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Back to basics: an evaluation of NaOH and alternative rapid DNA extraction protocols for DNA barcoding, genotyping, and disease diagnostics from fungal and oomycete samples

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Abstract

The ubiquity, high diversity and often-cryptic manifestations of fungi and oomycetes frequently necessitate molecular tools for detecting and identifying them in the environment. In applications including DNA barcoding, pathogen detection from plant samples, and genotyping for population genetics and epidemiology, rapid and dependable DNA extraction methods scalable from one to hundreds of samples are desirable. We evaluated several rapid extraction methods (NaOH, Rapid one-step extraction (ROSE), Chelex 100, proteinase K) for their ability to obtain DNA of quantity and quality suitable for the following applications: PCR amplification of the multicopy barcoding locus ITS1/5.8S/ITS2 from various fungal cultures and sporocarps; single-copy microsatellite amplification from cultures of the phytopathogenic oomycete *Phytophthora ramorum*; probe-based *P. ramorum* detection from leaves. Several methods were effective for most of the applications, with NaOH extraction favored in terms of success rate, cost, speed and simplicity. Frozen dilutions of ROSE and NaOH extracts maintained PCR viability for over 32 months. DNA from rapid extractions performed poorly compared to CTAB/phenol-chloroform extracts for TaqMan diagnostics from tanoak leaves, suggesting that incomplete removal of PCR inhibitors is an issue for sensitive diagnostic procedures, especially from plants with recalcitrant leaf chemistry. NaOH extracts exhibited lower yield and size than CTAB/phenol-chloroform extracts; however, NaOH extraction facilitated obtaining clean sequence data from sporocarps contaminated by other fungi, perhaps due to dilution resulting from low DNA yield. We conclude that conventional extractions are often unnecessary for routine DNA sequencing or genotyping of fungi and oomycetes, and recommend simpler strategies where source materials and intended applications warrant such use.

Keywords: microsatellite genotyping, Moorea Biocode Project, *Phytophthora ramorum*, plant pathology, rDNA internal transcribed spacer, Sudden Oak Death

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Introduction

Fungi are nearly ubiquitous constituents of ecosystems as saprotrophs, pathogens, mutualists, and commensal symbionts, and play important roles in local and global nutrient cycles. Because fungi often grow within substrates (soil, plant tissues, etc.) without producing distinguishing morphological characters, and those isolated in culture may grow without producing spores or other distinguishing features, molecular methods have become the standard means of identifying specimens in many contexts. Applications such as DNA barcoding, molecular genotyping and disease diagnosis often require high-throughput approaches to data acquisition, and DNA extraction is arguably the primary rate-limiting step in laboratory workflows.

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A typical DNA extraction protocol involves suspending macerated tissue in a buffer containing a detergent to disrupt the cell and nuclear membranes, treatment with phenol and chloroform to remove contaminating proteins, ethanol precipitation with high salt, ethanol washing, and resuspension in a buffered solution. Although yielding DNA with high purity, such a protocol is time-consuming and requires the use of hazardous chemicals. Methods that minimize time input while simultaneously reducing cost and environmental impact are desirable where they can be used without compromising the accuracy of downstream processes. A number of rapid extraction protocols have been developed for plant

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tissues (having cellulose-rich cell walls) and bacteria (having peptidoglycan-rich cell walls); these protocols may also be applicable to fungi (with chitin-rich cell walls). Dentinger *et al.* (2010) evaluated two methods for high-throughput extraction of fungal DNA from mushroom specimens. The first consisted of sample storage on Whatman FTA® cards followed by extraction using a commercial (Sigma Extract-N-Amp™ Plant PCR) kit; this procedure yielded high-quality DNA and has the advantage of room temperature storage of the original specimen on the cards, but requires the purchase of two commercial products, thereby adding expense. A similar method but using Whatman's recommended extraction protocol was successfully used for yeasts and filamentous fungal cultures by Borman *et al.* (2006). The second method was a modification of the glass fiber filter extraction of Ivanova *et al.* (2006), consisting of an overnight incubation in lysis buffer containing proteinase K followed by a binding step, wash step using a protein wash buffer, two washes using wash buffer, and elution. While this procedure is low-cost compared to commercial kits and yields high-quality DNA, the overnight incubation and five liquid additions make this procedure somewhat laborious and time-consuming.

For many applications, obtaining a rapid molecular identification may be of higher importance than obtaining the purest or highest-yield extracts possible. Therefore, it is useful to determine those sample types and applications for which a rapid, cruder extract may be sufficient, and to determine protocols that yield a high rate of success. The goal of the present study was to survey previously published – but often neglected – rapid extraction protocols on an increased number of sample types, loci and downstream applications than have been examined in previous studies related to DNA barcoding, molecular diagnostics, and genotyping of fungi and oomycetes. We determined that several of these methods performed well for most, but not all, of our sample types and applications. Our results validate rapid extraction, particularly an alkaline (NaOH) method, for obtaining molecular barcode or genotype data from a variety of fungal and oomycete sample types while simultaneously reducing costs and increasing throughput.

