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Non-lethal DNA sampling for caecilian amphibians

Simon T. Maddock[1,2], Claire J. Lewis[1,2], Mark Wilkinson[1,2], Julia J. Day[2], Charles Morel[3], Marcel T. Kouete[4] & David J. Gower[1]

[1]*Department of Life Sciences, The Natural History Museum, London, SW7 5BD, UK,*

[2]*Department of Genetics, Evolution and Environment, University College London, London, WC1E 6BT, UK,*

[3]*Natural History Museum, Victoria, Seychelles,*

[4]*Project Cameroon Herpetology, Conservation Biology Foundation, Yaoundé, Cameroon and Zoological Society of London, London NW1 4RY, UK*

For amphibians, non-lethal sampling methods have been developed and evaluated for only two of the three extant orders, with the limbless caecilians (Gymnophiona) thus far overlooked. Here we assess 16 different methods in five caecilian species representing five families with differing morphologies and ecologies. DNA was successfully extracted and amplified for multiple genetic markers using all tested methods in at least some cases although yields are, unsurprisingly, generally substantially lower than for DNA extractions from (lethally sampled) liver. Based on PCR performance, DNA yield and sampling considerations, buccal swabs, skin scrapes, blood pricks and dermal scale-pocket biopsies performed the best.

Key words: Amphibia, conservation, Gymnophiona, molecular, population genetics

It is now commonplace to use molecular genetic techniques to address diverse and important questions in molecular ecology, systematics and conservation. Some effort has been made recently to develop non-lethal methods for genetic sampling in non-mammalian and non-avian taxa (e.g., Beebee, 2008; Gallardo et al*.,* 2012; Mendoza et al., 2012a, b; Pidancier et al., 2003; Scriven et al., 2013). Although natural history specimen collections underpin many aspects of biology, including conservation (e.g., Arnold, 1991, 1998), non-lethal sampling is particularly important for studying populations that are at risk of extinction, or for projects that require large numbers of individual samples.

For frogs and toads (Anura) and salamanders and newts (Caudata), toe clipping (e.g., Taylor et al., 2012), tail clipping (e.g., Polich et al., 2013), blood puncture (Mendoza et al., 2012a), skin swabbing (Mendoza et al., 2012b; Müller et al., 2013; Prunier et al., 2012) and buccal swabbing (e.g., Gallardo et al., 2012 and references therein) have been used to obtain tissue samples for DNA non-lethally. As far as we are aware, there are no reported trials of non-lethal sampling for members of the third extant order of living amphibians, the caecilians (Gymnophiona). Instead, to our knowledge, all published molecular analyses of caecilians have been based on DNA extracted from tissues (typically liver or muscle) dissected from euthanised voucher specimens (e.g., see Hedges et al., 1993; Gower et al., 2002; Kamei et al., 2012; San Mauro et al., 2012).

In general, caecilians are much less studied than other living amphibians, probably because they are less speciose, are restricted to the moist tropics and adjacent areas, and are less readily encountered by virtue of their mostly soil-dwelling lifestyle (e.g., Gower & Wilkinson, 2005; Wilkinson, 2012). In addition, the divergent anatomy of caecilians prevents or substantially restricts application of the non-lethal sampling methods used for other amphibians. All extant caecilians are limbless and most lack or have greatly reduced tails (e.g., Taylor, 1968), preventing toe or tail clipping as used with other amphibians. Adult caecilians have a unique, dual jaw-closing mechanism with a powerful bite (Nussbaum, 1983; Kleinteich et al., 2008) and retrorse, pointed teeth, which makes inserting and removing a swab into the buccal cavity of a conscious caecilian without damage very difficult, especially given that it can be difficult to restrain their limbless and often slippery bodies. Additionally, caecilians rely upon high internal pressure to operate a hydrostatic locomotory system (O'Reilly et al., 1997) and so incisions into the coelom under anaesthesia to extract tissue for DNA would also be problematic.

It is advisable to test the feasibility and success of non-lethal methods prior to a full study being undertaken (Keyghobadi et al., 2009). Here we report the first development of non-lethal tissue sampling methods for caecilians and the results of our trials of them on field-collected animals from a range of taxa.

