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Good DNA from bat droppings

SEBASTIEN J. PUECHMAILLE[1, 2], GREGORY MATHY[1], and ERIC J. PETIT[1, 3]

[1]*Ethologie Evolution Ecologie, UMR CNRS 6552, Université de Rennes I, Station Biologique, 35380 Paimpont, France*
[2]*School of Biological and Environmental Sciences, University College Dublin, Belfield, Dublin 4, Ireland*
[3]*Corresponding author: E-mail: eric.petit@univ-rennes1.fr*

Amplification of a mitochondrial DNA fragment was used to compare the efficiency of five methods for extracting DNA from bat droppings. The Qiagen DNA Stool Kit, which yielded > 90% mtDNA amplification success, was chosen to extract DNA from 586 samples taken over two years in three French colonies of the lesser horseshoe bat (*Rhinolophus hipposideros*). Samples, for which mtDNA amplification was successful, were subject to the multiplex amplification of eight microsatellite loci. This resulted in > 95% amplification success over 12,592 PCRs. Allelic dropout (ADO) and false allele (FA) rates were low, and consequently, sample and locus quality indexes (QI) were high. These results demonstrate that large scale noninvasive studies of bat colonies are possible.

Key words: Chiroptera, error rate, faecal DNA extraction, microsatellite, mtDNA, noninvasive sampling, *Rhinolophus hipposideros*

INTRODUCTION

Noninvasive genetics is becoming an increasingly valuable tool in population biology, but obtaining good DNA from noninvasive material is still a challenge. Various methods have been used to extract DNA from faeces (see Eggert *et al.*, 2005 for a review) in a large variety of mammals (i.e., brown bear — Taberlet and Bouvet, 1992; dugong — Tikel *et al.*, 1996; seel — Reed *et al.*, 1997; wombat — Banks *et al.*, 2003; bats — Vege and McCracken, 2001; Zinck *et al.*, 2004; Carter *et al.*, 2006). The choice of the extraction method is very important in noninvasive genetics (Eggert *et al.*, 2005) as a non efficient method would furnish costly (many PCR replicates needed) and most probably unreliable results (inaccurate consensus genotypes) mainly because of high Allelic DropOut (ADO) and False Allele (FA) rates (for a review on genotyping errors — see Pompanon *et al.*, 2005). On the contrary, an efficient extraction method should provide reliable consensus genotypes with as few PCR replicates as possible.

We recently started a project on the conservation biology of the lesser horseshoe bat (*Rhinolophus hipposideros*) in Brittany. One aim of this project was to set up reliable noninvasive genotyping methods in this species. We first used the protocol published by Vege and McCracken (2001) to extract DNA from droppings, but it did not yield DNA of good quality in our species. In

addition to this protocol, we thus evaluated four other extraction methods. Then, the most efficient of those methods was adopted for further sample extractions and subsequent amplification of eight microsatellite loci, to evaluate the reliability of noninvasive genetics to provide accurate multilocus genotypes from faecal bats' DNA obtained from samples collected in natural populations.

Materials and Methods

Droppings Collection and Storage

Samples were taken from three different lesser horseshoe bat nurseries (Epiniac, Pluherlin, and Saint-Thurial in Brittany, France) in 2003 and 2004. Only bat droppings taken from St-Thurial in 2004 were used to compare the five extraction protocols. Samples from all colonies and both years were used to assess the quality of genotypes obtained after extracting DNA with the best protocol among the five tested.

Sampling consisted first in cleaning up the ground and placing newspaper under the main cluster of bats at the end of May, when most adults are at the colony. Collection of faeces occurred ten to 15 days later depending on colony and year, to maximise the number of individuals sampled (Puechmaille and Petit, In press). Droppings were placed individually in 2 ml microtubes, which were sent to the laboratory where silica gel fragments were added to each microtube. Newspapers and silica gel fragments were used in order to absorb humidity, thus avoiding DNA degradation (Wasser *et al.*, 1997; Taberlet *et al.*, 1999). Microtubes were then stored open in a dry room.

