

Application of an automated ribosomal intergenic spacer analysis database for identification of cultured Antarctic fungi

CALEB SLEMMONS, GREGORY JOHNSON and LAURIE B. CONNELL*

University of Maine, School of Marine Sciences, 5735 Hitchner Hall, Orono, ME 04468, USA

*corresponding author: laurie.connell@umit.maine.edu

Abstract: We utilized an automated ribosomal intergenic spacer analysis (ARISA) method as a more rapid alternative to classical morphological/nutritional identification and a less expensive alternative to sequencing for identification and grouping of isolates in culture-based fungal abundance studies. This method is well suited for the study of culturable Antarctic soil fungal communities where both abundance and diversity are relatively low. We optimized template concentration and verified the effect of primer selection from eight commonly used fungal polymerase chain reaction primers on ARISA chromatographs for 46 fungal species commonly isolated from south Victoria Land. A database of Antarctic fungal electropherograms was produced containing each of the species and was used as the first step in a tiered system for species identification. In addition, isolates containing more than one species were identified, allowing isolates not in the database to be sequenced for further analysis. This method unambiguously identified 78% of the fungal taxa in this study and we were able to rapidly determine which isolates should be subjected to further analysis by DNA sequencing. Using this approach, the cost of analysis for abundance studies can be greatly reduced compared to DNA sequencing of each isolate.

Received 6 February 2012, accepted 11 July 2012, first published online 6 November 2012

Key words: ARISA, ARISA-AFF, fungal abundance, McMurdo Dry Valleys, soil microbial community

Introduction

Microfungi have been cultured from a broad range of Antarctic habitats including, soil, moss and permanently frozen lakes (reviewed by Zalar *et al.* 2012). The relatively low diversity of (Fell *et al.* 2006) and potentially high percentage of fungi that are culturable from (Onofri *et al.* 2007) these Antarctic habitats can allow for a more thorough study of community structure than from highly complex lower latitude habitats. Because of the lower diversity and abundance of culturable fungi from each potential sample site, especially in the McMurdo Dry Valleys, identification of each isolate in abundance studies is a viable option.

All methods for the study of soil communities have biases. Identification of the members of a microbial community using culture-independent methods, via ribosomal RNA (rRNA) gene cloning and sequencing techniques, has become standard practice. Yet, despite the data and insights provided, even with second and third generation sequencing methods, a conclusive functional characterization of the community is not yet attainable (Kunin *et al.* 2010). One important bias for culture-independent methods is the efficiency of cell lysis and nucleic acid extraction from soil (Feinstein *et al.* 2009). The availability of cultured isolates opens the door to numerous microbiological, physiological, functional and other investigations, thereby allowing confirmation and

extension of culture-independent data. In addition, species abundance, for culturable species, can only be obtained from culture-based studies. Therefore, complementing microbiological culturing with culture-independent studies is recognized as a strategy to overcome the deficiency of using either method alone.

Fungal taxonomy can be challenging because of intraspecific morphological and physiological plasticity as well as a limited number of morphological markers. Further, the process of identification based on morphology and physiological traits can be time consuming. Sequencing of ribosomal genes (rDNA) and their internal transcribed spacer (ITS) or other variable regions within conserved genes (e.g. actin gene) are often necessary for accurate taxonomic identification. The ITS region of the nuclear rRNA gene has been used as a target for identification of microbial species because it is often variable enough to be used as a species-specific target region (Kurtzman 2006). The current cost of traditional sequencing places a substantial limitation on the number of isolates that may be analysed. Frequently in the process of identifying isolates, common species may be sequenced many times demonstrating the need for a lower cost method to screen a large number of isolates. Other molecular methods are available such as arbitrarily primed polymerase chain reaction (apPCR) (Rodriguez *et al.* 2004), three-primer PCR (Fell 1993) and direct hybridization (Kiesling *et al.* 2002). However, direct hybridization and three-primer PCR requires specific primers or probes be

developed for each taxa and apPCR is more appropriately used to determine intraspecific variation.

The automated ribosomal intergenic spacer analysis (ARISA) technique is a high-resolution automated capillary electrophoresis DNA fingerprinting method that is based on discriminating taxa by variation in the size of the target gene region. Since its development, along with similar methods such as denaturing gradient gel electrophoresis (DGGE) (Fisher & Lerman 1980), ARISA has been applied to a wide range of environmental samples from freshwater bacterial communities to soil fungi (Fisher & Triplett 1999, Slabbert *et al.* 2010). This technique, when applied to the ITS region, has shown promise as an accurate method for rapidly analysing the diversity and composition of microbial communities at large (reviewed by Popa *et al.* 2009) by comparing chromatogram peaks among samples. These peaks have been called operational taxonomic units (OTUs). Popa *et al.* (2009) proposed the term phylotypes over the use of OTUs as a description of taxa identified because more work still needs to be done to define phylogenetic resolution of data resulting from environmental ARISA. However, the ARISA technique can also be utilized to rapidly and relatively inexpensively determine species (phylotype) using cultured material. For example, ARISA has been used to measure intra-genomic diversity among *Shewanella* phylotypes (Popa *et al.* 2009).

