

RESEARCH ARTICLE

Diversity and Polycaprolactone-Degrading Activity of Endophytic Fungi Isolated from Marine Macroalgae in the Dadohae Coastal Region, Korea

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ABSTRACT

Marine macroalgae harbor diverse fungal communities; however, the taxonomic composition and physiological traits of their endophytes remain insufficiently characterized. In this study, endophytic fungi were isolated from three marine macroalgal species collected from the Dadohae coastal region of Korea and evaluated for their polycaprolactone (PCL)-hydrolyzing activity using the plate-clearing assay. Forty-three isolates representing 26 taxa were obtained, and representative strains of 11 taxa formed clear zones on PCL-containing medium. *Cladosporium anthropophilum* KNUE25S362 showed the largest mean halo zone, followed by *Aureobasidium proteae* KNUE25S381 and *Cladosporium tenuissimum* KNUE25S421. The isolates were identified on the basis of internal transcribed spacer (ITS) sequences and, for strains showing PCL-hydrolyzing activity, additional loci including the large subunit ribosomal DNA and protein-coding genes. These analyses supported species-level identification for most of the active strains. *Diaporthe* strain KNUE25S422, for which only ITS and β -tubulin sequences were analyzed, showed relatively low sequence identity to the type strain and was therefore conservatively treated as a *Diaporthe* sp. affiliated with the *Diaporthe eres* complex. These results highlight the diversity of culturable endophytic fungi associated with marine macroalgae in the Dadohae region and identify fungal strains showing extracellular PCL-hydrolyzing activity.

Keywords: Biodegradable polyester, Fungal diversity, Marine macroalgae, Polycaprolactone, Seaweed endophytes

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INTRODUCTION

Plastic accumulation is a persistent environmental problem in terrestrial and marine ecosystems. Global plastic waste generation increased from 156 million tonnes (Mt) in 2000 to 353 Mt in 2019 [1,2], and substantial quantities of discarded plastics continue to enter natural habitats [3–6]. Although biodegradable polymers can reduce long-term persistence in some applications, their degradation depends strongly on the polymer properties, environmental conditions, and microorganisms and enzymes present at the disposal site [7,8].

Fungi belonging to genera such as *Aspergillus*, *Penicillium*, *Trichoderma*, and *Fusarium* have been screened for their abilities to alter or hydrolyze the ester bonds of synthetic polyesters such as polycaprolactone (PCL), a semi-crystalline aliphatic polymer [9,10]. However, most fungal screening studies have focused on terrestrial isolates, whereas the corresponding functional diversity of marine-derived fungi remains poorly characterized [11].

Marine macroalgae provide structurally and chemically distinct habitats for endophytic fungi, which produce extracellular enzymes such as esterases, lipases, and cutinase-like hydrolases to colonize the algal tissues [12–14]. As some of these enzymes act on ester-containing synthetic polymers, macroalgal endophytes are a relevant source of fungi for physiological screening.

The objectives of this study were to isolate and identify culturable endophytic fungi from the macroalgal species *Ulva australis*, *Gracilaria vermiculophylla*, and *Sargassum thunbergii* collected from the Dadohae coastal region of Korea; screen representative fungal isolates that possess PCL-hydrolyzing capability; and characterize the active strains using internal transcribed spacer (ITS) sequences and additional taxonomically informative loci. The plate-clearing assay was used in this study, and although it does not establish complete polymer biodegradation, it is useful for detecting extracellular hydrolytic activity and selecting strains for subsequent quantitative analyses.

MATERIALS AND METHODS

Sampling of macroalgae and isolation of fungal strains

Healthy asymptomatic specimens of *Ulva australis* (Chlorophyta), *Gracilaria vermiculophylla* (Rhodophyta), and *Sargassum thunbergii* (Ochrophyta) were collected from the intertidal zones of Aphaedo, Jaeundo, and Chupodo in Shinan-gun, Jeollanam-do, Republic of Korea, between June 17 and 18, 2025 (Fig. 1). The thalli were stored in sterile bags with ambient seawater and transported to the laboratory within 12 h at 4°C.

