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# Intragenomic variation in the ITS rDNA region obscures phylogenetic relationships and inflates estimates of operational taxonomic units in genus *Laetiporus*

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***Abstract:*** Regions of rDNA are commonly used to infer phylogenetic relationships among fungal species and as DNA barcodes for identification. These regions occur in large tandem arrays, and concerted evolution is believed to reduce intragenomic variation among copies within these arrays, although some variation still might exist. Phylogenetic studies typically use consensus sequencing, which effectively conceals most intragenomic variation, but cloned sequences containing intragenomic variation are becoming prevalent in DNA databases. To understand effects of using cloned rDNA sequences in phylogenetic analyses we amplified and cloned the ITS region from pure cultures of six *Laetiporus* species and one *Wolfiporia* species (Basidiomycota, Polyporales). An average of 66 clones were selected randomly and sequenced from 21 cultures, producing a total of 1399 interpretable sequences. Significant variation (≥ 5% variation in sequence similarity) was observed among ITS copies within six cultures from three species clades (*L. cincinnatus*, *L.* sp. clade J, and *Wolfiporia dilatohypha*) and phylogenetic analyses with the cloned sequences produced different trees relative to analyses with consensus sequences. Cloned sequences from *L. cincinnatus* fell into more than one species clade and numerous cloned *L. cincinnatus* sequences fell into entirely new clades, which if analyzed on their own most likely would be recognized as ''undescribed'' or ''novel'' taxa. The use of a 95% cut off for defining operational taxonomic units (OTUs) produced seven *Laetiporus* OTUs with consensus ITS sequences and 20 OTUs with cloned ITS sequences. The use of cloned rDNA sequences might be problematic in fungal phylogenetic analyses, as well as in fungal bar-coding initiatives and efforts to detect fungal pathogens in environmental samples.

***Key words:*** evolution, Fungi, internal transcribed spacer region, intragenomic variation, molecular drive, sulfur shelf

## INTRODUCTION

Genus *Laetiporus* Murrill (Basidiomycota, Polyporales) contains important polypore species with worldwide distribution and the ability to produce cubical brown rot in living and dead wood of conifers and angiosperms. Ribosomal DNA sequences, including sequences from the internal transcribed spacer (ITS) and large subunit (LSU) regions, have been used to define species and infer phylogenetic relationships in *Laetiporus* and to confirm the existence of cryptic species (Lindner and Banik 2008, Ota and Hattori 2008, Tomsovsky and Jankovsky 2008, Ota et al. 2009, Vasaitis et al. 2009) described with mating compatibility, ITS-RFLP, morphology and host preference data (Banik et al. 1998, Banik and Burdsall 1999, Banik and Burdsall 2000, Burdsall and Banik 2001). Currently at least eight described *Laetiporus* species worldwide clearly fall in the core *Laetiporus* clade as defined by Lindner and Banik (2008) and ITS data indicate the existence of four undescribed *Laetiporus* species (Banik et al. 2010).

The regions of rDNA used to infer evolutionary relationships in genus *Laetiporus* also have been used to infer phylogenetic relationships among a wide range of fungal species (Bridge et al. 2005), as well as among diverse lineages of plants (Baldwin et al. 1995, Chaw et al. 1997), animals (Mallatt and Winchell 2002) and prokaryotes (Ludwig et al. 1998). Due to the high copy number of rDNA and extensive datasets available, these regions also are used commonly to detect and identify species. For fungi the ITS region is widely regarded as the preferred region for species identification and detection (Horton and Bruns 2001, Kõljalg et al. 2005, Peay et al. 2008, Avis et al. 2010) and this region most likely will be chosen as one of the universal barcodes for fungal species (Seifert 2009, Begerow et al. 2010).

