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Extraction of high-quality host DNA from feces and regurgitated seeds: a useful tool for vertebrate ecological studies

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ABSTRACT

DNA extraction methods for genotyping non-invasive samples have led to great advances in molecular research for ecological studies, and have been particularly useful for analyzing threatened species. However, scarce amounts of fragmented DNA and the presence of Taq polymerase inhibitors in non-invasive samples are potential problems for subsequent PCR amplifications. In this study we describe a novel technique for extracting DNA from alimentary tract cells found on external surfaces of feces and regurgitated seeds. The presence of contaminants and inhibitors is minimized and samples are preserved intact for use in other ecological research (e.g. trophic studies). The amplification efficiency and purity of the extracted DNA from feces were significantly higher than in commonly used extraction procedures. Moreover, DNA of two bird species was identified from seeds expelled by regurgitation. Therefore, this method may be suitable for future ecological studies of birds, and other vertebrate groups.

Key terms: DNA extraction, ecological studies, guanidine thiocyanate, non-invasive method, pigeons, seed dispersal.

INTRODUCTION

Numerous scientific publications have documented the advances that non-invasive sampling has allowed in the field of molecular ecology over the past decade (see reviews by Piggott and Taylor, 2003; Eggert et al., 2005; Waits and Paetkau, 2005). According to the definition of Taberlet et al. (1999), non-invasive samples are those that do not require capture and handling of animals. This sampling has even greater value when threatened and rare species are the objects of study. Today, DNA can be successfully recovered from diverse animal sources such as hair, feathers, feces and shed skins. However, potential problems with these samples stem from the low quantity and quality of the DNA obtained from them (Eggert et al.,

2005). Fecal samples from a target species contain epithelial cells from the digestive tract walls, but also other contaminant DNA, such as those from bacteria and prey. In herbivores, secondary compounds from plant foods also represent a source of inhibition of the PCR enzymatic reaction (Khanuja et al., 1999). Therefore, DNA amplification rates will increase with the reduction of the inhibitor concentration (Morin et al., 2001). Moreover, usually entire fecal samples or large portions of them are used during the DNA extraction protocol, which means that valuable information about the diet of endangered species can be lost after molecular analysis.

In addition, frugivores have important effects on plant community composition since they influence species diversity and gene flow in seed banks (McDonnell and

Stiles, 1983; McClanahan and Wolfe, 1993). Recent molecular approaches have succeeded in accurately determining seed sources, and thus, the dispersal distance from maternal plants (Godoy and Jordano, 2001; Jones et al., 2005). However no direct method to identify the dispersal agent is currently available. Indirect methods include field observation (Yumoto, 1999), radio-tracking (Westcott and Graham, 2000; Westcott et al., 2005) and the use of camera traps (Otani, 2002), but none of these methods can provide the exact identification of a particular dispersal event (Nathan, 2006; Jordano, 2007).

The main goal of this study was to isolate mitochondrial DNA (mtDNA) of a high-quality from two sympatric frugivorous pigeons (*Columba bollii* and *C. junoniae*) using only the external surfaces of their fecal samples and regurgitated seeds. The novel non-destructive extraction method will allow the entire fecal sample to be used for both molecular and dietary analysis. Frugivorous pigeons are able to swallow whole fruits and, after a short period of time, they regurgitate large intact seeds. If digestive track cells from the gizzard, esophagus or oral cavity have adhered to the seed coat, their DNA may be extracted for identification of the species that ejected the seed.

MATERIALS AND METHODS

1. DNA extraction from fecal samples and regurgitated seeds

Fresh fecal samples (n = 45) and regurgitated seeds (n = 24) were collected in roosting areas of Bolle's Laurel Pigeon (*Columba bollii*) and White-tailed Pigeon (*C. junoniae*) in Los Tiles laurel forest (La Palma, Canary Islands). Transparent plastic sheets were placed under perches, and only recent samples (maximum 24 hours after deposition) were collected and carefully stored at -20ºC prior to genetic analysis.

Each seed was separately wrapped in fine filter paper (approximately 2 x 2 cm) soaked in 50-100 μl of guanidine thiocyanate (GuSCN). The filter paper

became tightly adhered to the seed coat, absorbing the avian DNA. After 30 min, each piece of paper was transferred into 0.6 ml eppendorf tubes containing 100-150 μl GuSCN (Boom et al., 1990). These tubes had been previously perforated at the bottom with a sterilized needle, and were then placed into 1.5 ml eppendorfs and centrifuged at 13,000 x *g* for 10 min, in order to recover the aqueous phase from the paper. Then, 15 μl of silica was added to clean and concentrate the DNA extracts (Boom et al., 1990; Höss and Pääbo, 1993). The mixture was kept at room temperature with vortex mixing for 15 min. After centrifuging for 2 min at 13,000 x *g*, the supernatant was discarded and the silica pellets were washed with 200 μl of buffer (20 mM Tris-HCl pH 8.0; 1 mM EDTA; 200 mM NaCl and 50% ethanol), then 1 ml of ether: chloroform (v: v). DNA was eluted at 60ºC for 10 min in 50 μl TE pH 8.0.

