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# Detection and Identification of Fungi Intimately Associated with the Brown Seaweed *Fucus serratus*▽†

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**The filamentous fungi associated with healthy and decaying *Fucus serratus* thalli were studied over a 1-year period using isolation methods and molecular techniques such as 28S rRNA gene PCR-denaturing gradient gel electrophoresis (DGGE) and phylogenetic and real-time PCR analyses. The predominant DGGE bands obtained from healthy algal thalli belonged to the *Lindra*, *Lulworthia*, *Engyodontium*, *Sigmoidea*/*Corollospora* complex, and *Emericellopsis*/*Acremonium*-like ribotypes. In the culture-based analysis the incidence of recovery was highest for *Sigmoidea marina* isolates. In general, the environmental sequences retrieved could be matched unambiguously to isolates recovered from the seaweed except for the *Emericellopsis*/*Acremonium*-like ribotype, which showed 99% homology with the sequences of four different isolates, including that of *Acremonium fuci*. To estimate the extent of colonization of *A. fuci*, we used a TaqMan real-time quantitative PCR assay for intron 3 of the beta-tubulin gene, the probe for which proved to be species specific even when it was used in amplifications with high background concentrations of other eukaryotic DNAs. The *A. fuci* sequence was detected with both healthy and decaying thalli, but the signal was stronger for the latter. Additional sequence types, representing members from the Dothideomycetes, were recovered from the decaying thallus DNA, which suggested that a change in fungal community structure had occurred. Phylogenetic analysis of these environmental sequences and the sequences of isolates and type species indicated that the environmental sequences were novel in the Dothideomycetes.**

Coastal macrophytes are part of highly productive ecosystems and have essential functions in nutrient cycling and habitat structuring (8, 30). Helgoland intertidal marine seaweed populations are dominated by fucoids with diverse associated biocenoses. The complex communities that develop in these systems provide models for investigating alga-fungus interactions in a natural environment. Pathogens and parasites are the predominant fungi in seaweed communities that have been described (47); however, most of these organisms cannot be cultured in the laboratory and are known only from herbarium specimens (26, 39). Other algicolous fungi include saprobes and mycobionts, and there is little information on the autecology of these organisms. Studies of the interactions between these fungi and their algal hosts, therefore, can be effectively undertaken only by using a molecular approach to detect and differentiate between environmental signal sequences.

In a preliminary study of fungi associated with *Fucus serratus*, Zuccaro et al. (48) developed and described a PCR-denaturing gradient gel electrophoresis (DGGE) system, using novel fungus-specific primers that amplified the second domain (D2) of the nuclear large rRNA region. Fungal sequences retrieved from algal tissue matched sequences from ascomycetous groups known to be active in marine environments, as well as sequences from a group of isolates belonging to the genus *Emericellopsis* and their mitosporic form, the genus *Acremonium* (49). These organisms are primarily recognized as fungi that are active in terrestrial environments and include known endophytes and pathogens (9, 16, 34). The current study examined the consistency of fungal associations with *F. serratus* over 1 year, and this paper describes a real-time PCR detection system based on sequences of intron 3 of the beta-tubulin gene. It also addresses questions related to the seasonal occurrence and tissue localization of these fungi. In addition, sequences derived from environmental samples, isolates, and a herbarium specimen were combined in phylogenetic analyses to provide a basis for assessing the identities of novel marine fungal lineages. In particular, the fungi belonging to the Dothideomycetes, which contains many of the algal parasites, pathogens, and mycobionts (47), were targeted.

## MATERIALS AND METHODS

**Sampling site and collection of algae.** The sampling site and sampling strategies used have been described previously in detail (48). Submerged healthy-looking and decaying *F. serratus* tissues were collected on five independent sampling occasions over the course of 1 year (April 2002, July 2002, October 2002, January 2003, and April 2003) from a rocky-shore site on the northeastern side of Helgoland Island, Germany.

**Herbarium specimen.** Specimens of *Didymella fucicola* on *Fucus vesiculosus* were kept frozen in seawater from September 1971 until September 2005 and

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then air dried (United Kingdom: Cornwall: West Looe, 17 September 1971, J. Kohlmeyer [J.K.2932] [Institute of Marine Science-IMS]).

**Fungal isolation, identification, genomic DNA extraction, and PCR amplification.** Fungi were isolated from algal parts in pure culture by mycelial transfer onto agar plates and, where possible, by single-conidium isolation. For conventional isolation from different parts (receptacles, growing tips, and blade and holdfast tissues) of healthy *F. serratus*, approximately 2,100 segments were surface sterilized with bleach and 1,000 segments were rinsed with sterile water; approximately 400 segments from decaying material were cleaned with sterile water. Segments were plated on different media as described by Zuccaro et al. (48). Emerging fungi were isolated in pure cultures and identified on the basis of morphology when possible. The genomic DNA of isolates selected on the basis of morphological characteristics for further phylogenetic analysis, including mycelia sterilia, was extracted using a FastDNA spin kit for soil (Bio 101 Systems or Q-Bio gene) by following the company's protocol. DNA was amplified using primers NL209 and NL912, purified with a Geneclean III kit (Q-Bio gene), and sequenced using the fluorescent method and a Li-COR 4200 DNA sequencer (Amodia Bioservice GmbH, Braunschweig, Germany).

**Extraction of DNA from the dried herbarium specimen, PCR amplification, cloning, and sequencing for phylogenetic analysis.** DNA was extracted from a 35-year-old herbarium specimen of the marine obligate parasite *D. fucicola*. Five ascomata were picked off the decaying midribs of the brown algal host *F. vesiculosus*, and DNA was extracted using a FastDNA spin kit from Q-Bio gene. PCR amplification of the large-subunit (LSU) rRNA gene was performed using a seminested approach with the fungus-specific primers NL209 and NL912, followed by primers NL359 and NL912GC, as previously described (48). Internal transcribed spacer (ITS) regions were amplified using primers ITS1f (14) and NL209r (5′-CTGTTGGTTTCTTTTCCTCCGCTT-3′) under the following conditions: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and a final extension for 7 min at 72°C. PCR products from the herbarium specimen were purified with a Geneclean III kit (Q-Bio gene) and then ligated into the vector pCR 2.1 (TA cloning kit; Invitrogen). DNA from seven plasmids was extracted with a QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and was sequenced using primers M13f and M13r.

**Environmental DNA extraction.** Total environmental DNA was extracted from 21 10-g samples of healthy *F. serratus* thalli which were previously sectioned into different parts (blade, receptacles, holdfast, and growing tips) and six 10-g samples of decaying algal material. The extraction procedure, including a CsCl centrifugation step, was performed using the protocol previously described by Zuccaro et al. (48). The environmental DNA was then diluted to a final concentration of 5 μg/μl.

**PCR amplification and DGGE conditions.** A total of 57 PCR amplifications, consisting of two or three replicates for each independent DNA sample, were performed using a seminested approach with primers NL209 and NL912, followed by primers NL359 and NL912GC, and the products were separated on LSU rRNA gene DGGE gels with the Bio-Rad D-Code system (Bio-Rad Laboratories, Hercules, CA). Detailed descriptions of the primer efficiency, PCR conditions, DGGE gel reagents, denaturant range, and running and gel staining conditions have been provided elsewhere (48).

