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Genetic variation within the cosmopolitan aquatic fungus *Lignincola laevis* (Microascales, Ascomycota)

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Abstract Recent estimates suggest that there are as many as 10,000 marine fungal species, although the current number stands at just over 500. Previous studies were predominantly surveys of marine fungal diversity on various substrata in Europe, north American and Asia, while little research exists on the possible processes leading to their current distribution. Therefore, this study was initiated to assess the genetic structure and geographical distribution of the cosmopolitan aquatic fungus *Lignincola laevis*. The internal transcribed spacers and partial 28S region of the rRNA gene clusters, and the partial MCM7 protein gene were sequenced and analysed using maximum parsimony and Bayesian likelihood methods. Three distinct lineages were present in the sampled *L. laevis* isolates with a mean pairwise distance between the groups >10 %. However, no geographical assemblages could be identified in the phylogeny. The high genetic divergence suggests that *Lignincola laevis* is a species complex that consists of a group of closely related species with subtle morphological differences. The lack of geographical structure in the sampled isolates of *L. laevis* may suggest a high dispersal capacity for *L. laevis*.

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Keywords Genetic structure · Species complex · Aquatic fungus · Halosphaeriaceae · Phylogeny

Introduction

Marine fungi form an ecological group, and their entire life cycle is completed in some type of marine environment. Research on marine fungi has focused mainly on their taxonomy, phylogenetic relationships and diversity (Jones et al. 2009; Jones 2011; Sakayaroj et al. 2011). Inventories of marine fungi over the last 150 years have resulted in the recognition of 530 species, with Ascomycota as the dominant taxonomic group (Jones et al. 2009). While many marine fungi have a restricted distribution, others appear to be widespread (Jones and Pang 2012). Schmit and Shearer (2003) summarised information on the diversity and distribution of marine mangrove fungi in the Atlantic, Pacific and Indian Oceans, and found that many fungi are co-distributed along with their host substrates. Using ordination analysis, Schmit and Shearer (2004) further concluded that microfungal communities are more similar within a single ocean basin than between different ocean basins, although many mangrove-associated fungal species have wide longitudinal distributions. However, processes leading to the observed diversity and distribution of marine fungi are largely unknown.

Phylogeography is the study of historical processes that may have resulted in the current distribution of individuals (Avise et al. 1987). Genetic data have played a major role in testing hypotheses of biogeography. This enables relationships among groups of closely related taxa to be correlated with their distribution (Martin et al. 2002; Chapela and Garbelotto 2004; Hosaka et al. 2008), including population structure within widely distributed species (Hanna et al. 2007; Linzer et al. 2008; Pringle et al. 2009). A number of

terrestrial fungi with cosmopolitan distributions have been examined for their genetic variation and phylogeographic patterns, e.g. the cellar fungus *Coniophora puteana* (Kauserud et al. 2007), the ectomycorrhizal fungus *Tricholoma scalpturatum* (Carriconde et al. 2008) and the plant pathogenic *Fusarium* spp. (Cai et al. 2011). On the other hand, genetic variation and phylogeography of marine fungi have rarely been investigated. *Corollospora maritima* is the only marine fungus that has previously been shown to have within-species genetic variations amongst geographical isolates (Roberts et al. 1996).