Despite the ITS region's reputation as useful for both species identification and phylogenetics, it has recognized problems. One significant problem is intraspecies ITS variations, which have been identified for some fungal species (Kårén et al. 1997, Aanen et al. 2001, Smith et al. 2007), thus making it necessary to consider a range of ITS sequence variants

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across a population of individuals (Horton 2002, Nilsson et al. 2008). In addition intragenomic variation (sometimes referred to as intrastrain or intra-individual variation) among the various copies of the ribosomal array has been recognized in a number of fungal species (O'Donnell and Cigelnik 1997, Lachance et al. 2003, Pawlowska and Taylor 2004, Avis et al. 2005, Ganley and Kobayashi 2007, Smith et al. 2007, Simon and Weiss 2008, James et al. 2009, Connell et al. 2010, Huang et al. 2010, Santos et al. 2010), and this same phenomenon has been observed in a wide range of organisms, from animals (Harris and Crandall 2000, Leo and Barker 2002, Wörheide et al. 2004, Sánchez and Dorado 2008, Elderkin 2009) to dinoflagellates (Thornhill et al. 2007) to prokaryotes (Stewart and Cavanaugh 2007).

Intragenomic variation is believed to be due to a relaxation of concerted evolution, the process that homogenizes variation among the ribosomal DNA repeats in tandem arrays. Evidence suggests that concerted evolution acts through unequal crossing over between repeating units, gene conversion or gene amplification, although the exact mechanisms that govern concerted evolution are largely unknown (Dover 1993, Elder and Turner 1995, Liao 1999). In fungi tandem arrays may contain 45–200 copies of the ribosomal region (Maleszka and Clark-Walker 1990, Ganley and Kobayashi 2007) and these copies may be distributed across one or more chromosomal locations (Pasero and Marilley 1993), thus allowing for significant variation within the genome of one individual. Both intragenomic variation in the ITS region, as well as intraspecies variation in ITS at the population level, present significant challenges for phylogenetic analyses and species identification.

While intraspecies and intragenomic variation in ribosomal units has been recognized for some time in fungi (e.g. Kårén et al. 1997, O'Donnell and Cigelnik 1997), the issue of intragenomic variation recently has received increased attention due to the availability of whole genome data. Whole genome data let Rooney and Ward (2005) analyze many copies of the 5S region from four species of Ascomycota, *Aspergillus nidulans*, *Fusarium graminearum*, *Magnaporthe grisea* and *Neurospora crassa*. Rooney and Ward (2005) found multiple 5S gene types and pseudogenes within individual genomes and concluded that the 5S gene is characterized by ''birth-and-death'' evolution under strong selection pressure. Ganley and Kobayashi (2007) used whole genome data to examine intragenomic rDNA variation in four species of Ascomycota (*Ashbya gossypii*, *Aspergillus nidulans*, *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*) and one species of Basidiomycota (*Cryptococcus neoformans*). Although polymorphisms were observed in all species examined, Ganley and Kobayashi (2007) concluded that concerted evolution generally acts in a highly efficient fashion to eradicate variation.

The small amount of intragenomic variation observed by Ganley and Kobayashi (2007) in *Saccharomyces cerevisiae* and the conclusion that concerted evolution acts efficiently is in contrast to the findings of James et al. (2009), who observed significant intragenomic variation among 34 strains of *S. cerevisiae* for which whole genome data were available. James et al. (2009) observed significant variation throughout the ribosomal region but noted that the highest variation was confined to the intergenic spacer (IGS) region. James et al. (2009) also noted that many single nucleotide polymorphisms (SNPs) were unresolved, meaning that specific SNPs occurred on some copies of the ribosomal array and introduced the term pSNP to describe these polymorphisms. pSNPs were more common in strains with mosaic/hybrid genomes than in strains with typically structured genomes, suggesting that hybridization plays a role in intragenomic variation.

Based on whole genome fungal data, SNPs and pSNPs appear to be prevalent in the ribosomal array of some fungal species, which raises the question of whether these variations are detectible when high throughput cloning and sequencing or next generation sequencing (e.g. pyro-sequencing) techniques are applied to environmental samples. Cloning and sequencing of ribosomal regions has become popular as a culture-independent detection method in ecological and environmental studies, and this approach now has been applied to a wide range of environments and fungal groups (Jumpponen 2003, Anderson and Cairney 2004, O'Brien et al. 2005, Lindahl et al. 2007, Arnold et al. 2007, Fierer et al. 2007, Fröhlich-Nowoisky et al. 2009). Pyro-sequencing is a relatively new technique in fungal ecology but is being used more frequently on environmental samples (Buée et al. 2009, Gillevet et al. 2009, Jumpponen and Jones 2009, Öpik et al. 2009, Jumpponen et al. 2010, Tedersoo et al. 2010) despite a lack of information regarding the biases and limitations of this new technology (Nilsson et al. 2009, Kunin et al. 2010, Medinger et al. 2010, Tedersoo et al. 2010).