**Cloning and sequencing of 28S rRNA gene PCR products from decaying seaweed.** PCR products from decaying algal material, obtained using primers NL209 and NL912, were purified with a Geneclean III kit (Q-Bio gene) and then ligated into the vector pCR 2.1 (TA cloning kit; Invitrogen). Extracted plasmids were reamplified using primers NL209 and NL912 and were sequenced using primer NL912 and the fluorescent method with a Li-COR 4200 DNA sequencer (Amodia Bioservice GmbH, Braunschweig, Germany). The reamplified inserts were then subjected to seminested amplification using primers NL359 and NL912GC, and the products were electrophoresed in a DGGE gel together with the original sample to identify the corresponding environmental bands.

**Real-time quantitative PCR. (i) Design of TaqMan primers and probe.** Primers and a probe were designed for a TaqMan real-time quantitative PCR assay targeting the intron 3 region of the beta-tubulin gene from *Acremonium fuci* (GenBank accession number AY632690) using the Primer Express v2.0 software (Applied Biosystems, Foster City, CA). The *Emericellopsis*/*Acremonium*-like forward primer TUB1F (5′-GCGTCTACTTCAACGAGGTGAG T-3′) and reverse primer TUB2R (5′-ATGCTCATCCTCGCAGGC-3′) amplified a 68-bp fragment from base 108 to base 175. The 25-bp TaqMan probe AFP1 (5′-CGTCCGGAACAATGATACCCTAGCA-3′) was between bases 132 and 156 of this region. The probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein at the 5′ end and with the quencher dye 6-carboxytetramethylrhodamine at the 3′ end. The probe was obtained from Applied Biosystems, United Kingdom, and the primers were obtained from Invitrogen Life Technologies, United Kingdom. The specificities of the primers and probe were verified experimentally by using the marine fungi *Sigmoidea marina* and *Lindra obtusa* and the closely related organism *Emericellopsis minima*, all of which had been isolated from *F. serratus* samples. Additionally, a BLAST search (National Center for Biotechnology Information) was performed with the primer and probe sequences.

**(ii) Real-time PCR protocol.** The environmental samples were subjected to amplification using real-time quantitative PCR. The PCRs were performed in MicroAmp optical 96-well plates using an automated ABI Prism 7700 sequence detector (PE Applied Biosystems). Each 25-μl (total volume) reaction mixture contained TaqMan universal PCR master mixture, No AmpErase uracil-*N*-glycosylase (Applied Biosystems, Roche Molecular Systems, Inc., Branchburg, NJ), the primers at a final concentration of 900 nM, the probe at a final concentration of 200 nM, and 500 ng/μl of environmental DNA extracted from algal material. A standard curve was prepared for each run, using serially diluted genomic DNA extracted from *A. fuci* (8, 3, 1.5, 0.8, 0.3, 0.15, and 0.003 ng). The PCR cycling parameters were 50°C for 3 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and annealing at 60°C for 1 min. Data acquisition and threshold cycle values for each PCR were automatically calculated and analyzed by using the ABI Prism sequence detection system software (version 1.6; Applied Biosystems). A preamplification step was included for the healthy algal DNA samples using 0.5 μM primer T10 (31) and 0.5 μM primer Bt2b (15), which amplified an approximately 300-bp fragment. Amplification was performed using the following cycling conditions: 5 min at 95°C, followed by 25 cycles of 30 s at 95°C, 55 s at 55°C, and 45 s at 72°C, and a final extension for 10 min at 72°C. One microliter of the reaction mixture, including negative controls, was used as the substrate for the nested real-time PCR as described above.

**Phylogenetic analysis.** Isolates and environmental sequences used for phylogenetic analysis are listed in Table 1. The phylogenetic position of the obligate marine parasite *D. fucicola* on *F. vesiculosus* obtained from a 35-year-old dried herbarium specimen was determined using partial LSU DNA sequences in multigene phylogenies of related terrestrial and marine plant parasites and environmental sequences obtained from *F. serratus*. Herbarium material was used because the type material, *D. fucicola*, and living specimens from later collections (21, 24, 46) were unavailable for study. A neotype for this species was proposed by Kohlmeyer (21) and was fully illustrated by Kohlmeyer and Kohlmeyer (23).

A data matrix of 66 taxa representing a selection of major lineages in the Dothideomycetes was used (Table 1). We combined DNA sequence data obtained as part of the Assembling the Fungal Tree of Life (AFTOL) project (29) from ribosomal as well as protein-encoding genes. DNA sequences were obtained for the nuclear small subunit and LSU, as well as the sequence between domains 5 and 7 of the second largest subunit of RNA polymerase II (RPB2) and the transcription elongation factor 1 alpha gene (EF) (35). We chose 36 representative taxa following the Dothideomycetes phylogeny of Schoch et al. (35) in order to accurately place the environmental isolates obtained in this study. The data obtained were combined with a number of sequences obtained from GenBank. The final matrix used was deposited at treeBASE (http://www.treebase.org/treebase/index.html) under reference number SN3178. In order to incorporate differently weighted character sets, we used maximum likelihood as performed with RAxML-VI-HPC, version 2.2.0 (40), using the GTRMIX setting (applying a GTRCAT approximation of evolution with 25 rate categories but determining final likelihood values according to a gamma distribution with four rate categories). The data set was divided into eight parts, including nuclear small subunit, nuclear LSU, and each codon position of both RPB2 and EF. Nodal support in RAxML analyses was determined by 500 nonparametric bootstrap repetitions. Similarly, Metropolis coupled Markov chain Monte Carlo (B-MCMCMC) analyses were conducted using MrBayes 3.1.2 (http://mrbayes.csit.fsu.edu/index.php) with the same partition that was used with RAxML-VI-HPC and using the GTR model with a gamma distribution approximated with four categories and a proportion of invariable sites. Searches were conducted using four chains with trees sampled every 100 generations. Two independent 5 million-generation analyses were conducted, and likelihood values were examined to verify a "burn-in" parameter. The Bayesian analyses converged on the same topology and plateau of log likelihoods with harmonic mean values of −26,913.78 and −26,914.64 for runs 1 and 2, respectively. These runs were combined, and 10,000 trees were discarded as "burn in," yielding a single 50% majority rule tree with the proportions of trees in the final set of 90,000 expressed as percent posterior probabilities.

A second data matrix, comprising ITS-5.8S rRNA gene sequences, was constructed and represented 17 taxa that had a high level of homology with *D. fucicola*; these taxa included *Didymella* species and mitosporic forms (Table 1). Maximum parsimony and likelihood analyses were performed using this matrix. The maximum parsimony settings included heuristic searches with random se-

