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Direct Extraction and Assessment of Genomic DNA of Mycetoma Fungi from Black-grains Specimen

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Abstract:
Background: Direct isolation of genomic DNA of mycetoma fungi from black-grains achieve rapid diagnosis and may overcome culture disadvantages. **Objectives:** This study aimed to isolate and assess the DNA of mycetoma fungi using black-grains and to apply amplification of ITS region and nucleotide sequences. **Methods:** CTAB method was followed by manual homogenization alternatively to liquid nitrogen and glass beads disruption to obtain the genomic DNA. **Results:** Yielded DNA concentrations vary from 1.50 to 47.97 µg/ml (mean 10.09 µg/ml) while the optimum DNA purity recorded with 75.8% of specimens (n=69/91).Successful amplification of ITS region was done using pan-fungal primers (ITS4/5) with 90.1 (n=82/91)percentage. Species nucleotide sequences were detected with 67 (94.4%) amplicons from a total of 71.**Conclusion:** The study recommended using of black-grain specimens for DNA extraction of mycetoma fungi parallel with culture to insure rabid diagnosis and identification.

KEYWORDS Mycetoma, DNA, Black-grains, CTAB, Gezira, ITS, Sequencing

Direct Extraction and Assessment of Genomic DNA of Mycetoma Fungi from Black-grains Specimen

Introduction:

Early diagnosis and treatment of mycetoma; a neglected tropical disease; seem problematic. The accurate methods for identification of mycetoma fungi are the molecular-based techniques, such techniques use DNA[1,2], for this reason the efficiency of these tests depends on the quality and quantity of isolated DNA. Basically, fungal DNA could be obtained from different specimens including; culture, environmental and clinical samples, hence, a unique method for fungal DNA extraction is not prescribed until now, whereas methods for fungal DNA isolation include classical and kits methods. Enzymatic destructive methods of cell wall and mechanical agitation with metallic or glass beads are example of classical methods[3]. Recently, microwave radiation methods using lysis buffer was introduced[4].Extraction of DNA from pure culture of mycetoma fungi has some disadvantages because culture usually takes long time and even may be negative, in addition, maintenance of isolated strains become problematic[5]The genomic DNA of black-grain mycetoma could be obtained using different methods and specimens, relatively simple method, CTAB method, give sufficient quality of DNA from pure culture for PCR amplification, DNeasy Plant Kit method (Qiagen) is another used protocol[1].Rabid Whatman FTA filter matrices method also facilitate molecular identification of agents of black-grain mycetoma, although it is relatively expensive and involve several centrifugation steps[6]. Liquid nitrogen treatment help in the crushing of the black-grains is another used method[7].

Specimens for DNA extraction of black-grain mycetoma agents include pure culture of fungi, black-grains and tissue biopsies[8]. Environmental samples such as soil have been used for DNA isolation of eumycetoma fungi[9].

Standard CTAB method for isolation of fungal DNA as described by some researchers use hazardous liquid nitrogen beside glass beads, or pestle and mortar to accomplished the cell wall destruction from pure cultures[10].

From the literature one published finding could be observed in which several biopsies containing black-grain specimens were used for isolation of mycetoma agents DNA. The study used CTAB/chloroform-isoamyl alcohol method with glass beads disruption and other two kits methods; Qiagen DNeasy Plant and ZR Fungal /Bacterial DNA Mini pre, as a result, CTAB method gave DNA with bad quality[8].

The current study aimed to isolate the genomic DNA of black-grain mycetoma agents directly from clinical sample (grains) using CTAB protocol and manual homogenization and to assess the quality and quantity of isolated DNA by PCR amplification of ITS region and nucleotide sequencing.

Methods:

Reagents and Chemicals:

50 ml 2x CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer was prepared by transfer 1 gm of CTAB powder and 4.09 gms of NaCL to a glass beaker, then 2.5 ml of Tris-HCL stock solution (1 M, pH 8) and 1 ml of Na-EDTA stock solution were

added (0.5 M, pH 8) and gently mixed, then sterilized by autoclave. TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) was used for DNA suspending.