Due to the rate at which sequence data are generated with these high throughput technologies, ''non-consensus'' rDNA sequences (e.g. cloned or pyro-sequenced ITS regions) are becoming prevalent in public DNA databases. Approximately 33% of fungal ITS sequences in GenBank currently are derived from environmental samples (David Hibbett pers comm), and these sequences presumably will display intragenomic variation. The number of non-consensus ITS sequences likely will increase expo-

nentially as next generation sequencing is applied to environmental samples. Although non-consensus sequences are used to estimate the richness and diversity of fungal communities, such data will reflect interspecies, intraspecies as well as intragenomic variation in the ITS region and it is currently not known how this might affect analyses.

To understand the possible effects of intragenomic ITS variation on phylogenetic analyses and operational taxonomic unit (OTU) delimitation we concentrated on species within genus *Laetiporus*. We PCR amplified and cloned the ITS region from six established *Laetiporus* species (Banik et al. 2010) growing in pure culture and compared phylogenetic analyses with either the cloned ITS sequences or consensus ITS sequences obtained by direct sequencing. Sequences then were grouped into OTUs based on varying levels of similarity to determine whether cloned and consensus sequence data were equally capable of characterizing species richness.

## MATERIALS AND METHODS

*Isolate selection.*—Tissue isolates of six *Laetiporus* species (*L. cincinnatus*, *L. conifericola*, *L. gilbertsonii*, *L. huroniensis*, *L. sulphureus* clade E, and *L.* sp. clade J) were obtained by excising small pieces of context from the interior of fruiting bodies, placing on potato dextrose agar and then subculturing on 2% malt extract agar. We used tissue isolates from four collections of *L. cincinnatus* and *L. sulphureus*: two collections of *L. conifericola*, *L. gilbertsonii*, and *L.* sp. clade J; and one collection of *L. huroniensis* (TABLE I). Four single-spore isolates were obtained from *L. cincinnatus* collection DA-37 with techniques described by Banik et al. (1998). Two tissue isolates of *Wolfiporia dilatohypha* were included as an outgroup (Lindner and Banik 2008).

*DNA isolation, PCR amplification and cloning.*—DNA was isolated from cultures and the ITS region was amplified with primers ITS1F and ITS4 according to the methods of Lindner and Banik (2008). PCR was performed with GoTaq DNA polymerase (Promega, Madison, Wisconsin), a non-proofreading Taq polymerase with a reported error rate of $1–7 \times 10^{-4}$ incidents per base pair per cycle (Eun 1996) (see RESULTS for determination and further discussion of method-based errors). The resulting PCR products were cloned into JM109 competent cells with Promega (Madison, Wisconsin) pGEM-T Vector System II cloning kits with the protocols of Lindner and Banik (2009). Successfully transformed bacterial cells were re-amplified with primers ITS1F and ITS4. These amplification products were diluted 1 : 10 with molecular-grade water and sequenced with primer ITS-5 in the BigDye terminator (ABI Prism) system as described by Lindner and Banik (2008). Clones were selected randomly and sequenced from each culture. Each of the cloned ITS sequences was manually compared to the consensus sequence for that species, and the authenticity of DNA changes was determined by visually analyzing sequence trace chromatograms. Sequences varying > 5% relative to previously reported consensus sequences were deposited in GenBank (accession numbers HQ676133–HQ676155).

*Phylogenetic analyses and determination of OTUs.*—DNA alignment protocols followed Lindner and Banik (2008) for consensus sequences. Cloned sequence data were aligned with Clustal X 2.0.11 (Larkin et al. 2007) followed by manual alignment with Se-Al (2.0a9). Maximum likelihood phylogenies with bootstrap support were inferred with the RAxML Web-servers (http://phylobench.vital-it.ch/raxml-bb/) (Stamatakis et al. 2008). Trees were viewed with FigTree 1.3.1, and graphics were exported for final illustrations. Sequencher 4.8 (Gene Codes Corp.) was used to define the number of OTUs at different levels of percent similarity with the ''clean data'' algorithm.

