

DNA BARCODING

Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle

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Abstract

There is strong community-wide interest in applying molecular techniques to fungal species delimitation and identification, but selection of a standardized region or regions of the genome has not been finalized. A single marker, the ribosomal DNA internal transcribed spacer region, has frequently been suggested as the standard for fungi. We used a group of closely related blue stain fungi associated with the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) to examine the success of such single-locus species identification, comparing the internal transcribed spacer with four other nuclear markers. We demonstrate that single loci varied in their utility for identifying the six fungal species examined, while use of multiple loci was consistently successful. In a literature survey of 21 similar studies, individual loci were also highly variable in their ability to provide consistent species identifications and were less successful than multilocus diagnostics. Accurate species identification is the essence of any molecular diagnostic system, and this consideration should be central to locus selection. Moreover, our study and the literature survey demonstrate the value of using closely related species as the proving ground for developing a molecular identification system. We advocate use of a multilocus barcode approach that is similar to the practice employed by the plant barcode community, rather than reliance on a single locus.

Keywords: *Dendroctonus ponderosae*, DNA barcoding, fungi, identification, molecular diagnostics, Ophiostomataceae

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Introduction

Accurate species identification and delimitation is vital to understanding our natural world. Errors can have far-reaching consequences, impacting biodiversity assessment, ecological studies, and management decisions (Bortolus 2008). Less than 10% of an estimated 1.5 million fungal species have been described (Hawksworth 2001), impeding our understanding of fungal diversity and ecology. Closely related fungi can differ in their pathological effects, toxicogenic products, beneficial attributes and ecological niches (Lee *et al.* 2006b; Bleiker & Six 2007; Degenkolb *et al.* 2007). Morphological identification of such closely related fungi has remained elusive because

of the scarcity and ambiguity of diagnostic characters. Furthermore, morphology-based identifications are impossible for many species, because they cannot be cultured.

To counter difficulties in identification, use of DNA sequences has become increasingly popular for species delimitation and identification (e.g. Anderson & Cairney 2007; Shenoy *et al.* 2007; Giraud *et al.* 2008), allowing for the application of genealogical concordance phylogenetic species recognition (Taylor *et al.* 2000). Various diagnostic tools based on sequence variation have been developed for important groups of fungi (Geiser *et al.* 2004; Druzhinina *et al.* 2005; Kopchinskiy *et al.* 2005) but lack standardization. DNA barcoding (Hebert *et al.* 2003) is a currently common sequence-based approach that generally uses a single standard region of DNA to identify and recognize distinct animal species, but has received a wide

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range of responses among taxonomists (Tautz *et al.* 2002; Sperling 2003; Janzen 2004; Will *et al.* 2005).

Although there is now wide acceptance of the need for standardization of gene regions used in taxonomic studies (Caterino *et al.* 2000), it has proven more difficult to determine which particular gene regions and sequence length are most informative (Roe & Sperling 2007a,b; Rubinoff *et al.* 2006). Initially, a 658-bp segment of mitochondrial DNA from the cytochrome c oxidase gene (COI) was proposed as the single standard DNA barcoding region (Hebert *et al.* 2003). However, it has been demonstrated that COI is suboptimal for some groups of organisms, including fungi (Seifert *et al.* 2007; Seifert 2009) and plants (Chase *et al.* 2005). To develop a standardized molecular identification system like DNA barcoding in these groups, other genomic regions have been explored and suggested in place of COI (Rossman 2007; CBOL Plant Working Group *et al.*, 2009; Seifert 2009). Recently, application of DNA barcoding to fungi figured prominently in a special issue in *Molecular Ecology Resources*, in which Seifert (2009) encouraged adoption of the internal transcribed spacer regions 1 and 2 (ITS) as the standard fungal DNA barcoding gene region.

Accurate species identification is the essence of any molecular diagnostic system (Erickson *et al.* 2008), and potential genomic regions should be tested rigorously prior to adoption in a standardized system. Using five independent gene regions (elongation factor 1 alpha, beta tubulin, actin, internal transcribed spacer 2 + 28S rDNA and an anonymous nuclear locus), we examine the accuracy of each marker and combination of markers for species delimitation and identification in ophiostomatoid fungi associated with the mountain pine beetle [MPB; *Dendroctonus ponderosae* (Hopkins)]. MPB is an eruptive forest pest, attacking and feeding on pines across western North America. Western Canada is experiencing the largest outbreak on record, with over 7.8 million hectares of lodgepole pine (*Pinus contorta* Douglas var. *latifolia* Engelman) and lodgepole × jack pine hybrid (*P. contorta* Douglas var. *latifolia* Engelman × *Pinus banksiana* Lamb.) forest affected to date (British Columbia Ministry of Forests – <http://www.for.gov.bc.ca/hfp/health/overview/2008table.htm>, accessed Sept 01, 2009; Alberta Sustainable Resources Development – <http://www.mpb.alberta.ca/Resources/regionalmaps.aspx>, accessed Oct 16, 2009). MPB has several fungal symbionts (Rumbold 1941; Robinson 1962; Whitney & Farris 1970; Six & Bentz 2003; Lee *et al.* 2005, 2006a), and we focus on four closely related blue stain fungi (Ophiostomataceae): *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield, *Grosmannia aurea* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield, *Leptographium longiclavatum* Lee, Kim and Breuil and *Leptographium*

terebrantis Barras and Perry, collectively termed the *Grosmannia* clade (Zipfel *et al.* 2006). These fungi can be tree pathogens (Reid *et al.* 1967; Stobel & Sugarwara 1986; Owen *et al.* 1987; Lee *et al.* 2006b; Rice *et al.* 2007), aid in host suppression (Raffa & Berryman 1983) and provide nutrition to the beetle (Six & Paine 1998; Bentz & Six 2006; Adams & Six 2007). Species identification and delimitation in the *Grosmannia* clade is difficult because of interspecific overlap of morphological traits (Lim *et al.* 2004). Previous work on this group of fungi (Lim *et al.* 2004; Lee *et al.* 2005; Zipfel *et al.* 2006; Alamouti *et al.* 2009) has laid the foundation for an in depth exploration of species identification and delimitation in MPB-associated fungi using dense spatial and temporal sampling.

