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Streamlined and Cost-Effective Genomic DNA Extraction Method for Lichens, Mushrooms, and Endolichenic Fungi: Enabling DNA Barcoding and Molecular Research

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Abstract

Extraction of nucleic acids in pure form from organisms is of paramount importance for DNA based identification and other molecular studies. Over the past few decades, DNA-barcoding has emerged as a powerful technique, facilitating species identification across various 'difficult to identify' life-forms. Fungi, being an immensely diverse group of microorganisms, contribute significantly to global biodiversity, with estimates ranging from 2.2 to 3.8 million species. However, a vast majority of this diversity remains unidentified, and many fungal species are considered cryptic. Therefore, numerous large- and small-scale DNA-barcoding projects are being conducted worldwide to unravel this rich biodiversity. However, the rigidity and high complex polysaccharides content of fungal cell-wall presents a significant obstacle, making the extraction of high-quality genomic DNA a challenging task across varied fungal organisms. In this study, we employed a modified CTAB based method to isolate and purify high-quality PCR-amplifiable genomic DNA primarily from lichens and tested it on other fungal life forms as well, including, mushrooms, endolichenic fungi, and parasitic fungi. Remarkably, the isolated DNA proved successful as a template in PCR reactions, serving the purposes of DNA barcoding, RAPD as well as for metagenomic analysis effectively. This versatile protocol demonstrated its utility across all the fungal life forms investigated in this study, offering a universal, cost-effective, and efficient approach for fungal DNA isolation.

Keywords: ***CTAB, Cryptic species, DNA-barcoding, DNA extraction, Endolichenic fungi, Lichens, Mushrooms, Metagenomics***

1. Introduction

Fungi, being eukaryotic organisms characterized by their heterotrophic mode of nutrition, and membrane bound organelles, are not only one of the most abundant life forms on our planet but also play a pivotal role in a wide array of ecological, industrial, and medical contexts. The Earth is believed to host an astonishingly diverse fungal population, with estimates ranging from 2.2 to 3.8 million species (Hawksworth & Lücking, 2017). These fungi span various taxonomic groups and

niches, encompassing entities like lichens, mushrooms, coprophilous fungi, lichenicolous fungi, endophytic fungi, aquatic hyphomycetes, foliicolous fungi, lignolytic fungi, and many more. However, extraction of good quality genomic DNA for their identification remained a cumbersome task as they contain complex polysaccharides and secondary metabolites.

Hence, to facilitate comprehensive molecular systematic investigations on some of these fungal life-forms (lichens, mushrooms and endolichenic fungi), it is imperative to obtain high-quality genomic DNA suitable for PCR amplification, that can be achieved by disrupting the cell wall of fungi, which is mainly composed of glucans, chitin, mannans, chitosan, and glycoproteins (Ramya et al., 2021). Numerous cell disruption methods, including mechanical techniques (e.g., bead beating, sonication, liquid nitrogen grinding), enzymatic approaches (utilizing cell lysis enzymes like lyticase), and chemical procedures (employing detergents such as CTAB and SDS) (Kumar & Mugunthan, 2018), have been documented in various scientific publications (Klimek-Ochab et al. 2011; Kumar & Mugunthan 2018), which is a very crucial step prior to the fractionation and purification for the release of cellular content in the milieu of extraction buffer. . The extraction of high-quality, pure DNA is indispensable for molecular taxonomy, systematics, and downstream studies involving lichens, mushrooms, and other fungal entities. While the conventional CTAB (Cetyl-Trimethylammonium Bromide) method may not consistently yield high-quality DNA suitable for molecular investigations, several simplified and rapid protocols and commercial kits have been employed by lichen researchers and scientists studying various fungal forms, albeit with varying degrees of success (Grube et al., 1995; Cubero & Crespo, 2002; Aras & Duman, 2007; Werth et al., 2016); and still there is scope for improvement in the fungal DNA isolation protocols.

Generally, a single standardized protocol may not effectively extract DNA from all the lichen species and other fungi due to their biochemical and structural heterogeneity. The impurities, co-precipitating with DNA during extraction process can inhibit PCR and other enzymatic reactions (Park et al., 2014). Additionally, financial constraints, especially for researchers in developing countries with limited research funding, may hinder the use of commercial kits and expensive column-based methods.