## RESULTS

When maximum likelihood phylogenies were generated with consensus ITS sequences the expected six *Laetiporus* species clades were observed (FIG. 1) with four species clades displaying significant (> 70%) bootstrap support. Species clades with significant support were *L. cincinnatus*, *L. gilbertsonii*, *L. huroniensis* and *L.* sp. clade J. Two species clades, *L. conifericola* and *L. sulphureus*, did not receive significant bootstrap support, although isolates from both species consistently clustered in the expected fashion.

Maximum likelihood phylogenies with the cloned ITS sequences produced a complex tree (FIG. 2). The six consensus species clades could be identified in this tree (FIG. 2, vertical black lines) and close matches (> 98% similarity) to the consensus sequence were the predominant sequence type recovered from all species (TABLE I). Six clades in this tree received significant bootstrap support, but only three of these clades corresponded to recognized species clades. The three *Laetiporus* species clades receiving significant bootstrap support in this tree were *L. conifericola*, *L. huroniensis* and *L.* sp. clade J.

Numerous cloned sequences from *L. cincinnatus* cultures fell outside all recognized species clades and some cloned *L. cincinnatus* sequences fell within the *L. sulphureus* consensus clade (all cloned *L. cincinnatus* sequences that fell outside the *L. cincinnatus* consensus clade are indicated with gray arrows in FIG. 2). In addition long branches were observed within some species clades, especially in *L. cincinnatus*, *L.* sp. clade J, and *Wolfiporia dilatohypha*. These long branches represent respectively approximately 15 changes (six base pair changes and nine indels), 60 changes (31 base pair changes and 29 indels) and 22 changes (20 base pair changes and two indels)

TABLE I. Number of ITS sequence variants obtained from 1399 clones derived from 19 isolates of six *Laetiporus* species and two isolates of *Wolifiporia dilatohypha*

| Species | Clade designation from Banik et al. 2010 | Isolate | Number of clones sequenced | Number of sequence variants[a] | Percentage of clones within a given percent similarity of the consensus[b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | 98–100% similar to consensus | 95–98% similar to consensus | < 95% similar to consensus |
| *L. cincinnatus* | Clade K | DA-37 | 96 | 33 | 86.5% (83) | 1% (1) | 12.5% (12) |
| *L. cincinnatus* | Clade K | DA-37ss-1[c] | 24 | 15 | 75% (18) | 4% (1) | 21% (5) |
| *L. cincinnatus* | Clade K | DA-37ss-2[c] | 23 | 7 | 95.7% (22) | 0% | 4.3% (1) |
| *L. cincinnatus* | Clade K | DA-37ss-3[c] | 24 | 11 | 75% (18) | 0% | 25% (6) |
| *L. cincinnatus* | Clade K | DA-37ss-4[c] | 23 | 9 | 100% (23) | 0% | 0% |
| *L. cincinnatus* | Clade K | MAS-1 | 86 | 33 | 79.1% (68) | 12.8% (11) | 8.1% (7) |
| *L. cincinnatus* | Clade K | MO-4 | 93 | 39 | 89.3% (83) | 2.4% (2) | 8.6% (8) |
| *L. cincinnatus* | Clade K | IL-51 | 94 | 23 | 95.7% (90) | 0% | 4.3% (4) |
| *L. conifericola* | Clade B | NV-2 | 92 | 11 | 100% (92) | 0% | 0% |
| *L. conifericola* | Clade B | TR-5 | 2 | 1 | 100% (2) | 0% | 0% |
| *L. gilbertsonii* | Clade F | TJV-2000-101 | 87 | 25 | 100% (87) | 0% | 0% |
| *L. gilbertsonii* | Clade F | CA-7 | 94 | 22 | 100% (94) | 0% | 0% |
| *L. huroniensis* | Clade A1 | MI-5 | 29 | 3 | 100% (29) | 0% | 0% |
| *L.* sp. nov. | Clade J | GDL-1 | 95 | 17 | 100% (95) | 0% | 0% |
| *L.* sp. nov. | Clade J | PR6521 | 91 | 25 | 97.8% (89) | 0% | 2.2% (2) |
| *L. sulphureus* | Clade E | CT-1 | 62 | 18 | 100% (62) | 0% | 0% |
| *L. sulphureus* | Clade E | NAMA-2 | 95 | 22 | 100% (95) | 0% | 0% |
| *L. sulphureus* | Clade E | DA-11 | 92 | 18 | 100% (92) | 0% | 0% |
| *L. sulphureus* | Clade E | MAS-2 | 108 | 32 | 100% (108) | 0% | 0% |
| *W. dilatohypha* | | CS-63 | 45 | 11 | 100% (45) | 0% | 0% |
| *W. dilatohypha* | | FP72162 | 44 | 19 | 98% (43) | 2% (1) | 0% |

