Penicillium cecidicola, a new species on cynipid insect galls on *Quercus pacifica* in the western United States

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Abstract: A synnematous species of *Penicillium* subgenus *Biverticillium* was found inside emergence tunnels from insect galls (*Cynipidae, Hymenoptera*, the so-called gall wasps) on scrub oaks (*Quercus pacifica* Nixon & C.H. Muller) collected in the western United States. The fungus produces synnemata with white or creamish stipes, biverticillate conidiophores with lanceolate phialides typical of subgenus *Biverticillium*, and small, ellipsoidal, slightly roughened dark green conidia. In culture, the fungus produces velutinous, dark green colonies, with synnemata near the margins of the colonies, particularly in fresh isolates exposed to light after 10 days. The fungus produces the extrolite apiculide A and a series of unidentified extrolites also produced by *P. panamense*. The oak gall species is described here as *Penicillium cecidicola* and compared with similar species. An ITS phylogeny suggests that *P. cecidicola* is a sister species to *P. dendriticum*, an Australian species with yellow synnemata that also sometimes occurs on insect galls. Notes are included on other *Penicillium* species we have isolated from insect galls.

Taxonomic novelty: *Penicillium cecidicola* Seifert, Hoekstra & Frisvad sp. nov.
Key words: galls, *Penicillium glabrum*, *P. dendriticum, P. erythromellis, P. pseudostromaticum.*

INTRODUCTION

In 1989, fusiform insect galls on twigs of *Quercus pacifica* were received from Dr RJ Bandoni, after a collecting trip to south western Oregon. Synnemata of a *Penicillium* species grew from the interior surface of bore holes presumably made by the emerging morph of the gall-forming insect (unidentified *Cynipidae, Hymenoptera*). The fungus was isolated into pure culture and compared with previously described species using cultural characters, microscopy, extrolite profiling, and phylogenetic analysis of aligned internal transcribed spacer rDNA (ITS, including the 5.8S rDNA) sequences. It is described here as a new species in *Penicillium* subgenus *Biverticillium.*

MATERIALS AND METHODS

Isolations were made on malt extract agar with 100 mg/L tetracycline by removing conidial masses from synnemata in insect tunnels in the galls using a sterile needle. Other species of *Penicillium* from insect galls were isolated from conidiophores growing on the inside of various galls collected in North America and Australia, or from the exterior of damp-chambered galls.

For standardized descriptions, spore suspensions in semi-solid agar were inoculated at three points on Czapek Yeast Agar (CYA) with added micronutrients (TMS in Samson *et al*. 2004), Malt Extract Agar (MEA), and 25 % Glycerol Nitrate Agar (G25N) in 9 cm polystyrene Petri dishes, and incubated at 25 °C, 37 °C or 5 °C for 7 d in the dark (Samson & Pitt 1985) or for 7–14 d in incident light at room temperature. Colonies were also examined on creatine agar (CREA), oatmeal agar (OA), and yeast extract sucrose agar (YES) using the recipes in Samson *et al*. (2004). Capitalized colour names and codes used to describe colony colours refer to Kornerup & Wanscher (1978). For studies of extrolite production *in vitro*, cultures were grown on CYA, MEA, YES and OA. Extrolites were extracted and analyzed with HPLC-diode array detection following the methods of Nielsen & Smedsgaard (2003).

Sequencing of the ITS of the oak gall strain, other *Penicillium* species isolated from galls, and representatives of some other species of subgenus *Biverticillium*, was undertaken using DNA isolated from conidia produced on MEA using the FastPrep™ FP120 (BIO 101 Inc.) kit.