Materials and methods

Comparison of rapid extractions for PCR amplification from lyophilized mycelial cultures

Two tests comparing several rapid extraction protocols on lyophilized fungal mycelial cultures were conducted. In the first, DNA was extracted from cultures of tropical foliar endophytic fungi from Moorea, French Polynesia putatively assignable to the orders Xylariales, Diaporthales, Eurotiales, Capnodiales, and Hypocreales. Cultures obtained from surface-sterilized leaf tissue were subcultured on small discs of sterile nitrocellulose filter paper overlain on 1.5% malt extract plates. The filter papers were harvested, placed in microfuge tubes, lyophilized, and pulverized using a mixer mill. Thirty cultures were randomly assigned to each of the following extraction protocols:

1. Rapid one-step extraction (ROSE); Steiner *et al.* 1995). 200 μL extraction buffer (10 mM Tris-HCl, pH 8.0; 312.5 mM EDTA, pH 8.0; 1% sodium lauryl sarkosyl; 1% water insoluble PVPP) were added to the ground tissue. The mixture was incubated at 90 °C for 20 min, then placed on ice for 5 min. 10 μL of the extract were diluted in 1690 μL of sterile ddH_20, and 5 μL of the dilution were used as the DNA template in a 25 μL PCR reaction.
2. Chelex 100 extraction (de Lamballerie *et al.* 1992). 300 μL extraction buffer (10% w/w Chelex 100 (sodium form, 100–200 mesh); 0.1% w/v SDS; 1% v/v NP40; 1% v/v Tween 20) were added to the ground tissue. The mixture was incubated at 100 °C for 30 min and centrifuged for 5 min at 13 200 ***g***; the supernatant was removed and adjusted to a final concentration of 10 mM Tris-HCl and 1 mM EDTA, and 2.5 μL were used as template DNA.
3. Proteinase K extraction (Kawasaki 1990; Hiraishi 1992 modification). 200 μL of extraction buffer (40 mM Tris, pH 8.0; 1% Tween 20; 0.2% Nonidet P-40; 0.2 mM EDTA) and 40 μL of proteinase K solution (1 mg/mL) were added to the ground tissue. The mixture was incubated at 60 °C for 20 min, then at 95 °C for 10 min, centrifuged, and 1.25 μL of the supernatant were used as template DNA.
4. NaOH extraction (Wang *et al.* 1993). 200 μL of 0.5 M NaOH were added to the ground lyophilized tissue. 5 μL of the extract were diluted in 495 μL of 100 mM Tris-HCl, pH 8.0, and 1 μL of the dilution were used as template DNA.

For all procedures tested, the amount of buffer was increased if a sample absorbed all of the liquid. Original extracts were stored either at 4 °C or −20 °C; dilutions were stored at −20 °C. PCR reactions were conducted using the primers ITS1F (Gardes & Bruns 1993) and TW13 (White *et al.* 1990), amplifying the ITS1 + 5.8S + ITS2 portion of the nuclear ribosomal DNA repeat region – the proposed official DNA barcoding region for fungi (Schoch *et al.* 2012) – and an additional portion of the ribosomal large subunit gene. PCR reaction mixtures were prepared in 25 μL volumes including 5 μL 5× PCR buffer (GoTaq Flexi; Promega Inc., Madison, WI, USA), 2.5 μL dNTPs (2 mM/L), 2.5 μL

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bovine serum albumin (BSA) (2.5 mg/mL), 2 μL $MgCl_2$ (25 mM/L), 1 μL each primer (10 μM/L), 0.2 μL GoTaq Flexi DNA polymerase (Promega) (5 U/μL), template DNA in the amount noted for each protocol above, and sterile ddH_2O to reach 25 μL total. Thermocycling conditions followed Taylor *et al.* (2008).

In the second test, 10 cultures of the phytopathogenic basidiomycete *Heterobasidion irregulare* were grown in 1.5% malt extract broth, harvested by filtration, divided into three equal portions, lyophilized, and pulverized. The three replicate samples from each culture were assigned randomly to extraction by the ROSE, proteinase K, or NaOH procedures; extractions and PCR amplifications from the extracts using the primers ITS1F and TW13 were conducted as described above.

