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A fungal endophyte of black spruce (*Picea mariana*) needles is also an aquatic hyphomycete

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Abstract

An aquatic hyphomycete, *Dwayaangam* sp., was isolated from superficially sterilized black spruce (*Picea mariana*) needles submerged in aerated water in a small glass chamber (microcosm). The internal transcribed spacer (ITS) sequence of this fungus and of a commonly encountered foliar endophyte isolated from *P. mariana* showed a high degree of similarity. When sporulation was induced in the microcosm, both the aquatic hyphomycete and the endophyte isolate produced similar aquatic conidia after 30 days, which is longer than previously documented in similar studies. Without the use of molecular tools, the link between the aquatic and endophytic phases of the fungus would have gone unnoticed. This is the first time that a fungal endophyte of conifer needles has been shown to have an aquatic phase. Its presence both as a foliar endophyte and a sporulating aquatic fungus suggests an alternating life cycle between the two environments.

Keywords: aquatic hyphomycetes, black spruce, *Dwayaangam*, fungal endophyte, ITS, *Picea* spp.

Introduction

Foliar fungal endophytes live inside leaf tissue without showing any macroscopic symptoms of their presence (Stone & Petrini 1997). Most plants are colonized by leaf endophytes and forest trees are no exception (Carroll 1995). It is possible to detect and sample these fungi by plating a small amount of surface-sterilized leaf tissue onto nutrient agar. This technique usually yields the anamorphic (asexual) phase of the endophyte. By sequencing the ITS1, 5.8S and ITS2 regions of the nuclear ribosomal DNA of such isolated fungi, it is possible, in many cases, to identify the family, genus or even species in question (Haug 2002; Guo *et al.* 2003). Despite much research on the subject, the role or function of foliar endophytes is not well understood (Carroll 1995) and these fungi may be mutualistic inhabitants, latent pathogens or play a role in leaf degradation.

Aquatic hyphomycetes are mitosporic (asexual) fungi living and sporulating in flowing freshwater. They constitute an ecological group (Ingold 1942; Bärlocher 1992) rather than a taxonomical one. Over 300 species have been

described; however, only about 10% of these have a known sexual phase (Webster 1992; Sivichai & Jones 2003) and although most are Ascomycetes, the group also includes some Basidiomycetes. The aquatic hyphomycetes are responsible for the degradation of leaf litter in woodland streams (Gessner *et al.* 1993, 1997; Suberkropp 1998). Conidia of a few aquatic hyphomycetes are also found in other habitats such as humid litter and can survive for some time outside the aquatic environment (Sanders & Webster 1978; Bandoni 1981; Sridhar & Kaveriappa 1987; Maamri *et al.* 1998). Aquatic hyphomycetes have also been isolated from living roots of trees such as alder (Fisher & Petrini 1989; Fisher *et al.* 1991; Marvanová & Fisher 1991; Marvanová *et al.* 1992, 1997), poplar, willow (Iqbal *et al.* 1995) and white spruce (Sridhar & Bärlocher 1992a, b). However, to the best of our knowledge, no aquatic hyphomycete has been linked to the fungal endophytes found in needles or other aerial tissues of conifers.

Considering the known relationship between some aquatic hyphomycetes and root endophytic fungi (Sridhar & Bärlocher 1992a, b), and the fact that many of the fungi recorded from *Picea* spp. (Ellis & Ellis 1985) belong to families in which many aquatic anamorphic Ascomycotina are classified [e.g. Rhytismataceae (Descals & Webster 1982),

Leotiaceae, Dermateaceae, Hypocreaceae, Lophiostomataceae and Orbiliaceae (Pfister 1997; Sivichai & Jones 2003)], we hypothesized that endophytic fungi of black spruce needles [*Picea mariana* (Mill.) B.S.P.] could have an aquatic phase. To establish such a link, extensive surveys of both stream and needle inhabiting fungi were necessary (Sokolski 2005). We used both molecular and morphological methods to demonstrate links and at least one endophytic fungus was found to have an aquatic phase.

