Distribution and dispersal of *Xylaria* endophytes in two tree species in Puerto Rico

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Xylaria species are common endophytes in tropical plants. It is not known, however, whether transmission of *Xylaria* occurs horizontally or vertically, whether individual *Xylaria* strains have wide host ranges or are host-specific, or how they are dispersed. We compared frequency of *Xylaria* endophytes in leaves and seeds of two tree species in Puerto Rico, *Casuarina equisetifolia* (Australian pine) and *Manilkara bidentata* (ausubo). These trees were chosen because they differ markedly in morphology, habitat, distribution, and origin. In *C. equisetifolia Xylaria* was significantly more frequent in leaves than in seeds. *Xylaria* was isolated from seeds of trees in inland parks, but never from seeds of trees growing on beaches. This suggests that vertical transmission of *Xylaria* may be possible but is not necessary for infection. In *M. bidentata, Xylaria* was isolated from 97% of leaves but was never isolated from seeds, suggesting that transmission is entirely horizontal. Seedlings raised in a greenhouse far from other *M. bidentata* trees had a level of *Xylaria* infection as high as seedlings in the forest, suggesting that inocula can come from other sources and endophytic strains are not host-specific.

Xylaria species are common endophytes in many tropical plants, including palms, orchids, bromeliads, aroids, and ferns (Dreyfuss & Petrini, 1984; Rodrigues & Samuels, 1990; Rodrigues, 1994; Richardson & Currah, 1995). In Puerto Rico, *Xylaria* endophytes have been found in rain forest trees (Lodge, Fisher & Sutton, 1996) and orchids (Bayman *et al.,* 1997).

Many questions about the biology of endophytic *Xylaria* species remain unanswered, especially with regard to colonization of hosts (Lodge & Cantrell, 1995; Petrini, 1996). We address two such questions here. Firstly, does transmission of *Xylaria* to new host plants occur horizontally or vertically? Horizontal transmission is passage of inoculum from one individual to another; vertical (or seed-borne) transmission occurs from one generation to the next via the germ line (Voyles, 1993). Mode of transmission affects the efficiency of infection of new plants and the potential for co-evolution of plant and fungus (Clay, 1988, 1994). Vertical transmission has been established for some clavicipitaceous endophytes of grasses (Clay, 1994). Secondly, are *Xylaria* endophytes host-specific? Host specificity affects efficiency of infection, the ecology of both host and fungus, and could affect how host plants respond to habitat fragmentation if *Xylaria* endophytes are mutualists (Lodge & Cantrell, 1995).

Here we study transmission of *Xylaria* endophytes in two tree species in Puerto Rico, *Casuarina equisetifolia* L. and *Manilkara bidentata* (A.DC.) A. Chev. These trees were chosen because both are known to contain endophytes (Bose, 1947; Lodge *et al.,* 1996), yet they differ greatly in habitat, morphology, geographic origin, and reported diversity of the endophytic biota (Table 1).

Although *Xylaria* endophytes have been isolated from leaves of many tropical plants (Rodrigues & Samuels, 1990; Petrini, 1991; Rodrigues, 1994; Lodge *et al.,* 1996) and from roots of orchids (Richardson & Currah, 1995; Bayman *et al.,* 1997), this study is the first attempt, that we are aware of, to isolate *Xylaria* as endophytes from seeds.

MATERIALS AND METHODS

138 photosynthetic shoots and 320 seeds of *C. equisetifolia* were collected throughout NE Puerto Rico, from both coastal and inland trees. (The leaves of *Casuarina* are reduced to scales, and photosynthesis occurs in needle-like shoots. These photosynthetic shoots are equivalent to leaves in function and size.)