Various chaotropic salts, including sodium chloride, guanidinium hydrochloride, potassium chloride, ammonium acetate, potassium acetate, and silica, have been reported to be used for removing eliminating polysaccharides, proteins, and phenolic impurities from different biological materials during DNA extraction (Mishra et al., 2008; Heikrujam, 2020). In the present study, we have modified the CTAB method for genomic DNA isolation from lichens, which also has proven successful in case of mushrooms and ELFs; an overview of which is provided in **Figure 1**. The modified protocol not only consistently yielded high-quality and quantity DNA, suitable for reproducible PCR amplification, but also the estimated average cost of DNA extraction per sample is approximately 1.5 USD, and the procedure requires 3 to 4 hours of experimental time, demonstrating its cost and time-effectiveness. Hence, this method can be applied for processing a large number of samples, facilitating studies related to molecular diversity, identification, evolution, and marker-based research.

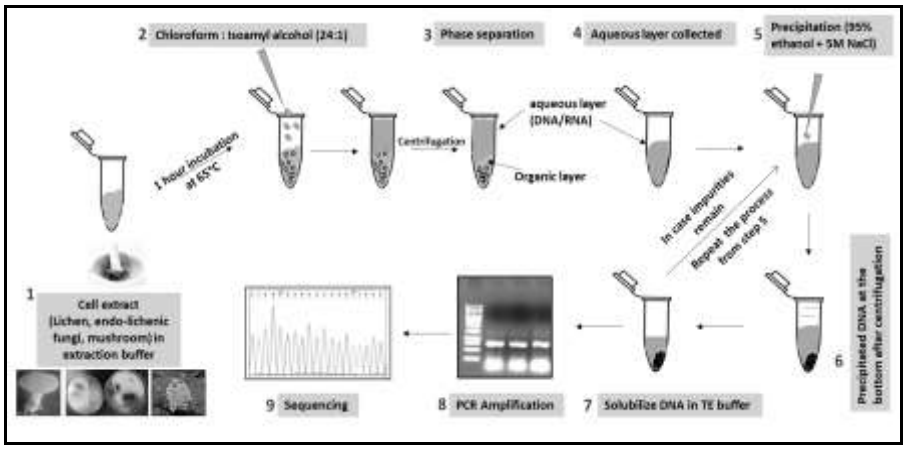

Figure 1: Overview of DNA extraction methodology used in the study.

2. Material and methods

Chemicals and Instruments

Potato Dextrose Agar (PDA), Cetyl-trimethylammonium bromide (CTAB), sodium chloride, EDTA, Tris-base, β-mercaptoethanol, ethanol, isoamyl alcohol, chloroform, proteinase K, RNase A, Taq polymerase, dNTPs, 6x loading dye, DNA ladder. All the chemicals were purchased from HiMedia Laboratories Pvt Limited.

Instruments: QIAxpert spectrophotometer (Qiagen), T100 PCR thermal cycler (Bio-Rad), Gel Doc XR+ System (Bio-Rad).

Sample collection

At the preliminary level lichens (viz. *Ramalina conduplicans* Vain., *Pertusaria leucosorodes* Nyl., *Candelaria indica* (Hue) Vain., *Usnea eumitrioides* Motyka, *Usnea orientalis* Motyka, *Hypotrachyna cirrhata* (Fr.) Divakar, A. Crespo, Sipman, Elix & Lumbsch, *Heterodermia diademata* (Taylor) D.D. Awasthi, *Parmotrema reticulatum* (Taylor) Choisy, *Flavoparmelia caperata* (L.) Hale, *Bulbothrix setschwanensis* (Zahlbr.) Hale, *Lobaria pindaransis* Räsänen) were collected from different regions of Uttarakhand, Central Himalaya and were identified as per classical taxonomy (microscopy and chemotaxonomy) and deposited in the herbarium of Kumaun University and accession numbers were obtained for lichen sample. Their DNA was extracted as per following protocol for their molecular characterization.

Similarly wild mushrooms (*Albatrellus* sp., *Hydnellum* sp., *Lactifluus* sp., and *Leccinellum* sp.) collected from the Nathuakhan forest area in Nainital district were identified up to generic level using classical taxonomy and their DNA was extracted for their molecular characterization.

Endolichenic fungi isolation

Endolichenic fungi (ELF) used in the present study were isolated from the lichen species viz. *Hypotrachyna cirrhata*, *Usnea eumitrioides*, and *U. nilgirica* as per Suryanarayanan et al. (2005, 2017) and Tripathi & Joshi (2019).

DNA extraction

Homogenization and cell lysis: Approximately 10 to 50 mg of each sample (lichen, ELF, and mushroom) was weighed and ground into fine powder using liquid nitrogen. Thereafter, preheated 3ml CTAB extraction buffer (1M Tris-HCl pH 8.0, 0.5M EDTA pH 8.0, 2% CTAB, 1M NaCl and 0.5% v/v β-mercaptoethanol) were added and mixed with the samples. The resulting solution was then carefully transferred to 2 ml microfuge tubes. To this solution, we added 10 µl of proteinase K (20 mg/ml), and the samples were incubated in a water bath at 65°C for duration of 45 minutes.

