Escaping introns in COI through cDNA barcoding of mushrooms: Pleurotus as a test case

Farhat A. Avin1,2 | Bhassu Subha3,4 | Yee-Shin Tan1,4 | Thomas W. A. Braukmann5 | Sabaratnam Vikineswary1,4 | Paul D. N. Hebert5

1Mushroom Research Centre (MRC), University of Malaya, Kuala Lumpur, Malaysia
2Department of Biotechnology, Faculty of Science, Lincoln University College, 47301 Petaling Jaya, Malaysia
3Centre for Biotechnology in Agriculture Research (CEBAR), Division of Genetics and Molecular Biology, University of Malaya, Kuala Lumpur, Malaysia
4Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
5Centre for Biodiversity Genomics, University of Guelph, Guelph, ON, Canada

Correspondence
Yee-Shin Tan and Farhat A. Avin, Mushroom Research Centre (MRC), University of Malaya, Kuala Lumpur, Malaysia.
Emails: tanyeeshin@um.edu.my and farhat.avin@gmail.com

Funding information
Mushroom Research Centre, Malaysia, Grant/Award Number: UM.0000117-KWI.AK; High Impact Research (HIR), Malaysia, Grant/Award Number: UM.C/625/1/HIR-MOHE/ASH/01 (H-23001-G000008); Ministry of Higher Education, Malaysia, Grant/Award Number: MOE, KT001-2014A; Institute of Research Management and Monitoring (IPPP), University of Malaya, Grant/Award Number: UMRG RP 005A – 13 AFR

Abstract
DNA barcoding involves the use of one or more short, standardized DNA fragments for the rapid identification of species. A 648-bp segment near the 5′ terminus of the mitochondrial cytochrome c oxidase subunit I (COI) gene has been adopted as the universal DNA barcode for members of the animal kingdom, but its utility in mushrooms is complicated by the frequent occurrence of large introns. As a consequence, ITS has been adopted as the standard DNA barcode marker for mushrooms despite several shortcomings. This study employed newly designed primers coupled with cDNA analysis to examine COI sequence diversity in six species of Pleurotus and compared these results with those for ITS. The ability of the COI gene to discriminate six species of Pleurotus, the commonly cultivated oyster mushroom, was examined by analysis of cDNA. The amplification success, sequence variation within and among species, and the ability to design effective primers was tested. We compared ITS sequences to their COI cDNA counterparts for all isolates. ITS discriminated between all six species, but some sequence results were uninterpretable, because of length variation among ITS copies. By comparison, a complete COI sequences were recovered from all but three individuals of Pleurotus giganteus where only the 5′ region was obtained. The COI sequences permitted the resolution of all species when partial data was excluded for P. giganteus. Our results suggest that COI can be a useful barcode marker for mushrooms when cDNA analysis is adopted, permitting identifications in cases where ITS cannot be recovered or where it offers higher resolution when fresh tissue is. The suitability of this approach remains to be confirmed for other mushrooms.

KEYWORDS
COI, DNA barcoding, internal transcribed spacer, oyster mushrooms, taxonomic verification

1 INTRODUCTION
DNA barcoding employs short, standardized DNA fragments for the rapid identification of species (Gilmore, Graefenhan, Louis Seize, & Seifert, 2009; Hebert, Cywinska, & Ball, 2003; Nguyen & Seifert, 2008; Vialle et al., 2009). This approach is particularly valuable for verifying species identification, and for the evaluation of taxonomic diversity in organisms with cryptic morphology such as fungi (Dentinger, Didukh, & Moncalvo, 2011). The use of molecular tools is essential for identifying and classifying the 90%–95% of undescribed fungi (Blackwell,
The ideal DNA barcode region is easy to amplify and variable enough to discriminate species, a condition that is best met when variation within species is low and divergence between species is high, a situation which creates a “barcode gap” (Hebert et al., 2003; Seifert et al., 2012). The ribosomal internal transcribed spacer (ITS), a highly variable region between the conserved sequences of the small subunit, 5.8S, and large subunit RNA genes, has been adopted as the primary DNA barcode marker for fungi (Schoch, Seifert, Huhndorf, et al., 2012).

COI and ITS generally delivered similar resolution, but the prevalence of introns and the apparent occurrence of nuclear pseudogenes, there is a need for more detailed study. In particular, given the prevalence of introns in the COI gene, the apparent occurrence of nuclear pseudogenes, it is critical to adopt RT-PCR to properly recover and evaluate the capacity of COI sequences to resolve fungal species.