In this study we assessed the ability of ARISA to provide taxonomic identification from cultured fungal material by development of a database consisting of culturable Antarctic fungal ARISA profiles, or ARISA Antarctic fungal fingerprints (ARISA-AFF). This method is also useful as a screening procedure to select isolates that will require sequencing for positive identification or to sort isolates into groups for further analysis. We tested the efficacy of ARISA-AFF in screening 203 isolates (46 species) from samples taken from lakewater, moss, soil and sponge from south Victoria Land, Antarctica. A total of 78% of the isolates in this study were unambiguously identified solely using ARISA-AFF, leaving fewer isolates to be identified by more expensive or time consuming protocols. DNA sequencing following sorting by ARISA-AFF analysis identified the remaining 22% of isolates. In addition, the ARISA-AFF technique helped to recognize

several previously unidentified species found in culture. These isolates were morphologically similar to others from the same sample and may have been missed in studies that did not identify each isolate.

Materials and methods

Field sites, collection and fungal isolation

Isolates for this study were from environmental samples collected in south Victoria Land, Antarctica (Table S1, which will be found at <http://dx.doi.org/10.1017/S0954102012000879>.) during field seasons spanning 2002–10. These samples include lakewater, soil and moss from Taylor Valley, soil from ‘the Labyrinths’ of Wright Valley and from fumarole caves on Mount Erebus and ancient sponge samples from the Ross Ice Shelf.

Wright Valley and Taylor Valley soil samples were collected using a sterile metal scoop and placed in Whirl-Pak bags (Nasco, Fort Atkinson, WI). Fumarole cave soil samples were collected by using a sterile metal scoop to scrape the soil from the bottom of the cave into sterile aluminium foil. Moss was collected by placing *c.* 20 g into a sterile Whirl-Pak bag using sterile forceps. Sponge samples were obtained from sponges in late Quaternary debris bands on top of the Ross Ice Shelf near Mount Discovery (Kellogg *et al.* 1990). Sterile forceps were used to place *c.* 100 g of sponge material into Whirl-Pak bags. Samples were transported frozen to the continental US (−20°C) then treated as previously described for fungal isolation (Connell *et al.* 2008). Water samples were obtained from various depths of the water column of Lake Fryxell during the 2008–09 field season. Holes were drilled through the ice with a seven-inch steel drill bit attached to a gasoline-powered mechanical Jiffy drill. Water was collected with a 5 l Niskin sampler and transferred to sterile 1 l bottles and stored at 4°C until filtering. Water samples were filtered within five hours on 47 mm, 0.45 µm Metricel Black membrane filters (Pall Corp, Ann Arbor, MI). The filters were transferred to 50 ml tubes, without sides touching and were shipped to the continental US at 4°C. Upon arrival in the US filters were placed on 50 mm petri plates containing one of two media

Table 1. Primer combinations tested for positive PCR amplification on select fungal isolates. Primer sources are as follow: ITS4, ITS5 (White *et al.* 1990), ITS1F (Gardes & Bruns 1993), EF3 (Smit *et al.* 1999), and NS11, NLB4, NLC2 (Martin & Rygielwicz 2005).

Forward primer	Reverse primer	Annealing temperature (°C)	Amplified all test isolates
ITS5	ITS4	52.3	+
ITS1F	ITS4	54.0	+
ITS1F	NLB4	54.0	-
NS11	NLB4	58.0	-
NS11	ITS4	58.0	-
NS11	NLC2	58.0	+
NS11	EF3	58.0	-
ITS1F	EF3	54.0	-

Table II. Fungal species used in the development of automated ribosomal intergenic spacer analysis (ARISA) database. Species that cannot be individually resolved using ARISA Antarctic fungal fingerprints (ARISA-AFF) are marked with an asterisk.