Jones and Pang (2012) listed seven cosmopolitan marine fungi, including *Aniptodera cheasapeakensis*, *Ceriosporopsis halima*, *Corollospora maritima*, *Lignincola laevis* (Halosphaeriaceae, Microascales), *Savoryella lignicola* (Savoryellales), *Torpedospora radiata* (*Torpedospora*/*Bertia*/*Melanospora* clade, Hypocreomycetidae *incertae sedis*) and *Zalerion maritima* (Lulworthiaceae, Lulworthiales). These widely distributed species are likely good candidates for phylogeographic studies. Among them, *Lignincola laevis* Höhnk (Halosphaeriaceae, Microascales) has the widest geographical distribution and has also been reported from diverse habitats (freshwater, brackish, marine) and substrata (wood, marsh grasses) (Jones 1993; Sarma and Hyde 2001; Schmit and Shearer 2003). *Lignincola laevis* can be identified easily by its long ascomatal necks, semi-persistent asci that swell in seawater and have a distinct thimble-like apex, and thin-walled ascospores without appendages (Fig. 1). Ascospore morphology of *L. laevis* from different collections is constant, whereas position and colour of the ascomata, even from the same piece of wood, can be extremely variable (Tan et al. 1995). Recently, Liu et al. (2011) described a new species of *Lignincola*, *L. conchicola*, from the adhesive pad of a marine invertebrate on submerged fronds of the intertidal palm *Phoenix paludosa*. Morphologically, this species is similar to *L. laevis* and may be congeneric. However, this fungus did not germinate in culture for a molecular study. The main objective of this study is to assess the genetic structure of the cosmopolitan aquatic fungus *Lignincola laevis* and its geographical distribution.

Materials and methods

Fungal cultures

The *L. laevis* isolates were collected from various geographical regions, including Asia (Hong Kong, Malaysia, Philippines, PR China, Taiwan), Australasia (Australia), North America (US), South America (Brazil) and Europe (Italy, UK). These isolates were cultured from previous field trips at these geographical locations and one isolate was selected from each sampling for use in this study. All cultures were deposited at the City University of Hong Kong Culture Collection, National Taiwan Ocean University Culture Collection and Portsmouth University Culture Collection or purchased from the American Type Culture Collection (Table 1). Cultures were grown in glucose-yeast extract-peptone (GYP) broth (4 g l^{-1} glucose, 4 g l^{-1} yeast extract, 2 g l^{-1} peptone) in filtered natural seawater, except for isolates ATCC200717, which was grown in a freshwater broth.

DNA extraction, PCR and sequencing

DNA was extracted using a DNeasy Plant DNA Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's

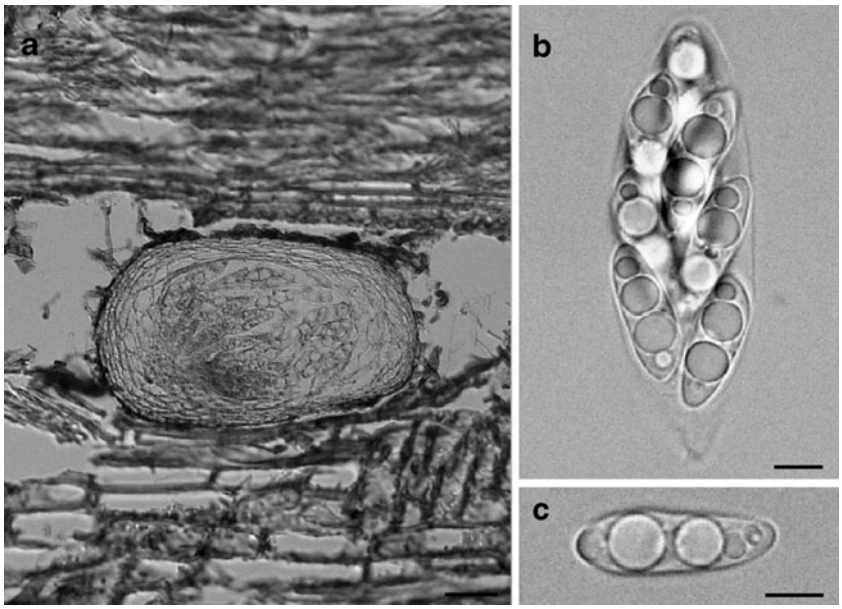

Fig. 1 **a**–**c** *Lignincola laevis*. **a** Ascoma inside mangrove wood. **b** Ascus. **c** Ascospore. *Bars* **a** 50 μm; **b**, **c** 10 μm

