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Four new *Penicillium* species having *Thysanophora*-like melanized conidiophores

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Five fungal isolates, tentatively identified as belonging to *Thysanophora*, were re-examined to confirm their generic placement. DNA sequences from multiple loci were determined and compared to homologous sequences from other fungi. The *Thysanophora*-like isolates belong in the genus *Penicillium* on the basis of phylogenetic analysis of their gene sequences. Because these isolates have unique phylogenetic positions within *Penicillium* and have unique phenotypes we describe them as the new species *P. boreae*, *P. subarcticum* and *P. canariense* spp. nov. In addition, *P. pullum* sp. nov. is described for NRRL 721, which served as the typical isolate of *P. fuscum* in the monographic studies of Raper & Thom and Ramirez; however, the name *P. fuscum* has been applied and neotypified using a different specimen and NRRL 721 is markedly different phylogenetically from the neotype of *P. fuscum*.

INTRODUCTION

The generic concept of *Penicillium* has been modified over the years to be more or less inclusive of different characteristics of hyphae, types of conidia produced, colony colours, complexity of the penicillus and other features, but the genus has been limited to species with hyaline hyphae and conidiophores. Recent studies have shown that very darkly pigmented species described within the genus *Thysanophora* are phylogenetically part of *Penicillium* (Iwamoto *et al*. 2002, Peterson & Sigler 2002). This prompted us to re-examine five isolates which had tentatively been identified as *Thysanophora* species based on their development of darkly pigmented conidiophores, but which in other features, more closely resemble *Penicillium* species. In order to determine their correct generic placement, we sequenced the ITS and LSU-rDNA regions, and the calmodulin and β-tubulin genes and compared them to published and unpublished homologous sequences from species of the *Trichocomaceae* and *Thysanophora*.

MATERIALS AND METHODS

Fungal isolates examined: *Penicillium boreae*, ex-type culture NRRL 31002 = UAMH 3896, isol. ex petroleum-contaminated soil, 1975, Norman Wells, Northwest Territories, Canada by D. Westlake; *P. boreae*, NRRL 31401, isol. ex soil, 1973, Swan Hills, Alberta, Canada, by J. W. Carmichael (NWF 154); *P. canariense*, ex-type culture NRRL 31003 = UAMH 6403, isol. ex soil, Canary Islands by A. T. Martinez (IJFM a-543); *P. pullum*, ex-type culture NRRL 721, isol. ex soil, 1930, near Austin, Texas by M. B. Morrow; *P. subarcticum*, ex-type culture NRRL 31108 (= UAMH 3897), isol. ex petroleum-contaminated soil, 1975, near Norman Wells, Northwest Territories, Canada, by D. Westlake (NWF 146); *Penicillium* sp., NRRL 28214, isol. ex soil in a *Sphagnum* bog, 1960, Vilas Co., Wisconsin, by Martha Christensen (WSF 3958). Other sequences used in Fig. 32 were obtained from GenBank and came from ex-type cultures of the species indicated.

All cultures were grown using the media and conditions specified by Pitt (1980) for the description of *Penicillium* species. Cultures were incubated in the dark for 7 d at 25 °C unless otherwise specified. Colour names for colonies are from Ridgway (1912). For light microscopy, fungal material was teased apart with needles and mounted on glass microscope slides in molten (*ca* 40 °) 0.5 % low melting temperature agarose. Microscopic examinations were made using a Zeiss microscope equipped with differential interference

contrast, phase contrast, and Koeller illumination. Photomicrography was performed using a Kodak 420B digital camera operated at ASA100 photo-sensitivity. For scanning electron microscopy (SEM), blocks of agar and mycelium (*ca* 5 × 5 mm) from 7 d old plate cultures were excised, fixed overnight in OsO_4 (1 % in neutral phosphate buffer), dehydrated in a graded series of acetone dilutions (20, 40, 60, 80, 95, 95 and 100 % acetone), critical-point dried, sputter coated with gold-palladium, and viewed in a JEOL scanning electron microscope. The contrast and brightness of digital images from light and SEM photography were adjusted and photographs were sized and fitted into plates using Adobe Photoshop (ver. 6.0.1).