By comparing multiple independent loci, we examined the contribution of independent character sets to the identification and delimitation of blue stain fungi associated with MPB. To relate our results to the larger body of ascomycete literature and assess the success of single- vs. multilocus species identification, we surveyed studies that used multiple, independent loci to delimit closely related species. We then contrasted single- and multilocus fungal species identification, examining the interaction between increased molecular sampling and successful species identification.

Methods

Field sampling

Sampling focused on pine stands in areas experiencing recent mountain pine beetle attacks in Alberta and eastern British Columbia (Fig. 1). Collections were made over two time periods: January–May 2007 (M001) and September 2007–May 2008 (M002). A 10-cm-hole-saw and chisel were used to obtain pine discs 10 cm in diameter and approximately 3 cm thick, containing both xylem and bark with beetle-infested phloem. Discs were placed in ziplock bags and transported to the laboratory on ice for processing.

Fungal culturing

Fungal cultures were obtained, following Rice & Langor (2009), directly from adult and larval MPB individuals or from wood samples taken from beetle galleries. Live individuals were allowed to crawl on malt extract agar for up to 24h, while dead individuals were wiped across the surface of the media. Fungi were allowed to grow for 2–5 days at room temperature (RT) and ambient light before subculturing onto malt extract agar. Subcultures were incubated at RT for 2–4 weeks then scored for cultural and microscopic traits and compared with

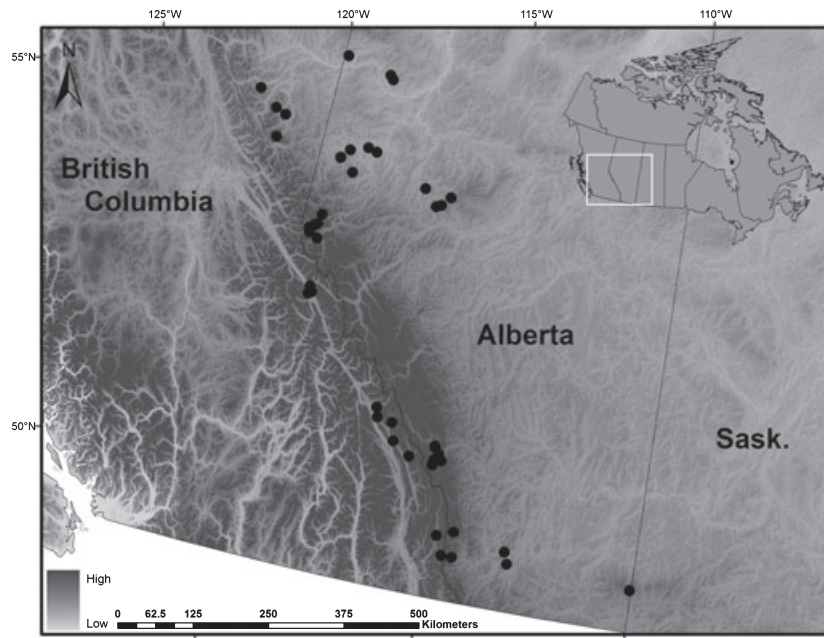


Fig. 1 Map of collecting localities surveyed for mountain pine beetle fungal associates.

previously authenticated strains by A. Rice and grouped according to colony morphology ('morphotype'). Representative cultures were deposited in the University of Alberta Microfungus Collection and Herbarium, with unique identifiers listed in Appendix S1, and images of strains deposited on MorphoBank project number P327 (<http://www.morphobank.org>).

DNA extraction, PCR and sequence analysis

Cultures were selected at random for multilocus typing within each collecting locality for each morphotype using a random number generator. Cultures with unusual morphology were also selected for typing. Single-spore isolates were grown for each selected strain.

Fungal tissue (~25 mg) was harvested for DNA extraction from single-spore isolates grown on 1.5% malt extract agar covered with a cellophane membrane and ground in liquid nitrogen. DNA was extracted using a CTAB (hexadecyltrimethylammonium bromide) protocol modified from Chang *et al.* (1993). CTAB buffer (700 μ L) was added to each sample, followed by 10 μ L of proteinase K (600 mAU/mL; Qiagen). Samples were incubated at 65 $^{\circ}$ C for 1 h and then cooled to RT before adding 600 μ L of 24:1 chloroform-isoamyl alcohol. Tubes were mixed vigorously then centrifuged for 5 min (all centrifugation steps performed at 17000 \times g and RT). The aqueous supernatant was mixed with 600 μ L isopropanol and chilled at -20 $^{\circ}$ C for at least 2 h. Samples were centrifuged for 15 min, supernatants were discarded, then 500 μ L 95% ethanol (v/v) added to the pellet, which was

vortexed briefly and centrifuged for 3 min. This process was repeated with 500 μ L 70% ethanol (v/v). Pellets were resuspended in 100 μ L Milli-Q water (Millipore, Billerica MA) with gentle agitation.

Five gene regions were amplified: actin, elongation factor 1 alpha (EF1a), beta tubulin (Btub), an anonymous nuclear locus (UFM) and ITS2 (partial 5.8S + internal transcribed spacer 2 + partial 28S). Primers used to amplify the above regions as well as their corresponding Polymerase chain reaction (PCR) profiles are listed in Table 1. Initially, amplification of the ITS region with universal primers resulted in nonspecific amplification as well as impediments to sequencing because of poly-A repeats in the ITS1 region. In fact, the UFM locus was obtained as a nonspecific product while trying to amplify ITS2 using primers ITS3 + LR3 (Vilgalys & Gonzalez 1990; White *et al.* 1990; Lim *et al.* 2004). ITS2 was eventually specifically amplified using primers ITS3 + TW13 (Table 1). The non-ITS identity of the UFM locus was confirmed by comparison with available ITS sequence and alignment with the *Grosmanina clavigera* genome sequence (whole genome shotgun sequence, GenBank ACXQ01001232.1, contig 29.6, 42669–43146 bp, Diguistini *et al.* 2009). Locus-specific internal primers were then designed to amplify the UFM region (Table 1).