DNA Extraction

Five protocols were evaluated for extracting DNA from lesser horseshoe bat droppings collected at Saint-Thurial in 2004. The first one is being used to extract DNA from tissue biopsies, while the other four have been used for difficult samples such as faecal material: (1) the NaCl method (Miller *et al.*, 1988); (2) the CTAB method (Launhardt *et al.*, 1998); (3) the DNeasy Tissue Kit (QIAGEN) modified following Vege and McCracken (2001); (4) the DNeasy Plant Mini Kit (QIAGEN); and, (5) the QIAamp DNA Stool Mini Kit (QIAGEN). We followed the authors' or manufacturers' instructions except for the Stool kit method for which some steps were modified as described below (step numbers correspond to the manufacturers' instructions handbook, pp. 22–24; ver. 08/2001).

Each dropping, weighing on average 5.3 mg (n = 80, SD = 0.98), was placed individually in a 2 ml microtube. Droppings were squashed with a sterile toothpick until the solution was homogenized. Steps 3 and 4 were discarded. Step 7 was run as follows; immediately after centrifugation, 600 µl of the supernatant was transferred into a new 2 ml microtube containing 25 µl Proteinase K. Incubation at step 11 lasted 15 minutes. From steps 13 to 17, centrifugations lasted 1 min. at 7200 rpm. DNA was finally eluted in 80 µl water (step 18). Eight extractions without dropping were run on eight different extraction batches to control for the absence of cross-contaminations during manipulations.

DNA Analysis

The ability of the various protocols to extract DNA from bat droppings was tested by amplifying a 338-bp fragment of the mitochondrial cytochrome *b* gene. PCR reactions were carried out in 15 µl volumes, each containing 1 µl of DNA extract, 0.4 µM of each primer (Rhcwtbf1: 5'-ATTCGCAAGTCCCACCCACT-3'; Rhcwtbr1: 5'-CCAATGTTTCATGTTTCTGAG-3'), 1 × Taq buffer (QIAGEN), 1.5 mM $MgCl_2$, 0.6 U HotStarTaq (QIAGEN) and 0.16 mM each dNTP. Amplification conditions were as follows: 15 min at 95°C; 50 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; 10 min at 72°C. DNA fragments, stained with Ethidium Bromide, were separated on 2% agarose gel electrophoresis (45 min, 120 V, 90 mA) and visualized using ultraviolet light. The presence of DNA was classified as positive or negative according to the intensity of the band, weak bands being conservatively classified as negative except for the negative controls that were run on each PCR to detect possible contamination.

Among the 586 samples extracted for further evaluation of the best extraction protocol, only those tested positive for the presence of mtDNA were typed for a set of eight tetra repeat microsatellites amplified together in a multiplex PCR (RHC108, RHD102, RHD103, RHD111, RHD113, RHD119, RHD2, RHD9 — Puechmaille *et al.*, 2005). Typing was processed following a comparative multiple tubes approach divided in three steps enabling to obtain highly reliable multilocus genotypes (Puechmaille and Petit, In press).

Error Rates and Samples Quality

Quality Index (QI), Allelic DropOut (ADO) and False Allele (FA) rates were estimated by comparing consensus genotypes to PCR replicates. Quality Indexes (QI) were estimated per sample and per locus according to formulas given by Miquel *et al.* (2006). A score of '1' was given to all PCR replicates that were exactly identical to the consensus genotype at a locus, otherwise they were given the score '0' whatever the difference (ADO, FA or negative PCR). When no consensus genotype was built for one locus, a score of '0' was attributed to each replicate. The per-replicate probability of ADO (p_j) and FA (f_j) at locus j were estimated according to Broquet and Petit (2004).

