Mycologia, 99(5), 2007, pp. 681–692.

Using ITS2 secondary structure to create species-specific oligonucleotide probes for fungi

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Abstract: Oligonucleotide microarray based on ITS2 rDNA sequences would be extremely useful in identifying fungi within soil samples. However ITS2 contains phylogenetic information and duplication of sequences among taxa make false positive detections likely unless a way could be found to identify taxon-specific portions of the ITS2 sequence a priori. Examination of component ITS2 sequences suggested one method of identifying species-specific probes. Analysis of 168 fungal ITS2 sequences showed that all 168 ITS2 rRNA sequences could be folded to produce similar secondary structures of 3–4 loops. Unique probes occurred most often in the second loop. While the loop 2 sequence was unique in all taxa, there were partial congeneric and intergeneric duplicates. Evidence for a decrease in duplicates with increasing phylogenetic distance was mixed. From the evidence, 2 or 3 disjunct oligonucleotide probes from the loop 2 sequence might be sufficient to identify most fungal species. This combination appears minimally susceptible to false positives and conceivably could be extended to design probes to identify any eukaryotic species.

Key words: ITS2 secondary structure, microarray design, oligonucleotide probes, soil fungi

INTRODUCTION

Environmental and clinical microbiologists have poured considerable work into developing microarrays for sampling prokaryotic communities in soil, water, medical and other samples (Zhou 2003, Bodrossy and Sessitsch 2004). Less research has focused on fungal microarrays despite a need for them to sample both true fungi and organisms such as oomycetes (all of which will be termed fungi here). In addition to medically and economically important fungal pathogens of humans, animals and plants, fungi have unique functions in ecosystems. As with prokaryotes (Schleifer 2004) fungi are well known for their essential role in nutrient cycling and other ecosystem processes, and fungi have renowned effects on plant community patterns. Members of the symbiotic Glomeromycota repeatedly have been shown to substantially affect plant community composition and diversity (Grime et al 1987, van der Heijden et al 1998, Hartnett and Wilson 2002, van der Heijden 2002, Landis et al 2004). Fungi can have even more dramatic community effects: Pathogenic ascomycete fungi ranging from chestnut blight (*Cryphonectria parasitica*) (Anagnostakis 1987) to Dutch elm disease (*Ophiostoma ulmi* and *O. novo-ulmi*) (Buisman 1932, Brasier 1991, Ingrouille 1995) destroyed billions of trees in North America and Europe, turning former forest dominants into rare species, while oomycetes such as *Phytophthora cinnamomi* have changed forests into savannas and grasslands in Australia (Wills 1993, Weste et al 2002). Sudden oak death caused by *P. ramorum* is a lurking threat for a broad range of plant species (Rizzo et al 2002, Rizzo and Garbelotto 2003). Given their profound ecological effects rapid fungal identification from environmental samples is needed crucially.

An obvious approach is to create an oligonucleotide microarray that contains taxon-specific probes. Such an array would be simple to use; DNA could be extracted from a soil sample, hybridized to the array and the fungi present could be read from the array (although admittedly only known fungi would be found). Because the sequences for the ITS2 region of many fungi and oomycetes are readily available and the sequences are easy to extract from samples, this region appears to be a good target for finding unique probes. However ITS2 sequences do carry some phylogenetic information (Coleman 2003, Schultz et al 2005) and fungi that share identical regions of their ITS2 sequences will share probes. The probes ideally should contain only autapomorphies for species or strains of interest, not synapomorphies at higher phylogenetic levels such as genera, families and phyla.

Finding appropriate autapomorphies within the ITS2 region is even more difficult than it first appears. Given that most species of soil fungi are unknown (Hawksworth et al 1995) the array designer has to create probes that not only will register the taxon of interest but will not respond to some unknown fungus, especially if the array is designed to sample wild soils. The only apparent solution is to find a region of ITS2 that contains many autapomorphies,

Accepted for publication 20 July 2007.
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probes based on that region would have a higher a priori probability of being taxon specific.

One possible solution is to use ITS2 loop structure to find regions containing many autapomorphies. The ITS2 region appears to fold in comparable ways across a number of species, including *Saccharomyces cerevisiae* (Joseph et al 1999), plants (Mai and Coleman 1997, Coleman 2003), green algae (Coleman and Mai 1997), *Drosophila* (Young and Coleman 2004) and recently in a broad survey of 5000 sequences across the eukaryotes (Schultz et al 2005). Numbers of autapomorphies appear to vary by position within the secondary structure (Coleman and Mai 1997, Mai and Coleman 1997, Coleman 2003, Young and Coleman 2004) and secondary structure has been used as a species-level character within the fungal genus *Polyporus* (Krüger and Gargas 2004).

Following their lead we tested whether folded structures could be used to find regions with high numbers of autapomorphies likely to generate unique probes among fungal taxa. Our analysis of the ITS2 secondary structures, sequences and probes focused on three questions: (i) Do fungal ITS2 sequences have a common folding pattern? (ii) Does the number of duplicates per microarray probe depend on its position in its parent ITS2 sequence and structure? and (iii) Is there a correlation between number of duplicates and phylogenetic distance? In other words, if two fungi share a probe, is it more likely that they are near relatives? This last feature might be useful in designing an array because it would indicate that any fungus generating a false positive would be more likely to have a near relative on the array, rather than being a random organism that happened to match a particular probe.

MATERIALS AND METHODS

Sequences.—One hundred sixty-eight sequences from GenBank were used in the analysis (TABLE I). These sequences were used to generate probes of 20 nucleotide oligonucleotides. Each probe's position within each ITS2 sequence was numbered by its position of its first nucleotide (5′) in the ITS2. For example probe 1 contained sequence nucleotides 1–20, probe 2 contained nucleotides 2–21, and so on. In a 200-nucleotide sequence there were 181 numbered probes. By convention a probe was in a secondary structure if its first nucleotide was within that structure. For instance if a hairpin loop covers nucleotides 20–40 then probes 20–40 are considered within that loop, even though probes 30–40 had most of their nucleotides outside that structure. This naming convention proved useful for locating ITS2 probes and mapping them onto secondary structures.

ITS2 foldings.—To determine ITS2 secondary structures, each sequence was submitted to Mfold version 3.1 (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) (Mathews et al 1999, Zuker 2003) to determine likely RNA folding structures. The sequence was submitted both in one piece and also as 2–4 overlapping pieces (depending on length) to find folding patterns (long sequences tended to generate several equally probable folding patterns, while shorter sequences tended to generate one). The Mfold output was aligned portions of the ITS2 sequence within each folding region using Sequencher 4.2.2 (2003, Gene Codes Corp., Ann Arbor, Michigan). It is important to note that every part of each sequence was assigned to a particular folding region.

