An evaluation of non-invasive sampling for genetic analysis in northern European reptiles

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Genetic studies of native herpetofauna populations are important for the conservation of European biodiversity, but previous studies have been largely dependent on invasive sample collection. Here we explore the efficiency of non-invasive sampling (NIS) for molecular studies and review the various potential sources of such samples. Snakes produce a multitude of by-products, such as sloughed skin, faeces and eggs or embryos, that, along with road kills, predated specimens and museum samples, could potentially be used in molecular studies. We describe a new method for obtaining snake faeces in the field and, using mitochondrial cytochrome *b* primers, we successfully amplified 500 and 758 bp sequences from a variety of tissues collected by NIS. The availability and degradation of such material differed greatly, and both DNA extraction and PCR success appeared dependent upon sample origin and storage. Nevertheless, for the first time we demonstrate that faecal, egg and foetal tissues, as well as sloughed skin and carcasses, represent valuable NIS source material permitting genetic studies with minimal disturbance to the individual and its population.

Key words: Coronella austriaca, mitochondrial DNA, *Natrix natrix*, snakes, *Vipera berus*

INTRODUCTION

The study of indigenous herpetofauna in the field is important for conservation and our understanding of reptile ecology in a changing landscape. Increasingly, prime reptile habitat in northern Europe is being modified, destroyed or fragmented (Beebee & Griffiths, 2000), with reptiles having to adapt to changes instigated by anthropogenic expansion. In general, translocation success rates for amphibians and reptiles are lower than those for mammals and birds (Griffith et al., 1989; Dodd & Seigel, 1991; Platenberg & Griffiths, 1999; Reinert & Rupert, 1999), and yet translocation exercises rarely employ genetic data. This is perhaps surprising, given the now seminal study of Madsen et al. (1999), who demonstrated unequivocally that the introduction of new genotypes into a severely inbred and isolated population of *Vipera berus* not only halted its precipitous decline towards extinction but resulted in dramatic population expansion. However, molecular ecological approaches are now more commonly used in the conservation of herpetofauna (e.g. Ciofi & Bruford, 1999; Madsen et al., 1999; Leaché & Reeder, 2002; Morrison & Scott, 2002) and cryptic snake taxa are being re-assigned following genetic analysis (Puorto et al., 2001; Burbrink, 2002; Wüster et al., 2002). Molecular characterization of individuals may also resolve novel insights into how genetic variation is partitioned within and among populations (e.g. Carlsson et al., 2004).

Invasively obtained material has tended to be the source for herpetological genetic studies to date (Keogh, 1998; Feldman & Spicer, 2002; Voris et al., 2002) but this is now less acceptable with the wide availability of commercial non-invasive sampling (NIS) DNA extraction kits. These offer affordable, alternative methods for molecular studies with minimal disturbance to the animal and its population (Morin & Woodruff, 1996). Below we summarize the various sources of NIS material that could potentially be utilized for molecular studies, concentrating on our target taxa, the three species indigenous to northern Europe: the adder (*Vipera berus*), the grass snake (*Natrix natrix*) and the smooth snake (*Coronella austriaca*).

1) Sloughed skin

Sloughs can be found fragmented or whole around hibernation sites, often entwined within gorse, bracken and other coarsely textured plants, and beneath tins or other such cover. With warm weather conditions, sloughs dry quickly in the field and can be folded into an envelope or sample bag on collection and stored dry.

2) Aborted embryo, egg, road kill and museum samples

Muscle is available for DNA extraction from semi-predated and stillborn carcasses, road kills and museum samples (Dallas et al., 2003). Stillborn offspring from both the adder and the smooth snake can be collected in the vicinity of hibernation sites. Grass snake eggs, laid from late June to July, are found in compost and manure heaps, decaying tree stumps, woodchip piles and even rotting seaweed. Around 30% of eggs fail to hatch in the autumn or are infertile (Beebee & Griffiths, 2000), therefore non-viable unfertilized eggs should only be collected after this period, being identifiable by their discoloured state. Although museums are a potential source of animal tissue, traditional specimen fixatives (commonly formalin) are optimized for morphological study, which degrades DNA (Chaw et al., 1980; Pääbo, 1989; Chang & Loew, 1994). Extracting DNA from formalin-fixed material is possible but laborious and PCR amplification success rates are low (Serth et al., 2000). The analysis of tissue stored in forma-

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Table 1. DNA extraction and PCR amplification success of 500 bp cytochrome *b* mtDNA products from non-invasively collected British snake samples (A.E.P.T. = Ancient Ethanol Preserved Tissue).