RESULTS

DNA Extraction Methods

Amplification success of the mitochondrial fragment was relatively low for extracts obtained with the NaCl method (16.6% — Fig. 1). The Plant and Stool kit methods yielded the best results (> 90% amplification success) and the CTAB and Tissue kit methods resulted in an intermediate level of mitochondrial DNA amplification (around 40%). All PCR controls for contamination were negative.

In the second experiment, the mitochondrial fragment could be amplified from 567 (96.8%) of the 586 samples extracted with the Stool kit. Eight extractions without dropping, run on eight different extraction batches, were negative. There was no difference in amplification success of the mitochondrial fragment between samples stored 18 vs. six months prior to extraction (χ^2 = 0.52, *d.f.* = 1, P = 0.47). All 567 positive samples except four (mixed during extraction due to pipetting error) have been repeatedly typed for a set of eight microsatellite loci. Finally, 534 samples yielded a reliable multilocus genotype at six to eight loci. In order to obtain a reliable multilocus genotype, the mean number of replicates per sample (negative reactions included) was

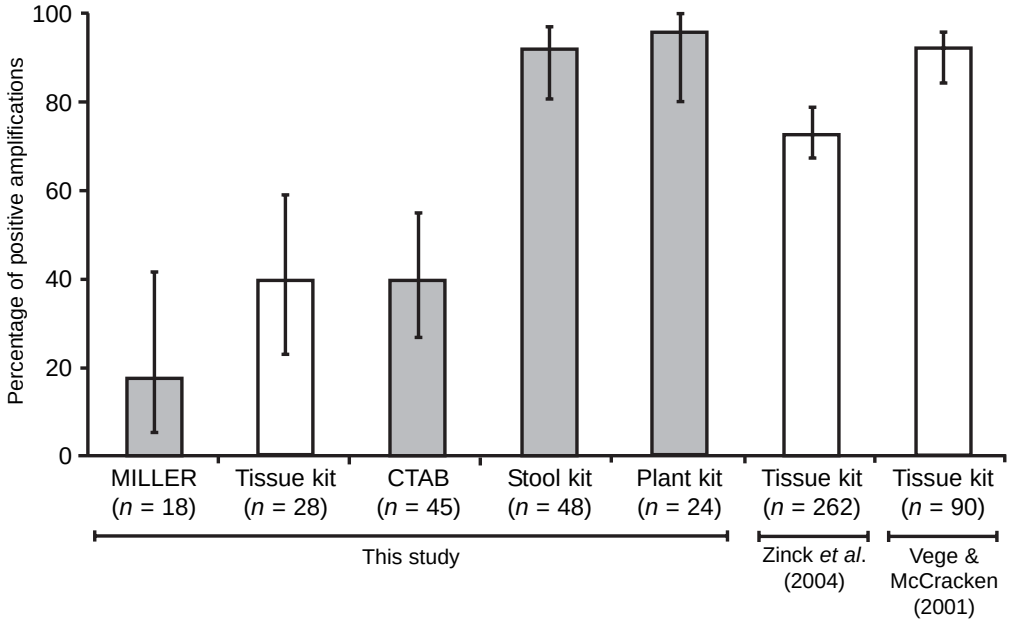

FIG. 1. Comparison of the amplification success of a mitochondrial fragment when DNA was extracted from bat droppings with five different methods. White bars give amplification successes for this study and two other studies that used the Tissue kit. Zinck *et al.* (2004) amplified a mitochondrial fragment while Vege and McCracken (2001) amplified an autosomal locus. Sample sizes are given in parentheses. Bars represent the lower and upper limits of the 95% confidence interval calculated from the binomial distribution

2.73 and varied between 2.54 and 3.09 according to colony and storage duration. Statistical analyses showed however that the effect of storage duration and colony were not significant (glm; family = poisson, $P = 0.16$ and $P = 0.17$, respectively).

Error Rates and Samples Quality

ADO probabilities ranged between 0.014 and 0.089 at Epiniac and Pluherlin and were higher at Saint-Thurial (range = 0.056–0.221). For the three colonies, FA probabilities ranged from 0.000 to 0.064 (Table 1).