Probes.—Numbers of probe duplicates within the dataset were calculated with Microsoft Access, both across the entire ITS2 sequence and by secondary structure. The number of duplicates was combined with probe numbering and mapping to determine the number of duplicates per nucleotide position along each ITS2 sequence. Mean number and standard deviation of duplicates per probe position were calculated for all sequences with Microsoft Excel. Because these ITS2 sequences were 130–334 nucleotides long their secondary structures also varied in length, and comparing duplicate numbers base pair by base pair was not practical. Thus we also compared the number of duplicates within each secondary structural feature with ANOVA with Type III sums of squares (to compensate for unequal sequence lengths within each structure) with differences compared by Tukey's HSD test. These tests were run with S-plus version 6.0 (2001, Insightful Corp., Seattle, Washington).

Probes and phylogenetic distance.—The ITS2 dataset could not be used to generate a phylogeny to test the correlation between the number of duplicates and phylogenetic distance because such a test would be circular. Phylogenetic distance therefore was determined in two ways.

The first approach was to use another gene to create the phylogeny and measure distances. Thirty-five representative species for the distance measurements and 35 proxy species (near relatives) were used to calculate phylogenetic distances (TABLE II). A distance matrix was obtained from an alignment of these 35 species with heuristic search of PAUP* 4.0b 10 (Swofford 2002) using default settings with the distance optimality criterion set to minimum evolution. The number of duplicates was regressed against phylogenetic distance with two methods, a linear regression and a correlation whose probabilities were calculated from 999 permutations of the two datasets. This second method was implemented by Dr Bret Larget (University of Wisconsin at Madison).

Second, because many taxa used for ITS2 sequences were not associated with sequences for other DNA regions, we simply counted numbers of conspecific, congeneric (same genus, different species) and intergeneric (between genera) probe duplications within the dataset because there were many accessions at each of these levels (TABLE I). However this accounting was complicated by the fact that many of the accessions were incompletely named, meaning that each unidentified taxon had to be designated as a separate species (e.g. the six *Rhizoctonia* sp. in TABLE I). In addition the dataset of fungal species included both teleomorphic (sexual) and anamorphic (asexual) genera. Although some, such as *Thanetophorus cucumeris* (teleomorph) and *Rhizoc-*

TABLE I. The 169 taxa used in our analysis. GenBank accession numbers are given in parentheses. Multiple sequences were used for a number of taxa to provide samples among conspecifics and near relatives

Phylum	Taxon (accession[s])
Ascomycota	*Acremonium obclavatum* (AJ292394), *Ajellomyces capsulatus* (AC*U*18363), *Ajellomyces dermatitidis* (AF038358), *Alternaria alternata* (AY160211), *Alternaria cheiranthi* (AF229457), *Alternaria dauci* (AF267130), *Alternaria longissima* (AF229489), *Alternaria zinniae* (AF267135), *Ascochyta lentis* (AY131201), *Aspergillus nomius* (AF338645), *Balansia cyperi* (U89369), *Bipolaris heveae* (AY004775), *Botryosphaeria corticis* (AF243397), *Botryosphaeria* sp. (AF283684), *Botrytis cinerea* (Z99665), *Botrytis porri* (Z99666), *Candida albicans* (AF455531), *Candida dubliniensis* (AJ311898), *Candida tropicalis* (L47112), *Cephalosporium curtipes* (AJ292404, AJ292405), *Cercospora apii* (AF163085), *Cercospora caricis* (AF284390), *Chaetomium funicola* (AJ279450), *Cladosporium cladosporioides* (AF455535), *Cladosporium cladosporioides* (AF455525), *Cladosporium oxysporum* (L25432), *Cochliobolus homomorphus* (AF071349), *Colletotrichum gloeosporioides* (Z18995), *Colletotrichum fragariae* (AB087221), *Colletotrichum* sp. (AJ300561), *Colletotrichum trifolii* (AB087223), *Corynascus sexualis* (AJ224202), *Corynespora olivacea* (AF163088), *Curvularia affinis* (AF071335), *Curvularia brachyspora* (AF212308), *Curvularia gladioli* (AF071337), *Curvularia trifolii* (AF455446), *Diaporthe ambigua* (AF046906), *Diaporthe phaseolorum* (AF001026), *Diaporthe phaseolus* (AJ312359), *Didymella bryoniae* (AF297228), *Drechslera dematioidea* (AY004790), *Dreschlera avenae* (X78123), *Drechslera phlei* (AY004807), *Embellisia* sp. DAR (AF212307), *Emmonsia crescens* (AF038340), *Engyodontium aranearum* (AJ292391), *Epichloe amarillans* (AF385200), *Epichloe clarkii* (L78299), *Epichloe festucae* (AF059729), *Epicoccum nigrum* (AF149928, AF455455, AY093413), *Epicoccum* sp. A9 A (AJ279452), *Eupenicillium bovifimosum* (AF263347), *Eurotium rubrum* (AF455528), *Fusarium arthrosporioides* (AF111065), *Fusarium buharicum* (U34581), *Fusarium* spp. (AF158303, AF178409, AF310977), *Gaeumannomyces* sp. (AJ010038), *Gelasinospora nigeriensis* (AJ002400), *Gibberella avenacea* (AF009187), *Gibberella fujikuroi* (AF117922, AF455460), *Helminthosporium velutinum* (AF120262), *Humicola fuscoatra* (AJ279444), Leaf litter ascomycetes (AF502897, AF502900, AF502834), *Leptosphaerulina trifolii* (AY131203), *Macrophomina phaseolina* (AF132795), *Magnaporthe grisea* (U17329), *Massarina eburnea* (AF383959), *Monilinia laxa* (Z73784), *Mycosphaerella populorum* (AF243392), *Myrothecium atroviride* (AF455507), *Myrothecium roridum* (AJ301995, AJ301994), *Nectria cinnabarina* (AF163025), *Nectria galligena* (AJ228662), *Nectria haematococca* (AF455451), *Nectria vilior* (U57673), *Neotyphodium chisosum* (AF385203), *Neotyphodium tembladerae* (AF385211), *Neurospora sitophila* (AF388926), *Nomuraea* cf. *viridula* (AF368500), *Paecilomyces variotii* (AF455416), *Paracoccidioides brasiliensis* (AF092903), *Penicillium aurantiovirens* (AJ005490), *Phoma destructiva* (AF268191), *Phoma herbarum* (AF218792), *Phomopsis amygdali* (AB017740), *Phomopsis longicolla* (AF000210), *Phomopsis oryzae* (AF079777), *Phomopsis vaccinii* (AF317573), *Podospora curvicolla* (AF486637), *Pseudocyphellaria episticta* (AF351152), *Pyrenophora teres* (AF163061), *Pyricularia grisea* (AB031347), *Saccharomyces cerevisiae* (AF219007, M87397) *Saccharomyces dairensis* (D89893), *Scleromitrula candolleana* (Z80878), *Sordaria macrospora* (AF246293), *Stachybotrys echinata* (AF205452), *Stachybotrys longispora* (AF081482), *Stemphylium callistephi* (AF229482), *Stemphylium solani* (AF426739), *Stemphylium trifolii* (AF442800), *Sticta martinii* (AF351155), *Thermoascus crustaceus* (U18353), *Thielavia hyrcaniae* (AJ271581), *Trichoderma aureoviride* (AF362108), *Trichoderma harzianum* (AJ507140), *Trichoderma inhamatum* (AF414302, AF414302), *Trichoderma* spp. (AF408107, AF408127), *Trichothecium roseum* (U51982), *Ulocladium botrytis* (AF267139), *Valdensinia heterodoxa* (Z81447), *Verticillium chlamydosporium* var. *catenulatum* (AJ292398), *Verticillium chlamydosporium* var *chlamydosporium* (AB100362)
Basidiomycota	*Armillaria mellea* (AF310329, U54818), *Armillaria ostoyae* (U54813), *Armillaria sinapina* (AF169646), *Ceratobasidium* sp. JTO078 (AF472293), *Ceratobasidium* sp. (AF472285), *Ceratobasidium* sp. CAG1 (AF354086), *Filobasidiella neoformans* (AF444444), *Rhizoctonia cerealis* (AF063019), *Rhizoctonia* spp. (AF200517, AJ242892, AJ242895, AJ318442, AJ318443, AJ419929), *Schizophyllum amplum* (AF141873), *Schizophyllum commune* (AF062633, AF249380, AF249385, AF280751), *Schizophyllum umbrinum* (AF249391), *Thanatephorus cucumeris* (AF354062)
Glomeromycota	*Gigaspora albida* (AF004702, AF004703), *Gigaspora decipiens* (AJ239119), *Gigaspora margarita* (AB048607), *Glomus coronatum* (X96844, X96845), *Glomus claroideum* (AF004687, AJ239126), *Glomus etunicatum* (AF004680), *Glomus fasciculataum* (X96843), *Glomus geosporum* (AF197918), *Glomus mosseae* (X84232), *Scutellospora cerradensis* (AB048684), *Scutellospora heterogama* (AF004691), *Scutellospora pellucida* (AJ239121)
Oomycota	*Phytophthora citricola* (L41375), *Phytophthora clandestina* (L76537), *Phytophthora erythroseptica* (AF339429), *Phytophthora nicotianae* (AF467087), *Phytophthora phaseoli* (AF266778), *Phytophthora ramorum* (AF429768), *Phytophthora richardiae* (AF271221), *Pythium aphanidermatum* (AF452146), *Pythium arrhenomanes* (AJ233444, AF330182), *Pythium inflatum* (AJ233446), *Pythium myriotylum* (AF452156), *Pythium terrestris* (AY039714), *Pythium ultimum* (AF339421)