Table 1 Collection details of the isolates and their GenBank accession numbers

Taxa	Number[a]	Habitat	Substrata	Locality	GenBank accession no.	
					rRNA gene	MCM7
Lignincola laevis	ATCC200717	Freshwater	Submerged reed	Lake Tuitman, Louisiana, USA	AY225488	
	ATCC62562	Marine	Branch	North Carolina, USA	AY225493	
	CY0236	Brackish	*Phragmites* culm	Mai Po, Hong Kong	AF539471	
	CY1491	Marine	*Kandelia* wood	Tingkok, Hong Kong	JQ838020	
	CY3364	Marine	Driftwood	Friday Harbour, USA	JQ838021	
	CY5099	Brackish	Mangrove wood	Futian, China	JQ838022	
	CY5281	Marine	Mangrove wood	Adelaide, Australia	JQ838023	
	NTOU137	Brackish	Mangrove wood	Chiayi, Taiwan	JQ838026	
	NTOU231	Brackish	Mangrove wood	Hsinchu, Taiwan	JQ838027	
	NTOU2946	Brackish	Mangrove wood	Samal, Philippines	JQ838033	KC417325
	NTOU2978	Brackish	Mangrove wood	Taoyuan, Taiwan	JQ838034	KC417326
	NTOU30	Brackish	Mangrove wood	Taipei, Taiwan	JQ838024	KC417320
	NTOU3603	Marine	Driftwood	Langstone Harbour, UK	JQ838035	KC417327
	NTOU3759	Brackish	*Phragmites* culm	Tainan, Taiwan	JQ838036	KC417328
	NTOU629	Marine	Driftwood	Taitung, Taiwan	JQ838028	KC417321
	NTOU664	Marine	Driftwood	Keelung, Taiwan	JQ838029	KC417322
	NTOU699	Brackish	Mangrove wood	Taichung, Taiwan	JQ838030	
	NTOU722	Brackish	Mangrove wood	Hsinchu, Taiwan	JQ838031	KC417323
	NTOU829	Brackish	Mangrove wood	Chiayi, Taiwan	JQ838032	KC417324
	NTOU98	Brackish	Mangrove wood	Tainan, Taiwan	JQ838025	
	PP2933	Brackish	Mangrove wood	Kuala Selangor, Malaysia	AY225489	
	PP3236	Marine	Test block	Naples, Italy	AY225490	
	PP3649	Marine	Mangrove wood	Morib, Malaysia	JQ838037	
	PP5129	Marine	Driftwood	Taipei, Taiwan	AY225491	
	PP5745	Brackish	*Rhizophora* wood	Mai Po, Hong Kong	AY225492	
	PP7099	Marine	Driftwood	Hainan, China	AY225494	
	PP7153	Marine	Driftwood	Friday Harbour, USA	AY225495	
	PP7520	Marine	Drifting bamboo	Brazil	AY225496	
Phaeonectriella lignicola	PP7008	Freshwater	Wood	Taiwan	AY150224	

[a] ATCC-American Type Culture Collection, CY-City University Culture Collection, NTOU-National Taiwan Ocean University Culture Collection, PP-Portsmouth University Culture Collection

instructions. A region from the small subunit (SSU) to large subunit (LSU) rRNA gene (up to 2,000 bp) was amplified with the primers NS5/JS8 or ITS4/ITS5 (White et al. 1990; Landvik 1996). Additionally, a fragment of the mini chromosome maintenance (MCM7) gene was amplified with the primers Mcm7-709for/Mcm7-1348rev (Schmitt et al. 2009) for nine isolates. PCR reactions were performed in a 25-μl volume containing ca. 20 ng DNA, 0.2 μM each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$ and 2.5 U *Taq* polymerase (Invitrogen, Carlsbad, CA). For the ribosomal marker, the PCR thermal cycling profile included a first denaturation step of 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 55 °C for 1.5 min and 72 °C for 2.5 min, and a final extension step at 72 °C for 10 min. For the MCM7 gene, the PCR thermal cycling profile included a first denaturation step of 94 °C for 10 min, 38 cycles of 94 °C for 45 s, 59 °C for 50 s and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Successful amplifications (a single band on a 1 % agarose gel stained with HealthView Nucleic acid stain [Cat. No.: GN-NAS-100, Genomics BioSci & Tech, Taipei, Taiwan)] were sent to Tri-I Biotech, Taiwan (http://www.tri-ibiotech.com.tw/front/bin/home.phtml) for direct sequencing of PCR products.

