

Original Article

Comparison of Different Methods for Extraction of Mitochondrial DNA from Human Pathogenic Yeasts

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SUMMARY: Methods of rapidly extracting chromosomal DNA from human pathogenic yeasts were used in mitochondrial DNA (mtDNA) studies. This paper is concerned with rapid and reliable methods of extracting mtDNA for sequence analysis for species or strain identification, and epidemiological study of medically important fungi and fungal infections. To determine the optimal method of mtDNA extraction without isolating mitochondria, we examined three commonly used methods: 1) boiling, 2) glass bead disruption, and 3) a commercially available kit. We assessed the amount and quality of DNAs using a spectrophotometer and specific polymerase chain reaction (PCR). The DNA yield depended on the extraction method used and the yeast species. An adequate amount of mtDNA was obtained with both glass beads and a commercially available kit to amplify the mitochondrial gene using PCR without isolating the mitochondria. These techniques are convenient for extracting DNA from a variety of small-scale samples.

INTRODUCTION

Opportunistic fungal infections have become an important medical problem in recent years because of the increasing number of immunocompromised patients (1,2). To control these life-threatening infections, quick and reliable methods of species or strain identification are essential.

During the past two decades many papers have been published on identification methods using molecular techniques (3), based on the sequences of nuclear DNA (nDNA). Previous studies using slow evolving nDNA were successful in comparing distantly related species; however, to identify closely related species or strains, mitochondrial DNA (mtDNA), is a target sequence candidate. Because of their rapid rate of evolutionary divergence, mtDNA have been used to identify individuals in the field of legal medicine and phylogeny in human evolution (4,5). Only limited sequences of mtDNA of medically important fungi are currently available, however (6,7).

The present paper is concerned with fast and reliable methods of extracting mtDNA for sequence analysis for species or strain identification, and also with an epidemiological study of medically important fungi and fungal infections. Due to the complexity, expense, and time cost involved with mitochondrial isolation, conventional methods of extracting mtDNA are inconvenient. We examined three commonly used quick methods of DNA extraction and evaluated the amount and quality of obtained by a spectrophotometer and specific polymerase chain reaction (PCR).

MATERIALS AND METHODS

Strains and DNA extraction: The strains used in this study are shown in Table 1; all strains were identified by their internal transcribed spacer 1 (ITS1) sequence (8). Genus *Malassezia* was grown at 32°C on slants of Leeming-Notman agar (9). *Candida* spp. and *Filobasidiella neoformans* (anamorph: *Cryptococcus neoformans*) were cultured on slants of YPD agar (2% peptone, 1% glucose and 0.5% yeast extract, 1.5% agar).

DNA was extracted from each strain using the following three common protocols for nDNA extraction. All procedures started with the same number of cells which were calculated using a hemacytometer. The number of loopful cells was approximately 2×10^8 .

Method 1 The first attempt to prepare DNA utilized a modified method of Makimura et al. (10). One loopful of cells was suspended in 100 μ l of lysis buffer 1 (200 mM Tris-HCl pH 7.5, 0.5% SDS, 30 mM EDTA), vortexed vigorously, and incubated at 100°C for 15 min. One hundred μ l of 2.5 M potassium acetate was added and the mixture was incubated at -20°C for 10 min and then centrifuged at $20,000 \times g$ for 10 min. The supernatant was extracted once with phenol:chloroform:isoamyl alcohol (P/C/I; 25:24:1) and subsequently

Table 1. Strains used in this research

Species	Strain	Note
<i>Malassezia furfur</i>	CBS 1878	Type strain
<i>Malassezia pachydermatis</i>	CBS 1879	Type strain
<i>Candida albicans</i>	TIMM 1768	Clinical isolate
<i>Candida tropicalis</i>	CN 02031401	Clinical isolate
<i>Filobasidiella neoformans</i>	CN 02032005	Clinical isolate

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extracted with chloroform:isoamyl alcohol (C/I; 24:1). Each sample was precipitated with one volume of cold 2-propanol and centrifuged at $20,000 \times g$ for 10 min. The DNA was washed with 70% ethanol, air-dried, resuspended in 100 μ l of Tris EDTA (10 mM Tris-HCl, 1 mM EDTA pH 8.0), and stored at -20°C until analyzed.

