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A "pocket-friendly" Dimethyl Sulphoxide (DMSO) technique for mushroom genomic DNA extraction suitable for DNA-based identifications

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ABSTRACT

DNA extraction from macrofungi is a prerequisite for all the downstream biotechnology-derived applications such as Sanger and genome sequencing, microarrays. However, extraction of high-quality DNA from macro-fungal tissues is often painstaking, challenging, and may pose a delay for high-throughput experiments, such as DNA sequence identification based biodiversity, invasion or impact assessment surveys. Commercial DNA extraction kits often prove to be either expensive or inconsistent in DNA extraction from diverse macro-fungal species samples, such as mushroom genera, where fruiting bodies vary greatly in texture, size, chemical composition and pigmentation. The present study implemented Dimethyl Sulphoxide (DMSO) to lyse cell walls of dried, grounded fungal samples for DNA extraction. The technique is efficient, rapid, and affordable and requires a low amount from the sample for DNA extraction. Furthermore, both the quantitative and qualitative data demonstrate superior grade DNA that yield products in subsequent PCR amplification of the ITS-5.8S phylogenetic marker region. This technique will allow researchers from underdeveloped and developing countries with varying levels of expertise to extract mushroom DNA even with a low research budget. It will also enable biodiversity, conservation, impact assessment, and invasion biology surveys in these countries, as well as fungorium based research.

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Introduction

Fungi are among the largest, most widely distributed, and diverse groups of organisms, ranging from single-celled yeasts to larger fungi with fruiting bodies that can be more than a meter in diameter (Bridge and Spooner 2001; Strauss et al. 2021). More than a million species play pivotal roles in all terrestrial ecosystems as decomposers, food sources, pathogens, and mutualists (Mueller et al. 2001; van Diepen et al. 2014). For instance, mycorrhizal fungi have uniquely beneficial relationships with most plant species and are essential in plant nutrition of terrestrial ecosystem (Mohan et al. 2014). The ectomycorrhizal group of fungi often produce large fruiting bodies such as mushrooms, boletes, and puffballs, of which many are sought after edibles while others are poisonous (Adl et al. 2005; Yamada et al. 2001). In recent years, studies on fungal communities and diversity increased rapidly. Conventionally, identification and taxonomical classification of these fungi are based on morphological characteristics, while molecular tools such as DNA sequencing of various genes are used to refine classifications and identifications (Koonin and Galperin 2003; Raja et al. 2017). Although some species can be identified convincingly using morphology alone, DNA sequence comparisons showed that other species represent complex cryptic species groups with unclear

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morphological differentiation, or novel species and genera (Jorger and Schrodl 2013; Xiao et al. 2010). Therefore, using DNA sequencing is especially advantageous when working with cryptic species complexes or fruiting bodies of deceivingly similar species or that lack key identification features (Shaffer et al. 2019). Furthermore, numerous fungal species in many countries are still undescribed, and DNA sequencing aids in placing these in the appropriate genera, families, and orders.

Genomic DNA extraction poses a bottleneck in DNA sequencing, especially when many samples need to be processed and when the specimen collection contains diverse morphological types of fungi. Fungi possess a rigid, chitinous cell wall with a high polysaccharide content that protects and provides integrity to the cell structures, posing difficulties in lysing the cell (Beauvais and Latge 2018). PCR (polymerase chain reaction) is a sensitive, precise, and accurate method, and high levels of pigmentation or molecules found in some fungal samples can inhibit this reaction affecting PCR performance (Jansson and Hedman 2019; Valones et al. 2009). Additionally, DNAse activity further makes it more challenging to isolate and preserve the DNA in the long term (Kumar and Mugunthan 2018; Zhou et al. 2007).