Phylogenetic analysis and pairwise distance

Sequence alignment was performed using the program MUSCLE (Edgar 2004). The program Gblocks Version 0.91b was used to improve the ITS alignment by removing divergent and ambiguously aligned blocks (Talavera and

Castresana 2007). The alignment is deposited in TreeBASE under accession no. 13775. A unique insertion (277 bases) in the LSU region of *Lignincola laevis* PP7153 was excluded from analysis. Any gaps, as well as the beginning and the end of the sequences, were treated as missing data. *Phaeonectriella lignicola* was designated as the outgroup taxon in all analyses. All analyses were performed using the programs BEASTv.1.7.2 (Drummond and Rambaut 2007; Drummond et al. 2012) and PAUP* 4.0b10 (Swofford 2002).

A partition homogeneity test was first performed in PAUP* 4.0b10 (Swofford 2002) to determine if the three gene regions could be analysed simultaneously. A combined dataset was then analysed using maximum parsimony (MP). All trees were determined through branch-and-bound searching algorithm (addition of sequence: furthest). Parsimony bootstrapping and mean genetic distance (missing data adjusted based on the proportion of the different nucleotide changes at other sites between the two sequences) were also analysed In PAUP* 4.0b10 (Swofford 2002). A total of 1,000 parsimony bootstrap analyses were used to determine support of the clades using branch-and-bound search. The complete internal transcribed spacer (ITS) region (ITS1, 5.8S and ITS2) was used to calculate the mean pairwise distance between the *L. laevis* isolates.

For Bayesian analysis, sequences were first entered into BEAUti v1.7.2 (Drummond et al. 2012) for prior settings and generation of XML files for Bayesian analyses in BEASTv.1.7.2. (Drummond and Rambaut 2007). The following analytical settings were implemented: GTR, estimated base frequency, gamma + invariant sites, number of gamma categories set at 4, a strict clock, coalescent tree prior for populations of constant size as the speciation model, running 50 million generations with parameters and trees sampled every 1,000 generations. The first 10 % of the trees were treated as the burn-in. Convergence of the analysis was checked in Tracer v1.5 (Drummond and Rambaut 2007) and an effective sample size (ESS) of the parameter statistics >200 was ensured. A summary tree was produced in TreeAnnotator v1.7.2 (Drummond and Rambaut 2007) and viewed and edited in FigTree v1.3.1 (Rambaut 2009). The MCM7 gene of the nine *L. laevis* isolates was analysed individually in BEASTv.1.7.2 using the same analytical settings.

Results

Phylogenetic relationships and pairwise distance between *Lignincola laevis* isolates

A *P*-value of 0.318 from the partition homogeneity test suggested that the three genes reflect the same underlying phylogeny and could be combined. As a result, a simultaneous analysis of the combined ITS and 28S regions of the rRNA and the partial MCM7 genes was run and the phylogenetic tree resulted from the Bayesian analysis is shown in Fig. 2b with posterior probabilities and MP bootstrap values. Three major well-supported clades (groups), labelled arbitrarily with the letters A, B and C, were discernible, with additional moderately supported sub-clades within each of these major lineages. The MCM7 tree (Fig. 2a) of the nine selected *L. laevis* isolates from the Bayesian analysis produced a similar tree with three well-supported clades, which correspond to the three major clades in the combined analysis. With a total of 336 parsimony-informative characters, the MP analysis resulted 28 equally parsimonious trees with a tree length of 848 steps (consistency index = 0.783; retention index = 0.866; and a rescaled consistency index of 0.678). The differences between the 28 MPTs are restricted within the major clades A and C, and the consensus tree also recovered the three major clades.