Method 2) The second method used glass beads for cell disruption (11,12). Cells were suspended in 400 μ l of lysis buffer 2 (100 mM Tris-HCl pH 8.0, 1.0 % SDS, 2.0 % Triton X-100, 10 mM EDTA, 100 mM NaCl) and vortexed. After adding 400 μ l of P/C/I, 400 mg of glass beads (0.5 mm in diameter) were added to disrupt the cells. The samples were mixed vigorously for 15 min, centrifuged at $20,000 \times g$ for 10 min, and the aqueous layer was then collected. It was extracted once with an equal volume of C/I. The subsequent steps were the same as those described in method 1.

Method 3) The third method utilized a commercial kit to extract DNA, the Gen Toru-Kun Kit (GenTLE) for yeast (Takara Bio, Shiga). One loopful of cells from each slant was added to 400 μ l of GenTLE Yeast Solution I and suspended well. Subsequent steps were according to the manufacturer's instructions.

Evaluation of nucleic acids: Each DNA solution was diluted with distilled water and its UV absorption spectrum was assayed using a Beckman Du 650 spectrometer (Beckman Coulter, Fullerton, Calif., USA). We estimated the concentration of nucleic acids based on the absorbance of 260 nm, and A260/A280 was calculated to evaluate the quality of extracted DNA. The suitability of DNA for a PCR template was compared among the three methods, using 1, 10, and 100 ng of nucleic acids.

Primers for PCR: Three primer-pairs were used in this study, two from mtDNA sequences and the other from nDNA. Oligonucleotide primers were designed as follows: (i) a large subunit of ribosomal region in mtDNA (mtLrRNA) (13): forward (ML7), 5'-GACCCTATGCAGCTTCTACTG-3', reverse (ML8), 5'-TTATCCCTAGCGTAACTTTTATGC-3'; (ii) cytochrome b gene in mtDNA (cyt b) (7): forward (E1mr2), 5'-TGRGGWGCWACWGTWATTACTAAYT-3', reverse (E2mr4), 5'-AGCACGTARWAYWGCRTAWAHGG-3', (iii) an ITS1 in nDNA (8): forward (18SF1): 5'-AGGTTT CCGTAGGTGAACCT-3', reverse (58SR1): 5'- TTCGCTGC GTTCTTCATCGA-3'.

PCR: Each PCR contained 10 μ l of diluted template (1, 10 or 100 ng), 0.5 units of Taq DNA polymerase, 30 pM of each primer, 100 μ M of each deoxynucleoside triphosphate, and 10 μ l of $10 \times$ PCR buffer (Amersham Biosciences, Piscataway, N.J., USA) in a total volume of 100 μ l. DNA amplifications were carried out in a PCR thermal cycler MP (Takara Bio). The following PCR conditions were used to amplify mtLrRNA and ITS1: 94°C for 4 min, followed by 30 cycles of 1 min at 94°C , 2 min at 55°C , 1.5 min at 72°C with a final extension of 10 min at 72°C . Amplification for cyt b was performed for initial denaturation at 94°C for 2 min, followed by 30 cycles with denaturation at 94°C for 1 min, annealing at 48°C for 2 min, and extension at 72°C for 2 min. After the last cycle, final extension was performed at 72°C for 10 min. Ten μ l of amplified solution was electrophoresed through a 2% agarose gel in TAE buffer and visualized by ethidium bromide staining under UV irradiation.