Comparison of rapid extractions for PCR amplification from dried sporocarps

Rapid extraction methods were tested using dried specimens of tropical sporocarps collected from Moorea, French Polynesia. Ten accessions were used: four agarics (*Galerina* sp., *Hypholoma* sp., *Pleurotus* sp., *Lentinus* sp.), three polypores (*Ganoderma* sp. and two unidentified taxa), one heterobasidiomycete (*Auricularia polytricha*), one resupinate basidiomycete, and one pezizomycete (Ascomycota). A small piece (8–64 cubic mm) of tissue was removed from each fresh sporocarp using a sterilized forceps. The tissue was lyophilized, then pulverized using a mixer mill. Three replicate samples were taken from each sporocarp and were assigned at random to extraction with either the ROSE, proteinase K, or NaOH protocols, followed by PCR amplification as described above.

Use of NaOH extracts for amplification of microsatellite loci from lyophilized cultures

Cultures of the phytopathogenic oomycete (Chromalveolata, Stramenopila) *Phytophthora ramorum* — the etiological agent of Sudden Oak Death — were isolated from rivers by stream baiting with *Rhododendron* leaves (Themann *et al.* 2002) followed by surface sterilization of the leaves, plating of the margins of symptomatic lesions on selective PARP agar (Erwin & Ribeiro 1996; Vettraino *et al.* 2009) and subculturing in 12% pea broth medium (Trione 1974; modified to 120 g frozen peas/L). Mycelia were harvested by filtration, lyophilized, and pulverized, then DNA was extracted using the NaOH protocol. Two microsatellite loci, M18 and M39, were amplified using primers and PCR conditions described by Ivors *et al.* (2006) and Prospero *et al.* (2007), respectively. Amplicon sizes were assessed by agarose gel electrophoresis and compared to the expected sizes of 218–278 bp for M18 (Ivors *et al.* 2006) and 129 and 248–252 bp for M39 (Prospero *et al.* 2007).

Comparison of ROSE and CTAB/phenol-chloroform extracts from symptomatic tanoak leaf tissue for PCR diagnostic identification of Phytophthora ramorum *infection*

DNA from asymptomatic leaves of tanoak (*Notholithocarpus densiflorus* — a *Phytophthora ramorum* host with sclerophyllous leaves from which DNA extraction is difficult) was extracted by both the ROSE and a CTAB-phenol-chloroform (PC) method (Ivors *et al.* 2004), in nine replicate extractions per method. The plant extracts were diluted as described for ROSE, or 100-fold in PCR water for PC. Extracts were then spiked with 5 pg of *P. ramorum* DNA, an amount approaching the lower limit for consistent detection using this method (Hayden *et al.* 2006). The plant extracts with added *P. ramorum* were amplified with a single round of diagnostic qPCR as described in Hayden *et al.* (2006), with or without addition of 3.75 ng of BSA. A dilution series of *P. ramorum* DNA ranging from 0.5 pg to 500 pg was amplified in quadruplicate in the same plate as a quantification standard. Observed starting quantities of pathogen DNA were estimated in each sample from the standard series and compared to the known value. Detection success was compared between treatments using a nominal logistic fit of detection by extraction method, addition of BSA, and the interaction of these factors using JMP v9 (SAS Institute, Cary NC).

To test whether additional post-extraction cleanup would improve *P. ramorum* detection in tanoak extracts, ROSE extractions of an additional five tanoak leaves were performed. From each sample three pieces, each 35–61 mg and including a portion of the tough midrib, were extracted using the ROSE protocol with 500 mL buffer. From each ROSE extract, 10 mL were diluted as previously described and the remaining extract was further purified using the OneStep^{TM} PCR Inhibitor Removal Kit (Zymo Research Corp.), followed by 100-fold dilution in PCR water. Pathogen detection and accurate quantification of 5 pg *P. ramorum* DNA were performed as described above, and compared to that in five PC extracts of tanoak leaf and midrib.

An additional test was conducted using leaves of California bay laurel (*Umbellularia californica*), another major host for *P. ramorum* with less recalcitrant leaf chemistry than tanoak (Hayden *et al.* 2004; Vettraino *et al.* 2009). Five samples shown by culture assay to be positive for *P. ramorum* infection were tested. From each sample, 3 pieces totaling 75 mg of leaf tissue were extracted in 500 mL ROSE buffer, with and without OneStep^{TM} column cleanup. Detection of *P. ramorum* DNA in the

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samples was assessed using the nested TaqMan qPCR assay described by Hayden *et al.* (2006), with the addition of 3.75 ng BSA to the first-round reaction. In all PCR tests, three negative controls were included with each reaction plate. The inclusion of a nested pre-amplification step in addition to the TaqMan PCR increases the assay sensitivity, but precludes template quantification.

