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Out of the rivers: are some aquatic hyphomycetes plant endophytes?

Ingoldian fungi, or aquatic hyphomycetes, are asexual microfungi, mostly ascomycetes, commonly occurring in running freshwater. They grow on dead plant material, such as leaves and twigs, and play a major role in nutrient flows in stream ecosystems (Bärlocher, 1992). They were discovered and first extensively

studied by Ingold (1942) and were thus named 'Ingoldian' fungi. Ingold described their abundant multicellular asexual spores of sigmoid or, more typically, tetra-radiate shape (Fig. 1). He recognized that they probably arose from multiple convergent evolutions, by secondary adaptation to aquatic life, as recently confirmed by molecular markers (Belliveau & Bärlocher, 2005; Baschien *et al.*, 2006). Because of their apparent lack of sexuality, Ingoldian species were placed in asexual genera, such as *Tricladium* or *Tetracladium*, based on conidial morphology and/or mode of conidiogenesis. Unexpectedly, several lines of evidence now suggest that some Ingoldian fungi are also plant endophytes, that is, they grow in plants without producing symptoms.

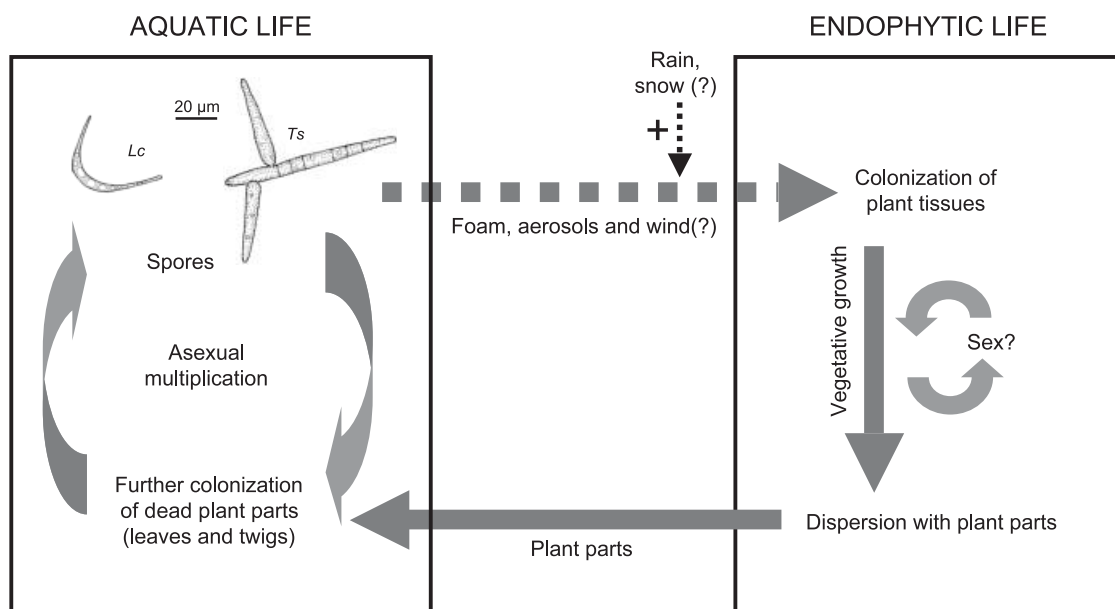


Fig. 1 Life cycle for aquatic fungi with an endophytic stage. The mechanism of colonization of plants from water is still unknown (dotted lines). Note the shape of aquatic asexual spores: sigmoid (*Lc*, *Lunulospora curvula*) or tetra-radiate (*Ts*, *Tricladium splendens*; modified from Webster, 1959).

