Endophytic species of *Xylaria:* cultural and isozymic studies

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Cultural descriptions of endophytic *Xylaria* species from an Amazonian palm, *Euterpe oleracea,* are presented. Eighty–one isolates representing 15 species of *Xylaria* were examined for isozyme variation by means of horizontal starch gel electrophoresis. Results from the isozyme analysis revealed a high degree of intra- and interspecific diversity among *Xylaria* species.

Keywords: endophytes, isozymes, tropical fungi, Xylariaceae, *Euterpe oleracea.*

The genus *Xylaria* and other members of the Xylariaceae are commonly isolated endophytes. The identification at the species level of putative endophytic *Xylaria* grown in culture is still a difficult task because they rarely produce morphologically diagnostic structures, and teleomorphs are seldom formed. Indeed, many of them may differ from free–living forms and some might not produce the teleomorph at all (Brunner & Petrini, 1992). Anamorphs produced in culture are relatively easy to identify to the genus because of their typical stromata, conidiophores and conidial development. However, accurate identification to species can be accomplished only when colonies are compared with descriptions or cultures derived from ascospores of reliably identified teleomorphs (Petrini & Petrini, 1985; Callan & Rogers, 1990). An analysis that combines morphology and cultural characters with biochemical methods such as the production of secondary metabolites (Whalley & Edwards, 1987), DNA sequences (Rogers, 1990) and isozyme and metabolite profiles (Brunner & Petrini, 1992) might lead to a precise identification.

Several accounts of tropical species of *Xylaria* growing in pure cultures have been reported (Martin, 1970; Rogers & al., 1987, 1988;

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Callan & Rogers, 1990; Rodrigues, 1992). However, the range of variability that is expected to occur within a species is as yet not known, thus limiting the ability to precisely identify sterile or anamorphic xylariaceous endophyte cultures. Under these circumstances, cultural morphology criteria alone would be inadequate for the taxonomic identification.

Electrophoretic techniques have been widely used to solve taxonomic problems within fungal taxa (Leuchtmann & Clay, 1990; Bonde & al., 1991; Oudemans & Coffey, 1991, Leuchtmann & al., 1992), to identify unknown fungal strains (Keller, 1992), and to study population genetics (Micales & al., 1986). The aim of this study was to combine cultural and biochemical data in order to determine taxonomic relationships of endophytic isolates of *Xylaria* species from the palm *Euterpe oleracea* Mart. These isolates represented the range of morphological variation observed in cultures of the endophytic *Xylaria* recovered from the palm. Isozyme analysis was undertaken to evaluate isolates of *Xylaria* species for isozyme activity; to determine the extent of intra- and interspecific variation within and among species; and to establish a suitable biochemical methodology for the characterization of *Xylaria* species.

Material and methods

Source of isolates

Seventy-six isolates of *Xylaria* spp. were obtained from surface sterilized healthy leaf tissues, following the methodology described in Rodrigues & Samuels (1992). Leaves were collected from adult trees and saplings of *Euterpe oleracea* naturally growing in the river island of Combu. The study site is a periodically inundated forest located in the floodplains of the Brazilian Amazon, in the vicinity of Belém (01°28' S, 48°27' W). Five additional strains were included in this study which derived from cultured teleomorphic species obtained from Prof. Jack D. Rogers (Department of Plant Pathology, Washington State University - WSU). Representative isolates are maintained at the New York Botanical Garden and the American Type Culture Collection.

Cultural work

Cultural characteristics of endophytic strains were studied on colonies growing on 9 cm plastic Petri dishes containing oatmeal agar (30 g oats, 12.5 g Bacto agar, 500 ml distilled H_2O; blended for 2 minutes, then autoclaved). The plates were incubated at 20 C under

a cycle of 12 h cool white fluorescent light and 12 h darkness. Color designations used throughout the taxonomic descriptions are from Kornerup & Wanscher (1978), and appear inside parentheses following the color names. The terminology used to describe texture of mycelia follow Stalpers (1978). Because cultures were sterile or presented only the anamorph they were identified by comparison with colonies known to have originated from ascospores of identified species grown at WSU.

Electrophoresis

Five mycelial plugs were taken from the edge of young fungal colonies actively growing on cornmeal dextrose agar (CMD, Difco) in Petri dishes by using a flame sterilized cork borer (11 mm diam), and then placed into sterile Eberbach blender bowls containing 100 ml CYM liquid medium (0.69 g KH_2PO_4, 1.50 g K_2HPO_4, 0.75 g $MgSO_4.7H_2O$, 30 g dextrose, 3 g yeast extract, 3 g peptone, 1.5 l distilled water). The plugs and medium were blended for 30 seconds at low speed in a Waring blender. Fifty ml of blended medium were poured into each of two 125 ml flasks. Flasks were placed on a rotary shaker at 150 rpm at 20 C in darkness. The isolates were incubated for 10 to 14 days, at which time there was abundant mycelium production. Mycelial mats were collected on No. 1 Whatman filter paper in a Büchner vacuum funnel and washed with 50 ml distilled water. Harvested mycelia were placed in plastic weighing boats, and stored in a freezer at –60 C. Frozen mycelia were transferred to plastic centrifuge tubes and covered with cheesecloth. Up to twelve tubes were placed in a stainless steel beaker, attached to a multi-dry/freeze-dryer (FTS Systems, Inc.), and lyophilized at –60C under 80 millitorr vacuum for approximately 18 h. The dry mycelia were then transferred to sealed plastic bags and kept at –60 C for 3 weeks.