Longevity of rapid extracts

Two trials were conducted to evaluate the longevity of rapid extracts. In the first, NaOH or ROSE extracts, either refrigerated or frozen for a period of 32 months (Table 1), were PCR amplified using the primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) to amplify the ITS1 + 5.8S + ITS2 rDNA region. The NaOH and ROSE tests used extracts from the *Heterobasidion irregulare* test described above; in addition, NaOH extracts from samples of the whitefly *Bemisia tabaci* infected with an entomopathogenic fungus were tested. PCR reaction mixtures were prepared as in the test using lyophilized mycelia, with the exception of the difference in primers. For original extracts, aliquots were removed and diluted 1:100 (NaOH) or 1:170 (ROSE) prior to use in PCR reactions in order to match the concentration of the stored dilutions. Thermocycling conditions consisted of an initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 30 s/55 °C for 30 s/72 °C for 1 min, and a final elongation at 72 °C for 7 min. Amplification success was evaluated using agarose gel electrophoresis.

In the second trial, NaOH extracts from *Phytophthora ramorum* cultures were tested for longevity after various storage times. Longevity was assessed using PCR amplification of the microsatellite loci M18 and M39 with culture, extraction, and PCR conditions as described above. Randomly selected 1:100 dilutions stored at −20 °C for approximately 6 months, approximately 1 year, and approximately 2 years (10 samples for each age class) were tested. Two fresh NaOH extracts and a CTAB/phenol-chloroform extract from *P. ramorum* cultures were used as positive controls. Amplicon sizes were assessed by agarose gel electrophoresis and compared to expected sizes as described above.

Results

Comparison of rapid extractions for PCR amplification from lyophilized mycelial cultures

DNA extracted from cultures of endophytic fungi using the ROSE, Chelex 100, Proteinase K, and NaOH methods yielded 76.67%, 0%, 80%, and 83.33% PCR success rates, respectively (Fig. 1a). Amplicon quality was highest for the NaOH extractions (76.67% strong and 6.67% weak amplification as visualized by agarose gel electrophoresis), followed by Proteinase K (63.33% strong, 16.67% weak), then ROSE (53.33% strong, 23.33% weak). Following the failure of the Chelex 100 procedure in the first trial, the remaining methods were tested on the *Heterobasidion irregulare* cultures. The ROSE and Proteinase K methods yielded slightly higher PCR success than the NaOH method (100% vs. 90%; Fig. 1b).

Comparison of rapid extractions for PCR amplification from dried sporocarps

DNA extracted from dried sporocarp tissue using the ROSE, Proteinase K, and NaOH methods yielded 100%, 50%, and 100% PCR success rates, respectively (Fig. 1c). No taxonomic trends were evident in PCR failure of the Proteinase K extracts, with failure occurring for two agarics (*Galerina* sp., *Hypholoma* sp.), *Auricularia polytricha*, the resupinate basidiomycete, and the Pezizomycete. In subsequent work involving sequencing from sporocarps where specimens extracted using the CTAB/phenol-chloroform method exhibited low quality chromatographs containing many overlapping peaks, clean sequences were often obtained by re-extracting from the

Table 1 Samples used to evaluate the longevity of rapid DNA extracts for successful PCR amplification of the fungal DNA barcoding locus ITS1 + 5.8S + ITS2. The extraction method, sample type, dilution and storage condition of extract, length of storage period, and number of samples are listed for each sample type

Method	Sample type	Extract storage	Storage (mo.)	N	PCR Success (percent)
NaOH	Insect samples colonized by entomopathogenic fungus	1:100 dilutions, −20 °C	19.5	4	100 (50 weak)
NaOH	Insect samples colonized by entomopathogenic fungus*	Original extracts, 4 °C	19.5	4	50
NaOH	Lyophilized *Heterobasidion irregulare* cultures	1:100 dilutions, −20 °C	32	10	90
NaOH	Lyophilized *H. irregulare* cultures*	Original extracts, −20 °C	32	10	30 (20 weak)
ROSE	Lyophilized *H. irregulare* cultures	1:170 dilutions, −20 °C	32	10	100
ROSE	Lyophilized *H. irregulare* cultures*	Original extracts, −20 °C	32	10	0

ROSE, rapid one-step extraction.
*Denotes same samples as entry immediately above.