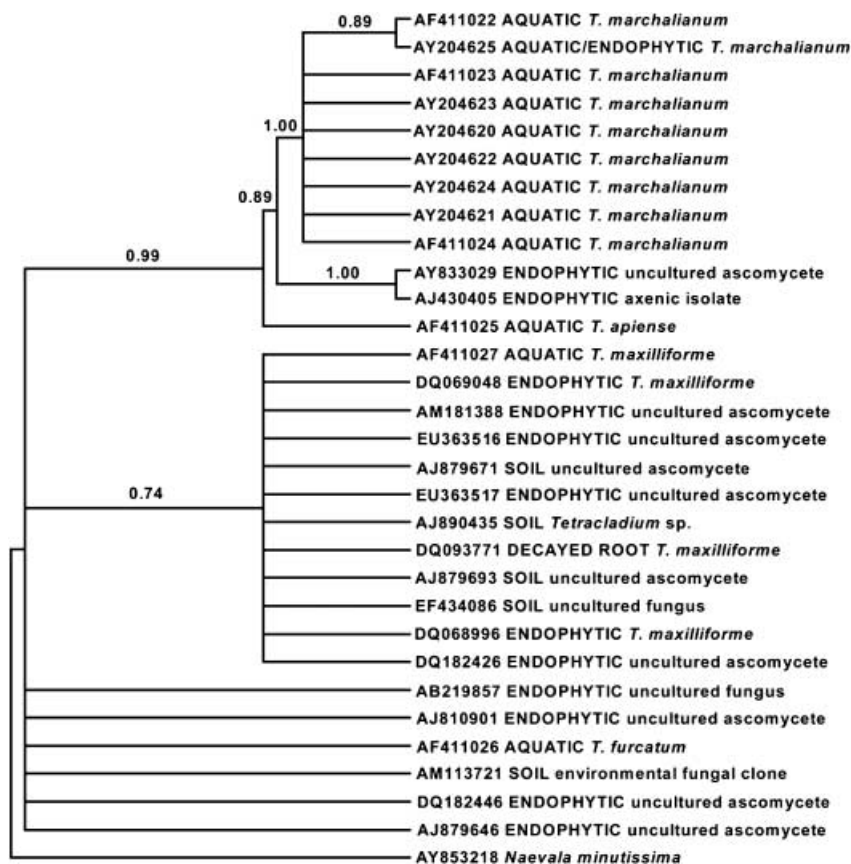


Fig. 2 Phylogenetic relationships within *Tetracladium* spp., according to a Bayesian analysis of an alignment of internal transcribed spacer (ITS) sequences available in GenBank. The reported ecology (aquatic, endophytic or from soil) and name are indicated. The topology was rooted with *Naevaia minutissima*. Numbers on branches are estimates for a *posteriori* probabilities that the respective groups are monophyletic given the data (whenever > 0.7; less supported branches were collapsed). Note a well-supported (0.99) clade with a mix of endophytic and aquatic sequences.

Aquatic fungi in terrestrial plants!

DNA sequences related to Ingoldian fungi were recently recovered from healthy-looking plant tissues by several 'naïve' researchers, including the authors, who were identifying plant-associated fungi using molecular tools (Fig. 2). *Tetracladium*-related sequences were found during an investigation of ectomycorrhizal fungal communities in *Tuber magnatum* producing stands (Murat *et al.*, 2005), in the liverwort *Marchantia foliacea* (Russell & Bulman, 2005), and in roots of the orchid *Cephalanthera longifolia* (Abadie *et al.*, 2006) and of ericaceous plants (Tedesoo *et al.*, 2007). Interestingly, in these studies, Ingoldian sequences were found among cloned polymerase chain reaction (PCR) products, that is, among other more dominant sequences. Several strains related to *Gyoeffiyella* (a genus known to encompass river fungi; Czezuga & Orłowska, 1997) were isolated from healthy ectomycorrhizal *Picea abies* roots (GenBank accession numbers EF093184-5, EF093150; M. Vohník *et al.*, unpublished). Such unexpected findings probably often went unpublished, as happened with dark septate endophytes found in roots before the 1990s (Jumpponen & Trappe, 1998). The major aim of this paper is to call for more reports, and to convince referees and editors of their potential validity.

The occurrence of Ingoldian fungi in plant tissues is neither really surprising nor a newly reported finding. First, their presence has been shown in roots growing in water or on riversides: in all, more than 35 aquatic fungal species were recovered from submerged roots of 13 different plant species (Sati & Belwal, 2005; Bärlocher, 2006). Secondly, without added inoculum, Ingoldian fungi appear on leaves collected from trees and submersed in distilled water: this suggested to Bandoni, more than three decades ago, that some Ingoldian species occur as endophytes in aerial plant tissues (see Bandoni, 1972, and references therein). In streams, the well-known rise in diversity and abundance of Ingoldian fungi in early autumn (Fabre, 1998) may at least partly be caused by dead leaves falling into streams. Indeed, the role of this inoculum versus that of profuse spores released by decomposing leaves still requires testing.