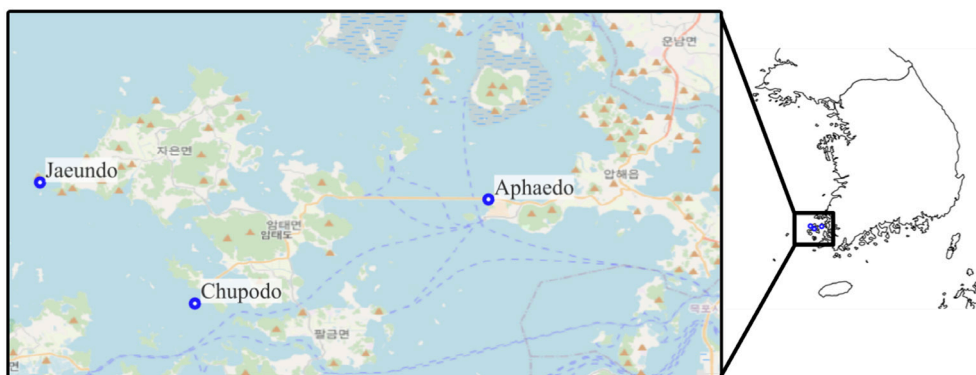


Fig. 1. Sampling sites of marine macroalgae in Shinan-gun, Korea.

The thalli were first surface sterilized to eliminate epiphytic microorganisms. The samples were washed under running tap water, cut into 1.0×0.5 cm segments, immersed in 70% ethanol for 15 s, and rinsed with sterile seawater. Then, the segments were dried on sterile tissue paper and plated onto dichloran rose bengal chloramphenicol agar (MBcell, Seoul, Korea) prepared with natural seawater and potato dextrose agar (PDA; Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with chloramphenicol. The plates were incubated at 25°C in the dark. Emerging hyphae were subcultured on fresh PDA to obtain pure axenic cultures.

Genomic DNA extraction, PCR, sequencing, and phylogenetic analysis

Genomic DNA was extracted from 14-day-old cultures grown on PDA, using the HiGene Genomic DNA Prep Kit (Biofact, Daejeon, Korea). For all isolates, the ITS region was amplified using ITS1F and ITS4 [15,16]. For isolates exhibiting PCL-hydrolyzing activity, additional loci were amplified using the following primers: ACT-512F/ACT-783R for actin (*ACT*) [17], EF1-728F/EF1-986R [17] or EF1-983F/EF1-1567R for translation elongation factor 1- α (*TEF1*) [18], TUB2Fd/TUB4Rd for beta-tubulin (*TUB2*) [19], CMD5/CMD6 for calmodulin (*CaM*) [20], and LR0R/LR7 for large subunit 28S nuclear ribosomal DNA (LSU) [21]. The sequences generated in this study were deposited in the GenBank database under the accession numbers listed in Table 1.

Table 1. GenBank accession numbers currently available for PCL-hydrolyzing fungal isolates obtained in this study

