and quantity, we compared migration patterns of extracted DNA at 20 ng/μL on a 3% agarose gel (Fig. 1).

Results

Skin sampling efficiency

In *I. alpestris*, buccal swab sampling provided a large quantity of DNA (152.96 ± 66.32 ng/μL, mean ± SD, n = 23) and enabled genotyping of all individuals (Table S1). Dorsal swab sampling provided a smaller quantity of DNA (18.13 ± 11.88 ng/μL, mean ± SD, n = 23, see Fig. 1). The mean difference per locus between genotypes from dorsal and buccal swabs was e_{1d} = 7.69% (n = 23) after the first amplification. Differences were mainly due to allelic dropouts, PCR failing to amplify some alleles;

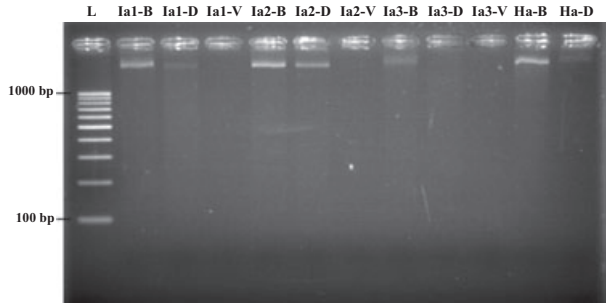

Fig. 1 Migration patterns of extracted DNA at 20 ng/μL visualized by ethidium bromide on 3% agarose gel, in *Ichthyosaura alpestris* and *Hyla arborea*. L: 100 bp DNA ladder; Ia1, Ia2 and Ia3 correspond to samples from three alpine newts (one female and two males respectively); Ha corresponds to samples from a male tree frog; -B, -D and -V correspond to Buccal, Dorsal and Ventral samples. Only buccal and dorsal swabs show visible bands, corresponding to high quantity of non-degraded native DNA; lower intensity in dorsal samples indicates lower DNA concentrations of DNA. Absence of a clear band in ventral swabs indicates that observed DNA concentrations are very low. See text for details on concentrations.

only four differences of 30 were due to allelic mismatch or contamination. This value decreased to e_{ld} = 0.51% (n = 23) after a second independent amplification of loci showing inconsistent genotypes. Then, dorsal swabs gave genotypes consistent with that obtained from buccal swabs for all individuals across all markers but CopTa3, the second independent amplification failing to reveal the shorter allele (75 bp) for two individuals. Ventral swab skin sampling provided the smallest quantities of DNA (6.48 ± 5.45 ng/μL, mean ± SD, n = 8, see Fig. 1) and, after the first amplification, led to significantly higher inconsistencies between genotypes (e_{Lv} = 52.21%, n = 8) than dorsal swab skin sampling (e_{ld} = 7.69%, n = 23, Wilcoxon rank sum test, V = 24, P = 0.0014). Ventral swab sampling was less efficient than dorsal sampling in this species, and thus not considered for new independent amplifications. The correlation between the mean error rates e_l and the concentrations of DNA in skin swab samples was negative and highly significant (Kendall rank correlation test, $\tau = -0.601$, 2-sided P-value $<10^{-5}$), indicating that genotyping efficiency notably depends on the quantity of collected DNA in skin swab samples.

In *H. arborea*, buccal and dorsal swab sampling provided identical genotypes for all individuals from the first amplification (e_{ld} = 0%, n = 4) across all 11 loci. Buccal swab sampling tended to provide a higher quantity of DNA than dorsal swab sampling (respectively 43.69 and 10.95 ng/μL, mean, n = 4, see Fig. 1). The eight individuals that had been sampled using both buccal and skin swabs and kept in observation did not show any visible side-effect.

