Comparison of extraction methods applicable to fungal spores in faecal samples from small mammals

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Mycophagy is often underestimated as dietary strategy, but forms an important part of the webs of life, especially in forest ecosystems. The identification of consumed fungal species is crucial to gain more knowledge about food web structures. DNA based methods are the way of choice to overcome limitations of species determination by microscopic analysis. DNA extraction from fungal spores in faecal samples requires specific methodology, due to the resistance of fungal spores and due to the properties of the faecal matrix.

We tested two open source extraction buffers, as well as one widely used commercial extraction kit in combination with a mechanical disruption procedure optimised for fungal spores in faecal samples. The efficiency of the DNA extraction was assessed by comparing DNA yield after amplification by PCR with fungus-specific primers. All three protocols were successful in extracting amplifiable fungal DNA. The CTAB protocol yielded the highest amounts of DNA after PCR and gave the most constant results, but the differences among protocols were not significant. The establishment of reliable protocols for DNA extraction from small quantities of fungal spores in faecal samples paves the way for high resolution non-invasive studies in the dietary ecology of wild-living mammals.

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Today we know that many forest dwelling animals, in particular small mammals, deer, wild boar, and many invertebrates use a food source, which has often been neglected due to its ephemerality and cryptic nature. This source are fungi (Cázares & Trappe 1994, Johnson 1996, Reddel *et al*. 1997) of different phylogenetic and ecological groups, many of them forming a key part of forest ecosystems, the "wood wide web" (Fogel & Trappe 1978, Carey 1999). The relationship of animals and fungi has been analysed more intensely in recent years (Shchipanov *et al*. 2003, Hanya 2004, Hanson *et al*. 2006), but detailed assessments of fungal species consumed by animals remain rare for

temperate European forests. Blaschke & Bäumler (1989) and Bertolino *et al.* (2004) investigated small mammal mycophagy in Central Europe, but they focused on single species only and did not complement microscopic analysis of faecal samples with DNA based methods. Thus, the degree of mycophagy of most European small mammals as well as the majority of fungal species consumed remains unknown.

The diversity of fungi consumed by different animal species can be investigated in two ways: (1) taking faecal samples and preparing microscopic slides, then counting and identifying fungal spores with a light microscope (Cázares & Trappe 1994, Colgan III *et al.* 1997, Vernes & Dunn 2009), or (2) taking faecal samples, extracting total DNA, amplifying selected gene fragments (`barcodes´) of fungal DNA with specific primers and applying strategies to obtain DNA fragments which can be sequenced (Kuske *et al.* 1998, Cubero *et al.* 1999, Manian *et al.* 2001).

Both approaches have their advantages and drawbacks. Obtaining faecal samples is non-invasive and usually the easiest part as small mammals are easily live-trapped and faeces from larger mammals can be directly obtained from their habitats. Preparation of microscopic slides is not difficult, neither, but identification of fungal spores in the faecal matrix as well as classification at the supra-generic, generic or even species level and the quantification of spores from mixed species samples requires expertise and thorough working (Colgan III *et al.* 1997). Spores can be detached from organic debris present in the faecal pellets by thorough homogenisation of the samples and adequate dilution. Keys and monographs of fungal genera (Castellano *et al.* 1989) assist in the microscopical determination of fungal taxa. The direct observation method requires time and careful observation (Castellano *et al.* 1989), as the reproducible quantification requires a sufficient number of observations of random fields of view in the microscope, but is the ideal way to record the diversity of fungal species and the proportions of spore types present in the sample. However, given the diversity of potentially ingested fungal species and the wide range of spore sizes, errors due to overlooking small or inconspicuous spores and misidentifications can not be excluded.