Isolate ID	Species	Estimated size (bp)	GenBank accession number	GenBank closest match	Percent match
ANT CW08-162	<i>Acremonium</i> sp.	619.6	JX171161	AB540571.1	94
G439-13B	<i>Alternaria alternata</i> (Fr.) Keissl., 1912	610.0	JX171162	JQ676196	100
LKF08-138	<i>Aureobasidium pullulans</i> (de Bary) Arnaud, 1918*	622.0	JX171163	JF422784.1	99
ANT03-087	<i>Chaumopycnis</i> sp.	616.4	JX171164	DQ402530.2	99
G439-03	<i>Cladophialophora</i> sp.	856.9	JX171165	AY843173.1	95
ANT03-399	<i>Cladosporium langeronii</i> (Fonseca, Leão & Nogueira) Vuill., 1931	589.0	JX171166	HQ115727.1	100
ANT03-415	<i>Cladosporium sphaerospermum</i> Penzig, 1882*	594.5	JX171167	EU570256.1	100
ANT03-107	<i>Clavispora lusitaniae</i> Rodrigues de Miranda, 1979	418.6	EU149777	FJ183442	100
LKF08-127	<i>Cryptococcus albidosimilis</i> Vishniac & Kurtzman, 1992*	663.3	JX171168	AF145331.1	100
ANT03-073	<i>Cryptococcus carnescens</i> (Verona & Luchettii) Takashima, Sugita, Shinoda & Nakase, 2003	561.7	EU149786	EU149785.1	100
ANT03-044	<i>Cryptococcus magnus</i> (Lodder & Kreger-van Rij) Baptist & Kurtzman, 1976	669.5	JX171169	AF190009.1	100
ANT03-190	<i>Cryptococcus saitoi</i> Á. Fonseca, Scorzetti & Fell, 2000a*	676.8	JX171170	FJ183436.1	100
LKF08-077	<i>Cryptococcus victoriae</i> M.J. Montes, C. Belloch, M. Galiana, M.D. Garcia, C. Andres, S. Ferrer, J.M. Torr.-Rodr. & J. Guinea, 1999	559.9	JX171171	AF444447.1	100
ANT03-052	<i>Cryptococcus vishniacii</i> Vishniac & Hempfling, 1979*	663.3	EU149788	AB032691.1	100
ANT03-111	<i>Dioszegia antarctica</i> Connell, Redman, Rodriguez & Fonseca, 2010	522.3	EU149800	FJ643481	100
ANT03-096	<i>Dioszegia cryoxerica</i> Connell, Redman, Rodriguez & Fonseca, 2010	518.3	EU149792	FJ640565	100
ANT03-067	<i>Geomyces</i> ribotype 1*	602.2	JX171172	AY873967.1	99
G439-11	<i>Geomyces</i> ribotype 2*	602.2	JX171173	DQ189229.1	99
ANT03-026	<i>Geomyces</i> ribotype 3	1005.9	JX171174	DQ402527.1	100
ANT03-428	<i>Geomyces</i> ribotype 4*	602.2	JX171175	DQ189225.1	100
LKF08-112	<i>Glaciozyma watsonii</i> Turchetti, Connell, Thomas-Hall & Boekhout, 2012	684.2	JX171176	EU149803.1	99
ANT03-039	<i>Glaciozyma</i> sp. nov 2	687.6	EU149805	AF444529	97
ANT03-061	<i>Glaciozyma martinii</i> Turchetti, Connell, Thomas-Hall & Boekhout, 2012	660.7	EU149807	AY040664	100
ANT03-033	<i>Glaciozyma antarctica</i> (Fell, Statzell, Hunter & Phaff) Turchetti, Connell, Thomas-Hall & Boekhout, 2012	690.0	EU149808	AY040663.1	99
ANT03-093	<i>Guehomyces pullulans</i> (Linder) Fell & Scorzetti, 2004	662.1	JX171177	AF444417.1	100
LKF10-013	<i>Heydenia alpina</i> Fresen, 1852	649.0	JX171178	HQ688653.1	100
ANT03-014	<i>Holtermanniella nyarrowii</i> (Thomas-Hall & K. Watson) Libkind, Wuczkowski, Turchetti & Boekhout, 2011	606.2	JX171179	DQ402536.1	100
ANT CW08-165	<i>Meira</i> sp.	665.1	JX171180	AM905091.1	99
ANT03-085	<i>Mrakia stokesii</i> (Fell, Statzell, I.L. Hunter & Phaff) Y. Yamada & Komag, 1987	681.2	EU149810	AF144486	100
LKF08-061	<i>Mrakiella aquatica</i> (E.B.G. Jones & Sloof) Margesin & Fell, 2008	676.8	JX171181	GQ911548.1	99
ANT03-172	<i>Nematotonus</i> sp.	743.2	JX171182	DQ402531.1	99
LKF10-002	<i>Penicillium commune</i> Thom, 1910*	622.0	JX171184	AJ004813.1	100
ANT T30-03-507	<i>Penicillium corylophilum</i> Dierckx, 1901*	622.0	JX171185	JF731268.1	100
ANT03-010	<i>Penicillium citreomigrum</i> (Frisvad, Filt. & Wicklow) Frisvad, 2000	617.8	JX171183	AY373908.1	99
LKF08-135	<i>Penicillium dipodomycicola</i> (Frisvad, Filt. & Wicklow) Frisvad, 2000*	622.0	JX171186	GQ161752.1	100
ANT T30-03-506	<i>Penicillium thomii</i> K.M. Zalesky, 1927	613.6	JX171187	FR670338.1	100
ANT03-009	<i>Phaeosphaeria</i> sp.	625.5	JX171188	AY345346.1	100
ANT03-414	<i>Pichia</i> sp.	644.2	JX171189	FJ381694	85
G439-31	<i>Psiloechia leprosa</i> Coppins & Purvis, 1987	633.5	JX171190	AY756496.1	99
ANT03-424	<i>Ramularia</i> sp.	578.4	JX171191	EU019284.2	96
LKF08-062	<i>Rhodotorula mucilaginoso</i> (Jørgensen) F.C. Harrison, 1928	657.7	JX171192	EU149812.1	99
ANT T30-03-512	<i>Sistotrema brinkmannii</i> (Bresàdola, 1903) Eriksson, 1948	675.3	JX171193	DQ899094.1	100
ANT CW08-156	<i>Tetracladium</i> sp.*	594.5	JX171194	EU883433.1	94
ANT03-417	<i>Thelebolus ellipsoideus</i> Brumm. & de Hoog, 2005	599.3	JX171195	AY957550.1	99
ANT03-221	<i>Thelebolus globosus</i> Brumm. & de Hoog, 2005	604.3	JX171196	DQ028268.1	99
ANT03-035	<i>Yarrowia lipolytica</i> (Wick. Kurtzman & Herman) Van der Walt & Arx, 1980	397.9	JX171197	GQ376068.1	100