Microsatellite markers

None of the 14 developed microsatellite markers (Table 1) appeared to be sex-linked, as heterozygote individuals were found in both sexes at each tested locus. The number of alleles ranged from 2 to 16. Observed heterozygosities (H_o) ranged from 0.04 to 0.96 and expected heterozygosities (H_e) ranged from 0.04 to 0.91 (Table 3). No locus departed significantly from Hardy–Weinberg equilibrium, following sequential Bonferroni correction. A trend towards allelic dropout was detected in CopTa3 for the two shorter alleles (75 and 77 bp): In electropherograms, peaks located at 75 or 77 bp are actually systematically smaller than peaks corresponding to longer alleles. For each individual, deciding whether a peak at 75 or 77 bp is an allele or an artefact may be challenging at first sight: any ambiguous genotype, i.e. any individual that was not clearly heterozygous for that locus (with two peaks at more than 77 bp), was thus checked with a new amplification that always led to the same electropherogram profile, showing that the DNA concentration (20 ng/μL) enabled a high detection level of these alleles from the first amplification. The presence of null alleles was detected in two markers: CopTa5 and CopTa6. Preliminary work on six other populations confirmed both the trend towards allelic dropout for the two shorter alleles in CopTa3 and the presence of null alleles in CopTa5 and CopTa6 (data not shown). Significant gametic disequilibrium was detected between CopTa7 and markers CopTa9 and CopTa10.

Using the PCR conditions optimized for *I. alpestris*, cross-species amplification tests revealed only two polymorphic markers for *L. helveticus* (CopTa3 and CopTa13), each exhibiting four alleles for eight individuals. No amplification was obtained at any locus for *T. cristatus*.

Discussion

There are key differences between the skin sampling protocol discussed in that study and the one used to gather and extract chytrid DNA in Kriger *et al.* (2006). To optimize the detection of *B. dendrobatidis*, skin has to be swabbed by firmly running the tip of a swab over different body parts (dorsal and ventral surface, and also body sides, underside of thighs and webbing of feet), at least once, but sometimes up to ten times (Kriger *et al.* 2007); swabs have then to be frozen at −20 °C. To get host DNA, which can be found obviously on any part of the body, we advise to firmly run the tip of a swab only three times over the skin and to concentrate the sampling on a single surface (dorsal or ventral surface) so as to limit the handling of animals. This protocol also offers practical advantages in the field: as genetic studies often rely on species-specific markers, cotton buds do not necessarily require to be acquired sterile (Poschadel & Moller 2004),

Table 3 Summary data for 12 microsatellite loci developed for *Ichthyosaura alpestris*

Populations	*N*	*A*	H_o	H_e	*f*	*N*	*A*	H_o	H_e	*f*
	CopTa1					CopTa9				
CW	19	3	0.421	0.496	0.155	19	8	0.842	0.845	0.004
CN	22	2	0.364	0.304	−0.200	22	8	0.864	0.886	0.026
X43	25	3	0.640	0.541	−0.187	25	14	0.960	0.912	−0.054
Z23	24	3	0.417	0.430	0.032	24	13	0.875	0.900	0.028
Total	90	3 (2.76)				90	16 (11)			
	CopTa2					CopTa10				
CW	19	4	0.737	0.681	−0.084	19	8	0.632	0.788	0.203
CN	22	4	0.545	0.697	0.221	22	5	0.455	0.673	0.330
X43	25	3	0.800	0.615	−0.310	25	10	0.800	0.811	0.013
Z23	24	4	0.542	0.691	0.219	24	9	0.583	0.801	0.276
Total	90	4 (3.72)				90	15 (8.09)			
	CopTa3					CopTa11				
CW	19	8	0.684	0.767	0.110	19	5	0.947	0.781	−0.220
CN	22	6	0.773	0.686	−0.130	22	5	0.636	0.747	0.152
X43	25	7	0.640	0.681	0.061	25	7	0.760	0.673	−0.132
Z23	24	8	0.792	0.769	−0.031	24	4	0.708	0.741	0.045
Total	90	11 (7.23)				90	8 (5.29)			
	CopTa4					CopTa12				
CW	19	2	0.158	0.149	−0.059	19	2	0.579	0.508	−0.145
CN	22	2	0.273	0.241	−0.135	22	2	0.455	0.507	0.106
X43	25	3	0.600	0.561	−0.071	25	2	0.160	0.150	−0.067
Z23	24	3	0.500	0.526	0.050	24	2	0.458	0.467	0.019
Total	90	3 (2.54)				90	2 (2)			
	CopTa7					CopTa13				
CW	19	10	0.789	0.883	0.109	19	2	0.316	0.478	0.346
CN	22	11	0.818	0.874	0.066	22	3	0.545	0.513	−0.066
X43	25	8	0.760	0.780	0.027	25	4	0.480	0.543	0.118
Z23	24	11	0.833	0.853	0.023	24	4	0.625	0.495	−0.271
Total	90	13 (9.96)				90	4 (3.33)			
	CopTa8					CopTa14				
CW	19	3	0.474	0.525	0.100	19	3	0.316	0.317	0.005
CN	22	3	0.727	0.606	−0.207	22	3	0.182	0.172	−0.057
X43	25	2	0.040	0.040	0.000	25	3	0.640	0.575	−0.116
Z23	24	5	0.500	0.483	−0.036	24	3	0.500	0.613	0.188
Total	90	5 (3.26)				90	3 (3)			