Lyophilized mycelia were ground to a fine powder, placed in glass vials and kept at –35 C. Gels were prepared with 12.8% hydrolyzed starch (Sigma), poured into an acrylic gel tray, covered with a glass plate to avoid desiccation, and left overnight at room temperature to solidify. Twenty–five mg of lyophilized mycelia were suspended in 0.3 ml Tris-HCl-PVP extraction buffer (pH 7.5) of Soltis & al. (1983), and extracted in Eppendorf tubes at 4 C overnight. Before electrophoresis, samples were centrifuged at 15,000 ***g*** for 15 minutes. The contents of each Eppendorf tube were absorbed onto filter paper wicks (2 x 13 mm, 0.8 mm thick; Whatman), quickly blotted on paper towels, and then completely inserted into the slit. Up to 32 samples were loaded onto each gel. 0.04% bromophenol blue was applied as a dye marker to either side of the samples. The gel tray was then put inside an electrode tray containing electrode buffer, and electrophoresis

run at 4 C. Three buffer systems (Soltis & al., 1983) were used: I: electrode buffer citric acid, pH 7.0; gel buffer histidine-HCl. – HCl, pH 7.0; 75mA constant current until marker dye migrated 6 cm. II: electrode buffer Tris-citric acid, pH 8.0; gel buffer Tris-citric acid, pH 8.0; 50mA constant current until marker dye migrated 9 cm. III: electrode buffer Tris citric acid, pH 7.2; gel buffer Tris citric acid, pH 7.2; 50mA constant current until marker dye migrated 9 cm. Enzyme staining solutions were prepared according to the protocols outlined by Soltis & al. (1983). Enzyme names with respective enzyme commission (E.C.) number (I.U.B., 1984), abbreviations, and buffer systems used for this work are given in Table 1. Initially, 21 enzyme stains were tested for activity with the three buffer systems. Five enzymes were selected based on results from previous experiments, which showed clear bands in most of the isolates examined. The following enzymes were examined but not included here, due to their streaking bands or inconsistent activity: acid phosphatase (E.C. 3.1.3.2), aconitase (E.C. 4.2.1.3), alcohol dehydrogenase (1.1.1.1), alkaline phosphatase (3.1.3.1), aspartate aminotransferase (2.6.1.1), β–D–glucosidase (3.2.1.21), diaphorase [NADH form] (1.6.4.3), fructose–bisphosphatase (3.1.3.11), fumarase (4.2.1.2), glucose–6–phosphate dehydrogenase (1.1.1.49); hexokinase (2.7.1.1), isocitrate dehydrogenase (1.1.1.42), leucine aminopeptidase (3.4.11.1), malate dehydrogenase (1.1.1.37), malic enzyme (1.1.1.40), shikimate dehydrogenase (1.1.1.25). The electromorphs assigned to each enzyme were determined by repeated side by side comparisons and expressed as numbers that reflected their increasing mobility on the gel, i.e., from the most cathodal to the most anodal edge. In PGM and 6PG several isolates exhibited a double–banded electromorph. Since the genetic basis of these electromorphs is not known, the isozyme data were interpreted phenotypically, i.e. each banding pattern was scored as one phenetic character. This procedure follows studies previously done by Leuchtmann & Clay (1990), and Leuchtmann & al. (1992).

Tab. 1.– Enzyme names with enzyme commission (EC) number, abbreviations, and buffer systems used.

Enzyme	EC No.	Abbreviation	Buffer system*
Aldolase	4.1.2.13	ALD	II
Phosphoglucose isomerase	5.3.1.9	PGI	III
Phosphoglucomutase	5.4.2.2.	PGM	III
6–Phosphogluconate dehydrogenase	1.1.1.44	6PG	I
Triosephosphate isomerase	5.3.1.1	TPI	III

* for details see text

Data analysis

Cluster analysis was applied to the resulting electromorphic matrix containing all isozyme phenotypes. A normalized percent of disagreement distance was used in order to measure dissimilarities. Intercluster distances were computed by means of average linkage clustering (unweighted pair-group mean average – UPGMA: Sneath & Sokal, 1973) with the software SYSTAT 5.2 (Wilkinson, 1990).

Results

Characteristics in culture of endophytic *Xylaria* species

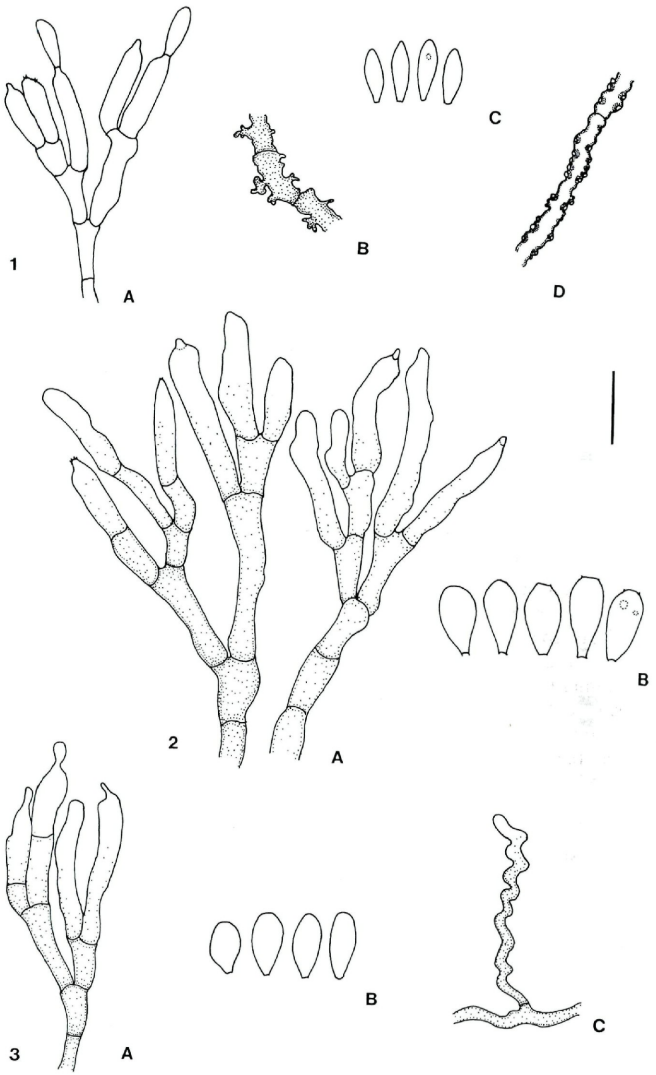
Xylaria adscendens (Fr.) Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 128. 1851. (anamorph). – Fig. 1.

References. – Rogers (1984a), Callan & Rogers (1990).

