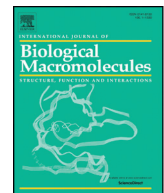
International Journal of Biological Macromolecules 121 (2019) 364–372

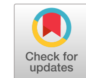
Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac



Characterization and comparison of the mitochondrial genomes from two *Lyophyllum* fungal species and insights into phylogeny of *Agaricomycetes*



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A R T I C L E I N F O

Article history:
Received 29 June 2018
Received in revised form 12 September 2018
Accepted 10 October 2018
Available online 11 October 2018

Keywords:
Lyophyllum
Mitochondrial genome
Protein coding gene
Repeat sequence
Gene rearrangement
Phylogenetic analysis

A B S T R A C T

Lyophyllum decastes and *Lyophyllum shimeji* are the two primary species within the *L. decastes* complex, and they differ considerably in their nutritional lifestyles and preferred growth environments. However, differences in their mitogenomes have not yet been investigated. In this study, the mitogenomes of the two species were sequenced by next-generation sequencing technology, successfully assembled, and compared. The two mitogenomes of *L. decastes* and *L. shimeji* comprised circular DNA molecules of sizes 50,643 bp and 73,678 bp, respectively. The lengths and base compositions of their core protein coding genes (PCGs) and tRNA genes varied considerably between the two mitogenomes. Further, gene collinearity analysis indicated a large-scale gene rearrangement between the two mitogenomes. Of the 15 core PCGs, the genetic distance of the *atp9* gene was the smallest between the two species, indicating that this gene was highly conserved in the two *Lyophyllum* species. Phylogenetic analysis based on a combined mitochondrial gene dataset resulted in a well-supported topology, wherein the two *Lyophyllum* species were most closely related to *Tricholoma matsutake*. This study represents the first report of mitogenomes for the *Lyophyllum* genus. As such, the results will provide a basis for understanding the differentiation and evolution of mitogenomes in the *Lyophyllum* genus.

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1. Introduction

The *Lyophyllum* genus of fungi belongs to the *Agaromycetes* class of the *Basidiomycetes* phylum and is widely distributed among northern temperate ecosystems [1]. >40 species have been described within the genus, of which the *Lyophyllum decastes* complex is the most intensively studied group [2]. Several species have been assigned to the complex, including *L. shimeji*, *L. decastes*, *L. fumosum*, and *L. loricatum* [3]. Some species within the complex have been recognized as delicacies for several centuries, especially in Japan [2,3]. In particular, *L. shimeji* is considered to have the best taste among all edible fungi, and its price is second only to *Tricholoma matsutake* in Japan [3]. Species within the *L. decastes* complex also exhibit many biological activities [4–7], including radioprotective, antitumor, antifungal, antidiabetic, and antioxidant effects. These effects are believed to arise from the presence of bioactive components of their fruiting bodies [8,9], including those of ribonucleases, beta-D-glucan, and others. However, changes in forest management, habitat loss, and pine wilt disease have caused declines of wild *L. shimeji* in Japan recently [3]. Consequently, artificial cultivation of *Lyophyllum* species has frequently been attempted to relieve the demand for the commercial production of *L. shimeji* [2,3,10].

Species within the *L. decastes* complex are morphologically very similar at the macroscopic and microscopic levels [3]. Further, they exhibit high intraspecific plasticity in the size and form of fruiting bodies, gill attachment, and cap color [3]. All of the above sources of variation have led to problems in the precise classification of the *L. decastes* complex. The use of molecular marker genes including large subunit (*LSU*) rRNA [11], internal transcribed spacer (*ITS*) [2], intergenic spacer (*IGS*) [2], and elongation factor 1-α (*EF1*-α) [2], have increased our understanding of phylogenetic relationships for the *L. decastes* complex. In addition, isozyme analysis and culture studies have aided the classification of species within the *L. decastes* complex [1]. *L. shimeji* and *L. decastes* are the primary species of the *L. decastes* complex, and have been assigned to the *Lyophyllum* section *Difformia* [2]. Phylogenetic investigations of the *Difformia* have indicated that species exhibiting different ecotypes also differ genetically [1]. *L. decastes* are commonly found in arable soils, soils of deciduous and coniferous forests, and within those along roadsides [2]. In contrast, *L. shimeji* typically grows in soils of mixed *Pinus densiflora–Quercus serrata* forests or in those of pine forests [2]. The two species also exhibit differing nutritional lifestyles [1,3]. *L. shimeji* is a facultative mycorrhizal fungi, whereas *L. decastes* is saprotrophic, and can form ectomycorrhizae *in vitro* with *Pinus pinaster* [3].

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https://doi.org/10.1016/j.ijbiomac.2018.10.037
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Mitochondrial genomes have been widely used in phylogenetic and evolutionary studies, due to its many advantages, including maternal inheritance, rapid evolution, and numerous available molecular markers [12–14]. In addition, the arrangement of mitochondrial genes and the structure of tRNAs can also provide useful information for determining phylogenetic relationships among species [15,16]. The rapid development of next generation sequencing (NGS) technologies has resulted in increasing numbers of mitochondrial genomes, thereby promoting the study of phylogenetics and evolutionary biology of eukaryotes [17–19]. However, the mitochondrial genomes of fungi, and especially of *Basidiomycetes*, have been less studied than its animal counterparts. <100 mitogenomes for *Basidiomycetes* have been published, which is an exceedingly small fraction of the *Basidiomycetes* found in nature (https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/). Moreover, no mitochondrial genomes have been reported for fungi in the *Lyophyllaceae* family, which limits our understanding of the "second genome" of this important fungal group. Identifying the differences between mitochondrial genomes of the two primary *L. decastes* species would lay the foundation for understanding the genetic evolution, species differentiation, and ecological adaptation of *Lyophyllum* species.