In an attempt to test the new methods on a range of caecilians with different morphologies and ecologies, eighteen field-collected individuals of five caecilian species were used in this study, including representatives of five of the ten currently recognised families (Kamei et al., 2012; Wilkinson et al., 2011). The rhinatrematid *Rhinatrema bivittatum* (*n*=1 for non-lethal sampling,

Correspondence: David J. Gower (d.gower@nhm.ac.uk)

n=2 for lethal sampling) and caeciliid *Caecilia* sp. (*n*=1 and 2, respectively) were sampled in French Guiana, the dermophiid *Geotrypetes seraphini* (*n*=1 and 2, respectively) and herpelid *Herpele squalostoma* (*n*=1 and 2, respectively) in Cameroon, and the indotyphlid *Grandisonia alternans* (*n*=2 and 2, respectively) in Seychelles.

Sixteen tissue-sampling approaches were used (Table 1). Methods varied with respect to whether each caecilian was anaesthetised or not, the type of tools used, the degree of effort applied and whether tissue was stored dry or in absolute ethanol. Animals were anaesthetised by immersion in an aqueous solution of MS222 (Sandoz, UK). Immediately after sampling procedures anaesthetised animals were either sacrificed (*G. alternans*) or rinsed in clean water and transferred to moist paper towels for 24 hours, after which they were transferred to single-animal boxes of soil and fed *ad libitum* with earthworms (*G. seraphini, H. squalostoma*). Dermal scale pockets were opened following the method reported by Wilkinson et al. (2013). Each dry-skin rub and blood sample was stored at 4°C on a Whatman FTA Classic Card. For two *G. alternans*, tissues obtained using Methods 13 and 14 (Table 1) were combined in a single vial of ethanol and a composite DNA extraction made.

For swabbing we used fine, sterile, rayon-tipped swabs (MW100-100; Medical Wire & Equipment Co., Crosham, UK); for brushing we used TePe (Malmö, Sweden) xxxx-fine interdental brushes sterilised by exposure to UV light. Sterile scalpels and forceps were used for other procedures. Except for the work on the Seychelles *G. alternans*, tissue-sampling procedures were carried out while handling each caecilian with clean, disposable latex gloves. Liver samples have often been used in molecular analyses of caecilians (e.g., San Mauro et al., 2012) and so we compared DNA yields and PCR performance of non-lethally sampled extracts with those from ethanol-stored fresh liver samples; only in the case of *G. alternans* were these the same individuals that we also used for the non-lethal sampling. All liver sample extracts were more than two years old except for *G. alternans*, which were freshly extracted.

DNA was extracted using the Qiagen DNeasy™ Tissue Kit. Extractions followed standard protocols except that all samples were ligated overnight and only 100µl of buffer AE was used to suspend final extracts. DNA was extracted from non-lethally obtained tissues within six weeks of sampling. For samples stored on FTA card, extractions were carried out using two 2mm diameter circles. We quantified 4µl of extracted DNA elutions using a Qubit® 2.0 Fluorometer with a dsDNA High Sensitivity Assay kit.

To test the utility of the different sampling techniques the polymerase chain reaction (PCR) was used to amplify partial sequences of one mitochondrial and one nuclear gene. PCRs were carried out no more than one week after extraction of non-lethally obtained DNA. We opted for the non-coding 16s rRNA (*16s*) mitochondrial marker because it is the most readily amplified and sequenced marker that we have used previously for caecilians (DJG, unpublished data) and because the universal vertebrate primers that are available allowed us to test whether contamination from other organisms may have occurred. The nuclear gene used in PCR tests was the protein

Table 1. Sixteen non-lethal sampling methods trialled for caecilians. For further details see the text.

	Anaesthetic?	Tool	Action	Storage
1	no	Swab	10 longitudinal strokes, around body circumference, rotating swab	Dry, 4°C
2	no	Swab	10 longitudinal strokes, around body circumference, rotating swab	EtOH
3	no	Swab	5 longitudinal strokes, around body circumference, rotating swab	Dry, 4°C
4	no	Swab	5 longitudinal strokes, around body circumference, rotating swab	EtOH
5	no	Brush	10 longitudinal strokes, around body circumference, rotating brush	EtOH
6	no	FTA card	Rubbing a midbody bend on ca. 25 mm diameter circle of FTA card until skin tacky	FTA card, dry
7	yes	Swab	10 longitudinal strokes, around body circumference, rotating swab	Dry, 4°C
8	yes	Swab	10 longitudinal strokes, around body circumference, rotating swab	EtOH
9	yes	Brush	10 longitudinal strokes, around body circumference, rotating brush	EtOH
10	yes	FTA card	Rubbing a midbody bend on ca. 25 mm diameter circle of FTA card until skin tacky	FTA card, dry
11	yes	Swab	10 strokes covering all surfaces of head, rotating swab	EtOH
12	yes	Swab	20 strokes covering disc around the anus, rotating swab	EtOH
13	yes	Swab	Rotating swab inside buccal cavity	EtOH
14	yes	Pin, forceps	Removing scales and soft tissue from middorsal part of dermal scale pocket	EtOH
15	yes	Scalpel	Longitudinal scraping of outer part of lateral section of skin between one pair of adjacent midbody annular grooves	EtOH
16	yes	Needle	Obtaining blood by superficial pricking of lateral and dorsal surfaces of post-anal terminus of body, blood dabbed onto FTA card	FTA card, dry