TABLE 1. Fungal strains and clones used in the molecular analyses

| AFTOL no. | Taxon | Source[a] | Accession no.[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | Small-subunit rRNA gene | LSU rRNA gene | RPB2 | EF | ITS |
| Isolates | | | | | | | |
| 267 | *Allewia eureka* | DAOM 195275 | DQ677994 | DQ678044 | DQ677938 | DQ677883 | |
| 1610 | *Alternaria alternata* | CBS 916.96 | DQ678031 | DQ678082 | DQ677980 | DQ677927 | |
| 931 | *Bimuria novae-zelandiae* | CBS 107.79 | AY016338 | AY016356 | DQ470917 | DQ471087 | |
| 946 | *Botryosphaeria dothidea* | CBS 115476 | DQ677998 | DQ678051 | DQ677944 | DQ767637 | |
| 1586 | *Botryosphaeria tsugae* | CBS 418.64 | AF271127 | DQ767655 | DQ767644 | DQ677914 | |
| 939 | *Capnodium coffeae* | CBS 147.52 | DQ247808 | DQ247800 | DQ247788 | DQ471089 | |
| 1289 | *Cladosporium cladosporioides* | CBS 170.54 | DQ678004 | DQ678057 | DQ677952 | DQ677898 | |
| 54 | *Cochliobolus heterostrophus* | CBS 134.39 | AY544727 | AY544645 | DQ247790 | DQ497603 | |
| | *Colispora elongata* | F-08382 | | | | | AY148102 |
| 1379 | *Coniothyrium palmarum* | CBS 400.71 | DQ678008 | DQ767653 | DQ677956 | DQ677903 | |
| 1568 | *Cucurbitaria elongata* | CBS 171.55 | DQ678009 | DQ678061 | DQ677957 | DQ677904 | |
| 1591 | *Davidiella tassiana* (as anamorph *Cladosporium herbarum*) | CBS 399.80 | DQ678022 | DQ678074 | DQ677971 | DQ677918 | |
| | *Decorospora gaudefroyi* | pp4723 | | **EF1778459** | | | |
| 1599 | *Delitschia winteri* | CBS 225.62 | DQ678026 | DQ678077 | DQ677975 | DQ677922 | |
| 995 | *Dendryphiella arenaria* | CBS 181.58 | DQ471022 | DQ470971 | DQ470924 | DQ677890 | |
| | *Didymella bryoniae* | | | | | | AF297228 |
| | *Didymella cucurbitacearum* | IMI 373225 | AY293779 | AY293792 | | | |
| | *Didymella cucurbitacearum* | | | | | | AY293804 |
| 2111 | *Didymella exigua* | CBS 183.55 | | **EF177845** | | | **EF192139** |
| | *Didymella fabae* | af1 | | | | | DQ383952 |
| | *Didymella lentis* | AL1 | | | | | AY131201 |
| | *Didymella pinodes* | | | | | | AY152551 |
| | *Didymella rabiei* | | | | | | DQ383949 |
| | *Didymella* sp. | hka9 | | | | | DQ092504 |
| | *Didymella* sp. | Hkb1 | | | | | DQ092514 |
| 919 | *Dothidea hippophaës* | DAOM 231303 | U42475 | DQ678048 | DQ677942 | DQ677887 | |
| 274 | *Dothidea sambuci* | DAOM 231303 | AY544722 | AY544681 | DQ522854 | DQ497606 | |
| 1359 | *Dothiora cannabinae* | CBS 737.71 | DQ479933 | DQ470984 | DQ470936 | DQ471107 | |
| | *Epicoccum andropogonis* | | | | | | AJ400905 |
| | *Epicoccum nigrum* | CBMAI 65 | | | | | DQ123608 |
| 1618 | *Guignardia bidwellii* | CBS 237.48 | DQ678034 | DQ678085 | DQ677983 | | |
| 1608 | *Herpotrichia juniperi* | CBS 200.31 | DQ678029 | DQ678080 | DQ677978 | DQ677925 | |
| 277 | *Leptosphaeria maculans* | DAOM 229267 | DQ470993 | DQ470946 | DQ470894 | DQ471062 | |
| 1081 | *Magnaporthe grisea* | Broad | AB026819 | AB026819 | Genome | Genome | |
| 1734 | *Montagnula opulenta* | CBS 168.34 | AF164370 | DQ678086 | DQ677984 | | |
| 1615 | *Mycosphaerella graminicola* | CBS 292.38 | DQ678033 | DQ678084 | DQ677982 | | |
| 942 | *Mycosphaerella punctiformis* | CBS 113265 | DQ471017 | DQ470968 | DQ470920 | DQ471092 | |
| 1078 | *Neurospora crassa* | Broad | X04971 | AF286411 | XM_324476 | Genome | |
| 1569 | *Ophiosphaerella herpotricha* | CBS 620.86 | DQ678010 | DQ678062 | DQ677958 | DQ677905 | |
| 1595 | *Ophiosphaerella herpotricha* (as synonym *Ophiobolus herpotrichus*) | CBS 240.31 | DQ767650 | DQ767656 | DQ767645 | DQ767639 | |
| 280 | *Phaeosphaeria avenaria* | DAOM 226215 | AY544725 | AY544684 | DQ677941 | DQ677885 | |
| | *Phaeosphaeria nodorum* | Broad | Genome | Genome | Genome | Genome | |
| | *Phaeosphaeria olivacea* | CBS 118420 | | | | | |
| 2206 | | J.K.5540Q | | **EF177847** | | | |
| 1441 | *Phaeosphaeria orae-maris* | J.K.4730 | | **EF179158** | | | |
| 1575 | *Phoma herbarum* | CBS 276.37 | DQ678014 | DQ678066 | DQ677962 | DQ677909 | |
| 1600 | *Pleomassaria siparia* | CBS 279.74 | DQ678027 | DQ678078 | DQ677976 | DQ677923 | |
| | | CBS 118380 | | | | | |
| 2205 | *Pleospora avicenniae* | J.K.5326A | | **EF177846** | | | |
| 940 | *Pleospora herbarum* var. *herbarum* | CBS 541.72 | DQ247812 | DQ247804 | DQ247794 | DQ471090 | |
| | | CBS 118219 | | | | | |
| 2207 | *Pleospora* sp. | J.K.5184D | | **EF177848** | | | |
| 283 | *Pyrenophora phaeocomes* | DAOM 222769 | DQ499595 | DQ499596 | DQ497614 | DQ497607 | |
| | Sarcosomataceae species | sd2bN1c | | | | | AY465503 |
| 1594 | *Scorias spongiosa* | CBS 325.33 | DQ678024 | DQ678075 | DQ677973 | DQ677920 | |
| | *Sporidesmium obclavatulum* | HKUCC 10834 | | DQ408556 | | | |
| 1256 | *Sporormiella minima* | CBS 524.50 | DQ678003 | DQ678056 | DQ677950 | DQ677897 | |
| | *Strumella griseola* | CBS433.59 | | | | | AF485078 |
| 1300 | *Sydowia polyspora* | CBS 116.29 | DQ678005 | DQ678058 | DQ677953 | DQ677899 | |

*Continued on following page*

TABLE 1—*Continued*

| AFTOL no. | Taxon | Source[a] | Accession no.[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | Small-subunit rRNA gene | LSU rRNA gene | RPB2 | EF | ITS |
| | *Tumularia aquatica* | MUCL28096 | | | | | AY265337 |
| | Uncultured ascomycete | dfmo0690_230 | | | | | AY969660 |
| | Uncultured fungus from cow rumen | | | AY464875 | | | |
| 1037 | *Westerdykella cylindrica* | CBS 454.72 | AY016355 | AY779322 | DQ470925 | DQ497610 | |
| Sterilia mycelia from *F. serratus* | MC340 | TUB340 | | **EF177836** | | | |
| | MC363 | TUB363 | | **EF177837** | | | |
| | MC541 | TUB541 | | **EF177838** | | | |
| | MC545 | TUB545 | | **EF177839** | | | |
| | MC555 | TUB555 | | **EF177840** | | | |
| | MC556 | TUB556 | | **EF177841** | | | |
| | MC564 | TUB564 | | **EF177842** | | | |
| | MC565 | TUB565 | | **EF177843** | | | |
| | MC190 | TUB190 | | **EF177844** | | | |
| Clones | hclone2, *Didymella fucicola* | Herbarium J.K.2932 | | **EF177850** | | | |
| | hclone5, *Didymella fucicola* | Herbarium J.K.2932 | | **EF177851** | | | |
| | hclone9, *Didymella fucicola* | Herbarium J.K.2932 | | **EF177852** | | | |
| | hcloneITS, *Didymella fucicola* | Herbarium J.K.2932 | | | | | **EF192138** |
| | eclone7 | Environment | | **EF177832** | | | |
| | eclone15 | Environment | | **EF177833** | | | |
| | eclone20 | Environment | | **EF177834** | | | |
| | eclone24 | Environment | | **EF177835** | | | |