Leaves of saplings and seeds of mature trees of *M. bidentata* were collected at the El Verde Field Station, Caribbean National Forest, Puerto Rico. Sixty seeds were used for fungal isolations and 16 were planted in commercial potting mix in the JGD greenhouse, UPR-Río Piedras; no additional seeds

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Table 1. Comparison of *Casuarina equisetifolia* and *Manilkara bidentata*[a]

Characteristic	*Casuarina equisetifolia*	*Manilkara bidentata*
Family	Casuarinaceae (Hamamelidae)	Sapotaceae (Dilleniidae)
Origin	Australia	Neotropics
Distribution in PR	Widespread	Restricted
Habitat	Dry, sandy coastal areas	Moist forests
Stand size	Mostly small, scattered	Large, continuous
Photosynthetic organs	Needle-like stems; leaves reduced to scales; xerophytic	Mesophytic leaves to 100 cm^2
Seeds	< 0.1 g, no endosperm	> 5 g
Seed dispersal	Wind	Bats
Economic products	Wood	Wood, balatá latex
English names	Red beefwood, Australian pine	Bulletwood
Spanish names	Pino australiano	Ausubo, balatá
Reported endophyte diversity	*Phomopsis casuarinae* only[b]	17 species in a single leaf[c]

[a] Most information from Liogier (1985), Heywood (1993).
[b] Bose (1947).
[c] Lodge *et al.* (1996).

were found. Seedlings in the greenhouse were sampled when less than two years old. These leaves were compared to leaves of 20 seedlings of similar age from the El Verde forest.

Shoots and seeds of *Cusuarina* were washed under running water, surface-sterilized in 75 % ethanol for 1 min, 65 % Clorox (= 3·4% NaOCl) for 10 min, and 75 % ethanol for 30 s (Rodrigues, 1994). Six 3-5 mm pieces were cut from each *Casuarina* shoot, and seeds were cut in half. Half of each seed and three pieces of each shoot were plated on each of two different culture media.

Leaves and seeds of *Manilkara* were washed vigorously in water containing approx 0·05 % Tween 80, surface-sterilized in 10 % Clorox (= 0·5 % NaOCl) for 20 minutes, and rinsed in sterile distilled water (Bills, 1996). Ethanol and concentrated NaOCl were not used to avoid possible interactions with the latex in the leaves. Four 5 × 5 mm pieces were cut from each *Manilkara* leaf after sterilization, and two pieces were plated on each isolation medium. Seeds were cut in quarters and two quarters were plated on each medium. For 35 seeds, the entire seed was surface-sterilized before removal of the seed coat; the seed coats were plated separately. For other seeds, seed coats were removed prior to surface sterilization of the embryos.

All isolations were replicated on two media: half-strength malt extract agar (MEA, Difco Inc.) with 35 µg ml^{-1} rose bengal added after autoclaving, and half-strength potato dextrose agar (PDA, Difco Inc.) with 50 µg ml^{-1} streptomycin and 50 µg ml^{-1} chloramphenicol added after autoclaving. Antibiotics restricted the growth of bacteria; rose bengal restricted colony diameter of most fungi, allowing slowly growing fungi to be isolated with less interference from rapidly growing fungi. Cultures were incubated at 22 °C with 12 h fluorescent light/12 h dark. Fungal colonies were transferred to PDA or MEA without rose bengal for identification.

Xylaria isolates were transferred to oatmeal agar (60 g ground oatmeal, 15 g agar l^{-1}) and incubated for 2 mo to allow stromatal initials to develop. Ten *Xylaria* isolates from *Casuarina* were identified to species by colony colour and by location and shape of stromatal initials. Isolates were compared to Callan & Rogers (1990, 1993) and photographs of cultures obtained from teleomorphs in D.J.L.'s collection.

Statistics were calculated with two-tailed Fisher's exact tests for categorical data using the program Fisher6 (Engels, 1988). These tests were used since the values of some cells were < 5, causing a high probability of Type 1 error (Sokal & Rohlf, 1981).