None of the major clades in the tree (Fig. 2b) corresponds to geographical assemblages of *L. laevis* isolates. Although most isolates in these clades were collected from the South China Sea, they interspersed in the tree and grouped with temperate isolates from different oceanic regions. For instance, Clade A consisted of isolates from four distinct bodies of water, i.e. South China Sea (CY1491, NTOU98, NTOU231, NTOU629, NTOU2946, PP3649, PP5129, PP7099), Southern Ocean (CY5281), Atlantic Ocean (ATCC62562, NTOU3603, PP7520) and Pacific Ocean (CY3364, PP7153).

The three major clades (A, B and C) were also distinguished by mean pairwise genetic distance of the ITS marker (Table 2). Within-clade variation of mean pairwise distance was between 0 and 10.16 %, while between-clade variation was at least over 10 % and some sequences differed by over 20 %. The variable regions of the ITS1 and ITS2 are shown in Fig. 3 (prior to using the program Gblocks to remove the ambiguous regions). Five indel-rich regions are present in the ITS regions: 3 in ITS1 and 2 in ITS2. Identical ITS sequences were present from isolates collected at different geographical locations: (1) CY1491 (Tingkok, Hong Kong), CY5281 (Adelaide, Australia) and NTOU231 (Hsinchu, Taiwan), (2) CY3364 (Friday Harbor, WA, USA) and NTOU3603 (Langstone Harbour, Hayling Island, UK), (3) CY5099 (Futian, China) and NTOU699 (Taichung, Taiwan), and (4) NTOU137 (Chiayi, Taiwan), NTOU829 (Chiayi, Taiwan), NTOU2978 (Taoyuan, Taiwan) and PP3236 (Naples, Italy).

Discussion

This is the first study to investigate the intraspecific variation of ITS, 28S rRNA and MCM7 gene sequences

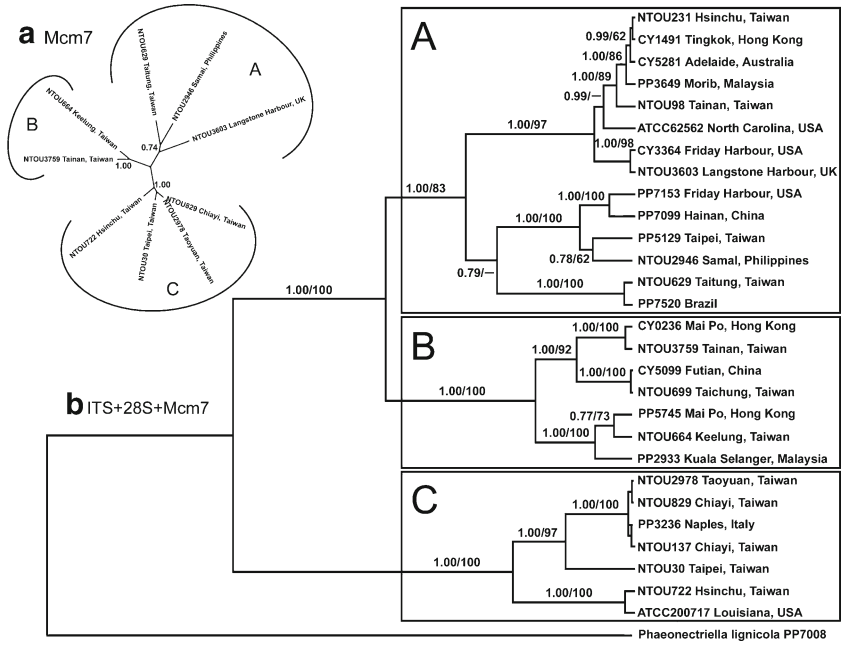

Fig. 2 The phylogenetic tree based on the Bayesian analysis of the ITS/28S ribosomal RNA and the MCM7 genes. Posterior probabilities and maximum parsimony bootstrap support values are shown on the branches. '–' Not supported or bootstrap value <50

of an aquatic fungus. *Lignincola laevis* is an ideal aquatic fungus for a phylogeographic study, since it has world-wide occurrence in terms of both longitudinal and latitudinal distributions (Fig. 4).