Phase separation: After incubation, an equal volume of Chloroform: Isoamyl alcohol (24:1) was added to each tube and mixed thoroughly by gentle inversion. The tubes were then centrifuged at 12,000 rpm (RCF = 13520g) for duration of 10 minutes, which resulted in the separation of two distinct phases: an aqueous phase and an organic phase. The aqueous phase was removed carefully and transferred with precision into fresh microfuge tubes.

Precipitation: To each sample tube, 300µl NaCl (5M) solution and 700 µl chilled ethanol (95%) were added, allowing the DNA to undergo precipitation. The tubes were incubated at -20˚C for 1 hour. After incubation, DNA was pelleted by subjecting the tubes to centrifugation at 12,000 rpm (RCF = 13520g) for 10 minutes.

Washing: The supernatant was discarded and DNA pellet was washed with ethanol (70%). The pellet was allowed to air dry after washing until the ethanol odor dissipated completely.

DNA Resuspension: The DNA pellet was allowed to resuspend in a buffer of 50 to 100 µl TE (1X; 1M Tris HCl and 0.5 M EDTA pH = 8.0). To eliminate any remaining RNA, 10 µl RNase solution (20mg/ml) was introduced to the suspension and incubated at 37˚C for 30 minutes. Thereafter, for deactivation of RNase, the solution was incubated at 65˚C for 5 minutes.

***Quantification of DNA*:** The purity of the DNA was determined by gel electrophoresis in 0.8 % agarose gel. Before quantifying DNA, the extracted DNA samples were diluted (dilution factor: 50) and the DNA yield was determined by using the following formula:

Concentration of DNA (µg/ml): 50 µg/ml × OD_{260} × dilution factor.

PCR amplification: PCR amplification of standard barcodes, including the Internal Transcribed Spacer region (ITS), Large Subunit ribosomal RNA (LSU), Small Subunit ribosomal RNA (SSU) and largest subunit of RNA Polymerase II (RBP1) were carried out using the genomic DNA isolated from samples of lichens, ELFs and mushrooms. Each PCR reaction mixture (with a total volume of 20 µl) consisted of the following components: 10X assay buffer A (2µl), 50mM $MgCl_2$ (0.4µl), 10mM dNTPs mix, (0.4µl), Taq polymerase 1U (0.5µl), 2µl Forward and Reverse primers (10pM), DNA template (2µl or ~20 to 200ng), and nuclease free water. The PCR amplification program for the barcodes was as follows: initial denaturation at 95˚C for 5 minutes, denaturation at 94˚C for 1 minute, primer annealing at 58 to 53˚C (gradient) for 45 seconds, extension at 72˚C for 55 seconds, and a final extension at 72˚C for 5 minutes. The reaction cycle was repeated 38 times. After completion of the cycles, the amplified products were visualized by agarose gel electrophoresis. Table 1 displays the primers used in the present study with their optimum annealing temperatures.

Table 1: List of primers used in this study and their nucleotide sequences

Primer Name	Sequence (5'-3')	Standardized optimum annealing temperature (range)	References
ITS Forward primer	5'TCCTCCGCTTATTGATATGC3'	54 to 57˚C	Raja et al., 2017
ITS Reverse primer	5'GGAAGTAAAAGTCGTAACAAGC3'		
LSU Forward primer	5' ACCCGCTGAACTTAAGC3'	53 to 57˚C	Raja et al., 2017
LSU Reverse primer	5'TCCTGAGGGAAACTTCG3'		
RBP1 Forward primer	5' GARTGYCCDGGDCAYTTYGG3'	53 to 57˚C	Schocha et al., 2012
RBP1 Reverse primer	5'CCNGCDATNTCRTTRTCCATRTA3'	50 to 57˚C	
SSU Forward Primer	5'GTAGTCATATGCTTGTCTC3'	50 to 57˚C	Raja et al., 2017
SSU Reverse Primer	5'CTTCCGTCAATTCCTTTAAG3'		