Black-grain samples collection:

A total of 91 black-grain specimens were collected from mycetoma biopsies from patients underwent surgical intervention in Gezira Mycetoma Center, University of Gezira, Sudan. Black grain specimens were collected in wide-neck plastic containers, and to make samples free of contaminants, grains were washed several times using sterile normal saline to remove blood, inflammatory exudates and tissues remnant(11). Storage of black-grain specimens was done at -20°C until time of use. Different grams amount of black-grains were collected according to mycetoma stages.

Ethical considerations:

Ethical approval for the study obtained from Ministry of Health and Faculty of Medical Laboratory Sciences, University of Gezira, Sudan. Verbal consents were taken from each enrolled patients. The consent was taken from parents in case of children containment.

DNA extraction:

The CTAB (Cetyl Trimethyl Ammonium Bromide) /chloroform-isoamyl alcohol method was used for isolation of genomic from black-grains with little Modification(5,12,13, 14).

In procedure; suitable amount of homogenized black-grains were added to an eppendorf tube containing 490 µl of sterile, freshly prepared CTAB buffer and 10 µl of proteinase K (iNtron). The mixture was mixed for a few minutes and incubated at 60 °C for one hour in an incubator. After incubation the tubes were re-mixed again to ensure disruption of mycelia. An emulsion formation done by adding 500 µL of chloroform : isoamyl-alcohol (CDH) (24 : 1) to the samples followed by shaking for several minutes. The tubes were spun in a microfuge at 14000 rpm for several minutes. The aqueous layer which formed in the upper part was collected in sterile eppendorf tubes contain 0.55 volume ice-cold iso-propanol (stored at 20-°C) and spun again at 14000 rpm for several minutes..

Finally, obtained pellets of DNA were washed with 70% ethanol and dried by air. The DNA was re-suspended in 100 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)and stored frozen at -20°C.

Measuring DNA concentration and purity:

DNA concentration(µg/ml) and purity were examined by using Nano Drop spectrophotometer (Bibby Scientific, UK)at 260 nm wavelength and optical density ratio at wavelength of 260/280 respectively. Additionally, The DNA quality was assessed by agarose electrophoresis after amplification of ITS region using Pan-fungal primers and *Madurella mycetomatis* species-specific primers as well as nucleotide sequencing.

Polymerase Chain Reaction:

Resulted DNA was amplified using Pan fungal primers ITS4/ITS5 (Macrogen, Seoul Korea) (The primers sequences shown below in Table 1). Primers were prepared as instructions of the manufacture. The test was accomplished using iNtRON's Maxime PCR PreMix Kit for 20 µL reaction volume containing 3 µL genomic DNA, 0.8 µL reverse primer, 0.8 µL forward primer and 15.4 µL distilled water. Cycling was done using PCR system 9700 thermocycler (Singapore) as the following: primary denaturation (95 for 2 minutes), then 35 cycles of alternating denaturation (94°C for 30 seconds), annealing of primers (58°C for 30 seconds), and extension by the thermostable polymerase (72°C for 30 seconds) with final extension of 2 minutes.

Preparation of 1.5% agarose gel and visualization of DNA:

Amount of 1.5gms of agarose powder were dissolved in 100 ml 1X TE buffer and heated until became clear. After cooling of the mixture 5 µl of (20mg/ml) ethidium bromide were added, mixed well and poured in a casting tray, and left to solidify at room temperature. DNA amplicons were visualized using 1.5% agarose gel covered with 1X TBE buffer. Selected voltage was 100V for 20 minutes. Amount of 4 µL of tested amplicons, 4 µL negative control and 2 µL of DNA marker were added to agarose wells. Imaging was done using Cleaver Scientific Ltd. gel documentation system (Model: OMNIDOC).