Colony 4.5 cm diam in 7 days, velvety to appressed, eventually forming radial strands, with white, abundant cottony mycelium around point of inoculum, and reaching lid of Petri dish; faintly zonate; at first white, then covered by a layer of warty canary yellow (2B7) mycelium (fig. 1, D), darkening to olive yellow (3D7), to blackish olive (2G6) from the center outwards; margin white to canary yellow, minutely plumose; dark brown, long stromatic hyphae with short protuberances (fig. 1, B); reverse golden blonde (5C4); no pigment diffusing. – Stromata formed after 1 week at point of inoculum or peripherally; cylindrical, robust, to 3.5 cm high x 4 mm wide, eventually branching when touching Petri dish lid, at first pale yellow (2A3) to canary yellow (2B7) or orange white (5A2), then darkening to black with yellowish white (4A2) to cream (4A3) or orange white (5A2) tips, villose at base; colorless to yellowish droplets forming along length of stromata. – Conidiophores forming after three to four weeks, in palisades at tip of stromata, to 31.5 µm long x 3.6 µm wide, hyaline, smooth, branching near base. – Conidiogenous cells, solitary, terminal, cylindrical, 13.5–22.5 x 3.6–4.5 µm, with denticulate secession scars. – Conidia ellipsoidal, (8.1–)9.9–11.7 x 2.7–3.6 µm, one-celled, with flat basal abscission scar, hyaline, smooth.

Xylaria allantoidea (Berk.) Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 127. 1851. (anamorph). – Fig. 2.

References. – Rogers (1984b), Callan & Rogers (1990).



Figs. 1–3. – Conidiophores, conidia, and hyphae of *Xylaria* species. – 1. *Xylaria adscendens*. A. conidiophore. B. segment of stromatic hypha with short protuberances. C. conidia. D. segment of hypha with warty excrescences. – 2. *Xylaria allantoidea*. A. conidiophores. B. conidia, with apical corona. – 3. *Xylaria* cf. *anisopleura*. A. conidiophore. B. conidia. C. coiled stromatic hypha. Scale bar = 10 µm.

Colony 2.5 cm diam in 5 days, cottony with hyphal radial strands, azonate to irregularly lobed, white to pale salmon then overlain with cocoa (6E6) mycelium; margin minutely plumose; stromatic hyphae not seen; yellowish exudate forming in the aerial mycelium; reverse greyish orange (6B5) to Sahara (6C5); no pigment diffusing. – Stromata produced after 3 weeks at center, or at periphery of colonies; cylindrical, to 3.5 cm high x 3 mm wide, unbranched, at first pale orange (6A3) then sunburn (6D5) except pale salmon at tip. – Conidiophores produced along the length of stromata as well as in tufts on surface of colony, forming in a palisade, to 72 µm long x 2.7–3.6 µm wide, subhyaline to pale brown, smooth, branching near base. – Conidiogenous cells solitary, terminal, cylindrical, 31.5–45 x 3.6–4 µm, hyaline, with discoid to denticulate secession scars. – Conidia clavate to ellipsoidal, few with a corona-like structure at apex, (6.3–)7–10(–11) x 3.6–4.5 µm, one-celled, with flat basal abscission scar, hyaline [dust (5D2) in mass], smooth.

Xylaria cf. *anisopleura* (Mont.) Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 127. 1851. (anamorph). – Fig. 3.

Reference. – Callan (1988).

Colony 1.5 cm diam in 5 days, velvety to appressed, occasionally whitish floccose sectors forming, azonate to faintly zonate; first white, then overlain with café-au-lait (6D3) to greyish brown (6E3) mycelium formed of subhyaline, coiled stromatic hyphae (fig. 3, C); margin plumose; surface of older cultures radially furrowed; reverse nonpigmented or greyish orange (5B4). – Stromata developing at edges of colony, and in more or less concentric zones, cylindrical, to 1 cm high x 1 mm wide, unbranched, at first white, then greyish brown (6E3) with bright orange exudate along the length. – Conidiophores located at tip of stromata as well as on compact hyphal tufts forming on colony surface, hyaline to pale brown, smooth, irregularly branched near base. – Conidiogenous cells solitary, terminal, cylindrical, 22.5–40.5 x 4.5–5.4 µm, with round to conical secession scars. – Conidia ellipsoidal, (7–) 8–9 x 3.6–4.5 µm, one-celled, with flat basal abscission scar, hyaline, smooth.

Xylaria arbuscula Sacc., Michelia 1: 249. 1878. (anamorph). – Fig. 4.

References. – Martin (1970), Callan & Rogers (1990).

Colony 3 cm diam in 7 days, velvety to appressed, faintly zonate; at first white, then darkening to cocoa (6E6) to burnt umber (6F6)

but remaining white close to the point of inoculum; margin white, minutely plumose; stromatic hyphae long with short, lateral protuberances (fig. 4, B) in the aerial mycelium; colorless to yellowish exudate forming in the aerial mycelium; reverse pale orange (5A3) to brownish orange (6C8), eventually forming a dark brown ring at center. – Stromata produced after 10 to 15 days in concentric zones, cylindrical, 1.5–3.5 cm high x 2 mm wide, unbranched to 2–3 times branched towards apex, at first white then darkening to brownish grey (6F8) to almost black with white apices, base villose with colorless exudate; or pulvinate, ca. 1 mm tall x 1 mm wide, white. – Conidiophores formed in palisades at apex of stromata, to 59.5 x 2.7–3.6 µm, smooth, branching near base. – Conidiogenous cells solitary, terminal, cylindrical, 6–15 x 3.6–4.5 µm, with round secession scars. – Conidia ellipsoidal, (4–)4.5– 6.3(–7) x 2.7–3.6 µm, one-celled, with flat basal abscission scar, hyaline, smooth.

Xylaria cf. *castorea* Berk. in Hooker, Flora Novae-Zelandiae II. p. 204. 1855. (anamorph).

Reference. – Rogers & Samuels (1986).

Colony 3.5 cm diam. in 5 days, cottony to appressed, azonate; pale orange (5A3) to pale salmon, then darkening to brownish orange (6C3) towards edge; margin entire; stromatic hyphae not seen; reverse non pigmented. – Stromata produced after 10 days, scattered, cylindrical, with broad bases and acute apices, to 3 cm high x 3 mm wide, unbranched, pale orange (6A3) then darkening to sunburn (6D5) to cinnamon (6D6) from the base upwards, villose. – No conidiogenous cells observed.

Description of cultures and photograph of colonies grown on oatmeal agar of *X. castorea* given by Rogers & Samuels (1986) are very similar to those of our isolates. Their cultures also remained sterile.

Xylaria coccophora Mont., Ann. Sci. Nat. Bot. Ser. 4, 3: 109. 1855. (anamorph). – Fig. 5.

References. – Callan (1988), Rogers et al. (1988).

