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Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America

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Abstract

Leptographium pyrinum, *Leptographium terebrantis*, *Ophiostoma aureum*, *Ophiostoma clavigerum*, and *Ophiostoma robustum* are very similar in morphology, host trees choice, and the way they are disseminated by bark beetles. Their phylogenetic relationships were clarified using rDNA and protein coding genes including actin, β-tubulin, and translation elongation factor-1α. Protein coding gene trees showed better resolution than the rDNA tree, which generated three clades: *O. clavigerum*, *L. terebrantis*/*L. pyrinum*, and *O. robustum*/*O. aureum*. A combined gene phylogenetic tree, which was supported by high bootstrap values, showed that *O. aureum*, *L. pyrinum*, *O. robustum*, and *O. clavigerum* each formed distinct clades while *L. terebrantis* was paraphyletic to *O. clavigerum*. The higher variability of the protein coding genes and the congruity in their phylogenetic results suggested that these genes may be better markers for identifying closely related species. These gene trees have also facilitated the description of the evolutionary relationships among these species.

Keywords: Bark beetle; Ophiostoma clavigerum; β-Tubulin; rDNA; Actin; Translation elongation factor 1α; Multigene phylogenies

1. Introduction

Many species of the genus *Ophiostoma* H. & P. Sydow are economically important bark beetle-associated fungi that cause blue stain and/or mortality in conifers [1–3]. These fungi belong to the class Pyrenomycetes in the phylum Ascomycota. Most Ascomycetes, including the genus *Ophiostoma*, are characterized by a life cycle that includes both teleomorph (sexual) and anamorph (asexual) reproductive states. In the sexual state the sexual spores (ascospores) are produced in a sac (ascus) contained inside a fruiting body, the perithecium. During the asexual state the vegetative haploid mycelium produces one or two types of conidiophores bearing asexual spores or conidia that germinate into haploid mycelia. Until recently, *Ophiostoma* species have been identified primarily by perithecium and ascospore shapes, and by anamorph morphology. For example, *O. clavigerum*, *O. robustum*, and *O. aureum*, which were isolated from *Pinus* species infested by bark beetles, were originally classified by their teleomorphs and anamorphs [4]. However, because teleomorphs have rarely been reported for these three species since they were originally described, their identification typically has been based on their anamorphs, mainly *Leptographium* and *Pesotum*. *Leptographium* conidiophores are formed from a single hypha that divides into branches that form a brush-like head with slimy conidia. In contrast, *Pesotum* conidiophores are formed by a bundle of hyphae that support a mass of sticky conidia at their apices.

The genus *Leptographium* is a heterogeneous group of species including several anamorphs of *Ophiostoma* spp., as well as many other species that have never

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shown a sexual state. It is not known whether those species have lost their sexual state during evolution, or whether the sexual state has simply not yet been observed. The genus includes species whose anamorphs show similarities to anamorphs in *Ophiostoma* and other genera. *Leptographium* spp. are often highly pleomorphic with unstable morphological characters, and so they are frequently confused with other closely related species [5,6]. Tsuneda and Hiratsuka [5] reported five distinct anamorphs for *O. clavigerum* which have wide ranges of conidiophore and conidium morphologies that overlap with those of other species such as *L. pyrinum*, *L. terebrantis*, *O. robustum*, and *O. aureum* [7,8].

Molecular identification using isozymes and DNA sequences is now widely used when identification based on fungal morphology is inconclusive. For the ophiostomatoid group, DNA identification often relies on a single genetic locus. Using one gene can be effective, but it can be insufficient when used for the phylogeny of closely related species. The species listed above appeared to be highly similar to one another based on isozyme markers [9]. Given mtDNA RFLPs and nDNA fingerprints, it has been suggested that *L. terebrantis* is very closely related to *O. clavigerum* and *L. pyrinum*, and that the non-mycangial fungus, *L. terebrantis*, might be a common ancestor of mycangial fungi, *L. pyrinum* and *O. clavigerum* [8]. However, results of phylogenetic studies of these species have not been conclusive, and more work was necessary to determine which individual loci are most effective and how many loci should be used for such analysis.