RESULTS

The DNA yield depended on the extracting method and

Table 2. Comparison of DNA yields of the three extraction methods

Method	Basidiomycota	Ascomycota
Boiling	$30.36 \pm 9.95 \mu\text{g}$	$49.60 \pm 21.6 \mu\text{g}$
Glass beads	$12.87 \pm 6.35 \mu\text{g}$	$38.47 \pm 24.77 \mu\text{g}$
GenTLE	$1.94 \pm 1.85 \mu\text{g}$	$19.45 \pm 16.57 \mu\text{g}$

species. The amount of nucleic acids was 100 ng-50 μ g. DNA from basidiomycetous species, *Malassezia* spp. and *F. neoformans*, was less than that from ascomycetous species, *Candida* spp., with these results being independent of the method used (Table 2). The protocol using GenTLE (method 3) was the quickest and simplest procedure, but the yield of each species was lowest, especially basidiomycetous species, which yielded only 1/15th of that by the boiling method (method 1). Nonetheless, all three techniques provided an adequate quantity.

PCR analyses were performed using three primer sets employing different quantities of the DNA as a template to test the quality of DNA. Figure 1 shows the ethidium bromide-stained gel images of the mtLrRNA and ITS1 amplicons. When PCR was performed with a mitochondrial specific primer set, ML7 and ML8, the amplified products differed in amount. The amount of PCR products also depended on the method of DNA extraction. Although the yield of total nucleic acids was relatively high, the boiling method often led to a failure of amplification of mitochondrial gene products. Even 100 ng of template DNA extracted using the boiling method was not enough for mtDNA amplification, especially for *F. neoformans*. When the template DNA using the boiling method was 1 ng, no amplification product was observed using mitochondria-specific primers. In spite of this, 100 ng of DNA

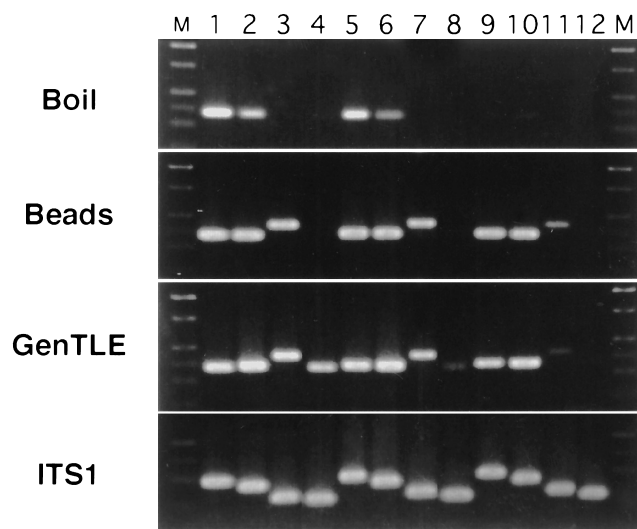


Fig. 1. Sensitivity of the PCR using two primer sets. Extracted DNAs were diluted in water and PCR was performed with 1, 10, or 100 ng of DNA template from four yeasts. The amplification patterns obtained using the mtLrRNA and the ITS1 primer sets are shown. The upper three were PCR products amplified using the ML7/ML8 primer set. Templates for PCR assay were extracted by means of three methods: boiling, beads, or GenTLE, respectively. The lowest was amplified using the ITS1 primer set. The template DNA was extracted by boiling. Lanes 1-4: 100 ng DNA as a template; 5-8: 10 ng DNA as a template; 9-12: 1 ng DNA as a template. M: DNA marker. 1, 5, 9: *Malassezia furfur* CBS 1878; 2, 6, 10: *M. pachydermatis* CBS 1879; 3, 7, 11: *Candida albicans* TIMM1768; 4, 8, 12: *Filobasidiella neoformans* CN02032005.

Table 3. Summary of the three extraction methods

Method	Yield		Time*	A260/A280	Cost/sample
	Total DNA	PCR sensitivity of mtDNA primers			
Boiling	high	less sensitive	60 min	2.0-2.1	< 1 yen
Glass beads	high	sensitive	90 min	2.0-3.0	<10 yen
GenTLE	low	sensitive	60 min	1.1-1.9	360 yen

*Average lengths of preparation time to extract DNA from eight fungal samples.