A few studies have compared the resolution of ITS and COI in sets of closely allied species. COI was more effective than ITS in Penicillium (Seifert et al., 2007), while COI and ITS were equally effective in Leohumicola, (Nguyen & Seifert, 2008). In the Agaricomycotina, COI and ITS generally delivered similar resolution, but the prevalence of introns resulted in COI not being recovered from many taxa (Dentinger et al., 2011). Conversely, COI sequences showed low divergences in Fusarium (Gilmore et al., 2009) and Aspergillus (Geiser et al., 2007), although data interpretation was complicated by the apparent presence of multiple copies of COI, perhaps reflecting the recovery of nuclear pseudogenes. The strong performance of COI as a DNA barcode in animals (Hebert et al., 2003) suggests the value of exploring its use as a marker in mushrooms. Similar to the multi-locus barcode approach used in plants, COI could be used in conjunction with ITS for the identification of fungal species. There is one barrier to the implementation: the prevalence of introns in the COI gene of many fungal species including mushrooms is well documented (Seifert, 2009; Vialle et al., 2009). For example, nine introns occur in Pleurotus ostreatus (Wang, Zeng, Hon, Zhang, & Leung, 2008), 19 in Agaricus bisporus (Féron et al., 2010), 15 in Trametes cingulata (Haridas & Gantt, 2010) and four in Agrocybe aegerita (Gonzalez, Barroso, & Labarère, 1998). These introns are often long, leading to extreme variation in length of the COI gene from approximately 1,584 bp in species lacking introns to over 22 kb in those with many introns (Féron et al., 2010; Gonzalez et al., 1998; Haridas & Gantt, 2010; Wang et al., 2008). The presence of these introns impedes sequence recovery by conventional PCR (Seifert, 2009; Seifert et al., 2007), a factor which has supported the adoption of ITS as the sole DNA barcode for mushrooms (Schoch & Seifert, 2012; Vialle et al., 2009).

In this study, we examine the ability of the COI gene to discriminate six species of Pleurotus. We test amplification success, sequence variation within and among species, and the ability to design effective primers. We also recover ITS sequences from all isolates to allow their comparison with the sequences recovered through the analysis of cDNA from COI.

## 2 MATERIALS AND METHODS

### 2.1 Sample collection

The 24 strains examined in this study included representatives of six species of Pleurotus (Table 1). They were mostly obtained from mushroom farms in Malaysia or from the University of Malaya collection. A few isolates were newly collected from Malaysia, while others were imported from China or Iraq (Table 1). The species assignment for each isolate was verified by comparison of morphological traits of basidiocarps and mycelial cultures.

### 2.2 DNA and RNA extraction and cDNA synthesis

Total genomic DNA was extracted from fresh mycelium by a rapid protocol (Avin, Bhassu, & Sabaratnam, 2013). Briefly, after adding sufficient 2% SDS buffer, the samples were homogenized at 65°C for 30 min. The mixture was purified twice with phenol: CHCl3: Isoamyl alcohol (25: 24: 1). DNA was precipitated with cold
TABLE 1  List of species and strains used in this study and length of amplicons for COI and ITS. Bold process IDs for the samples sequenced in this are also indicated and are publically available.