Strain	Identification	Genetic markers	GenBank accession numbers
KNUE25S357	<i>Alternaria alternata</i>	ITS, LSU	PZ467978, PZ463299
KNUE25S381	<i>Aureobasidium proteae</i>	ITS, LSU	PZ463285, PZ463300
KNUE25S347	<i>Phlebia acerina</i>	ITS, LSU	PZ463280, PZ463284
KNUE25S362	<i>Cladosporium anthropophilum</i>	ITS, <i>TEF1</i> , <i>ACT</i>	PZ467979, PZ468812, PZ468816
KNUE25S404	<i>Cladosporium halotolerans</i>	ITS, <i>TEF1</i> , <i>ACT</i>	PZ467980, PZ468813, PZ468817
KNUE25S425	<i>Cladosporium rectoides</i>	ITS, <i>TEF1</i> , <i>ACT</i>	PZ467984, PZ468815, PZ468819
KNUE25S421	<i>Cladosporium tenuissimum</i>	ITS, <i>TEF1</i> , <i>ACT</i>	PZ467981, PZ468814, PZ468818
KNUE25S422	<i>Diaporthe</i> sp. (<i>D. eres</i> complex)	ITS, <i>TUB2</i>	PZ467982, PZ468808
KNUE25S444	<i>Paraphoma vinacea</i>	ITS, <i>TUB2</i>	PZ467986, PZ468810
KNUE25S424	<i>Penicillium olsonii</i>	ITS, <i>TUB2</i>	PZ467983, PZ468809
KNUE25S434	<i>Stemphylium vesicarium</i>	ITS, <i>CaM</i>	PZ467985, PZ468811

ITS, internal transcribed spacer; LSU, large subunit 28S nuclear ribosomal DNA; *TEF1*, translation elongation factor 1- α ; *ACT*, actin; *TUB2*, β -tubulin; *CaM*, calmodulin.

The consensus sequences were compared with sequences in the GenBank database using BLASTN. Maximum-likelihood phylogenetic analyses were performed using IQ-TREE v3.0.1 [22]. For multilocus datasets, the loci were concatenated and analyzed using the partition scheme specified for each dataset. The best-fit substitution model for each partition was selected using ModelFinder Plus [23], and branch support was estimated using 1,000 ultrafast bootstrap replicates [24].

Polycaprolactone agar plate-clearing assay

The PCL-hydrolyzing activity was evaluated for one representative isolate per taxon using the emulsified agar plate-clearing assay [25]. The assay medium contained 1% (w/v) PCL diol ($M_w \approx 530$; RHAWN, Shanghai, China) dissolved in acetone and incorporated into mineral-salts agar in 90 mm Petri dishes. A 6 mm mycelial plug was inoculated at the center of each dish, and the plates were incubated at 25°C for 7 days. The radial distance from the margin of the fungal colony to the outer boundary of the clear zone was measured using a digital caliper. To account for asymmetrical halo formation, the longest and shortest radial distances from the colony margin to the outer boundary of the clear zone were measured and averaged. All assays were performed in triplicate, and the results are presented as the means $\pm$ standard deviations.

RESULTS AND DISCUSSION

Polycaprolactone-hydrolyzing activity of marine macroalgal endophytes

In total, 43 endophytic fungal isolates were obtained from the three macroalgal species and assigned to 26 taxa on the basis of their colony characteristics and ITS sequence comparisons. These isolates were predominantly ascomycetous. Among the strains showing PCL-hydrolyzing activity, *Phlebia acerina* was the only basidiomycete. The sampling sites are shown in Fig. 1.

One representative strain of each taxon was screened on PCL-containing agar. Eleven of the 26 taxa examined (~42%) formed visible halo zones (Table 2). Active strains were recovered from all three macroalgal hosts. *Ulva australis* yielded the highest number of active taxa, although differences among hosts were not statistically tested because the study was designed as a culture-based survey rather than a standardized comparison of isolation frequencies.

Table 2. Algal host, isolation site, and PCL halo-zone size (mean $\pm$ SD; $n = 3$) of 11 active marine endophytic fungi

