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Research report

Evaluating non-invasive sampling as a monitoring tool for the Ethiopian wolf in northern Ethiopia

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Abstract

With an estimated global population of less than 450 individuals, the Ethiopian wolf *Canis simensis* is the world's rarest canid. Its distribution is restricted to a few remote and isolated mountain ranges of the Ethiopian highlands where the populations are threatened by habitat destruction and fragmentation, disease outbreaks and persecution. Wolf populations of the northern highlands are under enormous pressure from high altitude agriculture and there is an urgent need to establish efficient methods for monitoring these populations. To this end we conducted a pilot-study evaluating the use of faecal DNA as a tool for individual identification of wolves and population genetic analysis. Faecal samples were collected from Menz in central Ethiopia, the main factors affecting amplification and genotyping success were determined, and additional samples were collected. We experienced a significant increase in the amplification rate between the first and second sampling event. The method improvement was mainly attributed to factors such as sampling strategy and storage method. A set of eight microsatellite markers was analysed for total of 42 samples, resulting in 23 unique multilocus genotypes. We conclude that by following the methodological suggestions proposed here, non-invasive sampling is an efficient way of obtaining population data for the small, elusive Ethiopian wolf populations of northern Ethiopia.

Introduction

The Ethiopian wolf *Canis simenis* is the rarest canid in the world, with a global population size estimated to fewer than 450 individuals and listed as IUCN Endangered (Sillero-Zubiri and Marino 2011). This specialist hunter is endemic to the Afroalpine ecosystem (>3,000m above sea level) of the Ethiopian highlands, where it preys almost exclusively on the endemic Afroalpine rodent fauna (Sillero-Zubiri and Gottelli 1995).

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The Ethiopian wolf is hypothesized to be a descendant of a grey wolf-like Eurasian ancestor, that colonised the Ethiopian highlands relatively recently and occupied the wider Afroalpine range available at the end of the Pleistocene (Gottelli et al. 1994, 2004). The process of population fragmentation started with the onset of the current interglacial period (approximately 18,000 years ago), when Afroalpine habitats were pushed upwards as a consequence of warmer climate (Gottelli et al. 2004). More recently, human impacts have contributed to further reduce the Afroalpine habitat to less than 10% of its former size (Marino 2003, Marino et al. 2011).

These factors have together led to a major decline and fragmentation of the Ethiopian wolf population over the last several thousand years (Gottelli et al. 2004). Moreover, rabies and canine distemper transmitted by ubiquitous domestic dogs pose an immediate threat to the wolves (Haydon et al. 2002; Randall et al. 2004). The Ethiopian wolves are at present restricted to a handful of populations on either side of the Great Rift Valley (Figure 1), and a previous study showed that these are genetically distinct populations segregated into a southern, central and a northern plateau (Gottelli et al. 2004). More than half the population is found in Bale Mountains National Park (BMNP) south of the Great Rift Valley (Marino 2003), where the wolves have been studied extensively for two decades (e.g. Gottelli et al. 1994, 2004; Randall, 2006; Randall et al. 2011; Sillero-Zubiri and Gottelli, 1995; Sillero-Zubiri and Macdonald 1996, 1997, 2004).

North of the Great Rift Valley five small (< 50 individuals) wolf populations remain in isolated mountain enclaves. Detailed demographic information on these populations is much harder to attain, with the wolves occurring at seemingly lower densities and proving more elusive to human observers (Ashenafi et al. 2005). Moreover, considering their conservation status and small numbers, handling and manipulation should be avoided if possible. Conventional population monitoring of the northern Ethiopian wolf populations is therefore challenging and a non-invasive sampling technique would be of great benefit for population monitoring.

Non-invasive genetic sampling of faecal samples is increasingly being applied to monitor rare carnivore species (wolverines *Gulo gulo*, Flagstad et al. 2004; Arctic fox *Alopex lagopus*, Meijer et al. 2008; Eurasian otters *Lutra lutra*, Hajkova et al. 2009; brown bear *Ursus arctos*, De Barba et al. 2010). However, the quality of faecal DNA may be affected by a number of factors such as field environmental conditions and sample age (Murphy et al. 2007; Santini et al. 2007; Brinkman et al. 2010), the diet of the target animals (Murphy et al. 2003), storage method (Piggot and Taylor, 2003) and DNA-extraction method (Wasser et al. 1997).