Sequencing and strains identification:

71 selected amplicons of ITS region was sequenced using the same primers; ITS4 and ITS5. Standard Sanger sequencing method was conducted by Macrogen (Seoul, Korea).
Sequence data were assembled manually to maximum homology. Nucleotide sequences were searched for sequences identity and similarity using nucleotide BLAST[15] (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). Then multiple-sequence alignments were analyzed using BioEdit software[16].

Statistical analysis:

Descriptive statistics was followed. Analysis was done using Statistical Package for Social Sciences (SPSS version 24).

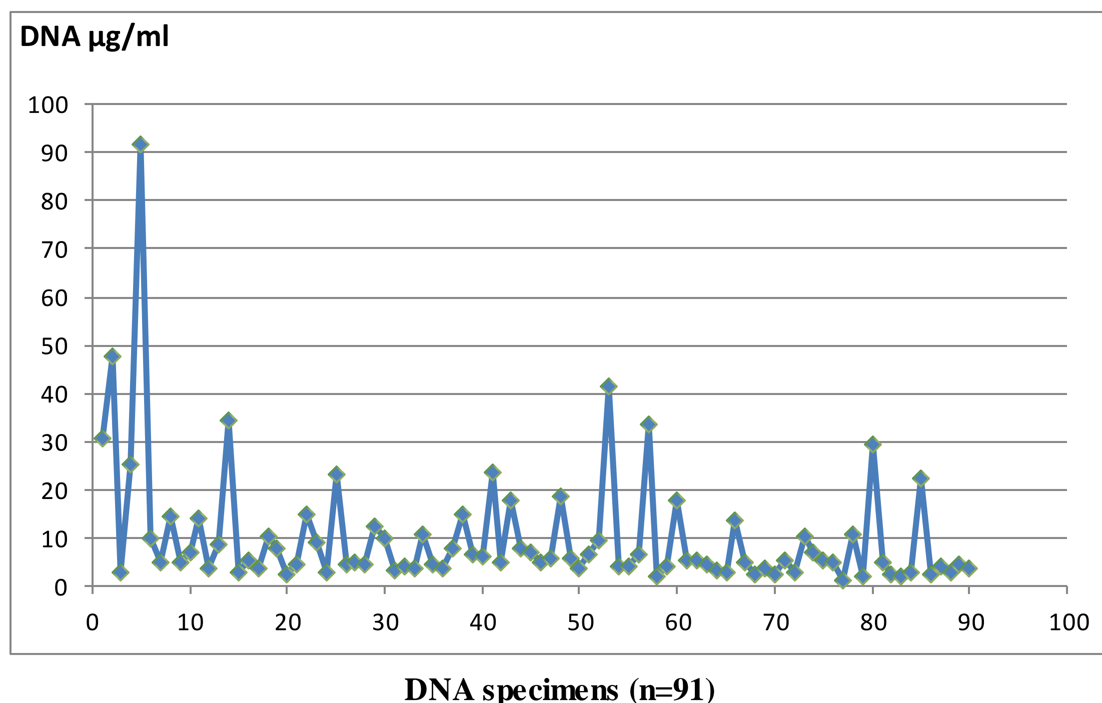
Table (1): Pan-fungal and *Madurella mycetomatis* species-specific primers used in the study

Primer	Location	Direction	T_m (°C)	Sequence
ITS5	SSU	Forward	58.4	5′-GGAAGTAAAAGTCGTAACAAGG-3′
ITS4	LSU	Reverse	56.4	5′-TCCTCCGCTTATTGATATGC-3′

Results:

Genomic DNA extraction from black-grains:

In the current study, a total of 91 black-grain specimens extracted were processed for fungal genomic DNA isolation. Time for extraction accomplished by CTAB/chloroform-isoamyl alcohol DNA extraction protocol was approximately 1½ hrs. The concentrations and purity of yielded DNA specimens varied from 1.5 to 91.65 µg/ml with 75.8% of DNA samples in the optical density of 1.6 to 2. as shown in (Figure1) and (Table 2) respectively.