Colony 2 cm diam in 5 days, velvety, zonate; at first white, then covered by a layer of warty canary yellow (2B7) hyphae (fig. 5, B), finally darkening to greyish yellow (3C4), to olive yellow from the center outwards, with pale yellow (3A3) zonation; margin pale yellow (3A3), plumose; dark brown, short stromatic hyphae formed in the aerial mycelium (fig. 5, D); reverse nonpigmented to orange grey

(5B2); no pigment diffusing. – Stromata produced in concentric zones, narrowly cylindrical, to 2 cm high x 1 mm wide, unbranched, at first white, then black, wrapped in canary yellow (2B7) hyphae, with a colorless exudate at base of stromata. – Conidiophores forming in palisades over surface of stromata and in zonations on surface of colony, branching near base. – Conidiogenous cells solitary, terminal, cylindrical, 18–27 x 4.5–6.3 µm with denticulate secession scars. – Conidia ellipsoidal, (7.2–)8.1–9 x 2.7–3.6 µm, one-celled, with flat basal abscission scar, hyaline, smooth.

Xylaria cubensis (Mont.) Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 126. 1851. – Fig. 6.

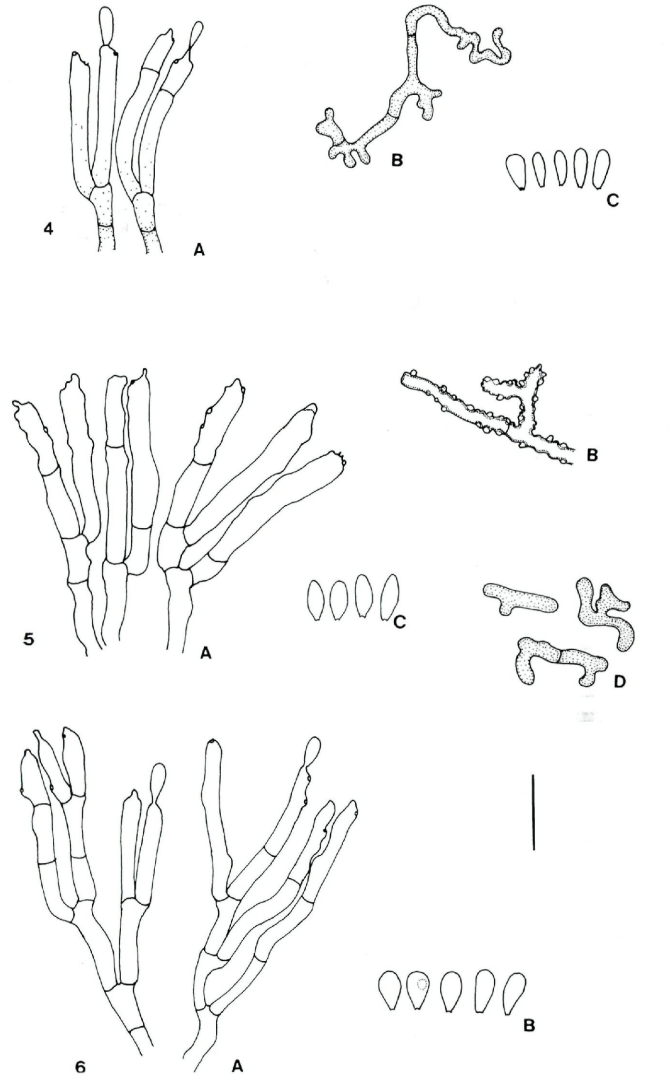
Anamorph: *Xylocoremium flabelliforme* (Schw.: Fr.) J. D. Rogers, Mycologia 76: 914. 1984.

References. – Jong (1970), Martin (1970), Rogers (1984b), Rogers & Samuels (1986), Nagasawa (1988), Callan & Rogers (1990).

Colony 4 cm diam. in 5 days, cottony with appressed sectors, azonate to faintly zonate; pale orange (6A3) to salmon (6A4); margin white, minutely plumose to even; exudate colorless forming in the aerial mycelium; stromatic hyphae in the aerial mycelium lacking; reverse pale orange (5A3). – Stromata forming after 5 days, first located in the center then abundant over whole colony; cylindrical or flabelliform, to 2.5 cm high x 4 mm wide, occasionally villose at base, salmon (6A4), sometimes darkening to light brown at base. – Conidiophores in palisades, developing abundantly on upper part of stromal surface, appearing floccose; to 36 µm long x 3 µm wide, hyaline, smooth, several times branching. – Conidiogenous cells solitary, terminal, cylindrical, 9–18 x 2.7–3.6 µm, with round to denticulate secession scars. – Conidia obovoid to ellipsoidal, (3.6–)5–6.3 x 1.8–3.6 µm, one–celled, with flat basal abscission scar, hyaline, smooth.

X. cubensis is a morphologically variable species, indicating that it is a complex comprising more than one taxon. Resolution of this complex will require morphological and biochemical studies of more cultures from ascospores than have been done so far.

X. cubensis was the most abundant endophytic species isolated from leaves of *Euterpe oleracea* (Rodrigues, 1992). This species has been reported living endophytically in another palm, *Licuala ramsayi* (Muell.) Domin. (Rodrigues & Samuels, 1990) and in *Chamaecyparis thyoides* (L.) B. S. P. (white Atlantic cedar; Bills & Polishook, 1992).



Figs. 4–6. – Conidiophores, conidia, and hyphae of *Xylaria* species. – 4. *Xylaria arbuscula*. A. conidiophore. B. long stromatic hypha with protuberances. C. conidia. – 5. *Xylaria coccophora*. A. conidiophores. B. segment of hypha with warty excrescences. C. conidia. D. short stromatic hypha. – 6. *Xylaria cubensis*. A. conidiophores. B. conidia. Scale bar = 10 µm.

Xylaria curta Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 126. 1851. (anamorph). – Fig. 7.

References. – Martin (1970), Rogers (1983), Callan & Rogers (1990).