coding pro-opiomelanocortin (*pomc*) because it has been successfully amplified and sequenced for caecilians (STM, unpublished data) and other vertebrates (e.g, Vieites, 2007).

Reaction volumes for PCR were 15µl: 1.5µl of Bioline Buffer (BioTaq), 0.75µl of MgCl2 (50mM), 0.15µl of dNTPs (10mM), 0.15µl of Taq (5u/µl), 0.6µl of each of the forward and reverse primers (1mM) (*16s*: 16sAL (5'-CGCCTGTTT ATCACG-3') and 16sBH (5'-CCGGTCTGAACTCAGATC ACG-3')(Palumbi et al., 1991); *pomc*: POMC_DRV_F1 (5'-ATATGTCATGASCCAYTTYCGCTGGAA-3') and POMC_ DRV-R1 (5'-GGCRTTYTTGAAWAGAGTCATTAGWGG-3') (Vieites, 2007), 0.6µl of template DNA, and 10.65µl ddH_2O. Cycling conditions were: denature at 94°C for 60s; followed by 35 (*16s*) or 40 (*pomc*) cycles of denaturing at 94°C for 30s, annealing at 50°C (*16s*) or 58°C (*pomc*) for 30s, and extending at 72°C for 30s; and a final extending step of 72°C for 5 min. PCRs that had successfully amplified were cleaned and sequenced by the Natural History Museum, London's in-house sequencing facility. All sequences were subject to an NCBI nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov).

All non-lethal sampling approaches that were trialled were successful in at least some instances (Table 2). DNA yields varied considerably among the different methods (Table 2). Those consistently producing relatively high DNA yields were buccal swabs (Method 13), scale-pocket biopsies (14), skin scrapes (15), and blood pricks (16). Lowest yields were obtained by rubbing an FTA card over the surface of the skin (6 and 10). Liver samples (lethally obtained) generally produced much higher DNA yields, up to two orders of magnitude in some cases, than the best non-lethal methods. Those extractions from liver that had surprisingly low quantities of DNA (for one specimen of *Caecilia* sp., and to a lesser extent one specimen of *Geotrypetes seraphini*) may have undergone some degradation because they were among those extracted more than two years prior to PCR.

Successful amplifications were achieved using all 16 methods for at least one sample obtained (Table 2). Unsuccessful PCR amplifications were generally associated with the DNA extractions of lowest yield (Table 2). Although we were able to amplify the *16s* marker from DNA extractions from most non-lethally obtained samples, fewer successful amplifications of the *pomc* marker occurred (Table 2). In terms of successful PCR amplification, the best results were obtained from extractions from buccal swabs (Method 13), scale-pocket biopsies (14), skin scrapes (15) and blood pricks (16), although scale-pocket biopsies produced variable results.

Sequences of *16s* derived from Method 2 swabbing of *G. seraphini* demonstrated human contamination, although it is unclear whether contamination occurred pre- or post-extraction. The more invasive methods (buccal swabbing, scale-pocket, skin scraping and blood pricks) were robust to contamination, even for the Seychelles samples that were obtained without wearing gloves.

As far as we can tell, the tissue sampling procedures and anaesthesia did not unduly affect live caecilians, at least in the short term in captive conditions. The French Guiana and Cameroon animals had all eaten and appeared healthy three weeks after sampling. There is a need for research into the effectiveness, dosage and consequences of anaesthesia by MS222 in caecilians. We do not have precise data but we have noted widely varying dosage and/or immersion time requirements in different species. Exposure to strong solutions of MS222 and/or for prolonged periods can result in caecilian skin becoming wrinkled and we are unaware, for example, of the consequences of exposing a resuscitated animal to non-sterile soil, especially given that skin biochemistry is the first line of defence for amphibians against many pathogens (e.g., Rollins-Smith et al., 2005) and at least some caecilians can be killed by chytridiomycosis (Gower et al., 2013). Releasing resuscitated caecilians may have an increased predation risk, but this remains unstudied.