Materials and methods

Sampling sites

The foliar fungal endophytes were isolated from fresh apparently healthy *Picea mariana* needles collected from mature trees (Sokolski 2005). Twenty sampled sites were located between 46°56'N and 53°45'N, and 69°00'W and 72°00'W, which covers the Québec boreal and mixed wood forests. The samples were collected in late August in 2002 and 2003.

The needles used to investigate a possible aquatic phase of foliar fungal endophytes were collected at the following locations: Valcartier [46°56'N, 71°29'W, 177 m above sea level (a.s.l.)], the Forêt Montmorency (47°20'N, 71°05'W, 750 m a.s.l.) and the Parc-des-Grands-Jardins (47°42'N, 70°51'W, 830 m a.s.l.). The Valcartier site is located in a mixed deciduous/coniferous forest. The Forêt Montmorency site is surrounded by predominantly coniferous species [40% *P. mariana*, 30% *Picea glauca* (Moench) Voss., 20% *Abies balsamifera* (L.) Mill.] with a few deciduous companion species. The Parc-des-Grands-Jardins site is located in a spruce-lichen forest. Trees were sampled at a minimum of 20 m from any stream, four trees were sampled per site, and 18 needles were collected per tree, giving a total of 72 needles per site. Needles were collected monthly from June to September in 2002 and 2003.

The streams sampled for aquatic conidia were the: Valcartier, Noire (Forêt Montmorency), Aux-Canots (Parc-des-Grands-Jardins), Écorces (48°11'N, 71°38'W), Chigoubiche (48°58'N, 73°14'W), Asasuch (52°56'N, 77°17'W), Castor (53°24'N, 77°35'W), Kapichinikaw (53°27'N, 77°35'W) and Mintuwataw (53°42'N, 78°01'W). All stream banks are lined with alder [*Alnus rugosa* (DuRoi) Sprengel]. The Asasuch, Castor, Kapichinikaw and Mintuwataw streams are in black spruce-lichen forest, while the Écorces and Chigoubiche streams are in spruce-feathermoss forest.

Needle disinfection

Needles were briefly rinsed under running distilled water and surface sterilized using a modified method from Bernstein & Carroll (1977) and Arnold *et al.* (2001). Needles were submerged in a 20% bleach (6% sodium hypochlorite,

commercial formulation) solution, to which a drop of Tween 80 (Polyoxyethylene sorbitan monooleate, Sigma Laboratory) was added as a wetting agent, for 2 min; the needles were then transferred to a 75% ethanol solution for 1 min, a 20% ethanol solution for 30 s and then rinsed three times (1 min each) in sterile dH₂O. The solutions were changed after processing the samples from a given site.

Isolation of aquatic fungi from *P. mariana* needles

An aerated (forced sterile air) sterile dH₂O-filled microcosm was used to stimulate the potential development of an aquatic phase of needle endophytes. The microcosms comprised a small glass chamber (diameter, 3.5 cm; height, 12 cm) with a removable lid (Suberkropp 1991). Thirty surface sterilized apparently healthy green needles were placed in each unit and the air-flow through the system was adjusted to provide a gentle bubbling. This isolation method selects for aquatic hyphomycetes and discriminates against terrestrial fungi. Needles were incubated in the microcosm in a growth chamber (15 °C, 8 h light : 16 h dark photoperiod). To assess the efficacy of the sterilization process, 10 needles per site were incubated on 2% malt agar (Becton Dickinson). Microcosms with sterile water only and microcosms with fully autoclaved needles served as controls. The water in the microcosm was first changed after 24 h and then twice a week. A 1-mL aliquot of the liquid removed from each microcosm was placed on a glass microscope slide, dried on a hot plate, stained with acid fuchsin (Descals 1997) and observed using a phase-contrast microscope (Polyvar, Reichert-Jung). If aquatic hyphomycete-type conidia were present, a further 1 mL aliquot was used to inoculate standard Petri plates containing 0.1% malt agar amended with penicillin and streptomycin sulphate (Descals 1997) and a 4-mL aliquot was stabilized in FAA (formaldehyde : acetic acid : ethanol, 1/3/16, v/v/v) for future reference. Further observations were made using a stereomicroscope (Nikon SMZ800) and an inverted microscope (Olympus CK2) at ×100 and ×250. Within 4 h, individual conidia from the inoculated Petri dishes were located using an inverted microscope at ×100 and collected using a micropipette (modified Pasteur pipette, 0.5 mm diameter) (Descals 1997). The isolated conidium attached to the 0.5-mm agar plug was transferred to a standard Petri dish containing 2% malt agar. Growth of isolates was regularly monitored.