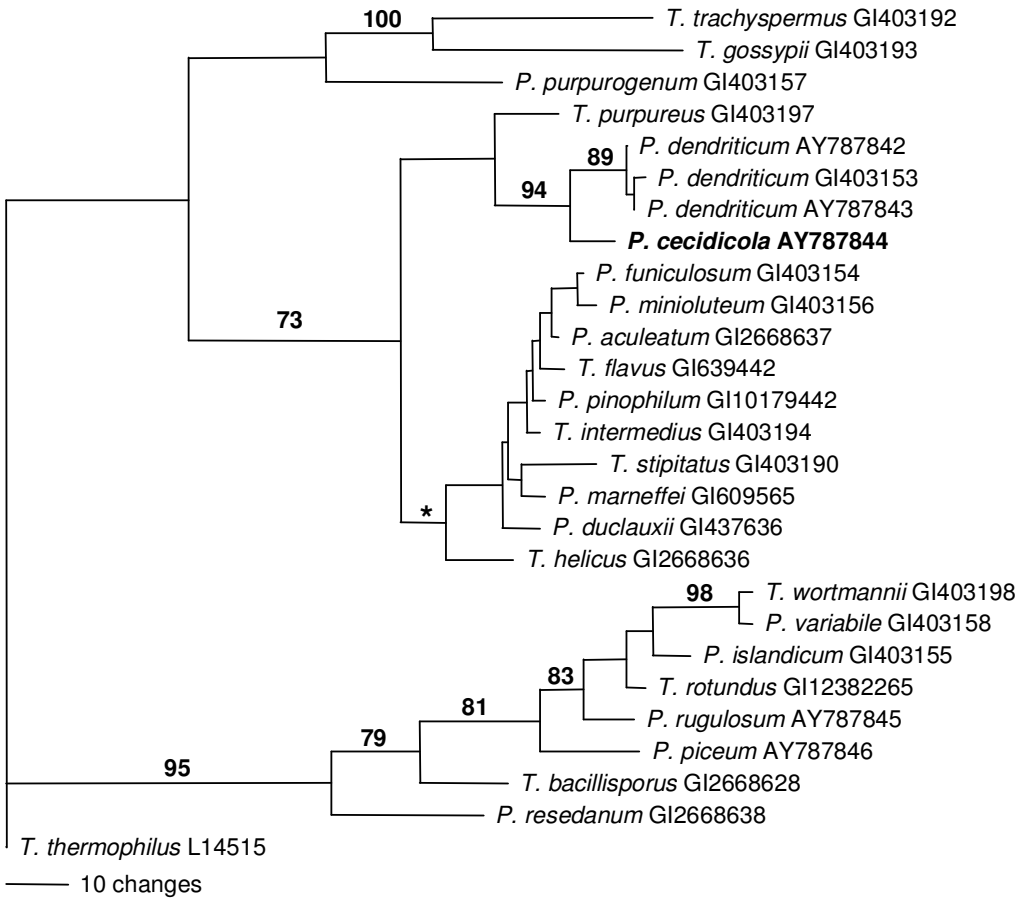
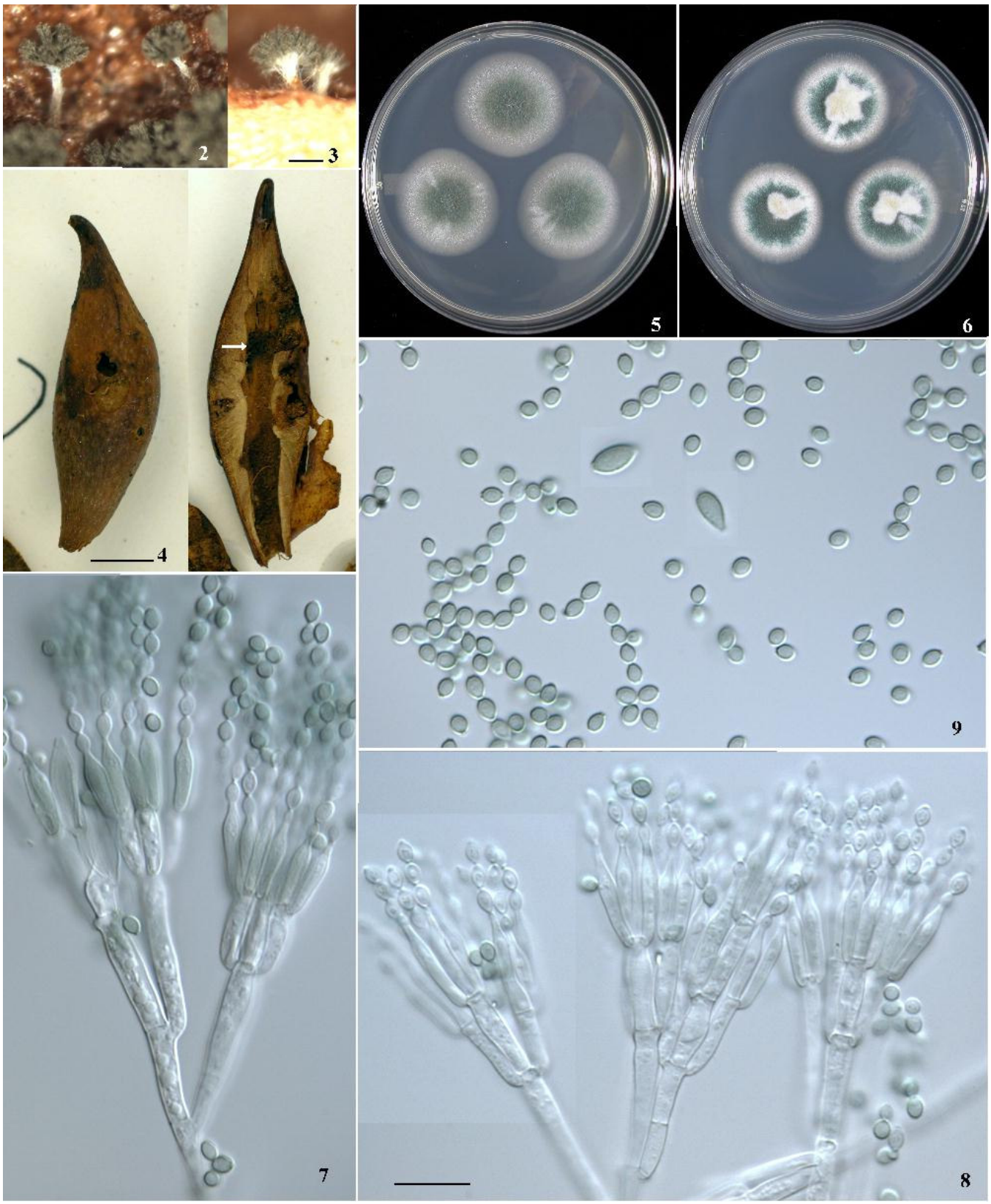