TABLE II. Accessions used to calculate number of duplicate probes and phylogenetic distance. Where the accession number is listed, that accession is for the sequence from GenBank. Where another species is listed, that species' accession was used as a proxy for the probe sequence. In all cases the proxy species is a known near relative and, where they are of different genera, typically one is an anamorphic species and one is a teleomorph

Species for Probe	Phylogeny accession and proxy species
Ascomycota	
Ajellomyces capsulatus (AC*U*18363)	(Z75306)
Alternaria alternata (AY160211)	(AF218791)
Alternaria longissima (AF229489)	*Paraphaeosphaeria quadriseptata* (AF250826)
Aspergillus nomius (AF338645)	(AB008404)
Botryosphaeria corticis (AF243397)	*Botryosphaeria ribis* (U42477)
Candida albicans (AF455531)	(AB013586)
Chaetomium funicola (AJ279450)	*Chaetomium globosum* (AY545725)
Cladosporium cladosporioides (AF455525)	(AF548071)
Engyodontium aranearum (AJ292391)	*Torrubiella confragosa* (AB079127)
Epichloe amarillans (AF385200)	*Nomuraea rileyi* (AB100361)
Epicoccum nigrum (AF149928)	(AJ295235)
Eurotium rubrum (AF455528)	(U00970)
Gibberella fujikuroi (AF117922)	*Cordyceps sinensis* (AB067700)
Helminthosporium velutinum (AF120262)	(AF120254)
Magnaporthe grisea (U17329)	(AB026819)
Mycosphaerella populorum (AF243392)	*Anguillospora rubescens* (AY357266)
Myrothecium atroviride (AF455507)	(AJ302002)
Nectria cinnabarina (AF163025)	(AB003949)
Paecilomyces variotii (AF455416)	(AB023948)
Paracoccidioides brasiliensis (AF092903)	(AF238302)
Phoma herbarum (AF218792)	(AF218792)
Phomopsis longicolla (AF000210)	*Leucostoma persoonii* (M83259)
Saccharomyces cerevisiae (AF219007)	(*J*01353)
Trichoderma harzianum (AJ507140)	*Hypocrea rufa* (AJ301991)
Trichothecium roseum (U51982)	*Paecilomyces lilacinus* (AB124670)
Ulocladium botrytis (AF267139)	(AF548106)
Basidiomycota	
Ceratobasidium sp. (AF472285)	(AY293122)
Filobasidiella neoformans (AF444444)	*Cryptococcus neoformans* (AJ560332)
Schizophyllum commune (AF062633)	(X54865)
Thanatephorus cucumeris (AF354062)	*Rhizoctonia solani* (D85643)
Glomeromycota	
Glomus mosseae (X84232)	(AY635833)
Gigaspora decipiens (AJ239119)	*Gigaspora rosea* (X58726)
Scutellospora cerradensis (AB048684)	(AB041345)
Oomycota	
Phytophthora nicotianae (AF467087)	(AY744947)
Pythium aphanidermatum (AF452146)	(AY742755)

tonia solani (anamorph), are known to be different names for the same taxon, the linking of anamorphic and teleomorphic genera is incomplete because there is no one-to-one concordance between sexual and asexual species names. For many species only one of the two forms is known (or even exists). Because of these discrepancies anamorphic and teleomorphic genera were treated as separate taxa, although this artificially inflated the number of intergeneric duplication events. This inflation more severely tests the utility of the analytic method because the method is designed to minimize the number of intergeneric matches.