Simple, cheap, and easy techniques enable even non-specialists to extract DNA from large numbers of dried fungal mushroom samples collected in the field, especially when expensive DNA extraction kits are not an option. The present study aimed to optimize a reliable genomic DNA extraction technique yielding a good concentration of genomic DNA from a highly diverse collection of fungal samples dried as fungorium specimens, and from which genomic regions could successfully be amplified. More specifically, the technique highlighted the extraction from dried pulverized fungal fruiting body samples by using an affordable and easily available cell-lysing agent, namely Dimethyl Sulphoxide (DMSO). The hydrophilic sulfoxide group and two hydrophobic methyl groups of DMSO makes it a small amphiphilic molecule (Nocca et al. 2012) that result in water pores in the dipalmitoyl-phosphatidylcholine bilayers of fungal cell membranes, causing active molecules to penetrate through the lipid membranes (Kashino et al. 2010). Furthermore, DMSO can break down tissues and membranes chemically when coupled with heat, loosening the inner layers of fungal cells and making DNA accessible (Fernandez and Reigada 2014) . It is anticipated that this technique can be employed across a diversity of macrofungal families and by numerous research laboratories, especially when funds, infrastructure or resources for projects are low.

Materials and methods

DNA extraction of mushroom samples

Diverse types of mushroom samples (Table 1) were collected from the Western Cape, Gauteng, Mpumalanga and Limpopo provinces of South Africa during June 2019. They were dried using low heat or silica gel, and packed in zip lock bags during. Following collection, specimens were transported to the Department of Genetics, University of the Free State, South Africa, for genomic DNA extraction. Subsamples from the dried mushroom specimens which were about 10-20 grams per sample were pulverized in a tissue homogenizer (QIAGEN, TissueLyser ll, Germany). In the absence of a tissue homogenizer, samples can be crushed with a mortar and pestle (preferably with liquid Nitrogen) or alternative methods to damage material.

From these, approximately 25-30 mg were aliquot in 1.5 mL Eppendorf tubes. Four hundred microliter of DMSO (Glantham Life Sciences Ltd., Corsham, UK) was added until the samples were fully immersed and mixed gently. The samples were incubated at 65°C for 1 hour to induce cell lysis. Following incubation, 100 µL of 5 M Sodium Chloride (NaCl) was added followed by 80 µL CTAB/NaCl buffer [2% CTAB, 100 mM Tris(hydroxymethyl)aminomethane, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl] to remove cell wall debris, denatured protein and polyssacharides, and mixed by inversion. The samples were re-incubated at 65°C for 10 min to complete the lysis of cells in the suspension. DNA precipitation was performed by adding 1X vol of 3M $C_2H_3NaO_2$ (sodium acetate) and 2X vol of isopropanol, mixed by inversion, followed by incubation for 30 min at 4°C or on ice. The genomic DNA pellet obtained after centrifugation at 12 000 g was washed with 70 % ethanol to remove low molecular weight contaminants like salt . After more centrifugation for 15 min at high speed the DNA was air-dried overnight or heated at 30°C by placing them on a heat block for 1 hour. The genomic DNA was re-suspended and dissolved in 40 µL of pre-warmed distilled water. The genomic DNA samples were resolved on 1.5% agarose (Cleaver Scientific Ltd, UK) gels containing Condasafe (Condalab, Madrid, Spain) and a 100bp ladder (NewEngland BioLabs Inc., USA), and quantified with a NanoDrop® Spectrophotometer ND-1000 (ThermoFisher Scientific).

PCR amplification of ITS-5.8 rRNA region

To determine the suitability of the extracted genomic DNA for PCR, the Internal Transcribed Spacer (ITS)-5.8S gene region of the ribosomal operon was targeted, which is the region most commonly used to identify fungi and that also represents the primary fungal DNA barcode region (Begerow et al. 2010; Bellemain et al. 2010; Dentinger et al. 2011; Schoch et al. 2012). The PCR amplification

reaction was performed on a T100™ Thermal Cycler (Biorad Laboratories (Pty) Ltd, South Africa) using the universal ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer set (White et al. 1990) with an Onetaq PCR kit (Biolabs, USA) with an annealing temperature of 54°C. PCR reactions were visualized on 1.5% agarose gels.