DNA sequencing

Erlenmeyer flasks (500 ml) containing 100 ml of sterilized YM broth (Peterson 1992) were inoculated with conidia from an agar slant culture of each isolate. The cultures were incubated on a rotary shaker (200 rpm) at 25 ° for 1–2 d until 1 g or more of biomass had accumulated. The cells were collected by filtration, freeze-dried, ground to a powder, rehydrated and DNA was purified by phenol–chloroform extraction of proteins and ethanol precipitation of nucleic acids (Peterson *et al*. 2001). The ITS-LSU rDNA (ID region) was amplified in a PCR procedure using the protocol and primers of Peterson (2000) except a modified primer D2R (5′-ttggtccgtgtttcaagacg) was used. Calmodulin was amplified using the procedure of Peterson *et al*. (2001), with $MgCl_2$ concentration varied between 1.5 and 2.75 mM. Beta tubulin was amplified using primers B1055 (5′-gcttgttgagcactccga) and B1244 (5′-ggagggaggtggtcaca) in an amplification using standard buffer and conditions (White *et al*. 1990) and a thermal profile of 96 ° for 2 min followed by 35 cycles of 96 ° for 30 s; 57 ° for 30 s; 72 ° for 90 s. Nucleotide sequences were determined using an ABI 377 or ABI 3100 DNA sequencer and fluorescent dye labels as prescribed by the manufacturer.

DNA sequence analysis

Homology searches were performed using BLAST (Altschul *et al*. 1997) as implemented at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Sequences for some species were obtained from GenBank. Alignment of sequences with related *Penicillium* species sequences was performed using CLUSTALW (Thompson, Higgins & Gibson 1994) followed by manual adjustments with a text editor. Aligned sequences were analyzed using the maximum parsimony criterion in PAUP* ver. 4β8 (Swofford 1998). Tree diagrams generated using PAUP* were formatted in TreeView (Page 1996) and final trees for publication were redrawn in CorelDraw® Ver 9.0.