Now, molecular data further support this view and suggest that relatives of Ingoldian fungi occur endophytically far from riversides. However, does the close similarity of endophytic and Ingoldian sequences actually demonstrate that aquatic fungi can grow in plants? Can we rule out the possibility that ungerminated spores may account for the successful isolation of a fungal strain or DNA, without endophytic growth? Indeed, these fungi are more abundant in peripheral tissues, such as bark

(Bärlocher, 2006), a feature expected for surface contaminations. Sokolski *et al.* (2006) recently provided direct evidence for dual ecology of aquatic hyphomycetes, as well as for the colonization of aerial parts of plants. They isolated strains of a *Dwayaangam* sp. from surface-sterilized spruce (*Picea mariana*) needles both after culture on solid agar medium and after incubation in a flow chamber designed to mimic a running water environment. Similar ribosomal DNA sequences (internal transcribed spacers (ITS)) were obtained from the various strains. Furthermore, after incubation in flow chamber, solid agar strains produced asexual spores identical to those of aquatic strains.

Although limited DNA-related data are currently available, ITS sequences of Ingoldian fungi from endophytic and aquatic environments are intermingled in phylogenetic trees, as shown here for *Tetracladium* (Fig. 2). If the related fungi did not have dual ecological abilities, this would imply repeated ecological transitions during evolution – an unlikely scenario. Although some species are exclusively endophytic or aquatic, ancestors with both abilities present a more parsimonious explanation. Endophytic life can be a more or less active ‘waiting stage’, allowing faster colonization of dead plant material under wet conditions. A similar scenario was hypothesized for a major group of terrestrial plant-decaying fungi, the Xylariales, when they turned out to be common plant endophytes (Davis *et al.*, 2003). However, it is currently difficult to establish the ancestral niche of these clades: we may well be dealing with either aquatic saprotrophs that secondarily colonized living plants or a niche extension of biotrophic endophytes to saprotrophy. In both cases, niche enlargement improves the utilization of host resources and allows temporary relief from intraspecific competition. Indeed, ascomycetes often associate with plants, making the second scenario parsimonious: for example, *Tricladium* and several *Anguillospora* species belong to the Helotiales, which are well known as plant endophytes (Vrålstad *et al.*, 2002).

More direct evidence for dual ecological abilities is needed for other Ingoldian species, as well as visualizations of these fungi in plant tissues. It is now time to look for Ingoldian fungi as endophytes in terrestrial plants – as early as 1972, Bandoni speculated that ‘many more [Ingoldian] species will be found on land’. It is also relevant to determine how many independent fungal lineages underwent convergent evolution to this dual mode of life. This raises an intriguing question: Ingoldian fungi can be introduced to water by dead plants, but how do they ‘go back’ from water to plants? This question largely remains to be tested using experimental and theoretical approaches.

Flying from water to plants?

Perhaps the most exciting issue is the adaptive value of the four-branched or sigmoid shape of asexual spores of many Ingoldian fungi (Fig. 1). This was a subject of debate in the

1950–1960s, and Ingold (1953) suggested three possibilities: (i) improved buoyancy, favouring aquatic dispersal; (ii) improved anchoring on potential substrata; and (iii) decreased palatability to aquatic animals. Although elaborate appendages increase surface area and buoyancy, as in many planktonic organisms, they are unlikely to be of much use in flowing freshwater where sedimentation is slower than flow, and impaired by turbulence. Webster (1959) experimentally demonstrated that tetradiate spores, having three-point attachment, are better trapped by various substrates on which they can develop. Several works showed that Ingoldian fungi increased the palatability of plant remains (Bärlocher, 1992), suggesting that the shape of the profuse conidia produced on leaf surfaces is not deterrent. Thus, the ‘better trap’ hypothesis seems the most probable of these three possibilities.