Genomic DNA Extraction

Reagents needed for the extraction included Tris (100 mM), EDTA (10 mM), SDS (2%), Chloroform:Isoamyl alcohol (24:1) and Ethanol (100%). The method was based on Avin et al. (2012) but with modifications. The dried fungal mass was homogenised with a tissue homogeniser (©QIAGEN, TissueLyserll, Germany). Only 1.5mL Eppendorf tubes were used and the lysis buffer was prepared in a mass master mixture from which was allocated to each sample. A minute amount of pulverised subsample (0.04g) was added to 900 µL of Tris (100mM)-EDTA (10 mM)-SDS (2%) buffer and briefly mixed by inversion and sharp probes. The lysis buffer was then incubated for 30 minutes at 65°C. Centrifugation (13,000 rpm at 4°C for 5 min) of the lysis buffer resulted in cellular debris that was discarded, and supernatant, which was carefully pipetted out. This step was repeated to reduce cellular debris. To the supernatant, 600 µL Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 13,000 rpm at 4°C for 5 min. The aqueous phase containing DNA was carefully pipetted out. The Chloroform: Isoamyl alcohol step was repeated as a modification to ensure the further removal of cellular debris and PCR inhibiting chemicals. Finally, the DNA was precipitated with cold ethanol (100%) after centrifugation at 16.000 rpm for 30 min at 4°C. The DNA pellet was resuspended in 40µL of sterile pre-warmed nuclease-free water.

Qualitative assessment of the DNA was done with gel electrophoresis in a 1.5% agarose gel (Cleaver Scientific Ltd, UK) containing Condasafe (Condalab, Madrid, Spain), visualised with a geldoc (Vacutec, Roosevelt Park, South Africa). The DNA concentration (ng/µL) was quantitatively measured using a NanoDrop® Spectrophotometer ND-1000 (ThermoFisher Scientific). The purity of the DNA was assessed at 260/280 nm absorbance. Measurements were performed in duplicates, and the averages of the two measurements were calculated. The DNA was stored at -20°C for subsequent use.

PCR amplification of 5.8S-ITS region

In order to evaluate the quality of DNA, PCR was employed to amplify the ITS-5.8S region. The two universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (19bp) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (20bp) were used for this study (Romanelli et al., 2014; White et al., 1990). Each 25 µL PCR reaction included 1µL of template DNA (≈ 100-200ng), 1.25µL of each primer (10 µM), 12.5µL One Tag® 2X MM w/standard buffer (New England BioLabs, inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) and 9µL nuclease free water.

PCR was performed in a BioRad T100 Thermal Cycler (BIO-RAD, Johannesburg, South Africa). The thermal cycling conditions were set with an initial denaturation temperature at 94°C for 2 min 30 s, followed by 40 cycles of denaturation at 94°C for 30s, annealing at 54°C for 30s and extension at 72°C for 40 s. A final extension was performed at 72°C for 10 min. The amplicons obtained were resolved on 1.5% agarose gels.

Results and Discussion

Fungal genomic DNA was successfully extracted with the simple and effective DMSO technique developed in this study without requiring any additional enzymes or chemicals besides CTAB related chemicals. The method is quick, taking on average four hours, and requires no hazardous materials or enzymes. Although most techniques provide satisfactory levels of DNA both qualitatively and quantitatively, many of these involve the usage of expensive chemicals or enzymes, or especially DNA extraction kits, which becomes expensive when a large number of specimens needs to be processed (Griffin et al. 2002; O'Neill et al. 2020). Good bands were observed in the gel (Figure 1), although RNA bands were also present because the DNA samples were not treated with RNAse. The DNA concentrations were all higher than 100 ng/µL except for samples T01 and T03 (Table 1).

All of the PCR reactions were successful the first time around, with strong amplicon bands observed at a range of 500 – 800 bp (Figure 2). This is despite the fact that the DNA was not treated to remove RNA. Molecular applications using the extracted DNA, such as PCR amplification of a target gene and gene sequencing may also fail if extracted DNA contain impurities or inhibiting compounds. However, the DMSO method developed here had a 100% PCR success rate for the first attempt.

Genomic DNA extraction yielding a good concentration of pure DNA is an essential step in DNA-based studies and encompasses two significant steps. The first step involves lysis of the cell wall and membrane to release the DNA, and the second step is purification of the DNA from organic matter and the chemicals used (Dairawan and Shetty 2020). For fungi, numerous extraction techniques have been established to date for breaking fungal cell walls, for example, using enzyme digestion (Moller et al. 1992; Shaolan et al. 2002), grinding of the mycelia with liquid nitrogen and a glass rod (Zhi-Hong et al. 2001), brief