DNA specimens (n=91)

Figure (1): Genomic DNA concentration of mycetoma fungi from black-grain samples using CTAB method (n=91).

Table (2): Genomic DNA purity obtained from black-grain specimens using CTAB method (n=91).

OD 260/280	No of samples	Percentage %
> 1.6	15	16.5
1.6 – 2.0	69	75.8
< 2.0	7	7.7
Total	91	100

OD: Optical density.

PCR results using pan-fungal primers (ITS4 and ITS5):

The internal transcribed spacer (ITS) region was successfully amplified using ITS4 and ITS5 primers. The target region include partial part of SSU, ITS1, 5.8S, ITS2 and partial part of LSU. Of the 91 DNA specimens 90.1 % (n=82) were amplified. The length of the resulted amplicon, approximately, was of 624 bp. The appearance of the DNA bands vary from clear to very clears with sharp ends (Figure 2).

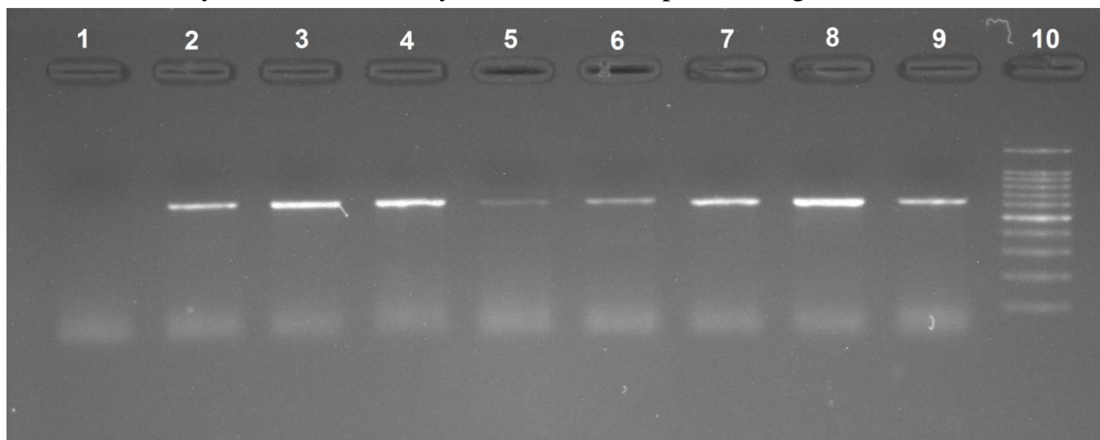


Figure (2): PCR amplification of the ITS region of black-grain mycetoma agents using ITS4/ITS5 universal fungal primers. Lanes 1: is negative, 2-9 are positive. And lane 10: 100-bp DNA ladder.

Nucleotide sequences of ITS region using pan-fungal primers (ITS4/ITS5):

The standard Sanger sequence analysis was done for 71 selected amplicons, both strands of the DNA were sequenced using the same pan-fungal primers. Sequences for 67 amplicons were successfully obtained, and 4 amplicons gave no signals; one of them was appeared as double band on agarose gel while the others were lost during processing. *Madurella mycetomatis* is the predominant identified mycetoma agent, one strain of *Madurella tropicana, Madurella fahalii* and *Sphaerulina rhododendricola* were identified. Accession numbers of 67 nucleotide sequences from GeneBank were shown in Table 3.

Table (3): GenBank accession numbers of 67 identified mycetoma agents form black-grain specimens

No	Name	Source	Origin	GenBank accessions
1	*Madurella mycetomatis*	Mycetoma	Sudan	MF980615
2	*Madurella mycetomatis*	Mycetoma	Sudan	MF980616
3	*Madurella mycetomatis*	Mycetoma	Sudan	MF980617