PCRs for all regions were performed in 25 μ L final volume. Each reaction contained 50 ng DNA, 0.1 μ M of each primer, 1 \times modified Thermopol buffer (M0273S; New England BioLabs, Pickering ON) with bovine serum albumin fraction V added to the buffer to give a final concentration of 1 ng/ μ L bovine serum albumin, 1.25 U

Gene	Direction	Sequence (5'-3')	Sources
actin			
LepactF	F	TACGTCGGTGACGAGGC	Lim <i>et al.</i> 2004
LepactF_2	F	ACAGTCCAAGCGTGGTATCC	This study
LepactR	R	CAATGATCTTGACCTTCAT	Lim <i>et al.</i> 2004
EF1a			
EF3E	F	GTCGYATCGGCCACGTCTGA	Lim <i>et al.</i> 2004
TEF1-rev	R	GCCATCCTTGGAGATACCAGC	Samuels <i>et al.</i> 2002
Btub			
T1	F	AACATGCGTGAGATTGTAAGT	O'Donnell & Cigelnik 1997
BT12	R	GTTGTCAATGCAGAAGGTCTCG	Kim <i>et al.</i> 2003
BT12_internal	R	CCTCMGTGTAGTGMCCCTTG	This study
ITS2			
ITS3	F	GCATCGATGAAGAACGCAGC	White <i>et al.</i> 1990
TW13	R	GGTCCGTGTTTCAAGACG	White <i>et al.</i> 1990
Anon. Locus			
UFM1_F	F	AGTATGTGCCCGAATCAG	This study
UFM1_R	R	ATCTGTTGCCGATGCTTG	This study

Table 1 Primers used in this study

Taq DNA polymerase (New England BioLabs), 200 μ M each dNTP, 1% DMSO (dimethyl sulfoxide) (v/v) and 4 mM MgCl₂. In the case of ITS amplification, 2 mM MgSO₄ was used in place of 4 mM MgCl₂.

PCR products were purified in 10- μ L reactions using an EXO-SAP reaction with Exonuclease I and Shrimp Alkaline Phosphatase (70073Z and 70092Y; USB Corporation, Cleveland OH). Purified PCR products were sequenced with ABI Big Dye Terminator v 3.1 cycle sequencing reaction kit (Applied Biosystems, Foster City, CA) and purified using an ethanol/ethylene-diaminetetraacetate/sodium acetate precipitation protocol (ABI Big Dye Terminator v3.1 cycle sequencing kit protocol manual; Applied Biosystems). Sequencing was performed on an Applied Biosystems 3730 DNA Analyzer. Initially, all reactions were sequenced bidirectionally to identify common haplotypes. Following the initial survey, reactions were sequenced unidirectionally to streamline data collection, with all new haplotypes confirmed by bidirectional sequencing. Sequence data and associated chromatograms for unique multilocus haplotypes were submitted to GenBank and the NCBI Trace Archive under accession numbers GU370130-GU370344.

Phylogenetic analyses

Parsimony haplotype networks. Sequence data were initially aligned in Sequencher 4.8 (Gene Codes, Ann Arbor, MI) with manual adjustments made by eye. Representative GenBank sequences from previous studies of closely related members of the *Grosmanina* clade (*G. clavigera*, *G. aurea*, *G. robusta*, *L. longiclavatum*, *L. terebrantis* and *L. pyrinum*) were included (Appendix S2). These

representatives helped to confirm morphotype identifications and to identify species not diagnosed during morphotyping. Unique haplotypes were determined for each individual locus and a concatenated multilocus data set, and haplotype networks were calculated using TCS 1.21 (Clement *et al.* 2000), which infers a haplotype network using a statistical parsimony framework (Templeton 1998). Gaps were treated as missing data, and the connection limit was set to 95%.

Neighbour-joining. Following sequence alignment, a neighbour-joining tree was produced using PAUP* v. 4.0b10 for each gene region and the concatenated multilocus data set. Two additional taxa were used as outgroups (*Leptographium koreanum* Kim and Kim and *Leptographium yunnanensis* Zhou, Jacobs, Wingfield and Morelet). Uncorrected 'p' distances were used to generate pairwise distances, and missing data (including gaps) were distributed proportionally to unambiguous changes.

Maximum likelihood. Following sequence alignment, a maximum likelihood (ML) tree was estimated for each gene region and the multilocus data set. The ML trees were calculated using a maximum likelihood framework as implemented in RaxML v. 7.0.4 (Stamatakis 2006) hosted by CIPRES portal v. 1.0 (Cyberinfrastructure for Phylogenetic Research – <http://www.phylo.org/portal/Home.do>, accessed June 30, 2009). RAXML, or 'randomized accelerated maximum likelihood' for high performance computing, uses a simultaneous optimized ML tree-space search method and rapid bootstrapping algorithm (Stamatakis *et al.* 2008) that allows a full ML analysis in a single run. Matrices and associated ML trees for each individual locus and the multilocus data set were

submitted to TreeBASE (<http://www.treebase.org>; accession number SN4846).

Multilocus power analysis

To determine whether species identification is improved by the addition of data for multiple genes, we compared the congruence of species limits in single-locus data sets to successively larger multilocus data sets. These comparisons were made on three data sets that consisted of several closely related individuals which had sequence data for five independent loci: our study (four species), *Mycosphaerella* (five species, Crous *et al.* 2004) and *Cercospora* (three species, Groenewald *et al.* 2005). For each study, we produced neighbour-joining trees for each individual locus, as well as all combinations of two, three, four and five loci. The average proportion of species identification success was calculated for each combination. Congruence with species limits was scored as in the literature survey below.

Literature survey of multilocus species identification

To relate our study to broader patterns of single-locus vs. multilocus species identification in the mycological literature, we used the literature survey approach described by Roe & Sperl (2007b). We selected fungal studies that examined at least two independent loci and sampled at least five individuals from two closely related species. For inclusion, studies had to provide results from single-locus analyses (e.g. phylogenetic or distance trees) either in the publication or in TreeBASE. To summarize each study, loci were grouped into different character classes (ribosomal, mtDNA, autosomal and unknown), and their haplotype fixation and phylogenetic/cluster congruence were compared to the multilocus species delimitations preferred by the authors. Haplotype fixation was defined as either fixed (species do not share haplotypes) or shared (species share haplotypes). Congruence with species limits was scored based on the type of analysis used. Phylogenetic results (parsimony, likelihood and Bayesian) were scored as reciprocally monophyletic, paraphyletic or polyphyletic. Distance- or cluster-based methods (neighbour-joining) were scored as congruent (CO) or noncongruent (NC) with species limits.