DNA based methods promise to provide a more objective procedure for the assessment of the diversity of ingested fungi and, potentially, accurate identification at the species level. Despite the widespread routine use of DNA extraction techniques, the optimal recovery and purification of fungal DNA from faecal samples remains challenging, because: (1) The available fungal DNA is packed up in the spores, well protected to survive gut passage (Trappe & Maser 1976, Colgan III & Claridge 2002) . Therefore, extraction of DNA requires the disruption of spore cell walls. In order to provide a representative analysis, spore wall disruption needs to be effective for all target species. (2) Low quantities of target DNA require minimization of losses during

extraction. (3) Faecal samples are potentially rich in inhibiting substances or DNA degrading components (DNAses, food components, etc.) of various origins, so these need to be efficiently inactivated to avoid loss of target DNA (Rossen *et al*. 1992, Eggert *et al*. 2005).

Thus, a reliable, inexpensive, and easy to use method for extraction of fungal DNA from faecal samples could boost knowledge on mycophagy of animals and shed light onto the feeding ecology of animal species, species connections, fungal distribution, and possibly also related conservation issues.

Up to date, researchers can choose from a variety of commercially available DNA extraction kits for fungal DNA as well as from a galore of other extraction protocols (e.g. Kuske *et al*. 1998, Cubero *et al*. 1999, Schwarzott & Schüßler 2001), but comparisons between any of them remain rare. Due to the variety of approaches it can be difficult to find the most suitable method available for a particular research question.

We therefore attempt to provide another piece of the puzzle by comparing a popular commercially available DNA extraction kit with two open source extraction protocols. The results can be of significance to any researcher, who wants to assess the feeding ecology and mycophagy of more than one animal species or a species community at the same time, since our favoured method is applicable to and successfully used for different small mammal species with various feeding habits.

Materials and methods

Sampling procedure and storing

We live trapped small mammals according to international standards (Kirkland Jr. 1998, Powell & Proulx 2003, Gannon & Sikes 2007) and obtained faecal samples from each newly captured animal by taking faecal pellets from the traps. We collected the faecal material during five live trapping sessions in 2006 and 2007 in the Dürrenstein Wilderness Area (Austria). Upon collection, samples were transferred into Eppendorf reaction tubes (1.5 mL) filled half with silica gel beads for rapid drying and storage. For further analysis (microscopic and DNA-based), we separated pellets from coarse plant material as well as from silica beads, added 600 µL distilled water and homogenized them mechanically with a conical pistil after short soaking in the Eppendorf reaction tubes. We kept aliquots of the resuspended samples frozen at –20 °C for DNA analysis.

Samples

We selected 16 faecal samples from six species of small ground dwelling mammals (*Myodes glareolus* Schreber 1780, *Apodemus flavicollis* Melchior 1834, *Microtus agrestis* Linnaeus 1761, *Glis glis* Linnaeus 1766, *Sorex araneus* Linnaeus 1758 and *Sorex minutus* Linnaeus

1766) for the comparison, based on amount of fungal spores (spore numbers ranging from 0.5 to 72.7 spores per field of view), but randomly chosen regarding collection time and sample size (total dry weights between 10 mg and 70 mg) (Table 1). We thoroughly resuspended samples by vortexing and divided each sample into three aliquots of 200 µL each (pipetting 100 µL twice using a cut 200 µl pipette tip), to ensure the best possible equality of the aliquots.

Tab. 1. – Faecal samples used for comparison of extraction methods. Spores per 50fov = Number of fungal spores counted in 50 random fields of view (= fov) with 400× magnification, Fluorescence signal = intensity of band with correct size range as calculated with ImageJ, GR – 1 kb GeneRuler; mos = months.