Strain	Sampling site	Host macroalga	Identified taxon	Halo zone (Mean $\pm$ SD, mm)
KNUE25S362	Jaen-do	<i>Ulva australis</i>	<i>C. anthropophilum</i>	13.50 $\pm$ 2.65
KNUE25S381	Aphae-do	<i>Gracilaria vermiculophylla</i>	<i>A. proteae</i>	8.83 $\pm$ 2.75
KNUE25S421	Chupo-do	<i>Sargassum thunbergii</i>	<i>C. tenuissimum</i>	8.83 $\pm$ 1.44
KNUE25S357	Aphae-do	<i>Ulva australis</i>	<i>A. alternata</i>	6.33 $\pm$ 4.49
KNUE25S434	Chupo-do	<i>Sargassum thunbergii</i>	<i>S. vesicarium</i>	4.67 $\pm$ 2.08
KNUE25S425	Chupo-do	<i>Ulva australis</i>	<i>C. rectoides</i>	5.83 $\pm$ 3.34
KNUE25S424	Chupo-do	<i>Ulva australis</i>	<i>P. olsonii</i>	3.00 $\pm$ 2.65
KNUE25S404	Chupo-do	<i>Ulva australis</i>	<i>C. halotolerans</i>	3.67 $\pm$ 1.04
KNUE25S444	Jaen-do	<i>Sargassum thunbergii</i>	<i>P. vinacea</i>	1.67 $\pm$ 1.15
KNUE25S347	Aphae-do	<i>Gracilaria vermiculophylla</i>	<i>P. acerina</i>	1.50 $\pm$ 0.87
KNUE25S422	Chupo-do	<i>Sargassum thunbergii</i>	<i>Diaporthe</i> sp.	1.67 $\pm$ 0.58

The size of the halo zone differed among the fungal strains. *Cladosporium anthropophilum* KNUE25S362 produced the largest mean halo zone (13.50 ± 2.65 mm), whereas *Aureobasidium proteae* KNUE25S381 and *Cladosporium tenuissimum* KNUE25S421 produced mean halo zones of 8.83 mm. *Alternaria alternata*, *Stemphylium vesicarium*, and *Cladosporium rectoides* showed intermediate values, whereas *Paraphoma vinacea*, *Phlebia acerina*, and a *Diaporthe* sp. affiliated with the *Diaporthe eres* complex produced relatively small halo zones (Table 2). Representative culture plates are shown in Fig. 2.

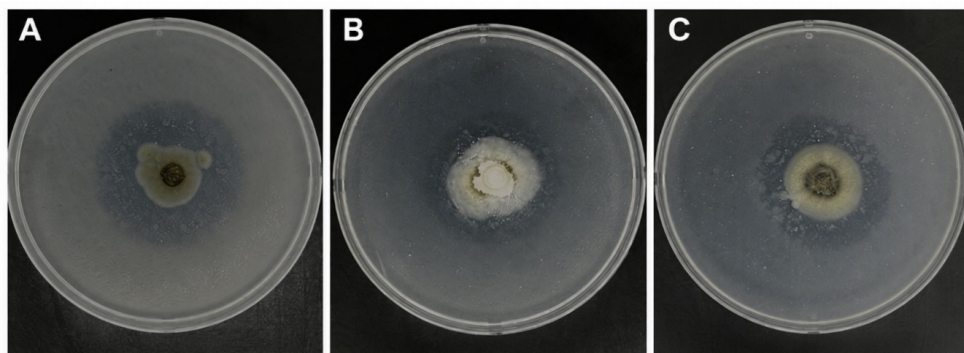


Fig. 2. Representative fungal strains exhibiting high polycaprolactone (PCL)-degrading activity on PCL-containing agar medium. A, *Cladosporium anthropophilum* KNUE25S362; B, *Aureobasidium proteae* KNUE25S381; C, *Cladosporium tenuissimum* KNUE25S421.

Four *Cladosporium* taxa produced halo zones, including the strain with the highest value. This is consistent with the broad ecological distribution and extracellular enzymatic capacity of *Cladosporium* species. A marine isolate of *Cladosporium halotolerans* was previously shown to degrade polyurethane [26], supporting the production of polyester-hydrolyzing enzymes by the genus *Cladosporium*. *Aureobasidium* species also produce extracellular esterases and lipases [27], which may explain the activity observed from *Aureobasidium proteae*. Considerable variation in PCL clear-zone formation has also been reported among fungi recovered from marine plastic debris [28]. However, because the size of the halo zone can be influenced by colony growth, enzyme secretion, diffusion through the agar, and substrate emulsification, the enzymatic activity observed in this study should be interpreted as a comparative screening response rather than a direct measure of the mass of polymer degraded [10,29].

