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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Bryophyte DNA sequences from faeces of an arctic herbivore, barnacle goose (*Branta leucopsis*)

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Abstract

We tested DNA extraction methods and PCR conditions for the amplification of bryophyte DNA from barnacle goose (*Branta leucopsis*) faeces collected from Spitsbergen (Svalbard). Both the Qiagen stool kit and a silica-based extraction method received sufficient DNA from fresh and older droppings, as indicated by successful amplification of the plastid *psbA-trnH* spacer. Standard Taq polymerase outperformed two hot start polymerases. Sequencing of cloned PCR products revealed at least ten moss and two angiosperm sequences. This first example of identifying bryophyte DNA from faeces will allow analysing moss diets of arctic herbivores with a DNA barcoding approach.

Keywords: Arctic herbivores, barnacle goose, bryophytes, DNA barcoding, faeces

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Because of their low nutrition value, bryophytes are rarely used as the main food source by herbivores (Prins 1981; see also Speed *et al.* 2009). In the Arctic, however, mosses are probably foraged because of their high biomass and availability within the tundra vegetation rather than because of their nutritional quality (Speed *et al.* 2009). Arctic herbivores foraging on mosses include both mammals (e.g., ruminants, van der Wal 2006; Ihl & Barboza 2007; rodents, Klein & Bay 1994) and migratory birds such as barnacle geese (*Branta leucopsis* Bechstein 1803). In the Kongsfjorden area on Spitsbergen (Svalbard), for example, bryophytes make up more than 90% of the diet composition of barnacle geese during pre-incubation time in May and still >40% during incubation in June and July (Prop & Vulink 1992), with nonbreeding individuals also relying strongly on mosses as food during moult (Stahl & Loonen 1998). So far, undigested plant fragments have formed the basis for identifying bryophytes in herbivore faeces such as barnacle geese (e.g., Owen 1975). However, microscopic analysis of droppings alone is not sufficient for determination of moss species (Fox *et al.* 2007; Kuijper *et al.* 2009). We, therefore, intend to develop a DNA barcoding approach for molecular identification of bryophyte species in arctic herbivore faeces. We test the methods of DNA extraction and PCR amplification with different polymerases to develop a protocol for bryophyte DNA analysis from faeces. Barnacle geese are used as a model system, as the chance of recovering DNA fragments suitable for sequencing is high in this bird species with a simple digestion tract, and fast and incomplete digestion (cf. Prop & Vulink 1992; van der Wal & Loonen 1998).

Barnacle goose droppings were collected in July 2009 on the tundra around Ny-Ålesund (Kongsfjorden, Svalbard). Both fresh droppings (*c.* 1–2 days old) and older droppings (*c.* 2–4 weeks old) were collected, air-dried and stored in paper bags until the molecular analysis. For DNA extraction, the outer layer of each dropping was removed with a razor blade to minimize contamination by environmental components from the tundra. Extractions were carried out under sterile conditions in the joint ancient-DNA facility of the Netherlands Centre for Biodiversity Naturalis and the Institute of Biology of Leiden University. Two extraction methods were used, the QIAamp DNA Stool Mini kit (Qiagen) and a silica-based ancient-DNA extraction method. The former has also been used for analysing vascular plant DNA from faeces of wild primates (Bradley *et al.* 2007), while the latter was originally developed by Rohland & Hofreiter (2007) for bone and teeth and slightly modified by one of us (KV) as outlined as follows.

Six samples were extracted with the Qiagen kit. Based on the amounts of faecal material recommended in the

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manufacturer's protocol, *c.* 180 mg of one fresh dropping (sample V1) and one old dropping (O1) were extracted without further grinding. To test the effect of mechanical grinding (better disruption of cells vs. possible DNA damage), two further samples (V2 and O2) were ground for 1 min at maximum speed in a mixer mill MM200 (Retsch) prior to applying the extraction buffer. The amount of material was limited to 98 mg (V2) and 87 mg (O2), respectively, to leave enough space for one 5-mm glass bead per tube. A fifth sample (V3) consisted of 94 mg of a second fresh dropping (without grinding), and finally, a negative control (blank) with only extraction buffer was included as well. We followed the manufacturer's protocol (stool isolation for pathogen detection) apart from the following exception: After applying 1.4 mL lysis buffer ASL, vortexing and heating the suspension for 5 min at 70 °C, the faecal material had absorbed most of the buffer. To receive a sufficient amount of supernatant for the further procedure, another 700 μL buffer ASL was applied after centrifugation, and the tube was vortexed and centrifuged again. Dilutions of 1:5, 1:10, 1:100 and 1:1000 in distilled H_2O were made from the extracted DNA for subsequent PCR, which was carried out immediately as freezing may deteriorate the DNA.