The number of successful PCRs at the first and second replicate were highly correlated (Spearman rank correlation, $r = 0.69$, $d.f. = 561$, $P < 0.001$). Nevertheless, in the final data set, seven samples had seven or eight positive PCRs for each repetition except one which totally failed, suggesting that the negative result was due to manipulation errors (e.g., no transfer of DNA template into the PCR well) and not bad sample quality. These negative PCRs where thus omitted when calculating QIs. The mean QI per sample was 0.940 (Fig. 2A) and the mean QI per locus ranged between 0.925 and 0.954 (Fig. 2B).

Discussion

DNA Extraction

In our study, the Tissue extraction method provided poor results compared to those obtained by Vege and McCracken (2001) or Zinck *et al.* (2004) (see Fig. 1). Our amplification success of the mtDNA fragment was 39.3% whereas the success rate for amplifying nuclear DNA (microsatellites), which is more difficult to amplify due to its lower number of copies, was 92% in Vege and McCracken (2001). Zinck *et al*. (2004) reached a success of 72.5% with a mitochondrial fragment. These differences can be due to different factors which are not mutually exclusive; (1) variability in sample quality, with bat droppings collected from humid places potentially harbouring more degraded DNA (Wasser *et al.*, 1997; Taberlet *et al.*, 1999); (2) amplified fragments were longer in our study, involving a lower amplification success because longer fragments are more degraded than shorter (Buchan *et al.*, 2005; Broquet *et al.*, 2007); (3) less material was used at the beginning of the extraction [5.3 mg vs. 20–25 mg in the study by Vege and McCracken (2001)], involving less DNA in the extract

Table 1. ADO (p_j) and FA rates (f_j) per colony, per year and per locus (respectively calculated after eqn 1 and 3 — Broquet and Petit, 2004) are presented. Means, minimal and maximal values are indicated at the bottom of the table

Locus	Epiniac				Pluherlin				Saint-Thurial			
	2003		2004		2003		2004		2003		2004	
	p_j	f_j	p_j	f_j	p_j	f_j	p_j	f_j	p_j	f_j	p_j	f_j
C108	0.024	0.011	0.038	0.012	0.024	0.007	0.030	0.000	0.214	0.029	0.188	0.028
D102	0.037	0.009	0.048	0.033	0.045	0.011	0.021	0.000	0.109	0.000	0.147	0.019
D103	0.064	0.034	0.026	0.032	0.077	0.014	0.014	0.012	0.070	0.014	0.212	0.055
D111	0.070	0.026	0.072	0.013	0.089	0.042	0.056	0.023	0.136	0.064	0.141	0.029
D113	0.070	0.009	0.050	0.047	0.037	0.017	0.021	0.031	0.173	0.014	0.146	0.028
D119	0.029	0.031	0.028	0.023	0.018	0.014	0.031	0.039	0.090	0.050	0.056	0.000
D2	0.037	0.034	0.050	0.030	0.072	0.059	0.028	0.031	0.087	0.058	0.113	0.046
D9	0.063	0.025	0.043	0.034	0.067	0.028	0.044	0.031	0.146	0.064	0.221	0.047
Mean	0.049	0.022	0.044	0.028	0.054	0.024	0.031	0.021	0.128	0.037	0.153	0.031
Min.	0.024	0.009	0.026	0.012	0.018	0.007	0.014	0.000	0.070	0.000	0.056	0.000
Max.	0.070	0.034	0.072	0.047	0.089	0.059	0.056	0.039	0.214	0.064	0.221	0.055

(Wehausen *et al.*, 2004); (4) differences in diet, involving presence or absence of PCR inhibitors (Idaghdour *et al.*, 2003). Variability in the quality of the bat droppings could explain why Zinck *et al.* (2004) had less success than Vege and McCracken (2001) because they collected droppings from natural roosts with some samples that were exposed more than ten years to temperature and humidity variations. In our case however, samples were collected in conditions that avoided their exposition to humidity. Vege and McCracken (2001) amplified microsatellites shorter than 200 bp, and Zinck *et al.* (2004) a mitochondrial fragment of 190 bp, while we amplified

