Benchmarks

DNA Analyses from Biological Material Previously Used for Scanning Electron Microscopy Studies

BioTechniques 27:274-276 (August 1999)

DNA from different types of biological material can be obtained through a range of sources, from living and from dead tissues. Only a minimal amount of material is required for a standard polymerase chain reaction (PCR) analysis of a particular DNA sequence. However, during studies of extremely scarce material, the investigator might have to choose whether the material is better utilized for DNA studies or for other analytical techniques such as electron microscopy scanning of the surface structure. Instead of making these choices, it would be desirable to combine the techniques, and thus be able to analyze the DNA from the very objects that were documented with a microscope. Taxonomic-type specimens, archeologic and forensic case samples are examples of valuable material for which combinations of techniques could be useful.

Biological material assigned for scanning electron microscopy (SEM) studies often requires pretreatments such as fixation and absolute dehydration before the surface is coated with a conductive agent to withstand the vacuum environment and the electron beam in the microscope. In these cases, the aldehyde fixatives can degrade the DNA in the material, which then could not be used for subsequent DNA analyses. In other cases, when the material is hard or thick-walled, and basically free from water, the specimen only needs to be coated with a conductive layer before the SEM studies. For instance, this can apply to fungal spores, plant pollen, hairs and bones. I speculated whether it would be possible to extract and sequence DNA from such material, previously studied in SEM.

Many fungi possess spores with an ornamented surface. This character has been widely used in classification and identification of different species, and

Table 1. Spores from Fungal Species Studied

Species	Collection	GenBank Accession No.
<i>Trichophaeopsis bicuspis</i> (Boud.) Korf & Erb	1992.10.17., RK 92.08	Identical to U53391
<i>Lamprospora kristiansenii</i> Benkert	1997.10.11., RK 97.45	AF121075
<i>Ascobolus denudatus</i> Fr.	1998.07.06., RK 98.51	AF121076
<i>Ascobolus viridis</i> Currey	1998.06.13., RK 98.29	AF121077

Spores from the following species were first studied in the scanning electron microscope and subsequently used for DNA extraction, PCR amplification and sequencing of partial SSU rDNA gene. All fungal specimens were collected in Østfold, Norway, and are stored in the private herbarium of R. Kristiansen, Norway (RK).

Figure 1. DNA from spores previously studied in the SEM yielded SSU rDNA sequences that were identical or similar to other reported sequences from the same species or genus. Bars = 5 µm.

descriptive works on fungal taxa often include SEM images of the spores. For SEM studies, the spores can be collected onto microscope coverslips, which can be stored for an extended time before the spores are studied in the electron microscope. Here, I report successful extraction and sequencing of DNA from fungal spores from the order Pezizales (Ascomycota), which previously had been coated with gold-palladium and studied in SEM.

Discharged ascospores (ca. 30 to several 100 spores) were collected on glass coverslips at the time of collection. The coverslips were mounted by double-sided conductive tape onto aluminum stubs. The specimens were coated by gold-palladium in a Model E5000 Diode Sputter (Polaron Instruments Ltd., London, England, UK) before examination in a JSM T20 Scanning Electron Microscope (JEOL,

Tokyo, Japan) at 80 kV. After examination, the coverslips were carefully removed from the tape and put into separate microcentrifuge tubes, to which 200 µL DNA lysis buffer were added. Two different DNA extraction methods were tested: (i) the Dynabeads® DNA Direct™ Kit (Dynal AS, Oslo, Norway) (*Trichophaeopsis bicuspis* and *Lamprospora kristiansenii*) and (ii) the QIAamp® Tissue Kit (Qiagen, Mississauga, ONT, Canada) (*Ascobolus denudatus* and *A. viridis*). The samples (one coverslip per fungal specimen) were ground using a pestal while in the cell lysis buffer, and the subsequent DNA extraction procedures followed the instructions of the respective kits.