RESULTS

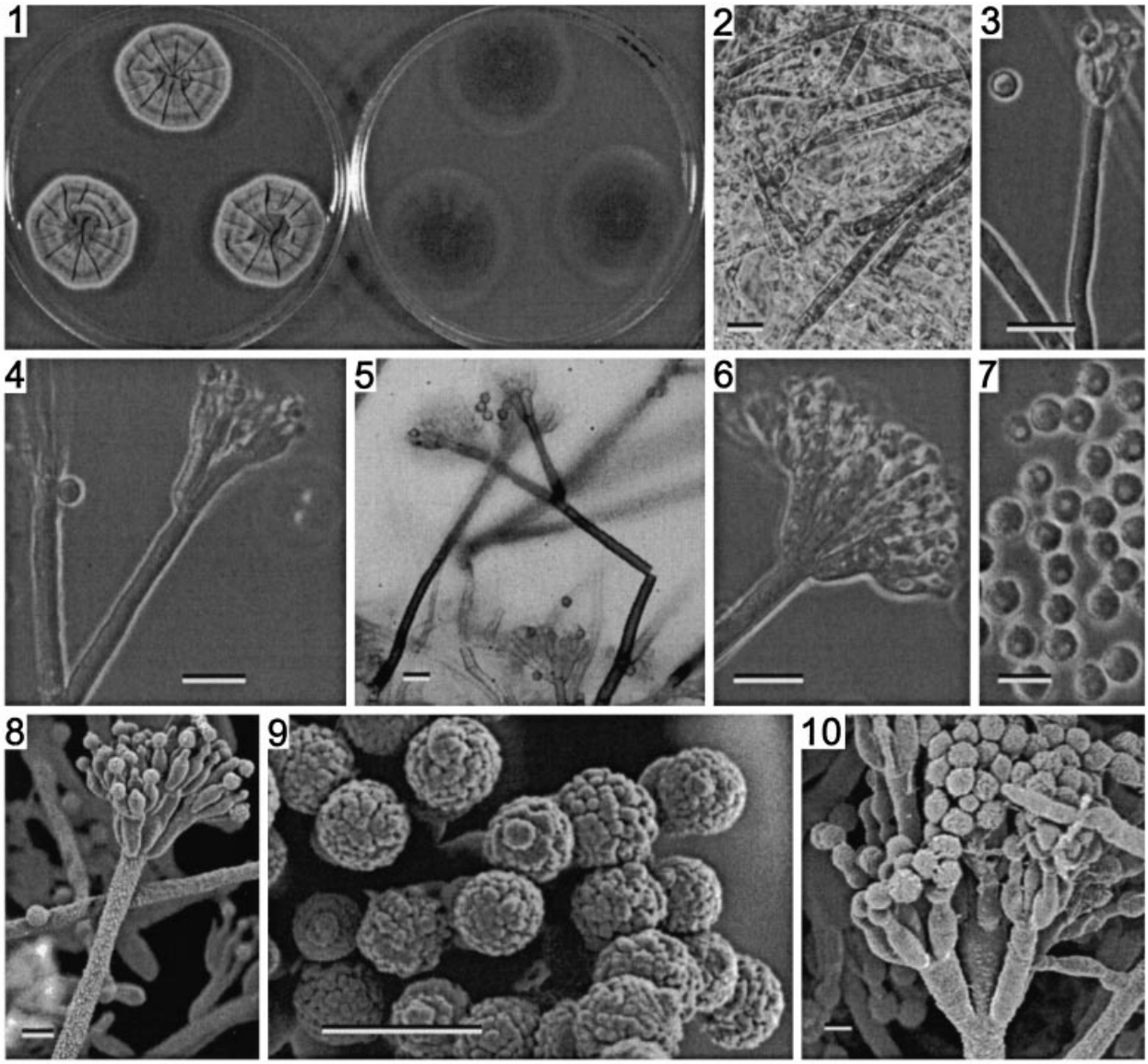
Molecular systematics

Alignment of the broader set of ID region DNA sequences resulted in a data set containing 44 species and 1185 base positions. Out of the 1185 base positions, 128 were eliminated because of insertion–deletion events (indels), 770 characters were constant, 65 characters were parsimony non-informative and 222 base positions were parsimony informative. Heuristic search of the data using maximum parsimony resulted in 959 equally parsimonious trees of 689 steps. One of those trees, with consensus branches, is presented in Fig. 32. Tree statistics are consistency index (CI) = 0.5743, homoplasy index (HI) = 0.4257, CI excluding uninformative characters = 0.5234, HI excluding uninformative characters = 0.4766, retention index (RI) = 0.7550, and rescaled consistency index (RC) = 0.4336. Bootstrap values were calculated using maximum parsimony with 1000 bootstrap samples and values above 60 % are placed on nodes of the tree (Fig. 32).

The five isolates having pigmented conidiophores occurred on one strongly supported branch (98 % of the bootstrap samples) along with *Penicillium donkii*, *Eupenicillium stolkiae* and an unclassified isolate *Penicillium* sp. NRRL 28214. This branch will be referred to as the *E. stolkiae* clade. This group of isolates with melanized conidiophores is separated from another group of darkly pigmented species placed within the genus *Thysanophora*, including the type species, shown as *T. penicillioides* (Fig. 32).

The β-tubulin alignments for the eight isolates included in the *E. stolkiae* clade had 219 characters, of which 4 were eliminated because of indels, 170 were constant, 34 were parsimony non-informative and 11 characters were parsimony informative. The amplified fragment is about 200 bp in length starting in exon 6, spanning all of intron 6 and ending in exon 7 (based on the complete *Aspergillus nidulans* gene sequence, GenBank M17520). Heuristic search using parsimony criterion yielded 3 equally parsimonious trees of 51 steps (not shown). The tree statistics were CI = 0.9412, HI = 0.0588, CI excluding uninformative characters = 0.8125, HI excluding uninformative characters = 0.1875, RI = 0.7692, and RC = 0.7240. Nucleotide substitutions were in the intron area, with no differences in the amino acid coding region.