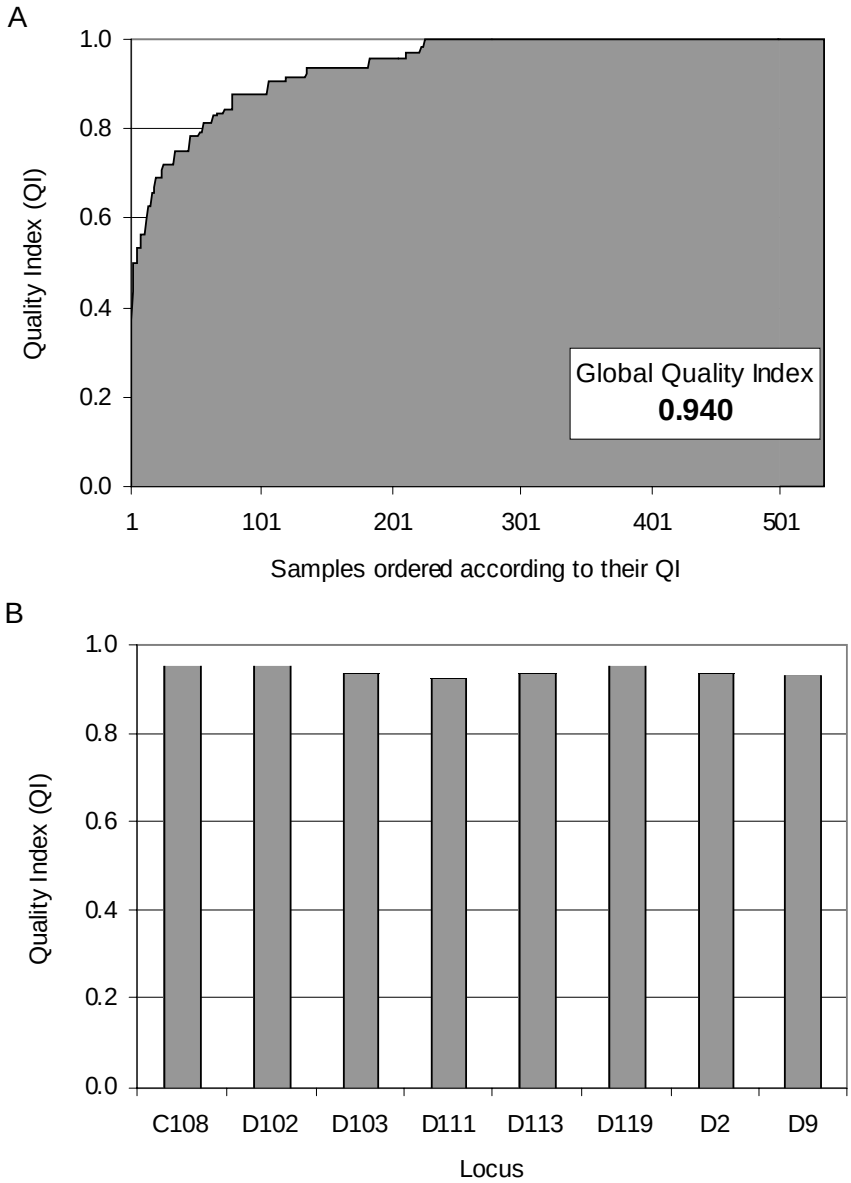

FIG. 2. Distribution of Quality Indexes (QI) among the 534 genotyped samples (A) and mean Quality Index for the eight loci (B)