Collection, observation and isolation of conidia of aquatic hyphomycetes from streams

Conidia of aquatic hyphomycetes are trapped in naturally occurring foam formed at the surface of fast-flowing streams. Samples of foam were collected using a fine-mesh net (10 cm diameter) attached to a wire frame. For observation,

a 15–20 mL aliquot of foam was placed in a small jar with a few drops of FAA. A 1-mL aliquot of this solution was placed on a glass microscope slide, dried on a hot plate and stained with acid fuchsin (Descals 1997). For conidia isolation, a 1- mL aliquot of foam was used to inoculate standard Petri dishes containing 0.1% malt agar and antibiotics (as above) (Descals 1997). Conidia were isolated as described above. The ITS region of the resulting pure cultures were sequenced in order to gain an insight into the diversity of aquatic hyphomycetes present in streams surrounded by *P. mariana*.

Induction of the aquatic phase of endophytic fungi grown on solid media

To induce the production of aquatic conidia from cultures of pure foliar fungal endophytes grown on malt agar, pieces of agar (5–8 × 20 mm) cut from the colony were incubated in aerated sterile distilled water in the microcosm system described above (Descals 1997). Pure cultures of over 100 morphotypes of needle endophytes collected from *P. mariana* (Sokolski 2005) were routinely submitted to conditions suitable for induction of conidia by aquatic hyphomycetes. The microcosms were incubated for 14 days, which is twice that of most other studies (Fisher *et al.* 1991). In the case of isolates showing strong internal transcribed spacer (ITS) region sequence similarity (see below) with aquatic hyphomycetes, the incubation period was extended. Conidia isolation was as above. To avoid cross-contamination, induction trials were never done concurrently with the incubation of surface sterilized needles. The entire process is outlined in Fig. 1.

Spore trapping

To investigate possible correlations between aquatic fungi isolated from surface sterilized needles incubated in the microcosm system and the fungi colonizing and degrading similar needles in forest streams, small traps containing needles were submerged in two of the sampled streams: the Valcartier and the Noire. The traps were made by removing and discarding a 6-cm disc from the centre of the top and bottom sections of a standard Petri dish. The two sections were placed back to back. In between these was inserted a sandwich comprising a sheet of coarse nylon netting (1.25 mm mesh), a sheet of finer nylon netting (0.5 mm mesh), 100 fresh *P. mariana* needles (10–12 mm long), a further sheet of fine netting and a sheet of the course netting. The system was then screwed together (Sokolski 2000). The resulting framed bag offered a better contact with the surrounding environment and reduced clumping of the needles, which is a common problem when mesh bags alone are used. The trap was submerged in the two streams in mid-September at a depth of about 30 cm. The

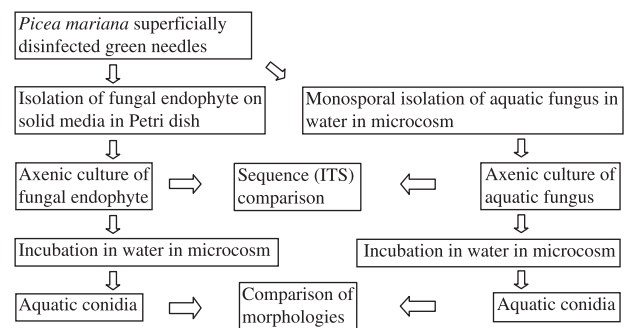


Fig. 1 Protocol used to demonstrate the aquatic and endophytic phases of a fungus.