[a] Abbreviations for culture collections, herbarium, and database: ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM, National Mycological Herbarium, Department of Agriculture, Ottawa, Ontario, Canada; J.K., culture collection of Jan Kohlmeyer and Brigitte Volkmann-Kohlmeyer, Department of Marine Sciences, University of North Carolina, Morehead City; pp, culture collection of Portsmouth School of Biological Sciences, University of Portsmouth, Portsmouth, United Kingdom; TUB, culture collection of Technische Universität Braunschweig Department of Microbiology, Braunschweig, Germany; Broad, Broad Institute, Cambridge, MA.

[b] Bold type indicates that data were obtained in this study.

quence addition (10 to 50 replicates) using the tree bisection-reconnection algorithm, while the maximum likelihood analysis used the GTR + G + I model with estimates of the nucleotide frequency, substitution rate matrix, among-site variation, and shape parameter from the matrix.

## RESULTS

**Fungal isolates from *F. serratus*.** The fungal reference library obtained from *F. serratus* contained 336 isolates representing 35 genera of the Ascomycota and Zygomycota (Table 2). A total of 56 strains had sterile mycelia and could not be identified morphologically (Table 2). The most commonly encountered isolates belonged to the Ascomycota, in agreement with our previous study (48). Representatives of the following five taxa were the predominant organisms in this study: *S. marina*, with 56 strains; *Acremonium* spp., with 36 strains, 29 of which were identified as *A. fuci* (49); *Cladosporium* spp., with 31 strains; *Dendryphiella salina*, with 26 isolates; and *Fusarium* spp., with 19 representative isolates. *S. marina* was isolated only from healthy surface-sterilized samples, whereas the other taxa were present in surface-sterilized and water-treated tissues, as well as in segments from decaying *F. serratus* (Fig. 1). Members of additional genera were isolated, but the numbers were lower and the organisms were isolated mainly from water-treated samples; these organisms included representatives of *Trichoderma*, *Alternaria*, *Phoma*, *Penicillium*, and *Paecilomyces* (Table 2). *Corollospora angusta*, *Corollospora intermedia*, and *L. obtusa* were obtained sporadically from water-treated living and decaying samples. *Microascus*, *Chaetomium*, and *Arthrinium* spp. were isolated only from surface-sterilized living *F. serratus* (Table 2 and Fig. 1).

Isolates were recovered from all thallus parts. Fewer isolates were cultured from growing tips and receptacles than from blades and holdfast tissues. *S. marina* was the predominant isolate obtained from receptacles and growing tips (23 strains recovered), followed by *D. salina* (three isolates) and a single *A. fuci* isolate (data not shown).

**PCR-DGGE analysis of the nuclear LSU rRNA gene in fungal sequences obtained from whole and sectioned algal thalli sampled seasonally.** Analyses of replicates of an individual sample generally resulted in similar profiles, although occasionally one or two additional bands were observed, indicating that there was a very small amount of DNA for some of the fungi detected. The profiles obtained for all of the living thalli of *F. serratus* tissues comprised a total of 87 bands, representing seven different ribotypes with one to six bands per sample (Fig. 2 and 3). Bands corresponding to bands amplified from *S. marina* were the bands that were observed most frequently, accounting for 34.5% of the total, followed by bads from *Emericellopsis*/*Acremonium* (19.5%), *L. obtusa* (17.2%), *C. angusta* (ca. 8%), *Engyodontium* sp. (ca. 8%), and the molecular ribotype for *Lulworthia* sp. (ca. 8%) (Fig. 2). One extra ribotype was recovered twice on one sampling occasion in January, and it was identified as an *Iodophanus*-like sequence after BLAST searches. *S. marina*

TABLE 2. Fungi isolated from specimens of decaying and submerged, attached, healthy *F. serratus* thalli on five different sampling occasions

| Taxon | No. of isolates obtained from surface-sterilized and water-treated *F. serratus* | |
|---|---|---|
| | Living disks (total no., 3,100) | Decaying disks (total no., 400) |
| *Acremonium* sp. | 5 | |
| *Acremonium murorum* | 1 | |
| *Acremonium fuci* | 21 | 8 |
| *Acremonium tubakii* | 1 | |
| *Acroconidiella* sp. | | 1 |
| *Alternaria* sp. | 5 | 6 |
| *Arthrinium* sp. | 3 | |
| *Aspergillus* sp. | 4 | 1 |
| *Asteromyces cruciatus* | 1 | 1 |
| *Botrytis cinerea* | 2 | |
| *Chaetomium funicola* | 3 | |
| *Chaetomium* sp. | 2 | |
| *Cladosporium* sp. | 17 | 14 |
| *Coniothyrium* sp. | 3 | |
| *Corollospora angusta* | 2 | 3 |
| *Corollospora intermedia* | 1 | 1 |
| *Dendryphiella salina* | 18 | 8 |
| *Emericellopsis minima* | 1 | |
| *Epicoccum purpurascens* | | 1 |
| *Epicoccum* sp. | 1 | 2 |
| *Fusarium* sp. | 15 | 4 |
| *Geomyces* sp. | 2 | |
| *Gliocladium* sp. | 1 | |
| *Humicola fuscoatra* | 1 | |
| *Lindra obtusa* | 1 | 5 |
| *Microascus* sp. | 3 | |
| *Mucor* sp. | 2 | |
| *Mycosphaerella* sp. | 1 | |
| *Nodulisporium* sp. | 1 | |
| *Oidiodendron* sp. | 1 | |
| *Paecilomyces* sp. | 7 | |
| *Penicillium* sp. | 11 | 3 |
| *Periconia* sp. | 1 | |
| *Phialophora* sp. | 2 | |
| *Phoma* sp. | 8 | 2 |
| *Phomopsis* sp. | | 2 |
| *Scopulariopsis* sp. | 3 | |
| *Sigmoidea marina* | 56 | |
| *Tetracladium maxilliformis* | | 1 |
| *Trichoderma* sp. | 7 | 1 |
| *Verticillium cinnabarinum* | 1 | 1 |
| Sterilia mycelia | 37 | 19 |
| Total | 252 | 84 |

and *Emericellopsis*/*Acremonium* ribotypes were obtained at all five sampling times over the course of the year, whereas the other ribotypes were recovered sporadically. An analysis of variance ($P > 0.05$, Kruskal-Wallis test) revealed no seasonal patterns for these signals but a significant prevalence of *S. marina* sequences compared with those retrieved sporadically ($P = 0.003$, pairwise multiple comparison procedure, Holm-Sidak method). This indicated that there was a predominant association between this fungus and *F. serratus* over the year (Fig. 2).