[a] Number of variants based on 100% sequence similarity.
[b] Percentage is given first, followed by the raw number of sequences in parentheses.
[c] Single-spore isolate derived from the fruiting body of DA-37.

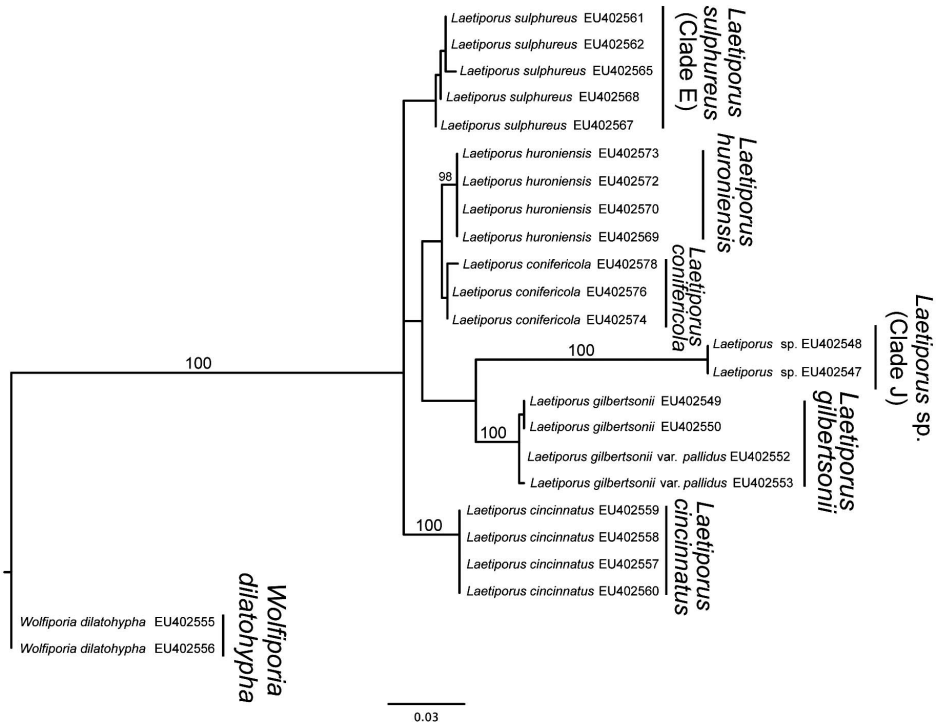


FIG. 1. Maximum likelihood phylogeny of *Laetiporus* isolates based on consensus ITS sequences from 22 cultures of six *Laetiporus* species and two cultures of *Wolfiporia dilatohypha*. Each sequence is labeled with the corresponding GenBank accession number, and species clades are indicated with vertical black lines. Bootstrap values greater than 70% are reported above branches.

from the consensus for *L. cincinnatus*, *L.* sp. clade J and *W. dilatohypha*.