RESULTS

All ITS2 sequences shared a secondary structure: two well defined hairpin loops (coded as ''loops'') with well defined sequences joining them (coded as ''joins''), and a complex structure that in different species was modeled by Mfold as a single loop, as two loops or as a complex forking structure (FIG. 1). Although many ITS2 secondary structures have been reported to have four well defined loops, some of our sequences lacked

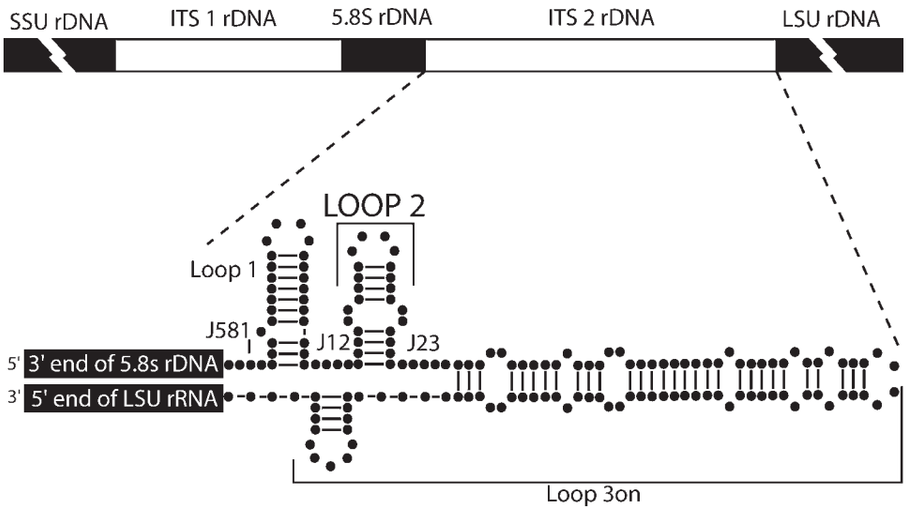

FIG. 1. Labeled ITS2 folding structure. The diagram shows the folding structures and labels we found in ITS2. Loops 1 and 2 are labeled, joined to the 5.8s region by the J581 structure and joined to each other by J12. In this example loop 3on shows two loops, this fourth loop was missing, while in others loop 3 had several subloops. Due to this complexity, the end simply was labeled L3on, as described in the text.

the fourth loop or it was included only in one of many equally probable structures. To describe the secondary structures, the regions were coded as either joins (J) or loops (L) with a number corresponding to their relative placement: J581 is the short sequence between the 5.8s ribosomal subunit and loop 1, L1 is loop 1, and so on. The structures in order were J581, L1, J12, L2, J23 and L3on. The last structure, L3on, abstracts the complexity of the 3′ end without further subdividing it into loops and joining regions. The loop 2 sequences used in this analysis are provided (APPENDIX 1).

Mean numbers of probe duplicates between fungal taxa per ITS2 sequence position showed a pronounced dip in the region corresponding to L2 in most sequences (FIG. 2a, at tip of arrow). In this region each probe had roughly 2.0 ± 2 duplicates and 80.8% of probes were unique. The similar low number of duplicates at the 3′ end of the L3on structure likely reflects the low number of sequences in this analysis which included this region (FIG. 2b); only *Armillaria* had ITS2 sequences that long. L2 was a well defined secondary structure present in all samples, although it was somewhat variable in length (mean 36.04 ± 8.81 nt). ANOVA unambiguously showed that J12 and L2 both have significantly fewer probe duplicates ($df = 7$, $P < 0.0001$) than other regions (FIG. 3). Therefore subsequent analyses focused on the L2 region.

Do close relatives share more probes? For the 35 species pairs used for the phylogenetic analysis there were five pairs of interspecific duplicates. With 630 possible pairs (not counting species paired to themselves) this means that less than 1% of the species shared probes. Regressions based on the 35 taxa datasets were both not significant, no matter what method was used. An examination of the pattern of duplications across phylogenetic distance provides an explanation (FIG. 4). Five pairs of species (out of a possible 630 pairs) were not unique, and there is no pattern to their distribution.

Looking at shared probes within the sample, within the L2 region, 7.4% of probes were unique to a single accession, 36.1% of the duplicates were found among conspecifics, 37.5% among congenerics and 19% apparently were shared among genera. The number of congenerics included fungi identified only to genus (such as *Rhizoctonia* sp.) so these congenerics probably included unrecognized conspecifics. Of the intergeneric duplicates, two-thirds occurred among five genera, the anamorphic *Rhizoctonia*, *Fusarium* and *Epicoccum*, and the teleomorphic *Ceratobasidium* and *Gibberella*. In this sample *Rhizoctonia* and *Ceratobasidium* were probably the same genus (and as noted above occasionally the same species), as were *Fusarium* and *Gibberella*. While there were certainly true intergeneric copying events, the anamorph/teleomorph pairing holds for many of the other 53 genera on this list. Therefore 19% was undoubtedly a gross overestimate of probe duplication among genera, resulting from a suboptimal dataset. This number is higher than the result from the phylogenetic survey because that used no close relatives. Overall it appeared that close relatives (conspecifics and congenerics) shared more probes.

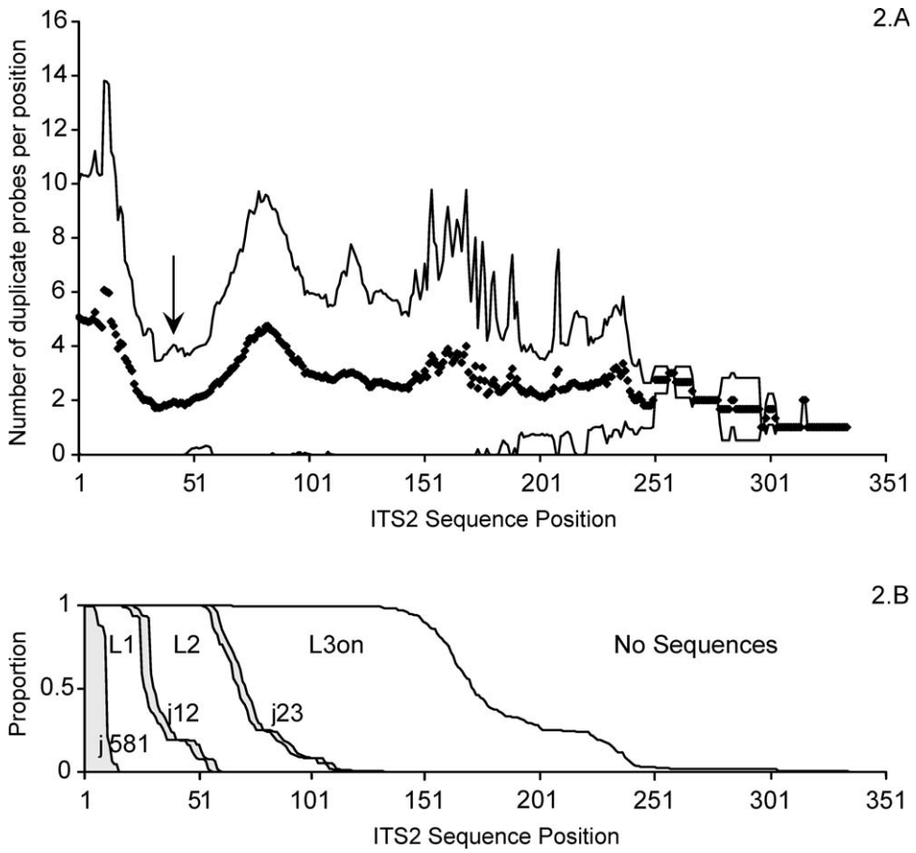

FIG. 2. ITS2 probe duplicates and sequence structures. A. Mean number of probes per ITS2 sequence position, based on a survey of 147 sequences. Black diamonds are the means, whereas the lines indicate ±1 s.d. The arrow indicates a region starting at ITS position 38, where 66% of probes are unique. B. Secondary structures mapped onto ITS2 sequence position. Hairpin loops (L1, L2, L3on) are white, while joining regions (J581, J12, J23) are light gray. Sequences were 130–334 nucleotides long. Top to bottom this graph shows the proportion of accessions that contain a particular secondary structure at that sequence location. Note that the region marked by the arrow in A. falls within L2 (loop 2).