Low quality DNA templates may result in critical genotyping errors, primarily by the occurrence of allelic drop-out (i.e. when one of the alleles at a heterozygous profile fails to amplify, producing a false homozygote) and false alleles (spurious amplification of a non-existing allele). A pilot study is therefore recommended in order to evaluate whether a non-invasive approach is feasible for a population genetic study of a particular species or population. Here we report on a pilot study from Menz in northern Ethiopia, where we have evaluated the use of non-invasive sampling as a tool for population monitoring of the Ethiopian wolf. We wanted to determine the optimal sampling, storage and DNA extraction methods and test the performance of a set of microsatellite markers for individual identification. The resulting microsatellite data were used to estimate standard population genetics parameters for the Menz population. Finally, we included data from the BMNP population to estimate the level of genetic differentiation between the two sides of the Great Rift Valley.

Methods and Methods

Sample collection

A total of 89 faecal samples of assumed wolf origin were collected in Menz (latitude 10°15`-10° 27`N and longitude 39°45`- 39°49`E) in North Shoa, Ethiopia, in June and July 2006. Another 45 faecal samples were collected in December 2008 (Figure 2). The Guassa range of Menz is an area of 111km^2 of suitable wolf habitat with an altitudinal range of 3,200-3,700m a.s.l. Wolf droppings were collected opportunistically, largely focusing on roads, 'dig-outs' made by wolves searching for rodents, and the vicinity of wolf dens. Approximately one third of each dropping collected was transferred to a plastic container, and stored in 70% (2006) or 96% (2008) ethanol. Clean disposable gloves and sterile blades were used for each new sample handled in order to prevent contamination between different samples. All samples were uniquely labelled with date and GPS position and stored at room temperature for up to five months prior to DNA extraction.

DNA-extraction

DNA from all samples collected in 2006 was initially extracted with the QIAamp DNA Stool Mini Kit (QIAGEN, GmbH, Hilden, Germany). DNA-extracts that failed to amplify for a test-marker were re-extracted by various other protocols; PowerMaxTM Soil DNA Isolation Kit (MO BIO Laboratories, Inc.), standard Phenol: Chloroform (Sambrock et al. 1989) and the procedure described in Ball et al. (2007).

To standardize the DNA extraction process and at the same time keep costs per extraction to a minimum, we developed an automated DNA-extraction protocol using a BioMek2000 robotic workstation. DNA from samples collected in 2008 was extracted using this protocol.

A small amount of the surface of each pellet was mixed with lysis buffer. As DNA extraction from bacterial cells requires much harsher lysis conditions than what is necessary from animal intestinal cells we used a mild lysis buffer: 500mM Tris (pH 9) 10mM NaCl and 50mM EDTA in order to increase the ratio target to non-target DNA. The lysates were incubated at 55°C for at least two hours with shaking at 800 rpm. DNA was precipitated by isopropanol and then absorbed to magnetic beads (Dynabeads Myone Silane, Invitrogen) followed by two washing steps in 70% ethanol. The DNA was finally eluted in distilled water. This simple and straightforward protocol, that does not require centrifugation, is performed in plates for up to 96 samples simultaneously. It is thus suitable for studies where the same sample needs to be re-extracted in a standardized manner. Furthermore, by minimizing the steps of manual handling, the chance of error (e.g. from pipetting) is reduced.

Test of sample quality

We performed an initial screening of sample quality by amplification of the microsatellite marker Pez17 (see below), followed by visualization of the PCR products on a 2% agarose gels containing EtBr. If no product was obtained after three replicates the sample was discarded. Lack of amplification products is often due to the high content of PCR-inhibiting substances (e.g. salts and bacteria) found in faeces. For a subset of 20 samples where no amplification product or any sign of primer-dimer was observed we performed an inhibitor-control PCR. We prepared an amplification reaction of a control DNA sequence (see details below), and added 0 - 100 x dilutions of wolf DNA as well as a negative control to the reaction tubes. If inhibitors were present in the wolf extract, amplification of the control DNA would be prevented. The reaction mix was prepared in 10µl volumes containing 5.41µl H2O, 0.1µl HDV control template (Hepatitis delta virus DNA, 10-7 dilution), 1µl 10x PCR buffer, 1.75mM $MgCl_2$, 0.08µM of dNTP, 0.2µl of each primer, 4µg BSA, 0.1 units of HotStarTaq DNA polymerase (QIAGEN) and 1.5µl of undiluted wolf DNA extract. In addition, two replicas were added milliQ H_2O instead of DNA. PCR was initiated by 15 minutes at 95°C, followed by 45 cycles comprising ten seconds at 94°C and one minute at 60°C. The last step was extension for ten minutes