Species	Tissue type	No. of samples	Preservation method	No. of extracts	No. of PCRs	Positive PCR
Coronella austriaca	Slough	5	Dried	9	17	5
	Foetus	2	Ethanol	2	2	2
	Carcass	2	Ethanol	2	2	2
	Road kill	3	Ethanol	2	7	3
Natrix natrix	A.E.P.T	3	Ethanol (one stored for 50–100 y)	9	26	3
	Faeces	1	Ethanol	1	2	0
	Faeces	1	Frozen	1	2	1
	Slough	5	Dried (one fresh)	6	8	5
	Egg	2	Frozen	3	4	2
	Carcass	2	Ethanol	2	2	2
	Road kill	3	Frozen	4	5	3
Vipera berus	Faeces	1	Ethanol	1	2	0
	Faeces	1	Frozen	2	3	1
	Slough	5	Dried (one 2 y old)	8	15	5
	Foetus	2	Ethanol	2	2	2
	Carcass	2	Ethanol	2	2	2
	Road kill	2	Frozen	3	4	2
	Road kill	1	Ethanol	1	1	1

lin pH <7 for longer than 12 months should be restricted to analysis of short (<100–200 bp) DNA fragments (Bucklin & Allen, 2003).

3) Faeces

Faecal samples typically contain low quantities of degraded target DNA (Gerloff et al., 1995; Taberlet et al., 1999), but have proved to be a valuable source of DNA from avian and mammalian samples (e.g. Robertson et al., 1999; Taberlet et al., 1999; Regnaut et al., 2006) and should also be useful in herpetological studies. Snake faeces are challenging to obtain in the field non-invasively but can potentially be found throughout the snakes' active period from late April to October. As faecal material contains a range of micro-organisms and is particularly prone to deterioration by endogenous nucleases, the highest quality DNA is found in freshly collected faeces (Taberlet et al., 1999; Wehausen et al., 2004). Faecal matter gathered in the field should be immediately sealed and cooled in a collection bag. This can either be frozen at –20 ºC or stored in ethanol (>95% molecular grade), RNALater (Ambion) or silica gel (Nsubuga et al., 2004) at 4 ºC.

4) Cloacal and buccal swabs

Cloacal and/or buccal swabbing is a rapid, inexpensive and potentially easy to implement field method for obtaining reptile DNA samples (Miller, 2006). However, in addition to the delicate bone structure of British reptiles, there are obvious hazards associated with buccal swabbing from both venomous and non-venomous reptiles. Therefore, for both these reasons this procedure is not recommended for British snakes.

5) Teeth and bone

Teeth and bone samples, from semi-predated and still-born carcasses, shed teeth, road kill and museum samples, might yield DNA of sufficient quality for certain studies, but because of the time involved in sample preparation and the number of replicates required (see Wandeler et al., 2003; Rohland et al., 2004), they should only be considered in the absence of other more suitable tissues.

This study aimed to demonstrate the utility of a range of non-invasively collected samples for mitochondrial DNA PCR in the three native UK snake species.

MATERIALS AND METHODS

Origin of samples

Table I shows the preservation method of the samples collected during the current study. An additional 20 sloughs and 20 carcasses from *Vipera berus* were collected 1–30 days prior to DNA extraction. Field collected carcasses, road kills and foetal samples were directly frozen at –20ºC or preserved in 95% ethanol. Ancient ethanol preserved tissue (A.E.P.T.; collected pre-1907 to 1969) consisted of museum samples donated by the National Museum of Wales, Cardiff. Non-viable grass snake eggs, located in compost heaps, were preserved at –20ºC. Sloughed skins, mostly collected at the entrance of identified adder domains, were preserved dry at room temperature for up to two years. An additional fresh slough was obtained from an adder observed in the process of ecdysis. Snake faeces (associated with sloughs)