The recovery of active strains from green, red, and brown macroalgae indicated that extracellular PCL-hydrolyzing activity was not restricted to a single host group in the present collection. Eleven of the 26 taxa examined produced clear zones, whereas a previous screening of 30 terrestrial fungal strains in Korea detected PCL- or polylactic acid-hydrolyzing activity in five strains [10]. These proportions should not be directly compared, because the fungal assemblages, substrates, and strain selection procedures differed between the two studies. Nevertheless, the findings from both studies, together with the results of a survey of marine plastic-associated fungi by Kim et al. [27], confirm that plate assays can be used to identify taxonomically diverse fungi with extracellular activity toward biodegradable polyesters. Confirmation of polymer biodegradation by the present isolates requires quantitative measurements such as polymer mass loss or molecular weight reduction, chemical analyses of the degradation products, and characterization of the responsible enzymes.

Taxonomic characterization of the active strains

The ITS sequence-based phylogenetic relationships among representative isolates are shown in Fig. 3, and the multilocus trees used for the identification of selected strains are presented in Supplementary Fig. S1. Although the ITS sequences were sufficient for the preliminary placement of the isolates, the additional analyses of protein-coding loci improved the discrimination within species complexes and among closely related taxa. Multilocus sequence analysis is generally recommended for the accurate species delimitation of genera such as *Diaporthe* and *Cladosporium*, for which the ITS region alone often lacks sufficient resolution power [28–30].

Diaporthe strain KNUE25S422 shared 96.34% ITS and 97.66% *TUB2* identities with the type strain within the *Diaporthe eres* complex. These values, together with the phylogenetic position of strain KNUE25S422, did not justify its identification at the species level. As mentioned above, species delimitation within *Diaporthe*, particularly the *Diaporthe eres* complex, generally requires the analyses of multiple protein-coding loci and morphological comparisons because the ITS sequence can show limited discriminatory power or intragenomic variation [29–31]. Therefore, because only the ITS and *TUB2* data were available for strain KNUE25S422, it is conservatively reported as a *Diaporthe* sp. affiliated with the *Diaporthe eres* complex.

Strain KNUE25S444 was identified as *Paraphoma vinacea* on the basis of the ITS and *TUB2* comparisons and its placement with reference strains in the multilocus analysis. This isolate was recovered from *Sargassum thunbergii*. Although this finding documents the occurrence of this strain among culturable fungi associated with the sampled macroalgal species, it does not establish host specificity or a new geographic record.

Within the genus *Cladosporium*, strains KNUE25S421 and KNUE25S362 grouped together with *Cladosporium tenuissimum* and *Cladosporium anthropophilum*, respectively. Although their ITS sequences closely matched those of the reference strains, the additional *TEF1* and *ACT* sequence comparisons supported their phylogenetic placement. The use of *ACT* and *TEF1* together with ITS for the species-level identification of *Cladosporium* is well established, whereas ITS alone often provides insufficient resolution among closely related species [28].

In conclusion, the three marine macroalgal species collected from the Dadohae coastal region harbored taxonomically diverse assemblages of culturable endophytic fungi. Eleven taxa produced clear zones on PCL-containing medium, with *Cladosporium anthropophilum* KNUE25S362 showing the strongest response. Multilocus analyses improved the identification of active strains and supported conservative identification of the divergent *Diaporthe* isolate. The strains identified in this study can be used as culture resources for subsequent quantitative studies of fungal enzymes and polyester biodegradation.