The molecular methods used did not detect an association between any particular ribotype and a specific part of the thallus. A significant difference ($P = 0.049$, Tukey's test) in the number of bands associated with the tissue types was observed, however, and the highest number of bands was obtained for the DNA extracted from blades and the lowest number of bands was obtained for the DNA extracted from the growing tips. This observation is consistent with observations made in the culture study, where, except for *S. marina*, fewer isolates were recovered from apex tissues (data not shown).

The molecular profiles obtained for the decaying tissues contained a total of 25 bands. *L. obtusa* was the predominant ribotype (25% of the bands), followed by *C. angusta* (20%), *S. marina* (16%), *Emericellopsis*/*Acremonium* (12%), and a *Lulworthia*-like sequence (8%). Since some of the bands resolved by DGGE were too diffuse to be analyzed further, PCR products from decaying material were cloned and sequenced. *Phoma*, *Mycosphaerella*, *Pleospora*, and *Didymella*-like ribotypes resulted from this cloning-sequencing analysis (data not shown).

**Design, specificity, and sensitivity of *A. fuci*-specific primers and probe.** The real-time PCR system was developed in order to differentiate between the environmental signals for the *Emericellopsis* and *Acremonium* sequences. The fragments amplified with primers TUB1F and TUB2R were in the range expected based on the sequence data for *A. fuci*. No amplification from DNA of *L. obtusa* or *S. marina* was observed. Amplification was obtained for the closely related organism *E. minima*, as expected from the BLASTn search, even though the reaction efficiency was lower than that for *A. fuci*. In the real-time PCR, all dilutions of DNA from *A. fuci* tested gave strong positive fluorescent signals after 20 to 26 cycles with 8, 3, and 1.5 ng of DNA and after 40 cycles with 0.003 ng of DNA. No signal was detected for the other fungi tested using these DNA concentrations.

DNA from *E. minima* gave a weak fluorescent signal after 40 cycles with higher concentrations of genomic DNA, but the reaction never reached exponential amplification (see Fig. SA2 in the supplemental material). The AFP1 probe, therefore, proved to be specific or highly enhanced for the *A. fuci* sequence.

**Detection of *A. fuci* beta-tubulin sequences in environmental samples.** The routine retrieval of a sequence belonging to *Emericellopsis*/*Acremonium* using the 28S rRNA gene PCR-DGGE system and the high isolation ratio of *A. fuci* were in general agreement with the real-time PCR results. Two of six environmental decaying *F. serratus* samples gave strong positive amplification using the 28S rRNA gene system for the *Emericellopsis*/*Acremonium* ribotype with an intense DGGE band (Fig. 3a). The same samples resulted in strong positive fluorescent signals after 32 and 36 cycles using real-time PCR. The four other environmental samples were negative for this ribotype using both methods. Of the 21 samples of healthy *F. serratus* analyzed, 12 resulted in positive amplification using the 28S rRNA gene system, which was visualized as low-intensity DGGE bands (Fig. 3b). Two of these samples gave weak fluorescent amplification signals after 40 cycles for *A. fuci* using the beta-tubulin real-time PCR system. At higher concentrations of DNA (600 to 1,000 ng/μl algal DNA) or when a nested approach was used with primers T10 and Bt2b followed by primers TUB1F and TUB2R, positive amplification of *A. fuci* was obtained with some of the living algal samples.

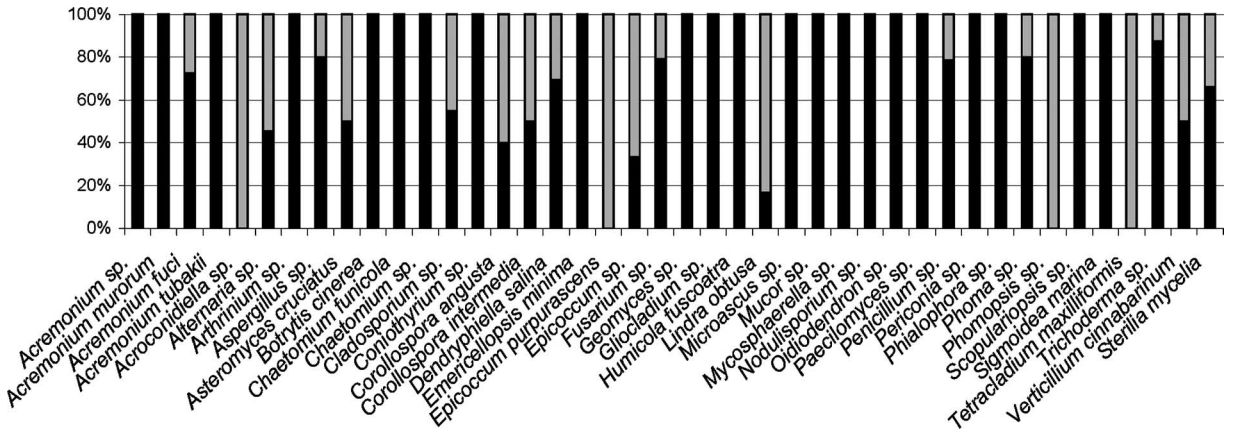


FIG. 1. Proportions of fungi isolated from surface-sterilized (gray bars) and water-treated (black bars) *F. serratus* thalli.

**Phylogenetic analysis of environmental isolates and signal sequences retrieved from healthy, decaying, and herbarium *Fucus* thalli.** To better characterize the Dothideomycetes sequences obtained from the diversity study, a four-gene combined phylogenetic analysis was performed. Five clades in the Pleosporales were identified (Fig. 4). The first clade is a well-supported group of isolates clustered around *Didymella* and *Phoma* species and includes all of the clones obtained from the herbarium sample of *D. fucicola*. The clade labeled *Leptosphaeria* is poorly supported and consists of disparate species. In contrast, the Pleosporaceae clade is well supported and contains one environmental clone (Eclone15 in Fig. 4) that did not cluster closely with any known species. This sequence could not be identified accurately after BLAST searches. More than 100 matches with E values of 0 were obtained for taxa, including species of *Phoma*, *Pleospora*, *Setosphaeria*, *Cochliobolus*, *Leptosphaeria*, *Phaeosphaeria*, and *Dendryphiella*. The greatest number of matching hits, however, was for sequences representing the Pleosporaceae. Likewise, the Phaeosphaeriaceae is a well-supported clade containing two environmental sequences, one of which (clone 24) shows a strong affinity to *Ophiosphaerella herpotrichia* and *Phaeosphaeria oraemaris*. Clone 20 grouped with other *Phaeosphaeria* members but at an uncertain position. In general, the marine species in this group formed a clade with good support, which was separated from the terrestrial *Phaeosphaeria* species. The fifth clade comprised only nuclear LSU sequences belonging to the four environmental isolates that formed a supported clade with *Sporidesmium* (37). The latter organism was isolated from leaf litter and often occurs on dead branches of woody plants (B. D. Shenoy, personal communication). Within the Capnodiales one environmental clone (Eclone7 in Fig. 4) showed a very close relationship with the ubiquitous *Cladosporium* species (Davidiellaceae).