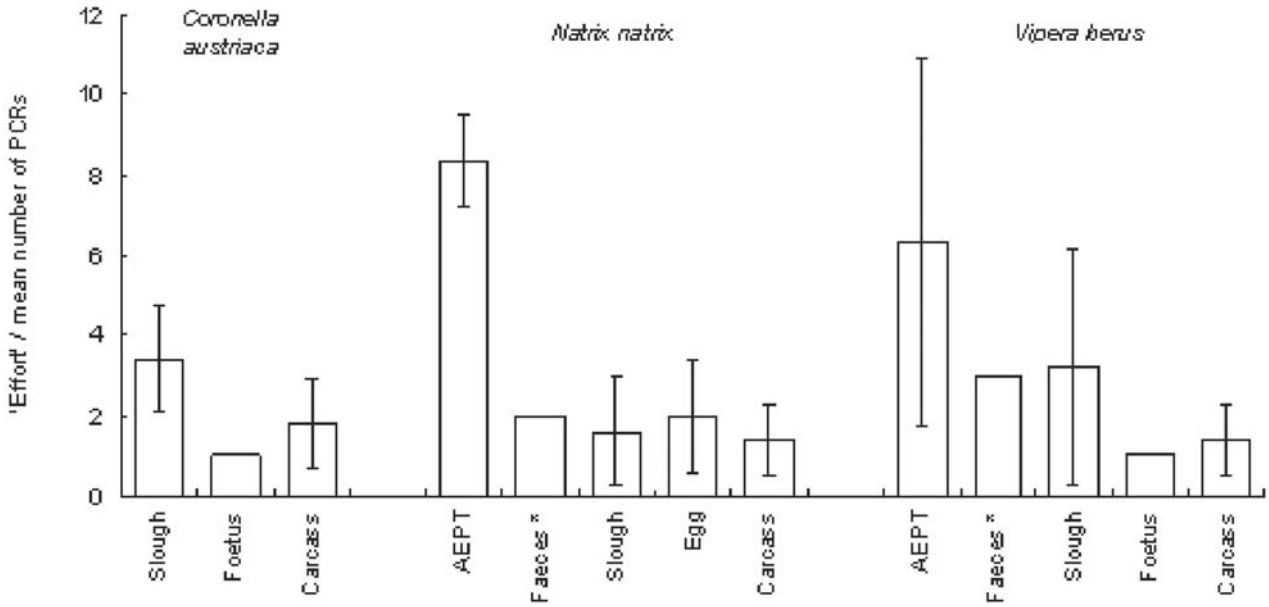

Fig. 1. Mean number (±SE) of PCR reactions required to generate a positive 500 bp partial cytochrome *b* gene amplification product from non-invasive snake sample material. Road kill and other carcasses are grouped together. *Error bars are not shown for the faecal samples as for both species only one of the two PCRs successfully amplified.

collected from a range of UK sites were either immediately frozen at –20ºC or preserved in 95% ethanol. A 10 µl blood sample, obtained by caudal extraction from an adder and stored in 90 µl of Seutin's buffer (Seutin et al., 1991) at room temperature, was collected as a positive PCR control.

DNA extraction

Sloughed skin required a rehydration step to remove impurities prior to DNA extraction. A fragment (1–2 cm^2) of slough was placed in a 1.5 ml eppendorf tube containing 1 ml of double distilled (dd) water at 55 °C in a rocking incubator. After 4–6 h the water was removed and a further 1 ml of dd water added to each sample prior to incubation at 55 °C for a further 8–12 h. DNA extraction was performed on these rehydrated samples, egg yolks (approximately 0.2 cm^3), NIS muscle (1 cm^3) and blood (5 µl in Seutin's buffer) following the manufacturer's protocols for Qiagen DNeasy® (Cat. # 69504) tissue extraction kit. Faecal material (1 cm^3) was extracted following the manufacturer's protocol for the QIAamp® DNA stool mini kit (Cat. # 51504). DNA extraction was not attempted from teeth or bone in the current study but relevant protocols and commercial kits are available (see QIAamp® DNA minikit protocol). A maximum of three extraction attempts were performed for each sample, with second and third extractions only prepared on failure to successfully amplify a product after three PCR attempts from the previous extraction.