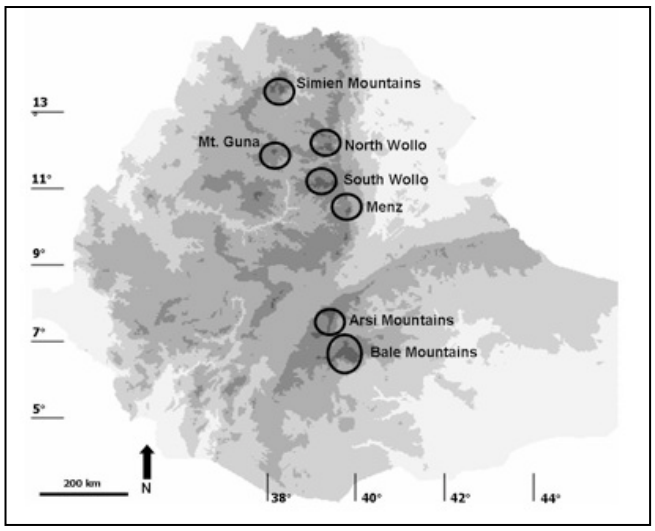

Figure 1. The current distribution of the Ethiopian wolf (Map from Marino 2003)

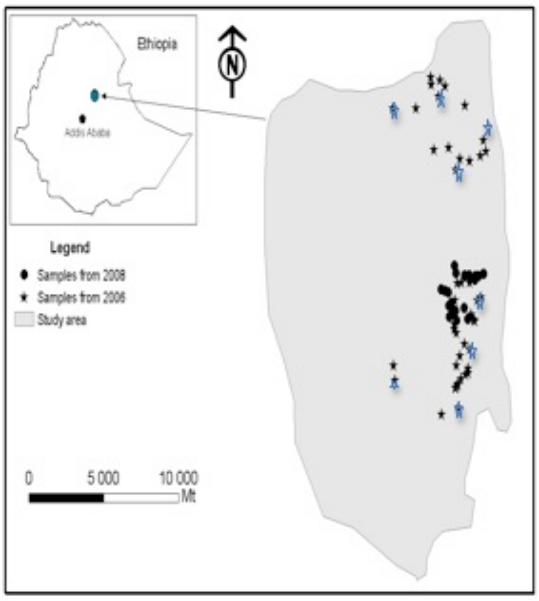

Figure 2. Menz study area with sample locations for 2006 (stars) and 2008 (circles). Blue stars indicate eight samples successfully genotyped for eight loci.

Species determination

The Cytochrome b gene has been demonstrated to be an appropriate marker for species identification in mammals (e.g. Naidu et al. 2012; Rueness et al. 2011; Tobe et al 2010). In order to determine whether the the collected faecal samples originated from Ethiopian wolves we amplified a 389 bp fragment of the Cytochrome b gene (Cytb) using the primers Cytb-1 and Cytb-2 (Janczewski et al. 1995). PCRs were performed in 50µl reactions with 5µl DNA template (x 20 dilution). The master mix contained 2mM $MgCl_2$, 1.6pmol of each primer, 5µl dNTP, 4µg of BSA, and 2.5 units of HotStarTaq polymerase. The PCR consisted of 40 cycles (15 minutes at 95°C was followed by 40 cycles of 30s at 94°C, 30s at 50°C and 1min at 72°C and a final extension step at 72°C for 10 min). DNA sequencing was performed using an ABI 3730 instrument (Applied Biosystems), according to the manufacturer's protocol. Sequences were submitted to GenBank (accession number HQ845261) and assigned to species using BLAST (Altschul et al. 1990).