obtained by the two other extraction methods worked successfully as a PCR template, and even 1 ng of DNA obtained by those methods gave good results. When template quantities were increased, the amount of ML7/ML8 PCR products also tended to become greater. No PCR products were amplified with the mtLrRNA PCR primers for any DNA isolated from *C. tropicalis* (data not shown), however. Therefore an additional PCR assay was done in order to evaluate the three extraction methods. DNAs (*M. furfur*, *M. pachydermatis*, and *C. tropicalis*) were amplified using cyt b primers; these primers are also mitochondrial-specific. When PCR was performed using cyt b primers, the amount of PCR products depended on the extraction method, and this was the same as when using mtLrRNA primers. As mentioned above, the amount of PCR product increased in proportion to the quantities of template (data not shown). On the other hand, PCR performed using a nDNA-specific primer set, 18SF1 and 58SR1, showed a clear single band in each sample lane. The amount of PCR product does not depend on the method of DNA extraction or the concentration of DNA. PCR with the primer pair 18SF1 and 58SR1 is able to amplify products when the template DNA is 1 ng. All PCR including *C. tropicalis* as a template worked successfully with ITS1 PCR primers. A summary of the three extraction methods is shown in Table 3.

In addition to the five medically important yeast-like fungi mentioned above, DNA which worked as a template for mitochondrial-specific primer sets has been successfully obtained using glass beads and/or GenTLE from more than 20 species including *Aspergillus fumigatus*, *A. niger*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *Geotrichum candidum*, *Schizosaccharomyces pombe*, and *Trichosporon asahii* (data not shown).

DISCUSSION

PCR has several potential clinical roles in the identification of fungal infections. Several typing methods able to differentiate fungal isolates have recently been applied as epidemiological tools (14). During the past two decades, sequence data have been gathered on fungi from nDNA; however, there has been little from mtDNA. One reason for this is the difficulty in obtaining mtDNA from fungi. The key to amplifying a target sequence by PCR is obtaining an ample quality of DNA. Some reports have described obtaining mtDNA using techniques based on the preparation of spheroplasts or isolation of mitochondria (6,15,16), followed by mtDNA isolation. However, these techniques cannot be used for routine analysis or diagnosis because they require a relatively large amount of cells and are time-consuming, labor-intensive, and expensive.

In the present study, rapid DNA extraction methods for chromosomal DNA were adapted for mtDNA extraction. To

select an optimal means of quick and reliable extraction of mtDNA for PCR analysis, we used three different methods of DNA isolation from medically important yeast-like fungi: all were originally established for chromosomal DNA extraction. A fundamental requirement for PCR detection is DNA of sufficient quality and quantity. We obtained nucleic acid fractions which included nDNA, mtDNA, and RNA; therefore, the ratio of mtDNA to nDNA might have been dependent on the extraction method used. Since the amount of ML7/ML8 PCR products tended to become greater when quantities of template were increased, this speculation was supported. DNA using the boiling method might also include less mtDNA.

None of the DNAs from *C. tropicalis* have been amplified using the ML7/ML8 primers. We tested *C. tropicalis* and several species of DNA using 38 mitochondrial specific primer sets (7,13,17) including ML7/ML8 and E1mr2/E2mr4 in order to amplify the target sequence (data not shown). When the template DNA was *M. furfur*, PCR products were obtained with 20 primer sets. However, when DNA was extracted from *C. tropicalis* using the glass beads or the GenTLE extraction method as a template, seven primer sets worked. DNA of *C. tropicalis* obtained using method 1 was amplified by only two primer sets. Nonetheless, no PCR products were amplified with ML7/ML8 primers using two species, *C. tropicalis* and *S. pombe* DNA. The sequences of the ML7 or ML8 primer region of these strains differ from other species; therefore, amplification reactions could not be performed successfully. As *C. tropicalis* could be amplified using a few of these sets, not only ML7 or ML8 but the sequence of various universal fungal primers for the mitochondrial ribosomal RNA region might be not conserved in the sequence of *C. tropicalis*.

In conclusion, these results show that we can obtain adequate mtDNA using glass beads and GenTLE to amplify the mitochondrial gene by PCR without isolating mitochondria. The sequences of mtLrRNA amplicons were confirmed and will appear elsewhere. These methods are also convenient for the extraction of DNA from many different small-scale samples. All extraction methods can be completed within 90 min. Even when many fungal isolates were extracted simultaneously, there was little increase in processing time.

