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BUCCAL SWABS AS A NON-DESTRUCTIVE TISSUE SAMPLING METHOD FOR DNA ANALYSIS IN AMPHIBIANS

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This study describes a non-destructive DNA sampling method for genetic studies on amphibians using buccal swabs. We assessed the quantity and quality of DNA collected in each species by amplifying a part of the cytochrome *b* gene (381-1060 bp) and microsatellite markers. Buccal swab sampling is a useful alternative method for DNA sampling for both mtDNA and nDNA markers in amphibians. However, only frozen storage allowed microsatellite genotyping. We conclude that this method could greatly increase the accessibility of genetic studies in small vertebrates and could be preferred in the field of conservation genetics.

Key words: sampling, mtDNA, nDNA, conservation genetics

INTRODUCTION

Non-invasive and non-destructive tissue sampling methods for DNA analysis are preferred in the field of conservation genetics. Non-destructive sampling involves the catching of animals to obtain samples (tissue, biopsy) for genetic analysis, whereas with non-invasive methods samples are collected without the need for animal contact (Taberlet *et al.*, 1999). Non-invasive sampling of hair, faeces, feathers or sloughs is currently used for molecular genetic studies of endangered species. This kind of genetic sampling is less stressful for animals than the non-destructive sampling of blood or tissues. Although non-invasive sampling may limit the number of subsequent genetic applications, it is often appropriate for protected, vulnerable or endangered species. Non-destructive methods are currently used for all vertebrates but non-invasive strategies are restricted to mammals (e.g. hair, faeces) and birds (feathers). Sloughs in reptiles and shed skins in amphibians can be found in the field and used as sources of DNA, but because the availability of these tissues is often low, they are of limited value for most studies.

Obtaining DNA from amphibians has until now only been possible with invasive methods, but recently Davis *et al.* (2002) proposed another method to obtain DNA from amphibian skin secretions. Common practices are to take dead or living individuals (eggs, larvae, juveniles or adults) or tissue samples from individuals (e.g. toe-clip, tail-clip, crest-clip). Tissue sampling causes disturbance and stress to animals, and may also affect subsequent survival rates, breeding behaviour, and reproductive success (Clarke, 1972; Golay & Durrer, 1994; Van Gelder & Strijbosch, 1996; Arntzen *et al.*, 1999). When sampling endangered, vulnerable, or declining amphibian species it is preferable to use the least destructive and least invasive method whenever possible. We tested a novel method, isolating DNA from both anuran and urodele species, using tissues obtained non-destructively with a buccal swab. The quantity and quality of the isolated DNA was determined, and how this was affected by storage conditions of the buccal swabs.

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MATERIALS AND METHODS

SAMPLE COLLECTION AND PROCESSING

Buccal cells were taken using swabs with a plastic tip (14.5 cm) and a cotton bud (length 13.5 mm and width 3 mm). These swabs are commercially available in sterile individual package. Buccal samples were taken for three anuran (*Bufo bufo*, *Rana temporaria* and *Rana esculenta* sk.) and three urodele species (*Triturus cristatus*, *Salamandra salamandra* and *Salamandra atra*).

Collecting buccal cells requires catching and handling the animals. Each collector must choose the least stressful catching and handling method according to the species studied. It was relatively easy to open the mouths of the larger species used in this study (i.e. the frogs *R. esculenta*, *R. temporaria*, toad *B. bufo* and salamander *S. salamandra*) by levering open the upper and lower jaw with a rigid sterile plastic tape. In smaller species (*S. atra* and *Triturus cristatus*), we used a smaller tape. A very limited amount of bleeding sometimes occurred during mouth sampling. The method had to be adapted to each species according to its size, mouth anatomy and behaviour.

Three samples were taken from each animal over an eight hour period. The first swab (fresh sample) was immediately used for a DNA genomic extraction. The second swab was stored for nine weeks at room temperature (room temperature sample) and the third swab was stored at –18°C for nine weeks (frozen sample) before DNA extraction. After sampling, the swabs were put in a

TABLE 1. Mitochondrial primers used and microsatellite markers tested. For the cytochrome *b* sequences, length of the amplified fragment is indicated between brackets. [a] personal primer (available on request to the authors), [b] Taberlet *et al.*, 1992, [c] Kocher *et al.*, 1989, [d] Irwin *et al.*, 1991.