and then incubated at 15°C. The media were yeast peptone dextrose (YPD) (BD, Sparks, MD) or 1/10 YPD, both with antibiotics as described in Connell *et al.* (2008). Filters were inspected twice weekly for two years and colonies were isolated as previously described for soil (Connell *et al.* 2006).

Culture and ITS region identification of fungal isolates

Isolates were grown on YPD agar and single colonies selected. Single colonies of yeast and yeast-like isolates were grown in 5 ml YPD broth until an optical density (OD) at 600 nm reached 0.6 (Ultraspec 10; GE Lifesciences, Piscataway, NJ). A cell pellet was produced by centrifugation at 1×10^4 rpm (8624 x g) for 5 min (Eppendorf 5418 microcentrifuge, Eppendorf North America, Hauppauge, NY). Filamentous fungi were scraped off the growth medium into 1.5 ml of MasterPure Yeast DNA Purification Kit lysis buffer (Epicenter, Madison, WI) and then frozen at -80°C for at least 30 min before further processing. DNA was extracted from cell pellets (yeast and yeast-like isolates) or thawed lysate (filamentous fungi) with MasterPure Yeast DNA Purification Kit using the manufacturer's protocol. DNA concentrations were determined using a Nanoview spectrophotometer (GE Lifesciences). Specific ITS region amplicons were produced by PCR completed with 100 ng genomic DNA in 25 µl reactions using Illustra PuReTaQ Ready-To-Go™ PCR Beads (GE Lifesciences). PCR primer set ITS5-ITS4 (White *et al.* 1990) were used to target the ITS region for sequencing (Table I). Initial denaturation completed for 2 min at 95°C and 35 cycles with a PTC-200 thermal cycler (MJ Research, Watertown, MA) under the following conditions: 30 sec at 95°C, 30 sec at 52.3°C, 1 min at 72°C with a final 72°C 10 min extension. The resulting PCR products were cleaned using Promega SV Gel and PCR Clean-up System (Promega, Madison, WI) and were visualized using standard gel electrophoresis as previously described (Connell *et al.* 2008). The purified DNA was then used as a double-stranded sequencing template with primers ITS5 and ITS4 in sequencing reactions. Both strands were individually sequenced using Big Dye Terminator Cycle Sequencing in an ABI 377 autosequencer (Applied BioSystems, Foster City, CA). Resulting sequence chromatograms were analysed using Sequencher v 4.9 (GeneCodes, Ann Arbor, MI). Taxonomic identification of each isolate was made by comparison of ITS sequence with GenBank BLASTn (NCBI) data as well as by using physical characteristics of the isolate. Isolate ITS sequences from each species used in this project were submitted to GenBank (Table II).

ARISA-AFF primer selection and PCR parameters

To determine if selected primer pairs were able to amplify the ITS region of each fungal species in this study, eight

sets of primer combinations including ITS4, ITS5 (White *et al.* 1990), ITS1F (Gardes & Bruns 1993), EF3 (Smit *et al.* 1999), and NS11, NLB4, NLC2 (Martin & Rygiel 2005) (Table I) were tested to ensure positive amplification with a selection of fungal isolates: *Clavispora lusitanae*, *Cryptococcus carnescens*, *Dioszegia cryoxerica*, *Dioszegia antarctica*, *Geomyces* sp., *Glaciozyma* sp., *Rhodotorula graminis* and *Thelebolus ellipsoideus*. PCR was performed as described above with the exception of annealing temperature, where a 30 sec target annealing temperature for each primer pair was used. PCR products generated were visualized on a standard agarose gel as previously described (Connell *et al.* 2008). When no PCR amplicon was produced the cycling conditions used in the publications listed in Table II were used.