In the work reported here, we compared single gene and combined gene sequences as a basis for establishing phylogenetic relationships among *L. pyrinum*, *L. terebrantis*, *O. aureum*, *O. clavigerum*, and *O. robustum*. We used four loci: rDNA region, actin, β-tubulin, and translation elongation factor-1α (EF-1α) genes. We describe the different trees obtained from each gene, and show that protein coding genes provide more robust markers than rDNA. We also discuss the evolutionary relationship of these closely related species.

2. Materials and methods

2.1. Isolates

The information about eighteen isolates representing the seven named species included in this work is listed in Table 1. Isolates are maintained in Breuil's culture collection at the Faculty of Forestry of the University of British Columbia (Canada). Cores from actively growing cultures in 1.5% malt extract agar have been stored in 20% glycerol at −80 °C.

2.2. DNA extraction, PCR and sequencing

All isolates grown for DNA extraction were cultured on 1.5% malt extract agar at room temperature. DNA from mycelia was extracted following the method described by Kim et al. [10]. The extracted DNA was stored at −20 °C until further use. The rDNA (ITS 2 region and D1/D2 region of the large subunit rDNA) region and three protein coding genes (actin, β-tubulin, and EF-1α) were used as molecular markers. To amplify the rDNA region, primers ITS3 [11] and LR3 [12] were used. We designed the primers, amplified and sequenced a partial region of the actin gene, based on the sequence of *N. crassa*, GenBank Accession No. X345566. The forward primer is Lepact F (5′-TAC GTC GGT GAC GAG GC) and the reverse primer is Lepact R (5′-CAA TGA TCT TGA CCT TCA T). The β-tubulin gene was amplified using the primer set T10 [13] and BT12 [14], and EF-1α using the primer set of EF3E (5′-GTC GTY ATC GGC CAC GTC GA), which was designed in the present work, and TEF1-rev [15]. The PCR amplification was performed as described by Lee et al. [7]. All loci were successfully amplified for all the isolates. PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen, Inc.) and sequenced with an ABI 3700 automated sequencer (Perkin–Elmer, Inc. USA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). Genbank accession numbers of sequences obtained are shown in Table 1.

2.3. Phylogenetic analysis

Sequences analysis and alignment were performed manually using the program PHYDIT version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/). The localization of introns and exons in the gene fragments were approximated by performing blast searches in GenBank, and comparing the sequences to homologous characterized genes from other species. Phylogenetic analyses of the individual loci and all four loci combined were performed with PAUP*4.0b10 [16]. A neighbor-joining (NJ) tree was constructed using the Kimura 2-parameter model. The stability of clades was evaluated by bootstrap tests with 1000 replications. Maximum likelihood (ML) analysis under the HKY'85 model was performed with asis addition sequence, transition/transversion ratio = 2, assumed nucleotide frequencies set to empirical frequencies, number of substitution type = 2, rate heterogeneity following the discrete gamma approximation, with four categories and $\alpha = 0.5$. In addition, one hundred bootstrap replicates were run with ML. Maximum parsimony (MP) trees were identified by heuristic searches using the tree bisection reconnection branch-swapping algorithm. All characters were of equal weight and unordered. Statistical support for phylogenetic grouping for individual genes as well as for the com-