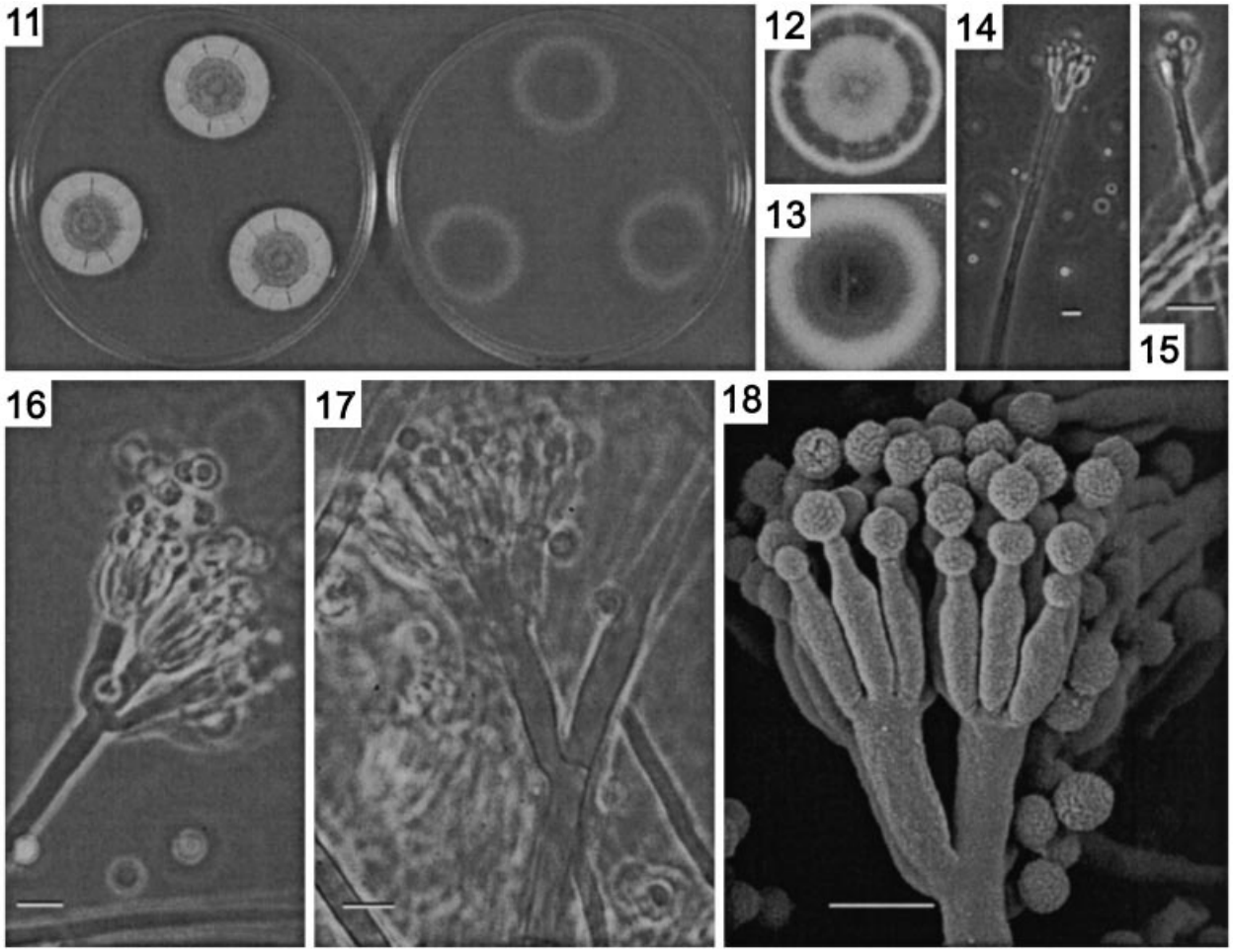
The calmodulin sequence alignment for the *E. stolkiae* clade was composed of 457 aligned base positions. Of these, 8 were eliminated due to indels, 376 were constant, 61 were parsimony non-informative and 12 were informative. Heuristic search using parsimony criterion resulted in 3 equally parsimonious trees of 83 steps (trees not shown). Tree statistics were CI = 0.9518, HI = 0.0482, CI excluding uninformative characters = 0.7895, HI excluding uninformative characters = 0.2105, RI = 0.8000, and RC = 0.7614. Nucleotide substitutions were in the intron area, with no differences in the amino acid coding region.