traps were positioned parallel to the direction of water flow, and attached within hollow concrete blocks to avoid movement and accumulation of debris. Three traps per site were removed after 4, 8, 12, 30, 40 and 52 weeks. The traps were disassembled, and the needles rinsed with distilled water and incubated for 2 weeks in aerated microcosms containing sterile dH₂O. The microcosms were emptied and refilled twice a week, the liquid removed was filtered (Whatman Nucleopore, 5 µm) and any conidia remaining on the filters were stained using a solution of 0.1% trypan blue in 60% lactic acid (Gessner *et al.* 2003).

DNA extraction

DNA was extracted from a 3–5 mm² plug cut from each axenic fungal culture using a 2% cetyltrimethyl ammonium bromide (CTAB) procedure modified from Zolan & Pukkila (1986). Briefly, each plug was crushed in 300 µL of a 2% CTAB solution containing 0.2% β-mercapto-ethanol and incubated at 65 °C for approximately 1 h. The mixture was then emulsified with a 300 µL aliquot of phenol : chloroform : isoamyl alcohol (25/24/1, v/v/v), and centrifuged at 10 000 r.p.m. 9352 g for 10 min. The supernatant was collected, precipitated with 300 µL of cold isopropanol, centrifuged at 5000 r.p.m. 2338 g for 10 min, rinsed with ethanol 70%, dried and resuspended in 50 µL TE-8.

Polymerase chain reaction and sequencing

rDNA was amplified using the universal primer ITS4 (White *et al.* 1990) and the universal fungal primer ITS1F (Gardes & Bruns 1993). The amplification was done on a MJ Research PTC-100 thermocycler. The polymerase chain reaction (PCR) mixture (25 µL) contained 20 mM Tris (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 0.25 mM of each dNTP, 1 µM of each oligonucleotide primer, 1 U *Taq* polymerase (Invitrogen), and 3 µL of a 1 : 10 dilution of c. 10 ng/µL genomic DNA. PCR was as follows: 3 min at 94 °C; 40 cycles at 92 °C for 1 min, 56 °C for 1 min, 72 °C for 1.5 min,

Table 1 Conidia of aquatic hyphomycetes observed or isolated in 2002 and 2003 from surface-sterilized green *Picea mariana* needles incubated in aerated sterile distilled water in microcosms

Sampling month	Site (stream)	<i>Dwayaangam</i> sp.		<i>Tripospermum</i> spp.		<i>Retiarius</i> sp.	
		2002	2003	2002	2003	2002	2003
June	Valcartier	+	isolated	Tc+	Tc+	-	+
	Montmorency	+	isolated	-	Tc+	-	+
	Grands Jardins	NS	NS	NS	NS	NS	NS
July	Valcartier	-	+	-	Tm+	-	+
	Montmorency	-	-	-	-	-	-
	Grands Jardins	NS	+	NS	Tc+	NS	+
August	Valcartier	-	-	-	-	-	-
	Montmorency	+	-	-	-	-	-
	Grands Jardins	+	-	-	-	-	-
September	Valcartier	-	-	-	-	-	-
	Montmorency	-	-	-	-	-	-
	Grands Jardins	-	-	-	-	-	-

Tripospermum camelopardus (Tc), *T. myrti* (Tm), not sampled (NS), present but not isolated (+), absence (-).

followed by 10 min at 72 °C. Electrophoresis gels [1.4% agarose LE (Roche Diagnostics) in TAE 1× (Gibco)] were stained with ethidium bromide and photographed under UV light. The amplified DNA was then purified using QIAquick PCR Purification Kit (QIAGEN) for sequencing.