Table 1
Cultures used in this work and GenBank accession numbers for sequences

Species	Isolate[a]	Host[b]	Origin	Isolation source	Collector	GenBank Accession No.[c]			
						Actin	ITS2	LSU & β-tubulin	EF-1α
L. lundbergii	UAMH9584	PS	Uppland, Sweden	Board	A. Mathiesen-Käärik	AY544585	AY544603	(AY263184)	AY544626
L. pyrinum	DLS879	PA	Pinaleno Mtns., AZ, USA	*Dendroctonus adjunctus*	D.L. Six	AY544586	AY544604	(AY263185)	AY544627
	CMW3889	PJ	CA, USA	Unknown	D.L. Six	AY544587	AY544605	AY544621	AY544628
L. terebrantis	UAMH9722	PC	Sooke, BC, Canada	Unknown	J. Reid	AY544588	AY544606	(AY263192)	AY544629
	C418	PP	Blodgett, CA, USA	Associated with *D. brevicomis*	T.C. Harrington	AY544589	AY544607	(AY263191)	AY544630
	AU98Pr2-155	PC	Princeton, BC, Canada	Sapwood	A. Uzunovic	AY544590	AY544608	AY544622	AY544631
	AU156-12-13	PC	Prince George, BC, Canada	Sapwood	A. Uzunovic	AY544591	AY544609	AY544623	AY544632
O. aureum	ATCC16936	PC	Invermere, BC, Canada	Ascocarps in bark beetle-infested tree	R.C.R.-Jeffrey/R.W. Davidson	AY544592	AY544610	(AY263187)	AY544633
	AU98Pr2-128	PC	Princeton, BC, Canada	Sapwood	A. Uzunovic	AY544593	AY544611	(AY263186)	AY544634
	AU98Pr2-169	PC	Princeton, BC, Canada	Sapwood	A. Uzunovic	AY544594	AY544612	(AY263188)	AY544635
O. clavigerum	ATCC18086	PP	Cache Creek, BC, Canada	Tree attacked by *Dendroctonus* sp.	R.C.R.-Jeffrey/R.W. Davidson	AY544595	AY544613	(AY263194)	AY544636
	C843	UN	Nevada Mtns., CA, USA	*D. jeffreyi*	D.L. Six	AY544596	AY544614	(AY263196)	AY544637
	SL-Kw1407	PC	Kamloops, BC, Canada	Sapwood associated with *D. ponderosae*	S. Lee	AY544597	AY544615	(AY263195)	AY544638
	AU98Pr3-18	PC	Princeton, BC, Canada	Sapwood	A. Uzunovic	AY544598	AY544616	AY544624	AY544639
O. huntii	UAMH4997	PC	Invermere, BC, Canada	Bark beetle galleries	R.C.R.-Jeffrey	AY544599	AY544617	AY349023	AY544640
	UAMH4825	PC	Westcastle, Alta., Canada	Sapwood between beetle galleries	A. Tsuneda	AY544600	AY544618	AY544625	AY544641
O. robustum	CMW668	PP	McCall, ID, USA	Ambrosia and *Dendroctonus* spp.	R.C.R.-Jeffrey/R.W. Davidson	AY544601	AY544619	(AY263190)	AY544642
	CMW2805	PP	ID, USA	Unknown	T. Hinds	AY544602	AY544620	(AY263189)	AY544643

[a] UAMH, the University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Canada; DLS, the culture collection of D.L. Six, University of Montana, USA; CMW, Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; C, Culture Collection of T.C. Harrington, Iowa State University, USA; ATCC, American Type Culture Collection, Manassas, VA, USA; AU- and SL-isolates, Breuil's culture collection, University of British Columbia, Canada.

[b] PS, *Pinus sylvestris*; PP, *Pinus ponderosa*; PA, *Pinus arizonica*; PJ, *Pinus jeffreyi*; PC, *Pinus contorta*; UN, Unknown.

[c] Accession numbers of sequences obtained from GenBank presented in parentheses.

bined dataset, was assessed by bootstrap analysis using 1000 replicate datasets with the random addition of sequences during each heuristic search. Concordance of the four different gene datasets was evaluated with the partition homogeneity test implemented with PAUP*4.0b10, using 1000 replicates and the heuristic general search option [17]. Based on the previous studies [7,9,18] *Ophiostoma huntii* and *L. lundbergii* were selected as outgroups.

3. Results and discussion

3.1. The rDNA region phylogeny

The molecular systematics of the ophiostomatoid fungi have largely relied on nuclear rDNA (ITS2 and partial LSU region) [18,19]. Because the ITS regions have wide interspecific and less intraspecific variability, they can be used to identify some fungi at the species level [20,21]. The ITS2 region has sub-regions with fairly high conservation, and so seems appropriate for comparing isolates at family, order and higher levels [22,23]. The LSU rDNA region is also sufficiently conserved for determining relationships between families or genera, and sometimes between species [24,25].