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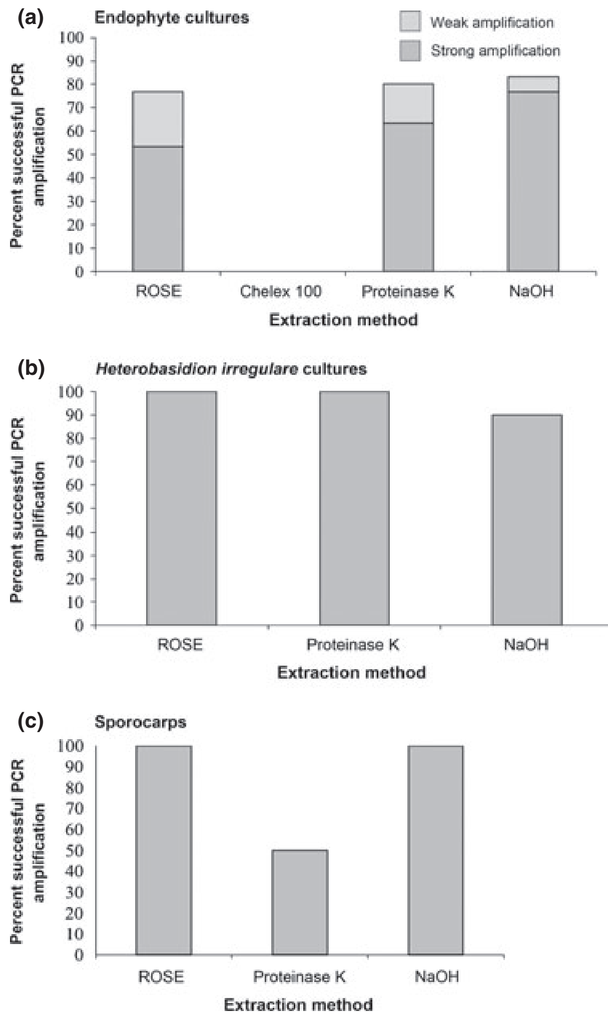

Fig. 1 Comparison of PCR success rates between rapid extraction methods for (a) endophyte cultures ($N = 30$ for each method), (b) *Heterobasidion irregulare* cultures ($N = 10$ for each method). and (c) sporocarps ($N = 10$ for each method).

dried specimens using an NaOH extraction, then repeating the PCR and cycle sequencing steps.

Use of NaOH extracts for amplification of microsatellite loci from lyophilized cultures

PCR amplification of NaOH extracts from *Phytophthora ramorum* cultures was successful for all isolates tested for both primers. Extracts maintained viability from 0–24 months post-DNA extraction for both primer sets (see results under 'Longevity of rapid extracts').

Use of rapid extracts from symptomatic leaf tissue for PCR diagnostic identification of Phytophthora ramorum

In the first trial, PCR detection of *Phytophthora ramorum* in tanoak leaf extracts was significantly greater in PC than in ROSE extracts (Table 2, $X^2_{1,1} = 6.29$, $P = 0.01$). There was a non-significant trend toward increased detection with the addition of BSA ($X^2_{1,1} = 1.43$, $P = 0.23$).

In the second trial, detection was lower in ROSE and column-cleaned ROSE extracts than PC extracts, with borderline significance (Fisher's exact test, $P = 0.06$, based on 10 000 simulated replicates). Column cleanup further lowered detection frequency. The frequency and accuracy of pathogen detection were considerably greater in the second trial than the first; indeed, the lowest Trial 2 detection rate was greater than the highest from Trial 1. Where there was detection, quantification was more accurate in Trial 2 than Trial 1. The confidence intervals for all observed pathogen template starting quantities overlapped the known value, and there was no significant difference in means among extraction methods (Table 2).

Phytophthora ramorum qPCR assays from culture-positive *Umbellularia californica* leaves were positive with 100 percent frequency from ROSE extracts with or without subsequent treatment with the PCR inhibitor removal kit. No noticeable difference in qPCR threshold cycle was present between treatments for each sample.

Longevity of rapid extracts

Frozen 1:100 Tris-HCl dilutions of NaOH extracts yielded high rates of PCR success from both the insect

Table 2 Detection frequency and accuracy of qPCR-based detection of 5 pg of *Phytophthora ramorum* DNA within a matrix of DNA extracted from tanoak leaves including midrib tissue.

Extraction method	Trial	N	Detection	Observed starting quantity (pg)
Phenol/ chloroform	1	9	56%	0.34 (0.09–0.59)
Phenol/ chloroform with BSA	1	9	67%	1.98 (0.83–3.13)
Phenol/ chloroform with BSA	2	5	100%	7.44 (1.89–12.99)
ROSE	1	9	11%	0.30 (n/a)
ROSE with BSA	1	9	33%	0.87 (0.17–1.58)
ROSE with BSA	2	5	80%	8.74 (2.94–14.54)
ROSE with BSA, column -cleaned	2	5	60%	7.54 (3.57–11.51)

In trial 1, detection was compared among extractions with and without the addition of bovine serum albumin (BSA) to the reaction mixture; in trial 2, an additional column cleanup step after ROSE extraction was employed (see text). Parentheses denote 95% confidence intervals.
ROSE, rapid one-step extraction.

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samples containing entomopathogenic fungi (100% success, though 50% exhibited weak amplification) and from the lyophilized *Heterobasidion irregulare* mycelium (90%) (Table 1; Fig. 2a). Frozen 1:170 H_2O dilutions of ROSE-extracted DNA from *H. irregulare* mycelium yielded 100% amplification success (Fig. 2b). For both extraction methods, new dilutions made from the stored extracts yielded no (ROSE extraction, stored at −20 °C for 32 months) to low (20% weak amplification and 10% strong amplification for NaOH extracts stored at −20 °C for 32 months; 50% amplification for NaOH extracts stored at 4 °C for 19.5 months) PCR success (Table 1; Fig. 2a, b).