The well-supported phylogenetic structure (Fig. 2b) and the high genetic divergence (mean pairwise distance over 20 % between them) within *L. laevis* (Table 2) provide strong evidence for the presence of previously unrecognised lineages, even under very conservative estimates of genetic distance. It has been observed previously that intraspecific pairwise genetic distances of ITS in other fungal species do not exceed 10 % (Martin et al. 2002, Table 3). Smith et al. (2007) suggested that ITS (ITS1, 5.8S and ITS2) sequences between populations differing by ≤4 % were considered to be the same species. This 4 % difference already includes the PCR/sequencing error rates and reported 1.5 % difference of ITS sequence between isolates of the same species in community studies (Izzo et al. 2005). Schoch et al. (2012) proposed adoption of the ITS as the primary fungal barcode marker and suggested that high divergence of infraspecific ITS sequence may indicate the presence of cryptic species. The evidence provided in this study clearly indicates that *L. laevis* is a species complex, and a detailed morphological study together with multilocus sequencing will help to delimit species within this complex. Morphological differences, including the dimension of asci and ascospores, have been observed in collections of *L. laevis* (authors' personal observation). Current taxonomy of broadly distributed aquatic fungi likely underestimates species diversity. Jones (2011) suspected that many cosmopolitan marine fungi, e.g. *Ceriosporopsis halima*, *Corollospora maritima* and *Haiyanga salina*, are species complexes and may represent more than one species. The data presented here for *L. laevis* tend to support this view.

Table 2 Mean pairwise distance within and between groups corresponding to clades A, B and C in the phylogenetic tree

Clades	A	B	C
A	0–10.16 %	–	–
B	10.10–14.46 %	0–6.43 %	–
C	16.90–19.49 %	19.72–21.44 %	0–6.26 %

No geographical structure was found from the sampled isolates of *L. laevis* and transoceanic isolates were found to have identical ITS sequences. The data presented here suggest an effective and broad dispersal capacity for *L. laevis*. Increased sampling, especially from freshwater habitats, will be crucial to adequately assess distribution patterns and dispersal capacity for this group. Here we propose four possible reasons to account for the long-distance dispersal and worldwide distribution in the *L. laevis* complex. These hypotheses are speculative and additional research will be required to test their validity:

Ascospores of *L. laevis* may be dispersed by water currents. However, this species would appear morphologically to be ill-equipped for transoceanic dispersal of ascospores alone because they lack ascospore appendages for flotation. Rees and Jones (1984) demonstrated that ascospores of marine fungi lacking appendages (removed by sonication) sank more rapidly than those with appendages.

Dispersal can alternatively be achieved through agencies such as driftwood or by birds. Tropical fruits of *Cocos* and seedlings of *Rhizophora*, have been shown to