Fig. 1. Phylogenetic tree based on aligned internal transcribed spacer sequences of selected *Talaromyces* species and related species of *Penicillium* subgenus *Biverticillium*, showing the sister group relationship between *P. dendriticum* and the new species, *P. cecidicola*. The outgroup is *T. thermophilus.* Accession numbers starting with "AY" are for sequences obtained as part of this study. One of two equally parsimonious trees, differing in the position of *P. purpurogenum* GI403157 (alternate position in the 2nd ept designated by *). Numbers above branches represent 'fast' bootstrap values above 70 % (141 steps, CI = 0.535, RI = 0.743, RC = 0.396, HI = 0.467).

Polymerase chain reaction profiles, purification and cycle sequencing methods were those given by Seifert *et al.* (2004), with the sequences determined by an ABI PRISM™ 310 DNA automated sequencer (Applied Biosystems, Foster, CA).The complete ITS and 5.8S rDNA were amplified using primers ITS1 and ITS4, and cycle-sequenced using primers ITS1, ITS2, ITS3 and ITS4 (White *et al.* 1990). A data set was compiled of sequences of our new species, species of *Penicillium* subgenus *Biverticillium* and related *Talaromyces* species from GenBank, based primarily on the studies of LoBuglio *et al.* (1993, 1994), Peterson (2000) and Heredia *et al.* (2001), and some newly derived sequences representing additional species in the subgenus. Accession numbers are included on Fig. 1. An alignment was calculated using Clustal-W, and adjusted to maximize homology. The alignment is available online from TreeBase (Study S1198, Matrix M2067). Parsimony analysis of alignments were performed with PAUP* v.4.0b10 (Swofford 2000) using heuristic searches with uninformative characters removed. The robustness of the phylogeny was tested using bootstrap analysis (1000 replications, 'fast' stepwise addition).

RESULTS

The GenBank accession number for the ITS sequence of the new species from oak galls is given in Fig. 1. For the phylogenetic analysis of subgenus *Biverticillium*, the alignment of ITS sequences was 602 bases long, which included 141 phylogenetically informative positions. *Talaromyces thermophilus* was chosen as a suitable outgroup based on the results of Heredia *et al.* (2001). The heuristic search yielded two equally parsimonious trees 430 steps long, which differed in the placement of *P. purpurogenum* GI403157 (Fig. 1). The results confirmed the placement of the gall species (described below as *P. cecidicola*) in the *Talaromyces/Penicillium* subg. *Biverticillium* clade, as a sister species to *P. dendriticum*. These two species formed a well-supported monophyletic group with *T. purpureus* as the closest neighbour. The oak gall fungus produced a distinctive profile of extrolites, namely apiculide A and series of unidentified extrolites some of which are also produced by *P. panamense*.