No.	Spores per 50fov	Small mammal species	Collection and storage time	Fluorescence signal		
				AnDNA	CTAB	Kit
1	280	Myodes glareolus	Oct 2007, 30 mos	1279.31	12431.41	18001.21
2	311	*Microtus agrestis*	Aug 2007, 32 mos	546.09	14500.38	8805.92
3	3633	*M. glareolus*	May 2007, 32 mos	10138.12	11548.82	11684.36
4	1552	*M. glareolus*	Aug 2007, 32 mos	15516.23	11333.48	11126.24
5	29	*Sorex minutus*	Oct 2007, 30 mos	2949.88	12381.31	11219.12
6	1127	*M. glareolus*	Aug 2007, 32 mos	9964.60	11309.14	2288.95
7	631	*M. glareolus*	Aug 2007, 32 mos	19940.20	17758.33	10967.65
8	596	*Glis glis*	Aug 2007, 32 mos	8484.17	868.40	461.16
9	1140	M. glareolus	Oct 2007, 30 mos	572.09	6328.49	3534.23
10	389	*M. glareolus*	May 2007, 32 mos	12999.39	15329.12	13140.46
11	381	*M. glareolus*	Oct 2006, 42 mos	3874.40	5940.25	5439.18
12	380	*Apodemus flavicollis*	Oct 2006, 42 mos	4894.59	3688.23	6053.76
13	320	*A. flavicollis*	Oct 2006, 42 mos	4039.25	3770.23	5530.13
14	217	*Sorex araneus*	Aug 2007, 32 mos	4704.47	8051.61	3721.00
15	39	*M. glareolus*	Jul 2006, 45 mos	144.95	112.95	72.95
16	27	*A. flavicollis*	Oct 2006, 42 mos	6908.63	13743.26	7525.10
GR	mean	500 bp band			1316.47	

Extraction kit and buffers

The tested commercial kit was the QIAamp DNA Stool Mini Kit (Catalogue no. 51504, No. of preps: 50). We followed the manufacturers protocol for extraction, but added three scoops of fine quartz sand (SIGMA, Catalogue no: S-9887) and five glass beads (diameter 2–3 mm) during the initial lysis steps.

We compared the QIAamp kit with one commonly used extraction buffer for fungal DNA – CTAB (Cubero *et al.* 1999, Izzo *et al.* 2005, Zhang *et al.* 2006) and one very simple buffer established for extracting ancient DNA from paleontological samples – AnDNA (Rohland & Hofreiter 2007).

The CTAB extraction buffer consisted of 0.01 M Tris, pH 8.0, 3 M NaCl, 0.02 M EDTA pH 8.0, and 0.5 M CTAB (Cetrimonium bromide), with 2 % (wt/vol) PVP (polyvinylpyrrolidone), and 0.5 % (vol/vol) ß-

mercaptoethanol added before use. The AnDNA buffer is composed of 400 mM EDTA pH 8.0 and 0.5 mg/mL proteinase K in distilled water.

Extraction protocol (CTAB, AnDNA)

We resuspended each sample aliquot in 600 µL extraction buffer in a 2-mL tube and added 3 scoops of quartz sand as well as five glass beads (diameter 2–3 mm). After sealing with parafilm, we placed the tubes in the grinding mill (Retsch, MM301, Düsseldorf, Germany) and shook them for 20 min at 30 Hz (maximum frequency). Finally, we checked all samples microscopically for the degree of disruption of the spore walls.

We then incubated the samples for one hour in an Eppendorf Thermomixer compact (Eppendorf, Hamburg, Germany) at 55 °C and 800 rpm, transferred the supernatant into a new 1.5-mL tube after centrifugation for 1 min, and added an equal volume chloroform-isoamylalcohol (24:1). After vortexing, we centrifuged samples for 10 min. We transferred the upper phase into a new 1.5-mL tube and added 1/10 volume of 3 M sodium acetate and 2/3 volume of isopropanol to precipitate DNA.

We pelleted DNA by centrifugation for 30 min after incubation for 5 min at room temperature, discarded the supernatant, and washed the pellet twice with 100 µL 70 % EtOH and centrifuged for 10 min. Subsequently we let the pellet air-dry at 40 °C and dissolved it in 50 µL 10 mM Tris-HCl (pH 7.5) at 55 °C and 1000 rpm in an Eppendorf Thermomixer compact (Eppendorf, Hamburg, Germany). We carried out all centrifugation steps at top speed (13.000 rpm ~ 16.100 g) using an Eppendorf Centrifuge 5415D (Eppendorf, Hamburg, Germany).