RESULTS

Casuarina. *Xylaria* was the most common genus isolated from *Casuarina* shoots. *Xylaria* was found in 54% of shoots but in only 8 % of seeds. The difference between shoots and seeds in frequency of *Xylaria* was significant ($P < 0{\cdot}001$) (Fig. 1). Other fungi (combined) were, however, significantly more common in seeds (79%) than in shoots (62%) ($P < 0{\cdot}001$). Of the other genera identified, the most common in shoots were *Penicillium* and *Colletotrichum*. The other common genera identified from seeds were *Penicillium* and *Pestalotia. Phomopsis casuarinae* Tassi, present in all *Casuarina* shoots and seeds from India and Australia studied by Bose (1947), was not found.

Tree location was related to frequency of *Xylaria* in *Casuarina* seeds. *Xylaria* was recovered from 16% of seeds from inland trees but was never found in seeds of trees on beaches (Fig. 2). Other fungi were significantly more common in seeds from trees on beaches than in seeds from trees in parks - the reverse of what was found for *Xylaria* ($P < 0{\cdot}001$). Frequency of other fungi in shoots was not significantly different in the two locations ($P > 0{\cdot}05$).

Manilkara. *Xylaria* was isolated from 97% of tree leaves and 0% of embryos and seed coats; the difference between

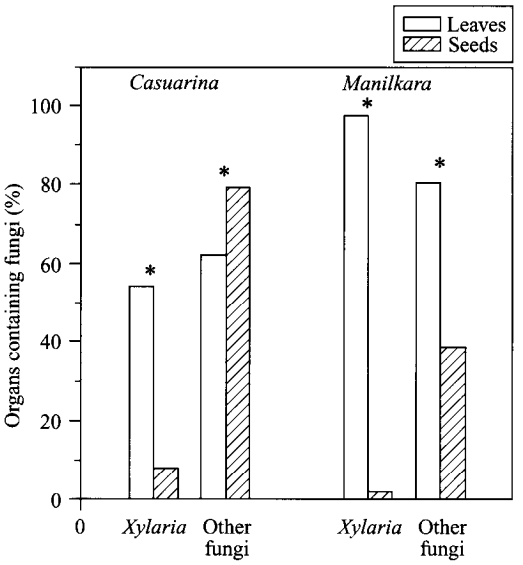

Fig. 1. Differences in endophytes between leaves and seeds of *Manilkara bidentata* and *Casuarina equisetifolia*. (*) *indicates significant difference at the P = 0·05 level.*

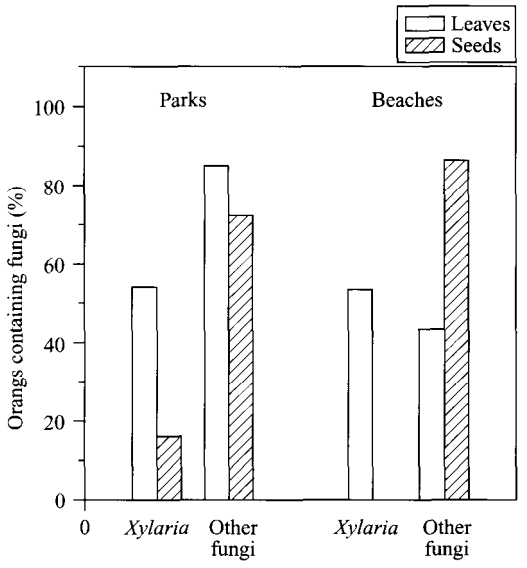

Fig. 2. Differences in endophytes in *Casuarina* trees from parks U. beaches.