The second extraction method was performed with the same three droppings, but with respect to the remaining amount of faeces material only three samples (plus a blank) were prepared, namely V4 (107 mg, corresponding to V2), O3 (108 mg, corresponding to O2), and V5 (80 mg, corresponding to V3). In contrast to the original protocol in Rohland & Hofreiter (2007), the material was ground in the mixer mill MM200 in 2-mL tubes as described previously. Furthermore, the amounts of buffers added, and the duration of the centrifugation steps was adjusted to the smaller amount of material used. The precise changes are as follows, with numbers referring to the steps in Rohland & Hofreiter (2007): 6. add 2 mL extraction buffer, 9. centrifuge for 1 min, 10. transfer supernatant to 8 mL binding buffer with 40 μL silica suspension, 12. centrifuge for 1 min, 15. centrifuge for 5 s, 18. centrifuge for 10 s, 22. dry at RT for 10 min with open lids, 23. add 70 μL 1× TE buffer and elute at RT for 8 min with closed lids, 24. centrifuge for 1 min. Dilutions of 1:5, 1:10 and 1:100 were made for subsequent PCR, which was carried out immediately as freezing may deteriorate the DNA.

To test the success of DNA extraction and to compare both extraction methods, the plastid DNA *psbA-trnH* spacer was amplified. This marker complies with the DNA barcode requirements of (i) short length in bryophytes (e.g., approximately 100–200 bp in mosses: Stech & Frey 2008) and (ii) universal PCR amplification. The *psbA-trnH* spacer has been proposed as universal barcode marker for land plants (Kress *et al.* 2005), with potential for species discrimination especially in angiosperms (e.g., Lahaye *et al.* 2008) but potentially also in bryophytes (Liu *et al.* 2010). At present, no optimal stand-alone barcoding marker for bryophytes is available, and species delimitation in some bryophyte groups will probably only be possible by combining two or three DNA regions (cf. Stech & Quandt 2010). For a preliminary assessment of the moss diversity in a difficult template such as degraded DNA from faeces, however, we consider the *psbA-trnH* spacer a suitable marker. Amplification was carried out as 25 μL reactions in Biometra thermocyclers (TrioBlock or Tgradient, respectively), using primers psbAF (5′-GTT ATG CAT GAA CGT AAT GCT C-3′, Sang *et al.* 1997) and trnHR-2 (5′-CGC GCA TGG TGG ATT CAC AAT CC -3′; slightly modified after Sang *et al.* 1997). PCR protocols varied according to the three polymerases that were tested, namely AmpliTaq Gold® DNA Polymerase LD (Applied Biosystems), Phire® Hot Start DNA Polymerase (Finnzymes) and standard Taq DNA Polymerase (Qiagen). AmpliTaq Gold DNA Polymerase is recommended for PCR applications that require low background levels of bacterial DNA; its use minimizes false positive DNA products arising from DNA contamination. Phire Hot Start DNA Polymerase combines high yields with very fast amplification times and zero-time initial reactivation.

PCRs with AmpliTaq Gold contained 2.5 μL 10× Amplitaq Gold PCR buffer, 1.5 μL 25 mM $MgCl_2$, 1.5 μL 10 mM dNTP's, 0.25 μL 10 mM bovine serum albumin (BSA), 1 μL 10 mM of each primer, 1 μL polymerase and 1 μL DNA template. The PCR program consisted of 95 °C for 10 min, 35 cycles (95 °C for 30 s, 48 °C for 1 min and 72 °C for 1 min 40 s) and 72 °C for 7 min (cf. Stech & Frey 2008). The Phire Hot Start PCRs contained 4 μL 5× Phire reaction buffer including $MgCl_2$, 4 μL 2.5 mM dNTP's, 1 μL 10 mM of each primer, 1 μL polymerase and 1 μL template. The PCR conditions were set according to the manufacturer's protocol, but three annealing temperatures were tested as the recommended annealing temperature is generally much higher (≥60 °C) than for PCRs with other polymerases: 98 °C for 30 s, 30 cycles (98 °C for 5 s, 60/65/70 °C for 5 s and 72 °C for 20 s) and 72 °C for 1 min. The Qiagen Taq polymerase reactions contained 2.5 μL Taq Polymerase PCR buffer including $MgCl_2$, 1 μL 10 mM dNTP's, 1 μL 10 mM of each primer, 1 μL polymerase (5 U/μL) and 1 μL template. The PCR program was the same as for the AmpliTaq Gold reactions, except an initial denaturation at 95 °C for 5 min.