Species	Mitochondrial primers	Microsatellite locus studied
Rana temporaria	CytbF[a] H15573[b] (421 bp)	RtU4 (Berlin *et al.*, 2000)
Rana esculenta	CytbF[a] H15573[b] (421 bp)	RlCA1 (Garner *et al.*, 2000)
Bufo bufo	CytbF[a] H15573[b] (393 bp)	Bbuμ1 (Scribner *et al.*, 1994)
Triturus cristatus	CytbF[a] H15573[b] (381 bp)	Tri96b (Jehle *et al.*, 2001)
Salamandra salamandra	L14841[c] H15915[d] (1050 bp)	No microsatellite loci available
Salamandra atra	L14841[c] H15915[d] (1000 bp)	No microsatellite loci available

1.5 ml sterile Eppendorf tube. For comparison with traditional methods, a toe-clip sample was also taken. All animals were kept in captivity for 15 days before release. The DNA genomic extractions were conducted with a Qiagen Tissue Kit following the manufacturer's conditions. DNA was eluted in a 250 μl volume (TE buffer) for all extractions and stored at –18°C.

DNA Quantification and PCR based Assays

Total DNA was quantified with the Picogreen® dsDNA quantification kit (Molecular Probes). The DNA quantity can be overestimated due to bacterial growth during storage. To test the quality of the DNA in the samples, mitochondrial and nuclear markers were amplified by PCR. Specific primers were used to reveal the presence and the quality of amphibian DNA. For the mitochondrial marker, a fragment of cytochrome b was amplified for all species. The PCR reaction was performed in a 25 μl tube containing 10mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.5 μM of each primer, 0.1 mM of each dNTP, Bovine Serum Albumin (5 μg), AmpliTaq Gold DNA polymerase (0.5 U) and 6-20 ng DNA. The primers used are presented in Table 1. Forty-five cycles were performed with 10 min at 95°C followed by 30s at 95°C, 30s at 45°C (except for Salamandra sp. 60s at 50°C), 40s at 72°C (except for Salamandra sp. 60s) and a 5 min final extension at 72°C. The PCR products were purified with a Qiaquick purification kit (Qiagen) and were double strand sequenced with a Big Dye terminator sequencing Kit (Perkin Elmer).

Microsatellite markers were amplified only for *Bufo bufo*, *R. temporaria*, *R. esculenta* and *T. cristatus* because microsatellite markers are currently still unavailable for the *Salamandra* genus. The microsatellite loci studied are presented in Table 1. The PCR reaction components were the same conditions as for sequencing (see above), and forward primers were labelled with fluorochrome. Forty cycles of amplification were performed and PCR products were run on 6%