Jacobs et al. [18] have suggested that the rDNA region (ITS2 and partial LSU) is useful for identifying and distinguishing the morphologically similar *Leptographium* species. However, our present data suggest that rDNA may be unreliable for the identification and phylogeny of in-group taxa. Out of 713 bp of aligned sequences within the rDNA region, comprising ITS2 and partial LSU regions, a total of 16 were variable and 4 (0.56%) were informative (Table 2). However, within in-group taxa, only two informative sites were found in the ITS2 region and only one was found in the partial LSU region. There was no conflict among the trees resulting from NJ, ML ($-\ln = 1096.0411$) and MP analyses. The phylogenetic trees based on three phylogenetic analyses recognized three clades: *O. clavigerum*, *L. terebrantis*/*L. pyrinum*, and *O. robustum*/*O. aureum*. The MP tree is presented in Fig. 1(a). The rDNA region exhibited very few informative characters (Table 2), and so failed to separate *L. pyrinum* from *L. terebrantis* and *O. aureum* from *O. robustum*.

3.2. The protein coding region phylogeny

The actin gene encodes actin, a cytoskeletal filament, and is present as a single copy in the majority of fungi [26]. The EF-1α gene is usually present in a single copy and encodes the translation elongation factor that controls the rate and fidelity of protein synthesis [27]. These genes have been used to determine evolutionary relationships in eukaryotes, including fungi [28–31]. Although the β-tubulin gene has several copies [32], its introns and exons have been an excellent source of phylogenetic information for *Leptographium* and other ascomycetous fungi [7,13,32–34].

In this work only partial genes were used. The sequences of the actin and EF-1α genes included two exons and one intron, while the β-tubulin sequence contained five exons and four introns (Table 2). Variations were highest in the introns of the β-tubulin and the EF-1α genes and in the exons of the actin gene. Most of the polymorphic positions of the coding regions were found in the synonymous third position. Two synonymous first position C–T transitions were found in the actin gene (Fig. 2). These suggest that the synonymous substitution rate of the actin gene is higher than in the other genes. For the in-group taxa the β-tubulin and EF-1α introns had separate 1- to 4-bp indels; in contrast, the actin intron lacked in-

Table 2
Sequence and tree information of the four loci and the combined dataset

	Locus				
	Actin	β-tubulin	EF-1α	rDNA	All
Sequence information					
No. of aligned characters	841 (841)[a]	800 (786)	670 (591)	713 (712)	3024 (2930)
No. of constant characters	761 (818)	705 (765)	592 (578)	697 (706)	2755 (2867)
No. of variable characters	80 (24)	95 (21)	78 (13)	16 (36)	269 (63)
No. of informative characters	53 (21)	60 (18)	46 (13)	4 (3)	163 (55)
% of informative characters	6.30 (2.49)	7.50 (2.29)	6.87 (2.20)	0.56 (0.42)	5.39 (1.87)
No. of exon nucleotide	756	555	306		
No. of exon/intron	5/4	2/1	2/1		
Tree information					
No. of most parsimonious trees	4	1	6	3	1
Tree length	94	116	97	16	329
Consistence index (CI)	0.9043	0.9310	0.9278	1.000	0.9088
Retention index (RI)	0.9135	0.9344	0.9136	1.000	0.9065

[a] Sequence statistics from in-group comparison are shown in parentheses.