Results

Morphotyping

A total of 5063 strains were isolated, morphotyped and preserved for future examination from 45 stands in Alberta and eastern British Columbia (Fig. 1). Strains were initially identified as three common MPB fungal

associates: *Grosmannia clavigera* ($n = 2162$), *Leptographium longiclavatum* ($n = 675$) and *Ophiostoma montium* (Rumbold) von Arx ($n = 1991$) based on diagnostic characters from original species descriptions (Rumbold 1941; Robinson-Jeffery & Davidson 1968; Lee *et al.* 2005), as well as via comparisons with previously authenticated strains. Some strains appeared to be morphologically intermediate between *G. clavigera* and *L. longiclavatum* and were identified as 'intermediate' ($n = 235$). A subset of morphotyped strains was selected for multilocus typing.

Phylogenetic analyses

For the remainder of this study, we focused on strains belonging to the *Grosmannia* clade (*G. clavigera*, *L. longiclavatum* and intermediate strains). *Ophiostoma montium* was clearly genetically divergent from the *Grosmannia* clade, although not always morphologically distinct, and phylogenetic analysis for this species will be reported elsewhere. In total, 350 single-spore isolates from the *Grosmannia* clade were selected for multilocus characterization (see Appendix S1 for a complete list of all cultures and collecting localities). In addition to these strains, sequence data were included for six previously published representative species within the *Grosmannia* clade and two outgroup species (Appendix S2).

Five loci (actin, EF1a, Btub, UFM and ITS2) were amplified for all isolates. Unique haplotypes for each locus were selected for phylogenetic analysis, treating insertions and deletions as missing data. For each locus and the combined multilocus data set, parsimony haplotype networks (Figs 2 and 3), ML and neighbour-joining trees (Figs 4 and 5) were produced. A summary of the phylogenetic data and ML model parameters for each data set is presented in Table 2.

Single-locus analyses

Generally, similar patterns of tree topology and species limits were found for each locus, with the exception of ITS2. ITS2 had several haplotypes that were shared between species (*L. terebrantis*, *L. pyrinum*, *G. robusta* and *G. aurea*, Fig. 2), while no haplotypes were shared between species for the remaining loci (actin, EF1a, Btub and UFM). Monophyly of individual species was well supported where species were represented by multiple haplotypes, with the exception of species sharing ITS2 haplotypes. There was no evidence of paralogous ITS copies or interspecific hybridization, suggesting that shared ITS haplotypes resulted from incomplete lineage sorting. *L. terebrantis* was paraphyletic in actin, EF1a and Btub, and *G. clavigera* was paraphyletic in UFM. While monophyly of individual species was generally well supported, internal nodes within and between species

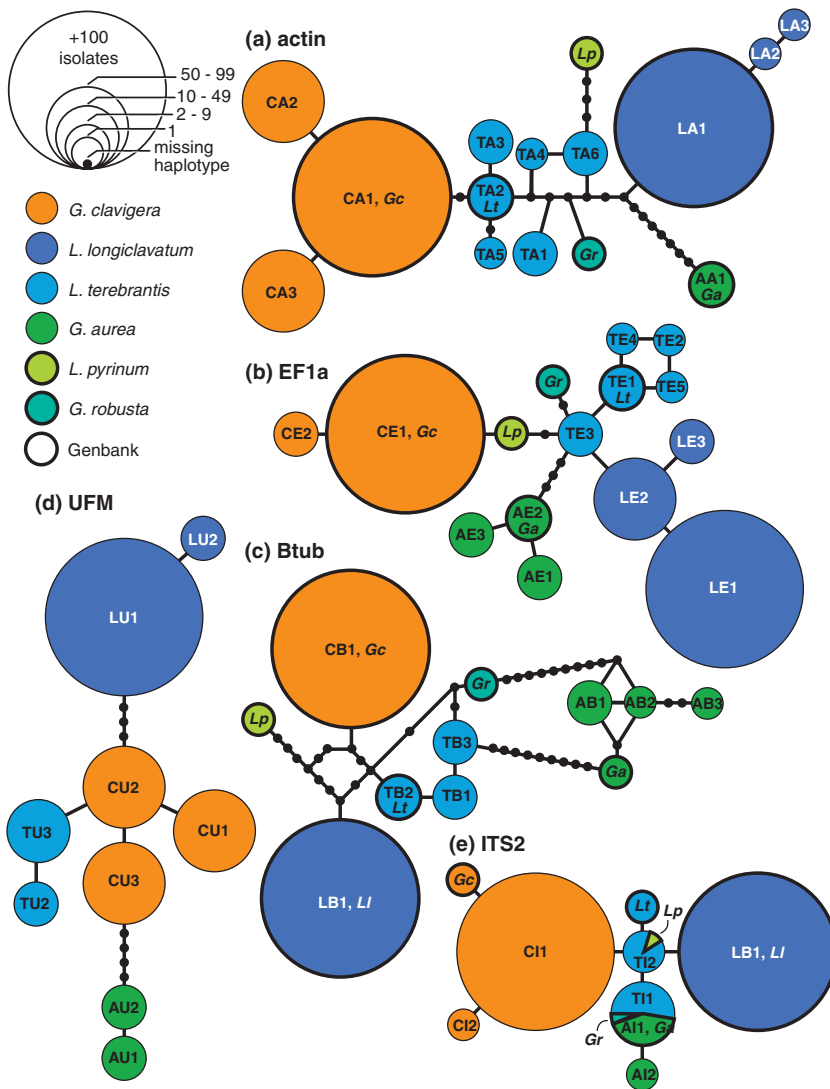


Fig. 2 Parsimony haplotype networks for five independent loci of six closely related members of the *Grosmannia* clade. Coloured circles represent individual haplotypes, with circle size proportional to the number of isolates. Lines linking haplotypes represent single nucleotide differences. Black circles indicate missing haplotypes. Haplotypes with thick black borders are identical to representative GenBank sequences. (a) actin; (b) elongation factor 1 alpha; (c) beta tubulin; (d) anonymous locus; (e) internal transcribed spacer + partial 28S.

received little bootstrap support. Monophyly of the *Grosmannia* clade was well supported for each locus (Fig. 4), although this could not be assessed for UFM, because sequences were unavailable for outgroup taxa.