Partial cytochrome b gene amplification and sequencing

Two different snake PCR primer sets were used that generated approximately 500 and 758 bp amplicons. The first primer pair consisted of a forward primer (UKsnakecyto_F, 5' CAACATCAACTTAGCCTTCTC 3') adapted from cytochrome *b* primer, 703bot (Pook et al., 2000) and a reverse primer (UKsnakecyto_R, 5' GTGGAATGGGATTTTATCG 3') designed from an alignment of partial cytochrome *b* gene from *Vipera berus* (GenBank accession number AJ275728) and *Natrix natrix* (AF471059). The second primer set, 5' TCAAACATCTCAACCTGATGAAA 3' and 5' GGCAAATAGGAAGTATCATTCTG 3', were previously used by Pook et al. (2000) to generate a 758 bp cyt *b* fragment. Primer set 1 was tested on all tissue samples, whereas set 2 was only tested on the additional 20 sloughs and 20 carcasses from *Vipera berus*. Each PCR was performed in 25 ml comprising 1 ml of DNA, 1 × Invitrogen buffer (200 mM Tris-HCL, pH 8.4, 500 mM KCl), 3 mM $MgCl_2$, 250 mM of each dNTP, 0.5 mM of each primer and 1 U of Invitrogen *Taq* polymerase. DNA amplification was performed at 96 ºC for 4 min and then 35 cycles of 94 ºC for 1 min, 50 ºC for 1 min and 72 ºC for 2 min with a final extension cycle of 72 ºC for 3 min in an Applied Biosystems GeneAmp® PCR system 9700 thermocycler. Both negative (dd water) and positive (adder blood) controls were included with each PCR. PCR success was determined by running products on an agarose gel. Fragment length of PCR product was determined by interpolation using a 100 bp ladder.

PCR products were purified using the GeneClean® Turbo for PCR kit. For each DNA sequencing reaction, 3 µl of PCR product, 2.5 µl Better Buffer (Webscientific Ltd, Cat. # 3BB-10), 0.5 µl ABI Big Dye Vs. 3 and 2.4 pmol of primer was made up to a final volume of 11 µl volume with deionised dd water. Sequencing of the isopropanol purified products was performed in both forward and reverse directions. Sequencing PCR entailed a step of 94 ºC for 90 s followed by 25 cycles of 96 ºC for 15 s, 50 ºC for 10

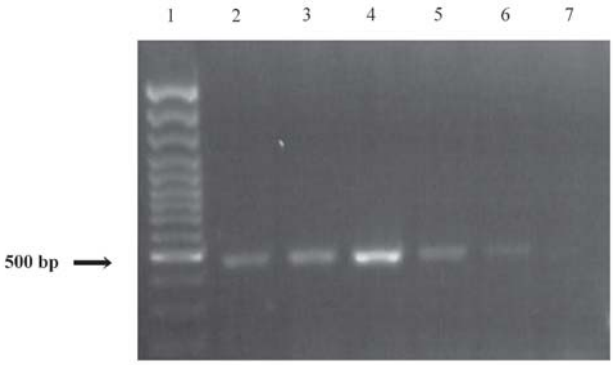

Fig. 2. Gel electophoresis of partial cytochrome *b* amplification products from *Vipera berus* and *Natrix natrix* non-invasively collected tissues and blood. Lane 1) 100 bp DNA size ladder; 2) blood; 3) sloughed skin; 4) carcass (road kill); 5) Ancient Ethanol Preserved Tissue (museum sample); 6) egg; 7) faeces.

s and 60ºC for 4 min. Samples were run on an ABI 3100 DNA semi-automated DNA analyser (Perkin Elmer) and sequences were aligned using Sequencer™ and corrected by eye.

RESULTS

Faecal sample collection

Although potentially accessible, snake faeces are challenging to identify and rarely located in the field. During the course of this study, we developed a simple semi-invasive method of faeces collection following the observation that snakes often defecate in response to direct disturbance or handling. In a series of trials, individual snakes were carefully scooped from the ground in an upright motion whilst the tail was quickly transferred into a small plastic collection bag. As adders are venomous and have particularly delicate cervical vertebrae, extreme care is necessary when securing a hold on these animals and the mid-body should also be supported while the snake is held aloft and the tail placed into the collecting bag. However, unless suitably qualified we strongly advise the use of restraint tubing to secure the adder allowing safer handling. Repeated sampling (n=100) revealed that the majority of British snakes handled in this fashion would defecate in the bag, usually within 20 s of capture. This procedure was most reliable with grass snakes (about 95% defecation) compared to smooth snakes (about 75%) and adders (about 70%). These animals could then be released after photography, measurement and other details had been recorded. The sample bag was quickly cooled *in situ* before storage at –20 ºC.

DNA extraction and amplification

Table 1 displays the success rates of extraction and amplification of the 500 bp mtDNA products from non-invasively collected sample material. These tissue types were sampled for all three British snake species with the exception of faeces and ancient ethanol preserved tissue (A.E.P.T.) for *Coronella austriaca*, which were not available in the current study. All different types of tissue samples eventually amplified and sequenced, but with varying rates of success both between tissue types and species (Fig. 1).

