High throughput barcoding of fungal species: a feasibility analysis

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INTRODUCTION

Fungi constitute a large, diverse, and economically important group of organisms. Due to a paucity of morphological characters and many microscopic species, identification of fungal species is often difficult. As a result, only 10% of estimated ~2M fungal species are formally described.

Molecular approaches have been extensively used in fungal biosystematics, both for phylogenetic reconstructions and for establishing species boundaries. Several loci have been utilized in fungal biosystematics but the most common genes used at species-level are the large subunit ribosomal RNA (LSU: 25-28S) and the Internal Transcribed Spacer (ITS). However, both of these markers have limitations. In many cases LSU is not variable enough to provide species-level resolution. On the other hand, ITS shows greater variation but in some cases it is heterogeneous and shows intra-individual variation, making it a difficult marker to work with. In addition, ITS is not a protein-coding gene and can show a great deal of length variation in different taxa, thus building sequence alignments in global species libraries for this marker is challenging.

Earlier work has established the feasibility of COI in high-throughput DNA barcoding of animal species (Hajibabaei et al., 2005) as a short 650-bp segment of mitochondrial cytochrome *c* oxidase I (COI) gene has successfully been used as a DNA barcode for species identification and discovery in many animal and several protist groups (Hebert et al., 2003; Hajibabaei et al., 2007). Two recent studies have also suggested that COI-barcodes can provide species-level resolution in fungal species (Seifert et al., 2007; Minn and Hickey, 2007).

We have initiated a research program to test the feasibility and to optimize the conditions of a high-throughput barcoding system for fungi. In this poster, we present preliminary results of this work. We compare the amplification and sequencing of two fragments of COI as well as LSU and ITS in a wide range of fungal species from Basidiomycota, Ascomycota, and Glomeromycota (Arbuscular Mycorrhizal Fungi, AMF).

Figure 2: Sampling localities across Canada. Future sampling sites are shown in blue.

RESULTS

DNA was extracted successfully from all specimens using a silica-based 96-well format commercial kit, Macherey Nagel (MN) Nucleospin 96 plant.

Table 1 summarizes the sequencing success for two COI amplicons (short and long), as well as for LSU and ITS, in samples from culture collections and field specimens. Overall, we found varied success in the amplification of COI barcode region in different sets of specimens. Shorter fragments of COI-barcode provided a much higher number of amplicons as compared to full-length barcode region (COI-long). The absence of amplification of full-length barcodes in many fungal specimens may be due to the presence of intronic regions within the barcode region. In addition, primers used for COI amplification were more successful in Basidiomycota as compared to other taxonomic groups tested. As a consequence, primer match can also be a limiting factor in the amplification of COI barcodes in fungi.

LSU and ITS amplifications were generally robust using available universal primer pairs (Table 1). Sequences obtained from LSU were clean and easy to align and edit. In contrast, because ITS sequences showed substantial length variation and several sequences included heterogeneous peaks in their electropherograms, editing and aligning ITS sequences was much harder.

Preliminary analysis of sequences obtained from COI as compared to LSU and ITS suggest a general congruence among these loci. However, species resolution is variable across different set of samples. For example, short fragments of COI may not provide enough variable sites to resolve species-level identifications when used in dense sampling within genera (i.e. Agaricus collection) but they may provide resolution for taxa collected in fields (i.e. Churchill samples) (Figure 3).

Serpula sp.
Boletales

Arbuscular Mycorrhizal Fungi
Glomeromycota

Nawawia filiformis
Aquatic Hyphomycetes

Figure 3: NJ tree based on COI short sequences

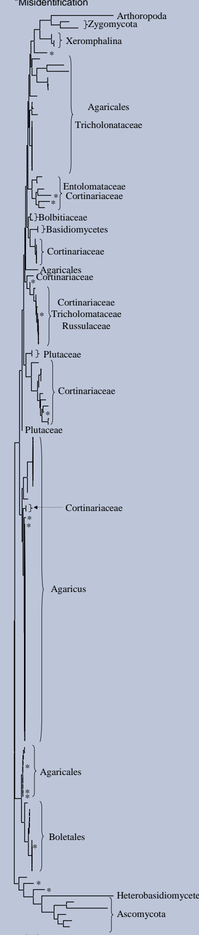

Table 1: Sampling regimes and sequencing results for COI short, COI long, LSU and ITS.

	Culture Collection Specimens			*Field Collected Specimens*	
	Agaricus Spp.	**Glomeromycota (AMF)**	**Aquatic Hyphomycetes**	**Thunder Bay**	**Churchill**
Collaborator	Dr. D. Royse	Dr. P. Antunes	Dr. M. Solé	Alison Fischer	Tiarella T. Hanna
Specimens	38 DNA samples representing at least 4 species	8 spores representing 8 species	30 DNA samples From 21 species	282 fruiting bodies in SDS	182 fruiting bodies in ETOH
DNA extraction		NuceoSpin plant kit (MN)		96 NucleoSpin plant (MN)	96 NucleoSpin plant (MN)
COI short	36	1	0	61	100
COI long	8	1	11	5	41
LSU	36	2	28	245	149
ITS	35	3	30	206	135

CONCLUSIONS

Our preliminary results indicate that fungal specimens of various sources, especially field collected specimens, are feasible targets for high throughput DNA barcoding. Fungal DNA isolates in 96-well format can be readily amplified/sequenced for multiple markers. While robust COI amplification and sequencing may require taxonomically targeted primer pairs (similar to animal barcoding), development of "primer cocktails" may facilitate the amplification process. We are now using these strategies to enhance the recovery of COI in fungal barcoding.

Furthermore, the presence of intronic sequences within COI barcode DNA, which may reduce barcode recovery, can be alleviated by using RNA (in RT-PCR) as template for barcode amplification. We have started investigating the feasibility of this approach in a wide range of specimens. As a consequence, sampling efforts are underway to collect fresh specimens from Ontario, Quebec, and Manitoba. The RT-PCR system will also be tested on various culture collections specimens including samples from ATCC.

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METHODS

Primer design strategy

- Alignment of available COI sequences were used to find conserved amino acids.
- Agaricus sequence was used as template for primers sequences (Figure 1)
- 2 sets of primers were designed: COI short: 110bp in the 5' region of COI-barcode avoiding probable introns
  COI long: 650bp covering the entire barcoding region

Sampling approach

- Culture collections: Agaricus spp, Glomeromycota (AMF), and Hyphomycetes spp (Cf. Table 1 for sources)
- Field collected: Basidiomycota and Ascomycota communities from Thunder Bay and Churchill (Figure 2)

PCR and sequencing

- COI short: Ag-3F/Ag-3R
- COI long: Ag-3F/Ag-Long2R
- LSU: LROR/LR5
- ITS: ITS5/ITS4

Figure 1: Amino acid alignment of COI used for primer design and the position of primers used in this study.