To compare the efficiency of this new DNA extraction method with the commonly used direct method, which uses part of or the entire feces, each fecal sample was subdivided into two equal portions by weight. The first portion was wrapped in filter paper soaked in GuSCN, while the second was used directly in the extraction procedure. After GuSCN incubation for 30 min, the second portion was centrifuged for 2 min and fecal debris removed. The supernatant was transferred to a new eppendorf and the protocol described above was followed for both samples. Instruments were sterilized between sample analyses and all extraction procedures were carried out in a separate pre-PCR room in order to prevent contamination.

2. DNA quantity and quality

The quantity and quality of DNA obtained was recorded by absorption spectroscopy using an Ultrospec 1100 pro, Amersham Pharmacia Biotech. The purity of nucleic acid samples was determined by measurement of absorbance at the wavelengths of 260 nm and 280 nm. An A_{260}/A_{280} ratio > 1.8 indicates low amounts of protein contamination in the samples. Due to the differences in pigeon DNA sample

sets, the DNA yield was expressed in µg/100 mm^2 area for seeds and as µg/100 mg of fresh weight in the case of fecal samples.

The PCR amplification efficiency was tested on a fragment of about 196 base pairs (bp) from the mtDNA Control Region for *C. bollii* and *C. junoniae*. The primers RA3 (5'-AAACCAGCAACTCGACGCGAGA-3') and CR2R were used for 10s at 94ºC, 15s at 58ºC, and 15s at 72ºC for a total of 35 cycles (see Marrero et al., 2008).

RESULTS AND DISCUSSION

1. Fecal and regurgitated seeds

Our results indicate that the DNA yield was significantly greater using a direct method of extraction from fecal samples compared to the method that involved transferring the sample to the filter paper (Kruskal-Wallis test; χ^2 = 16.46; df = 2; p < 0.001; Fig. 1). No significant differences were observed between the two types of samples (external surfaces of feces and regurgitated seeds) extracted by filter paper (Mann-Whitney test; Z =-0.73; df = 1; p = 0.46). However, when the degree of DNA purity was estimated by A_{260}/A_{280} ratio, a statistically significant difference was recorded between the methods: filter paper from seeds > filter paper from feces > direct extraction (Kruskal-Wallis test; χ^2 = 67.24; df = 2; p < 0.001; Fig. 1). A significantly higher number of fecal samples (80%) and regurgitated seeds (87.5%) extracted by filter paper yielded successful amplifications compared to the directly extracted DNA samples (53.3%) (Likelihood ratio test; G = 11.87; df = 2; *p* = 0.003). The inhibition of the *Taq*-polymerase in the PCR reactions was detected by the lack of primer dimer formation, while its detection was clear in the negative controls. Of the samples amplified from regurgitated seeds, 85.7% corresponded to *C. junoniae* and 14.3% to *C. bollii*, while the total fecal samples yielded 60% *C. junoniae* and 40% *C. bollii*. Given the pigeon abundances recorded in the study area (3.01 birds/10 min for *C. junoniae* and 14.83 birds/10 min for *C.

bollii*), the pseudo-replication of samples should be very low (Martín et al., 2000).