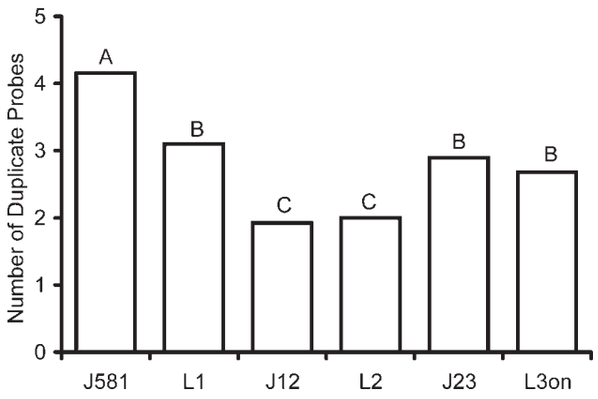

FIG. 3. Mean number of duplicates per ITS2 secondary structure. Structure codes follow FIG. 2 and are explained in the results. Letters indicate values that are significantly different, following Tukey's HSD test with 95% confidence intervals.

Overall in no case was the entire L2 region shared between truly different genera. Although some parts of the L2 region might be shared, duplication events inevitably were clustered at either the beginning or end of the ITS2 sequence. In a few cases up to half of the sequence was duplicated among species, but in those cases sequences at the other end of the loop were unique.

DISCUSSION

The analysis demonstrated that fungal ITS2 rRNA have consistent secondary structures with a region in the second loop structure rich in autapomorphies suited for unique probe design. While we used a three-loop structure (rather than the four-loop secondary structure found for other taxa) our L2 corresponds roughly to the tip of the second loop demonstrated in plants (Coleman and Mai 1997, Coleman 2003) and

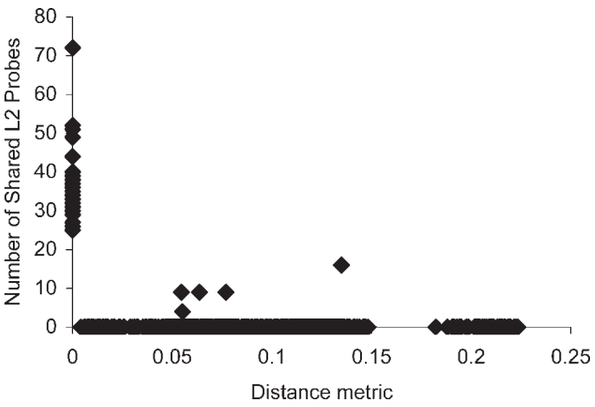

FIG. 4. Mapping numbers of probe duplicates among accessions and phylogenetic distance for 35 species. Diamonds on the Y axis show numbers of duplicate probes within a single species (phylogenetic distance = 0), whereas diamonds on the X axis show where there are no duplicate probes between species. Only five pairs of species (out of a possible 630 pairs) have duplicate probes between species. No pattern was found for the duplication; numbers of duplicate probes did not increase or decrease with phylogenetic distance.

green algae (Schultz et al 2005). The second loop also was identified as a region of high autapomorphies by other researchers (Coleman 2003).

Interspecific and intergeneric duplications did occur within L2, but this presents no major problems. A single probe cannot unambiguously identify a fungal species. Multiple, separated probes, for instance from the beginning, middle, and end of L2, likely will be sufficient to identify a species because we found no evidence of species that share entire loop 2 sequences. It is possible that groups of species share entire loop 2 sequences, but they did not appear in this sample. However any such groups identified in the future likely will appear in the literature and future researchers will be able to avoid problematic taxa. Searches of GenBank and other databases for related sequences should be a preliminary part of any probes designed with this technique.

The regression results do not unequivocally support the hypothesis that the number of duplicate probes decreases with increasing phylogenetic distance. The results of the duplicate counts in the entire dataset suggested otherwise, but that dataset could not be used to calculate phylogenetic distances in our research. More thorough sampling of sequences outside the ITS2 region likely will provide the accessions necessary for future re-examination of this hypothesis.

The fundamental conclusion is that ITS2 sequences can be used to design probes that are, if not species-specific, close to that level. The second loop of ITS2 has been shown to contain a high number of autapomorphies. By using a number of probes based on the loop 2 sequence, it should be possible to detect any known fungal species. Moreover a set of 2–3 loop 2 probes is resistant to false positives, a benefit given that unknown fungal species likely will be present in most environmental samples. While this analysis focused on probes for microarray design, these results are adaptable to any technology that uses oligonucleotide probes to sample communities. Finally, because eukaryotes in a number of phyla have been shown to have ITS2 sequences with similar secondary structures, ITS2 loop 2 sequences could be suitable for creating probes for any eukaryote.

ACKNOWLEDGMENTS

The authors thank the University of Wisconsin at Madison and UW Graduate School for financial support; Luigi Vitiritti, Steve Smith and Emile Nuwaysir of Nimblegen Systems Inc., Madison, Wisconsin, for microarray design and creation; and Sandra Splinter, David Frish and John Luecke of the Gene Expression Center at UW-Madison for microarray hybridization and analysis advice. Dirk Krüger and Paula T. DePriest provided helpful insight. We thank Bret Larget for patient guidance and designing a neat and useful permutation-based statistical test

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APPENDIX I.