Sequencing and sequence analysis

Sequencing was done on a Genetic Analyser ABI PRISM 3100 (Applied Biosystem) sequencer. Resulting sequences were aligned with SE-AL (Rambaut 1996) and CLUSTAL_X version 1.83 (Thompson *et al.* 1997), and the lengths adjusted to 484 bp to include part of the 18S, the complete ITS1, the 5.8S, the ITS2 and part of the 28S. Molecular identification was performed with maximum parsimony using PAUP version 4.0b10 (Swofford 2002). The PAUP settings comprised a heuristic search with default parsimony settings, a random starting tree was obtained by 100 random stepwise-additions and TBR (tree-bisection-reconnection) branch-swapping; the number of trees saved automatically was increased by 100; all characters were unordered, equally weighted and gaps treated as missing; bootstrap analysis was done with 1000 resamplings and 100 additional sequence replicates to estimate the robustness of the trees. Bayesian analysis was performed using MRBAYES version 3.0b4 (Huelsenbeck & Ronquist 2001), four Markov chain were run over 1 million generations, sampling trees every 100th generation (10 000 trees saved) and the burn-in value was set to 1000. The number of substitution type (nst) was set to 6 (GTR model). Sequences of fungal endophytes and aquatic hyphomycetes were deposited in GenBank/EMBL. They were compared in a BLASTN with sequences in GenBank and those from the authors' aquatic hyphomycetes collection.

Results

Aquatic fungi isolated from surface sterilized Picea mariana needles

Surface sterilized *Picea mariana* needles incubated in microcosms yielded four aquatic hyphomycetes: *Dwayaangam* sp. (description in progress), *Tripospermum camelopardus* Ingold, Dann & McDougall, *T. myrti* (Lind.) Hughes, and a *Retiarius* sp. The first staurosporous conidium of *Dwayaangam* sp. appeared after 10 days of incubation and a monosporal culture (V3.13) was successfully subcultured. Another conidium (V3.10B) was isolated 5 months later in the same microcosm. Isolates V3.13 and V3.10B shared identical ITS sequences (Fig. 2) and colony morphology. Conidia of *Dwayaangam* sp. were not observed in microcosm from needles collected after late August (Table 1). Occurrence of the encountered aquatic conidia from needles is listed in Table 1.

The axenic culture of *Dwayaangam* sp. V3.13 was re-incubated in a microcosm to re-initiate the production of conidia. Twenty conidia were collected over a 28-day period. These conidia exhibited the same morphology as that used to establish the original isolate.

Comparison of ITS region sequences from endophytic and aquatic fungi

The ITS sequences (approximately 600 bp long with a cleaned section of 484 bp) of the aquatic fungi V3.13 and V3.10B, isolated from surface sterilized needles incubated in the microcosm system, showed a very high degree of similarity (482/484 bp) with 10 isolates (4.37, T5.2, T5.20, T5.26, 7.37, T7.18, 10.44, 10.48, T24.8, T24.16 [Table 2]) of