An analysis also was performed to determine how many OTUs would be recognized with varying levels of percent similarity (TABLE II). A large number of small variants differing less than 1% were observed, although the majority of these errors might be attributable to the DNA extraction, PCR (*Taq* error) and cloning procedures. ITS sequences from multiple clones were PCR amplified, cloned and sequenced, similar to the ''*Taq* test'' run by Simon and Weiss (2008), to determine the overall error rate of our molecular methods. Four initial clones were used for this test and 48 clones were generated from each of these clones (192 total sequences). This test indicated that the molecular methods accounted for less than 1% of variation (approximately one error in 7000 bp sequenced). Grouping sequences by 99% similarity therefore should remove this error, a result also observed in bacterial community analyses (Speksnijder et al. 2001, Acinas et al. 2005).

The number of OTUs increased when the percent similarity used to define OTUs was increased (TABLE II). For consensus ITS sequences the number of OTUs remained constant (seven) but increased to 16 when 100% similarity was used. For cloned ITS sequences the number of OTUs increased gradually 20–35 when similarity was increased 90–99%, then increased dramatically at 100% similarity to 338 OTUs.

The spatial distribution of changes relative to the consensus sequence also was mapped for the *L. cincinnatus* ITS region (FIG. 3). This analysis was restricted to *L. cincinnatus*, the species where the most variation was observed. Changes observed in all isolates of *L. cincinnatus* were superimposed on this map, thus giving an overview of changes across multiple isolates. Only changes observed in more than one clone were mapped. In some cases it was difficult to fully represent all data because multiple changes occurred (pSNPs) at a single location, thus producing superimposed transitions and transversions. This map indicates that all insertions/deletions and all transversions occurred in the ITS1 and ITS2 regions; the 5.8S region displayed 21 transitions and no insertions/deletions or transversions.

## DISCUSSION

Significant intragenomic ITS variation was common among the fungal isolates examined in this study, thus raising the question of whether this type of

