Amplification success of multilocus genotypes from feathers found in the field compared to feathers obtained from shot birds

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Effective extraction methods from bird's feathers have been used as a source for DNA to allow non-invasive sampling and therefore are a great source for genetic information. However, although the utility of feathers in genetic studies have been shown repeatedly, few studies have addressed whether all feathers can be used or give equal amounts of useful template. In this study feathers collected in various ways from Irish Red Grouse (*Lagopus lagopus hibernicus*) were examined to establish the quality of DNA extracted. Individual samples were classified into two categories i.e. shot and collected. DNA was extracted from all samples and they were genotyped at 19 microsatellite loci. PCR products were analysed on a MegaBACE 1000 and output was analysed. A total of 93% of the 'shot' category produced a genotype that was considered successful (i.e. 15 of 18 loci) and 23% of the 'collected' category produced successful genotypes under the same criteria. There was a significant difference between shot and collected samples in genotyping success and the observed number of missing loci. Recommendations and best practices are discussed along with utility of bird feathers as a source of DNA for population and conservation biology.

Keywords: Red Grouse *Lagopus lagopus hibernicus*, PCR amplification, genotyping success

Several protocols for extraction of DNA from moulted or otherwise lost bird feathers have been published over recent decades (Taberlet & Bouvet 1991, Morin *et al*. 1994, Eguchi & Eguchi 2000, Bello *et al*. 2001, Horvath *et al*. 2005, Bayard de Volo *et al*. 2008). Feathers have been used as a source for DNA to allow non-invasive sampling and therefore are a great source for genetic information particularly for bird species that are difficult to catch or where catching and treatment of the birds should be avoided. Smith *et al*. (2003) postulated feather sampling as a unique option to gather information from birds which would otherwise be easily available. However, although the utility of feathers in genetic studies have been shown repeatedly, few studies have addressed whether all feathers can be used or give equal amounts

of useful template (Segelbacher 2002, Gebhardt *et al*. 2009). Here we highlight the potential pitfalls of planning studies based on feather samples and argue for a carefully planned study design and evaluation of different materials for the respective research question.

It is known that DNA quality can be affected by a number of sources that can be found in nature. These include varying temperatures including repeated cycles of thawing and freezing and damage imposed by UV-light (Pompanon *et al*. 2005). Although measures for checking the validity of genotypes ad hoc (Taberlet *et al*. 1999, Pompanon *et al*. 2005) often are and should always be employed, it would be useful to quantify the possible bias introduced by the varying times between when the feather was shed and collected.

In this study we took advantage of feathers collected in various ways from Irish grouse *Lagopus lagopus hibernicus* in the Republic of Ireland. In particular we ask whether PCR-amplification and genotyping success varied among samples that come from shot birds and thus were collected immediately and minimally affected by the extrinsic factors mentioned above and feathers that were spontaneously moulted and may thus have been in the field for various lengths of time before being found by the collector.

METHODS

Through the efforts of birdwatchers, hunters and hikers, feathers from 145 individuals of red grouse were collected all over Ireland between the years 2006 and 2009. Of these, 80 were from shot birds, 65 were shed feathers collected in the field. The collected feathers were of unknown age and had been in the field for an unspecified period of time. Samples were sent to Dr. Barry John McMahon at the UCD School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland. Feathers were stored dry at room temperature

and away from direct sunlight for a period of time that ranged from 2 years to 6 months before extraction. Each sample was given an ID (1 – 145) and as much additional information as possible about the sampler and where the sample was collected (e.g. location, date, whether it was shot or collected etc.). In some cases the contributor chose to be anonymous and provided only a vague reference to the location. Often an Irish map grid reference (*e.g.* S08 07) or a town name was given and rarely a GPS coordinate.

We classified each individual sample in two categories: 1) birds that were shot and from which feathers were immediately plucked and sent to us. These samples are hereafter referred to as 'shot'. 2) Samples that came from birds that were found opportunistically in the field. These samples are referred to as 'collected'.