Multilocus analyses

Minimal topological conflict existed among supported clades in each single-locus analysis; accordingly, the five data sets were combined into a single concatenated data set. No haplotypes were shared between species (Fig. 3), similar to the individual loci, although contrasting with the results obtained for ITS2 (Fig. 2). *G. clavigera*, *L. longiclavatum*, *L. terebrantis* and *G. aurea* were all represented by multiple haplotypes and were monophyletic, with strong support for all except *L. terebrantis*. As with the single-locus analyses, internal nodes within and between species received little bootstrap support. Monophyly of

the *Grosmannia* clade was also strongly supported by the concatenated data set (Fig. 5).

Multilocus power analysis

Using three multilocus data sets, including the one from our study, we examined how species identification success changed with the inclusion of additional loci. Based on single loci, average proportion of identification success ranged from 0.4 to 0.7 (40–70%) for *Grosmannia*, *Mycosphaerella* and *Cercospora* species. The proportion of successful identification increased as additional loci were added (Fig. 6). Eighty per cent success was attained in the *Grosmannia* data set using as few as two loci, while four loci were needed for both *Mycosphaerella* and *Cercospora*. Furthermore, the addition of more loci resulted in an increase in identification success to 100% in both the *Grosmannia* and *Mycosphaerella* with three and five loci,

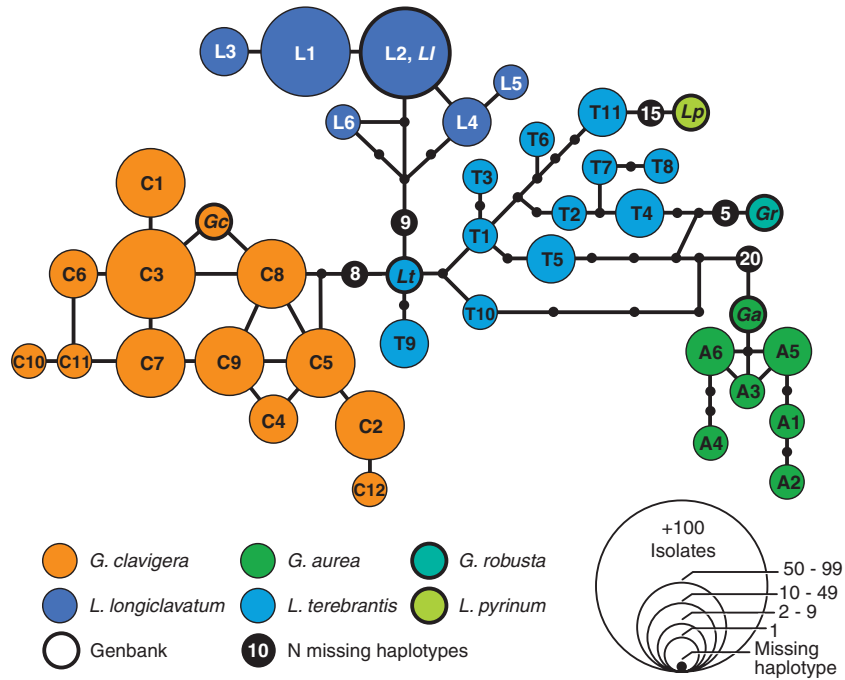


Fig. 3 Parsimony haplotype network for the concatenated multilocus data set of six closely related members of the *Grosmannia* clade. See Fig. 2 caption for description of haplotype network.

