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A new *Vestigium* on *Abies balsamea*

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Abstract: A second species of *Vestigium* is described and contrasted with *V. felicis* known from Oregon and B.C. on *Thuja plicata*. The new species occurs on needles of *Abies balsamea* in New Brunswick and is compared with *Rhizothyrium abietis*, the anamorph of *Rhizocalyx abietis*. Molecular studies of the new species show affinity with Chlorencheliaceae, Dermateaceae, Hemiphacidiaceae or Hyaloscyphaceae.

Key words: Endophytes, *Vestigium trifidum*, *Vestigium felicis*, *Rhizothyrium abietis*, *Rhizocalyx abietis*, *Chlorenchelia torta*, *Cistella spicicola*, *Cryptosporiopsis actinidiae*, *Colipila masdugwana*, *Phialia strobilina*, *Tetracladium* spp., *Cetenulifera* sp., *Heyderia abietis*, and *Phaeocryptopus nudus*.

An intriguing fungus was found in New Brunswick on the underside of balsam fir needles, unconnected with the lines of stomata. At low magnifications, the fruitbodies appear as tiny brown exposed pycnothyria with a granular surface (Fig. 1). The fruitbodies are easily removed with a needle and are superficial on the outer surface of the cuticle without obvious penetration into the needle cells. When mounted in water, a large mass of conidia swells out from underneath a small (220-250 μm diam.), brown, shield, (Figs. 2, 3) which is made up of radiating, septate, brown, sparsely-branched hyphae 8 μm diam. near centre and 5 μm at the blunt ends, as in *Microthyrium* but with less regular cell structure and without a pore and thereby resembling more closely *Rhizothyrium*. There are also some finer hyphae radiating from the tips of the shield that may help to hold the pycnothyrium to the needle. The conidia are mostly four-celled, applanate, hyaline, echinulate, finely guttulate, 11-15 x 10-11 μm with a basal somewhat rectangular cell and (2) 3 (4) obovoid toe-like cells (Fig. 5). There may be a mass of gel around the spores. Within the mass of conidia are a few conspicuous brown, thin, almost straight, distantly septate, rarely branched, smooth hyphae, 1.5-3 μm diam. The conidiophores are hyaline, straight, distantly septate, thin walled, echinulate in some regions, di- or trichotomously branched one to three times near the apex, 50-70 x (2)3(4) μm (Fig. 4). The conidiogenous cells occur in twos or threes, are slightly curved, 10-12 x 3 μm , verrucose and are crowded close together on the terminal part of the conidiophore. The attachment appears pore-like but is inconspicuous and unpigmented.

Conidium germination occurs from the basal cell with broad, brown, septate, somewhat curved hyphae, 50-70 (120) x 5-6(7) μm after 4 days on PDA at 20°C in dim light (Figs. 7, 8). Occasionally, one of the other cells germinates later. Growth is extremely slow, 1 mm diam. in 7 days on PDA, and 3-4 mm in 28 days on oat agar. The colony appears as a black mound with some

short, projecting, hyaline mycelium. After 28 days on oat agar, the colony appeared as a spherical structure, somewhat sclerotium-like. The surface was of brown, interwoven hyphae, 4-7 μm diam. with brown pigment in the walls and intercellular spaces (Fig. 9). The central interior consisted of perpendicular, slightly twisted, widely spaced, brown hyphae, 1-2 μm diam. (Fig. 6). Colonies on autoclaved *Ilex* stems and berries or alfalfa stems did not sporulate; only a small, black, spherical colony was produced like that formed on PDA or oat agar.

The paw-like, applanate conidia resemble those of *Vestigium felcis* Pirozynski & Shoemaker (1972), which occurs on *Thuja plicata* foliage in Oregon and B.C. (Figs. 10-12). The new species is readily distinguished by its verrucose, regularly 3-toed conidia and its superficial pycnothyrium whereas *V. felcis* has smooth 3- to 6-toed conidia formed intraepidermally (Fig. 10). Because of the great similarity in the conidia, the fungus is described as a new species of *Vestigium*. Further studies may attach greater significance to the nature of the pycnothyrium versus the intraepidermal acervulus and support a revised generic placement.