Microsatellite analysis

We selected ten microsatellite loci from the domestic dog genome including seven tetranucleotide microsatellite loci: FH2001, FH2137, FH2140, FH2096, FH2159 (Fransisco et al. 1996), FH2226 (Melleresh et al. 1997), Pez17 (Neff et al. 1999) one hexanucleotide locus: vwf (Shibuya et al. 1994), and two dinucleotide loci: Cxx466, c253 (Ostrander et al. 1993). We selected markers previously shown to be polymorphic in the BMNP population (Randall, 2006). Two specific primer pairs MS34 and MS41 were used to amplify two microsatellite loci, located within a 658-bp sequence at the dog Y-chromosome. This sequence has been demonstrated not to amplify for wild canids other than dogs and wolves (Olivier et al. 1999). The PCR products were screened on an agarose-gel, and positives interpreted as males. The PCR reactions were performed in a final volume of 10µl containing 10x PCR-buffer, 1.5-3.0µM $MgCl_2$, 0.5pmol of each primer, 0.2µM dNTP, 4µg of BSA, 1.5µl of DNA extract and 0.11 units of HotStarTaq Polymerase. PCR was performed according to the following protocol: 95°C for 15 minutes, continued with a touch down procedure with the annealing temperature starting at 58°C and gradually reduced by 0.5°C for each of the initial cycles (13) followed by amplification for 36 cycles at the lowest temperature in the gradient (52°C). The rest of the 36-40 cycles comprised denaturation for 30s at 94°C, annealing for 30s at 58°C, and extensions at 72°C for 1 min, plus a final extension at 72°C for 10 min. We followed the multiple tube approach recommended for reliable genotyping from degraded DNA sources (Navidi et al. 1992; Taberlet et al. 1996). The predefined criteria for accepting a single-locus genotype were at least three independent amplifications of a homozygote genotype and two identical heterozygote genotypes (Flagstad et al. 2004). Allelic dropout was interpreted when at least one replicate showed a heterozygous profile, while the others showed homozygous profiles. To estimate the possibility of having accepted false homozygous genotypes, we used the formula P (false homozygote) = (K)*(K/2)n-1 as suggested by Gagneux et al. (1997). K is the observed frequency of false homozygotes averaged over all loci and n

is the average number of repeated amplifications. PCR products from multiple primers were pooled together after independent PCRs and electrophoresis run on an ABI3730 sequencer. Fragment sizes were subsequently scored using the GeneMapper Software (Applied Biosystems, Foster City, California).

Individual identification

Probability of Identity (PID) (Paetkau and Strobeck 1994) and PIDsibs (Waits et al. 2001), the probability that two individuals drawn at random from a given population share identical genotypes at all genotyped loci, were calculated from multilocus genotypes using Gimlet version 1.3.3. (Valiere 2002).

Genetic variability

The microsatellite data was used to estimate standard genetic diversity parameters such as the number of alleles (NA), observed (HO) and expected (HE) heterozygosities for each locus, exact test of deviations from Hardy-Weinberg expectations (HWE) using the software package Arlequin version 3.0 (Excoffier et al. 2005).

Genetic differentiation

Microsatellite data from the BMNP population has previously been analysed by Randall (2006). When microsatellite alleles are scored automatically, there may be a discrepancy in allele sizes from different sequencing machines. For a reliable comparison of data sets, five of the BMNP samples were run on the sequencer (ABI3730) and analysed in the same manner as the rest of the samples analysed in the current study. The differences in allele sizes observed in these five individuals were then used to calibrate the rest of the dataset. The calibrated dataset was used to estimate population differentiation between the northern and southern population by calculating FST as implemented in Arlequin.

Results

Performance of non-invasive sampling

The results of amplification success and genotyping are summarized in Table 1. The PCR inhibition test performed on a subset of 20 samples indicated that the lack of amplification product from PCR was due to inhibiting substances in the extract. When diluted x 10-100 amplification of the virus DNA was restored for 18 samples. However, due to initial low concentration of wolf DNA in samples, the diluted DNA was not sufficient as a template for the microsatellite PCRs.

Altogether, 23 different individuals were identified in the two sampling trials (Table 1), 12 males and 11 females. Eight loci produced PID 0.00024 and PIDsibs 0.02. When adding the two extra loci (FH2159 and FH2096) for analysis of the 2008 samples we obtained PID 0.00005 and PIDsibs 0.006. For both sampling trials we found a low probability of false homozygotes in our dataset after more then three replicates (Table 1).