Further work is in progress to analyze mtDNA sequences in various species and strains of fungi, and these results will be reported in another paper.

REFERENCES

1. Pinner, R. W., Teutsch, S. M., Simonsen, L., Klug, L. A., Graber, J. M., Clarke, M. J. and Berkelman, R. L. (1996): Trends in infectious diseases mortality in the United States. *JAMA*, 275, 189-193.
2. Simonsen, L., Conn, L. A., Pinner, R. W. and Teutsch, S. (1998): Trends in infectious disease hospitalizations in

- the United States, 1980-1994. Arch. Intern. Med., 158, 1923-1928.
3. Iwen, P. C., Hinrichs, S. H. and Rupp, M. E. (2002): Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med. Mycol., 40, 87-109.
 4. Linch, C. A., Whiting, D. A. and Holland, M. M. (2001): Human hair histogenesis for the mitochondrial DNA forensic scientist. J. Forensic Sci., 46, 844-853.
 5. Garritsen, H. S., Hoerning, A., Hellenkamp, F., Cassens, U., Mittmann, K. and Sibrowski, W. (2001): Polymorphisms in the non-coding region of the human mitochondrial genome in unrelated plateletapheresis donors. Br. J. Haematol., 112, 995-1003.
 6. Sanson, G. F. and Briones, M. R. (2000): Typing of *Candida glabrata* in clinical isolates by comparative sequence analysis of the cytochrome c oxidase subunit 2 gene distinguishes two clusters of strains associated with geographical sequence polymorphisms. J. Clin. Microbiol., 38, 227-235.
 7. Biswas, S. K., Yokoyama, K., Nishimura, K. and Miyaji, M. (2001): Molecular phylogenetics of the genus *Rhodotorula* and related basidiomycetous yeasts inferred from the mitochondrial cytochrome b gene. Int. J. Syst. Evol. Microbiol., 51, 1191-1199.
 8. Makimura, K., Mochizuki, T., Hasegawa, A., Uchida, K., Saito, H. and Yamaguchi, H. (1998): Phylogenetic classification of *Trichophyton mentagrophytes* complex strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J. Clin. Microbiol., 36, 2629-2633.
 9. Leeming, J. P. and Notman, F. H. (1987): Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. J. Clin. Microbiol., 25, 2017-2019.
 10. Makimura, K., Murayama, S. Y. and Yamaguchi, H. (1994): Detection of a wide range of medically important fungi by the polymerase chain reaction. J. Med. Microbiol., 40, 358-364.
 11. van Burik, J. A., Schreckhise, R. W., White, T. C., Bowden, R. A. and Myerson, D. (1998): Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med. Mycol., 36, 299-303.
 12. Sambrook, J. and Russell, D. W. (2001): Rapid isolation of yeast DNA. p. 6.31-6.32. In J. Sambrook and D.W. Russell (eds.), Molecular Cloning, a Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, New York.
 13. White, T. J., Bruns, T. D., Lee, S. and Taylor, J. W. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315-322. In M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego.
 14. Franzot, S. P., Hamdan, J. S., Currie, B. P. and Casadevall, A. (1997) : Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both local genetic differences and a global clonal population structure. J. Clin. Microbiol., 35, 2243-2251.
 15. Wang, L., Yokoyama, K., Miyaji, M. and Nishimura, K. (1998): The identification and phylogenetic relationship of pathogenic species of *Aspergillus* based on the mitochondrial cytochrome b gene. Med. Mycol., 36, 153-164.
 16. Xu, J., Vilgalys, R. and Mitchell, T. G. (2000): Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. Mol. Ecol., 9, 1471-1481.
 17. Bruns, T. D., Szaro, T. M., Gardes, M., Cullings, K. W., Pan, J. J., Taylor, D. L., Horton, T. R., Kretzer, A., Garbelotto, M. and Li, Y. (1998): A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Mol. Ecol., 7, 257-272.