Figs 2–9. *Penicillium cecidicola*, photographs from holotype and ex-type material. 2, 3. Synnemata on holotype. 4. Fusiform cynipid wasp gall showing emergence hole (left) and split to show presence of synnemata (arrow) in tunnels. 5. Ex-type culture on MEA after 7 d. 6. Ex-type culture on CYA after 7 d. 7, 8. Conidiophores from ex-type culture on MEA after 7 d. 9. Conidia from ex-type culture on MEA after 7 d. Scale bars: 3 = 100 µm for Fig. 2, 3; 4 = 500 µm; 8 = 10 µm for Figs 7–9.

Taxonomy

Penicillium cecidicola Seifert, Hoekstra & Frisvad, **sp. nov**. MycoBank MB500150. Figs 2–10.

Etymology: *cecidium* (L)- gall. *cola* (L)- living on.

Coloniae in agaro CYA post 7 dies 25 °C 19–31 mm diam Coloniae in agaro MEA post 7 dies 25 °C 32–40 mm diam Synnemata determinata, 150–1250 × 20–75 µm; stipites albi vel cremei. Conidiophora solitaria vel e synnematibus oriunda, stipites leves. Penicilli biverticillati vel quaterverticillati, metulis appressis, 9–12(–17) × 2.5–3 µm. Phialides acerosae, (8–)11–15 × 1.5–3 µm. Conidia ellipsoidea, plusminusve levia, (2–)2.5–3.5 × 1.5–2.5 µm. Holotypus DAOM 233329.

Conidiophores arising from the agar surface, from aerial mycelium or funicles, forming synnemata towards the margin or in sectors in some colonies after 7–14 d in the light. *Synnemata* determinate, 250–1250 µm tall, with an unbranched stalk 20–75 µm wide (to 250 µm wide at the base), white, often becoming cream-coloured or light orange in the basal 1/2–2/3 with age, and a terminal, compact to slightly divergent capitulum comprising conidiophores and a powdery green conidial mass, in some transfers mostly produced near the junctions between the colonies. Mononematous and synnematous conidiophores biverticillate, symmetrical, sometimes with an extra whorl of metulae arising from the stipe below the terminal whorl, or sometimes terverticillate (Figs 7, 8), the stipes smooth-walled, 3–4 µm wide, 20–80 µm long when arising from funicles, and 200 µm or longer when arising directly from the agar. *Metulae* in whorls of 3–7, usually rather appressed, sometimes slightly divergent when forced apart by larger whorls, about 9–13(–20) µm across the top, individually more or less cylindrical, 9–12(–17) × 2.5–3 µm; branches, when present, 15–22.5 × 2.5–3 µm. *Phialides* acerose, or narrowly ampulliform, (8–)11–15 × 2–3 µm, slightly green, conidiogenous aperture 0.5–1.5 µm wide, lacking an obvious periclinal thickening or collarette. *Conidia* (Fig. 9) ellipsoidal, smooth-walled or slightly roughened, (2–)2.5–3.5 × 1.5–2.5 µm (mean +/– standard error = 3.1 +/– 0.05 × 2.3 +/– 0.03, n = 25), inconspicuous remnants of connectives sometimes visible, walls rather thick, often with sparse larger conidia up to 7 × 3 µm.

Synnemata produced on the host (Figs 2, 3) more clearly delimited than in culture, determinate, 150–350 µm tall, with a white to creamish or pale brown, unbranched stipe 20–75 µm wide and terminal, slightly divergent capitulum comprising conidiophores and a powdery, green conidial mass. *Conidiophores* biverticillate, symmetrical, the stipes smooth-walled, 2.5–3.5 µm wide. *Metulae* shorter than in culture, sometimes slightly swollen, 8–9 × 2.5–3 µm. *Phialides* shaped as in culture but shorter, 9–13 × 1.5–2 µm. *Conidia* as in culture, but tending to adhere in chains with inconspicuous but visible connectives between the conidia.

Colonies on CYA 19–31 mm diam after 7 d at 25 °C (Fig. 6), dense, planar to convex, with sparse aerial mycelium, or with white to pale Orange-White (5A2) floccose to funiculose aerial mycelium in the centre of the colony of some transfers, most of the colony velutinous, covered with Greyish Green to Greyish Turquoise conidia (24E6, 27D5), exudate absent or sparse clear drops, the reverse Reddish Brown (8CF5–8), the margin entire, soluble pigment absent or pink to red.