Species identification

All the faecal samples resulting in complete multilocus genotype were confirmed by sequencing to originate from Ethiopian wolves (Table 1). The Ethiopian wolf sequences differed from domestic dog sequences by 4-5% (see Rueness et al. 2011).

Genetic diversity

The characteristics of the ten microsatellite loci analysed in this study are summarized in Table 2. All ten loci were polymorphic, with an average of three alleles per locus (2-5 alleles per locus). Observed and expected heterozygosities from 0.136 to 0.846 and 0.130 to 0.713 respectively. Two loci, FH2137 and FH2159, deviated significantly from Hardy-Weinberg equilibrium due to heterozygote deficiancy and excess respectively (Table 1). The test for genetic differentiation between the northern and the southern part of Ethiopia resulted in FST = 0.27 (p = 0), supporting the presumption of no current gen-flow across the Great Rift Valley.

Discussion

The marginally small northern populations of the Ethiopian wolf make them particularly at risk of falling into an extinction vortex (Caughley 1994). At least two wolf populations have gone extinct within the last century (Marino 2003), stressing the urgency of establishing efficient ways of collecting population data for conservation purposes. The northern Ethiopian highlands are less accessible than the Bale Mountains, thus posing a challenge for population studies of wolves inhabiting these areas. Demographic studies based on non-invasive genetic sampling have been shown to be a suitable method for population monitoring of elusive carnivores. There are however, methodological problems related to this procedure; hence we conducted a pilot study to evaluate its feasibility for studying the elusive northern populations of the Ethiopian wolf.

Sampling procedure

By modifying the collection protocol used in 2006, we experienced a significant increase in both amplification success (37% and 86% respectively) and genotyping success (9% and 86% respectively) in 2008. Some essential differences between the two sampling events may explain this improvement. Firstly, in 2006, samples were collected from a large area (see Figure 2) where the wolves' whereabouts were unknown, resulting in samples often being several days old when collected. Sample age has been found to negatively affect PCR amplification success (e.g. Murphy et al. 2007; Santini et al. 2007) due to the rapid degradation of DNA under field conditions. Moreover, with sampling conducted at the onset of the rainy season most droppings were exposed to heavy rain prior to sampling. Rain has been shown to greatly reduce the quality of faecal samples (Brinkman et al. 2010; Farrel et al. 2000; Piggot, 2004) because it washes away the surface layer and as the humidity provides a better climate for bacteria and enzymes (Lampa et al. 2008). For both trials samples were stored in ethanol, but we increased the concentration in 2008 to 96% compared to 70% used in 2006. The effect of increased EtOH concentration has been documented previously (e.g. Wasser et al. 1997) and may partly explain our results.

Table 1. The results of amplification success and genotyping from the two sampling events. * number of samples identified as Ethiopian wolf using Cytb **Number of PCR replicates necessary to obtain a reliable genotype at a locus *** Number of false homozygotes estimated using the methods of Gagneux et al. (1997).

N samples (Year)	Storage (% EtOH)	Pez17	*Cytb**	Complete multi-locus genotype	Unique genotype	PCR reps.**	Allelic drop-out	P (false homozygotes)***
N = 89 (2006)	70%	33(37%)	8(9%)	8(9%)	8	7	10.8%	0.000314
N = 45 (2008)	96%	32(86%)	32(86%)	32(86%)	15	4	5.4%	0.00003

Table 2. Characteristics of the ten microsatellite loci analysed in this study. N number of individuals, NA number of alleles, HO observed and HE expected heterozygosities, H-W Hardy-Weinberg P-values, * significant p<0.01

Locus	Size range (bp)	N	N_A	H_O	H_E	HWE
Cx253	101-107	23	3	0.636	0.513	0.475
FH2137	158-174	23	4	0.455	0.621	0.001*
FH2001	126-128	23	3	0.428	0.396	0.757
Pez17	199-212	22	3	0.364	0.439	0.242
FH2140	112-154	23	3	0.500	0.439	1.000
VWFx	166-174	22	2	0.727	0.500	0.080
Cxx466	142-144	23	2	0.136	0.130	1.000
FH2226	192-236	23	2	0.364	0.495	0.372
FH2096	93-101	14	3	0.642	0.632	0.222
FH2159	136-190	13	5	0.846	0.713	0.007*

DNA extraction, amplification and individual identification

Several different DNA extraction protocols were applied in 2006. We observed that for most of the samples that did not amplify when extracted with one method, applying a different extraction method did not make any difference. This indicates that the initial quantity and quality of the DNA present in the samples was already marginally low.