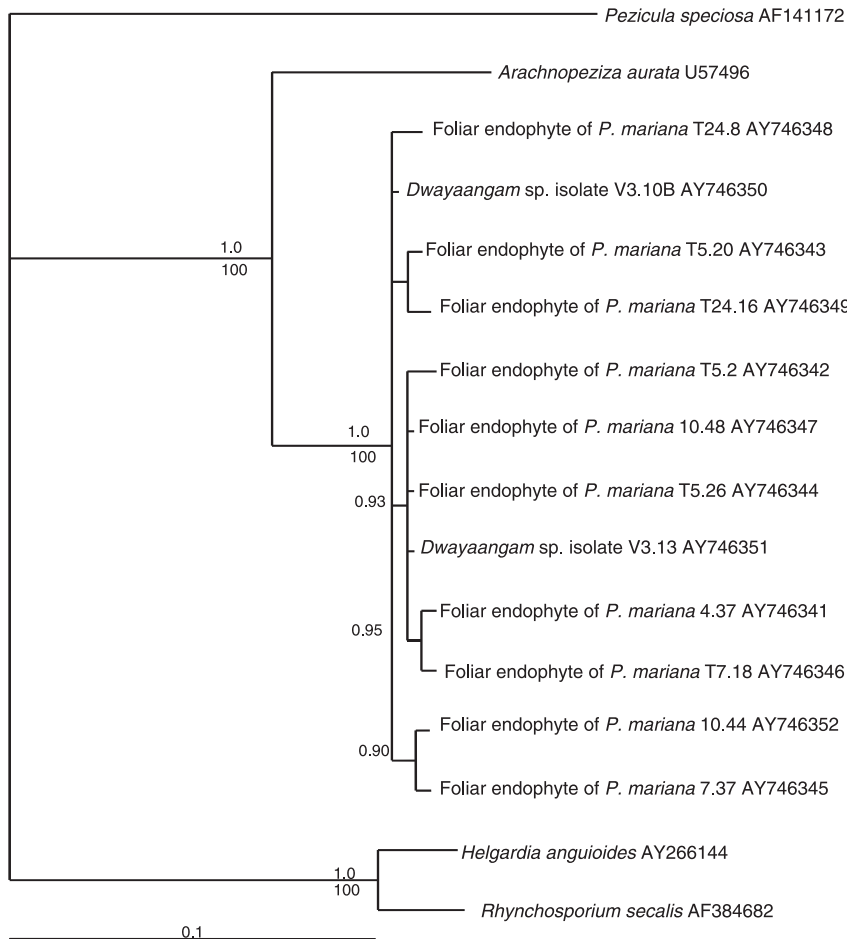


Fig. 2 Phylogenetic analysis of nuclear ribosomal DNA ITS1-5.8S-ITS2 sequences of *Dwayaangam* sp. and fungal needle endophytes using MRBAYES (1 000 000 generations) and PAUP heuristic search (1000 replicates). The Bayesian analyses resulted in a consensus tree of $-ln = 1685.95$. The heuristic search conducted under the maximum-parsimony criteria resulted in 1200 equal trees of 213 steps (RI = 0.8960, CI excluding uninformative characters = 0.8960). Bayesian values ($> 90\%$) are indicated above and the bootstrap percentage ($> 80\%$) below each branch of the Bayesian tree. V3.13's closest ITS sequences available in GenBank/EMBL (November 2005) are included. Scale indicates 0.1 base substitution per site.

Table 2 Strain number, isolation method and GenBank sequence accession number of ITS region sequences of aquatic hyphomycetes and fungal endophytes isolated from superficially disinfected *Picea mariana* needles

Strain	Staurosporous conidia observed in microcosm	Isolation method	GenBank accession number
4.37	Yes	Malt agar (solid media)	AY746341
T5.2	Yes	Malt agar (solid media)	AY746342
T5.20	No	Malt agar (solid media)	AY746343
T5.26	No	Malt agar (solid media)	AY746344
7.37	Yes	Malt agar (solid media)	AY746345
T7.18	No	Malt agar (solid media)	AY746346
10.44	Yes	Malt agar (solid media)	AY746352
10.48	No	Malt agar (solid media)	AY746347
T24.8	No	Malt agar (solid media)	AY746348
T24.16	No	Malt agar (solid media)	AY746349
V3.10B	Yes	Microcosm (water)	AY746350
V3.13	Yes	Microcosm (water)	AY746351

the third most frequently isolated black spruce needle endophyte in 2002 and in 2003 (Sokolski 2005). The endophyte in question occurred in 60% of the sites sampled and accounted for 9% of the isolations. This endophyte shows a 90% ITS similarity (446/484 bp) with that of an *Arachnopeziza* sp. in GenBank. The ITS sequences closest to V3.13 in GenBank/EMBL were included in the phylogenetic analysis (Fig. 2). They were: *Helgardia anguioides* AY266144 (231/484 bp similarity), *Rhynchosporium secalis* AF384682 (231/484 bp similarity). *Pezicula speciosa* AF141172 was used as the outgroup. The unnamed fungus R20 AY699674 (326/484 bp similarity) was not included. More distant sequences were too different and too difficult to align in the ITS1 and ITS2 sections and were not included. The Bayesian tree and the parsimony tree showed identical topologies (Fig. 2) when nodes with bootstrap values $> 50\%$ and Bayesian posterior probabilities > 0.95 were considered.