The PCR template concentration and extension time were optimized to maximize the fluorescence recorded from the ABI 3730 DNA Analyser (Applied Biosystems, Carlsbad, CA) without over-saturation of the instruments charged couple device (CCD) digital image sensor. An excess of PCR product will over-saturate the detector, resulting in broad, flat-topped peaks and therefore reduce the precision of size calling. To optimize the sample loading concentration for precision sizing of fragments, eight isolates were tested in duplicate with between 3 ng and 60 ng of PCR product (3 ng, 6 ng, 19 ng, 32 ng, 35 ng, 40 ng, 52 ng, 60 ng). Replicate samples with a final PCR extension step of 30 min compared with 10 min were also compared on ARISA-AFF. Samples were run and analysed as described in the following section.

ARISA-AFF database construction and analysis

Genomic DNA (gDNA) from fungal isolates (Table II) was amplified using the primer set ITS1F-ITS4. In cases where this primer set did not resolve closely related species using ARISA-AFF two other sets were used (ITS5-ITS4 and NS11-NLB4) to determine if they would resolve the species. The reverse primer of each pair was labelled with 6-FAM carboxyfluorescein, succinimidyl ester and cycling parameters used are as described by Okubo & Sugiyama (2009). The resulting PCR products were cleaned using Promega SV Gel and PCR Clean-up System (Promega, Madison, WI) and amplicon concentrations were determined using a NanoVue spectrophotometer. Samples were diluted to $1.5 \text{ ng } \mu\text{l}^{-1}$ with 2 µl placed into duplicate wells of a 96-well, half-skirt plate. Each well contained 0.5 µl of ROX rhodamine labelled standard Mapmarker 1000 (BioVentures, Murfreesboro, TN) and 7.5 µl formamide. One isolate (ANT03-026) required the use of a custom ROX standard that included a 1050 bp fragment (BioVentures) as its size exceeded the largest standard in MapMarker 1000. Sample plates were then heated for 3 min at 90°C and placed on ice. Capillary electrophoresis was applied to samples using a ABI 3730

DNA Analyser run for 40 min at 15 kV, with 1.6 kV injection for 15 sec on a POP7 polymer in a 50 cm array at 60°C. Resultant chromatograms were viewed and analysed using GeneMarker version 1.91 (SoftGenetics, State College, PA) and fragments sized using default analysis parameters with local southern selected as a peak sizing method and large-size fragment algorithm. These parameters included smoothing, corrections for peak saturation, pull-up, baseline subtraction and spike removal. Binning was not used for sizing fragments for identification of fungal isolates. Fragments were sized visually using the peak centre for sizing or for isolates with multiple peaks a mean of the peak sizes was used as an estimate of fragment size. Isolates were considered un-resolvable as distinct peaks when they were overlapping (typically < 1 bp in size difference). Each sample was loaded into three wells per instrument run and three independent PCR reactions were analysed per species. There was very high replication among samples and instrument runs.

Results

ARISA chromatographs for 46 fungal species commonly isolated from south Victoria Land were compared to compile a fingerprint database for rapid identification of fungal isolates. Of these 46 species, ARISA analysis of 34 species (78%) resulted in clearly identifiable chromatograms (Table II). Only three primer combinations amplified all templates (Table I) from test isolates using cycling parameters specified above. Primer pair NSI1-NLC2 yielded fragments that were too large to be sized with the internal lane standard. A commonly used fungal primer, EF3 did not amplify all of the isolates under conditions tested. Fragment sizes using ITS1F-ITS4 ranged from 397.9 bp (*Yarrowia lipolytica*) to 1006.5 bp (*Geomyces ribotype 3*) with most clustered between 589 and 690 bp. Signal peaks for many of the isolates showed a single, sharp, diagnostic peak that allowed easy characterization of the isolate as a narrow range of sizes (Table II). Isolates that were not positively identified using ARISA-AFF were identified by isolate morphology and DNA sequencing of the ITS region.

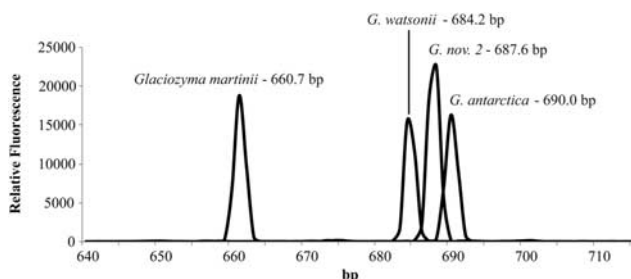


Fig. 1. Combined electropherograms from four species of *Glaciozyma* demonstrating clear separation of species. The x-axis shows estimated peak size in base pairs (bp).