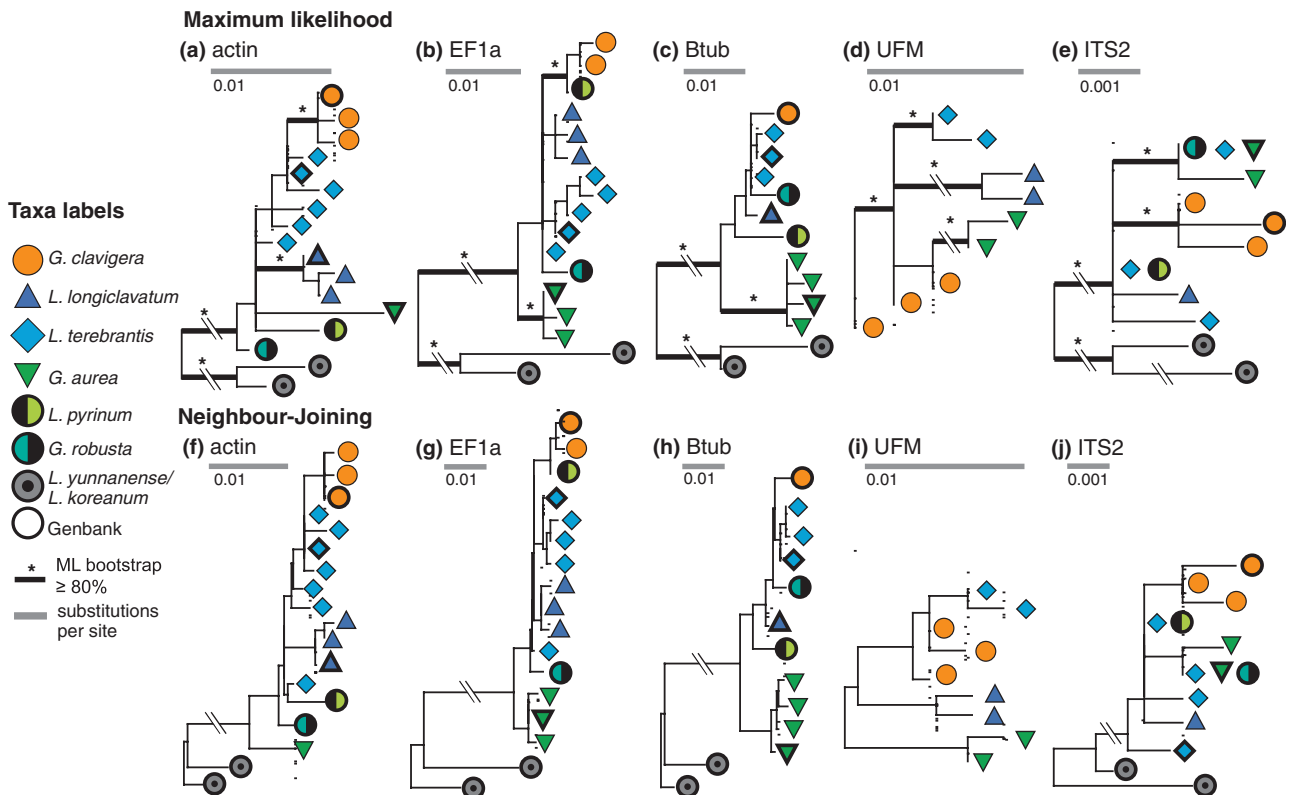


Fig. 4 Maximum likelihood (ML) and neighbour-joining (NJ) trees for five independent loci of six closely related members of the *Grosmannia* clade.

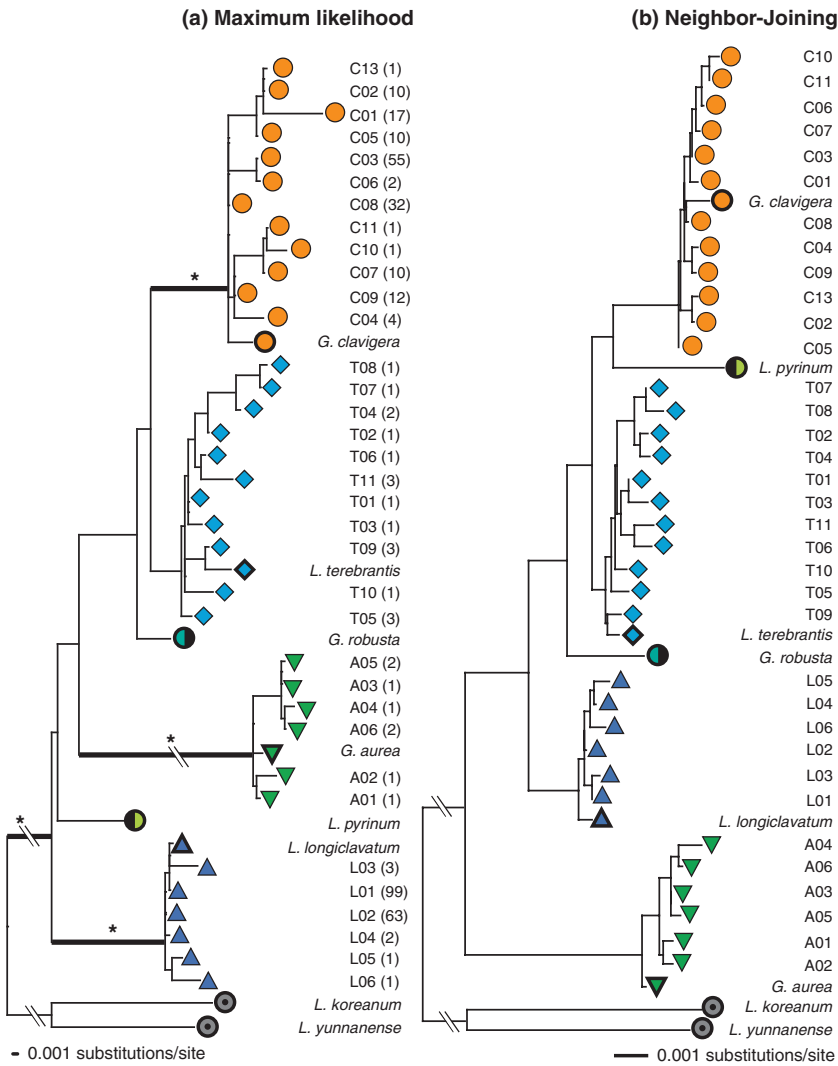


Fig. 5 Maximum likelihood and neighbour-joining trees for the concatenated multilocus data set. (a) Maximum likelihood phylogram. (b) Neighbour-joining tree. Legend as in Fig. 4.

respectively, while *Cercospora* never surpassed 80% success, even with the entire five locus data set.

Literature survey

We obtained multilocus data for 22 closely related species pairs of ascomycetes from 21 studies (Appendix S2). Species pairs occurred in six orders: Ophiosmatales (two pairs), Hypocreales (eight pairs), Capnodiales (five pairs), Calosphaerales (one pair), Eurotiales (two pairs), Botryosphaerales (three pairs) and one unplaced Sordariomycetidae genus. This was not an exhaustive review, but we believe that it represents the overall patterns in the literature. Results of this survey are summarized in Table 3 and presented in greater detail in Appendix S3. Gene regions examined in the studies fall into five major categories: ribosomal (ITS1, 5.8S, ITS2, 28S and intergenic spacer region), mtDNA (mitochondrial small subunit rDNA, adenosine triphosphatase 6), autosomal (actin,

beta tubulin, elongation factor 1 alpha, calmodulin, histone H3, RNA polymerase II subunit and chit18-5), anonymous (UFM, BotF15) and amplified fragment length polymorphisms.