3. Results and Discussion

DNA extraction from lichens and other fungal sources is often a more challenging endeavour compared to extracting DNA from bacteria, plants, animals, and viruses, primarily due to the intricate and robust architecture of their cell walls (Mishra et al., 2008). The conventional CTAB method, initially designed for DNA isolation from plant sources by Doyle & Doyle (1990), has been widely utilized. However, over time, many changes have been made to the original Doyle and Doyle method to make it more effective and adaptive for use with other organisms (Aboul-Maaty & Oraby, 2019). During the DNA extraction process, CTAB (amphipathic cationic detergent) forms micelles in aqueous environment. Tissues rich in complex polysaccharides and various secondary metabolites can pose challenges by impeding DNA co-precipitation during the purification process (Heikrujam, 2020). The effectiveness of CTAB varies depending upon the ionic strength of the buffer. High ionic strength is required to remove the polysaccharides efficiently. In lower ionic strength buffers, CTAB tends to co-precipitate with DNA along with acidic polysaccharides, while neutral polysaccharides and proteins remain in solution. High salt (NaCl) concentration provides the necessary ionic strength for CTAB to bind and form complexes with polysaccharides and other impurities, which are removed in

the subsequent chloroform: Isoamyl alcohol treatment (Heikrujam, 2020; Paterson et al., 1993; Murray & Thompson, 1980; Park et al., 2014).

We successfully isolated and purified genomic DNA from a diverse range of biological samples, including 11 distinct lichen species, 5 different mushrooms, and 5 separate ELF isolates (**Figure 2**), with DNA purity ranging from 1.2 to 2.02 ($OD_{260/280}$**)** and the DNA yield ranging from 25.17 to 155.3 ng/µl **(Table 2)**. Besides, we also isolated and successfully PCR-amplified the DNA of *Ophiocordyceps sp.* an endoparasitic (entomopathogenic) fungus of high medicinal value (also known as, 'Caterpillar fungus' and 'Yartsa Gunbu') by using this method (*data not shown*).

Figure 2: Lichens, Mushrooms and Endolichenic fungal isolates used for DNA extraction. 1. *Ramalina conduplicans*; 2. *Pertusaria leucosorodes*; 3. *Candelaria indica*; 4. *Usnea eumitrioides*; 5.

Usnea orientalis; 6. *Hypotrachyna cirrhata*; 7. *Heterodermia diademata*; 8. *Parmotrema reticulatum*; 9. *Flavoparmelia caperata*; 10. *Bulbothrix setschwanensis*; 11. *Lobaria pindaransis*; 12. *Hydnellum* sp.; 13. *Lactifluus* sp.; 14. *Leccinellum* sp.**;** 15. *Albatrellus* sp.; 16. *Mucor* sp.; 17. *Aspergillus* sp.; 18. *Epicoccum* sp.; 19. *Preussia* sp.; 20. *Nigrospora* sp.

Table 2: List of samples (Lichens, Mushrooms and Endolichenic fungi) used for DNA extraction in the present study

S. No.	Sample species of Lichens, Mushrooms, and ELFs	Herbarium (Fungarium) Accession no.	$OD_{260/280}$	DNA yield (ng/µl)	Barcode(s) amplified	NCBI acc. no. (Representative sequences)	PCR amplifiability
Lichen Species							
1	*Ramalina conduplicans* Vain.	55567	1.81	65.3	RBP1, ITS, SSU, LSU	ITS: OP315027	Yes
2	*Pertusaria leucosorodes* Nyl.	55377	1.5	53	LSU, ITS	LSU: OP669345 ITS: OQ155036	Yes
3	*Candelaria indica* (Hue) Vain.	55418	1.6	45	LSU, ITS	ITS: OP647850 LSU: OP647770	Yes
4	*Usnea eumitrioides* Motyka	55568	1.2	40.5	RBP1, LSU, ITS,	LSU: OQ160791	Yes
5	*Usnea orientalis* Motyka	55545	1.4	38.1	LSU	OQ152453	Yes
6	*Hypotrachyna cirrhata* (Fr.) Divakar, A. Crespo, Sipman, Elix & Lumbsch	55376	1.5	55	SSU, ITS, LSU	ITS: OQ152489	Yes
7	*Heterodermia diademata* (Taylor) D.D. Awasthi	55570	1.4	42	ITS, LSU, RBP1	ITS: OQ152493 LSU: OQ152495	Yes
8	*Parmotrema reticulatum* (Taylor) Choisy	55341	1.3	32	ITS, LSU,	ITS: OQ152503	Yes
9	*Flavoparmelia caperata* (L.) Hale	55394	1.7	78	LSU, ITS, SSU	LSU: OR073644 ITS: OR077294	Yes
10	*Bulbothrix setschwanensis* (Zahlbr.) Hale	55395	1.4	56.8	LSU, ITS	LSU: OR077299 ITS: OR077318	Yes
11	*Lobaria pindaransis* Räsänen	55318	1.9	50.7	ITS	OQ006744	Yes
Mushrooms							
12	*Lactifluus deceptivus* (Peck) Kuntze	NA	1.72	51.1	ITS	OQ152539	Yes