Aquatic hyphomycetes from streams

Staurosporous conidia from *Dwayaangam* sp. were observed in foam from the following streams: Chigoubiche, Asasuch,

Castor and Kapichinikaw. Isolation of these conidia from the foam was unsuccessful.

Aquatic phase induction of endophytic fungi

None of the endophyte isolates obtained on solid malt agar media produced conidia in the microcosm system after the normal 14-day incubation period. Most showed little or no sign of growth. Those isolates that did grow exhibited only swollen cells and hyphae.

Because of the ITS sequence similarity between V3.13 and V3.10B, and the black spruce needle endophytes 10.44, 4.37, 7.37, T5.2, the latter were incubated in microcosms for a longer period. Conidia were produced; however, these appeared after an average of 31 days, 16 days beyond our routine programme, and 28 days after the usual time in other studies (Marvanová *et al.* 1997). The production of typical aquatic conidia lasted for 1 month. The rather low sporulation rate gave a peak production of two conidia per mL of water per day, with an average of one conidium per mL. No conidia were produced after 2 months incubation.

Comparisons of conidial morphology

The staurosporous conidia of V3.13 and V3.10B isolated from surface sterilized *P. mariana* needles in the microcosm system were identical to the aquatic conidia produced by the foliar endophyte cultures 10.44, 4.37, 7.37 and T5.2 incubated in the same system. They were also morphologically identical to the conidia observed in stream-collected foam. Axenic cultures on malt agar showed similar growth rate, morphology, texture and colour.

Aquatic hyphomycetes observed on stream-incubated P. mariana needles

During the first 3 months of incubation in the streams, the dominant aquatic hyphomycete obtained from the needles was *Heliscus lugdunensis* Sacc. & Therry and *Flagellospora curvula* Ingold was occasionally observed. After 1 year, the following species (in decreasing order of importance) were found: *H. lugdunensis*, *F. curvula*, *Lunulospora curvula* Ingold, *Flagellospora minuta* SH Iqbal & Bhatti, *Culicidospora aquatica* RH Petersen, *Alatospora acuminata* Ingold, *Tricladium attenuatum* SH Iqbal, *Tricladium fuscum* Nawawi, *Anguillospora crassa* Ingold, *Anguillospora longissima* (Sacc. & P Syd.) Ingold, *Anguillospora* sp. and *Filospora* sp. Conidia of *Dwayaangam* sp. were not observed on needles incubated in the spore-trapping devices in the two streams.

Discussion

The third most common needle endophyte of black spruce in the boreal forest of Québec shows a very high ITS rDNA

sequence similarity (99%) with an aquatic hyphomycete in the genus *Dwayaangam* (V3.13 and V3.10B; Fig. 2). The phylogenetic analysis placed the isolates together in a well-supported clade representing a single species (the species is presently being described). Without the use of sequence comparisons, this link would have gone unnoticed, demonstrating the usefulness of DNA sequences as a tool for comparing unknown fungi isolated from different habitats. Furthermore, testing a large collection of endophytes for their possible ability to form an aquatic phase in microcosms over a long incubation period is, practically speaking, unfeasible. Once the link between the needle endophyte and the aquatic hyphomycete was established, it was easy to confirm using morphological methods, as both produced similar conidia under the microcosm system if given a sufficiently long incubation period. Specific primers are valuable tools allowing the detection of a known fungus that is difficult or impossible to isolate. However, the use of these tools to detect the presence of the fungus in the fresh needles was not necessary as the *Dwayaangam* sp. was easily isolated on solid growth media. The only known teleomorph of a *Dwayaangam* species is an *Orbilium* sp. (Kohlmeyer *et al.* 1998). None of the available sequences of *Orbilium* spp. were close enough to those of the V3.13 and V3.10B isolates to be aligned and they were not included in the phylogenetic analysis.