Colonies on MEA 32–40 mm diam after 7 d at 25 °C (Fig. 5), less dense than on CYA, low, 1–2 mm deep, planar, the surface velutinous to funiculose, covered with Dark Green conidia (26–27F6–7), with sparse aerial mycelium, with minute, embedded clear exudate droplets in the centre of the colony, or exudate lacking, the reverse Green (27C2–3), Orange (6–7A8) or Reddish Brown (8D8), the margin even to slightly uneven, soluble pigment not produced.

No growth on G25N. Conidia germinating but with no linear growth at 37 °C on CYA, or growing up to 10 mm diam. No growth on CYA at 5 °C. Colonies on YES 22–28 mm diam, cream to yellow, reverse dark red brown. Colonies on OA 28–32 mm diam. Colonies on CREA growing poorly, 3–5 mm diam, no acid production.

Specimen examined: U.S.A., Oregon, 2.5 km N. of O'Brien (*ca.* 42 °N, 123 °W), on Hwy 199 N., from cynipid insect galls on twigs of *Quercus pacifica*, United States, Oregon, specimen leg. A.A. & R.J. Bandoni, 11 Mar. 1989, R.J.B. 8348, culture isol. Keith A. Seifert. (**Holotype** DAOM 233329, ex-type culture CBS 101419, DAOM 233329, IBT 21679, IBT 26282).

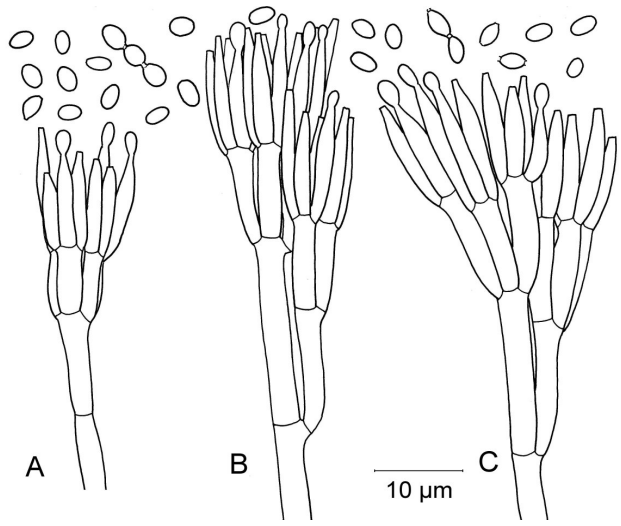


Fig. 10. *Penicillium cecidicola*, line drawings from holotype and ex-type material. A, conidiophores from synnemata on holotype. B, C. Conidiophores from ex-type on MEA after 7 d.

DISCUSSION

Penicillium species are usually considered soil fungi (Pitt 1979), but many species inhabit well-defined habitats other than soil. The species described here, *P. cecidicola*, was collected on fusiform galls of unidentified cynipid wasp on *Quercus pacifica* in the western United States, and has not been found on other substrates. Most collections of cynipid galls that we examined had species of subgenus *Biverticillium* present. These included two other species that we have yet to conclusively identify, which produce tufted, caespitose conidiophores rather than synnemata *in vivo* and have distinctive extrolites. Insect galls have not been examined often for *Penicillium* species, but we have isolated several species from the interiors and exteriors of several kinds of cynipid galls (Table 1). The idea that at least some *Penicillium* species are associated with insects and that their extrolites may affect this fauna is further supported by the recent description of *P. brocae* S.W. Peterson *et al*. (2003) from borer infested coffee berries in Mexico.

The synnematous species of subgenus *Biverticillium* have the typical lanceolate phialides that characterize the subgenus. Often, the conidiophores have an extra level of branching and are terverticillate and asymmetrical, rather than biverticillate and symmetrical, especially when grown in culture. Most have dark green conidia in the approximate size range of 3–4 × 2–3 µm, and phialides are generally 10–15 µm long. The species are distinguished mostly by cultural characters, in combination with lengths and colours of synnemata, the disposition of the conidiophores on synnemata, and the presence or absence of sterile hyphae among the conidiophores. Apart from its unusual substrate, *P. cecidicola* is characterized by the production of short, determinate, synnematous conidiomata with white to creamish stipes, biverticillate to terverticillate conidiophores, lanceolate phialides, and relatively smooth-walled, ellipsoidal, dark green conidia. As revealed by the ITS analysis, *P. cecidicola* is a sister species of *P. dendriticum*. The latter species produces similar synnemata but with sulphur-yellow stipes, and is mostly associated with *Eucalyptus* in Australia (Pitt 1979). The two species are otherwise very similar, having similar synnemata, metulae, phialide, and conidial dimensions. We have also found *P. dendriticum* on the surface of unidentified insect galls on *Eucalyptus* leaves (Table 1). The recently described *P. aureocephalum* Munt.-Cvet., Hoyo & Gómez-Bolea is also similar to these species and was isolated from leaves of *Quercus*. However, the synnemata have yellow stipes that turn red in KOH, and the conidial head is covered with a mass of sinuous, sterile hyphae (Muntañola-Cvetković *et al.* 2001). Cultures of the latter species have not yet been sequenced.