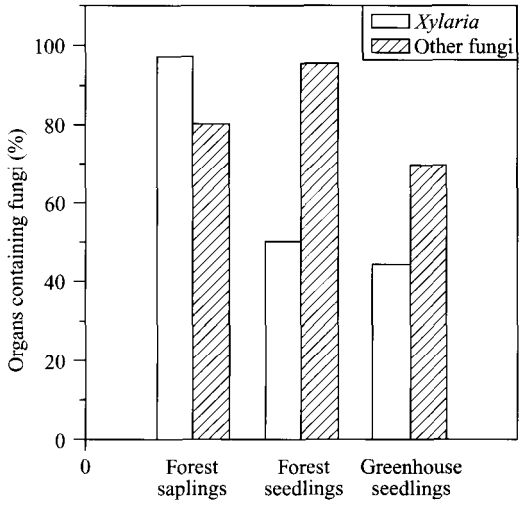

Fig. 3. Effect of location and plant age on endophyte infection of *Manilkara* leaves.

leaves and seeds was significant ($P < 0{\cdot}03$). Other fungi (combined) were also significantly more common in leaves than in embryos (80% *v.* 38%; $P < 0{\cdot}001$). Of the other fungi identified, the most common in leaves were *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk, *Pestalotia,* and *Colletotrichum crassipes* (Speg.) Arx. The most common genus identified from embryos was *Pestalotia.* The most common fungi identified from seed coats were *Penicillium, Aspergillus,* and *Papulospora.* A Gram positive bacterium was also commonly isolated from *Manilkara* embryos.

To determine whether infection of young plants depended on the density of the same species nearby, we compared year-old leaves of *Manilkara* seedlings grown in a greenhouse on the UPR campus with those in the forest. In the forest seedlings, *Xylaria* was isolated from 50% of leaves (Fig. 3). In the greenhouse, *Xylaria* was isolated from 46 % of leaves. This difference was not significant ($P > 0{\cdot}5$), but both groups of seedlings had a significantly lower infection rate than leaves from forest saplings ($P < 0{\cdot}001$). Other fungi were isolated from 100% of leaves from seedlings both in the forest and in the greenhouse. The other common genera identified from the forest seedlings were *Aspergillus* and *Colletotrichum.* The most common fungus identified from the greenhouse seedlings was *Colletotrichum,* found in 54% of leaves; it was more common than *Xylaria.*

Diversity of Xylaria species. Ten *Xylaria* isolates from *Casuarina* were identified to species. Eight belonged to the *X. arbuscula* Sacc. / *X. mellisii* Berk. complex, and one each to *X. enteroleuca* (Speg.) P. M. D. Martin and *X. obovata* (Berk.) Fr. These species have previously been reported as endophytes of *M. bidentata* and of orchids in Puerto Rico (Lodge *et al.,* 1996; Bayman *et al.,* 1997). Xylaria isolates from *Manilkara* appeared to comprise of at least 15 different species or species complexes, including *X. arbuscula/X. mellisii, X. enteroleuca,* and *X. obovata,* based on colony morphology and stromatal initials in culture (Callan & Rogers, 1990, 1993; Rodrigues, Leuchtmann & Petrini, 1993). Of these, the *X. arbuscula/X. mellisii* complex appeared to be the most common, although most isolates were not identified to species. More than one morphologically distinct species was frequently isolated from a single leaf.

Comparison of isolation media. All isolations were done on two media in parallel: MEA with rose bengal and PDA with streptomycin and chloramphenicol. In *Casuarina,* the two media did not differ significantly in the numbers of isolates of *Xylaria* ($P = 0{\cdot}77$), but PDA yielded a significantly higher number of colonies of other fungi than did MEA ($P = 0{\cdot}01$). From leaves of Manilkara, significantly more Xylaria isolates were produced on MEA than on PDA ($P = 0{\cdot}04$), but other fungi were more common on PDA than on MEA ($P = 0{\cdot}05$). Thus for both fungi, MEA with rose bengal was a more effective selective medium for *Xylaria* than was PDA with antibiotics.

DISCUSSION

Endophytic *Xylaria* species were isolated from more than half of the shoots of *C. equisetifolia* sampled, and from nearly all of the mature *M. bidentata* leaves sampled. The pronounced differences between these hosts (Table 1) suggests that distribution of *Xylaria* endophytes is not restricted by host habitat, morphology, or origin.