Vestigium trifidum sp. nov. Shoemaker & Malloch

Mycobank # 564890

Etymology: Latin epithet, trifidum, cleft in three, referring to the resemblance to a footprint with a central pad and three claws.

Pycnothyria hypofolia, superficialis, scutiformis, 200-250 μm diam.; *lacuna absentia*; *hyphae radians, decrescens, pauciramosae, septatae, brunneae*, 8-5 μm diam. *Conidiophorae hyalinae, rectae, pauciseptatae, tenuitunicatae, ± echinulatae, apicaliter bi- trifurcatae*, 50-70 x (2)3(4) μm . *Cellulae conidiogenae* 2-3 *aggregatae, curvatae, verrucosae*, 10-12 x 3 μm , *cum hilo poriformo hyalino*. *Conidia quadripartita, plana, hyalina, echinulata, guttulata*, 11-15 x 10-11 μm ; *cellula basale ± squarrosa; cellulae apicale*, (2) 3 (4),

obovoideae; in gel, inter hyphae brunneae, rectae, pauciseptatae, pauciramosae, glabrae, 1.5-3 µm diam.

TYPUS: “*Vestigium* sp. On dead needles of *Abies balsamea*, 1.5 km W of Little Lepreau, Lepreau Parish, Charlotte Co., New Brunswick, 45° 08' 05" N 66° 29' 33" W, 21 April 2009, Coll. David Malloch. DAOM 240321.” ISOTYPUS: NBM F-04448 (same data).

The holotype was collected on dead needles still attached to branches. These branches had fallen from the parent tree and had come to rest against the trunk of the same or different tree in an upright position. It is likely that some of the needles had been attached high enough on the branch to have been above the snowline. It is not known when the conidia had matured, but the time of collection suggests that they had done so either in the spring or the preceding autumn.

The type locality was a relatively disturbed and rather senescent forest dominated by *Abies balsamea*, *Picea rubens* and *Betula cordifolia* with an understory of scattered *Alnus viridis* and *Sorbus americana*, located about 140 m from the shore of the sea. It is classified within the Fundy Coast Ecoregion, an area characterized by relatively high summer precipitation (May–September: 450–500 mm) and persistent summer fog (Zelazny, 2007).

Order and Family placement based on morphology is tentative. Pycnothyriales is in the latest Dictionary of the Fungi (Kirk et al. 2008) but Pycnothyriaceae seems not to be accepted by Cannon & Kirk (2007). See the discussion of orders and families by Darker (1965). Superficially, the pycnothyrium is like that of *Rhizothyrium abietis* as observed from its type (Figs. 13, 14). *R. abietis*, as indicated by the generic name, has a pycnothyrium and a central stalk that is rooted in a large complex of hyphae in the substomatal chamber. There is no ostiole. The central column supports the covering layer from which 3-septate, terete, ellipsoidal, smooth

conidia are produced (Fig. 14). See also Naoumoff (1915 Figs. 17–19). The short conidiophores arise from the inner surface of the external shield. The dehiscence of conidia is from the margin. It is well illustrated by Darker (1965). The teleomorph connection to *Rhizocalyx abietis* Petrak (1928) was established in culture by Smerlis (1967) who illustrated and fully described the teleomorph. In contrast, the new fungus though hypophyllous, is not stomaticolous, lacks a central stalk, and has applanate, paw-like, echinulate conidia. The conidiophores are few, hypha-like below, and branch several times and produce many closely clustered conidia in the upper part. In water, the shield is quickly raised by a mass of hydrophilic conidia in *Vestigium trifidum* whereas in *R. abietis*, a fringe of still-attached conidia emerges from under the periphery of the shield. For *R. abietis*, Smerlis (1967) reported a colony diameter of 22–25 mm on 3% malt agar at 15°C after 4 weeks. In contrast, *Vestigium trifidum* on 2% malt agar at 20°C attained only 3–4 mm after 4 weeks.