In testing various template concentrations for ARISA-AFF analysis we found 3 ng of gDNA to be adequate for maximizing instrument signal and all subsequent samples were run at this concentration. The effect of longer PCR final extension times on replication of chromatogram peaks and detection of any “false peaks” was tested by running a subset of samples with a 30 min extension. There was no effect with additional extension times in discriminating false peaks within replicates. Therefore, all subsequent samples were processed with the 10 min final extension. Signal “pull-up”, which may appear in one or more dye channels, occurs as a result of strong fluorescence in a particular dye channel and a sharp peak in the resulting electropherograms. An increased response in the detector, sometimes referred to as signal “bleed through”, in the automated sequencer often occurs resulting in a false fluorescent signal in other dye channels. There was more signal pull-up in lane standard dye channel in these samples but the GeneMarker program compensates and corrects this effect. Otherwise, we did not find any effect on accurately sizing fragments (data not shown) and had excellent replication of major diagnostic peaks with little background signal.

This ARISA-AFF technique was successful in discriminating among some closely related taxa (Table II). For example, the four species of *Glaciozyma* were clearly

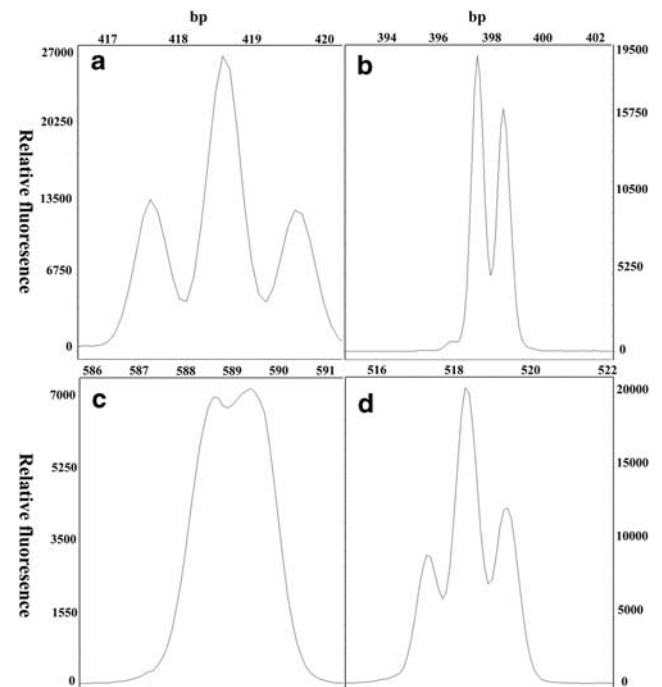


Fig. 2. Automated ribosomal intergenic spacer analysis (ARISA) profiles of four individual fungal species that demonstrate diagnostic peak patterns using primer pair ITS1F and ITS4. The x-axis shows peak size in base pairs (bp) of each species. **a.** *Clavispora lustaniae*, **b.** *Yarrowia lipolytica*, **c.** *Cladisporium lageronii*, and **d.** *Dioszegia cryoxerica*.

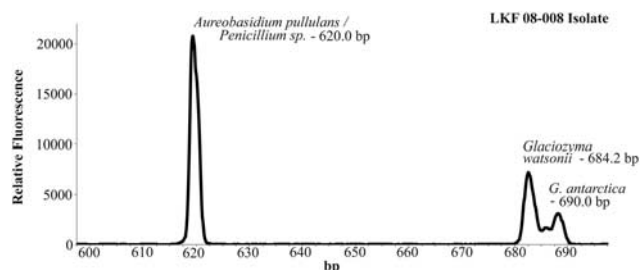


Fig. 3. Automated ribosomal intergenic spacer analysis (ARISA) profile of a mixed Lake Fryxell fungal culture generated via PCR amplification with ITS1F-ITS4 primers. The peaks show species names based on Table II. The two yeasts, *Glaciozyma watsonii* and *G. antarctica* were co-isolated with *Aureobasidium pullulans*.

resolved (Fig. 1). In addition, the *Glaciozyma* and *Dioszegia* species in this study were all separated to the species level along with some closely related Tremellales *Cryptococcus* species (e.g. *C. carnescens* and *C. victoriae*). Many of the more distantly related Filobasidiales *Cryptococcus* species were able to be resolved from one another (e.g. *C. magnus*, *C. satoi* and *C. vishniacii*/ *C. albidosimilis* group) but the *C. vishniacii*/*C. albidosimilis* group itself did not separate.

Heterogeneous ITS sequences within a species may create broad or multiple peaks that showed diagnostic peak sets. Several species in this study exhibited complex and diagnostic peaks using the ITS1F-ITS4 primer set. An example of this is shown in Fig. 2 where chromatograms of individual species (e.g. *D. cryoxerica*, *C. lusitaniae*, *Y. lipolytica* and *Cladosporium langeronii*) are shown to have more than one peak. For these species the size was estimated as the centre of the group of peak signals.