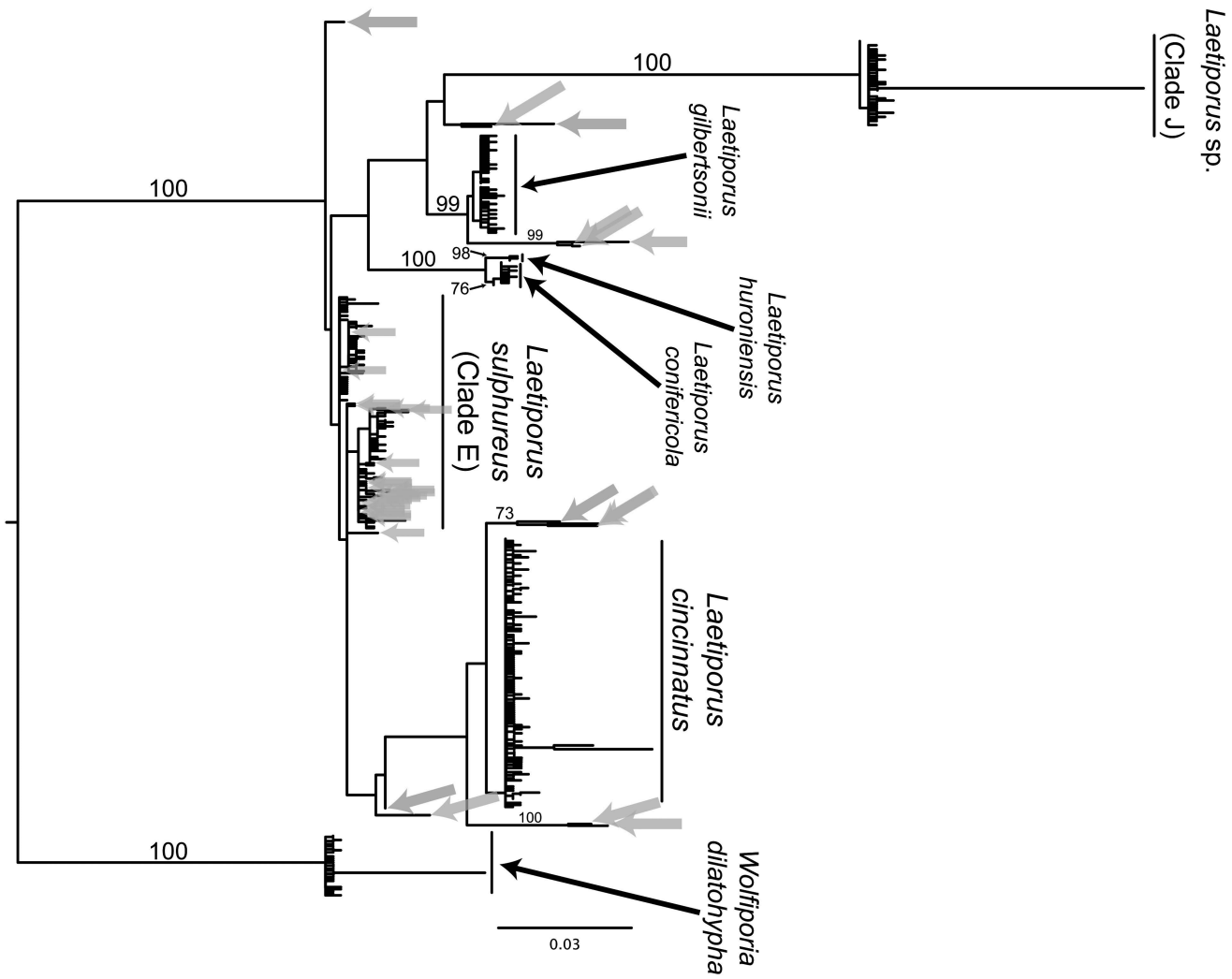


FIG. 2. Maximum likelihood phylogeny of cloned ITS sequences from 1399 clones derived from 19 isolates of six *Laetiporus* species and one *Wolfiporia* species. Species clades based on consensus sequences are indicated with vertical black lines. Gray arrows indicate the position of cloned *L. cincinnatus* ITS sequences that fell outside the *L. cincinnatus* consensus clade. Bootstrap values greater than 70% are reported above branches.

variation is confined to *Laetiporus* and closely related species or whether it is more widespread in kingdom Fungi. The small amount of work conducted with whole genome fungal data (e.g. Ganley and Kobayashi 2007, James et al. 2009) suggests intragenomic variation in the rDNA regions might be more widespread in fungi than previously recognized. Follow-up studies with next generation sequencing are under way on a wide range of Ascomycota and Basidiomycota species to determine the prevalence of intragenomic variation across a greater phylogenetic breadth of fungal species. These data might be valuable for determining how to best analyze the vast amount of environmental rDNA sequence data currently being generated with high throughput cloning and next generation sequencing.

TABLE II. Number of *Laetiporus* OTUs based on cloned and consensus ITS sequences from cultures of six *Laetiporus* species

| Percent similarity used to define OTUs | Number of *Laetiporus* OTUs observed | |
|---|---|---|
| | Consensus ITS sequences | Cloned ITS sequences |
| 90% | 7 | 20 |
| 95% | 7 | 20 |
| 97% | 7 | 21 |
| 98% | 7 | 28 |
| 99% | 7 | 35 |
| 100% | 16 | 338 |

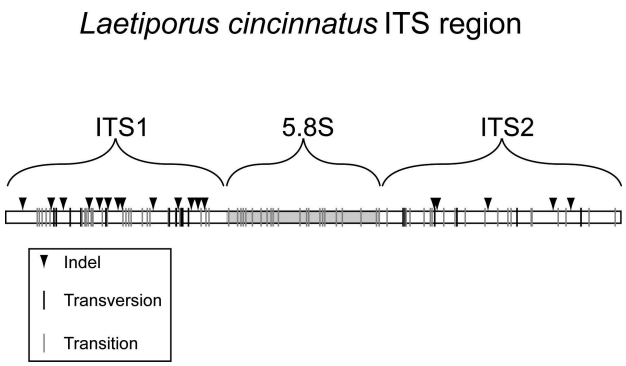


FIG. 3. Distribution of changes observed in cloned ITS sequences of *L. cincinnatus* isolates relative to the consensus *L. cincinnatus* ITS sequence. Changes were mapped only if observed in more than one clone.