The nuclear ribosomal small subunit DNA (SSU rDNA) gene from the different samples were amplified using Ready-To-Go® PCR Beads (Amersham Pharmacia Biotech, Uppsala,

Sweden) and the primers NS1 and NS8 (5). The PCR parameters included an initial 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 45 s and a final extension at 72°C for 7 min. Three microliters of the PCR products were visualized by agarose electrophoresis and ethidium bromide staining. The products, if not well visible on the agarose gel (PCR product concentration less than ca. 10 ng/µL), were purified and diluted 300× and re-amplified before sequencing, using the same PCR settings and the amplification primers NS1 and MB2 (5'-gga gta tgg tcg caa ggc tg-3'; Mary Berbee, personal communication). PCR products chosen for re-amplification or for direct sequencing were purified with QIAquick™ PCR Purification Kit (Qiagen) and eluted in 30 µL of elution buffer. To obtain approximately 1100 bp from both strands, NS1, NS2

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(5), NS19 (2) and MB2 were used as primers for sequencing with AmpliTaq® DNA Polymerase FS Dye Terminator Cycle Sequencing (PE Biosystems, Foster City, CA, USA) (25 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 45 s). The products were ethanol-precipitated and analyzed on an ABI PRISM® 377 DNA Automatic Sequencer (PE Biosystems).

The sequences obtained were manually aligned with 17 homologous sequences from GenBank® (acknowledged with species name and accession number in Figure 1). This matrix was subjected to a parsimonious, heuristic, phylogenetic analysis [PAUP 4.0b1 (4)] including a bootstrap test of 1000 replicates. In the resulting tree, all sequences from the specimens studied in SEM grouped with previously reported sequences from other members of the

same species or genus, with high bootstrap supports (77%–100%) (Figure 1). The partial SSU rDNA sequence from the *T. bicuspis* spores were identical with the reported sequence from the same species. The qualities of the obtained sequences were generally good, but ambiguous nucleotides (detected as multiple peaks in the chromatographs) occasionally showed up in the re-amplified material.

The spores of *T. bicuspis* were collected more than six years ago, but I had no difficulty obtaining the DNA from that material. The spherical spores from this species possess rather thin walls, as compared to those from *Ascobolus* and *Lamprospora*, and they collapse easily in the scanning microscope, as can be seen in Figure 1. However, even though this does affect the SEM image of the spore morphology, I could not detect any effect on the amplification and sequencing performances of the DNA.

It can be difficult to obtain DNA from thick-walled spores, as they do not easily disrupt in the lysis step of the DNA extraction, and the spores are often small enough to escape the effect of mechanical grinding. Several methods have been published on DNA extractions from thick-walled spores (1,3). I used standard methods to extract the DNA from all fungal specimen used in the experiments here, even though at least the spores from *Ascobolus* are considered thick-walled. Overall, the amplification of the DNA often resulted in rather weak PCR products, which then had to be re-amplified to produce a PCR product that could be directly sequenced. The use of a method that is optimized to break the spore walls would likely increase the yield of DNA.

This study shows that fungal spores coated with gold-palladium and studied in a scanning electron microscope are suitable for subsequent DNA analyses. It should be possible to apply the described technique not only to fungal spores but to any biological material that does not require fixation and rehydration before SEM analyses. Molecular biologists and electron microscopists could thus benefit from keeping each other in mind when handling their specimens. The method is certainly useful when the studied mate-

rial is scarce, but might also help the investigator to ensure that the material is free from contaminants. This can be especially crucial in forensic investigations and analyses of ancient material. In such studies, bones, teeth and roots of hairs and furs are routinely utilized as a source of DNA and are at the same time tissues that can be informative when studied by SEM.

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Thanks to R. Kristiansen for the gift of the fungal specimens and for determination of the same. I am grateful to Dr. Moss, Portsmouth University, England, for providing SEM facilities and to Prof. Berbee, University of British Columbia, Canada, for providing DNA analyses facilities. The work was supported by a grant from STINT, Sweden. Address correspondence to Dr. Sara Landvik, Department of Ecology and Environmental Science, Umeå University, SE-90187 Umeå, Sweden. Internet: sara.landvik@eg.umu.se

Received 28 January 1999; accepted 26 April 1999.

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