Some taxa had peaks that were very similar using the ITS1F-ITS4 primer set (Table II) yet were resolvable using additional primer pairs. For example, the NLB-NSII primer set separated *Aureobasidium pullulans* from *Penicillium commune*, *P. corylophilum* and *P. dipodomycicola*. In addition, *Cladosporium sphaerospermum* and *Tetracladium* sp. (isolate ANT CW08-156) were resolved using the ITS5-ITS4 primer set. Other species were not resolved to the species level with any primer set used (e.g. *Cryptococcus satoi* and *Mrakiella aquaticus*) and these isolates were then subjected to direct sequencing and/or morphological/physiological criteria for identification.

This ARISA database also allowed for the identification of unpurified cultures (Fig. 3). Six of the initial isolates from our Lake Fryxell samples were identified as “mixed” by the existence to two or more peaks that corresponded to previously identified taxa. Subsequent ARISA-AFF analysis following re-isolation and separation of the cultures confirmed the identification of each of the species from the mixed sample.

Discussion

In this work we developed an Antarctic fungal ARISA database suitable for rapid identification and sorting into groups of isolates generated from culture-based abundance studies. The use of ARISA-AFF for more cost effective identification of cultured fungal isolates has to be balanced with the consideration that new isolates not in the database may be cultured and therefore will require the same primary analysis before their chromatograms are entered into the ARISA-AFF database. Thus, this technique may be quite useful in initial screening of cultures into higher taxonomic groups for further analysis. In addition, ARISA peaks are viewed in context with isolate morphology. Therefore, gross morphology (e.g. filamentous vs yeast-like) that can clearly be easily discriminated visually help in identification as a second-tier of analysis. The identification of cultures that contain more than one species by comparison with library peaks has been helpful in several respects. First, if these cultures were thought to be single species isolates and were sent directly for sequencing no useable data would have been produced. This method provides identification and the option to re-culture and isolate component species if desired. Second, since many fungi can have similar gross morphology, identification of taxa that are not already in the database will allow for more rapid detection of potential new species. It remains possible that there may be species with very similar morphology and ARISA profiles. In this case, DNA sequencing of some isolates representing specific morphologies may be useful.

Accuracy of this method was higher than anticipated, based on similar studies, with *c.* 2 bp resolution up to 700 bp and 3 bp for > 700 bp. However, Fisher & Triplett (1999) found a similar level of accuracy in fragment sizing. The higher precision was probably due to a lower baseline resulting from the use of cleaned PCR products that removed non-specific targets (e.g. primer dimers) and excess reagents. Quantitation of individual PCR products and standardization of DNA concentration used for this ARISA technique helped achieve consistent high precision and sharp, unsaturated signals with a high relative fluorescence unit value (RFU). Nonetheless, much of the data analysis was done visually as complications with multiple peaks and peak “shoulders” did not lend to data analysis automation. Regardless, a large number of isolates can be screened and identified to the species level at a lower cost than sequencing.

The complex peaks found in several species (e.g. *D. cryoxerica*, *C. lusitaniae*) may be based on the fact that these species have intraspecific variation of the ITS sequence in each cell (Lachance *et al.* 2003, Connell *et al.* 2010). This variability in ITS does not affect identification of these isolates, in fact the peaks are very distinctive, but it does make future analysis automation more problematic.

The ARISA technique itself may be useful in discriminating fungal communities among treatments or habitats. This Antarctic fungal ARISA-AFF database can be used for identification of species in the traditional ARISA analysis of microbial communities, but it should be done so with some caution. For example, traditional peak calling would identify three phylotypes for *D. cryoxerica*. However, when environmental samples have few distinctive peaks (< 20) some of the peaks may be identified using this database, especially when combined with information obtained by culturing species from the sample. Therefore, the use of the ARISA-AFF for species identification from environmental samples is of limited utility and not its intended purpose.

Acknowledgements

The authors would like to thank Megan Altenritter, Katie Earle and Ben Segee for laboratory support, Regina Redmond, Rusty Rodriguez, Scott Craig, Amy Chiuchiolo, Tristy Vick, Andrew Barber and Hubert Staudigel for help in field sample collection as well as Raytheon Polar Services and PHI for logistical support. The authors also thank the anonymous reviews that helped improve this manuscript. This work was supported in part by NSF OPP-0125611, NSF ANT 0739696, as well as a University of Maine HEIR grant.

Supplemental material

A supplemental table will be found at <http://dx.doi.org/10.1017/S0954102012000879>.