Colony 3.5 cm diam in 7 days, velvety to appressed; becoming overlain by feathery, white radial hyphal strands, azonate; at first white, then darkening to mouse grey (5E3) to greyish brown (6E3) from the center outwards; margin white, highly plumose, later rising from colony surface after touching the Petri dish edge and then developing into stroma–like structures; colorless exudate forming in the colony surface; dark brown stromatic hyphae forming in the aerial mycelium (fig. 7, C); reverse nonpigmented to orange white (5A2); no pigment diffusing. – Stromata produced within 2 weeks at center of colony along rays or at periphery of colony; cylindrical, to 3 cm high x 2 mm wide, fragile, unbranched, to branched when touching Petri dish lid, at first white, then darkening to brownish grey (6E2) to black, with white apices, base villose, with a colorless exudate. – Conidiophores forming a compact palisade along the length of stromata after 3 weeks of incubation, to 72 x 4.5 µm, subhyaline, smooth, sparingly branched. – Conidiogenous cells solitary, terminal, cylindrical, 40–45 x 4–5.4 µm, with denticulate conidial secession scars. – Conidia obovate to ellipsoidal, (7.2–)7.3–8.9(–9.9) x 3.6–4.2(–4.5) µm, one–celled, with a flat basal abscission scar, hyaline, smooth.

This species has been isolated as an endophyte from *Licuala ramsayi* (as *Xylaria* sp.; Rodrigues & Samuels, 1990) and from *Chamaecyparis thyoides* (Bills & Polishook, 1992).

Xylaria cf. *microceras* (Mont.) Fr., Nova Acta Reg. Soc. Upsal. Ser. 3, 1: 128. 1851. (anamorph).

References. – Rogers & al. (1988), Callan & Rogers (1990).

Colony 2.5 cm diam in 5 days, cottony with radial strands, faintly zonate; white to orange white (5A2), darkening to birch grey (5C2) at center of colony; margin plumose; stromatic hyphae not formed; reverse greyish orange (6B5); no pigment diffusing. – Neither stromata nor conidiogenous cells produced.

These endophytic isolates were tentatively retained as *X. microceras* because of their resemblance to ascospore cultures of *X. microceras* originating from Taiwan, Puerto Rico, and Venezuela growing at

WSU. The only published descriptions of *X. microceras* were based on collections from Venezuela (Rogers & al., 1988), and from Puerto Rico and USA (Callan & Rogers, 1990). Cultures from the teleomorphs collected in localities other than Puerto Rico and USA did not produce stromata and remained sterile.

Xylaria cf. *multiplex* (Kunze) Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser.3, 1: 127. 1851. (anamorph).

Reference. – Martin (1970).

Colony 4.5 cm diam in 7 days, velvety to felty, faintly zonate with white, thin radial hyphal strands; at first white to orange white (5A2), then darkening to elephant skin (5E2) to beaver (5F4); margin white, thinly plumose; colorless to yellowish exudate produced in the colony surface; stromatic hyphae with short protuberances forming in the aerial mycelium; reverse beige (4C3); no pigment diffusing. – Stromata rarely forming after 3 weeks, in the center of colony, in a more or less concentric pattern; cylindrical, robust, to 2 cm high x 3 mm wide, unbranched to occasionally forked, first white then turning to black, white at tip, tomentose at the base, with a yellowish exudate. – Conidiogenous cells and conidia not observed.

The stromatic hyphae produced could not be distinguished from those of *X. adscendens* and *X. obovata*. Identification was possible by comparison with cultures of *X. multiplex* derived from teleomorphs collected in Taiwan and South America growing at WSU. Martin's (1970, plate IV, fig. 6) photograph of a culture of *X. multiplex* is strikingly similar to our isolates. Martin's isolates and those growing at WSU also remained sterile.

Xylaria cf. *obovata* (Berk.) Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 127. 1851. (anamorph). – Fig. 8.

References. – Callan (1988), Callan & Rogers (1990).

Colony 4 cm diam in 7 days, velvety to appressed, faintly zonate at center of colony; at first white then overlain with dark brown (6F7) to negro (6F3) layer of mycelium; margin white, plumose; exudate not produced; stromatic hyphae dark brown (fig. 8, C); reverse brownish orange (6C8). – Stromata formed at periphery of colony after 2 weeks, cylindrical to clavate, fragile, to 2.5 cm high x 5 mm wide, unbranched, at first white, then turning teak [brown] (6F5); colorless exudate at base of stromata. – Conidiophores forming palisades at tip of stromata, 45–54 µm long x 2.7–3.6 µm

wide, subhyaline, dichotomously branching near base. – Conidiogenous cells solitary, terminal, cylindrical, 18–27 x 3.6–4.5 µm, with conical secession scars. – Conidia ovoid to obclavate, (8.4–)9.4–12(–11.9) x 2.8–3.5 µm, one-celled, with flat basal abscission scar, hyaline, smooth.

Xylaria cf. *palmicola* Winter, Grevillea 15: 89. 1887. (anamorph).

Reference. – Rogers & Samuels (1986).

Colony 3.5 cm diam in 7 days, velvety to appressed, irregularly zonate; white first, then darkening to old silver (4E2), becoming overlain with scattered patches of white to cream (4A3) velvety mycelium; margin minutely plumose; exudate not produced. Colony surface developing a greenish ring of more or less appressed mycelium around the center as colonies mature. Reverse greyish orange (6B4). – Stromata produced after 10 days, in more or less concentric zones; cylindrical, 1 (–2) cm high x 2 mm wide, robust with acute, white apex, erect to less frequently curved, unbranched, villose at base; first white then greyish beige (4C2) to dark grey (1F1), becoming clothed in olive green (3D7) hyphae, forming in more or less regular rings around stromata. – Conidiogenous cells and conidia not observed.