Amplification of *Phytophthora ramorum* microsatellite loci from frozen 1:100 NaOH extract dilutions exhibited complete success regardless of storage period for both loci M18 (Fig. 3a) and M39 (Fig. 3b). Although differences in band intensity on agarose gels were noted between samples, there does not appear to be a clear relationship between amplification efficiency and storage period: M18 exhibited a higher degree of weaker bands for the 24^{+}-month-old samples (50% vs. 10% for 12-month-old and 20% for 6-month-old extracts); the opposite was true for M39 (20%, 10%, and 60%, respectively).

Discussion

A wide array of methods have been developed for DNA extraction, producing numerous tradeoffs between cost, ease of use, time required, materials including hazardous chemicals used, and quantity and quality of extracted DNA. Several considerations are particularly important in choosing an extraction method: type of source material, type of downstream applications and procedures, and need for long-term archival storage of extracts. In our work on identification, population biology, and systematics of plant-associated fungi and oomycetes, we often encounter situations where rapid diagnosis is required. For example, in providing routine sample identification for Cooperative Extension services, positive identification is required from one or several fungal sporocarps or cultures obtained from diseased plant material; in contrast, when obtaining genotype data for molecular epidemiology studies, many cultures need to be handled simultaneously. In the first case, further use of DNA extracts is unlikely; in the second, cultures are retained for long-term storage so a separate archival DNA extract is often not necessary. For such applications, standard extraction procedures require unnecessary expenditures of money, time, and materials. In the present study, we evaluated the utility of several rapid, low-cost extraction procedures for a variety of sample types and downstream procedures. We determined that several previously described procedures yield DNA from lyophilized mycelium and dried sporocarp tissue suitable for DNA barcoding, molecular identification, and microsatellite genotyping. An additional protocol tested, the Chelex 100 protocol of de Lamballerie *et al.* (1992), did not yield PCR amplicons in our first trial; however, we did not conduct tests sufficient to determine whether this failure was a function of the extraction method itself or of our specific, single PCR trial.

We determined that a simple sodium hydroxide extraction based on the procedure of Wang *et al.* (1993) performed most reliably over the range of samples and applications that we tested. This method yielded DNA

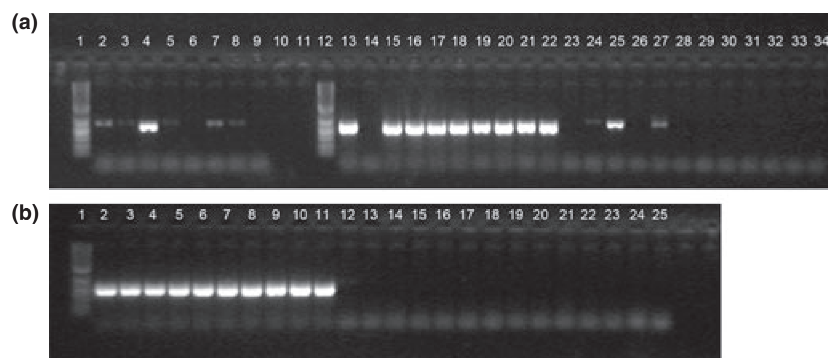

Fig. 2 Agarose gel images showing results of PCR amplification of the rDNA ITS1 + 5.8S + ITS2 locus from stored rapid extracts. (a) NaOH extracts. Lanes 1–9: Samples of the whitefly *Bemisia tabaci* infected with an entomopathogenic fungus. Lane 1: molecular size marker (1 kb DNA ladder); lanes 2–5: NaOH dilutions (1:100) stored −20 °C for 19.5 months; lanes 6–9: original NaOH extracts stored 4 °C for 19.5 months; lanes 10–11: blank; Lanes 12–34: *Heterobasidion irregulare* lyophilized mycelia. Lane 12: molecular size marker; lanes 13–23: dilutions (1:100) stored at −20 °C for 32 months (extraction negative control in lane 23); lanes 24–34: original extracts stored at −20 °C for 32 months (extraction negative control in lane 34). (b) ROSE extracts of *Heterobasidion irregulare* lyophilized mycelia. Lane 1: molecular size marker; lanes 2–12: dilutions (1:170) stored at −20 °C for 32 months (extraction negative control in lane 12); lanes 13–23: original extracts stored at −20 °C for 32 months (extraction negative control in lane 23); lanes 24–25: PCR negative controls.