Mode of transmission and frequency of infection: Casuarina. *Xylaria* was isolated from 8% of *Casuarina* seeds, significantly lower than the 54% isolated from shoots. It is thus possible that the fungus can be transmitted vertically, i.e. inherited from the female parent via the seed, though it is clearly not the sole mode of transmission.

Xylaria was never, however, isolated from seeds of *Casuarina* trees growing on beaches, the most common habitat for *Casuarina* in Puerto Rico. Trees on beaches were presumably infected horizontally. The cause of the difference between trees on beaches and trees inland is not clear. *Casuarina* seeds from beaches contained other fungi at

frequencies as high as seeds from inland parks, and shoots from the two locations did not contain significantly different numbers of *Xylaria.* Thus it is unlikely that the drier, windier conditions on the beaches affected the survival of *Xylaria* in fruits or seeds but not in shoots. Also, xylariaceous anamorphs have been found in cacti in seasonally dry areas (Fisher *et al.*, 1994). It is also unlikely that seeds of *Casuarina* contain compounds that inhibit the growth of *Xylaria* since other fungi were apparently not inhibited. Other studies have shown that the same species in different environments can have different endophytes (Fisher & Petrini, 1992).

We had hypothesized that, if endophytes were transmitted horizontally, *Casuarina* trees on beaches would have a *lower* incidence of infection than inland trees. The north east trade winds cross the Atlantic Ocean, and presumably do not carry many viable fungal spores when they arrive at the beaches of NE Puerto Rico. It was, therefore, surprising that trees on the beaches were as heavily infected with endophytes as leaves of trees farther inland.

It is unlikely that rain splashing from infected shoots is the source of inoculum rather than wind. Rain has been shown to disperse two endophytic fungi in Oregon, *Rhabdocline parkeri* (Sherwood, J. K. Stone & G. C. Carroll) and *Discula quercina* (Westd.) von Arx (Stone, 1987; Wilson & Carroll, 1994). These fungi, however, produce fruiting bodies on green plant parts, whereas *Xylaria* does not. Thus the source of the inoculum remains unclear.

In one of the earliest published studies on endophytes of tropical plants, Bose (1947) reported that *Phomopsis casuarinae* was found in every *C. equisitifolia* plant examined from India and Australia. He found *Phomopsis* in seeds as well as roots, shoots, and fruits, and concluded that infection was vertical, in his words hereditary. Bose stated that *Phomopsis* was a mycorrhizal symbiont of young, healthy plants, but was a parasite of weak plants. Pycnidia of *Phomopsis* were not mentioned, and no other fungi were reported.

In contrast, no *Phomopsis* was isolated in this study from either seeds or shoots, and most plants contained a variety of fungi. The discrepancy between our results and those of Bose agrees with other studies of endophytes of trees removed from their native habitats. Naturalized stands of *Quercus ilex* L. in Britain contained fewer host-specific endophytes and more non-specific endophytes than native *Q. ilex* in Spain and Switzerland (Fisher *et al.*, 1994). Similarly, introduced *Eucalyptus nitens* (Deane & Maiden) Maiden trees in England contained a lower diversity of fungi than native *E. nitens* in Australia, and contained fewer host-specific fungi and more generalists (Fisher, Petrini & Sutton, 1993).

Since Bose's work was, however, based mainly on microscopic observation of hyphae in plant tissues, rather than on cultures, it is possible that some of the hyphae he assumed to be *P. casuarinae* were actually other fungi. The fact that *Phomopsis* is not considered to be mycorrhizal is further reason to suspect that the mycota of *Casuarina* from India and Australia was more complex than Bose realized.

Mode of transmission and frequency of infection: Manilkara. Transmission of Xylaria endophytes in *M. bidentata* appeared to be strictly horizontal; *Xylaria* was never isolated from seeds, even though it occurred in nearly every leaf (Figs 1, 3).