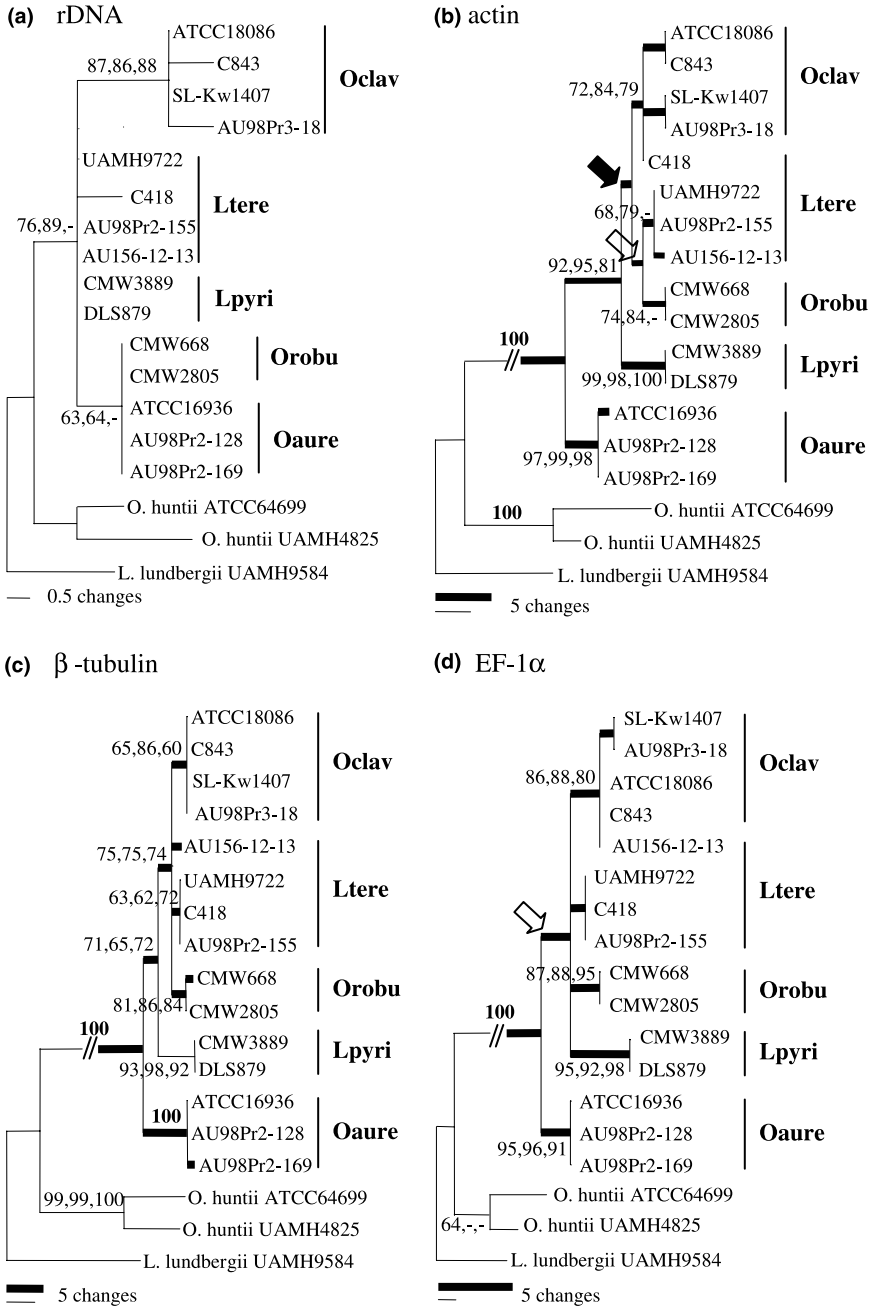

Fig. 1. One of the most parsimonious trees for each of the four nuclear gene datasets, using *L. lundbergii* and *O. huntii* as outgroup. (a) rDNA, (b) actin, (c) β-tubulin, and (d) EF-1α. Numbers above or below the branches are bootstrap values for MP, NJ and ML analyses when numbers are greater than 60%. Bootstrap values below 60% are marked with a hyphen (-) or not shown. Bold numbers indicate bootstrap values that were the same for MP, NJ and ML. Conflict branches between MP and NJ are shown by empty arrow and between MP and ML by bold arrow. Bold lines indicate an adjustment of scale.

dels. Intraspecific polymorphisms were found only in *O. clavigerum* and *L. terebrantis*.