Number of alleles (*A*; in brackets, effective number of alleles), observed and expected heterozygosity (H_o and H_e) and fixation index (*f*) are given for each locus for *N* individuals analysed for four populations in France: two populations from Seine-et-Marne (CW and CN) and two populations from Saône-et-Loire (X43 and Z23). No locus departed significantly from Hardy–Weinberg equilibrium, following sequential Bonferroni correction.

as long as inter-individual contaminations are avoided. Besides, samples do not need to be frozen, but can be stored at ambient temperature in a hermetic container with silica gel providing total desiccation.

In both *I. alpestris* and *H. arborea*, dorsal swabs provided satisfactory amounts of DNA, and correct genotypes for all individuals at all loci except one. Allelic dropouts were only observed at locus CopTa3. Collecting a lower quantity of DNA in superficial skin cells may exacerbate amplification difficulties already observed with the two shortest alleles at this locus. Overall, dorsal swab sampling proved to be an efficient method to provide satisfactory DNA quantity, drastically limiting the handling of individuals, but some markers may not work well with the limited amount of DNA recovered with this method.

Ventral swabs in *H. arborea* were not considered for DNA extraction, because their abdomen was too dry to enable recovery of skin cells from gentle sampling. Ventral swabs in *I. alpestris* failed to provide DNA in satisfactory quantities, when compared with dorsal swabs. Cotton swabs may slip over the smooth ventral skin and only collect dead sloughing cells with highly degraded DNA, whereas the grainy dorsal skin may enable better sampling of epidermic cells. Interestingly, although chytrid detection implies swabbing of the abdomen, this kind of sampling did not lead to satisfactory amounts of DNA in our study. As DNA quantity and quality may

vary between amphibian species according to the localization of the sampled skin (ventral or dorsal), we recommend (i) to sample preferentially grainy skin surfaces, ensuring a better abrasive rubbing, and (ii) to use preliminary sampling tests on the study species, to determine the best body region for skin swabs.

Although buccal swabs in amphibians remain the reference method in terms of amount and quality of recovered DNA, necessary in this study for calculation of genotyping efficiency, skin swabs may be an interesting alternative method when sampling small individuals (juveniles) or species likely to be hurt during handling or buccal swab sampling. Skin swabbing makes DNA sampling faster, and might considerably reduce handling and sampling time, which is useful when studying large numbers of individuals. To increase the utility of this protocol, and because the understanding of prevalence and intensity of chytridiomycosis is fundamental for the conservation of amphibians, we also advise researchers to take two skin swabs, the first one intended for host DNA studies, concentrating the swabbing on a grainy skin surface, the second one saved for chytrid detection, using the swabbing on various part of animal, following the standard protocol of Kriger *et al.* (2006). It might also be useful to consider that skin swab samples collected for chytrid detection may be a used to obtain host DNA for microsatellite studies.

The levels of polymorphism found in the new microsatellite markers developed in *I. alpestris* will increase the power of genetic studies in this species. Using the described amplification conditions, these markers will not be useful in *T. cristatus*. Two markers may be used in *L. helveticus*, but more specific PCR conditions may improve their performance. Markers CopTa5 and CopTa6 should be used with caution, as null alleles may lead to genotyping errors and erroneous interpretations of observed homozygote excess in some populations (Pompanon *et al.* 2005; Selkoe & Toonen 2006). Markers with significant gametic disequilibrium should be discarded from analysis in which loci are assumed to be independent samples of the genome (Selkoe & Toonen 2006). However, new descriptive methods, such as spatial principal component analysis, do not require linkage equilibrium or Hardy–Weinberg equilibrium (Jombart *et al.* 2008) and may thus benefit from an additional highly polymorphic marker such as CopTa7.

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Data Accessibility

DNA sequences: Genbank accessions JN048427–JN048440.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Values estimated for the 27 individuals used for the analysis of the skin sampling efficiency.

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