A total of 77 loci were examined across the included studies with two to six loci per species pair (Table 3, Appendix S3). Excluding amplified fragment length polymorphisms, 60 loci showed fixed haplotype differences between species pairs, while 16 loci had shared or partially shared haplotypes between species. All loci with shared or partially shared haplotypes (16 loci) were polyphyletic or non-congruent. Of the loci with fixed haplotype differences, the majority of haplotypes were reciprocally monophyletic (RM – 28 loci) or congruent (C – 22 loci) with species limits. The remaining fixed loci (10 loci) were paraphyletic or non-congruent. Of the surveyed studies, the majority of loci fall into two locus types, ribosomal (20 loci) and autosomal loci (52 loci). Proportion of haplotype fixation was higher in autosomal

Table 2 Locus information for single-locus analyses and concatenated multilocus maximum likelihood analysis

	actin	EF1a	Btub	UFM	ITS2	Combined
# haplotypes*	15	15	11	10	8	40
# sites	765	630	1045	393	887	3720
# constant char.*	735	615	1007	379	880	3616
# variable char. (uninformative)*	9	2	13	2	3	30
# pars. inform. char.*	21	13	25	12	4	74
% informative*	2.75	2.06	2.39	3.05	0.34	1.99
ML Model	GTR+ Γ	GTR+ Γ	GTR+ Γ	GTR+ Γ	GTR+ Γ	GTR+ Γ
-ln	-1453.2358	-1264.0232	-2020.8777	-638.2299	-1315.2930	-6947.2673
α gaps/missing	0.003862	0.1160	0.1580	0.0000	0.03908	0.09674
Base freq.						Partitioned
A	0.2079	0.2179	0.2111	0.2203	0.2124	—
C	0.3039	0.3036	0.2790	0.3022	0.2974	—
G	0.2721	0.2267	0.2783	0.2960	0.3187	—
T	0.2162	0.2518	0.2316	0.1814	0.1714	—
Rate Matrix						Partitioned
A-C	0.0721 (0.8423)	0.3959 (0.4898)	0.8292 (1.2951)	0.000017	0.4284 (0.2233)	—
A-G	0.8200 (2.5714)	1.2255 (1.8538)	1.9061 (2.8402)	4.1214 (7.1599)	0.1973 (0.1053)	—
A-T	2.6728 (0.000017)	1.5994 (3.1258)	0.5950 (0.8616)	0.000017	0.000017	—
C-G	0.3688 (0.4131)	0.2826 (0.4170)	0.4735 (0.7996)	1.4752 (3.1950)	0.000017	—
C-T	5.3126 (4.2967)	4.2956 (7.2423)	4.3915 (6.2634)	5.6200 (9.4605)	2.4492 (2.1376)	—
G-T	1.0000	1.0000	1.0000	1.0000	1.0000	—
Γ (alpha)	0.02000	0.9497 (0.2318)	0.1820 (0.2457)	0.0200	29.5889 (0.0200)	Partitioned

*ingroup only.

Values calculated for the multilocus partitioned ML analysis are shown in brackets for each gene where values differ from single-locus analyses. Ingroup character information includes representative sequence data obtained from GenBank.

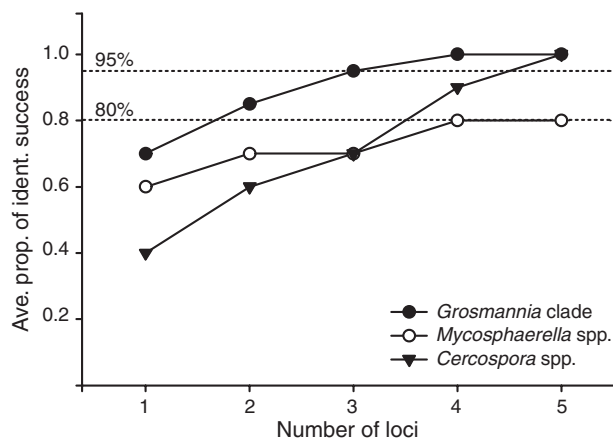


Fig. 6 Changes in species identification success with the addition of loci. Proportion of neighbour-joining haplotype clusters congruent with published species limits was used as a measure of accurate species identification. All possible locus combinations were examined and averaged for each locus category. Benchmark levels of identification success (80% and 95%) are indicated. Data for this analysis were obtained from the current study (*Grosmannia* clade), Crous *et al.* (2004) (*Mycosphaerella* spp.), and Groenewald *et al.* (2005) (*Cercospora* spp.).

loci (46 loci – 88%), compared to rDNA loci (11 loci – 55%) (Table 3, Appendix S3). Of the loci with fixed haplotype differences, levels of reciprocal monophyly and congruence were similar between autosomal loci (87%) and rDNA loci (82%).

Discussion

Accurate identification and delimitation of closely related species should be the proving ground for any diagnostic tool designed to elucidate biodiversity (Sperling 2003; Roe & Sperling 2007b; Hollingsworth 2008). Boundaries between closely related species are often indistinct, lacking clear limits and diagnostic characters (Sites & Marshall 2004; Bickford *et al.* 2007), because of processes such as introgressive hybridization and lineage sorting (Funk & Omland 2003). These evolutionary phenomena can lead to discordance between species limits and diagnostic traits, which are especially problematic – and undetectable – when a single character set is used. Currently, many molecular identification approaches, such as DNA barcoding, rely on a single standardized fragment of

Table 3 Literature survey of multilocus data for pairs of closely related ascomycete fungi

Location	Total	Fixed	Shared	Partial	RM	PA	PO	C	NC
rDNA	20	11	5	4	4	1	5	5	5
mtDNA	2	2	0	0	0	2	0	0	0
Autosomal	52	46	1	5	23	6	0	17	6
Anonymous	2	1	1	0	1	0	1	0	0
AFLP	1	—	—	—	—	—	—	1	0

Total number of loci (TOTAL) with fixed haplotype differences (FIXED), shared haplotypes (SHARED), or with a combination of fixed and shared haplotypes (PARTIAL) between a fungal species pair. Relationships between the species are classified as follows: for phylogenetic analyses RM, reciprocally monophyletic; M, monophyletic; PA, paraphyletic; PO, polyphyletic; for distance analyses C, congruent; NC, not congruent.

DNA for species identification, delimitation and discovery (Hebert *et al.* 2003; Rubinoff *et al.* 2006).