We recommend that, after tissue sampling, anaesthetised animals be rinsed in clean freshwater as soon as possible, that they are not returned to where they were collected until full resuscitation has seemingly occurred,and that when they are returned they are not visible to surface predators. Previously, Measey et al. (2001, 2003) used MS222 to anaesthetise terrestrial caecilians and returned resuscitated animals to non-sterile soil in the laboratory and in the wild to no ill effect. Their studies also included scarring or cutting into the skin and it was noted that caecilians seem to recover well from such superficial wounds. It is not uncommon to find caecilians in the wild (or captivity) with conspecific bite marks and/or heavy scars or fresh wounds (e.g., Measey et al., 2001; Wilkinson et al., 2013). Thus, we expect that opening a single scale pocket is unlikely to be notably detrimental to survival, especially considering that these pockets are folds of dermis (e.g., Fox, 1983) and that their contents can be removed without drawing blood. Opening of a scale pocket leaves a small, thin flap of skin, and clipping this might offer a further non-lethal tissue sampling method, perhaps one yielding more DNA than the contents of scale pockets.

Based on ease of sampling in field conditions and DNA yield and PCR results (especially for *G. alternans*), we suggest that the most suitable approach to non-lethal sampling is to combine buccal swabs and scale pocket biopsies. Although skin scrapes (Method 15) generally provided higher yields than scale-pocket contents, we suspect that skin scraping carries a higher chance of contamination and damage. Some methods of non-lethal sampling are taxon dependent. Notably, scale-pocket biopsies are not possible for all caecilians because not all taxa (and/or early life-history stages) possess scale pockets or scales (e.g., Taylor, 1972), and the swabs used in this study are too large for caecilians with small mouths.

Although the quantities of DNA obtained here from non-lethal sampling methods are generally suitable for Sanger sequencing protocols, extractions may not produce high enough DNA yields for high-throughput genomic approaches, such as RAD-seq that typically requires ~1µg of extracted DNA to produce the libraries (e.g., Nosil et al., 2012, but see Wang et al., 2012). In such cases, decisions would have to be made about the

Table 2. Quantities of DNA extracted and PCR success. For methods 1–16 see Table 1. Liver=lethally obtained liver sample stored in ethanol. Each cell contains three elements in the following order: extracted DNA quantity (ng/µl), PCR result for mt*16s*, PCR result for nu *pomc*. *=successful PCR as judged by gel electrophoresis and Sanger sequencing; -=unsuccessful PCR as judged by gel electrophoresis; H=sequenced PCR product identified as *Homo sapiens* (contamination); N=Qubit score too low to quantify DNA; X=PCR product that yielded poorquality sequence data. Shaded cells indicate those DNA extractions for which all PCRs were successful and not contaminated.

Method	*Herpele squalostoma*			*Geotrypetes seraphini*			*Caecilia* sp.			*Rhinatrema bivittatum*			*Grandisonia alternans*			
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	4
1							0.18, *, -			0.52, *, -						
2							0.27, *, -			0.18, *, -						
3	0.24, *, -			0.15, -, -												
4	0.33, *, -			0.07, *, -												
5	0.53, *, X			N, -, -												
6	N, *, -			N, -, -												
7	0.46, *, *			0.04, -, -												
8	0.11, *, -			0.05, H, -												
9	2.76, *, -			N, -, -												
10	N, *, -			N, -, -												
11	0.09, -, -			0.52, *, -												
12	0.07, *, -			0.14, *, -												
13	2.72, *, *			7.12, *, *									0.87, *, *			0.57, *, *
14	0.59, *, *			0.24, *, *									0.47, -, *			0.10, *, -
15	3.60, *, *			0.57, *, *									1.53, *, *			
16	0.54, *, *			0.76, *, *												
13+14														1.46, *, *	1.62, *, *	
Liver		9.32, *, *	3.90, *, *		2.02, *, *	8.70, *, *		0.43, *, *	8.94, *, *		8.88, *, *	8.32, -, *	6.46, -, *	6.72, *, *	6.50, *, *	6.46, *, *

extent to which multiple scale pockets could be opened (and perhaps clipped) and whether this could be further combined with other methods to achieve the desired yield. Future work might also explore additional non-lethal sampling methods for caecilians, for example by attempting to extract DNA from shed skin, skin secretions and faeces. There remains room for further research, but we have developed some initial options and have provided some insights into their performance and demonstrated the potential for non-lethal population-scale molecular genetic studies.

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