There are, however, two very unexpected series of observations. First, Ingoldian fungi spores are very frequent in river foam, perhaps because they are trapped at the surface of air bubbles as a result of lowered surface tension. Foam is indeed the best place to observe Ingoldian spores. Introduction of air bubbles to a suspension of Ingoldian spores removes them (Iqbal & Webster, 1973), especially those with four-part expansions. Secondly, spores of these fungi were found in unexpected terrestrial environments, such as snow (Tubaki, 1960; Czczuga & Orłowska, 1999) or rainwater (Czczuga & Orłowska, 1997, 1999)! We propose here to reconcile these observations within a single adaptation: the shape of Ingoldian asexual spores has evolved not only to disperse in water, but also to concentrate in bubbles and foam, to favour their capture by wind or aerosols. In this aerial phase, expansions would also limit sedimentation and final landing, which could sometimes be enhanced by rain or snow (Fig. 1).

This proposal accounts for the colonization of terrestrial plants, but also for another paradox. Simple dispersal by water stream would imply a progressive migration of population downstream and, at least, a diversity gradient (higher downstream than upstream). However, limited to undetectable spatial differentiation was observed along stream communities (a plateau in species diversity can be reached within 50 km downstream of the river source; Fabre, 1998) or populations (Peláez *et al.*, 1996). There are even claims for possible transfer between streams (Laitung *et al.*, 2004). A variety of compensatory mechanisms for the continuous drift of fungal propagules have been proposed, including transport by animals after ingestion, transport by wind of putative sexual spores or transport of asexual spores in water droplets (Iqbal & Webster, 1973; Bärlocher, 1992). The extent to which such processes occur in nature remains unknown.

While the survival of asexual spores in aerosols and air remains to be addressed, one should keep in mind that, with large spore numbers in stream water (up to 30 000 l⁻¹; Webster & Descals, 1981), even a low survival rate might facilitate aerial colonization of plants, as well as transport both upstream and between rivers.

Future directions

Ingold (1966) stated that, for Ingoldian fungi, the 'whole life history, vegetative growth, spore production and spore liberation and local dispersal, takes place in water.' We propose instead that some of them spend a part of their life in plants, and have a planktonic, aquatic and aerial, dispersal. It can be speculated that the use of water for dispersion preceded a truly aquatic life, and that species with true aquatic abilities arose secondarily. However, we need to know more, in a phylogenetic background, about the biology of several Ingoldian species – an ambitious goal. Clear testing of the hypothesis of a dual life style is needed for more strains of various phylogenetic origins by evaluating aquatic and endophytic growth in realistic conditions, as in the work by Sokolski *et al.* (2006). Finally, detection (and survival) of spores in air could support our model of the life cycle (Fig. 1).

What is the impact of Ingoldian fungi on host plants? Do they have some protective effects, at the tissue or whole-plant level, as described recently in *Theobroma cacao* for other endophytic fungi (Arnold *et al.*, 2003)? Where do they grow and how do they receive carbon in tissues? More studies on the endophytic phase of Ingoldian fungi are thus necessary to visualize and understand their interaction with the host, and we may observe a range between mutualism and parasitism, including neutral commensalism (Saikkonen *et al.*, 1998). On the fungal side, endophytic life may be the place for sex. Only 10% of Ingoldian fungi are currently connected to sexual species (Sivichai & Jones, 2003), but many more could be sexual. Interestingly, no linkage disequilibrium was found in *Tetracladium elegans* (Laitung *et al.*, 2004), suggesting that sexual recombination occurs in this species. Saprophytic growth, which has been thoroughly investigated, may not allow sexual differentiation, as for ectomycorrhizal fungi, which produce meiotic spores only when associated with their plant hosts. The design of microsatellites (e.g. on *Tetracladium marchalianum*; Anderson *et al.*, 2006) opens the way for comparison of endophytic and aquatic populations, as well as modes of reproduction.

A side issue is that we should not perhaps be so surprised to read about aquatic fungi in snow (Czeczuga & Orłowska, 1997) or in orchids (Abadie *et al.*, 2006); we simply have to imagine a broader niche. Ingoldian fungi challenge our simple views of fungal ecology and saprotrophy. This and other reports, such as those of wood-decay fungi in living fine roots of conifers (Vasiliauskas *et al.*, 2007), or of *Xylariaceae* as endophytes of various liverworts and angiosperms (Davis *et al.*, 2003), strongly question the (vanishing) separation between biotrophy and saprotrophy in fungi.

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