Table 1 Dried mushroom samples from South Africa used in this study

Sample #	Morphological ID	Fruiting body type	Area of collection	Collector	Date of collection
1	*Albatrellus* sp.	Polypore	Moreleta Kloof Nature Reserve, Pretoria, Gauteng	L. Popich	2019/01/28
CF1.1	*Amanita muscaria*	Gilled mushroom	Cecelia Forest, Cape Town, Western Cape	G. Goldman	2019/06/20
GF4	*Amanita rubescens*	Gilled mushroom	Groote Schuur Forest, Cape Town, Western Cape	G. Goldman	2019/06/21
T01	*Auricularia* sp	Jelly fungus	Magoebaskloof, Limpopo	L. Popich	2019/01/08
WP8.1	*Chroogomphus rutilus*	Bolete	Wynberg Park, Cape Town, Western Cape	G. Goldman	2019/06/20
WP 8.3	*C. rutilus*	Bolete	Wynberg Park, Cape Town, Western Cape	G. Goldman	2019/06/20
T05	*Cordyceps* sp.	Cup fungus	Sabie, Mpumalanga	L. Popich	2019/01/11
T06	*Cordyceps* sp.	Cup fungus	Sabie, Mpumalanga	L. Popich	2019/01/11
WP8.1	*C. rutilus*	Bolete	Wynberg Park, Cape Town, Western Cape	G. Goldman	2019/06/20
GF6	*Gymnopilus junonius*	Gilled mushroom	Groote Schuur Forest, Cape Town, Western Cape	G. Goldman	2019/06/21
CF6.5	*Leccinum duriusculum*	Bolete	Cecelia Forest, Cape Town, Western Cape	G. Goldman	2019/06/20
GF3	*Lactarius deliciosus*	Gilled mushroom	Groote Schuur Forest, Cape Town, Western Cape	G. Goldman	2019/06/21
CF6.1	*L. deliciosus*	Gilled mushroom	Cecelia Forest, Cape Town, Western Cape	G. Goldman	2019/06/20
WP 9.4	*L. deliciosus*	Gilled mushroom	Wynberg Park, Cape Town, Western Cape	G. Goldman	2019/06/20
T00	*Leucocoprinus* sp.	Gilled mushroom	Magoebaskloof, Limpopo	L. Popich	2019/01/08
T07	*Lindquistia* sp.	Flask fungus	Moreleta Kloof Nature Reserve, Pretoria, Gauteng	L. Popich	2019/01/26
T08	*Lindquistia* sp.	Flask fungus	Moreleta Kloof Nature Reserve, Pretoria, Gauteng	L. Popich	2019/01/26
T12	*Lysurus cruciatus*	Stinkhorn	Sudwala, Mpumalanga	L. Popich	2019/03/02
CF 6.3	*Paxillus involutus*	Gilled mushroom	Cecelia Forest, Cape Town, Western Cape	G. Goldman	2019/06/20
RC2	Possible *Clitocybe*	Gilled mushroom	Rondebosch Common, Cape Town, Western Cape	G. Goldman	2019/06/21
T04	*Rickenella* sp.	Gilled mushroom	Magoebaskloof, Limpopo	L. Popich	2019/01/08
CF 7.1	*Russula sardonia*	Gilled mushroom	Cecelia Forest, Cape Town, Western Cape	G. Goldman	2019/06/20
WP9.3	*Russula capensis*	Gilled mushroom	Wynberg Park, Cape Town, Western Cape	G. Goldman	2019/06/21
JK5	*Suillus luteus*	Bolete	Jonkershoek Nature Reserve, Stellenbosch, Western Cape	G. Goldman	2019/06/20
WP 9.1	*Suillus granulatus*	Bolete	Wynberg Park, Cape Town, Western Cape	G. Goldman	2019/06/20
JK14	*S. granulatus*	Bolete	Jonkershoek Nature Reserve, Cape Town, Western Cape	G. Goldman	2019/06/21
GF7	*Tricholoma saponaceum*	Gilled mushroom	Groote Schuur Forest, Cape Town, Western Cape	G. Goldman	2019/06/21
JK 10	*T. saponaceum*	Gilled mushroom	Jonkershoek Nature Reserve, Cape Town, Western Cape	G. Goldman	2019/06/21
T02	*Trogia* sp.	Gilled mushroom	Magoebaskloof, Limpopo	L. Popich	2019/01/07
T03	*Trogia* sp.	Gilled mushroom	Magoebaskloof, Limpopo	L. Popich	2019/01/07
T10	Unknown	Gilled mushroom	Moreleta Kloof Nature Reserve, Pretoria, Gauteng	L. Popich	2019/01/26

vortexing (10-20 s) with sterile sand (Ghosh et al. 2015) or glass/magnetic beads (Faggi et al. 2005), and microwave exposure (Goodwin and Lee 1993).