Species	Accession	Start	Sequence
Acremonium obclavatum	AJ292394	38	GGGACCCGGCGATCGGGGACTTTAGTTCCCCTGCCGGTCCCG
Ajellomyces capsulatus	ACU18363	31	GGGCCATCGTCCCCCTGACCGGTGGGACGTGCCCG
Ajellomyces dermatitidis	AF038358	26	GGGCCTTCGTCCCCCCGTGGACGTGCCCG
Alternaria alternata	AY160211	30	GGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTT
Alternaria cheiranthi	AF229457	30	GGGCGTCTTGTCTCCAGTTCGCTGGAGACTCGCCTT
Alternaria dauci	AF267130	30	GGGCGTCTTTTTGTCTCCCCTTGCGGGAGACTCGCCTT
Alternaria longissima	AF229489	30	GGGCGTCTTGTCCCGCGTTGTCGCGTGGACTCGCCTT
Alternaria zinniae	AF267135	30	GGGCGTCTTTTTGTCCCCCCCCCCTTGCGGGGAGACTCGCCTT
Armillaria mellea	AF310329	23	GGGGGTTTGCTGGTCTCTAACGAGATCAGCTCCTCTG
Armillaria mellea	U54818	23	GGGGGTTTGCTGGTTTCTAACGAGATCAGCTCCTCTG
Armillaria ostoyae	U54813	23	GGGGGTTTGCTGGTTTCTAACGAGATCAGCTCCTCTG
Armillaria sinapina	AF169646	53	GGGGGTTTGCTGGTTTCTAACGAGATCAGCTCCTCTG
Ascochyta lentis	AY131201	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTC
Aspergillus nomius	AF338645	32	GGGTCGTCGTCCCCCCCTCCGGGGGGGGGACGGGCCCT
Bipolaris heveae	AY004775	30	GGGCGTCTTTTTGTCTCTCCTTTGCGGGAGACTCGCCTT
Botryosphaeria corticis	AF243397	30	GGGCACCGTCCCTTGCGGGCGCGCCTC
Botryosphaeria sp.	AF283684	30	GGGCCCCGTCCTCTGTGGACGCGCCTC
Botrytis cinerea	Z99665	30	GAGTCTATGTCAGTAATGGCAGGCTCC
Botrytis porri	Z99666	30	GAGTCTATGTCAGCAATGGCAGGCTCT
Candida albicans	AF455531	32	GAGCAATACGACTTGGGTTTGCTTG
Candida dubliniensis	AJ311898	33	GAGCAATACGACTTGGGTTTGCTTG
Candida tropicalis	L47112	32	GAGCAANACCCTAGGTTTGTTTG
Cephalosporium curtipes	AJ292404	30	GGGGATCGGCGCCGCCCCCCCTCTGTCGTTCGCGGC AGGGCGGGAGGGTCGCCGCCCCCG
Cephalosporium curtipes	AJ292405	30	GGGGATCGGCGTTGCCTCCTCCGTCGTTCGCG GCGGGGTGAGGTCGCCGCCCCG
Ceratobasidium sp.	AF472285	52	GGAGGTCTTTGCGGATTAATATCTGCTCCTCTT
Ceratobasidium sp. CAG1	AF354086	52	GGAGGTTTTGCAGATTCACGTCTGCTCCTCTT
Ceratobasidium sp. JTO078	AF472293	52	GGGGGTTTGCAGATTCACGTCTGCTCCTCTT
Cercospora apii	AF163085	30	GGGCGCCGCGGTGTTCCGCGCGCBTC
Cercospora caricis	AF284390	30	GGGCGCCGCGGTGTTTCCGCGCGCCTC
Chaetomium funicola	AJ279450	31	GGGGACCTGCGGCACACCCGCAGGCCCTG
Cladosporium cladosporioides	AF455525	30	GGGCAACGCGGTCCGCCGCGTGCCTC
Cladosporium cladosporioides	AF455535	30	GGGCAACGCGGTCCGCCGCGTGCCTC
Cladosporium oxysporum	L25432	30	GGGCAACTCGGTCCGCCGGGTGCCCTC
Cochliobolus homomorphus	AF071349	24	GGGCGCTTGTCTCTCCTCTTTTTGGGGGAGACTCGCCTT
Colletotrichum fragariae	AB087221	30	GGGGCCCTACAGCTGATGTAGGCCCTC
Colletotrichum sp.	AJ300561	30	GGGGCCCCACGGCACACGTGGGCCCTTG
Colletotrichum trifolii	AB087223	30	GGGGCTTCCACGGCTGACGTGGGCCCTC
Corynascus sexualis	AJ224202	32	GGGGACCTGCGGCTGCCCGCAGGCCCTG
Corynespora olivacea	AF163088	30	GGGCGTCTGTCCCGCCTCCGCGCGTGGACTCGCCC
Curvularia affinis	AF071335	30	GGGCGTTTTTTGTCTTTGGTTTTGTCCAAAGACTCGCCTT
Curvularia brachyspora	AF212308	30	GGGCGTCTTTTTTGTCTTGGGCCTTTGTGCCCTGAGAC TCGCCTT
Curvularia gladioli	AF071337	30	GGGCGTCTTGTCTTTTGGCTTCCAGCCCAAAGACTCGCCTT
Curvularia trifolii	AF455446	30	GGGCGTCTTGTCTTTTGGCTCTTTGCCCAAAGACTCGCCTT
Diaporthe ambigua	AF046906	30	GGGGCACTGCTTCTTACCCAAGANGCAGGCCCTG
Diaporthe phaseolorum	AF001026	30	GGGGCACTGCGTCTCTCGCGGGATGCAGGCCCTG
Diaporthe phaseolus	AJ312359	30	GGGGCACTGCTTTCGTCCAGAAAGCAGGCCCTG
Didymella bryoniae	AF297228	30	GGGTGTTTGTCTCGCCTCTGCGCGCAGACTCGCCTC
Drechslera dematioidea	AY004790	30	GGGCGTTTTTGTCTCGGGTCCGCCCCGAGACTCGCCTT
Drechslera phlei	AY004807	30	GGGCGTCTTGTCTTGCGGGGTCCATCCCCCACGACTCGCCTT
Dreschlera avenae	X78123	30	GGGCGTTTTGTCTTGGGTCCGTCCCGAGACTCGCCTT
Embellisia sp. DAR	AF212307	30	GGGCGTCTTTTTGTCTCCGGCTTGCTGGAGACTCGCCTT
Emmonsia crescens	AF038340	31	GGGCCCTCGTCCCCCGTGGACGTGCCCG
Engyodontium aranearum	AJ292391	30	GGGGTCGGCAGCTACCGCCGGCCCCG