A small part of the tip (2 – 5 mm long) of the calamus of the feathers was split and placed into in a sterile 2 ml Eppendorf tube along with two sterile steel balls. Samples were then frozen in liquid nitrogen and shaken for one minute at 2500rpm to crush the feather. Afterwards DNA extraction followed the DNeasy Tissue Kit protocol (Qiagen, Hilden Cat No 69506) with the following changes, 20μl 1 MDTT was added to the digestions step and samples were digested for 2h at 55° before further processing. DNA was eluted in two separate washing steps in 100 μl AE buffer each.

The DNA concentration of a subset of samples was measured with a NanoDrop. In total, DNA concentration was measured for 45 shot and 36 collected samples. Concentrations over 10ng/µl were regarded as good and were run through PCR without further processing. The ratio of absorbance at 260nm and 280nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as 'pure' for DNA; a ratio of ~2.0 is generally accepted as

'pure' for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm. Samples containing lower amount of DNA were re-extracted if possible when a second feather was available. If the 260/280 ratio was regarded good (1.75 – 2.20) but the amount of DNA was poor, the concentration of the sample was increased by precipitating the DNA and solving the pellet in a smaller volume (50μl) than original (100μl).

All samples were genotyped at 19 microsatellite loci selected based on previous studies done on grouse species (Piertney & Dallas 1997, Sahlsten *et al*. 2008, see Table 1) and amplified using PCR (Table 1). The markers were labeled with fluorescent dye (HEX, FAM and NED). Using a QIAGEN Multiplex (Cat No 206145) mix PCR was performed on all samples with the following master mix: 5µl of multiplex mix; 2µl of ddH_2O; 1µl of Q-solution; 1µl of primer mix and 1µl of DNA. Primer mix was made by mixing 10µl of each 20µM primer stock (forward and reverse) and then adding ddH_2O to a total volume of 100µl. PCR conditions for each multiplex (as in Table 1) were as follows: denaturation at 95˚C for 15 min; 40 cycles of; 94˚C for 30s: annealing for 90s and 72˚C for 60s. This was followed by a final extension for 30 minutes at 60˚C.

PCR products were analyzed on a MegaBACE 1000 and output was analysed with the software Fragment Profiler (Fragment Profiler 1.2, Amersham Biosciences, 2003). Scoring of microsatellites was done automatically using a constructed peak filter but each score was verified manually. A successful multilocus genotype was called when homozygotes at each locus had been verified in at least 3 independent PCRs and when at least 15 loci could be successfully genotyped. To check for null alleles, stuttering and large allelic dropout the dataset was run through MicroChecker 2.2.3 (Van Oosterhout *et al*. 2004). A null allele is

generally defined as an allele that has not amplified during PCR. Stuttering is when there are slight changes in allele size and large allele dropout occurs when a large allele do not amplify as successfully as a small one. Such errors in the PCR will create a bias in any analysis based on genotypic data. The heterozygosity and number of alleles were calculated using Microsatellite Toolkit (Park 2001). The bootstrapping to estimate confidence intervals for the comparison of DNA concentration was done using R 2.12.2 and the package 'boot' (Angelo & Ripley 2010).

RESULTS

The only locus that was consistently found to contain null allele was TUT4 and was removed from further analyses. No stuttering or large allele dropout was reported for any other locus. In total 61 % of the sampled individuals could be successfully genotyped at least in 15 of 18 loci. A total of 93% (74 out of 80) of the 'shot' category produced a genotype that was considered successful (i.e. 15 of 18 loci) and 23% (15 out of 65) of the 'collected' category produced successful genotypes under the same criteria. Of the samples belonging to the 'collected' category only 23% were successfully genotyped. There was a significant difference between shot and collected samples in genotyping success (χ^2= 12.42, df = 1, P < 0.0001). Furthermore there was a difference among categories in mean genotyping success at individual loci (mean collected=30%; mean shot=86%, t = 11.9077, df = 111.574, P < 0.0001). The observed number of missing loci in the two categories was different and highly significant (mean collected=11.9; mean shot=2.4; t = 12.43, df = 111.442, P < 0.0001, Fig. 1). DNA concentration was higher in the subset of shot samples (n= 42) compared to the collected (n= 36) samples (Table 2). The shot samples had a mean concentration (± SD) of 40.79 ± 26.38 ng/ul and the collected samples had a mean of 15.80 ± 7.64 ng/ul DNA. The