13	*Hydnellum caeruleum* Hornem.) P. Karst.	NA	2.06	38.8	ITS	OQ170829	Yes
14	*Leccinellum* sp	NA	1.6	73.54	ITS	OQ170827	Yes
15	*Albatrellus* sp.	NA	1.9	47.6		OQ170828	Yes
Endolichenic fungi							
16	*Mucor fragilis* Bainier	NA	1.5	25. 17	ITS	OQ183441	Yes
17	*Aspergillus chevalieri* Thom and Church	NA	1.71	52.75	ITS	OQ152599	Yes
18	*Epicoccum nigrum* Link	NA	2.2	30.91	ITS	OQ152603	Yes
19	*Preussia sp.*	NA	1.49	61.70	ITS	OQ152639	Yes
20	*Nigrospora sp.*	NA	1.6	31	ITS	OQ152638	Yes

In our experiments, we observed that the initial extraction process of grinding the lichen material is critical. In separate trials, we utilized liquid nitrogen and preheated CTAB buffer to grind the sample, and found that the yield of DNA was higher in the liquid nitrogen grinded samples. Additionally, we noted a substantial increase in polysaccharide contamination in the samples extracted using the traditional CTAB approach and that dissolving the DNA pellet in 1 X TE buffer (pH-8.0) resulted in a jelly-like solution. To address these issues, we modified our CTAB procedure by incorporating 5M NaCl, which markedly reduced the formation of the gel-like consistency in the extracted DNA solution.

We also observed that during DNA precipitation with ice cold isopropanol, polysaccharide contaminants precipitated alongside the DNA **(Figure 3A).** Conversely, when we employed 95% ethanol in conjunction with 5M NaCl, the precipitation of contaminants was considerably reduced **(Figure 3B).** Other chaotropic salts, such as 3M Gd-HCl (Guanidine hydrochloride) and 3M potassium acetate (CH_3CO_2K), were also employed evaluate their effectiveness in removing impurities during the extraction process. DNA extracted from conventional CTAB method exhibited higher levels of impurities **(Figure 3A).** Similarly, when we utilized 3M Gd-HCl with 95% ethanol for DNA precipitation, results were comparable to those obtained using 5M NaCl with 95% ethanol. However, potassium acetate (3M) proved less effective in eliminating contaminants compared to both 5M NaCl and 3M Gd-HCl in combination with 95% ethanol. Furthermore, we noticed that the DNA extraction from fresh lichen samples yielded higher quality and quantity of genomic DNA as compared to older herbarium samples (*data not shown*).

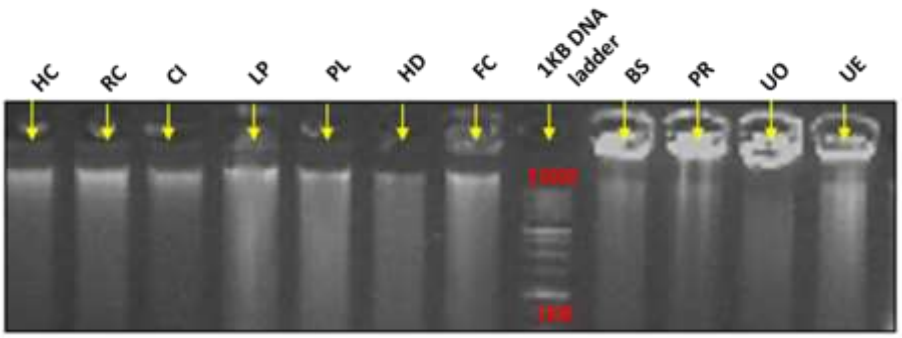

Figure 3: Agarose gel-electrophoresis of extracted DNA from lichen samples using the conventional CTABCTAB method without NaCl treatment and DNA precipitated with Isopropanol. From right to left. *Hypotrachyna cirrhata* (HC), *Ramalina conduplicans* (RC), *Candelaria indica* (CI), *Lobaria pindaransis* (LP), *Pertusaria leucosorodes* (PL), *Heterodermia diademata* (HD), *Flavoparmelia caparata* (FC), *Bulbothrix setschwanensis* (BS), *Parmotrema reticulatum* (PR), *Usnea orientalis* (UO), *Usnea eumitrioides* (UE).