APPENDIX I. Continued

Species	Accession	Start	Sequence
Epichloe amarillans	AF385200	36	GGGGACCGGCTCACCCGCCTCGCGGCGGCGGCCGCCCCTG
Epichloe festucae	AF059729	36	GGGGACCGGCTCACCCGCCTCGCGGCGGCGGCCGCCCCCG
Epicoccum nigrum	AF149928	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTT
Epicoccum nigrum	AF455455	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTT
Epicoccum nigrum	AY093413	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTT
Epicoccum sp. A9	AJ279452	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTT
Epicoccum sp. A9	AJ279452	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTT
Eupenicillium bovifimosum	AF263347	31	GGGCCCCGTCCTCCGATTCCGGGGGACGGGCCCG
Eurotium rubrum	AF455528	31	GGGCTTCCGTCCCTGGCAACGGGGACGGGCCC
Filobasidiella neoformans	AF444444	37	GGACTTGGATTTGGGTGTTTGCCGCGACCTGCA AAGGACGTCGGCTCGCCTT
Fusarium arthrosporioides	AF111065	33	GGGGATCGGCTCTGCCTTCTGGCGGTGCCGCCCCCG
Fusarium buharicum	U34581	33	GGGGATCGGCGAGTCTCTAGGGACGAGCCGGCCCCG
Fusarium sp.	AF158303	33	GGGGATCGGCGAGCCCTTGCGGCAAGCCGGCCCCG
Fusarium sp.	AF178409	33	GGGGATCGGCGGAGCCCTCCGTGGCACACGCCGTCCCCC
Fusarium sp.	AF310977	33	GGGGATCGGCGAGTCTCTCGGGACGAGCCGGCCCCG
Fusarium sp.	U61692	33	GGGGATCGGCGAGCCCTTGCGGCAAACCGGCGCCG
Gaeumannomyces sp.	AJ010038	31	GGGGCACCCGGCCGCCCAGCGGCCCGGGGCCCCC AAGTCAATCGGCGGGCTCGTCGGG ACCCTGAGCGCAGTAACACGCGGTCCCCC
Gelasinospora nigeriensis	AJ002400	27	GGGGATCCGCGGCTGCCCGCGGTCCCTC
Gibberella avenacea	AF009187	33	GGGGATCGGCTCTGCCTTACGGCGGTGCCGCCCCCG
Gibberella fujikuroi	AF117922	33	GGGGATCGGCAAGCCCTTGCGGCAAGCCGGCCCCG
Gibberella fujikuroi	AF455460	33	GGGGATCGGCGAGCCCTTGCGGCAAGCCGGCCCCG
Gigaspora albida	AF004702	38	TGGGTAATATCGATTTTATAAATCGATTACCT
Gigaspora albida	AF004703	38	TGGGTATTTTGATTTTATAAATCAATTACCT
Gigaspora decipiens	AJ239119	38	TGGGTATTCCGATTTTATAAATTGGTTACCT
Gigaspora margarita	AB048607	39	TGAGTATTCCGATTTTATTAATCAGTTACCT
Glomus claroideum	AF004687	40	GGGCTTTTATTTCATTTTAATGATTTATGGCCTT
Glomus claroideum	AJ239126	40	GGGCTTTTATTTCATTTTAATGATTTATGGCCTT
Glomus etunicatum	AF004680	41	GGGCCTTTATTTCATTAACGATTTATGGCCTC
Glomus geosporum	AF197918	41	GGGTCTTTATTTCATTAATGATTTATGGCCTC
Helminthosporium velutinum	AF120262	30	GGGCGTCTGTCCCGCCTCCGCGCGTGGACTCGCCCC
Humicola fuscoatra	AJ279444	26	GGGTGCCTGTCCCGCCTCCGCGCGTGGACTCACCTC
Leaf litter asco	AF502834	30	GGGGACCTGCGGCTGCCGCAGGCCCTG
Leaf litter asco	AF502897	30	GGGGCACTGCTCTCCCACGAGAGCAGGCCCTG
Leaf litter asco	AF502900	30	GGGGATCGGCGCGGCGCCCCCTCACCGGGGCGCTG CCGGCCCCG
Leptosphaerulina trifolii	AY131203	30	GGGTGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTT
Macrophomina phaseolina	AF132795	30	GGGCACCGTCCTTTGCGGGCGCGCCTC
Magnaporthe grisea	U17329	31	GGGGCGCCCGGGCCCTCCGCGGCCCGGGGCCCCC
Massarina eburnea	AF383959	30	GGGCGTCTGTCCCCTCTTCGGGGGGGACTCGCCCC
Monilinia laxa	Z73784	30	GAGTCTATGTCAGCAATGGCAGGCTCT
Mycosphaerella populorum	AF243392	31	GGGCGCCGCGGTGTTCCGCGCGCCTC
Myrothecium atroviride	AF455507	34	GGGGATCGGCCCAGCCTTCTCGCAAGGCCGCCGGCCCCG
Myrothecium roridum	AJ301994	34	GGGGATCGGCGTGGGCGGCGACGGCTCTCCGGA GCCCGAGCCAATGCCTGCCGGCCCCG
Myrothecium roridum	AJ301995	34	GGGGATCGGCGCGGGCCGGGGTCGTCCTCCGGGA CGGTCCCGCGCCTGCCGGCCCCG
Nectria cinnabarina	AF163025	35	GGGGATCGGCCTGCGGCGTGACGCGTGGCCGGCCCCG
Nectria galligena	AJ228662	33	GGGGATCGGCGTGCCCTTCGCGGCGCGCCGTCCCCT
Nectria haematococca	AF455451	34	GGGGATCGGCGGAAGCCCCCTGTGGGCACACGCCGTCCCTC
Nectria vilior	U57673	33	GGGGATCGGCCGCCCTCCGGCGCGCCGGCCCCG
Neotyphodium chisosum	AF385203	36	GGGGGCCGGCCCGCCCGCCTCGCGGTGGCGGCCGCCCCCG
Neotyphodium tembladerae	AF385211	36	GGGGGCCGGCTCACCCGCCTCGCGGCGGCGGCCGCCCCCG
Neurospora sitophila	AF388926	29	GGGGATCCGCGGCTGCCCGCGGTCCCTC