Maximum parsimony searches yielded from one to six MPTs for each locus (Table 2). NJ, ML and MP trees for protein coding genes were similar. Although there were a few conflicts on short branches for actin (Fig. 1(b)) and EF-1α (Fig. 1(d)), tree topology differed only in the degree to which certain clades were resolved (Figs. 1(b)–(d)). Clade resolution and stability identified the supported clades in the protein coding gene trees more strongly than in the rDNA tree. The in-group taxa resolved into five clades, although some strains of *L. terebrantis* showed ambiguous phylogenetic positions. AU 156-12-13 closely clustered with *O. clavigerum* in the β-tubulin and EF-1α gene analyses (Figs. 1(c) and (d)), and C418 grouped with *O. clavigerum* in the actin gene

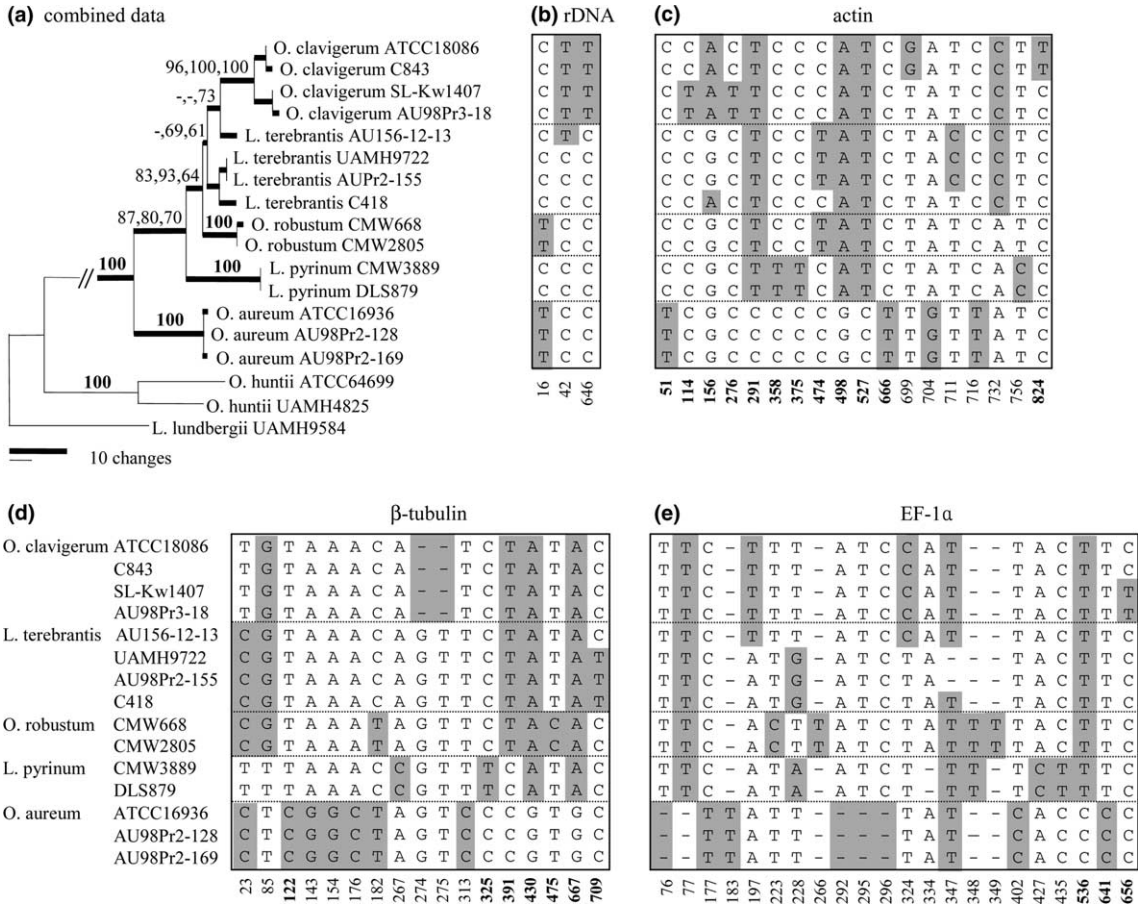

Fig. 2. Single most parsimonious tree based on the combined datasets of four loci and distribution of sequences polymorphisms in each sequence (a). Bootstrap values of MP, NJ and ML analyses above 60% are shown. Bold lines indicate an adjustment of scale. The position of the polymorphic sites in the aligned sequence matrices is written vertically below columns (b–e). Bold numbers indicate exon region position and the shaded characters indicate the derived characters.

analysis (Fig. 1(b)). The β-tubulin gene phylogeny provided more robust phylogenetic information for separating *L. pyrinum* from other in-group taxa and showed high bootstrap values (Fig. 1(c)). However, its separation was not supported in the two other gene phylogenies.