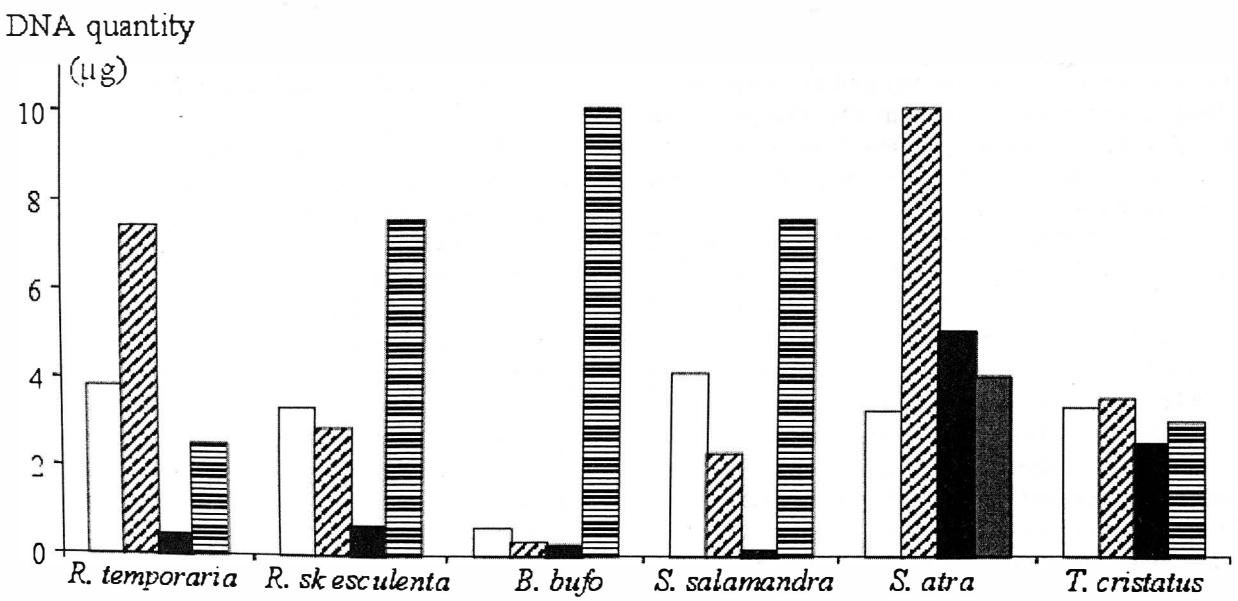

FIG. 1. Total yield of DNA (μg) for buccal swab sampling and toe-clip sampling for six amphibian species (*R. temporaria, R. esculenta* sk., *B. bufo, S. salamandra, S. atra* and *T. cristatus)*. Open bars, fresh samples; oblique shading, frozen samples; filled bars, room temperature; horizontal bars, toe-clips. Fresh samples were immediately extracted after collection. Frozen samples and room temperature samples were stored for 9 weeks at –18°C and at room temperature, respectively, before DNA extraction.

TABLE 2. Results of sequencing and microsatellite genotyping with buccal swabs and toe-clip samples. A successful sequencing or genotyping is indicated by "yes" and failure by "no".

Species	Cytochrome *b* sequencing				Microsatellite genotyping			
	Buccal swabs			Toe-clip	Buccal swabs			Toe-clip
	Fresh	Room temperature	Frozen		Fresh	Room temperature	Frozen	
Rana temporaria	yes	yes	yes	yes	yes	no	yes	yes
Rana esculenta	yes	no	yes	yes	yes	no	yes	yes
Bufo bufo	yes	no	yes	yes	yes	no	yes	yes
Triturus cristatus	yes	yes	yes	yes	yes	no	yes	yes
Salamandra salamandra	yes	no	yes	yes	-	-	-	-
Salamandra atra	yes	yes	yes	yes	-	-	-	-

polyacrylamide gels on an automated ABI 377 DNA sequencer. The allele size was determined with the Genotyper software version 2 (Perkin Elmer).

RESULTS AND DISCUSSION

As we estimated DNA concentrations using only one individual per species, the values are only indicative. The total DNA yields from the fresh and frozen samples were similar in *R. esculenta*, *S. salamandra*, *B. bufo* and *T. cristatus*, or higher from the frozen samples in *R. temporaria* and *S. atra* (Fig. 1). The total DNA yields from the fresh and frozen samples, were higher than those from the room temperature samples, in four of the six species. Nucleic acid degradation was observed with agarose gel images at room temperature, but was very limited at –18°C. In *Salamandra atra* a higher DNA yield was obtained at room temperature than in the other storage conditions. Bacterial growth and DNA contamination may explain this higher value. Moreover, because amphibians have nucleated red blood cells, the limited bleeding which occurred during the mouth sampling of some individuals could account for the variation in DNA quantity observed among the species.

We successfully amplified parts of the cytochrome *b* gene from fresh and frozen buccal swabs and from the toe-clip samples in all six species. PCR products for the room temperature samples were only obtained from *R. temporaria*, *T. cristatus,* and *S. atra* but not from *R. esculenta, B. bufo,* and *S. salamandra* (Table 2). For each species, the cytochrome *b* sequences obtained were identical across storage conditions and matched with the correct taxon. For microsatellite markers, a correct fluorescent profile was obtained for the four tested species for all samples except for the buccal samples stored at room temperature (Table 2). For each individual, allele sizes were identical with DNA extracted from the fresh and frozen buccal samples and from the toe-clip sample.