observed heterozygosity for all genotyped samples from the collected group was 0.61 (± 0.03), and 0.63 (± 0.01) for the shot samples. Expected heterozygosity for collected samples was 0.68 (± 0.04), for shot birds it was 0.67 (± 0.04). The mean number of alleles was 6.82 (±2.51) for the collected and 8.65 (±3.59) for the shot samples, respectively.

DISCUSSION

An important aspect in any conservation genetic study is the potential problem of inbreeding when small populations become isolated. Inbreeding can occur when only a few individuals are remaining and as inbreeding increases, the number of heterozygotes is expected to decrease (Frankham *et al*. 2007). A higher relatedness among individuals also requires a higher resolution of the given genetic marker set and the risk of false identified unique genotypes may further increase with genotyping errors due to low DNA quality. Any possible genotyping errors from false alleles or homozagotes due to allelic drop out need to be avoided. We recommend that feather quality should be examined at the time of collection and that feathers that show obvious evidence of long term exposure to the elements because they have been shed long before collection should be avoided. If possible, the time between shedding and collection should be standardised and kept as short as possible. If this is not possible, stringent measures, such as estimating deviations from theoretical expectations (Van Oosterhout *et al*. 2004) and multiple independent PCR amplifications to avoid the inclusion of false homozygotes need to be applied.

Molted feathers, if in good condition, are considered a good source of DNA (Segelbacher 2002, Bayard de Volo *et al*. 2008). If in bad condition, i.e. degraded (mainly through repeated freezing/thawing and exposure to UV-light), the success rate of DNA extraction goes down (Pompanon *et al*. 2005). We suggest that this may be the main reason for why only 23% of

the samples collected in the field were successfully genotyped at a threshold of at least 15 out of 18 microsatellite loci. However, when combining shot and collected samples an overall success rate of 61 % could be obtained. This success rate is comparable to that found in an earlier study where the estimated percent of reliable genotypes from collected molted feathers was ~50% (Segelbacher 2002). However, when the success of the 'collected' category is compared against Segelbacher (2002) 23% represents an inferior amplification success rate. The reasons for this could be many including, the condition of feathers may have been heavily degraded before they were collected. Samples in the study by Segelbacher (2002) werecollected during the high peaks of the molting season and likely consisted of relatively freshly moulted feathers. A success rate of 23% is, however, in accordance with another study of Ptargiman in the Yukon territory (Gernot Segelbacher, unpublished data) where similar low amplification success values were found. Amplification success of shed feathers sampled in the field is highly depend on the size of the feathers (Segelbacher 2002). Large feathers (wing or tail feathers) generally yield more DNA than coverts or body contour feathers. Studies on large birds generally achieve higher success rates (85% of shed feathers could be used in the Imperial Eagle *Aquila heliacea*, (Vili *et al*. 2009) and 95.5% of PCRs was positive from molted feathers in the Roseate Spoonbill *Plataela ajaja*, (Mino & Del Lama 2009). DNA concentration in our samples is relatively low compared to other species: ~ 115ng/μl in molted remiges of spoonbills (Mino & Del Lama 2009), and ~ 93ng/μl in Spanish Imperial eagles (Horváth *et al*. 2005)but higher than molted contour and body feathers (13ng/μl) and molted wing and tail feathers (0 ng/μl) of the Greater Sage Grouse *Centrocercus urophasianus* (Bush et al. 2005). Interestingly plucked body contour feathers from living Sage grouse yielded similar DNA concentrations of 40 ng/μl to our study and 100% DNA extraction and sexing success (Bush *et al*. 2005).