2. Methodological advantages

In summary, this methodology has four main advantages: (i) the efficiency of amplification and the purity of extracted target DNA using filter paper were significantly higher than when the whole feces were used. Consequently, the PCR inhibition problems were reduced by using only the external surfaces. Although the direct extraction protocol yielded greater amounts of total DNA, the strong inhibition caused a decrease in amplification efficiency. (ii) Once the feces surface has been tested in the molecular analysis, the entire intact fecal samples can be subsequently utilized for other scientific purposes, such as diet analysis. Thus, no food items are removed during the molecular procedure, avoiding the loss of valuable information. This approach is especially useful when small, rare or elusive bird species are studied. (iii) Amplifiable pigeon DNA from seeds was of high quality, enabling an unambiguous identification of the pigeon species that ejected the seed, based on comparison of mtDNA sequences. Although the DNA yield was low, the birds were successfully identified at species level. As far as we know, this is the first time that a molecular protocol has permitted the identification of bird species from recovered regurgitated seeds. DNA isolation from non-invasively collected samples of frugivorous birds provides important data to understand the roles that these species play in ecosystem dynamics. Identification of the roles of these birds, as seed-dispersers or predators, is crucial in the analysis of ecological networks. This is of particular importance in forested habitats, where frugivores are strongly associated with numerous fleshy-fruited plant species (Jordano, 2000). (iv) This method can also be extended to other vertebrates, such as mammals and reptiles. This may permit future studies, particularly in seed dispersal, which is a diverse ecological phenomenon where birds play the most important role in many systems.
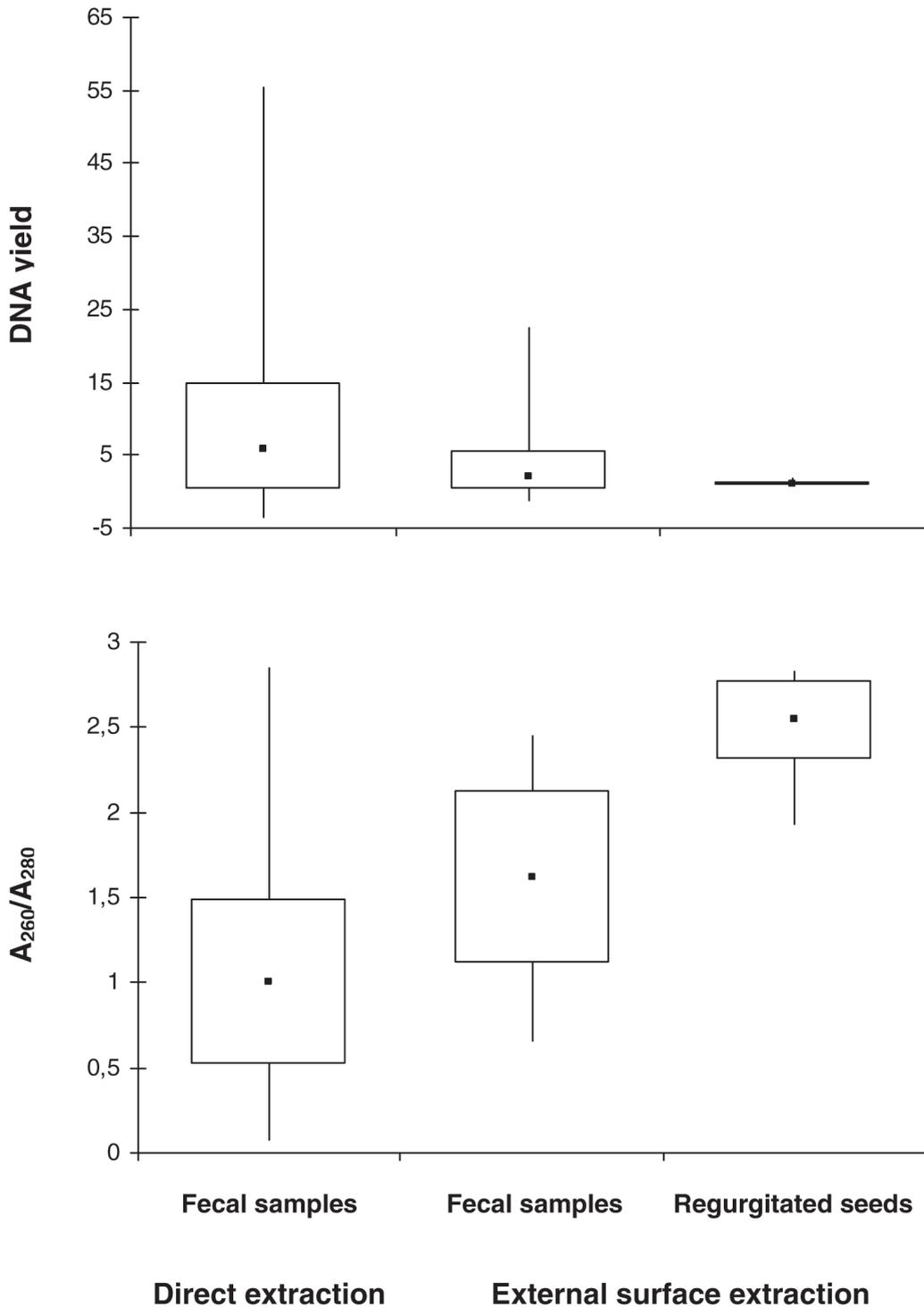
Figure 1: DNA concentration (μg/100 mg and μg/100 mm^2) for fecal samples and regurgitated seeds, respectively and A_{260}/A_{280} ratio for PCR products of two endemic Columbidae species (*Columba bollii* and *C. junoniae*), using two different DNA sources in the extraction procedure ("direct" whole fecal samples and external surfaces of feces and regurgitated seeds). The boxes represent the average ± standard deviation values; maximum and minimum values are also included.

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