The nuclear LSU rRNA gene analysis of *D. fucicola* revealed that this species was closely related to other members of *Didymella*. In order to confirm this, the ITS region was amplified from sectioned ascoma of herbarium material and sequenced. BLAST homology searches of the sequences indicated that the closest matches were with uncultured ascomycetes (E = 1e106) or mitotic species, such as *Tumularia aquatica* (E = 5e84) and *Strumella griseola* (E = 5e84). The greatest number of hits recorded

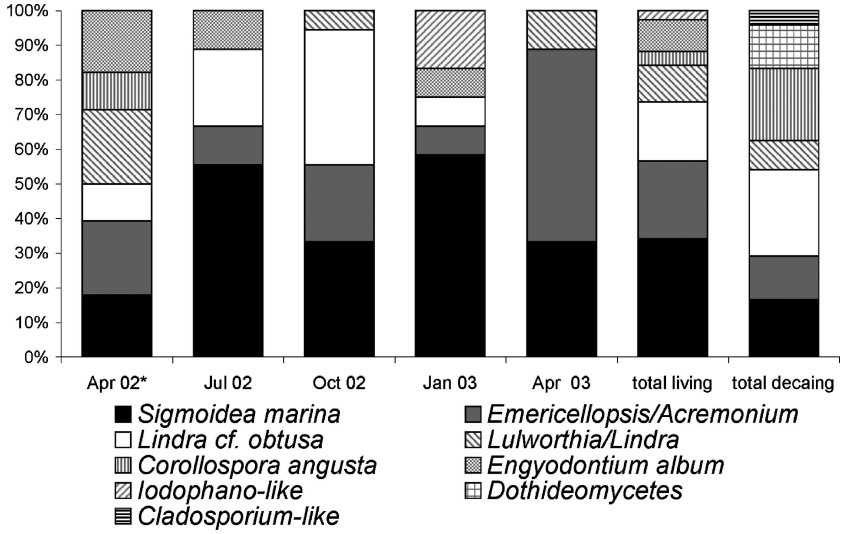


FIG. 2. Proportions of the predominant Ascomycetes phylotypes recovered from *F. serratus* tissues. Some of the April 2002 data were obtained from reference 48.

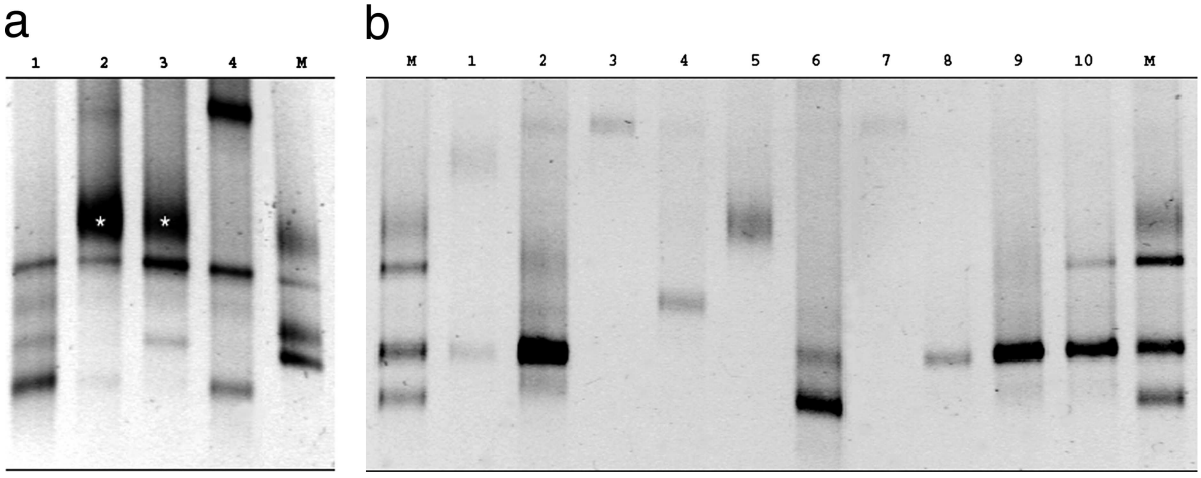


FIG. 3. DGGE gels of fungi associated with decaying and sectioned living algal tissues. (a) Separation of PCR products generated by NL359-NL912GC amplification of genomic DNA extracted from decaying thalli of *F. serratus* in a 38 to 60% denaturant gradient gel. Lane M, marker DNA consisting of NL359-NL912GC amplicons from *A. fuci*, *Lindra cf. obtusa*, *Verticillium cinnabarinum*, and *S. marina*, from top to bottom; lane 1, April 2002 collection; lane 2, July 2002 collection; lane 3, October 2002 collection; lane 4, January 2003 collection. (b) DGGE profiles of amplified 28S rRNA gene fragments (obtained with primers NL359 and NL912GC) of DNA extracted from sectioned living algal thalli collected in April 2002. Lanes M, marker DNA comprising NL359-NL912GC fragments from *A. fuci*, *Lindra cf. obtusa*, *S. marina*, and *C. angusta*, from top to bottom; lanes 1 and 2, profiles for holdfasts of *F. serratus*; lanes 3 to 7, profiles for blades of *F. serratus*; lane 8, profile for growing tips of *F. serratus*; lanes 9 and 10, profiles for receptacles of *F. serratus*.

was with sequences from members of the Dothideomycetes (881/1,500 hits) within the Leptosphaeriaceae. To further clarify the phylogenetic placement of this taxon, maximum parsimony and likelihood analyses were performed using a matrix of 17 taxa comprising sequences from *Didymella* species and the closest matches. The alignment revealed a strong similarity between the ITS1 and ITS2 regions of the *Didymella* species sampled; nonetheless, the sequences from *D. fucicola* had greater similarity with the sequences from *T. aquatica* and *S. grisolus* (see Fig. SA3 in the supplemental material).

Maximum parsimony analysis produced three trees with a length of 335 (consistency index, 0.81; retention index, 0.87; number of parsimony informative characters, 126) (Fig. 5). The topology of this trees was similar to the topology obtained from the maximum likelihood analysis (data not shown). In both analyses the *Didymella* and *Epicoccum* species, including two *Didymella* isolates cultured from coral tissue (*Didymella* sp. strains HKA9 and HKB1), formed a strongly supported clade (bootstrap value = 100%). *D. fucicola* was located at the base of this clade, followed by two environmentally derived sequences (Sarcosomataceae strain Sd2bN1c and uncultured ascomycete isolate dfmo0690_230) and the mitotic species *T. aquatica*, *Colispora elongata*, and *S. griseola*.

## DISCUSSION

The culture-based study and LSU rRNA PCR-DGGE analysis of healthy and decaying thalli revealed the presence of a number of fungal groups associated with *F. serratus* populations. These groups included isolates and rRNA gene ribotypes representative of the Halosphaeriaceae, Lulworthiaceae, Hypocreales, and Dothideomycetes, along with two distinct ribotypes of *S. marina* and *Emericellopsis*/*Acremonium* that were detected extensively throughout the year.

The hyphomycete *S. marina* is linked molecularly with the Halosphaeriaceae via its connection to *Corollospora* (48). In this study these organisms were consistently cultured after surface sterilization from all healthy tissue types, even in the winter (January) when the average water temperature was 2°C (17). At this time the *S. marina* environmental signal sequence represented 60% of the DGGE bands detected. It was found molecularly and culturally on the growing tips of the alga, which represented the youngest algal tissue. This suggests that there is systemic growth of the fungus within the algal tissues. This behavior resembles that of *Mycophycias ascophylli*, an endophytic mycophycobiont that grows mutually within its hosts, *Ascophyllum nodosum* and *Pelvetia canaliculata* (1, 22, 24). This endophyte remains associated with its algal host throughout its life cycle (41), has differential hyphal densities within the algal thallus (12), protects the photobiont from desiccation (13), and has a nutritional dependence on its partner (20). The failure to recover isolates of *S. marina* from decaying material in this study suggests that this fungus is a hemibiotroph that cannot thrive in the environment without the protection of the alga (Table 2).