Of the four potentially aquatic fungi isolated from disinfected black spruce needles, *Dwayaangam* sp. V3.13 and V3.10B were the only ones successfully cultured. The genus *Retiarius* has two species originally described as phyllosphere fungi, and parasitic on wind-borne pollen grains (Oliver 1978). It has been reported in stream foam (Descals *et al.* 1995). Isolation of the two *Tripaspermum* spp. failed. It was not surprising to find these species on conifers, as a *Tripaspermum* sp. has already been reported on decaying leaves of *Pinus densiflora* (Sinclair & Morgan-Jones 1979). However, its endophytic status is questionable.

Aquatic hyphomycetes were first reported to decompose conifer needles in streams by Bärlocher & Oertli (1978a). This finding was later supported by observations by Iqbal *et al.* (1980). Needles are a difficult substrate to breakdown: natural inhibitors such as phenolic compounds and a high lignin content slow fungal colonization (Bärlocher & Oertli 1978a, b; Bärlocher *et al.* 1978; Michaelides & Kendrick 1978; Bärlocher 1982; Robinson *et al.* 2000). The aquatic hyphomycete species observed on needles in the spore trapping devices in the present study are among the most common and follow the same colonization patterns as reported for white spruce needles (Bärlocher 1982). We did not detect the typical staurosporous conidia of the *Dwayaangam* sp. on needles in the spore traps but they were observed in foam samples from streams flowing through *Picea mariana* stands. Although molecular tools have been useful in the detection of aquatic hyphomycetes

(Nikolcheva & Bärlocher 2004), we did not use this approach to identify those species growing on the needles in the traps. Not finding the *Dwayaangam* sp. in the traps does not exclude this species from being a needle decomposer. It is a commonly encountered black spruce needle endophyte and it may take advantage of this by rapidly colonizing needle tissue just prior to needle drop or immediately after falling into a stream. However, heavily sporulating aquatic hyphomycetes made it impossible to isolate this fungus, which appeared to produce relatively few conidia. A common Norway spruce needle endophyte has been found as a minor saprophyte after needle cast (Müller *et al.* 2001). This may or may not be the case for the *Dwayaangam* sp. We can only hypothesize as to its potential role in the degradation of black spruce needles.

The present study using two different techniques (molecular and morphological) shows that *Dwayaangam* sp. V3.13 definitely occurs in *P. mariana* needles. It has been isolated from tissues collected from trees that were not adjacent to streams. Of the aquatic hyphomycetes previously reported to have an endophytic phase, all were isolated from roots of plants growing along riverbanks (Fisher & Petrini 1989; Fisher *et al.* 1991; Marvanová & Fisher 1991; Marvanová *et al.* 1992, 1997; Sridhar & Bärlocher 1992a, b; Iqbal *et al.* 1995). The needle endophyte phase of *Dwayaangam* sp. V3.13 has been found on *P. mariana* throughout its geographic range in Québec. Its aquatic phase has been observed in northern and southern rivers of the Québec boreal forest (Sokolski 2005). We cannot clearly establish the path of needle colonization, but we presume that, as many endophytes, this species is relatively persistent and may be found in needle, twig, branches and stem tissue. The fact that the *Dwayaangam* sp. was present both as a needle endophyte and as a sporulating aquatic fungus, suggests the occurrence of the following colonization pathways: colonization of living needles is initiated by the germination of ascospores (if a sexual phase exists) or aerial conidia, or through mycelial extension from tree top to lower branches as observed in *Pinus strobus* L. endophytes (Deckert *et al.* 2002). Senescent needles that have fallen into streams are then subject to further development of the fungus, as well as colonization by other saprophytic fungi, and aquatic conidia are released. The extent to which these conidia allow the colonization of new resource units within a stream is still unknown. It is not known if this *Dwayaangam* sp. actively participates in the decomposition of needles, but we may assume that its conidia, carried by water droplets, could themselves also be a new source of inoculation of living needles.

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