| Strain ID | Species                    | Source          | Length of amplicon (bp) | Sequence ID (The BOLD System) | NCBI GenBank accession number |
|-----------|----------------------------|-----------------|-------------------------|------------------------------|-------------------------------|
| FUM-077   | Pleurotus pulmonarius      | Farm Malaysia   | 759 757 1.516           | CDB0001-15 KY951484 KY951528 KY951506 |
| FUM-078   | Pleurotus pulmonarius      | Farm Malaysia   | 759 757 1.516           | CDB0002-15 KY951490 KY951534 KY951512 |
| FUM-079   | Pleurotus pulmonarius      | Farm Malaysia   | 759 757 1.516           | CDB0003-15 KY951489 KY951533 KY951511 |
| FUM-080   | Pleurotus giganteus        | Wild Malaysia   | 759 Partial X           | CDB0004-15 KY951479 X X     |
| FUM-081   | Pleurotus ostreatus        | Iraq            | 759 757 1.516           | CDB0005-15 KY951483 KY951527 KY951505 |
| FUM-082   | Pleurotus ostreatus        | Farm Malaysia   | 759 757 1.516           | X X CDB0006-15 KY951520 KY951498 |
| FUM-084   | Pleurotus giganteus        | Wild Malaysia   | 759 Partial X           | CDB0007-15 KY951477 X X     |
| FUM-085   | Pleurotus flabellatus      | Iraq            | 759 757 1.516           | CDB0008-15 KY951474 KY951516 KY951494 |
| FUM-086   | Pleurotus ostreatus        | UM collection   | 759 757 1.516           | CDB0009-15 KY951482 KY951525 KY951503 |
| FUM-087   | Pleurotus ostreatus        | Iraq            | 759 757 1.516           | CDB0010-15 X KY951523 KY951501 |
| FUM-088   | Pleurotus citrinopileatus  | Farm Malaysia   | 759 757 1.516           | CDB0011-15 KY951471 KY951513 KY951491 |
| FUM-089   | Pleurotus pulmonarius      | Farm Malaysia   | 759 757 1.516           | CDB0012-15 KY951488 KY951532 KY951510 |
| FUM-090   | Pleurotus ostreatus        | China           | 759 757 1.516           | CDB0013-15 KY951480 KY951521 KY951499 |
| FUM-091   | Pleurotus pulmonarius      | Farm Malaysia   | 759 757 1.516           | CDB0014-15 KY951487 KY951531 KY951509 |
| FUM-093   | Pleurotus flabellatus      | Farm Malaysia   | 759 757 1.516           | CDB0015-15 KY951475 KY951517 KY951495 |
| FUM-095   | Pleurotus citrinopileatus  | Farm Malaysia   | 759 757 1.516           | CDB0016-15 KY951473 KY951515 KY951493 |
| FUM-096   | Pleurotus pulmonarius      | Farm Malaysia   | 759 757 1.516           | CDB0017-15 KY951486 KY951530 KY951508 |
| FUM-099   | Pleurotus giganteus        | China           | 759 Partial X           | CDB0018-15 KY951478 KY951519 KY951497 |
| FUM-100   | Pleurotus ostreatus        | Farm Malaysia   | 759 757 1.516           | CDB0019-15 KY951481 KY951524 KY951502 |
| FUM-101   | Pleurotus ostreatus        | Farm Malaysia   | 759 757 1.516           | X X CDB0020-15 KY951526 KY951504 |
| FUM-102   | Pleurotus ostreatus        | UM collection   | 759 757 1.516           | X X CDB0021-15 KY951522 KY951500 |
| FUM-103   | Pleurotus pulmonarius      | UM collection   | 759 757 1.516           | X X CDB0022-15 KY951485 KY951529 KY951507 |
| FUM-104   | Pleurotus eryngii          | UM collection   | 759 757 1.516           | 600 CDB0023-15 KY951476 KY951518 KY951496 |
| FUM-105   | Pleurotus citrinopileatus  | UM collection   | 759 757 1.516           | 598 CDB0024-15 KY951472 KY951514 KY951492 |
isopropanol, and then pelleted by centrifugation at 4°C for 15 min at 11,000 × g. The resultant DNA pellet was dissolved in TE buffer and stored at -20°C.

Total RNA was isolated from fresh mycelium using Trizol (Invitrogen, USA). Briefly, sufficient Trizol was added to the homogenized mycelia and incubated at 25°C for 15 min, then purified by chloroform. RNA was precipitated with cold ethanol and the pellet was washed twice with 70% ethanol. The RNA pellet was then dissolved in RNAase free water and stored at -80°C. Samples that did not successfully amplify in the first round of RT-PCR were re-extracted using Nucleospin® RNA columns (Macherey-Nagel, Germany) following the manufacturers protocol. This included a DNAase treatment prior to elution in nuclease free water.

Total cDNA was synthesized from the RNA extracts using an Access One Step RT-PCR system kit (Promega, USA). The first mixture was generated by gently mixing 1.0 μl of total extracted RNA, 1.0 μl of Oligo dt primer, and 3.0 μl of Nuclease-free H2O that was incubated for 5 min at 70°C. The second mixture was prepared by mixing 6.1 μl of Nuclease-free H2O, 4.0 μl of Improm II reaction buffer, 2.4 μl of 25 mmol/L MgCl2, 1.0 μl of 10 mmol/L dNTP mix, 0.5 μl of Rnasin ribonuclease inhibitor and 1.0 μl of Improm II reverse transcriptase. Mixtures I and II were then combined for each sample and incubated for: 5 min at 25°C, 60 min at 42°C, and 15 min at 70°C before being stored at -20°C.