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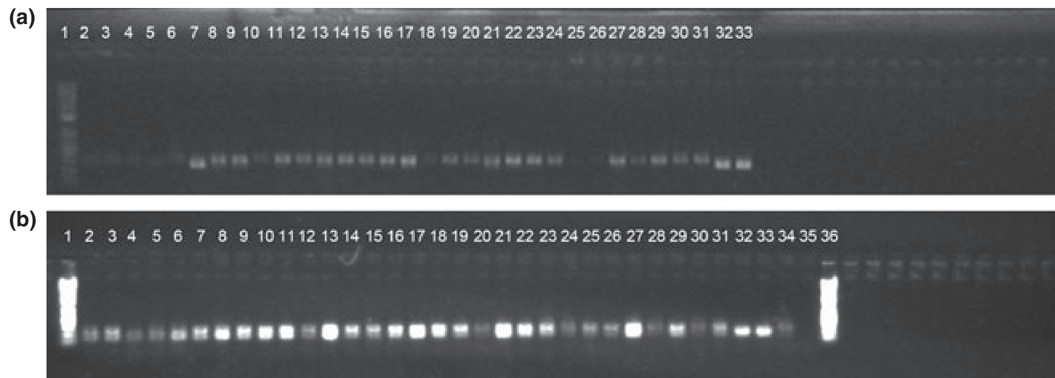

Fig. 3 Agarose gel images showing results of PCR amplification of the *Phytophthora ramorum* microsatellite loci M18 and M39 from NaOH rapid extract dilutions (1:100) stored for different time periods. (a) locus M18. Lane 1: molecular size marker (1 Kb DNA ladder); lanes 2–11: extracts stored for over 24 months; lanes 12–21: extracts stored for approximately 12 months; lanes 22–31: extracts stored for approximately 6 months; lanes 32–33: new extracts. (b) locus M39. Lane 1: molecular size marker; lanes 2–11: extracts stored for over 24 months; lanes 12–21: extracts stored for approximately 12 months; lanes 22–31: extracts stored for approximately 6 months; lanes 32–33: new extracts; lane 34: positive control, standard (CTAB/phenol-chloroform) extract stored for over 24 months; lane 35: H_2O negative control; lane 36: molecular size marker.

from dried sporocarps and lyophilized cultures suitable for PCR amplification of targeted loci, both multicopy (rDNA ITS) and single-copy (microsatellite loci). Subsequent use has also been successful for tissue taken directly from fresh sporocarps and cultures. This procedure offers several advantages: it is rapid (a set of 12 samples requires <10 min, assuming that the NaOH solution and Tris-HCl buffer are prepared in advance), economical, and reduces waste by requiring few and non-toxic reagents and a single transfer of material between tubes. Importantly for diagnostic applications, it is amenable to large numbers of samples prepared separately or in 96-well format, but its efficiency does not require an economy of scale; single samples can be extracted quickly and easily when necessary. Use of a similar protocol (using 50 mM rather than 0.5 M NaOH, followed by a 95 °C heating step) is routinely used in DNA extraction from buccal cells for genetic studies of human diseases (Richards *et al.* 1993; Vance 2006). We have subsequently used NaOH extraction on a variety of additional macrofungi including *Galerina*, *Laccaria*, gasteroid Agaricaceae, and *Lactarius*, and on cultures of *Seiridium* obtained from tree stem samples. While our results are specific to the materials and assays that we tested, the use of this technique on plants, human cells, and now fungi and protists (Stramenopila) suggests that it is broadly useful across taxa. Substrates, however, may show substantial variation in how well fungi and oomycetes present in low amounts can be detected. Substrates rich in lignin or humic acids present particular difficulties for extraction; we have achieved better results for wood samples using a commercial stool DNA extraction kit and for soil using a commercial soil DNA extraction kit.

Lower detection success of the ROSE method (compared to a standard CTAB/phenol-chloroform procedure) was observed in conducting quantitative PCR detection of *Phytophthora ramorum* infection in tanoak leaves. In this TaqMan diagnostic assay, the two most likely explanations for failure are the low amounts of *P. ramorum* tissue present in the sample relative to the plant tissue, or the presence of contaminating substances in the crude extract that inhibit the TaqMan chemistry. The disparity in results from the two tanoak trials suggests that inter-assay variation in reagents and techniques is also important. Because the tanoak extracts were spiked with a known quantity of *P. ramorum* DNA, the presence of PCR inhibitors was the most likely reason for detection failure in rapid extracts. The degree to which inhibition potential affects the choice of extraction method depends on the application; if maximum sensitivity in recalcitrant substrates is required, then rapid extraction may not be the optimal choice. In applications where the assay is more sensitive, leaf chemistry is less recalcitrant, or greater uncertainty can be tolerated in exchange for lower cost and greater speed, the ROSE method may suffice well. This conclusion is supported by our complete detection of *P. ramorum* in ROSE-extracted leaves of California bay laurel, a plant with leaf chemistry less recalcitrant to PCR amplification than that of tanoak. Use of the NaOH protocol for extracting DNA from cultures of *P. ramorum* for the same TaqMan diagnostic procedure resulted in high success rates when compared to known positive/negative results obtained from phenol-chloroform extracts from the same samples (C. Eyre, unpublished data). The high success of rapid extraction from cultures compared to leaves further suggests that inhibitory compounds present in tanoak leaves

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are responsible for the diminished success rates observed in ROSE extracts from leaf material. Due to differences in leaf chemistry between species, a degree of caution is warranted in using rapid extracts for plant disease diagnostics; some applications and/or substrates may require a more intensive extraction protocol, and careful optimization may be necessary in order to obtain good results.