In contrast, the *Emericellopsis*/*Acremonium* LSU signal sequence and isolates were retrieved from both living and decaying *F. serratus* fronds (Table 2 and Fig. 2). Three closely related isolates, *A. fuci*, *Acremonium tubakii*, and *E. minima*, produced sequences that matched this signal, although only *A. fuci* was routinely isolated in culture. All of these organisms are related to the Bionectriaceae within the Hypocreales, but their positions are uncertain (33). In order to distinguish between these sequences, the real-time PCR system was designed based on beta-tubulin sequence information from 22 related isolates (49). This gene has previously been used to estimate fungal phylogenies, including those of *Stanjemonium* and *Emericellopsis* (11, 49), and to detect species-specific transcripts in the environment (6). Intron 3 of *Emericellopsis* and related *Acre-*

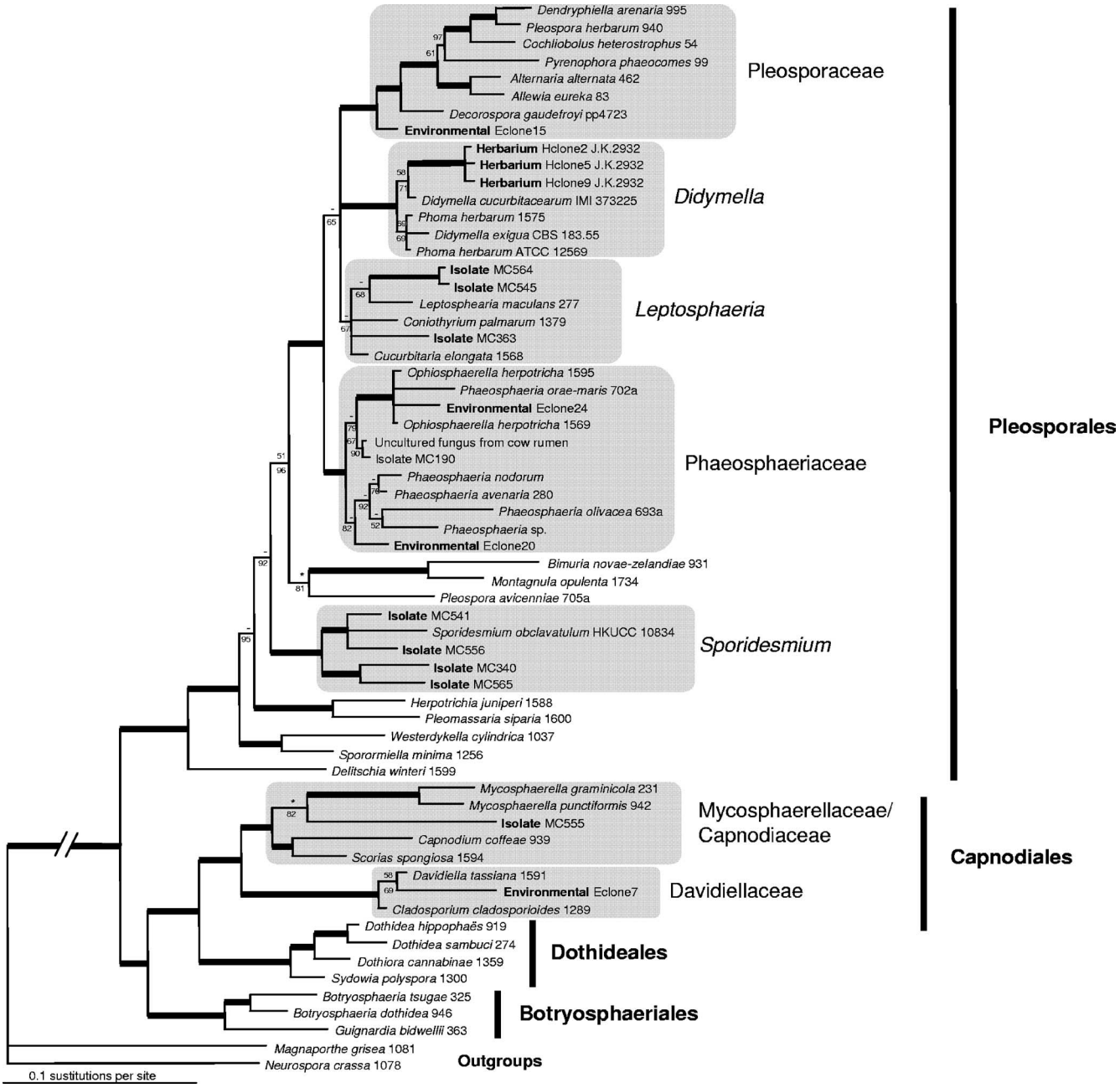


FIG. 4. Phylogenetic tree showing the relationship between the environmental sequences, the sequence from *D. fucicola* herbarium specimen J.K.2932, and the isolates recovered from *F. serratus* thalli. A 50% majority rule for 90,000 trees obtained by Bayesian inference was used. Nodes with >95% posterior probability and >70% bootstrap support are indicated by thick branches. For the other nodes the percentages of posterior probability are indicated below the nodes and the RAxML maximum likelihood bootstrap values are indicated above the nodes. Nodes with bootstrap values less than 50% are indicated by a minus sign, and nodes resolved differently in the RAxML consensus tree are indicated by an asterisk. Clades containing sequences obtained in this study are highlighted and named.

*monium* types is characteristically short, but it contained enough information to design a 25-bp hybridization probe that, unlike the ITS region, distinguished between the isolates (see Fig. SA1 in the supplemental material). *A. fuci* was chosen as the target organism after a series of physiological tests indicated that there was an interaction between the fungus and the brown algae (49).

The TaqMan primers and probe could detect *A. fuci* environmental sequences in decaying algal material without a nested PCR. The absence of a signal from healthy fronds contradicts the results obtained using the LSU rRNA gene PCR-DGGE system. The contradiction, however, can be explained by the higher algal DNA/fungal DNA ratio expected for healthy fronds than for decaying fronds and the predicted lower copy number associated with the beta-tubulin gene compared to the copy number for the rRNA genes. When a pre-