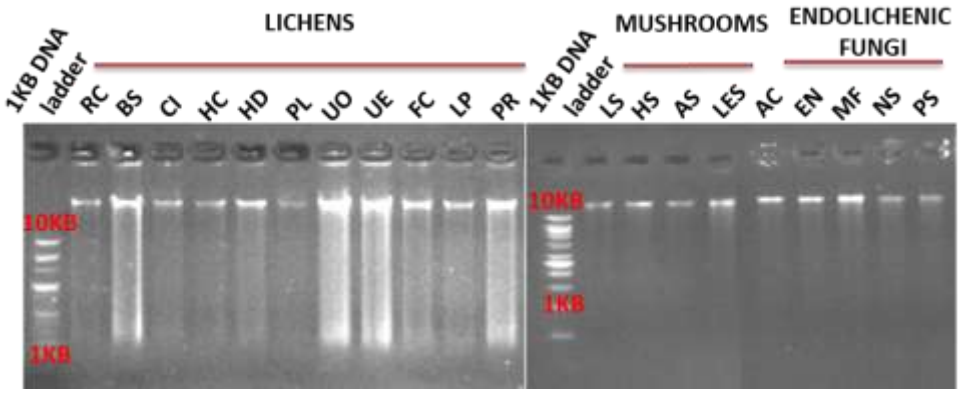

Figure 3B. Extracted genomic DNA using modified CTABCTAB method (DNA precipitation with 5M NaCl + 95% ethanol) of lichens, mushrooms and ELF isolates used in the study in 1 % agarose gel. Lichens RC, BS, CI, HC, HD, PL, UO, FC, LP, and PR (full names as given in figure 3A), *Lactifluus* sp. (LS), *Hydnellum* sp. (HS), *Albatrellus* sp. (AS), *Leccinellum sp.* (LES), *Aspergillus* sp. (AC), *Epicoccum* sp. (EN), *Mucor* sp. (MF), *Nigrospora* sp. (NS), *Preussia* sp. (PS).

It is generally believed that most of the secondary metabolites from lichens are supposed to be effectively eliminated during PCI (Phenol-Chloroform-Isoamyl alcohol) or CI (Chloroform-Isoamyl alcohol) treatment and does not cause much concern about co-precipitation with DNA. However, the presence of abundant polysachharides in lichen thalli poses a notable challenge by co-precipitating with the DNA during ethanol precipitation **(Common, 1991; Armaleo & Clerc, 1995)**. Furthermore, PCI/CI treatment may not be entirely sufficient in removing all polysaccharide impurities, as highlighted by **Common (1991) and Armaleo & Clerc (1995)**. The PCR amplifiability of DNA extracted through conventional CTAB method was found to be limited due to the presence of PCR inhibitors in the form of these impurities. Additionally, the DNA samples stored in refrigerator for extended period tended to degrade over time, resulting in the loss of PCR amplifiability. Similar issues were also discussed by Grube et al. (1995). For many lichen species, including *Bulbothhrix* sp., *Heterodermia* sp., *Parmotrema* sp. and *Usnea* sp., it was observed that impurities were particularly high and additional inhibitor removal strategies are required for these taxa. In contrast, our modified CTAB methods significantly enhanced the frequency of PCR amplification without any additional treatment for removal of PCR inhibitors **(Figure 4)**.