4	*Madurella mycetomatis*	Mycetoma	Sudan	MF980618
5	*Madurella mycetomatis*	Mycetoma	Sudan	MF980619
6	*Madurella mycetomatis*	Mycetoma	Sudan	MF980620
7	*Madurella mycetomatis*	Mycetoma	Sudan	MF980621
8	*Madurella mycetomatis*	Mycetoma	Sudan	MF980622
9	*Madurella mycetomatis*	Mycetoma	Sudan	MF980623
10	*Madurella mycetomatis*	Mycetoma	Sudan	MF980624
11	*Madurella mycetomatis*	Mycetoma	Sudan	MF980625
12	*Madurella mycetomatis*	Mycetoma	Sudan	MF980626
13	*Madurella mycetomatis*	Mycetoma	Sudan	MF980627
14	*Madurella mycetomatis*	Mycetoma	Sudan	MF980628
15	*Madurella mycetomatis*	Mycetoma	Sudan	MF980629
16	*Madurella mycetomatis*	Mycetoma	Sudan	MF980630
17	*Madurella mycetomatis*	Mycetoma	Sudan	MF980631
18	*Madurella tropicana*	Mycetoma	Sudan	MF980632
19	*Madurella fahalii*	Mycetoma	Sudan	MF980633
20	*Sphaerulina rhododendricola*	Mycetoma	Sudan	MF980634
21	*Madurella mycetomatis*	Mycetoma	Sudan	MH425455
22	*Madurella mycetomatis*	Mycetoma	Sudan	MH425456
32	*Madurella mycetomatis*	Mycetoma	Sudan	MH425457
24	*Madurella mycetomatis*	Mycetoma	Sudan	MH425458
52	*Madurella mycetomatis*	Mycetoma	Sudan	MH425459
26	*Madurella mycetomatis*	Mycetoma	Sudan	MH425460
27	*Madurella mycetomatis*	Mycetoma	Sudan	MH425461
28	*Madurella mycetomatis*	Mycetoma	Sudan	MH425462
29	*Madurella mycetomatis*	Mycetoma	Sudan	MH425463
30	*Madurella mycetomatis*	Mycetoma	Sudan	MH425464
31	*Madurella mycetomatis*	Mycetoma	Sudan	MH425465

32	*Madurella mycetomatis*	Mycetoma	Sudan	MH425466
33	*Madurella mycetomatis*	Mycetoma	Sudan	MH425467
34	*Madurella mycetomatis*	Mycetoma	Sudan	MH425468
35	*Madurella mycetomatis*	Mycetoma	Sudan	MH425469
35	*Madurella mycetomatis*	Mycetoma	Sudan	MH425470
37	*Madurella mycetomatis*	Mycetoma	Sudan	MH425471
38	*Madurella mycetomatis*	Mycetoma	Sudan	MH425472
39	*Madurella mycetomatis*	Mycetoma	Sudan	MH425473
40	*Madurella mycetomatis*	Mycetoma	Sudan	MH425474
41	*Madurella mycetomatis*	Mycetoma	Sudan	MH425475
42	*Madurella mycetomatis*	Mycetoma	Sudan	MH425476
43	*Madurella mycetomatis*	Mycetoma	Sudan	MH425477
44	*Madurella mycetomatis*	Mycetoma	Sudan	MH425478
45	*Madurella mycetomatis*	Mycetoma	Sudan	MH425479
46	*Madurella mycetomatis*	Mycetoma	Sudan	MH425480
47	*Madurella mycetomatis*	Mycetoma	Sudan	MH425481
48	*Madurella mycetomatis*	Mycetoma	Sudan	MH425482
49	*Madurella mycetomatis*	Mycetoma	Sudan	MH425483
50	*Madurella mycetomatis*	Mycetoma	Sudan	MH425484
51	*Madurella mycetomatis*	Mycetoma	Sudan	MH425485
52	*Madurella mycetomatis*	Mycetoma	Sudan	MH425486
53	*Madurella mycetomatis*	Mycetoma	Sudan	MH425487
54	*Madurella mycetomatis*	Mycetoma	Sudan	MH425488
55	*Madurella mycetomatis*	Mycetoma	Sudan	MH425489
56	*Madurella mycetomatis*	Mycetoma	Sudan	MH425490
57	*Madurella mycetomatis*	Mycetoma	Sudan	MH425491
58	*Madurella mycetomatis*	Mycetoma	Sudan	MH425492