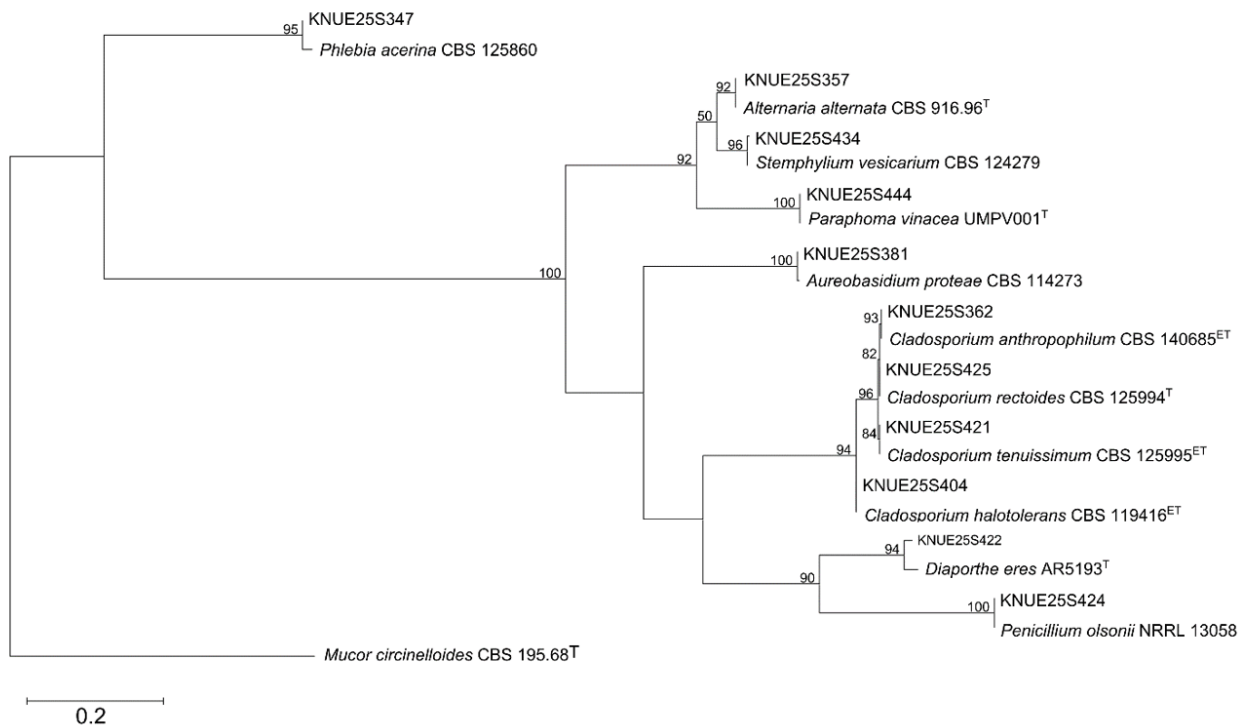


Fig. 3. ITS sequence-based maximum-likelihood phylogenetic tree of endophytic fungi isolated from marine macroalgae. Bootstrap values are shown at the nodes. Type (T) and ex-type (ET) strains are indicated. The scale bar represents nucleotide substitutions per site.