The PCR inhibition test revealed the presence of PCR-inhibitors in the samples collected in 2006, however, when diluting samples to overcome this effect, the DNA concentration ended up at extremely low levels and microsatellite amplification was unsuccessful. The higher amplification success of the 2008 samples could result from improved removal of inhibitors from using the magnetic bead DNA extraction protocol. Flagstad et al. (1999) argued that the magnetic beads reduce particle aggregation as compared to silica-based protocols, resulting in a more efficient washing of the DNA than the spin-column approach. However, several different extraction protocols were tested also in 2006, and it seems more likely that the main reason for amplification failure is the low DNA concentration resulting from the different factors discussed above.

Analyses of samples collected in 2006 revealed eight unique genotypes each represented by a single sample. This could be explained by the larger area sampled in 2006 (reducing the chance of resampling the same individual). Or by the fact that only a small fraction of the 2006 samples could be reliably genotyped and included in the analysis, thus potentially deflating the actual number of unique genotypes present, or masking resampling of individuals and/or individuals with the same genotype. Of those samples, only two were actually sampled in locations that overlapped with the 2008 sampling area (Figure 2). However, the quality and quantity of DNA in 2006 was generally low, which may have resulted in a higher level of genotyping errors than what was detected. Of the genotypes observed in 2008 (n = 15) approximately half were represented by two or more samples. These samples were collected within a small geographic area, thus we may have sampled the same individual multiple times.

Genetic variability

Considering that the Ethiopian wolf populations of northern Ethiopia have undergone a dramatic population decline, probably resulting in marginally small population sizes, a surprisingly high level of genetic variability is still retained (HE=0.130 to 0.713, Table 1). Comparatively, HE values for the larger southern population ranged between 0.58

and 0.61 (Randall 2006). High levels of genetic variability may be due to strong inbreeding avoidance behaviour. For example, in a population of African wild dogs *Lycaon pictus* from Kruger National Park, male and female dogs that formed new packs did so only with unrelated members of the opposite sex (Girman and Wayne 1997). Several studies have reported strong female-biased dispersal in Ethiopian wolves (Sillero-Zubiri et al. 1996; Randall et al. 2007), and this behaviour could potentially explain the higher than expected level of genetic variability retained in the Menz population.

Two loci showed significant deviations from Hardy-Weinberg expectations, locus FH2137 showed heterozygote deficiancy whereas locus FH2159 showed heterozygote excess. Analysis of more individuals is necessary to investigate whether actual processes or just technical artefacts cause this. The estimated genetic differentiation (FST = 0.27) between northern and southern Ethiopia support the notion of a significant genetic structuring found by Gottelli et al. (2004). Whether or not the high level of genetic differentiation reflects adaptive differentiation needs further investigation.

The wolves on each side of the Great Rift Valley share habitat preferences and look morphologically identical, however, one cannot exclude the possibility of local adaptations within the populations. There are some important ecological differences in the structure of rodent communities between the northern and the southern Ethiopian highlands (Ashenafi et al. 2005). For example, the giant molerat *Tachyroryctes macrocephalus* that comprises the wolf's main prey in BMNP is absent in the northern highlands (Ashenafi et al., 2005), which, in turn may influence feeding behaviour.

Concluding remarks

In this study we have evaluated the use of non-invasive sampling of Ethiopian wolf faecal samples, and based on our results we suggest the following guidelines: for the Ethiopian wolf populations in northern Ethiopia the low densities of animals demands some prior knowledge of the distribution of wolf territories in order to ensure the collection of fresh samples. Sampling during the peak of the dry season (January-February) is optimal because of rapid desiccation of samples. Samples should be stored in 96-100% EtOH and DNA should be extracted as soon as possible after returning from the field. We recommend the magnetic bead DNA-extraction method, manually or automated, in order to avoid PCR-inhibitor substances in the extracts.

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