PCR products were separated on 1% agarose gels prepared with 1× SB buffer. PCRs with AmpliTaq Gold gave relatively weak bands with a size of approximately 300 bp for the extraction with the Stool kit dilutions 1:10

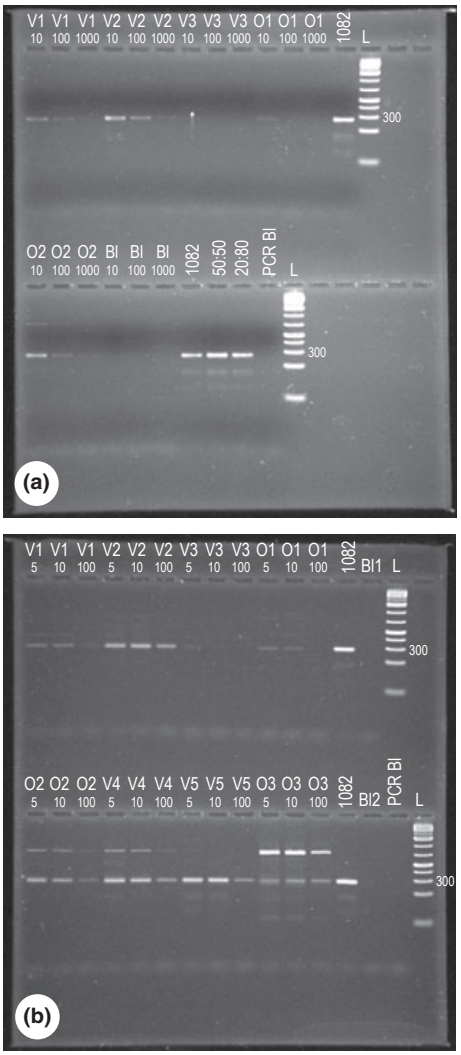

Fig. 1 Gel photographs of PCR products (plastid *psbA-trnH* spacer) obtained with (a) AmpliTaq Gold® DNA Polymerase LD and (b) standard Taq DNA Polymerase (Qiagen) from different dilutions of DNA extracted from barnacle goose faeces. Blanks refer to extraction blanks (Bl) or PCR blanks (PCR Bl), respectively. Ladder: GeneRuler™ 1 kb Plus (Fermentas); the 300-bp band is indicated. V2 = fresh dropping, O2 = older dropping. See text for further explanation.

and 1:100 (Fig. 1a). Size comparison with the band of the positive control (DNA No. 1082; *Sanionia uncinata* Hedw. (Loeske) from Spitsbergen, voucher no. *Stech & Kruijer 09-228*, deposited in herbarium L) indicated that these bands represent the desired target product. Mixtures of the positive control with the faeces extract V1 (control:extract 50:50 and 20:80) were also amplified (Fig. 1a), indicating that the PCR is not severely affected by inhibitors in the faeces. However, PCRs with AmpliTaq Gold were not consistently reliable. For example, no PCR products could be obtained from the samples of the silica-based extraction, and in the respective PCRs even the positive control could not be amplified (gel photo not shown). The Phire Hot Start DNA Polymerase did not amplify samples of the first extraction at the different annealing temperatures tested. As it soon became clear that the standard Taq polymerase performed best, we did not use Phire Hot Start DNA Polymerase for the samples of the second extraction. The Qiagen Taq polymerase yielded the products of similar length of all samples with dilutions 1:5, 1:10, and 1:100, except V3 (Fig. 1b). In addition, a longer product (*c.* 800 bp) became more clearly visible, which in O3 was even stronger than the target band.

To confirm that the amplification products of the target band included bryophyte DNA, two of them, V2 and O2 (AmpliTaq Gold, dilution 1:10), were cloned into the pCR®II vector using the TOPO® TA Cloning® kit (Invitrogen) according to the manufacturer's protocol. A total of 20 positive clones (10 from V2 and 10 from O2) were sequenced at Macrogen Inc., Europe, Amsterdam (http://www.macrogen.com). To identify the longer product (*c.* 800 bp), the respective band (O2, all three dilutions pooled) was excised from the agarose gel, extracted using the Promega Wizard® SV Gel and PCR Clean-Up kit and cloned as described earlier. Five positive clones were sequenced at Macrogen. All sequences were edited manually, and their identity inferred by a BLAST search of respective GenBank entries (nucleotide blast with algorithms 'discontinuous megablast' or 'blastn') and by comparison with our own unpublished sequence data from arctic mosses.