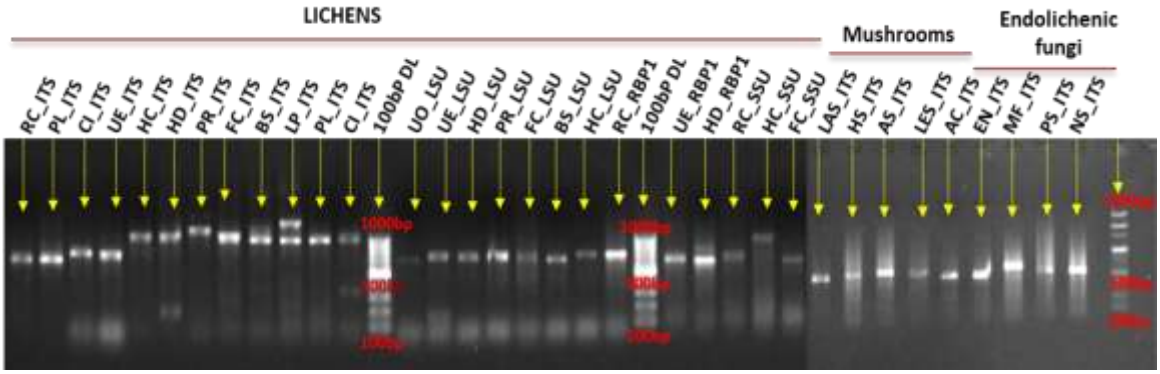

Figure 4. Gel image showing bands of amplified ITS, LSU, RBP1 and SSU amplicons of lichens "*Ramalina conduplicans* (RC_ITS, RC_RBP1, RC_SSU), *Pertusaria leucosorodes* (PL_ITS, PL_LSU), *Candelaria indica* (CI_ITS, CI_LSU), *Usnea eumetrioides* (UE_ITS, UE_LSU), *Hypotrachyna cirrhata* (HC_ITS, HC_LSU, HC _SSU), *Heterodermia diademata* (HD_ITS, HD_LSU, HD_RBP1), *Parmotrema reticulatum* (PR_ITS, PR_LSU), *Flavoparmelia caparata* (FC_ITS, FC_LSU, FC_SSU), *Bulbothrix setschwanensis* (BS_ITS, BS_LSU), *Usnea orientalis* (UO_LSU), *Lobaria pindaransis* (LP_ITS)," mushrooms "*Lactifluus* sp. (LAS_ITS). *Hydnellum* sp. (HS_ITS)., *Albatrellus* sp. (AS_ITS), *Leccinellum* sp. (LES_ITS), and ELF isolates "*Aspergillus* sp. (AC_ITS), *Epicoccum* sp. (EN_ITS), *Mucor* sp. (MF_ITS), *Nigrospora sp.* (NS_ITS), *Preussia sp.* (PS_ITS)" used in the study.

Furthermore, we also found that genomic DNA obtained through our modified CTAB approach provided high quality sequences when used for high throughput amplicon meta-barcode sequencing of *Ramalina* sp. (*unpublished data*). Additionally, to evaluate the efficacy of extracted DNA in RAPD amplification, we performed PCR for with universal 10mer RAPD primers for *Ramalina* sp. The results were consistent with our predictions, confirming the efficacy of our projected strategy for lichen DNA isolation using modified CTAB method **(Figure 5).**

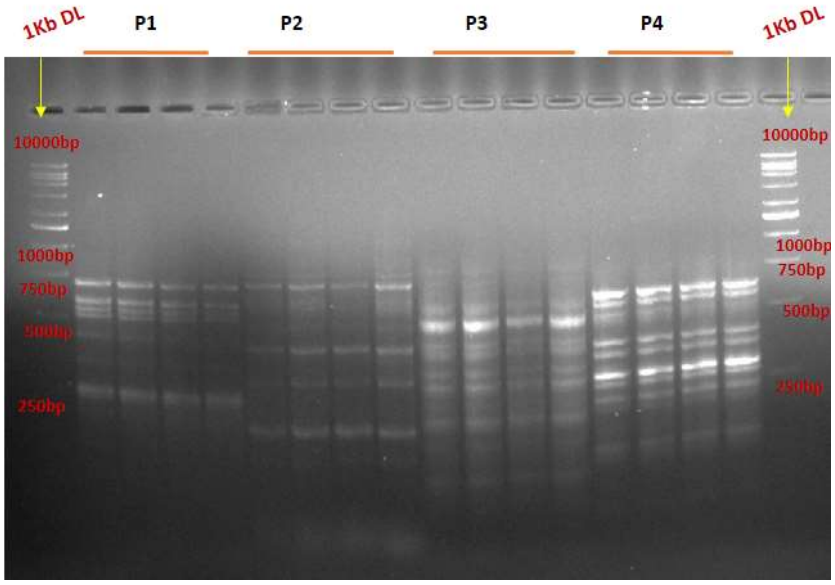

Figure 5. RAPD signature of *Ramalina sp.* used to check RAPD PCR amplifiability of extracted DNA using the protocol used in the study. The 10-mer universal primers used to generate RAPD signatures were P1 (5`ACCGCGAAGG 3`), P2 (5`TGCCGGCTTG 3`), P3 (5` AGGTGACCGT 3`) and P4 (5` GCACGGCGTT 3`).

We conducted a comparative analysis of previous reports detailing the methodologies employed by other research groups for genomic DNA isolation from fungi and lichens, and compared these with our current work (**Table 3**). Our proposed method (modified CTAB method), stands out as more efficient, user-friendly, rapid, and cost-effective when compared to the techniques used by other research groups. Notably, it eliminated the need of any mechanical cell disruption methods such as pulverization, or bead beating homogenizers for cell lysis and homogenisation. We have explored various chaotropic salts to find the most effective combination for isolating high quality gDNA from wide range of species. It's important to highlight that our proposed method is not limited solely to DNA isolation from lichens. It can be applied for isolating gDNA from a wide range of fungal isolates, different lichen taxa's and endolichenic fungi. Overall, our modified CTAB method consistently yields high quality genomic DNA, leading to the efficient production of PCR products and sequence data.