### 2.3 Primer design

The coding sequence of COI from the mitochondrial genome of *P. ostreatus* (19: EF204913) was used as a reference to design primers (Figure 1). Several criteria, including the generation of proper length fragments (800–900 bp) with enough conserved sites in the binding regions were employed to design primers. NCBI Primer-BLAST was used to design primer pairs for two cDNA regions that spanned the coding sequence of COI (Rozen & Skaletsky, 2000; Ye et al., 2012). Figure 1 shows the location and orientation of these primers on the open reading frames of COI. Primer ID, sequence and annealing temperatures are provided in Table 2.

### 2.4 PCR and reverse transcription (RT)-PCR conditions

PCR amplification of the COI cDNA employed an initial denaturation at 95°C for 5 min; followed by 30 cycles with denaturation at 94°C, annealing at 55°C and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. The 50 μl PCR reactions included 4.0 μl (~100 ng) of template DNA (cDNA), 1.0 mmol/L MgCl2, 0.4 μmol/L of each primer (Part I: P1COIF & P1COIR and Part II: P2COIF & P2COIR), 0.4 mmol/L of each dNTP, 10 μl of 5× Taq buffer, 2 units of GoTaq® Flexi DNA Polymerase (Promega, USA). We used genomic DNA to amplify and sequence the ITS region with primers ITS1 and ITS4 using standard protocols (White, Bruns, Lee, & Taylor, 1990), or with local primers ITS1-UM2 and ITS2-UM2 (Avin, Bhassu, Shin, & Vikineswary, 2014). Successfully amplified PCR products were purified using the Nucleospin Extract II Kit (Chemopharm), and bidirectionally sequenced using an ABI 3730XL automated sequencer. Sequences along with voucher information were deposited in the Barcode of Life Data System (BOLD Process IDs; CDB001-CDB024-15) (Ratnasingham & Hebert, 2007) and are publicly available in NCBI GenBank (Table 1).

### 2.5 Sequence alignment, barcode gap analysis, and phylogenetic analysis

Chromatograms were edited using ChromasPro version 1.7.6 (Technelysium Pty Ltd., QLD, Australia). Additional ITS sequences were retrieved from GenBank and included in our analysis (Accession Numbers AF465404, EU314927, HM590443, EF514248, KF681359, EU424300, KJ862075, KCS82641, KJ862073, JQ026939, EU233951, AY696300, AY450349, HM245782, KPI20919, KP012913, KF724509, JNO43316, KCS82636, JQ37487, EU424288 and AY265827). The sequences were aligned using MEGA ver. 6.0 (Pawlik et al., 2012) and BioEdit ver. 7.2.5 (Hall, 1999). Barcode gap analysis was performed in BOLD 3 (Ratnasingham & Hebert, 2007) under a Kimura two-parameter model with pairwise deletion of gaps and ambiguities on complete COI and ITS sequences. MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) was used to determine the model that best fit sequence evolution for COI and ITS datasets prior
to phylogenetic tree construction using maximum likelihood (ML). Phyllogenetic trees were constructed for COI data both with and without partial sequences (5′ end) for *Pleurotus giganteus*. ITS trees were constructed with only sequences obtained in this study, and after including GenBank sequences for the species in our data sets. According to Bayesian information criterion (Schwarz, 1978), a General Time Reversible model (Lanave, Preparata, Sacone, & Serio, 1984) with rate variation among nucleotides following a discrete gamma distribution (GTR+G) was selected as the best fit model for COI data, and a Tamura three parameter model (Tamura, 1992) with a proportion of invariable sites (TN92+I) for ITS data (with and without GenBank sequences). For both genes, ML trees were constructed in MEGA 6 (Tamura et al., 2013) under the selected model; branch topology was optimized using extensive subtree pruning and regrafting (SPR) with branch swap filter selected. The stability of nodes was inferred by non-parametric bootstrapping (Felsenstein, 1985), using 1,000 heuristic bootstrap pseudoreplicates. DNAsp ver. 5.10 was used to calculate the haplotype data file and genetic divergences (Librado & Rozas, 2009). To estimate the significance of variance within and among species, an AMOVA (analysis of molecular variance) was calculated using Arlequin ver. 3.50 (Excoffier, Laval, & Schneider, 2005).