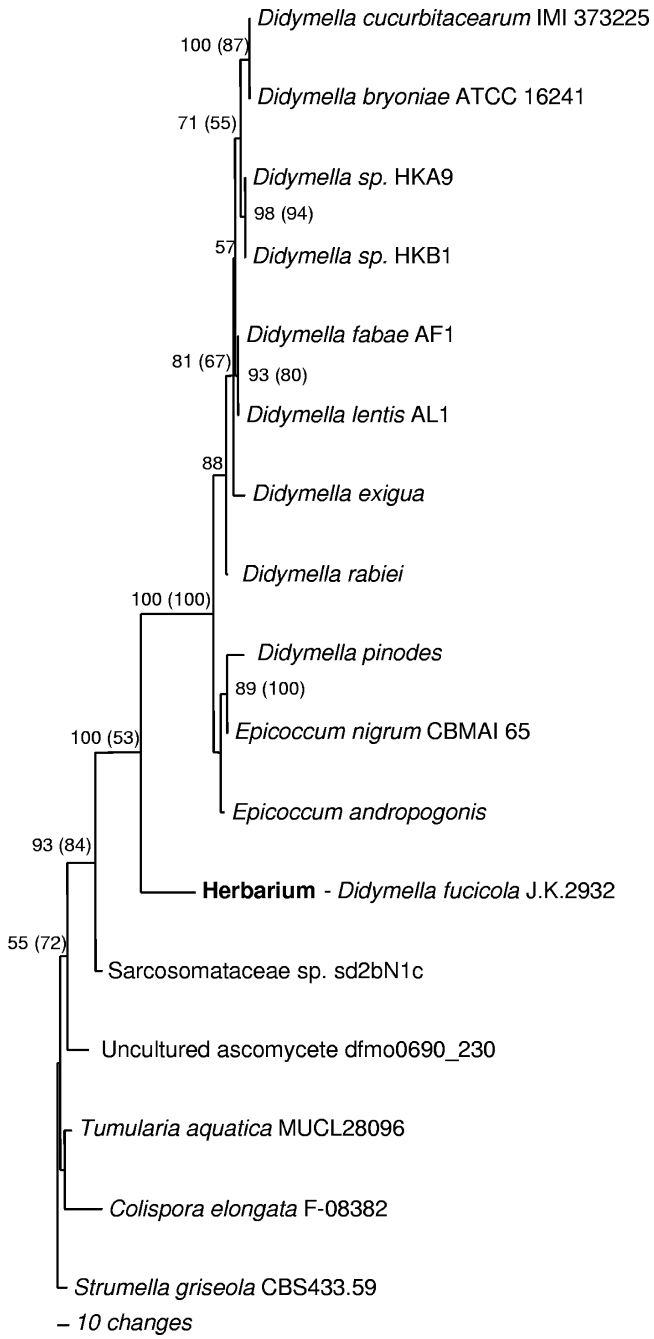


FIG. 5. Phylogram showing the relationship between *Didymella* species, based upon ITS and 5.8S rRNA gene sequences: one of three most parsimonious trees with 335 steps (consistency index, 0.81; retention index, 0.71; homoplasy index, 0.19) generated from a single tree island. A similar phylogram was produced after maximum likelihood analysis (−ln L [likelihood value] = 2,314.347). Bootstrap values are indicated above the branches, and the values generated by a maximum likelihood analysis are in parentheses. Values less than 50% are not shown.

amplification step or increased concentrations of environmental DNA were used, a signal was obtained for some living samples. These results confirmed that this fungus was associated with the thallus but that the amounts were small ($<1 \times 10^{-5}$ ng). The difference in signal detection levels between living and dead algae suggests that this fungus is latent in healthy tissues. This may represent an adaptive strategy of the saprobe for rapid colonization of the decaying material. The life history strategies of *A. fuci* may parallel those of endophytic fungi in higher plants (36). Some endophytes grow discretely in a healthy host, resuming saprophytic growth only during senescence of the host (e.g., *Rhabdocline parkeri* in needles of *Pseudotsuga menziesii*) (2, 42).

The other fungal LSU rRNA gene signals retrieved from living and decaying host tissue after DGGE analysis included signals for *L. obtusa* (Lulworthiaceae), *Engyodontium album*-like species (Clavicipitaceae), and *C. angusta* (Halosphaeriaceae) and a signal for an *Iodophanus*-like organism (Pezizaceae) (48). Further environmental sequences representing Dothideomycetes were amplified from decaying alga material using primers NL209 and NL912 and were separated by molecular cloning. Reamplified fragments from these clones were not resolved under the DGGE conditions that we employed. The absence of DGGE bands representing Dothideomycetes, therefore, does not reflect a primer bias (48) but most likely an electrophoresis band resolution problem.

The majority of the environmental signals and rRNA genes from sterile mycelia falling within the Dothideomycetes exhibited similarities with sequence representatives of families belonging to the Pleosporales. The majority of marine *Phaeosphaeria* species have been obtained from beach grass and salt marsh plants (27) but not from seaweeds. As the host is a phylogenetically important characteristic in defining species within this fungal group (7), the environmental signals may reflect undescribed lineages representing novel organisms.

An additional environmental sequence (represented by clone 7) belonging to a *Cladosporium* species was detected. Commonly, *Cladosporium* isolates can be cultured from many marine substrates (24, 25, 32). Some *Cladosporium* species exhibit physiological adaptations to saline conditions (19), while others can cause fish diseases (38) and are important producers of bioactive molecules (4, 18). Algal tissues have provided substrate material for *Cladosporium algarum* (24) and a large number of unidentified *Cladosporium* isolates from this study. The recovery of an environmental signal for this group obtained from decaying material is therefore not surprising.

The other cloned sequences included in this study were the sequences derived from ascocarps of *D. fucicola* (herbarium specimen J.K.2932). This fungus is an obligate parasite whose ascocarps are embedded in the central midribs of damaged lower side branches of living *Fucus spiralis* and *F. vesiculosus* and vegetative thalli of *P. canaliculata*, and it is often associated with the bases of *Elachista clandestina* and *Elachista fucicola* (24). The *D. fucicola* ITS sequences, although exhibiting a degree of similarity to the ITS sequences of other *Didymella* species, exhibited more similarity to the ITS sequences of *T. aquatica*, *C. elongata*, and *S. griseola* and unpublished environmental signals in the GenBank database. *T. aquatica* and *C. elongata* are aquatic mitosporic species (10) whose molecular lineage is uncertain. Bussaban et al. (5) noted that the ITS sequences of *T. aquatica* were similar to those of *Pyricularia variabilis*, which is separate from other *Pyricularia* species in the Magnaporthaceae. The teleomorphic form of *T. aquatica* is *Massarina aquatica* (44, 45), suggesting a link to the Pleospo-

rales, although the exact placement of the anamorph is unclear (3). *S. griseola* is another mitosporic species, but it has an affiliation with the Sarcosomataceae of the Pezizales. The taxonomic position of this species, however, is questionable. It lacks the ability to produce the hexaketide galiellalactone, which is believed to be a chemotaxonomic marker for the family sensu stricto (28). All of these mitosporic forms, therefore, represent nontypical members of their respective taxa. It is to this heterogeneous group that *D. fucicola* appears to be related based on ITS1 and the 5.8S rRNA gene data, although the nuclear LSU rRNA gene analysis identified it as a sister taxon of members of *Didymella*.

All of the nonsporulating isolates except one were members of the Pleosporales, but none of the environmental signals representing the Dothideomycetes exactly matched the signals amplified from these isolates or the herbarium specimen of *D. fucicola*. Therefore, their presence may reflect the existence of novel marine dothideomycete lineages, although it should be noted that many species belonging to this class have not been studied yet at the molecular level (43). Furthermore, the presence of these signals associated with decaying seaweeds suggests a change in fungal populations that could be related to the release of nutrients resulting from tissue breakdown.

Our current understanding of alga-fungus relationships is quite limited, yet a few algal ecological studies have included fungal associations as a significant research component. The adoption of large-scale projects, such as the AFTOL project (http://www.aftol.org) and the international barcoding initiatives (http://www.bolnet.ca/rp_fungi.php), which are rapidly improving the representation of known fungal lineages in sequence databases, provides a framework to link organisms to the processes that they control and to the molecular signals present in the environment. This is important from taxonomic, phylogenetic, and ecological points of view as the proportion of fungi that have been found to be actively associated with marine substrates is greater than previously thought.

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