Table 3: Comparison of different published DNA extraction methods used for lichen and other fungi

Extraction methods	Fungal organism	Yield	PCR amplification	Remark	References
Quick and safe (QS) DNA extraction method (Mechanical pulverization +	*Botryosperia* sp., *Colletotrichum acutatum*, *C. gloeosporioides*, *Fusarium oxysporum*, *F. verticilioides*, *F. graminearum*, *Magnaporthe oryzae*,	2 µg/mg fungal mass	Yes	Safety and time efficiency eliminating use of phenol and chloroform	Chi, Park & Lee, 2009

KCL	*Phomopsis* sp., *Phytophthora* sp.				
Polyvinylpyrrolidone + 7.5 M ammonium acetate used in precipitation step	*Fusarium oxysporum* f.sp. *lycopersici* (F-1322 and the isolate (RBS-1)	-	Yes	Efficient in reducing polysaccharides	Parmar et al., 2014
Bead beating homogenizer method	*Aspergillus oryzae, A. fumigatus, A. terreus, A. flavus, Cladosporium tenuissimum, Curvularia affinis, C. lunata, Emericella variecolor, Earliella scabrosa, Fusarium phaseoli, Fusarium* sp., *F. incarnatum, Inonotus pachyphloeus, Penicillium concentricum, P. oxalicum, Rigidoporus vinctus*	80-230 µg/200 mg fungal mass	Yes	High-throughput method, safe and time efficient	Aamir et al., 2015
ZnO-based fungal DNA isolation	*Aspergillus* sp.	-	Yes	High-throughput method, Safety and time efficient	Qiao et al., 2020
Optimised CTAB (Cetyl-trimethyl ammonium bromide) extraction Method	*Parmelia* spp., *Physconia* spp.	-	Yes	Use with herbarium specimens of both non-lichenized and lichenized fungi	Cubero et al., 1999
KCL method	*Flavoparmelia* sp., *Heterodermia* sp., *Lobaria* sp., *Myelochroa* sp., *Peltigera* sp., *Punctelia* sp., *Umblicilaria* sp.	16-213 ng/mg fungal mass	Yes	Rapid, consistent, and cost-effective method for DNA isolation appropriate for PCR and DNA sequencing of broad range of lichen taxa.	Park et al., 2014
Mini-column purification	Lichen species	0-1 and 1 µg/100 mg thallus	-	Rapid, consistent, and cost-effective method for DNA isolation	Armaleo & Clerc, 1995
Modified DNA isolation protocol with use of glass powder in precipitation step	Lichen ascomata	-	Yes	Rapid, consistent, and cost-effective method for DNA isolation appropriate for PCR and DNA sequencing of	Grube et al., 1995

				broad range of lichen taxa.	
Modified CTAB method (using 5M NaCl + 95% ethanol)	*Albatrellus* sp., *Aspergillus* sp., *Bulbothrix setschwanensis*, *Candelaria indica*, *Epicoccum* sp., *Flavoparmelia caparata*, *Hypotrachyna cirrhata*, *Heterodermia diademata*, *Hydnellum* sp., *Leccinellum* sp., *Lobaria pindaransis*, *Lactifluus* sp., *Mucor* sp., *Nigrospora* sp., *Preussia* sp., *Parmotrema reticulatum*, *Pertusaria leucosorodes*, *Ramalina conduplicans*, *Usnea orientalis*, *U. eumetrioides*,	25.17 to 155.3 ng/<50 mg	Yes	Rapid, consistent, and cost-effective method for DNA isolation appropriate for PCR and DNA sequencing of broad range of lichen taxa. Mushrooms and other fungi	Present work

4. Conclusion

The analysis of DNA from various fungal sources, mushrooms, endolichenic fungi (ELF) and the fungal components of lichens, requires selective protocols to isolate their DNA due to the presence of complex structure of their cell walls. In this study, we report a highly efficient DNA isolation method tailored for lichens, ELF, and mushrooms. This procedure, featuring DNA precipitation with 5M NaCl + 95% ethanol, proves exceptionally valuable even when working with limited sample quantities. The method works well for extracting high-quality DNA from herbarium specimens, cultivated fungus, and lichens with high polysaccharide content. This modified CTAB protocol now opens up possibilities for studying many fungi that were previously considered challenging to work with using DNA-based approaches.

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Conflict of Interest

Authors declare no conflict of interest.

Accessibility of data

Lichen Herbarium (Fungarium) specimens were submitted to Department of Biotechnology, Kumaun University Campus, Bhimtal, and Lichen Herbarium repository, CSIR-National Botanical Research Institute (NBRI), Lucknow. Nucleotide sequences were submitted to NCBI.

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