DNA purification

We purified the DNA solution of the 32 samples extracted with the open source buffers using the Invisorb® Spin PCRapid Kit (Invitek, Berlin, Germany) according to the manufacturer's protocol with slight modifications as recommended by the manufacturer – we increased centrifugation time at step two and four to 1.5 min and 10 min, respectively. We increased the volume of the elution buffer in step five to 40 µL and extended the incubation time at room temperature with elution buffer to 10 min. DNA extracts were stored at –20 °C.

Detection of DNA and PCR conditions.

We checked DNA contents of genomic DNA extracts and PCR products by loading 5 µL onto an 1 % agarose gel (0.5 x TAE buffer) stained with GelRed™ (Biotium, Hayward, CA, USA). We ran the gel electrophoresis for 25 min at 90 V, using the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) for approximate size determination.

We used the fungi specific primer pair ITS1F/ITS4 (ITS1F: CTTGGTCATTTAGAGGAAGTAA; ITS4: TCCTCCGCTTATTGATATGC, typical amplicon size: 650 bp) for amplification of fungal DNA by PCR. ITS1F binds close to the 3'-end of the nuclear 18S rDNA (nrSSU; Gardes & Bruns 1993), whereas ITS4 (White *et al.* 1990) anneals to a conserved region close to the 5'-terminal part of the nuclear 28S ribosomal DNA (nrLSU). Using the extracted samples as template DNA, we amplified a fragment of fungal DNA fragments ranging approximately between 550 bp and 700 bp in size. We prepared the following amplification mixture: 1X Taq Buffer + NH_3SO_4 (Fermentas), 2.5 mM $MgCl_2$ (Fermentas), 200 mM each dNTP, 0.8 µg/µL BSA, 1 % DMSO, 0.5 µM each primer, 2 µL Taq polymerase recombinant (Fermentas, Catalogue No: EP0404, 1 u/mL), and 1 µL of each extracted sample solution (undiluted), and distilled water to a total volume of 25 µL. PCR cycling parameters were initial denaturation 120 s at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 54 °C, 90 s at 72 °C, and final elongation for 10 min at 72 °C. To minimize the influence of external factors potentially affecting PCR efficiency, we amplified the DNA in a single PCR run, using one master mix and one PCR plate for all samples.

Evaluation criterion and statistics

We used PCR efficiency as criterion instead of genomic DNA yields, because (1) genomic DNA yields were generally very low, precluding a precise quantification after extraction, (2) stool sample genomic DNA is of various origins, the proportion of fungal DNA is a priori unknown, and (3) PCR efficiency is most important for all downstream applications.

We quantified PCR amplified DNA yields based on agarose gel band intensity using the open source program ImageJ (Rasband 1997). We applied square root + inverse transformation to the fluorescence signal data and inverse transformation to the No of spores to obtain a normally distributed data set. We examined potential differences in extraction efficiency between the three protocols applying (1) one-way ANOVA with repeated measurements (rmANOVA, model: transformed fluorescence signal ~ method + Error (sample/method)) with methods being AnDNA, CTAB and Kit and (2) two-way ANOVA (model: transformed fluorescence signal ~ method * transformed No of spores) to account for potential correlations between spore numbers and PCR efficiency. We used the statistical environment R (R Development Core Team 2011) for all calculations.

Results

We achieved an extraction/PCR efficiency of nearly 100 %, amplifying fungal DNA from 15 out of 16 samples in sufficient quantities. The extraction of fungal DNA from faecal samples of small mammals

was successful even after more than three years of storage at –20°C (Tab. 1). Sample no 15 did not show bright bands on the agarose gel (Fig. 1a), but the quantification program ImageJ did detect small amounts of DNA of the correct size (Tab. 1). However, as very small amounts of amplified DNA are generally not sufficient for downstream applications such as dye terminator sequencing, we classified this sample as failed and excluded it from statistical calculations.