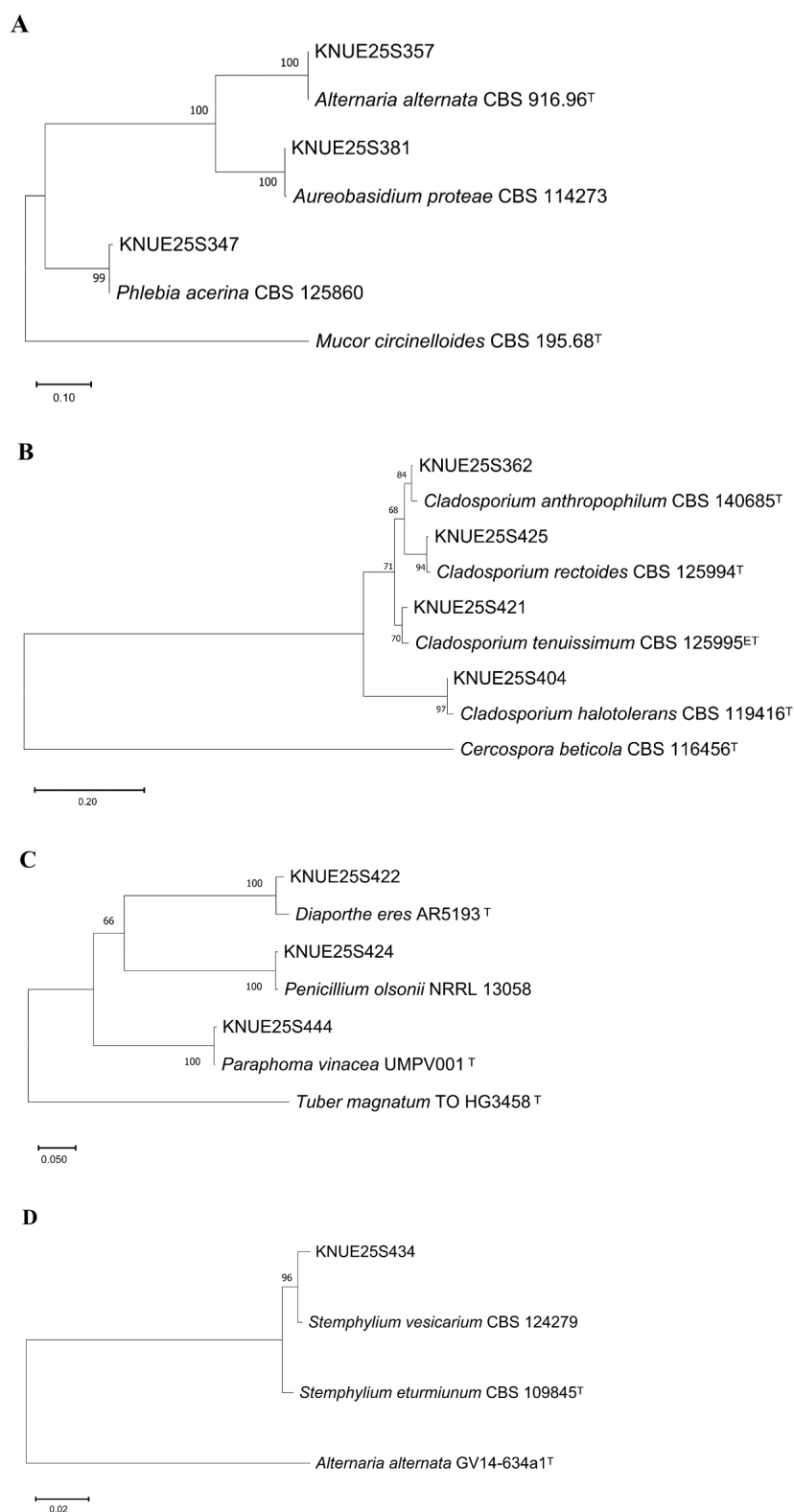


Fig. S1. Maximum-likelihood phylogenetic trees used to identify selected PCL-hydrolyzing fungal strains. Trees were inferred from (A) ITS and LSU, (B) ITS, *TEF1*, and *ACT*, (C) ITS and *TUB2*, and (D) ITS and *CaM* datasets. Bootstrap values are shown at the nodes. Type (T) and ex-type (ET) strains are indicated. Scale bars represent nucleotide substitutions per site. ITS, internal transcribed spacer; LSU, large subunit 28S nuclear ribosomal DNA; *TEF1*, translation elongation factor 1- α ; *ACT*, actin; *TUB2*, β -tubulin; *CaM*, calmodulin.

CONFLICT OF INTEREST

The authors declare that they have no potential conflict of interest.

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