In the present study, the complete mitochondrial genomes of *L. shimeji* and *L. decastes* were assembled and annotated. The content, structure, and organization of the mitochondrial genes were analyzed, and the two genomes were compared to identify variation and similarities in genome organization, gene content, and gene order. In addition, the phylogenetic relationships among various *Agaricomycetes* were analyzed based on combined mitochondrial gene sets. The mitochondrial genomes of the two *Lyophyllum* species further our understanding of the population genetics, taxonomy, and evolutionary biology of this important genus, and other related genera.

2. Materials and methods

2.1. Sampling, DNA extraction and NGS sequencing

L. shimeji and *L. decastes* strains were obtained from the China Forestry Culture Collection Center, under the accession numbers of cfcc 89096 and cfcc 85729, respectively. The obtained mycelia were first cultured in potato dextrose medium at 20 °C for five days. The mycelia were then collected for genomic DNA extraction using a fungal DNA kit (Cat. #D3390-00, Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions. NEBNext Ultra II DNA Library Prep Kits (NEB, Beijing, China) were used to construct sequencing libraries with the extracted genomic DNA, following the manufacturer's instructions. Whole genomic sequencing was conducted on the Illumina HiSeq 2500 Platform (Illumina, San Diego, CA, USA).

2.2. De novo genome assembly, and annotation of the mitogenomes

Illumina PCR adapter reads were removed from the raw reads using the AdapterRemoval v 2 [20]. Low quality reads from the paired-end were filtered using our own compling pipeline. *De novo* assemblies of the mitogenomes were performed on the clean reads using SPAdes 3.9.0 [21]. Gaps between contigs were filled using MITObim V1.9 [22]. The two *Lyophyllum* mitogenomes were annotated according to our previously described methods [14,15]. Briefly, complete mitogenomes for the two *Lyophyllum* species were first annotated by combining the results of MFannot [23] and MITOS [24], both using the genetic code 4. Protein coding genes (PCGs), rRNA genes, and tRNA genes were initially annotated at this step. Initial PCG annotations were then modified and predicted with the NCBI Open Reading Frame Finder [25], and further annotated by BLASTP searches against the NCBI non-redundant protein sequence database [26]. tRNA genes were also predicted with tRNAscan-SE v1.3.1 [27]. Graphical maps of the complete mitogenomes were drawn with OGDraw v1.2 [28].

2.3. Sequence analysis

The base compositions of the two mitochondrial genomes were analyzed using DNASTAR Lasergene v7.1 (http://www.dnastar.com/). Strand asymmetries of the two mitogenomes were assessed using the following formulas: AT skew = [A − T] / [A + T], and GC skew = [G − TC] / [G + C] [29]. The synonymous (Ks) and nonsynonymous (Ka) substitution rates for core PCGs in the two mitogenomes were calculated using DnaSP v6.10.01 [30]. MEGA v6.06 [31] was used to calculate the overall mean genetic distances between each pair of the 14 core PCGs (*atp6*, *atp8*, *atp9*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, and *cob*), and *rps3*, using the Kimura-2-parameter (K2P) substitution model. Codon usages within the two mitogenomes were analyzed using the Sequence Manipulation Suite [32], based on the genetic code 4. Lastly, genomic synteny of the two mitogenomes was analyzed with Mauve v2.4.0 [33].

2.4. Repetitive element analysis

To identify gene segments that may have been transferred between the mitochondrial and nuclear genomes of the two *Lyophyllum* species, local BLAST searches were performed of the two mitogenomes against the previously published *Lyophyllum* nuclear genomes (*L. decastes*: BCJR00000000.1; *L. shimeji* BCJS00000000.1). To identify intragenomic duplications of large fragments or interspersed repeats throughout the two mitogenomes, BLASTN searches of each mitogenome against itself were conducted [34] using an E-value of $<10^{-10}$. Tandem repeats within the two mitogenomes were detected using the Tandem Repeats Finder [35], with default parameters.

2.5. Phylogenetic analysis

In order to determine the phylogenetic relationships of the two *Lyophyllum* species among the *Agaricomycetes* class, a phylogenetic tree comprising 25 species was constructed with the combined mitochondrial gene set (14 core PCGs + *rps3* + two RNA genes). Individual genes were first aligned using MAFFT v7.037 [36]. Individual alignments were then concatenated in SequenceMatrix v1.7.8 [37]. Modelgenerator v851 [38] was used to determine the best-fit evolutionary model for the combined alignment. Phylogenetic trees were then constructed using Bayesian inference (BI) methods with the combined gene dataset in MrBayes v3.2.6 [39]. Two independent runs with four chains (three heated and one cold) were each conducted simultaneously for $2 * 10^6$ generations. Each run was sampled every 100 generations. Stationarity was assumed to have been reached when the estimated sample size (ESS) was >100, and the potential scale reduction factor (PSRF) approached 1.0. The first 25% of samples were discarded as burn-in, and the remaining trees were used to calculate Bayesian posterior probabilities (BPP) for a 50% majority-rule consensus tree [14]. We also used maximum likelihood (ML) method to construct phylogenies based on the combined alignment. Our ML analysis was performed using RAxML v8.0.0 [40]; bootstrap (BS) values were calculated using 1000 replicates to assess node support.

2.6. Data availability

The two newly sequenced *Lyophyllum* mitogenomes were deposited in the GenBank database under the following accession numbers: *L. decastes*: MH447974; *L. shimeji*: MH447975.

3. Results

3.1. Protein coding genes, rRNA genes, tRNA genes, and codon analyses of the two mitogenomes

The mitochondrial genomes of the two *Lyophyllum* species, *L. decastes* and *L. shimeji*, were composed of circular DNA molecules

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