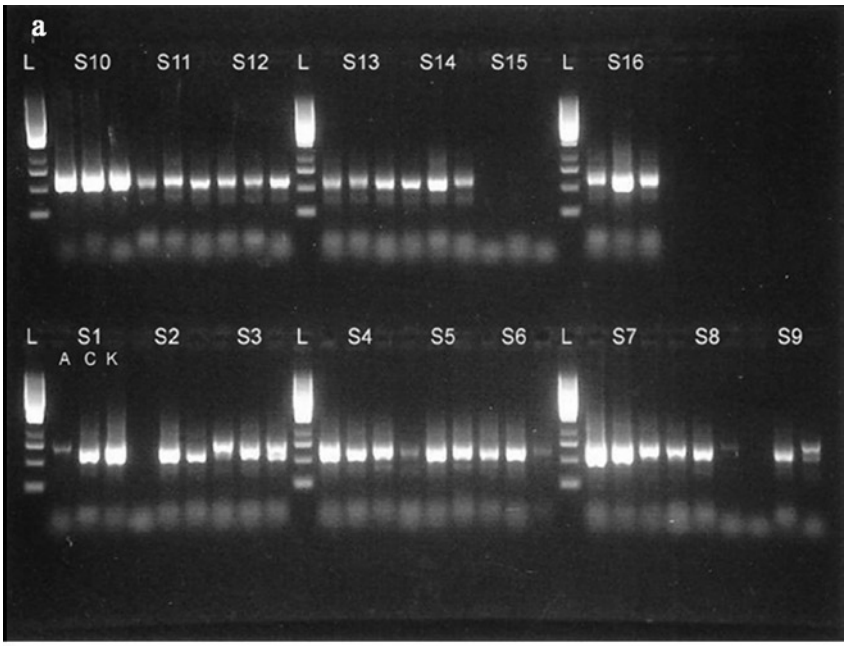
Calculation of mean and median fluorescence signal value (AnDNA = 6684.8 / 4799.5, CTAB = 9318.5 / 11321.3, Stool DNA Kit = 7473.2 / 6789.4) and visual method comparison showed CTAB to yield the highest amounts of DNA after PCR, but neither one way rmANOVA (P = 0.548) nor two-way ANOVA (P(method) = 0.374, P(NoOfSpores) = 0.139, P(method:NoOfSpores) = 0.743) did reveal significant differences.

We determined the number of fungal spores prior to extraction, but there is no obvious relationship between spore numbers in 50 random fields of view (400x magnification) and fluorescence signal after PCR for any extraction method (Fig. 1b). Nevertheless, we could visualise the lower average DNA yield for samples extracted with AnDNA buffer and the QIAamp kit compared to the CTAB buffer and the high scatter of the individual performances.

Discussion

All three tested extraction methods can be regarded as quite reliable, in most cases yielding DNA extracts suitable for the PCR amplification of nuclear ribosomal ITS sequences, a region commonly used in DNA based identification of many fungal groups, including ectomycorrhizal fungi (Köljalg *et al.* 2005). Furthermore, sequencing of the obtained PCR fragments revealed fungal species observed in the microscope prior to extraction, confirming the suitability of the extraction protocols.

Despite the high dispersion of the results, certain differences between the methods could be observed: AnDNA buffer gave the weakest and most variable results particularly when low numbers of fungal spores were observed in the sample. Fungal DNA fragments could be amplified in sufficient amounts from 75 % of the samples extracted with this protocol. The QIAamp DNA Stool Mini Kit led to satisfactory results in 80 % of the samples, and seemed to achieve more constant results in PCR efficiency than the AnDNA extraction protocol. The CTAB method, which is a common procedure for fungal DNA extraction effective for environmental samples (Izzo *et al.* 2005) shows the most stable results for small mammal faecal samples, especially for those with low numbers of spores. It failed only once in sample 15, but this sample failed with the other two methods as well. However, successful PCR amplification of fungal DNA from samples with few spores