Twelve different sequence types could be distinguished in the clones from the target product (seq1–seq12, Table 1). These were each represented 1–5 times among the 20 clones, with no sequence type shared between V2 and O2. All sequences could be attributed to mosses, except for seq1 that belongs to the arctic angiosperm *Saxifraga cernua* L. (Magnoliophyta). Most of the moss sequences could be assigned to species or genera occurring on the tundra around Ny-Ålesund where the goose droppings were collected (Table 1). For three sequence types belonging to pleurocarpous mosses of the Hypnales, the BLAST search indicated only species not present on Svalbard. These results may be biased, however, by the availability of sequences for comparison and by the short length and low sequence divergence of the *psbA-trnH* spacer in Hypnales (e.g., Stech & Frey 2008). The clones of the longer PCR product represented another angiosperm *psbA-trnH* spacer sequence, from the grass *Poa* sp., which also occurs on Svalbard. The length differences between the *psbA-trnH* sequences of *Saxifraga* and *Poa* inferred here may correspond to the fact that this region is one of the most variable intergenic spacers of the chloroplast genome in seed plants (Borsch & Quandt 2009).

Table 1 Frequency, Genbank accession numbers and taxonomic identification of 12 DNA sequence types obtained from 20 cloned *psbA-trnH* spacer sequences from two samples of barnacle goose faeces (V2 = fresh dropping, O2 = older dropping) from Svalbard

Sequence type	No. of times found in V2/O2	Genbank acc. no.	Identification (B = Bryophyta, M = Magnoliophyta)	Occurrence of identified taxon on Svalbard
Seq1	0/2	HQ331466	*Saxifraga cernua* L. (M)	Yes
Seq2	0/1	HQ331467	*Mnium* sp. or *Plagiomnium* sp. (B)	Yes
Seq3	0/1	HQ331468	*Paludella squarrosa* (Hedw.) Brid. (B)	Yes
Seq4	0/1	HQ331469	*Aulacomnium turgidum* (Wahlenb.) Schwägr. (B)	Yes
Seq5	0/1	HQ331470	*Philonotis* sp. (B)	Yes
Seq6	0/1	HQ331471	Hypnales (B)	Potentially yes
Seq7	0/5	HQ331472	Hypnales (B)	Potentially yes
Seq8	3/0	HQ331473	*Sanionia uncinata* (Hedw.) Loeske (B)	Yes
Seq9	1/0	HQ331474	Hypnales (B)	Potentially yes
Seq10	1/0	HQ331475	*Drepanocladus* sp. (B)	Yes
Seq11	2/0	HQ331476	*Bryum* sp. (B)	Yes
Seq12	1/0	HQ331477	*Plagiomnium* sp. (B)	Yes

The procedure described here for the arctic herbivorous barnacle goose is the first example of successful extraction and sequencing of bryophyte DNA from faeces. As the two considerably different extraction methods tested gave almost equally good results, extraction of bryophyte DNA from faeces seems to be rather method independent and straightforward, at least under ancient-DNA conditions. The relatively easy PCR amplification with standard polymerase and a standard protocol, and comparable relative success in amplification for both the fresh and old droppings, indicates that the DNA stays relatively intact after digestion of the moss plants. The success of amplification of longer markers, however, needs yet to be tested, as the *psbA-trnH* spacer alone will not be sufficient as a stand-alone barcoding marker for all arctic bryophyte species. Angiosperm sequences seem to be more prominent in the older droppings (cf. Table 1 and the stronger long band in O3, Fig. 1b), which contrasts with the field and microscopic observations of increasing angiosperm and decreasing moss components of the barnacle goose diet during the summer season (Prop & Vulink 1992). At the present stage, it can neither be ruled out that angiosperm DNA degrades faster than bryophyte DNA in the faeces, nor that it amplifies less reliably under the employed protocols.

The present results form the basis for detailed analyses of the moss diet and feeding behaviour of herbivores in the Arctic. Subsequent studies with barnacle geese are now straightforward, whereas DNA extraction and PCR amplification for mammal species with different digestive physiology needs to be tested and eventually adapted. Given the amplification success in this study, however, we expect that bryophyte DNA can also be detected in herbivorous mammals, as has been shown earlier for angiosperm DNA (e.g. Bradley *et al.* 2007). DNA barcoding will allow species determination of mosses which is limited microscopically and overcome the problem that the moss diversity is 'lumped' in ecological analyses, whereas vascular plant species can be treated separately (e.g., Fox *et al.* 2007; Kuijper *et al.* 2009).

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