3 | RESULTS

An interpretable ITS sequence was recovered from 20 of the 24 specimens, including at least one representative of each species with sequences varying in length from 592 to 625 bp (Table 1). A COI sequence was recovered from all specimens, but only a partial COI-3′ sequence was obtained from specimens of *P. giganteus*. Near full length COI sequences were generated by aligning and assembling a consensus of the 5′ and 3′ reads for the five species with reads for both regions (Table 1 and 3). Because the COI sequences were generated from cDNA template generated by RT-PCR they lacked introns, while ITS was amplified using standard PCR (Figure 2 and Table 3). The percentage of variable sites for all six species was computed for both genes (Table 3). Across all 1,516 sites for COI, 76.8% were conserved, while 23.1% were variable with 12.3% being parsimony informative and 10.9% singletons. By comparison, 37.7% of the 715 ITS sites were conserved, while 55.2% were variable with 38.4% being parsimony informative, and 17.0% singletons (Table 3). Due to the indels in ITS, the mean divergence for all 20 sequences was higher for ITS (0.199) than for COI (0.059). Intra-specific divergences were generally slightly higher for ITS than COI, but so too were inter-specific divergences. Barcode gap analysis supports higher interspecific and intraspecific distances for ITS than COI. Both markers indicate *P. ostreatus*, *P. eryngii*, and *P. pulmonarius* are relatively close (Table 4) and fall under the 2% divergence threshold for COI and ITS (except *P. eryngii*). However, the use of the closely related mushrooms in our analysis suggests that the use of small sample sizes may explain the low divergence threshold (below 2%). The maximum intraspecific distance was greater for both COI and ITS in *P. ostreatus*. Otherwise, intraspecific distances were low for the remaining *Pleurotus* species with multiple representatives per species.

Figure 2a–d shows ML trees for COI and ITS with bootstrap values for each node based on 1,000 replicates. ITS (Figure 2c,d) discriminated all six species with strong support, but sequences from four of eight specimens of *P. ostreatus* failed. COI sequences were recovered from all specimens, albeit just partial COI-5′ sequences for *P. giganteus*. COI failed to distinguish between *P. pulmonarius* and *P. giganteus* when partial 5′ sequences were included, but when partial sequences were excluded, COI distinguished between these two species with strong support (Figure 2). Overall, both markers readily distinguished between species with moderate to strong support. When sampling was improved for ITS with GenBank sequences, there was strong support for the monophyly of the six *Pleurotus* species, results confirming our morphological identifications (Figure 2d).

4 | DISCUSSION

In contrast to prior studies that failed to recover COI through conventional PCR-based approaches (Dentinger et al., 2011; Vialle et al., 2009), the cDNA approach employed in this analysis recovered full COI sequences from all six species of oyster mushrooms (barring a few incomplete recoveries for *P. giganteus*). The past failures of
TABLE 3  Comparison of four potential markers for DNA barcoding of Pleurotus

| Region                        | COI-5′ | COI-3′ | COI-whole | ITS   |
|-------------------------------|--------|--------|-----------|-------|
| Method                        | RT-PCR | RT-PCR | RT-PCR    | PCR   |
| No. of mushroom strains analyzed | 24     | 24     | 24        | 24    |
| No. with sequence record      | 24     | 21 + 3 partial | 21   | 20    |
| Number of introns             | 5      | 5      | 9         | N/A   |
| Number of exons               | 6      | 6      | 10        | N/A   |
| Final fragment length         | 759    | 757    | 1,516     | 592–625 |
| Gap                           | No     | No     | No        | Yes   |
| Number of haplotypes          | 7      | 10     | 13        | 13    |
| Conserved sites               | 583/759 (76.8%) | 576/757 (76.1%) | 1,165/1,516 (76.8%) | 271/719 (37.7%) |
| Variable sites                | 176/759 (23.2%) | 181/757 (23.9%) | 352/1,516 (23.1%) | 397/719 (55.2%) |
| Parsimony informative sites   | 87/759 (11.5%) | 99/757 (13.1%) | 186/1,516 (12.3%) | 276/719 (38.4%) |
| Singleton sites               | 89/759 (11.7%) | 82/757 (10.8%) | 166/1,516 (10.9%) | 122/719 (17.0%) |
| Total number of mutations     | 193/759 (25.4%) | 205/757 (27.1%) | 380/1,516 (25.1%) | 390/719 (54.2%) |
| Overall mean distance         | 0.050  | 0.064  | 0.059     | 0.199 |
| G+C content (%)               | 33.3%  | 34.4%  | 33.9%     | 43.4% |