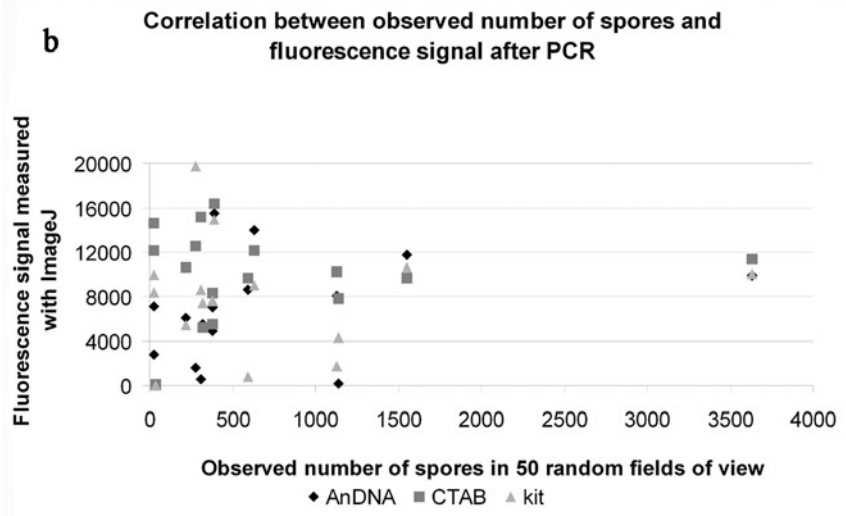


Fig. 1 – (**a**) Agarose gel stained with GelRedTM showing all 16 faecal small mammal samples after fungal DNA extraction and PCR with ITS1/ITS4 primers. Amplification using the same primers but no template (negative control) resulted in no PCR product (data not shown), L: 36 GeneRuler 1kb (Fermentas), S1-S16: sample numbers, A-AnDNA buffer, C-CTAB buffer, K37 QIAamp DNA Stool Mini Kit; same order for every sample. (**b**) Fluorescence signal of the extracted samples as calculated with ImageJ in relation to the total number of fungal spores observed in 50 random fields of view with 400× magnification.

frequently contain a high proportion of sequences from yeast genera such as *Cryptococcus* and *Rhodotorula*, and rare target fungi can be missed, even if a larger pool of clones is analysed (Urban *et al.*, unpub.). Therefore, it is more advisable to use samples rich in target spores. Possibly, the high proportion of positive PCR results from samples with few spores when using the CTAB and QIAmp protocols is due to efficient lysis of yeast cells, which might also explain the absence of a correlation between numbers of larger fungal spores (yeast cells were not counted) and DNA amplicon yields.

Selection of DNA polymerase seems to be essential, too. The taq polymerase chosen was compatible with all three extraction methods, which was not the case with certain other DNA polymerases, which were tested randomly in the earlier phases of protocol development. PCR with DNA extracts obtained with the commercial stool DNA extraction kit was least sensitive to DNA polymerase choice, suggesting that this method provides the lowest level of PCR inhibitors, and may be the best choice in case of very sensitive downstream applications.

There may be potential for further improvement of DNA extraction from fungal spores in faecal samples. The inclusion of additional washing and/or concentrating steps prior to the extraction procedure might further reduce PCR inhibitors and raise the relative proportion of target DNA. However, we found fungal spores distributed across different phases in all solutions to discard after initial fractionation and additional washing steps, so we prefer to work with the whole stool samples in order not to loose any information on certain (possibly rare) species.

As we suppose that any DNA contained in ingested fungal hyphae is likely to be too degraded for successful amplification, the disruption of the spore walls is crucial for extracting fungal DNA from spores out of faecal samples. The successful mechanical spore disruption applied in all extraction protocols contributed to the reliability of the three tested methods and is probably essential regardless of the extraction protocol chosen afterwards. The established method resulted in the identification of more than 30 species of fungi from small mammal scats (Urban *et al.*, unpubl.) and may help other scientists to shed more light onto the field of mycophagy and its role in the webs of life.

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