Preliminary phylogenies of the *Talaromyces* clade suggest that it is a monophyletic group distinct from *Eupenicillium*, and anamorphs presently classified in *Penicillium* subgenus. *Biverticillium* should probably be segregated in a distinct anamorph genus. Heredia *et al.* (2001) showed that *T. thermophilus* is relatively distant from and probably not congeneric with the rest of *Talaromyces.* In the analysis presented here, there are three or four clades within *Talaromyces* that might warrant recognition as subgenera. The variable position of *P. purpurogenum* in the two most parsimous trees, and the lack of bootstrap support for much of the phylogram, suggest that these preliminary results need to be confirmed with broader taxon sampling and analyses of additional genes

Extrolite profiling is a valuable means of phenotype characterization in *Penicillium*, and was applied in *Talaromyces* by Frisvad *et al*. (1990) and to subgenus *Biverticillium* by Samson *et al*. (1989). Our strain of *P. cecidicola* produced apiculide A, first found in another species of subgenus *Biverticillium, P. aculeatum* var. *apiculatum* by Breinholt *et al*. (1993). Our strain produced a series of unidentified extrolites that are also produced by *P. panamense* (Samson *et al.* 1989), and several other extrolites that are presently unidentified. In contrast, the sister species *P. dendriticum* produces a different profile of extrolites, including mitorubrinic acid and secalonic acid D (Samson *et al*. 1989).

Table 1. Other species of *Penicillium* isolated from insect galls during this study. These galls did not have evidence of bore holes.

Species	Strain	Gall type	Location
P. dendriticum	DAOM 233861	Unidentified insect gall on *Eucalyptus* leaf	Kalnura, NSW, Australia[1]
P. erythromellis	CBS 101415	*Diplolepis rosae* galls on *Rosa sitchensis*	Hornby Island, BC, Canada
P. glabrum	DAOM 233860	Cynipidae gall on *Quercus pacifica*	Julian, CA, U.S.A.
P. paxilli	DAOM 233863	Cynipidae gall on *Quercus macrocarpa* leaves	Baton Rouge, LA, U.S.A.[1]
P. pseudostromaticum	DAOM 233862	*Diplolepis rosae* galls on *Rosa sitchensis*	Hornby Isl., BC, Canada

[1]GenBank accession number for ITS of these two strains are AY787843 (DAOM 233861) and AY787847 (DAOM 233863).

Key to synnematous species of *Penicillium* subgenus *Biverticillium*

1. Synnemata indeterminate (i.e. producing conidiophores along the entire length).....2
1. Synnemata determinate (i.e. producing a distinct stipe and terminal sporulating region) 4

2. Growth rapid on CYA, 50–70 mm diam in 7 d, synnemata often more than 10 mm tall .....***P. isariiforme***
2. Growth slower on CYA, less than 35 mm diam in 7 d, synnemata shorter 3

3. Growth on CYA 15–20 mm diam in 7 d..... ***P. duclauxii***
3. Growth on CYA 25–35 mm diam in 7 d.....***P. palmae***

4. Synnemata often more than 2–5 mm tall 5
4. Synnemata usually less than 2 mm tall 6

5. Growth on MEA 16–25 mm diam in 7 d, metulae 8–10 µm.....***P. panamense***
5. Growth on MEA 35–45 mm diam in 7 d; metulae 10–15 µm.....***P. pseudostromaticum***

6. Growth on CYA more than 35 mm in 7 d.....***P. coalescens***
6. Growth on CYA less than 35 mm in 7 d.....7

7. Synnema stipes white; associated with insect galls on *Quercus* .....***P. cecidicola***
7. Synnema stipes yellow.....8

8. Synnemata resembling mycetozoan sporangia, the conidial head covered by sterile hyphae, on leaves of *Quercus* and *Cistus* .....***P. aureocephalum***
8. Synnema head not covered with sterile hyphae, associated with *Eucalyptus* ..... ***P. dendriticum***

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