FIGURE 2  COI and ITS phylogenetic analyzes. (a–d) Phylogeny reconstruction based on maximum likelihood under a GTR+G model for COI and a TN92+I model for ITS. Numbers at the nodes indicate the percentage of bootstrap replicates supporting a given topology, although bootstrap values below 50% are not indicated. Samples ID and species delimitations are indicated at the tips of the tree. One COI tree for Pleurotus giganteus is based on 759-bp COI-5′ fragments, while sequences for the other taxa were full length 1,516 bp. Twenty-two ITS sequences and two mitochondrial sequences were retrieved from GenBank; they are indicated in yellow.
standard PCR were undoubtedly due to the presence of several large introns in the COI gene of *Pleurotus* (Dentinger et al., 2011; Seifert, 2009; Seifert et al., 2007). However, cDNA barcoding escapes this problem, generating amplicons that are easily aligned. The present study generated a 1,516-bp COI sequences from 21 of the 24 specimens, failing only to recover full sequence information from the 3′ region of *P. giganteus*. Our failure to amplify the 3′ end of *P. giganteus* reflects the need to further optimize COI primers for *Pleurotus* (and other mushrooms) given the diagnostic ability of the 3′ end of this gene. Alternatively, more samples of different species should be sequenced and aligned to design appropriate primer pairs on the most conserved regions. ITS sequences were recovered from all six species, but results from four of the 24 specimens were uninterpretable due to sequence length variation. Although the number of species examined in this study was small, the success of COI in discriminating one pair of conspecific individuals that can complicate sequence alignment and subsequent data analysis. Although Schoch, Seifert, Huhndorf, et al. (2012) concluded that ribosomal markers (e.g., ITS) have fewer problems with PCR amplification than protein-coding markers (e.g., COI), the difficulties in generating a reliable alignment are an important drawback to the use of ITS as a DNA barcode marker (Dentinger et al., 2011; Seifert et al., 2007). Furthermore, sequence variation among paralogues can result in uncertain base calls. Despite these caveats, the availability of ITS sequences from a large number of fungal species in GenBank is a major advantage that often outweighs the complications introduced by alignment problems.

| Species          | Number of individuals | Mean intra-sp | Max intra-sp | Distance to NN | NN           |
|------------------|-----------------------|---------------|--------------|----------------|--------------|
| COI              |                       |               |              |                |              |
| *P. citrinopileatus* | 3                     | 0             | 0            | 7.7            | *P. djamor*  |
| *P. djamor*      | 2                     | 0.13          | 0.13         | 7.7            | *P. citrinopileatus* |
| *P. eryngii*     | 1                     | N/A           | N/A          | 1.47           | *P. ostreatus* |
| *P. giganteus*   | 1                     | N/A           | N/A          | 4.55           | *P. pulmonarius* |
| *P. ostreatus*   | 8                     | 0.45          | 1.2          | 1.2            | *P. pulmonarius* |
| *P. pulmonarius* | 7                     | 0.08          | 0.13         | 1.2            | *P. ostreatus* |
| ITS              |                       |               |              |                |              |
| *P. citrinopileatus* | 3                     | 0             | 0            | 12.06          | *P. giganteus* |
| *P. djamor*      | 2                     | 0.16          | 0.16         | 19.37          | *P. pulmonarius* |
| *P. eryngii*     | 1                     | N/A           | N/A          | 2.37           | *P. ostreatus* |
| *P. giganteus*   | 3                     | 2.33          | 2.68         | 12.06          | *P. citrinopileatus* |
| *P. ostreatus*   | 4                     | 12.18         | 17.89        | 1.89           | *P. pulmonarius* |
| *P. pulmonarius* | 7                     | 0             | 0            | 1.89           | *P. ostreatus* |
The current study suggests that the COI can be an additional barcode marker for particular taxonomic groups of fungi when ITS is unsuitable (e.g., some genera in Ascomycota or some species of mushrooms discussed in this study) or for examining fresh material through a cDNA based approach. However, this approach needs to be extended to determine its suitability for other fungi. Moreover, COI sequences generated phylogenetic groupings for Pleurotus similar to those for ITS while having the advantage of being easily aligned. These results justify the broader examination of cDNA-based analysis to test the potential of COI as a barcode marker that could complement ITS, in much the same fashion that two gene regions (rbcL, matK) have been adopted as the standard barcode regions for plants (Hollingsworth et al., 2009). Future efforts should explore the use of COI in groups where ITS is unable to deliver species-level resolution.

ACKNOWLEDGMENTS

This study was supported by UM.C/625/1/HIR-MOHE/ASH/01 (H-23001-G000008) from High Impact Research (HIR), MOE, KT001-2014A from Ministry of Higher Education, Malaysia, UM.0000117-KWJ.JAK, Mushroom Research Centre (MRC), University of Malaya and UMRG RP 005A – 13 AFR from Institute of Research Management and Monitoring (IPPPP), University of Malaya. This publication is a contribution to the Food From Thought research program supported by the Canada First Research Excellence fund.