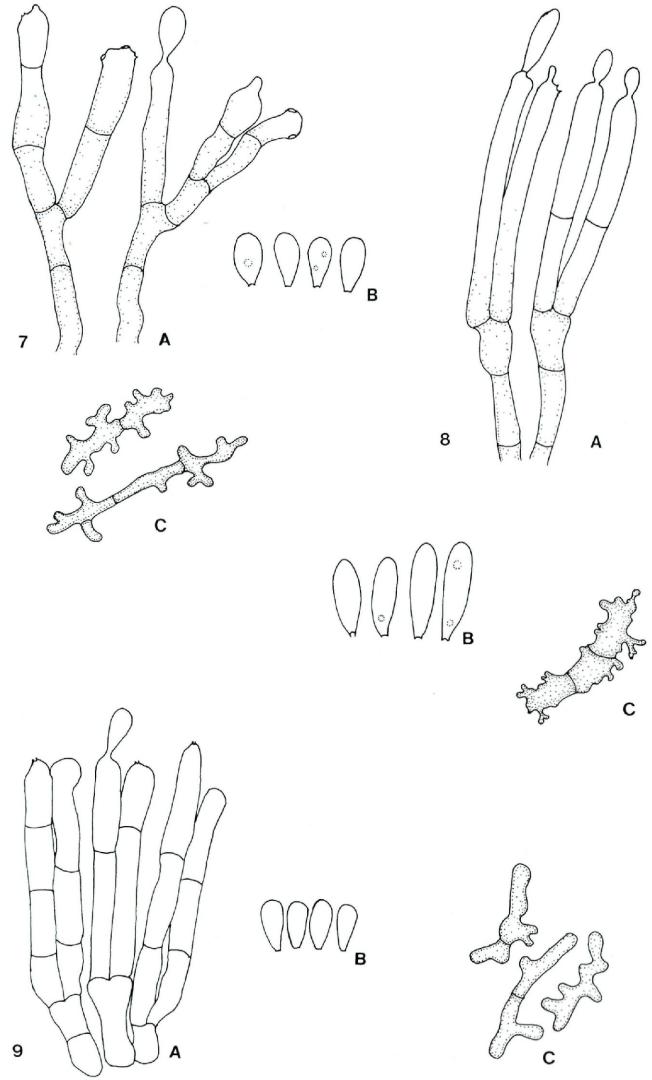
Rogers & Samuels (1986) described similar cultural characters for New Zealand collections of *X. palmicola*, where the presence of a "blue ring around the centre of colony" and stromata "clothed in green hyphae" were noted. Conidia formed along stromata in their cultures. In New Zealand the species was found only on seeds of the palm *Rhopalostylis sapida* (Wendl.) Drude.

Xylaria cf. *telfairii* Berk. & Fr., Nova Acta Reg. Soc. Sci. Upsal. Ser. 3, 1: 127. 1851. (anamorph).

Reference. – Callan & Rogers (1990).

Colony 2 cm diam in 5 days, velvety to appressed, faintly zonate; at first white, then becoming red-haired (6C4) in the center; coiled hyphae forming at surface of colony; colorless exudate forming in the aerial mycelium; margin minutely lobed, raised, developing into stromatal structures after 1 week; reverse non pigmented to pale orange (5A3); no pigment diffusing. – Stromata formed at periphery of colony, fan-like, to 0.5 cm high, white to orange white (6A2) to greyish orange (6B5). – Conidiogenous cells not observed.

Identification of these isolates was possible only after comparison with cultures of *X. telfairii* derived from teleomorphs that originated



Figs. 7–9. – Conidiophores, conidia, and hyphae of *Xylaria* species. – 7. *Xylaria curta*. A. conidiophores. B. conidia. C. stromatic hyphae with protuberances. – 8. *Xylaria* cf. *obovata*. A. conidiophores. B. conidia. C. stromatic hypha with short protuberances. – 9. *Xylaria* sp. 1. A. conidiophores. B. conidia. C. stromatic hyphae with protuberances. Scale bar = 10 µm.

in South America and kept at WSU, and which agree with the description given by Callan & Rogers (1990) for cultures from a specimen collected in French Guiana.

Xylaria sp. 1 (anamorph). Fig. 9.

Colony 3.5 cm diam in 7 days, velvety with few white radial hyphal strands, azonate; at first white, then darkening to negro (6F3); margin white, plumose; reverse nonpigmented. – Stromata developing after 1 week, at center of colony, narrowly cylindrical, unbranched to branched after touching Petri dish lid, to 2 cm high, at first white then becoming black except for white apex; colorless exudate at base. – Conidiophores forming in palisades at tip of the stromata, to 55 µm long x 4.5 µm wide. – Conidiogenous cells cylindrical, terminal, with conical secession scars. – Conidia (6–)6.4–7.4(–8.1) x 2.7–3.4(–4) µm, subhyaline, one-celled, with flat basal abscission scars.

Xylaria sp. 2 (anamorph).

Colony 4.5 cm diam in 7 days, at first velvety then appressed, azonate; at first pale orange (6A3), then overlain with brownish grey (6D2 to 6E2) mycelium; margin even; reverse orange grey (5B2). – Stromata forming abundantly throughout the colony within 2 weeks., filiform, unbranched, acute, to 2 cm high x 0.5 mm wide, at first pale orange (6A3), then darkening to black, with flesh (6B3 to 6C3) apices, glabrous. Colorless exudate at tip of stromata. – No conidiogenous cells or conidia observed.

The cultural and stromatal characteristics of this species resembles *X.* cf. *filiformis* (Alb. & Schw.: Fr.) Fr. described by Rogers & Samuels (1986).

Isozyme analysis

All enzymes, except phosphoglucomutase (PGM) and 6-phosphogluconate dehydrogenase (6PG), produced simple banding patterns. Although a few isolates exhibited one additional band in PGM and in 6PG, they were scored as a single character since we do not know whether they are of multiple or single locus origin. Most isolates presented enzyme activity in the five enzyme systems used (Tab. 2). There were 45 distinct electromorphs distributed among 15 species and five enzyme systems, which were found in 45 different combinations of electromorphs or phenotypes (Fig. 10). Seven to twelve electromorphs occurred for each enzyme system. The phenotypes are

Tab. 2.– Isozyme phenotypes with identity of electromorphs assigned to the *Xylaria* species investigated. Electromorph numbers as in Fig. 10.

Phenotype	Strain #	Source*	Enzyme electromorphs				
			ALD	PGI	PGM	6PG	TPI
X. adscendens							
A	39	A	6	3	8	3	12
B	22, 31	C	6	7	8	3	12
C	7	B	6	7	8	1	12
D	41	C	6	7	7	3	12
E	34	C	6	2	8	3	9
X. allantoidea							
F	52	C	3	5	1	4	1
G	2, 66	C	3	5	1	5	13
H	15	C	3	5	3	4	1
I	14, 1	C	3	4	1	5	13
X. cf. *anisopleura*							
J	56	B	6	5	10	2	11
K	138, 137	C	5	5	10	5	11
X. arbuscula							
L	55, 77, 82	B	4	6	6	4	12
M	83, 70, 78, 85, 86, 88	C	4	6	6	4	9
X. cf. *castorea*							
N	4, 47, 48	B	4	7	3	2	2
O	53, 65	B	4	7	3	5	3
X. coccophora							
P	58	C	1	6	4	3	7
Q	33, 59, 75	C	1	6	5	3	7
X. cubensis							
R	3	C	4	5	4	3	3
S	29	C	4	7	4	3	3
T	8, 20, 23, 63	C	4	9	4	3	3
X. curta							
U	13, 72, 64, 73, 74, 87, 35, 50, 62	B	6	1	6	2	12
V	6	B	6	1	6	2	10
W	71, 68	C	6	3	9	212	
X. cf. *microceras*							
X	11	B	9	10	9	6	12
Y	10	B	7	6	n.a.**	6	12
Z	40	B	4	3	1	6	12