As predicted, the highest success rates for DNA extractions and amplification were obtained from recently preserved muscle samples, namely fresh foetus and other carcasses including road kill. Theses samples consistently produced the strongest intensity 500 bp fragment (based on comparison to 100 bp ladder luminosity) with an amplification success of 84% (*V. berus* 90%; *N. natrix* 86%; *C. austriacas* 81%). There was no obvious difference between ethanol and frozen samples, but sample sizes were low. High amplification success (70%) was also obtained using a second primer set that consistently generated 758 bp of *cyt b* sequence from 20 *V. berus* carcasses. Three DNA extractions from eggs generated faint 500 bp bands in two out of four PCRs (Table 1). Dried sloughs, including a sample stored for two years, amplified strong 500 bp products with an overall 61% amplification success rate (*V. berus* 64%; *N. natrix* 85%; *C. austriacas* 40%), although the brightness of this product was more intense from the fresh slough (Fig. 2). Surprisingly, 758 bp fragments were also obtained from 10 of 20 *V. berus* dried sloughs. From faeces, 500 bp fragments were amplified from one of five samples for both *V. berus* and *N. natrix*, but products were weak in intensity and the resulting sequences were of extremely poor quality. A.E.P.T. snake samples required up to three extractions before positive amplifications were achieved in five of the six samples tested (Table 1), with only 41% and 11% amplification success rate for *V. berus* and *N. natrix*, respectively.

Sequences of 500 bp with very few or no ambiguous bases were obtained from all tissue types, apart from faeces. The 758 bp *cyt b* fragment was successfully sequenced from *V. berus* carcasses and sloughs; sequencing of this larger amplicon was not attempted from other tissues types. Interspecific sequence homology allowed alignment of partial *cyt* b sequences from all three species and yet sufficient base substitution existed to reliably determine and identify sequences at species level. BLAST searches revealed that all sequences generated during the current study matched with GenBank sequences of adder, grass snake or smooth snake.

DISCUSSION

This is the first study to demonstrate the feasibility of DNA amplification from snake faeces, egg and foetal material, as well as from other non-invasive samples including slough skin and non-invasively sampled (NIS) muscle. Although snake faeces have previously been used to identify the morphological remains of specific prey items (e.g. in the black rat snake; Weatherhead et al., 2003), their utility as an NIS material for DNA extraction from snakes had not previously been investigated. The most reliable NIS sources in this study were muscle tissue and slough skin, with ancient tissue samples being the least reliable. Some snake by-products can be easily lo-

cated in the field, but consideration must be given to exclude temporal duplication of samples from the same individual, for instance multiple skin sections from the same slough that have broken up and dispersed. If only a small number of samples are analysed, such non-random sampling can skew data and give a false representation of population genetic diversity. However, repeated sampling of the same individual over time can provide useful ecological data regarding movement and lifespan. Recording the exact location of collected samples is critical and advances in geographic information system (GIS) technology (Salem, 2003) allow patterns of genetic structure to be analysed in a geographical context (e.g. Kidd & Ritchie, 2006). It is also important to record the age of the sample and method of preservation. PCR from template DNA extracted from degraded tissue is problematic, due not only to general DNA degradation but also to the presence of inhibiting factors (Kohn & Wayne, 1997; Wehausen et al., 2004). In addition, using universal (highly conserved) mitochondrial PCR primers, there is an increased likelihood of inadvertently amplifying non-target organism DNA. Furthermore, even if DNA does amplify, decayed nuclear DNA is more commonly associated with genotyping errors, such as allelic dropout and false alleles (Taberlet et al., 1999).

In the current study, we also developed a new method for faeces collection from wild-caught snakes. In the field, British snakes can be secured and scooped vertically from the ground, initiating a defecation defence response. The faeces can be simultaneously collected and appropriately preserved for subsequent DNA extraction. There appeared to be a relationship between stool consistency and successful sample collection: grass snakes (that have loose stools) are more likely to defecate when handled that either smooth snakes (with intermediate stools) or adders (firmer stools). Such variation in faecal consistency is related to diet, with grass snakes preferring amphibians and fish, smooth snakes eating mostly reptiles and rarely small mammals, and adders consuming mostly small mammals. Typically, snakes with loose stools defecate more regularly than those with firm stools and so our observations are not surprising, but we demonstrate how this natural response can be exploited for semi-NIS.