APPENDIX I. Continued

Species	Accession	Start	Sequence
Nomuraea cf. *viridula*	AF368500	35	GGGGGCCGGCAATAGTGCCTCGCGTTGTATGATGC GAGCGCGCGCCGCCCCCG
Paecilomyces variotii	AF455416	32	GGGCCGCCGTCCCCCCTCCCCGGGGGACGGGCCCG
Paracoccidioides brasiliensis	AF092903	31	GGGCCCGCGTCCCCCCATGGACGTGCCCG
Penicillium aurantiovirens	AJ005490	31	GGGCCCCGTCCTCCGATTCCGGGGGACGGGCCCG
Phoma destructiva	AF268191	30	GGGTGTTTGTCTCGCCTTTGCGTGTAGACTCGCCTC
Phoma herbarum	AF218792	30	GGGTGTTTGTCTCGCCTCTGCGCGCAGACTCGCCTC
Phomopsis amygdali	AB017740	26	GGGGCACTGCCTTTGTGTAAAAGCGAAAGCAGCCCTG
Phomopsis longicolla	AF000210	30	GGGGCACTGCTCTCTGACGGGAGCAGGCCCTG
Phomopsis oryzae	AF079777	30	GGGGCACTGCTTTTCACGAAGCAGGCCCTG
Phomopsis vaccinii	AF317573	30	GGGGCACTGCCTTTACCCAAAGGCAGGCCCTG
Phytophthora citricola	L41375	59	GAAGTGTCTTGCAGGTGTCCTTCGGGTCGTCTGC GAGTCCTTTG
Phytophthora clandestina	L76537	59	GAAGTGTCTTGCGGTTGGTTTCCGGACCGACTG CGAGTCCTGTT
Phytophthora nicotianae	AF467087	59	GAAGTGTCTTGCGATTGGTCTTCGGACCGGCTGCGA GTCCTTTT
Phytophthora phaseoli	AF266778	59	GAAGTGTCTTGCGGTTGGTTTTCGGACCGACTGC GAGTCCTTTT
Phytophthora richardiae	AF271221	61	GAAGTGTCTTGCGGCGGCGGCTTCTGCCGGCTGCTG CGAGTCCTTTG
Podospora curvicolla	AF486637	32	GGGGACCTGCGTCCGACGCAGGCCCTG
Pseudocyphellaria episticta	AF351152	30	GGGCCCCGTCCCCCGGACGGGTCCG
Pyrenophora teres	AF163061	30	GGGCGTCTTTTGTCTCTCCCCCGAGACTCGCCTT
Pyricularia grisea	AB031347	30	GGGGCGCCCGGGTCCTCCGCGGCCCGGGGCCCCC
Pythium aphanidermatum	AF452146	59	GAGGTGTCTCGCTGGCTCCCTTTTCGGAGGAGAAGA CGCGAGTCCCTTT
Pythium arrhenomanes	AF330182	58	GAGGTGTCTCGCTGACTCCCTCTTCGGAGGAGAAGA CGCGAGTCCCTTT
Pythium arrhenomanes	AJ233444	58	GAGGTGTCTCGCTGACTCCCTCTTCGGAGGAGAAGAC GCGAGTCCCTTT
Pythium inflatum	AJ233446	59	GAGGTGTCTCGCTGGCTCCCTCTTCGGAGGAGAAGAC GCGAGTCCCTTT
Pythium myriotylum	AF452156	59	GAGGTGTCTCGCTGGCTCCCTCTTCGGAGGAGAAGA CGCGAGTCCCTTT
Pythium terrestris	AY039714	59	GAGGTGTCTCGCGGCTGTTGTGTGTAGAAGGTTTGTATG AACTTTGTATGCGAAGCTTCGAGTCCCTTT
Pythium ultimum	AF339421	60	GAAGTGTCTCGCTGTGGTTGGTATATTTGTTTATGC ACAACTTGCGAGTCCTTTT
Rhizoctonia cerealis	AF063019	53	GGAGGTTTTGCAGATTCACGTCTGCTCCTCTT
Rhizoctonia sp.	AF200517	50	GGAGGCTTGCAGATTTCACACTCTGCTCCTCTT
Rhizoctonia sp.	AJ242892	50	GGAGGTCTTGCAGATGTCACAGTCTGCTCCTCTT
Rhizoctonia sp.	AJ242895	50	GGAGGTCTGCAGATTCACGTCTGCTCCTCTT
Rhizoctonia sp.	AJ318442	50	GGAGGTCTTTTGCAGATTTCACGTCTGCTCCTCTT
Rhizoctonia sp.	AJ318443	50	GGAGGTCTTTGCAGATTTCACGTCTGCTCCTCTT
Rhizoctonia sp.	AJ419929	50	GGAGGTCTGCAGATTCACGTCTGCTCCTCTT
Saccharomyces cerevisiae	AF219007	31	GAGTGATACTCTTTGGAGTTAACTTG
Saccharomyces cerevisiae	M87397	31	GAGTTAACTTG
Saccharomyces dairensis	D89893	33	GAGTGATACTCTTGCGAGTTAACTTG
Schizophyllum amplum	AF141873	52	GGAGGTCTGCTGGAACCTAACAGTGCCAGCTCCTCTC
Schizophyllum commune	AF062633	52	GGAGGTCTTGCTGGAGCCTAACGGTGCCAGCTCCTCTT
Schizophyllum commune	AF249380	52	GGAGGTCTTGCTGGAGCCTAACGGAGCCAGCTCCTCTT
Schizophyllum commune	AF249385	52	GGAGGTCTGCTGGAGCCTAACGGAGCCAGCTCCTCTC
Schizophyllum commune	AF280751	52	GGAGGTCTGCTGGAGCCTAACGGAGCCAGCT
Schizophyllum umbrinum	AF249391	53	GGAGGTCTGCTGGAGCCTAACGGATCCAGCTCCTCTT
Scleromitrula candolleana	Z80878	25	GGGCCTCCGCCAGTAAAATGGCGGGCCTT

APPENDIX I. Continued

Species	Accession	Start	Sequence
Scutellospora cerradensis	AB048684	39	TGGGTTATCTGATATTATTAATCGGTTACCT
Scutellospora heterogama	AF004691	38	TGGGTTATCCGATTCTTAAGGTCGGTTACCT
Scutellospora pellucida	AJ239121	39	TGGGTTATCCGATTCTTAAGGTCGGTTACCT
Sordaria macrospora	AF246293	29	GGGGATCCGCGTCTGACGCGGTCCCTC
Stachybotrys echinata	AF205452	47	GGGGATCGGCCCGCCCCCGCGGCGGCGCCGTCCCCG
Stachybotrys longispora	AF081482	39	GGGGATCGGCCCGCCTCAGCGCGGTGCCGTCCCCG
Stemphylium callistephi	AF229482	30	GGGCGTCTTTTTGTCTCTCACGAGACTCGCCTT
Stemphylium solani	AF426739	26	TGTTGGGCGTCTTGTCTCTCACGAGACTCGCCTT
Stemphylium trifolii	AF442800	30	GGGCGTCTTTTGTCTCTCACGAGACTCGCCTT
Sticta martinii	AF351155	31	GAGCCGTGCGTCCCTCGGGGACGGGCTTG
Thanatephorus cucumeris	AF354062	53	GGAGGTTATTGCAGCTTCACACCTGCTCCTCTT
Thermoascus crustaceus	U18353	26	GGGCCGCCGTCCCCGCCCTCCGCGGGGGGACGGGCCCG
Thielavia hyrcaniae	AJ271581	32	GGGGACCTGCGGCTGCCCGCAGGCCCTG
Trichoderma aureoviride	AF362108	37	GGGGATCGGCCCTGCCTCTGGCGGTGGCCGTCTCCG
Trichoderma harzianum	AJ507140	37	GGGGATCGGCCCTCCTCTAGCGGGGGCCGTCTCCG
Trichoderma inhamatum	AF414302	37	GGGGATCGGCCCTCCCTTAGCGGGGGCCGTCTCCG
Trichoderma sp.	AF408107	37	GGGGATCGGCCCTCCTCTCGCGGGGGCCGTCTCCG
Trichoderma sp.	AF408127	37	GGGGATCGGGACCCCTCACACGGGTGCCGGCCCCG
Trichothecium roseum	U51982	60	GGGGCCCAGGCGTCCTCCAAGGGCGCCTGTCCCCG
Ulocladium botrytis	AF267139	30	GGGCGTCTTGTCTCCAGTTCGCTGGAGACTCGCCTT
Valdensinia heterodoxa	Z81447	25	GAGCCCATGTCAGCAATGGCAGGCTCT
Verticillium chlamydosporium var. *catenulatum*	AJ292398	34	GGGGACCGGCGAGTACAGAGGCGGATGGGGACTTGTG CCCCTTCTTCCTCGGCGCCGCCCCCG
Verticillium chlamydosporium var. *chlamydosporium*	AB100362	34	GGGGACCGGCGAGTACAGAGGCTTTGGGGACTTGTCCC CCTTCCCTCGGCGCCGCCCCCG