Although other studes have reported > 99% amplification success rates for feather samples plucked from birds (Gernot Segelbacher, own data) success rates of 93% as in the present study can be likely attributed to different procedures of taking feathers after the bird being shot and the following storage. Interestingly 7 out of the 8 unsuccessful samples taken from shot birds were provided by the same person. Feather plucked by a feather – trap (Maurer et al. 2010) and thus resembling somewhat plucking by humans, yielded only enough DNA for genotyping with 5 microsatellite loci in 71.4 % of the samples. There has been a recent debate if feathers are at all a good source of DNA quality when the bird has been caught and other material like tissue or blood would be available (McDonald & Griffith in press). Blood sampling has a number of advantages especially in the long term and blood or tissue can thus be regarded as a more reliable material as we would not have to deal with shortage of DNA, and less problems of amplifications. Our results indicate that plucking of feathers from shot birds has some limitations and we thus argue that future studies should rather aim at sampling blood or tissue whenever feasible. However, the advantage of feathers is the relative ease with which they can be collected and would thus be a viable option if the collectors are not experienced with collecting blood and / or tissue. This is especially true for endangered or elusive species, when catching birds is not an option.

Recommendations:

Field biologists still regard molted feathers found in the field as valuable source for genetic studies. Although, such material can be used as template for DNA extraction we here highlight that such an approach has serious limitations. Not every feather found in the field can be used and this restricts obviously the analysis and interpretation of the gathered data. Even feathers freshly plucked from shot birds are no guarantee for good DNA quality.

Thus we argue that field biologists and conservation geneticists should sit together during planning a study and consider the following things:

1) Plan the sampling design carefully (see also Segelbacher *et al*. 2010).
2) Teach all persons in the field how to handle samples and avoid contamination and provide a standardized sampling protocol.
3) Plan a pilot study to evaluate the amplification and genotyping success, estimate error rates and potential of allelic drop out or false alleles.
4) Estimate DNA concentration threshold to optimize the number of PCR to be done per each sample.
5) Evaluate which markers are useful for the given material and possibly design new microsatellite primers targeting shorter PCR products.

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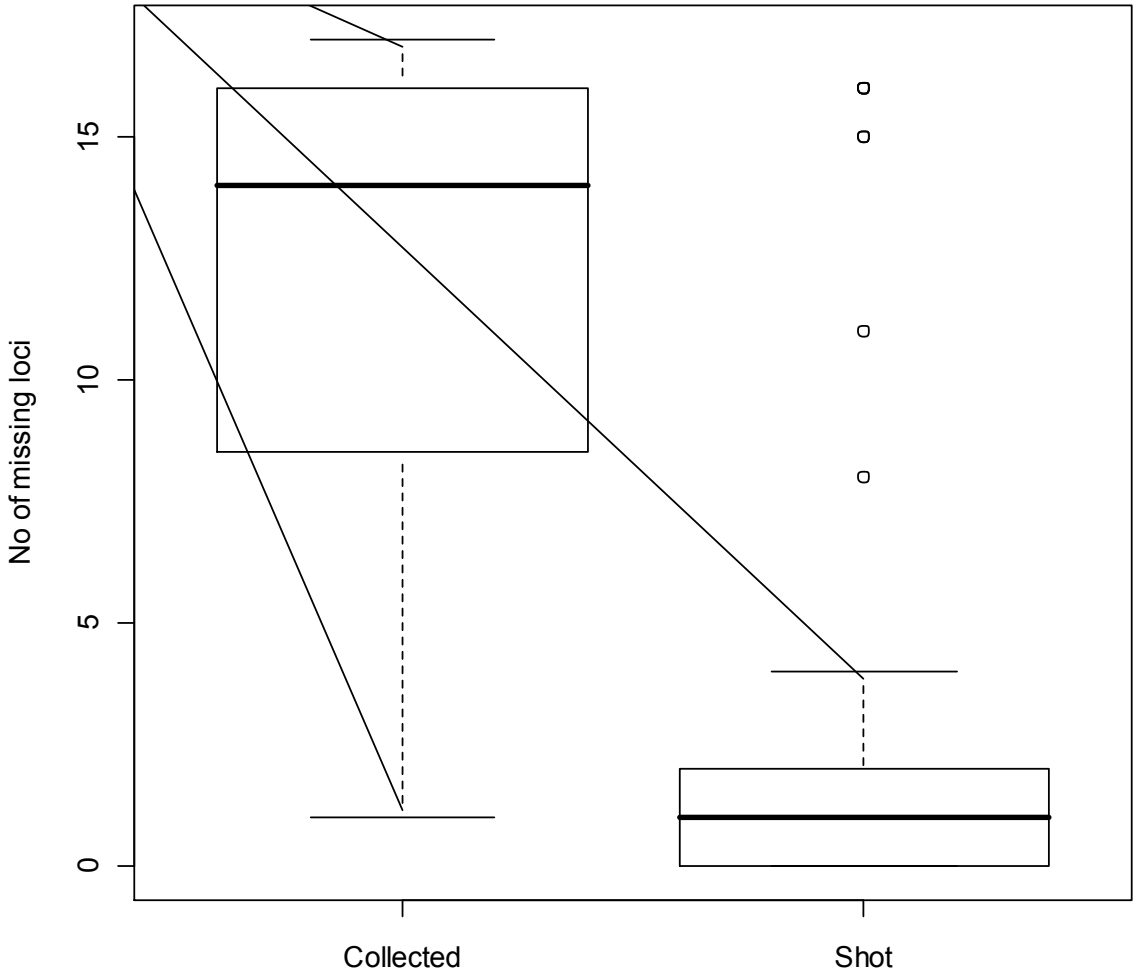