Transmission presumably occurs when ascospores or conidia infect leaves. A previous study on *M. bidentata* leaf endophytes also suggested that infection resulted from air- or water-borne spores; *Xylaria* was found in leaf blades of *M. bidentata* but not in petioles, so it presumably did not pass to the leaf from the shoot (Lodge *et al.,* 1996). The fact that other fungi were found in 38% of *Manilkara* seeds, even though *Xylaria* was never found, reduces the possibility that *Xylaria* was present in seeds but that its growth was chemically inhibited.

In *Manilkara,* leaves of seedlings raised in the greenhouse were as heavily infected with *Xylaria* as leaves of seedlings in the forest. These seedlings were grown in an urban area at least 15 km from the nearest stand of *M. bidentata.* This suggests that endophytic *Xylaria* strains were not host-specific, since the spores reaching the seedlings in the greenhouse were unlikely to have come from *M. bidentata* trees. Although *Xylaria* endophytes are thought to be generalists, two host-specific saprotrophic *Xylaria* species have been described from the forest we studied (Laessøe & Lodge, 1994). The speciose composition of tropical forests may select against host specificity in endophytic fungi (Lodge *et al.,* 1996). As with *Casuarina,* the data suggest a surprisingly high level of inoculum.

The source of the inoculum that infects *M. bidentata* leaves may be stromata formed on dead twigs of *M. bidentata* (Lodge *et al.,* 1996). Neither the teleomorph nor the anamorph of the *Xylaria* endophytes is, however, produced on fallen *M. bidentata* leaves. *Geniculosporium,* a *Xylaria* anamorph, was not among the 104 species found on fallen *M. bidentata* leaves (Polishook, Bills & Lodge, 1996). Thus the infection of *Manilkara* leaves may be a dead end for *Xylaria.* If this is true, it may preclude any coevolution between host and endophyte.

Significance of endophytic *Xylaria* species. *Xylaria* species have been isolated from almost all tropical plants in which fungal endophytes have been examined (Dreyfuss & Petrini, 1984; Rodrigues & Samuels, 1990; Rodrigues, 1994; Richardson & Currah, 1995 ; Lodge *et al.,* 1996; Bayman *et al.,* 1997). Although these published studies have looked at wild plants, we have also isolated *Xylaria* from two important crop plants in Puerto Rico, coffee and banana.

Although *Xylaria* has dominated cultural studies of the tropical endophyte mycota, other endophytes may turn out to be equally more important. This applies to bacteria as well as fungi (Fisher, Petrini & Lappin-Scott, 1992; Chanway, 1996); in fact, a Gram-positive bacterium was commonly isolated from *Manilkara* seeds. It is also likely that some endophytes do not grow well on agar media, and are thus overlooked in studies like this one. DNA-based studies may provide a better estimate of the total endophyte diversity within a leaf, and the relative frequencies of different microorganisms. Thus this study presents only a partial picture of the endophyte flora of *Casuarina* and *Manilkara.*

Distribution and dispersal of *Xylaria* in *Casuarina* and *Manilkara* appears to differ from that of many tree endophytes in temperate climates. Firstly, the most abundant endophytes

of temperate trees are often host-specific (Carroll, 1995), which does not appear to apply to endophytes of *M. bidentata* (Fig. 3). Secondly, isolated individuals of temperate trees tend to have lower rates of infection than trees in a conspecific stand (Carroll, 1995); we found that isolated *M. bidentata* seedlings in an urban greenhouse had rates of infection as high as seedlings in the forest. This suggests lack of host specificity and high levels of inoculum. Third, temperate trees in drier, more exposed locations generally have fewer endophytes than trees in humid or sheltered sites (Carroll, 1995), yet *Casuarina* trees on beaches had as high a frequency of *Xylaria* infection of shoots as trees inland.

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