Faecal samples have previously been an overlooked source of reptile DNA. However, faeces are routinely used to genotype protected species (e.g. Bayes et al., 2000; Goossens et al., 2000; Garnier et al., 2001; Chih-Ming et al., 2004). Although DNA from faecal samples is degraded, microsatellite analyses and sequencing of short amplicons is usually possible, but DNA fragments of greater than 500 bp are difficult to sequence, as observed in the current study. More in-depth studies are required to assess the maximum size of amplicons than can be sequenced from snake faeces. In addition, storage methods for snake faeces should be optimized, as has been done for large mammals. For example, Roeder et al. (2004) described a two-step method of preservation whereby gorilla faecal samples stored in ethanol for 24–36 h were subsequently transferred into silica for optimal DNA preservation.

The most reliable source of non-invasive sample material was muscle tissue from carcasses (road killed and semi-predated specimens), recent museum samples and foetal tissue. Roads provide excellent basking opportunities for snakes (under low-traffic conditions) as they heat up quickly, maintaining temperature throughout the day and into the cooler evening (Ashley & Robinson**,** 1996; Shine et al., 2004). Unfortunately, snakes are often not quick enough to evade vehicles, exhibiting momentary immobilization in response to traffic (Andrews & Gibbons, 2005), but no agency records snake road kill statistics for the UK, despite the fact that animal carcasses do provide high quality genetic data for a multitude of studies including phylogeography and phylogenetics (Keogh, 1998; Doyon et al., 2003; Piertney et al., 2005).

DNA from grass snake eggs was successfully extracted and amplified, but success rates could not be assessed during the current study due to the small sample size. Unhatched eggs should only be collected late in summer/autumn after all viable eggs have hatched, but such samples are likely to be contaminated by microbial PCR inhibitors (Fernando et al., 2003). Avian eggshell membrane is established as a non-invasive DNA source (Fernando et al., 2003; Strausberger & Ashley, 2001), allowing genotyping of an identified egg-laying female without disturbance; however, this is the first study to show that yolk tissue can also be used to identify British reptiles such as the grass snake or sand lizard. We have not yet tested whether DNA can be successfully extracted from the membranous egg shells of grass snakes.

Of the non-invasive materials tested in this study, sloughed skin was the easiest to collect and store directly from the field. DNA extraction from slough does require an initial rehydration step, but this is still a simple and quick method. The lower yield of DNA (compared to muscle) and its potentially fragmented condition can make amplification above 500 bp intermittent, especially in older samples. However, with relatively fresh sloughs it is possible to amplify DNA fragments in excess of 750 bp, although previous studies indicate the ability to amplify such products would diminish with time (Fetzner, 1999). Sloughed skins are often subjected to moisture, UV and microbial damage before collection, which reduces DNA quality. Generally, it is recommended that studies based on dry, room-temperature-stored sloughs collected over a year (in various states of decay) should not aim to target sequences over 700 bp (e.g. Ursenbacher et al., 2006). The inclusion of slough as a source of DNA is becoming more frequent for snakes (Vidal et al., 2000; Burbrink 2002; Clark et al., 2003) and other animals (e.g. Fetzner, 1999; Sigler et al., 2002; Valsecchi et al., 1998).

Ancient tissue sources were amongst the least viable non-invasively collected source tissues for reptiles. This could be due to degradation of DNA (Thomas et al., 2005; Krause et al., 2006) or contamination of the sample storage medium, e.g. in the event of evaporation, alcohol preserved sample bottles being “topped up” from neighbouring bottles containing related sample material. Although a single band may apparently be amplified, it is common to produce a recombinant or multiple sequence

comprising two or more individuals. Depending on storage media and sample age, extractions from preserved museum samples often prove labour-intensive (Pääbo, 1989; Serth et al., 2000).

Non-invasively collected British herpetofaunal samples reliably yielded DNA of sufficient quality and quantity for sequencing; in fact the current study is a conservative estimate of the value of snake NIS tissues for genetic studies as we intentionally targeted relatively long DNA fragments (500 and 758 bp). Populations and species of an endangered, threatened or highly protected nature, such as the British sand lizard or smooth snake, can now be studied non-invasively, producing viable data and valuable insight into the ecology of these reclusive animals.

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