59	*Madurella mycetomatis*	Mycetoma	Sudan	MH425493
60	*Madurella mycetomatis*	Mycetoma	Sudan	MH425494
61	*Madurella mycetomatis*	Mycetoma	Sudan	MH425495
62	*Madurella mycetomatis*	Mycetoma	Sudan	MH425496
63	*Madurella mycetomatis*	Mycetoma	Sudan	MH425497
64	*Madurella mycetomatis*	Mycetoma	Sudan	MH425498
65	*Madurella mycetomatis*	Mycetoma	Sudan	MH425499
66	*Madurella mycetomatis*	Mycetoma	Sudan	MH425500
67	*Madurella mycetomatis*	Mycetoma	Sudan	MH425501

Discussion:

Black-grain mycetoma constitute the most common type of mycetoma in Sudan where mycetoma is hyperendemic[17,18]. Phenotypic identification of mycetoma fungi does not ensure certainty[19]instead, molecular based methods delicately identify the causative agents to its strain level. In this study in order to obtain fungal genomic DNA rabidly, culture-free method with few modification of CTAB protocol was performed. CTAB method has been adapted to isolate fungal DNA from whole blood and skin biopsy materials with some modifications[20], but to our knowledge this study is the first trial deal with modified culture-free method for isolation of DNA of mycetoma fungi using big sample size of black-grain specimens.

The best time to collect fresh black-grain specimens is the time of surgical intervention because the complete excision of mycetoma lesion help in collection of sufficient grains in case of early infections or recurrences which are common. No standard amount of grains has been described for DNA isolation, so in this study we used black-grain specimens with different amount. During processing black-grains made a good contrast against excised biopsy and easy to collect. Clear DNA band was detected even with few number of black-grains (5 – 7 grains) which may enhance the sensitivity. Washing of grains several time to remove dead tissues and inflammatory exudates reduce the contamination during DNA extraction which may also result in good DNA quality[8]. Stored black-grains were aseptically homogenized using manual grinding without hazardous liquid nitrogen or glass beads. Resulted DNA pellet appeared clear after elusion step and only one specimen gave brown pigment which may indicate DNA contamination, and or/ inadequate purification due to the presence of melanin[8].

Our CTAB protocol yielded good sensitivity in isolation of the genomic DNA of eumycetoma fungi from black-grains which resulted in successful amplification of ITS region of 90.1 % (n=82/91) of extracted specimens using ITS4 and ITS5 primers, CTAB has ability to disrupt fungal cell wall as well as removing of polysaccharides; a PCR inhibitor[21].The negative results recorded with 9.9% (n=9/91) of black-grain specimens may occurred due to the losses of DNA during the purification step, and also knowing that, DNA contamination is another cause of PCR inhibition[22]. The variation in the concentration of yielded DNA because of either the amount of collected black-grains and or/the existent fungal elements.

The present study described a modified CTAB method for direct DNA extraction from black-grain specimens suitable for PCR of ITS using pan-fungal primers and nucleotide sequences identification. *Madurella mycetomatis* species-specific primers was used, beside diagnosis, for detection of DNA from soil and thorn specimens[23].In view of the above results DNA from black-grains could be testing for other molecular tagging such as RFLP and RAPD.

The only observed study dealt with small sample size of black-grains for DNA isolation use it within the biopsy specimens, CTAB method was used with glass beads and tissue lyser for homogenization[8].Our results of CTAB protocol achieve DNA with good quality and concentration and this not in agreement with that reported by Sarah and her colleagues[8]the main

differences between our studies which may contributed to this results are that we use black-grains alone and the manual homogenization instead of glass beads.

As conclusion, we recommend using of black-grain specimens for DNA extraction of mycetoma fungi parallel with culture to insure rabid diagnosis and identification.

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