Phenotype	Strain #	Source*	Enzyme electromorphs				
			ALD	PGI	PGM	6PG	TPI
X. cf. *multiplex*							
AA	38	A	6	2	7	5	12
AB	76	B	6	4	6	5	11
AC	17, 21, 43	B	6	4	11	5	n.a.
AD	24, 79	B	6	4	11	5	12
AE	80	B	6	4	12	5	12
X. cf. *obovata*							
AF	36	B	6	3	9	1	9
AG	32, 42, 67	C	6	3	10	2	12
X. cf. *palmicola*							
AH	12, 5	B	6	7	2	1	6
Xylaria sp. 1							
AI	49	C	6	3	3	2	12
AJ	84	B	6	3	4	2	12
Xylaria sp. 2							
AK	81	B	8	3	2	4	5
X. cf. *telfairii*							
AL	18	B	5	3	5	4	4
AM	27, 60	B	5	4	5	74	
AN	25	B	5	4	6	4	4
AO	26	B	5	4	5	4	4
AP	30, 133	B	10	5	5	4	4
AQ	9	A	5	4	10	3	4
AR	16	A	5	4	10	3	8
AS	46	A	n.a.	4	10	n.a.	n.a.

*A = strains derived from ascospore isolates obtained from Prof. J. Rogers (WSU):
X. adscendens: # 39: Hawaii, on *Sapindus saponaria*.
X. multiplex: # 38: SM 1491, Mexico, on unident. dicot.
X. telfairii: # 9, 16, 46: 7708–0307 NTU, Taiwan, on unident. dicot.
B = non–conidial endophytic strains.
C = conidial endophytic strains.

**n.a.= no enzyme activity.

listed in Tab. 2 by species and isolate numbers. Among the isolates included in the study two to eight phenotypes were found for each species. The less variable zymogram observed within species was produced by aldolase (ALD) and the most variable by phosphoglucomutase (PGM). Species that showed the highest phenotypic diversity were isolates identified as *X. adscendens*, *X.* cf. *multiplex* (each five phenotypes) and *X.* cf. *telfairii* (with eight distinct phenotypes).

In general, endophytic conidial strains had different enzymatic activities than non–conidial strains, as seen among isolates of *X.* cf. *anisopleura*, *X. arbuscula*, *X. curta*, *X.* cf. *obovata*, and *Xylaria* sp. 1.

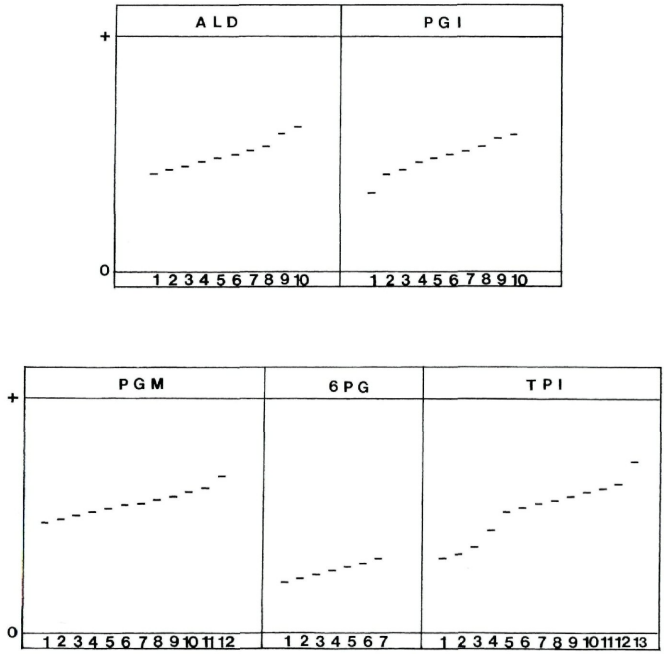


Fig. 10. – Diagrammatic representation of the electromorphs of the five enzyme systems found in 81 isolates of *Xylaria* species. Numbers are those used in Tab. 2.

At no time were banding patterns of conspecific isolates derived from cultured teleomorphs identical to those of the endophytic isolates. However, because the geographical origins of the cultured teleomorphs were distinct, and because they derived from different hosts, these differences may well reflect a large gene pool for these cosmopolitan species as well as host specialization (Petrini, 1993).

The dendrogram generated by the cluster analysis is presented in Fig. 11. In general, isolates assigned to a given taxon by morphological characterisation tended to form comparatively compact groups. Four main clusters were recognized. The first cluster (1) contained subclusters formed by isolates of *X. curta*, *X.* cf. *obovata*, *X. adscendens*, *X.* cf. *multiplex*, and *X.* cf. *palmicola*. Within the second cluster (2) five groups containing *X.* cf. *castorea*, *X. cubensis*, *X. coccophora*, *X. arbuscula* and *X.* cf. *microceras* were distinguished. The third

cluster (3) included three subclusters constituted by *X. allantoidea*, X. cf. *telfairii*, and *X.* cf. *anisopleura*. The last cluster contained only *Xylaria* sp. 2.

Discussion

The use of only five enzyme systems in the isozyme analysis does not allow too many firm conclusions to be drawn on the taxonomic relationships within and among morphological taxa. The isozyme banding patterns presented by isolates of *Xylaria* species confirmed the delimitation of most species by cultural morphology. However, some groups of isolates that were thought to represent distinct species on the basis of cultural characters were revised in the light of identical isozyme patterns.

The isozyme analysis showed a high degree of variation within and among the putative species examined, reflecting the morphological variation found in pure cultures. In addition, the results showed isozyme diversity within species, confirming the genetic diversity of the isolates. There were cases in which isolates presumed to belong to a single species shared no electromorphs. This phenomenon has already been reported to occur in congeneric species of unrelated fungal genera (Leuchtmann & al., 1992). The amount of genetic diversity present within species may be an indication that some of the more heterogeneous taxa actually consist of more than one species. It is at least conceivable that within a 'species' asexual endophytes and stromata forming strains represent different evolutionary lines and thus are differentiating into different taxa. Perhaps, as more information becomes available from larger samples and relies on many more biochemical as well as other characters justification will be given to split these species into several taxa.