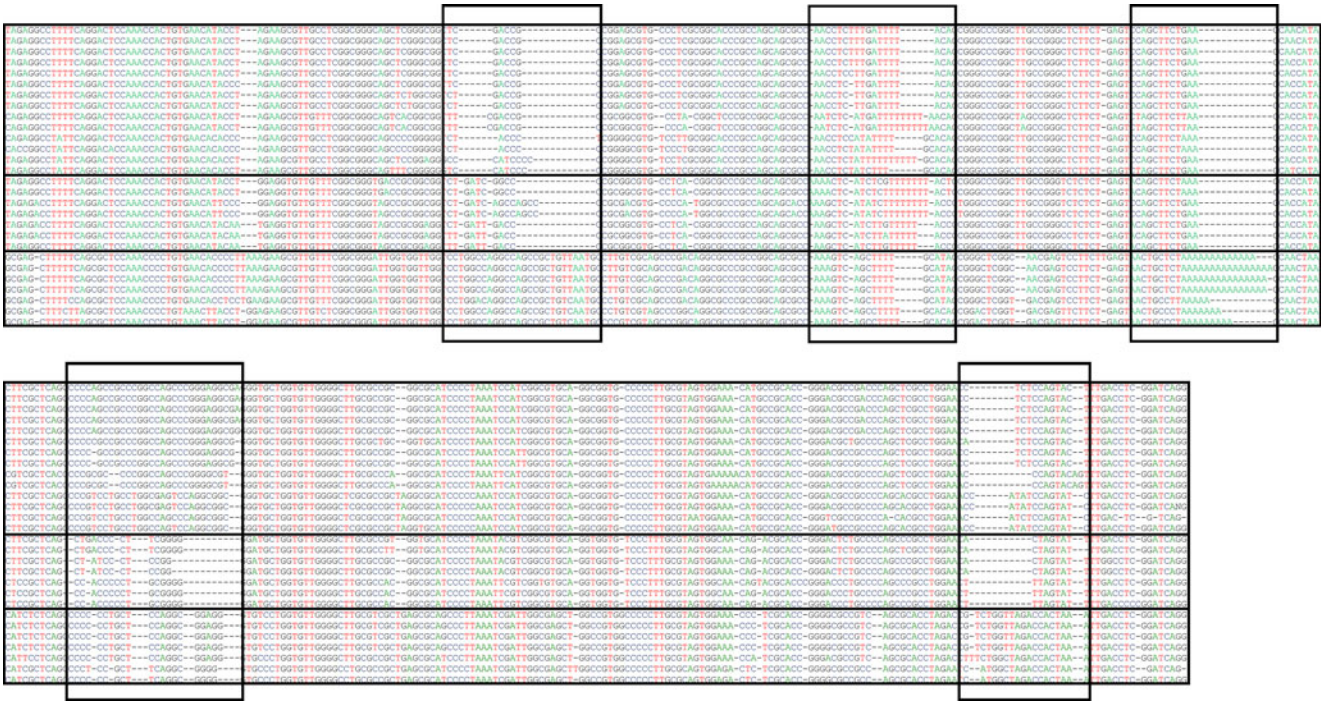

Fig. 3 Alignment of internal transcribed spacer 1 (ITS1) (*top*) and ITS2 (*bottom*) of *Lignincola laevis* isolates

be transported northward with the Gulf Stream and washed ashore on the coast south of Cape Hatteras (Kohlmeyer and Kohlmeyer 1971). *Rhizophora* seedlings were found specifically to be a good candidate because of their ability to float (Kohlmeyer 1969a), with those of *Rhizophora mucronata* remaining afloat for 87 days (Aleem 1980). This method of dispersal is more likely for *L. laevis* over relatively short distances, as it can be found on *Rhizophora* seedlings, but not for long-distance dispersal (Kohlmeyer 1969b). Transportation of marine fungi through mangrove seedlings may be more efficient as ascospores are protected against unfavourable conditions inside the ascomata (Kohlmeyer 1969a). Moreover, *L. laevis* is a primary coloniser on submerged wood panels

Fig. 4 Known geographical distribution of *Lignincola laevis*

Table 3 Interspecific and intraspecific variation of different fungi based on genetic distance

Taxa	Genetic distance	Interspecific variation	Intraspecific variation	Reference
Amanita muscaria	Mean character difference	–	0.79–4.64 %	Geml et al. (2008)
Epicoccum nigrum	Mean character difference	–	1.5 %	Fávaro et al. (2011)
Genea harknessii, Cenococcum geophilum, Cortinarius flexipes	Mean character difference	–	0.16 %–2.85 %,	Smith et al. (2007)
Hymenoscyphus pseudoalbidus, H. albidus	Mean character difference	0.02 %	–	Husson et al. (2011)
Lignincola laevis	Mean character difference	–	0 %–21.44 %	This study
Pisolithus spp.	Pairwise distance according to Jukes–Cantor model	7–36 %	0.1–9.7 %	Martin et al. (2002)
Tricholoma argyraceum, T. cingulatum, T. inocybeoides, T. scalpturatum	Pairwise distance according to Jukes–Cantor model	1.78 %–4.22 %	<0.2 %	Jargeat et al. (2010)
Tricholoma flavovirens	Mean character difference	–	0.14–6.25 %	Horton (2002)
Tricholoma scalpturatum	Mean character difference	3.9 %	0 %	Carriconde et al. (2008)