338 bp of the mitochondrial cytochrome *b* gene. However, using droppings extracted with the Stool kit, the microsatellite locus D111 (allele range = 280–304 bp), which was just slightly shorter than our cytochrome *b* fragment, had an amplification success of 97.6% (*n* = 1574 PCRs), suggesting that amplifying relatively long fragments (ca. 300 bp) from lesser horseshoe bat droppings is not problematic. Fragment size is thus unlikely to explain such a difference in amplification success for the Tissue extraction method. The second explanation is also unlikely to explain this large difference because extractions from 5.3 mg of material using other methods (cf. Fig. 1) provided very good amplification success, suggesting that DNA contained in such droppings is quantitatively sufficient to be easily amplified. The third explanation is more likely because differences in diet have proved to affect amplification success (Murphy *et al.*, 2003), most probably through the presence of inhibitors not removed during extraction (see above). In their study, Vege and McCracken (2001) fed their captive bats exclusively on mealworms, while in our case, bats were in natural conditions and fed on various kind of insects, mainly Lepidoptera, Diptera and Neuroptera (Schofield, 1996), increasing the chance of having ingested a prey containing inhibitors. Our presumption is supported by a study on great bustards by Idaghdour *et al.* (2003) who found that the presence of insect fragments in faeces reduced amplification success.

In our study, extracting DNA using the Tissue kit yielded significantly lower results than when using the Plant or Stool kits (see Fig. 1). These two kits provided the best results and as the extraction using the Stool kit was quicker (about three vs. six hours for a batch of 24 samples), this method was considered to be the most efficient among those tested.

The absence of a significant difference in amplification success rate after 6 vs. 18 months of storage duration suggests that the effect of storage duration is negligible. Obviously, if sample storage duration diminishes quality, the effect is quite limited when samples are kept in good conditions. This agrees with results published by Zinck *et al.* (2004) who successfully amplified and sequenced DNA from guano collected 10 years prior to genetic analysis.

Error Rates and Quality Indexes

Combining the three colonies over the two years, ADO (5.95%) and FA (2.61%) rates are among the lowest reported in non-invasive studies (Broquet and Petit, 2004), involving the need of only 2.73 PCRs per locus/sample to obtain a reliable genotype. Bat droppings thus contain enough DNA of good quality to obtain reliable multilocus genotypes at low laboratory costs (€ 6.64/sample, including extraction, amplification and genotyping).

The quality index can vary between 0, for samples of very low quality (involving ADO, FA, negative PCRs), to 1 for samples of high quality (all replicates are positive and match exactly the consensus genotype) (Miquel *et al.*, 2006). Thus, the high mean QI of our 534 samples (QI = 0.940 — see Fig. 2A) suggests that our samples were of very good quality. Furthermore, the eight loci typed had a high and quite similar mean QI (range = 0.925–0.954 — Fig. 2B), suggesting that none of the loci was problematic (Miquel *et al.*, 2006). This view is supported by a comparison with mean QI values reported from three other data sets based on the analysis of faecal DNA; Mean QI are 0.625 in a data set of French *Canis lupus*, 0.564 and 0.371 for *Ursus arctos* data sets from Sweden and the French Pyrenees, respectively (Miquel *et al.*, 2006). The value we computed here is however not

directly comparable to the values given by Miquel *et al.* (2006) because we discarded samples that did not have multilocus genotypes with at least six complete loci from our analyses (see Materials and Methods). As recommended by Miquel *et al.* (2006), we thus recomputed a mean QI that took into account all samples (n = 563) that were amplified with the multiplex, giving a conservative QI of 0 to all samples that did not yield a multilocus genotype. This new value is 0.89 and still lies far above the mean QI reported by Miquel *et al.* (2006). We conclude from this comparison that multilocus genotypes obtained from a multiplex amplification of DNA extracted from bat droppings are indeed data of very good quality. Such genotypes can be used to reconstruct pedigrees or to estimate demographic parameters with capture-mark-recapture models. This suggests that noninvasive genetics could become a highly valuable tool for the study of bats, because no capture/handling of these elusive and sometimes rare species is required to acquire data that could illuminate various aspects of their biology.

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