The purification of the genomic DNA involves the use of CTAB (cetyl trimethyl ammonium bromide) buffer (Doyle and Doyle 1987; Moller et al. 1992) that may or may not be followed by phenol/chloroform purification (Ghosh et al. 2015). Other types of purification buffers may also be used, such as guanidium thiocyanate (GITC) (Alberti and Fornaro 1990), before DNA is precipitated and cleaned further using 70% ethanol.

Samples used in this study varied in terms of morphology and taxonomy (Table 1), which indicates that this method will be ideal for large scale collection projects such as biodiversity surveys, DNA barcoding, impact assessments, and invasion biology surveys. The wide diversity of fungi with morphologically dissimilar fruiting bodies, such as fragile to robust mushrooms, boletes, puffballs, jelly fungi, and hard tissued bracket or polypore fungi produced by mostly Basidiomycota species (Alexopoulos et al. 1996), may be the reason that a single DNA extraction protocol often does not work across the various specimens. Furthermore, most extraction protocols have been developed mainly for cultured fungi and not precisely to extract directly from fresh or dried fungal fruiting bodies that usually have different textures, chemicals, and pigments (Al-Samarrai and Schmid 2000; Kumar and Mugunthan 2018).

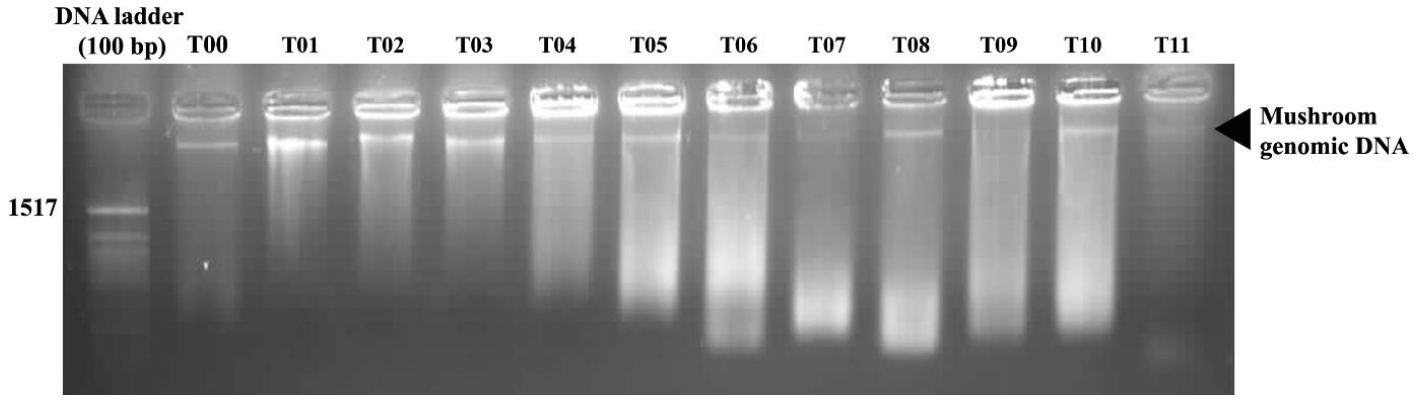

Fig 1. Subset of the fungal samples extracted with the new DMSO technique. The alphanumeric characters indicates sample numbers used for genomic DNA extraction. Three microliter of genomic DNA was loaded onto the agarose gel. The largest band size of the DNA ladder is indicated to the left.