References

- CONNELL, L.B., REDMAN, R.S., CRAIG, S.D. & RODRIGUEZ, R.J. 2006. Distribution and abundance of fungi in the soils of Taylor Valley, Antarctica. *Soil Biology & Biochemistry*, **38**, 3083–3094.
- CONNELL, L.B., REDMAN, R.S., CRAIG, S.D., SCORZETTI, G., ISZARD, M. & RODRIGUEZ, R.J. 2008. Diversity of soil yeasts isolated from south Victoria Land, Antarctica. *Microbial Ecology*, **56**, 448–459.
- CONNELL, L.B., REDMAN, R.S., RODRIGUEZ, R.J., BARRETT, A., ISZARD, M. & FONSECA, Á. 2010. *Dioszegia antarctica* and *D. cryoxerica* spp. nov., two novel psychrophilic basidiomycetous yeasts from polar desert soils in Antarctica. *International Journal of Systematic and Evolutionary Microbiology*, **60**, 1466–1472.
- FEINSTEIN, L.M., SUL, W.J. & BLACKWOOD, C.B. 2009. Assessment of bias associated with incomplete extraction of microbial DNA from soil. *Applied and Environmental Microbiology*, **75**, 5428–5433.
- FELL, J.W. 1993. Rapid identification of yeast species using three primers in a polymerase chain reaction. *Molecular Marine Biology and Biotechnology*, **2**, 174–180.
- FELL, J.W., SCORZETTI, G., CONNELL, L.B. & CRAIG, S.D. 2006. Biodiversity of micro-eukaryotes in Antarctic Dry Valley soil with < 5% soil moisture. *Soil Biology & Biochemistry*, **38**, 3107–3119.
- FISHER, M.M. & TRIPLETT, E.W. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology*, **65**, 4630–4636.
- FISHER, S.G. & LERMAN, L.S. 1980. Separation of random fragments of DNA according to properties of their sequences. *Proceeding National Academy of Sciences of the United States of America*, **77**, 4420–4424.
- GARDES, M. & BRUNS, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*, **2**, 113–118.
- KELLOGG, T.B., KELLOGG, D.E. & STUIVER, M. 1990. Late Quaternary history of the southwestern Ross Sea: evidence from debris bands on the McMurdo Ice Shelf, Antarctica. *Antarctic Research Series*, **50**, 25–56.
- KIESLING, T.L., DIAZ, M.R., STATZELL-TALLMAN, A. & FELL, J.W. 2002. Field identification of marine yeasts using DNA hybridization macroarrays. In HYDE, K.D., ed. *Marine mycology: the organisms, ecology and applied aspects*. Hong Kong: Fungal Diversity Press, 69–80.
- KUNIN, V., ENGELBREKTSON, A., OCHMAN, H. & HUGENHOLTZ, P. 2010. Wrinkles in the rare biosphere: pyrosequencing errors lead to artificial inflation of diversity estimates. *Environmental Microbiology*, **12**, 118–123.
- KURTZMAN, C.P. 2006. Yeast species recognition from gene sequence analysis and other molecular methods. *Mycoscience*, **47**, 65–71.
- LACHANCE, M.-A., DANIEL, M.H., MEYER, W., PRASAD, G., GAUTAM, S.P. & BOUNDY-MILLS, K. 2003. The D1/D2 domain of the large subunit rDNA of the yeast species *Clavispora lusitanae* is unusually polymorphic. *FEMS Yeast Research*, **4**, 253–258.
- MARTIN, K. & RYGIWICZ, P.T. 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, **5**, 1–11.
- OKUBO, A. & SUGIYAMA, S.-I. 2009. Comparison of molecular fingerprinting methods for analysis of soil microbial community structure. *Ecological Research*, **24**, 1399–1405.
- ONOFRI, S., ZUCCONI, L. & TOSI, S. 2007. *Continental Antarctic fungi*. München: IHW, 247 pp.
- POPA, R., POPA, R., MARSHALL, M.J., NGUYEN, H., TEBO, B.M. & BRAUER, S. 2009. Limitations and benefits of ARISA intra-genomic diversity fingerprinting. *Journal of Microbiological Methods*, **78**, 111–118.
- RODRIGUEZ, R.J., CULLEN, D., KURTZMAN, C.P., KHACHATOURIANS, G.G. & HEGEDUA, D.D. 2004. Molecular methods for discriminating taxa, monitoring species, and assessing fungal diversity. In MUELLER, G.M., BILLS, G.F. & FOSTER, M.S., eds. *Biodiversity of fungi: inventory and monitoring methods*. Burlington MA: Elsevier, 77–102.
- SLABBERT, E., VAN HEERDEN, C.J. & JACOBS, K. 2010. Optimization of automated ribosomal intergenic spacer analysis for the estimation of microbial diversity in fynbos soil. *South African Journal of Science*, **10.4102/sajs.v106i7/8.329**.
- SMIT, E., LEEFLANG, P., GLANDORF, B., VAN ELSAS, J.D. & WERNARS, K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient electrophoresis. *Applied and Environmental Microbiology*, **65**, 2614–2621.
- WHITE, T.J., BRUNS, T., LEE, S. & TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In INNIS, M., GELFAND, J., SNINSKY, J. & WHITE, T.J., eds. *PCR protocols: a guide to methods and applications*. Orlando, FL: Academic Press, 315–322.
- ZALAR, P., SONJAK, S. & GUNDE-CIMERMAN, N. 2012. Fungi in polar environments. In MILLER, R.V. & WHYTE, L.G., eds. *Polar microbiology: life in a deep freeze*. Washington, DC: ASM Press, 79–99.