Figure 1. Median number of missing loci (line though boxes) and 75% (boxes), 95% (error bars) and outlier observations (points) for shot and collected samples. (n = 80 and n = 65 respectively).

Table 1. Compilation of the microsatellite markers used arranged in multiplexes. Size is given in base pairs (bp); spacing is the repeat number of the microsatellite, also in bp. PCR Amplification success is given in percent for both sampling categories of feathers: shot/collected.

Marker	Dye	Size (bp)	Spacing (bp)	Annealing T(˚C)	% Amplification success (shot/collected)
Multiplex 1					
ADL 230	FAM	90 - 115	2	48	95.0 / 32.3
ADL 142	HEX	207 - 255	2	48	91.3 / 33.9
Multiplex 2					
ADL 184	NED	110 - 160	2	54	92.5 / 35.4
BG 15	HEX	130 - 173	4	54	92.5 / 35.4
BG 16	NED	130 - 170	4	54	92.5 / 26.2
BG 18	FAM	110 - 180	4	54	91.3 / 30.8
Multiplex 3a					
LEI098	NED	135 - 165	2	60	92.5 / 15.4
TUT2	HEX	135 - 175	4	60	93.8 / 32.3
Multiplex 3b					93.8 / 26.2
TUT1	NED	178 - 220	4	60	95.0 / 40.0
TUT3	FAM	150 - 180	4	60	
Multiplex 4					92.5 / 21.5
LLSD4	HEX	185 - 220	2	58	95.0 / 46.2
LLSD6	FAM	88 - 126	2	58	93.8 / 30.8
LLSD7	HEX	140 - 176	2	58	93.8 / 30.8
LLSD8	FAM	138 - 170	2	58	
Multiplex 5					75.0 / 20.0
LLST1	NED	120 - 170	3	54	97.5 / 67.7
LLSD2	HEX	100 - 117	2	54	93.8 / 47.7
LLSD3	HEX	123 - 145	2	54	95.0 / 32.3

Table 2. Bootstrapped mean (1000 iterations) of concentration (ng/μl) and ratio (260/280) for the two categories of feathers: Shot and Collected. The confidence intervals are the Bias Corrected intervals (BCa). The sample sizes for Shot and Collected are n = 42 and n = 36 respectively.

	Shot		Collected	
	Concentration	Ratio	Concentration	Ratio
Bootstrapped mean	40.97	2.76	15.73	3.49
BCa 95% CI	(33.99 , 49.93)	(2.64 , 2.95)	(13.20 , 18.11)	(3.03 , 4.05)