CONFLICT OF INTEREST

None declared.

DATA ACCESSIBILITY

The DNA sequences are available in the Barcode of Life Data System (BOLD) and National Center for Biotechnology Information (NCBI) which have shown in Table 1.

AUTHOR CONTRIBUTIONS

Farhat A. Avin: designed research, performed research, analyzed data, wrote the paper; Subha Bhassu: project adviser, edited the paper, analysis adviser; Dr. Tan Yee Shin: project adviser, edited the paper, analysis adviser; Thomas W. A. Braukmann: analyzed data, edited the paper; Vikineswary Sabaratnam: project leader, project financial leader, project adviser, edited the paper; Paul Hebert: project adviser, edited the paper, analysis adviser.

REFERENCES

Avin, F. A., Bhashu, S., Rameeh, V., Tan, Y. S., & Vikineswary, S. (2016). Genetics and hybrid breeding of Pleurotus pulmonarius: Heterosis, heritability and combining ability. Euphytica, 209, 85–102.
Avin, F. A., Bhashu, S., & Sabaratnam, V. (2013). A simple and low-cost technique of DNA extraction from edible mushrooms examined by molecular phylogenetics. Research on Crops, 14, 897–901.
Avin, F. A., Bhashu, S., Shim, T. Y., & Sabaratnam, V. (2012). Molecular classification and phylogenetic relationships of selected edible Basidiomycetes species. Molecular Biology Reports, 39, 7355–7364.
Avin, F. A., Bhashu, S., Shin, T. Y., & Vikineswary, S. (2014). DNA pedigree tracking to identify compatible mating partners of Pleurotus pulmonarius. Journal of Animal and Plant Sciences, 24, 89–97.
Avin, F. A., Bhassu, S., Tan, Y. S., Shahbaz, P., & Vikineswary, S. (2014). Molecular divergence and species delimitation of the cultivated oyster mushrooms: Integration of IGS1 and ITS. The Scientific World Journal, 2014, 1–11.
Bao, D., Ishihara, H., Mori, N., & Kitamoto, Y. (2004). Phylogenetic analysis of oyster mushrooms (Pleurotus spp.) based on restriction fragment length polymorphisms of the 5’ portion of 26S rDNA. Journal of Wood Science, 50, 169–176.
Begerow, D., Nilsson, H., Unterseher, M., & Maier, W. (2010). Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Applied Microbiology and Biotechnology, 87, 99–108.
Blackwell, M. (2011). The Fungi: 1, 2, 3… 5.1 million species? American Journal of Botany, 98, 426–438.
Dentinger, B. T. M., Didukh, M. Y., & Moncalvo, J. M. (2011). Comparing COI and ITS as DNA barcode markers for mushrooms and allies (Agaricomycotina), PLoS One, 6, e25081.
Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47–50.
Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 783–791.
Férandon, C., Moukha, S., Callac, P., Benedetto, J. P., Castroviejo, M., & Barroso, G. (2010). The Agaricus bisporus cox1 gene: The longest mitochondrial gene and the largest reservoir of mitochondrial group I introns. PLoS One, 5, e104048.
Geiser, D. M., Klich, M. A., Frisvad, J. C., Peterson, S. W., Varga, J., & Samson, R. A. (2007). The current status of species recognition and identification in Aspergillus. Studies in Mycology, 59, 1–10.
Gilmore, S. R., Graefenhahn, T., Louis Seige, G., & Seifert, K. A. (2009). Multiple copies of cytochrome oxidase 1 in species of the fungal genus Fusarium. Molecular Ecology Resources, 9, 90–98.
Gonzalez, P., Barroso, G., & Labarère, J. (1998). Molecular analysis of the split cox1 gene from the Basidiomycota Agrocybe aegerita: Relationship of its introns with homologous Ascomycota introns and divergence levels from common ancestral copies. Gene, 220, 45–53.
Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95–98.
Haridas, S., & Gantt, J. S. (2010). The mitochondrial genome of the wood-degrading basidiomycete Trametes circulata. FEMS Microbiology Letters, 308, 29–34.
Hebert, P. D. N., Cywinska, A., & Ball, S. L. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270, 313–321.
Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., Van Der Bank, M., ... Fazekas, A. J. (2009). A DNA barcode for land plants. Proceedings of the National Academy of Sciences, 106, 12794–12797.
James, T. Y., Kauff, F., Schou, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., ... Miadlikowska, J. (2006). Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature, 443, 818–822.
Lahaye, R., Van Der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., ... Savolainen, V. (2008). DNA barcoding the florals of biodiversity hotspots. Proceedings of the National Academy of Sciences, 105, 2923–2928.
Lanave, C., Preparata, G., Saccone, C., & Serio, G. (1984). A new method for calculating evolutionary substitution rates. Journal of Molecular Evolution, 20, 86–93.
Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25, 1451–1452.
Nguyen, H. D. T., & Seifert, K. A. (2008). Description and DNA barcoding of three new species of Leohumicola from South Africa and the United States. Persoonia: Molecular Phylogeny and Evolution of Fungi, 21, 57–69.