Although ITS consensus sequences are currently considered the standard for phylogenetic analyses and barcoding efforts, a consensus sequence is a theoretical construct. In the most extreme case the consensus sequence would not have to occur even once within a tandem array if small variations were consistently and evenly distributed across the different copies of the array. However the data in this study confirm that the vast majority of copies in the array do match the consensus sequence exactly or almost exactly (within 98% similarity). Nonetheless the intragenomic ITS variation observed in this study is sufficiently significant that it undoubtedly would affect environmental detection of *Laetiporus* and related species. This variation until now had been overlooked because even apparently clean consensus sequences can harbor unobserved sequence variants (e.g. Lindner and Banik 2009), as was the case for *Laetiporus cincinnatus*. All isolates examined to date of *L. cincinnatus* produce consistent consensus ITS sequences > 99% similar; yet this species harbored the most intragenomic variation of all species examined. Consensus sequencing averages over all copies in a genome, so that rare ITS types may not be easily observed if sequence variants consistently differ in unique ways, thus keeping the proportion of each particular variant low, as occurred in *L. cincinnatus*.

Environmental fungal ITS sequences are commonly grouped by 95–98% similarity to define OTUs. If these standards are applied to our cloned dataset, the number of OTUs is overestimated by 14–22 taxa relative to the six traditionally defined *Laetiporus* species. For consensus sequences grouping sequences by 95–98% similarity consistently overestimates the number of taxa by a single taxon (seven instead of six taxa). It is difficult to determine whether the consensus ITS sequences fail to accurately reflect the true number of *Laetiporus* taxa or whether traditional taxonomic methods have failed to recognize the true number of taxa in this group. Of interest, the additional taxon recognized by constructing OTUs from consensus sequences falls in the *L. sulphureus* clade E, a heterogeneous group that has yet to be fully resolved taxonomically, so it is possible that seven *Laetiporus* taxa were included in this study.

More data are needed before it will be possible to determine the percent similarity that best estimates fungal OTUs in environmental ITS datasets. Defining OTUs based on 100% similarity clearly gives biased results, most likely due to a combination of method-based errors and intraspecies variation. Based on the overall error rates of our molecular procedures (as determined in METHODS), grouping sequences by 99% sequence similarity appears to remove all method-based ITS variation. However some of this microvariation in fact might be real, a result observed by Simon and Weiss (2008) in four Ascomycota species when high fidelity *Taq* polymerase was used. To determine whether the microvariation observed in this study is biological or an artifact of molecular methods it would be necessary to employ *Taq* with greater proofreading capability than was used in the current study.

Some of the divergent sequences observed in this study were significantly greater than 5% different from the consensus and might represent pseudogenes located outside the rDNA tandem array and thus outside the influence of concerted evolution. Physical mapping of ribosomal regions is needed to determine whether the divergent ITS sequences observed in this study are separated from the other members of the array. Preliminary mapping of changes within the ITS region of *L. cincinnatus* (FIG. 3) indicated that all changes within the 5.8S coding region were transitions, many of which were A to G. Because A–G transitions should not greatly affect secondary structure this suggests that the ribosomal coding regions observed in this study are functional. However detailed analyses are needed to determine whether these variants exhibit significant changes in secondary structure that compromise fitness. Although not observed in filamentous fungi it also is possible that extrachromosomal rDNA (Meyerink et al. 1979, Sinclair and Guarente 1997, Simon and Weiss 2008) contributes to intragenomic variation, but little is known about the distribution and importance of this type of rDNA.

Additional work on intragenomic ITS variation in fungi is needed across a wider phylogenetic range of fungi, and more equal sampling needs to be conducted for each species. In the current work the number of clones sequenced from each species was based primarily on the number of clones successfully

generated for each species. In addition, if little variation was observed in a species, no further effort was made to generate more clones for that particular species. This unfortunately led to some species (e.g. *L. conifiericola* and *L. huroniensis*) being sampled less intensively than other species (e.g. *L. cincinnatus*). Work in progress with next generation sequencing should greatly expand both the number of species examined as well as the number of ITS copies sequenced per isolate. Further examination of intragenomic rDNA variation in fungi hopefully will lead to better analysis strategies for next generation environmental rDNA sequence data and also might shed light on the fundamental mechanisms of concerted evolution and speciation in fungi.

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