Molecular Studies: Ribosomal DNA sequence of 1,600 nucleotides covering ITS 1, 5.8s, ITS 2 and partial 28s was sequenced (GB number KC407777). A blast search of GenBank records for the 28s partial sequence of 1083 nucleotides revealed a 98% maximum identity with *Chlorenchelia torta* (Schwein.) J.R. Dixon (Chlorencheliaceae), 96% maximum identity with *Cistella spicicola* Huhtinen & Söderh. (Hyaloscyphaceae), *Cryptosporiopsis actinidiae* P.R. Johnst., M.A. Manning & X. Meier (Dermateaceae), *Colipila masduguana* Baral & Guy García (Helotiales *incertae sedis*), *Phialea strobilina* (Fr.) Gillet (Pezizales *incertae sedis*), 95% maximum identity with *Tetracladium* spp. (mitosporic Ascomycota) and *Cetenulifera* (Hyaloscyphaceae) etc. Another search using the ITS1, 5.8S and ITS2 complete sequence revealed two named strains: *Chlorenchelia* sp. (Chlorencheliaceae / Hemiphaciaceae fide CABI) and *Heyderia abietis* (Fr.) Link

(Hemiphacidiaceae fide CABI and Wang et al. (2006) as well as three unnamed strains with maximum identity for all in the range of 93% down to 83%. Wang et al. (2006) suggested a distinct order for the families Hemiphacidiaceae, Rutstroemiaceae and Sclerotiniaceae.

The partial ITS affinity with a sample labeled *Heyderia abietis* based on a culture derived from China, HMAS 71954 GenBank AY789297 (Wang et al. 2005) suggests a disposition somewhere in the Hemiphacidiaceae. Redhead (1977) monographed *Mitrula* and excluded *Heyderia abietis* (Fries) Link and gave *M. cucullata* (Batsch) Fr. as its synonym. *Heyderia abietis* did not group with *Mitrula* species in a molecular study (Wang et al. 2005).

The present molecular evidence aligns *V. trifidum* with a number of unnamed endophytes. There is no exact match to material of named strains, the closest being *Chlorencoelia* sp., and *Heyderia abietis* followed by *Cistella*, *Cryptosporiopsis*, *Colipila*, *Phialea*, *Tetracladium*, *Cetenulifera*. This gives a number of choices for the appropriate family: Chlorencoeliaceae, Dermateaceae, Hemiphacidiaceae or Hyaloscyphaceae. Unfortunately, sequences are not available in GenBank for *Rhizocalyx abietis* (Helotiaceae fide CABI) or its anamorph, *Rhizothyrium abietis*, or for *Vestigium felicis*.

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Figs. 1-9 *Vestigium trifidum* DAOM 240321, Fig. 1, Six hypophyllous pycnothyria X 27, scale = 0.1 mm, (also present under white flecks, *Phaeocryptopus nudus* (Peck) Petrak); Fig. 2, Pycnothyrium surface X 280, Scale = 10 μ m; Fig. 3, Pycnothyrium and exuded conidia X 100, Scale = 100 μ m; Fig. 4, Conidiophore, conidiogenous cells X 1100, Scale = 10 μ m; Fig. 5, Conidia X 1100, Scale = 10 μ m; Fig. 6, Internal structure of spheroid formed in culture X 300, Scale = 10 μ m; Figs. 7, 8, Conidia germinated first from central cell X 900, Scales = 10 μ m; Fig. 9, Surface cells of spheroid in culture X 750, Scale = 10 μ m.

Figs. 10-12 *Vestigium felicis* DAOM 144492, Fig. 10, fructification splitting outer epidermal layer X 200, Scale = 50 µm; Fig. 11, Three 6-toed conidia X 1300, Scale = 10 µm; Fig. 12, Conidia with uninucleate cells (Cotton Blue) X 1800, Scale = 10 µm; Figs. 13, 14, *Rhizothyrium abietis* Naumoff ex TYPE ex LE, DAOM 229764, Fig. 13, Pycnothyrium surface and conidia X 400, Scale = 10 µm; Fig. 14, Conidia X 900, Scale = 10 µm.