Pawluk, A., Janusz, G., Kosszerny, J., Małek, W., & Rogalski, J. (2012). Genetic diversity of the edible mushroom Pleurotus sp. by amplified fragment length polymorphism. Current Microbiology, 65, 438–445.

Ratnasingham, S., & HeBERT, P. D. N. (2007). BOLD: The barcode of life data system (http://www.barcodinglife.org). Molecular Ecology Notes, 7, 355–364.

Robert, V., Szöke, S., Eberhardt, U., Cardinali, G., Meyer, W., Seifert, K. A., ... Lewis, C. T. (2011). The quest for a general and reliable fungal DNA barcode. Open Applied Informatics Journal, 5, 45–61.

Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Molecular Biology, 132, 365–386.

Schoch, C. L., & Seifert, K. A. (2012). Reply to Kiss: Internal transcribed spacer (ITS) remains the best candidate as a universal DNA barcode marker for Fungi despite imperfections. Proceedings of the National Academy of Sciences, 109, E1812.

Schoch, C. L., Seifert, K. A., Caldeira, K., Myhrvold, N. P., Alvarez, R. A., Pacala, S. W., ... Mullins, R. D. (2012). Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for Fungi. Proceedings of the National Academy of Sciences, 109, 10741–10742.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., ... Crous, P. W. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences, 109, 6241–6264.

Schwarz, G. (1978). Estimating the dimension of a model. The Annals of Statistics, 6, 461–464.

Seifert, K. A. (2009). Progress towards DNA barcoding of fungi. Molecular Ecology Resources, 9, 83–89.

Seifert, K. A., Samson, R. A., DeWaard, J. R., Houbreken, J., Levesque, C. A., Moncalvo, J. M., ... Hebert, P. D. N. (2007). Prospects for fungus identification using CO1 DNA barcodes, with Penicillium as a test case. Proceedings of the National Academy of Sciences, 104, 3901–3906.

Stielow, J., Lévesque, C., Seifert, K., Meyer, W., Iriny, L., Smits, D., ... Chaduli, D. (2015). One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. Persoonia: Molecular Phylogeny and Evolution of Fungi, 35, 242–263.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Molecular Biology and Evolution, 9, 678–687.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725–2729.

Vialle, A., Feau, N., Allaire, M., Didukh, M., Martin, F., Moncalvo, J., & Hamelin, R. C. (2009). Evaluation of mitochondrial genes as DNA barcode for Basidiomycota. Molecular Ecology Resources, 9, 99–113.

Vilgalys, R., Moncalvo, J. M., Liou, S. R., & Volovsek, M. (1996). Recent advances in molecular systematics of the genus Pleurotus. In D. J. Royse (Ed.), Mushroom biology and mushroom products: Proceedings of the 2nd international conference (pp. 91–101). Pennsylvania State University: World Society for Mushroom Biology and Mushroom Products: PA (USA).

Wang, Y., Zeng, F., Hon, C., Zhang, Y., & Leung, F. (2008). The mitochondrial genome of the Basidiomycete fungus Pleurotus ostreatus (oyster mushroom). FEMS Microbiology Letters, 280, 34–41.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). PCR protocols: A guide to methods and applications. New York, NY: Academic Press.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 13, 134–144.

Zervakis, G., & Balis, C. (1996). A pluralistic approach in the study of Pleurotus species with emphasis on compatibility and physiology of the European morphotaxa. Mycological Research, 100, 717–731.

How to cite this article: Avin FA, Subha B, Tan Y-S, Braukmann TWA, Vikineswary S, Hebert PDN. Escaping introns in COI through cDNA barcoding of mushrooms: Pleurotus as a test case. Ecol Evol. 2017;7:6972–6980. https://doi.org/10.1002/ece3.3049