Single-locus delimitation and identification

The ideal barcode identification system recognizes distinct species, has universal primers and reliably produces high quality sequence (Erickson *et al.* 2008). The fungal community is currently working towards a standardized molecular identification system to improve the identification, delimitation, and discovery of cryptic fungal biodiversity. For example, the All Fungi Barcode Initiative (Rossman 2007) has promoted the adoption of ITS as the sole fungal barcode region, a recommendation also followed by Seifert (2009). ITS, in many respects, appears to fit the criteria of an ideal molecular marker. Given its successful usage in a range of fungal groups (e.g. Iwen *et al.* 2002; Druzhinina *et al.* 2005; Koljalg *et al.* 2005; Le Gac *et al.* 2007; Seifert *et al.* 2007; Shenoy *et al.* 2007; Summerbell *et al.* 2007; Feau *et al.* 2009), universal primers (White *et al.* 1990), manageable sequence length, high copy number and phylogenetic utility (Kohn 1992; Coleman 2003), selecting ITS as the molecular marker for fungi initially seems reasonable.

In addition to ITS, other loci have been suggested as potential molecular diagnostic markers. Mitochondrial genes, such as COI, work well for certain groups (Seifert *et al.* 2007; Vialle *et al.* 2009). As a database of COI sequence already exists for animals (Ratnasingham & Hebert 2007), there is a strong advantage to using the same region to avoid a 'Tower of Babel' (Caterino *et al.* 2000). Single-/low-copy nuclear genes have also been suggested (Lieckfeldt & Seifert 2000; Geiser *et al.* 2004). These types of loci can have a number of advantages, including easier alignment, fewer indels, more variable 3rd codon positions and known copy number (Bruns & Shefferson 2004). Of these, variability is the most important advantage, as indicated in our literature survey.

Despite their various positive attributes, each marker system has potential negative issues, and no single

marker is perfect. For example, low variability in ITS has resulted in failure to separate closely related species (Appendix S2, Bruns & Shefferson 2004; Crouch *et al.* 2009; Landis & Gargas 2007; Lieckfeldt & Seifert 2000; Varga *et al.* 2007), which is where identifications matter most (Sperling 2003; Hollingsworth 2008). As well, ITS may have multiple paralogous copies (O'Donnell & Cigelnik 1997; Simon & Weiss 2008), leading to inaccurate identifications, or variation in copy number (Vilgalys & Gonzalez 1990; Pukkila & Skrzynia 1993), preventing its use as a quantitative marker. Problems with COI have also been well documented, including problems with multiple paralogous copies (Gilmore *et al.* 2009), low species resolutions, mobile introns and limited historical usage (for a detailed discussion see Seifert 2009). Single-/low-copy nuclear genes, while more variable, experience rapid nucleotide saturation and may be more difficult to amplify in degraded material (Bruns & Shefferson 2004). Development of universal primer regions is important for standardization of a molecular identification system, which can be more problematic in nuclear genes. Universality problems can also occur with ITS (Taylor & McCormick 2008), as we saw in our study. Nonorthologous and inconsistent amplification of universal regions may cause missing or inaccurate sequence data, leading to errors in species identification.

Multilocus species delimitation and identification

Our data, and to an even greater extent our survey of the literature, demonstrate the limitations of relying on a single locus for identifying and inferring species limits among closely related fungal species. If we had relied on a single locus in this study, such as ITS, we would have underestimated fungal species diversity (Table 3, Appendix S3). This is similar to the findings of Seifert *et al.* (2007) where beta tubulin, a single-copy nuclear locus, provided diagnostic barcodes for 80% of the *Penicillium* species examined, outperforming both ITS

(25%) and COI (66%). Such failure to separate closely related species occurred in every type of gene class, with nuclear genes also being susceptible to incongruence. However, the inclusion of additional loci in a multilocus framework helps to minimize the risk of inaccurate species identification, increasing the proportion of successful species identifications relative to single-locus data sets. The number of additional loci needed to achieve a high proportion of successful identifications varied between groups.

Demonstration of the improvement in species delimitation and identification that can be obtained with multilocus data sets has prompted recommendations to move towards an integrative multilocus approach (Rubinoff *et al.* 2006; Roe & Sperling 2007b). A recent example of a newly developed multilocus diagnostic system is the 2-locus plant barcode proposed by the CBOL Plant Working Group *et al.* (2009). Rigorous testing of chloroplast gene regions in this study demonstrated that two or three regions are optimal, balancing universality, resolution and consistency. Using the plant barcode as a benchmark, we strongly support the adoption of a similar multilocus fungal identification system. Other studies with fungi have also demonstrated that multiple loci provide the most accurate species identification and delimitation (Summerbell *et al.* 2005; Dettman *et al.* 2006; Balajee *et al.* 2007; Petit & Excoffier 2009). Despite this, much of the fungal literature relies on a single locus (often ITS) and does not include additional characters to independently evaluate lineage diversity. It is difficult to know when additional characters are needed to clarify species limits, because species-level variability is not predictable (Nilsson *et al.* 2008). This unpredictability makes it nearly impossible to reliably include additional characters using only a targeted approach to counter single-locus invariability (Rossman 2007; Seifert 2009).

Conclusions

Any method that aims to achieve accurate species identification and delimitation should be capable of delimiting closely related species and identifying gene tree vs. species tree discordance (Olmstead 1995; Funk & Omland 2003; Fazekas *et al.* 2008; Lahaye *et al.* 2008). Examination of multiple, independent character sets in an integrative taxonomic framework (Rubinoff *et al.* 2006; Roe & Sperling 2007b) can minimize the risk of inaccurate species delimitation by inferring species boundaries through congruence among multiple character sets. With multiple character sets, a genealogical concordance phylogenetic species concept (Taylor *et al.* 2000) could provide a means to detect discordance between gene trees and species trees in

an identification framework. Discordance can be particularly problematic for closely related species as their lineages may have not had sufficient time to coalesce, compared to species with longer, independent evolutionary histories. Ultimately, the development of a molecular identification system like DNA barcoding is a compromise between universality, resolution, efficiency and consistency (Erickson *et al.* 2008; Vialle *et al.* 2009). Fungal biodiversity vastly exceeds that of many other taxa, so extensive testing will be needed to evaluate potential combinations of loci.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1 Table of specimen collection data, voucher information, and haplotype assignments.

Appendix S2 Representative GenBank sequences from closely related *Grosmannia* species.

Appendix S3 Literature survey of multilocus data for pairs of closely related ascomycete fungi (methodology described in Materials and Methods).

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