Table 2 Concentrations of the fungal genomic DNA measured by Thermo Scientific NanoDrop 1000 recorded at λ_{260}

Samples	DNA concentration (ng/µL)	260/280
T00	184.9	1.38
T01	66.1	0.95
T02	106.6	1.30
T03	73.4	1.30
T04	257.1	1.18
T05	389.9	0.49
T06	476.8	1.74
T07	451.3	1.33
T08	917.0	1.40
T10	506.2	1.76
T11	539.6	0.65
T12	567.2	1.4

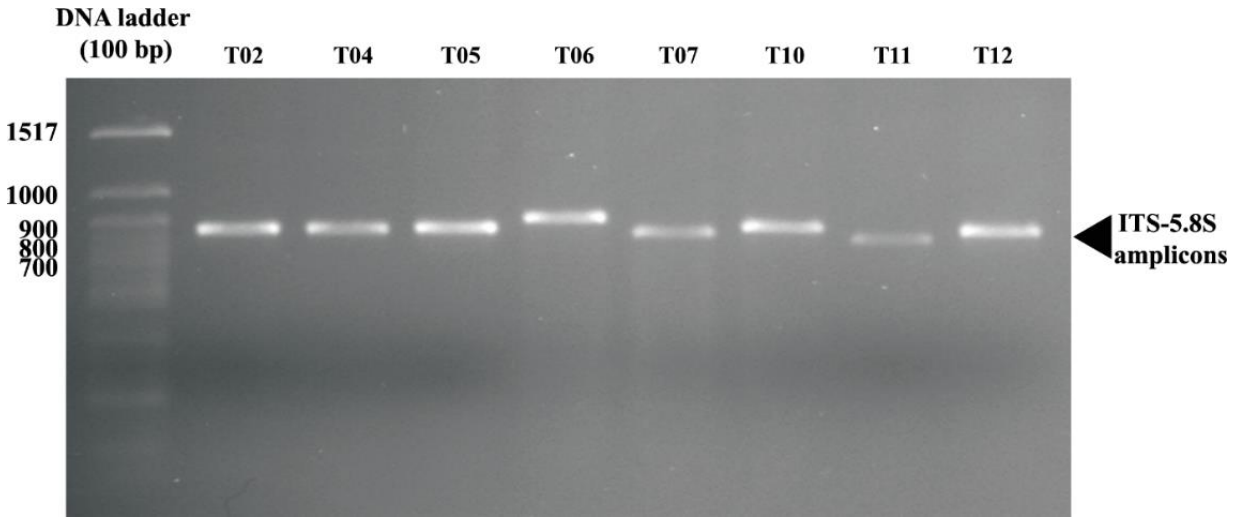

Fig 2. The ITS-5.8S PCR amplicons from the fungal genomic DNA. The black arrowhead indicates the amplicons' positions, while band sizes of the DNA ladder are indicated to the left. Three microliters of genomic DNA were loaded onto the agarose gel.

Over a thousand specimens can be collected during extensive biodiversity surveys (Brunbjerg et al. 2019; Heydari and Mahdavi 2009) and may include some samples that could not be identified morphologically, for instance novel taxa. Other types of studies investigating the conservation status of a fungal species (Buchanan and May 2003; Dahlberg et al. 2010; Dahlberg and Mueller 2011; Nascimbene et al. 2013) will require accurate identifications of the species forming the focus of the conservation assessment. Simultaneously, accurate identifications and verifications will also be needed to conduct an impact assessment where fungi are also(Andersen et al. 2004; Bartz and Kowarik 2019; Wilson 2014) included and that likely also include a large number of specimens. In cases of critical and worrying invasion biological studies of fungi, DNA extractions must be performed precisely and rapidly in order to identify the numerous samples from many places, hosts, or substrates needed to establish a checklist and database and for the number of surveys needed to generate the data for invasion assessments.

Conclusion

The DNA extraction approach used in this paper is sufficient for achieving outcomes, such as PCR amplification, without creating a bottleneck and requiring troubleshooting. In various fields, where swift and accurate findings are expected in a short period, this method will be advantageous. It can be used as a crucial step in discovering, tracking, and identifying fungi from a whole range of environmental samples, barcoding DNA, and can also support techniques such as DNA sequencing using DNA segments from a region of interest. For laboratories with technology that is not advanced, the existing approach is recommended and can result in high-throughput sample preparation for different molecular analytical methods. Additionally, this developed approach can also possibly be used to extract DNA from other organisms as well.

Declaration of interests

This study was supported by the University of the Free State and the Environmental and Biosafety Research Ethics Committee (UFS-ESD2019/0033). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's contribution

MG obtained the funding for the study. SG conceived the study and designed the experiment. MN and THM conducted all the experiments under the close guidance and supervision of SG. MN and SG analyzed the data and drafted the manuscript. SG and MG read and edited the manuscript.

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