As DNA quantity varies between amphibian species, we recommend initiating a pilot study to test the reliability of the buccal swab sampling DNA method before using this method on different species. Moreover, with very low DNA quantity, increasing genotyping errors with microsatellite markers can affect the reliability of genetic analysis (Taberlet *et al.*, 1999), and, a multiple-tube approach (i. e. repeating treatments several times for each locus and each extract) might be recommended to avoid such problems (Goossens *et al.*, 1998).

We did not test the effect of storage buffer on the preservation of DNA. It is sure that a storage buffer could improve DNA preservation by limiting nucleic acid degradation and bacterial growth.

This method for collecting tissue for subsequent amphibian genetic molecular studies presents several positive features compared to sampling methods such as toe-clipping. Buccal swab sampling is easy to perform, few materials are needed, there are no liquids to handle (storage buffer or disinfectant) in the field and it is as cheap as other sampling procedures. Moreover it may be easier to obtain sampling permits, especially for endangered species.

Here, we have shown that it is possible to collect DNA with buccal swabs and to perform subsequent genetic analysis, but there is not sufficient evidence to imply a general applicability of buccal swabs as a DNA source in large scale projects.

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REFERENCES

Arntzen, J. W., Smithson, A. & Oldham R. S. (1999). Marking and tissue sampling effects on body condition and survival in the newt (*Triturus cristatus*). *Journal of Herpetology* **33**, 567-576.

Berlin, S., Merila, J. & Hellegren H. (2000). Isolation and characterization of polymorphic microsatellite loci in the common frog, *Rana temporaria. Molecular Ecology* **9**, 1938-1939.

Clarke, R. D. (1972). The effect of toe clipping on survival in Fowler's toad (*Bufo woodhousei fowleri*). *Copeia* **1972**, 182-185.

Davis, A. Sluscher, L. B. & Beneski J. T. (2002). A novel method for the extraction and amplification of DNA from amphibians. *Herpetological Review* **33**, 104-105.

Garner, T. W., Gautschi, J. B., Röthlisberger, S. & Reyer H.-U. (2000). A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology* **9**, 2155-2234.

Golay, N. & Durrer, H. (1994). Inflammation due to toe-clipping in natterjacks toads (*Bufo calamita*). *Amphibia-Reptilia* **15**, 81-83.

Goossens, B., Waits, L. P. & Taberlet P. (1998). Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Molecular Ecology* **7**, 1237-1241.

Irwin, D., Kocher, T. & Wilson A. (1991). Evolution of the cytochrome *b* gene of Mammals. *Journal of Molecular Evolution* **32**, 128-144.

Jehle, R., Arntzen, J. W., Burke, T., Krupa, A. P. & Hödl W. (2001). The annual number of breeding adults and the effective population size of syntopic newts (*Triturus cristatus, T. marmoratus*). *Molecular Ecology* **10**, 839-850.

Kocher, T. D., Thomas, W. K., Meyer, A. Pääbo, S., Villablanca, F. X. & Wilson A. C. (1989). Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6196-6200.

Scribner, K. T., Arntzen, J. W. & Burke T. (1994). Comparative analysis of intra- and interpopulation genetic diversity in *Bufo bufo*, using allozyme, single-locus microsatellite, minisatellite, and multilocus minisatellite data. *Molecular Biology Evolution* **11**, 737-748.

Taberlet, P., Meyer, A. & Bouvet J. (1992). Unusual mitochondrial DNA polymorphism in two local populations of blue tit *Parus caeruleus*. *Molecular Ecology* **1**, 27-36.

Taberlet, P., Waits, L. P. & Luikart, G. (1999). Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution* **14**, 323-327.

Van Gelder, J. J. & Strijbosch H. (1996). Marking amphibians: effects of toe-clipping on *Bufo bufo* (Anura: Bufonidae). *Amphibia-Reptilia* **17**, 169-174.

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