The three protein coding genes had more parsimony-informative characters than the rDNA (Table 2). The β-tubulin gene produced the highest parsimony informative characters (7.50%), but the actin gene showed the highest (2.49%) in the in-group comparison (Table 2). The higher variability of the protein coding genes compared to the rDNA suggests that these genes have the potential to be robust markers for molecular identification and phylogeny.

3.3. Combined gene phylogeny

To resolve the alternative phylogenies, a dataset comprised of sequences that were obtained from rDNA, actin, β-tubulin, and EF-1α, was analyzed with *O. huntii* and *L. lundbergii* as outgroups. The partition-homogeneity test ($P = 0.045$) indicated that there were no significant conflicts between the datasets and that our four different gene trees also reflected the same underlying phylogeny. Therefore, these datasets were combined and analyzed using a tree-building program. The MP analysis produced one most parsimonious tree, which was the almost similar in topology to the NJ and ML trees. The phylogeny of the combined dataset was similar to the phylogenetic trees derived from individual genes (Fig. 2). Overall, bootstrap values increased when the combined dataset was used. The four in-group taxa (*O. aureum*, *L. pyrinum*, *O. robustum* and *O. clavigerum*) were separated into distinct groups with strong basal supports and *L. terebrantis* showed a paraphyletic relationship with *O. clavigerum*. *O. aureum* was placed at the primitive base of the other in-group clades with 100% bootstrap values, which strongly supports previous reports that separated this species from the rest of in-group species using anamorph states, particularly shape and color of conidia [4,35]. This was also consistent with the isozyme analysis by Zambino and Harrington [9].

When multiple phylogenetic trees were generated from a single genetic data set input, several evolutionary

histories appeared equally likely. The similar morphological, ecological and molecular characteristics of these species suggest that the separation of the five closely related species from a common ancestor may have occurred recently and may have been the most recent speciation event. *L. pyrinum* and *O. robustum* appeared to be differentiated species, while *L. terebrantis*' speciation to *O. clavigerum* may be ongoing. Six et al. [8] suggested that *L. terebrantis* might be the primitive species of *L. pyrinum* and *O. clavigerum*, and showed that several *L. terebrantis* isolates, including C418, were closer to *O. clavigerum* than other *L. terebrantis* isolates in their mtDNA RFLP profiles. Our phylogenetic data support the statement that *L. terebrantis* may be more primitive than *O. clavigerum* (Figs. 1 and 2). However, none of the phylogenies in this study indicated that *L. pyrinum* may have diverged from *L. terebrantis*. Rather, the highly resolved parsimony analysis of multiple gene phylogenies suggests that *L. pyrinum* is basal to *L. terebrantis*, *O. robustum*, and *O. clavigerum* (83% bootstrap value). *L. pyrinum*'s and *O. clavigerum*'s association with mycangia of bark beetles appeared to be independent events. This would be consistent with the absence of co-speciation among the beetles and their mycangial fungi [36].

In conclusion, the multigene analysis described here resolved the evolutionary relationships among the species tested and suggested that: (a) for identifying closely related species and their phylogenetic analysis, protein-coding genes were more effective than rDNA, (b) *O. clavigerum, O. robustum* and *L. pyrinum* are distinct species rather than morphological variants of the same species, (c) *L. pyrinum* appeared to be basal to *L. terebrantis* and *O. clavigerum*, and (d) mycangial association of *L. pyrinum* and *O. clavigerum* might have occurred by independent events. In addition, our results for intraspecific polymorphisms and paraphyletic relationship between *L. terebrantis* and *O. clavigerum*, support the suggestion by Six et al. [8] that *L. terebrantis* may be the primitive species of *O. clavigerum*, and that the two may still be diverging.

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