The results of the cluster analysis suggested some relationships among the taxa investigated. For instance, the near clustering of endophytic strains of *X. curta*, a species known to be related to the *X. polymorpha* species complex (Rogers, 1985), to those of *X.* cf. *obovata* might indicate some close taxonomic affinity among these taxa, despite the recent exclusion of *X. obovata* from this species complex (Callan & Rogers, 1990). Although *X. anisopleura* was considered to belong to the *X. polymorpha* complex (Rogers & al., 1987, 1988) on the base of morphological characters, its isozyme profile placed it in a separated cluster. *X.* cf. *anisopleura* was distinguished in culture by the presence of coiled hyphae in the aerial mycelia, morphology of the conidiogenous structures, stromata shape, and production of a bright orange exudate along the length of the stromata. Cultures of *X.* cf. *obovata* presented colonial morphology and conidiogenous structures typical of the species as described by Callan & Rogers (1990). Isolates identified as *X. curta* were divided among two morphologically

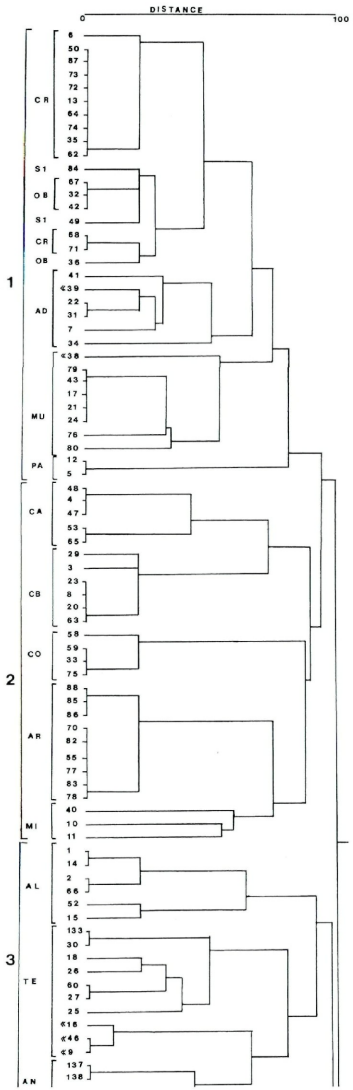


Fig. 11. – Dendrogram based on isozyme phenotypes showing the similarities among *Xylaria* species isolates. Distance is normalized percent of disagreement, analyzed by the average linkage method of clustering. « = isolates originated from ascospores. AD *X. adscendens*, AL *X. allantoidea*, AN *X.* cf. *anisopleura*, AR *X. arbuscula*, CA *X.* cf. *castorea*, CB *X. cubensis*, CO *X. coccophora*, CR *X. curta*; MI *X.* cf. *microceras*, MU *X.* cf. *multiplex*, OB *X. obovata*, PA *X.* cf. *palmicola*, S1 *Xylaria* sp. 1, S2 *Xylaria* sp. 2, TE *Xylaria* cf. *telfairii*.

distinct groups. Isolates of one group formed stromata abundantly and produced conidia. Isolates of the second group produced stromata scattered at the periphery of colonies, and cultures were always sterile. This variation in culture was reflected in the isozyme analysis, which isolates presented different enzymatic activity as shown by the three phenotypes produced. Conidial strains of *X. curta* were represented by one phenotype (W), whereas non-conidial strains presented two phenotypes (U, V). From the isozyme analysis, the isolates of *Xylaria* sp. 1 appear to be comparatively similar to those of *X.* cf. *obovata*. This suggests that the two taxa could be closely related and that *Xylaria* sp. 1 may even belong to *X.* cf. *obovata*. On the other hand, marked morphological differences do not warrant the inclusion of *Xylaria* sp. 1 isolates in the former species, at least at this stage.

Xylaria arbuscula and *X. coccophora* have been considered related to the *X. multiplex* group (Callan, 1988), and the inclusion of *X. microceras* in the same complex was suggested by Joly (1968). Cultures of *X. arbuscula* were basically characterized by the production of black stromata with white apices that form in concentric rings, and by the morphology of the conidiogenous structures, formed either on the cylindrical stromata or on pulvinate structures on the colony surface. Cultures of *X. coccophora* were characterized by the production of a canary yellow pigmentation on the surface of colonies and stromata. This, along with the formation of thin, wiry stromata and morphology of conidiogenous structures, is in accordance with the descriptions of *X. coccophora* given by Callan & Rogers (1990). *Xylaria* cf. *microceras* produced neither stromata nor conidiogenous structures in culture. According to the results of the cluster analysis, *X. arbuscula*, *X. coccophora*, and *X.* cf. *microceras* form a closely related group of species based on their isozyme profiles. However, endophytic strains as well as ascospore isolates identified as *X. multiplex* did not group together with the three species mentioned above, rather they appeared to be closely allied to *X. adscendens* and *X.* cf. *palmicola*.

Isolates of *X. cubensis* were grouped together with *X.* cf. *castorea* based on their isozyme profiles, however, these species are not thought to be related. The former is considered to be taxonomically closely related to *X. allantoidea* (Rogers, 1984b). Although some species are clustered in this analysis, this does not necessarily imply close taxonomic relatedness but rather a tendency that may have resulted from the small number of enzyme systems studied.

The isozyme electrophoresis placed isolates of *X.* cf. *telfairii* in the same group with *X. allantoidea* suggesting that these two species may be related. Cultures of *X. telfairii* were characterized by colony color, presence of coiled hyphae in the aerial mycelium, and fan-like stromata. All endophytic strains were sterile, in accordance with previous reports on cultures of this species (Callan & Rogers, 1990).

Although cultures of the three ascospore isolates of *X. telfairii* derived from different hosts were morphologically similar to the endophytic isolates, the isozyme profiles of cultured teleomorphs were not comparable to the endophytic strains. Isolates of *X. telfairii* derived from ascospores grouped close to endophytic isolates of *X.* cf. *anisopleura*.

Isozyme electrophoresis when used in combination with cultural characters may be a useful tool to differentiate endophytic *Xylaria* species. However, due to the high degree of enzymatic variation, the identification of *Xylaria* species cannot be accomplished solely based on direct comparison of zymograms.

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