Because the NaOH extraction procedure is simple and lacks additional wash steps, DNA yield, quality and long-term storage viability are important concerns. Our results indicate that extracts tend to have lower DNA concentration and a lower prevalence of high molecular weight DNA compared to CTAB/phenol-chloroform extracts; the former is at least in part due to the high dilutions required in the NaOH protocol. Lower DNA yield suggests that this technique may not be useful for specimens with low amounts of target DNA. However, we have found that sufficient DNA for targeted gene amplification was obtained from the same amount of starting material that we generally use for standard extractions using CTAB/phenol-chloroform separation followed by silica column purification, and we have obtained positive PCR and sequencing results from specimens of the puffball mushroom genus *Calvatia* collected as early as 1940. Direct sequencing of ITS amplicons from sporocarps frequently results in obtaining low-quality sequences appearing as multiple, juxtaposed chromatographs. Two major causes for this problem are (i) contamination by other fungi that grow on — or the spores of which land upon — the sporocarp and are subsequently co-amplified with the target organism; or (ii) because the ITS is part of a tandemly-repeated mutigene family, paralogous sequences with incompletely-concerted evolution may be co-amplified. An additional advantage that we observed during this study was that NaOH extraction led to a high degree of success in obtaining 'clean' sequences where sequencing from CTAB/phenol-chloroform/column extracts had previously shown evidence of contamination. Such success is presumably a result of relatively low extraction efficiency combined with high dilution (1:100) of the initial extract, leading to PCR amplification of the dominant (target) organism exclusive of contaminating DNA. Use of this technique has proven valuable in our work on DNA barcoding of tropical mushrooms, in which mold contamination can be a significant issue. Our results indicate that NaOH extracts consist of lower-molecular-weight fragments than would be expected under a conventional extraction protocol. While we found template quality to be sufficient for targeted gene amplification, caution should be employed for genome fingerprinting methods such as AFLP (Vos *et al.* 1995) that require large, intact genomic DNA fragments for accuracy.

In our tests of template longevity, frozen NaOH and ROSE dilutions yielded viable PCR templates for amplification of the ~700 bp rDNA ITS fragment and two shorter (<400 bp) microsatellite loci for a minimum of 2–3 years. It is important to note that the original extracts did not persist well, whether they were stored at 4 °C or −20 °C; in our trials, it therefore appeared critical to store samples as dilutions rather than original extracts. This result is in disagreement with several previously published results that reported longevity of stored, undiluted extracts. For example, DNA from human buccal cells extracted using NaOH was previously shown to yield successful PCR amplification following storage at 4 °C for 10 months (Richards *et al.* 1993). In a second example, undiluted samples stored at 4 °C for periods ranging from 12 to over 36 months did not exhibit statistically significant decreased PCR amplification success with storage age, though all of these storage periods exhibited at least 6% PCR failure compared to the same samples amplified shortly after initial sample collection (Walker *et al.* 1999). The primary difference in the extraction methods used in these previous studies is the use of 50 mM rather than 0.5 M NaOH; it seems likely that the higher NaOH concentration used in the Wang *et al.* (1993) method results in rapid DNA degradation in the original extract.

Finally, it should be noted that, while we favor the NaOH procedure when our own work necessitates rapid extractions, other procedures may be more suitable for other applications. In our case, we favor the NaOH procedure because it yielded a slightly higher success rate in initial trials, is faster and requires fewer ingredients, and because a high prevalence of high molecular weight DNA is not absolutely critical for targeted gene amplification as long as DNA is not overly degraded. Other rapid extraction procedures (Steiner *et al.* 1995; Kang & Yang 2004), as well as a variety of commercial rapid extraction kits, have been shown to yield high molecular weight DNA, and may be more suitable for genome fingerprinting applications. Overall, we found that conventional DNA extraction procedures are unnecessarily expensive, laborious, and waste-generating for routine DNA sequencing or genotyping of fungi and oomycetes. We recommend adopting NaOH, ROSE, or other suitable rapid extraction techniques as a first-line approach where source materials and intended applications warrant their use.

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Data Accessibility

Data used in drawing conclusions are presented within the article.

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