and exposed mangrove wood (Vrijmoed et al. 1986; Tan et al. 1989; Leong et al. 1991; Alias and Jones 2000). Ascospores of *L. laevis*, although without ascospore appendages, may possess sticky cell walls that can attach to substrata immediately after release from the ascus (Hyde and Jones 1988). Other than cellulolytic enzymes, *L. laevis* produces ligninolytic enzymes, which are uncommon among marine fungi, to degrade wood for nutrition (Pointing and Hyde 2000). *Lignincola laevis* has been shown to be able to colonise and sporulate early (6 weeks) on *Avicennia alba*, *Bruguiera cylindrica* and *Rhizophora apiculata* wood (Tan et al. 1995). Therefore, *L. laevis* could colonise mangrove wood when available and remain viable. The presence of sporulating structures is not essential as both marine ascomycetes and asexual forms can alternatively be present as mycelium within the wood. Birds may also be implicated in fungal dispersal or transportation (Davidson 1974; Hyde and Goh 2003).

The fungus could be distributed in ballast water, or on the timbers of ocean-going wooden ships (Williams et al. 1988; Carlton and Hodder 1995). While dispersal in ballast water would account for recent spread of marine fungi, it would not necessarily apply to geological time. Rao (2005) recorded 205 non-indigenous taxa of the Indian Seas using data prior to 1960. He attributed the fact that the spread of microbiota (fungi, bacteria, toxic algae) between geographically separated waters was likely due to anthropogenic activity, mainly through ballast water discharge. Rao (2005) also listed 14 obligate marine fungi (e.g. *Amylocarpus encephaloides*, *Arenariomyces majusculus*, *Nimbospora effusa*) which he regarded as non-indigenous. The lack of data on marine fungi before the 1960 period makes this deduction inconclusive.

Marine fungi could have been transported through historical ocean-going wooden ships, since the marine basidiomycetes, *Digitatispora marina* and *Nia vibrissa*, have been found on the hull of the English Tudor ship *Mary Rose* (Jones 2000; Jones and Jones 1993).

Marine fungi fall into distinct geographical (cold water, temperate, tropical) and ecological (oceanic waters, mangroves, salt marshes, algicolous, arenicolous) groups. However, a number of species appear to be adapted to a wider range of habitats and geographical distribution. Booth and Kenkel (1986), using ordination analysis, described a distribution model of marine fungi based on salinity and temperature regimes and concluded that temperature is the dominant factor influencing the worldwide distribution of marine fungi. Hughes (1974, 1986) also argued for a major effect of temperature in their geographical distribution and produced a series of maps based on isocrymal/isothermal lines. Physiological studies of selected fungi have indicated that tropical fungi have a higher temperature requirement when compared with their temperate counterparts (Boyd and Kohlmeyer 1982; Bebout et al. 1987; Panebianco 1994). The geographical limit of those fungi collected infrequently remains to be determined.

While most fungi have a restricted geographical distribution, others are cosmopolitan. Studies on the geographical distribution of specific marine fungi, including *Corollospora maritima* (Bebout et al. 1987; Roberts et al. 1996) and the data presented here on *Lignincola laevis*, remain very limited. Clearly, further research is required to determine the genetic variability between populations of cosmopolitan marine fungi. Such studies should be undertaken in the *Aniptodera*/*Halosarpheia* complex, where delimitation into genera remains problematic due to the lack of clear morphological characters to separate them (Campbell et al. 2003; Pang et al. 2003a, b).

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