Figs 1–10. *Penicillium boreae* (holotype). **Fig. 1.** Colonies grown on CYA (left) and MEA (right) for 7 d in darkness. **Fig. 2.** Darkened conidiophores arising from the basal mycelial mat. **Fig. 3.** Monoverticillate penicillus. **Fig. 4.** Subapically branched conidiophore with furcate penicillus. **Fig. 5.** Fractured, heavily melanized conidiophore, photographed with plain light, no staining. **Fig. 6.** Typical furcate penicillus with crowded metulae and phialides. **Fig. 7.** Conidia, showing fine roughening. **Fig. 8.** SEM micrograph of a furcate penicillus showing the coarse roughening of the stipe. **Fig. 9.** Conidia with roughened surface. **Fig. 10.** Highly branched furcate penicillus and ampulliform phialides. Bar Figs 2–7 = 10 μm, Figs 8–10 = 5 μm.

The ID sequence alignment of the *E. stolkiae* clade was composed of 1149 aligned base positions (*ca* 575 nt in ITS1-5.8s rDNA – ITS2 and 635 nt in lsu-rDNA). Of these, 4 were eliminated due to indels, 1125 were constant, 12 were parsimony non-informative and 8 were informative. Heuristic search using maximum parsimony resulted in 10 equally parsimonious trees of 27 steps (trees not shown). Tree statistics were CI = 0.8148, HI = 0.1852, CI excluding uninformative characters = 0.6429, HI excluding uninformative characters = 0.3571, RI = 0.6154, and RC = 0.5014.

None of the individual data sets provided full resolution of the *E. stolkiae* clade. In order to determine the compatibility of these data sets, the partition homogeneity test was performed (Geiser, Frisvad & Taylor 1998) and the different data sets were found to be compatible. The data from the three loci were combined into a single data set and analyzed using maximum parsimony. The combined data set contained 1825 base positions of which 16 were eliminated because of indels, 1671 were constant, 107 were parsimony non-informative and 31 were parsimony informative. Tree statistics were CI = 0.9255, HI = 0.0745, CI excluding uninformative characters = 0.7551, HI excluding uninformative characters = 0.2449, RI = 0.7391, and RC = 0.6840. Heuristic search resulted in a single most parsimonious tree of 161 steps (Fig. 33). Bootstrap values were calculated using maximum parsimony and



Figs 11–18. *Penicillium canariense* (holotype). **Fig. 11.** Colonies grown on CYA (left) and MEA (right) for 7 d in darkness. **Fig. 12.** CYA colony showing the radial sulcation of the outer third of the colony. **Fig. 13.** MEA colony showing the velutinous layer of conidiophores and conidia. **Fig. 14.** Furcate penicillus. **Fig. 15.** Monoverticillate penicillus. **Fig. 16.** Typical penicillus with uncrowded metulae and phialides. **Fig. 17.** Subapical branching of the melanized conidiophore developing a furcate penicillus. **Fig. 18.** SEM furcate penicillus with metulae and phialide, also showing the smooth conidiophore. Bar Fig. 15 = 10 μm, Figs 14, 16–18 = 5 μm.

1000 bootstrap samples and all nodes were supported in greater than 90 % of the bootstrap samples (Fig. 33). Evidence from this combined data set confirms the placement of these species with melanized conidiophores within *Penicillium* close to *E. stolkiae*, and strongly supports the distinction of four new species.

Taxonomy

Penicillium boreae S. W. Peterson & Sigler, **sp. nov.** (Figs 1–10)

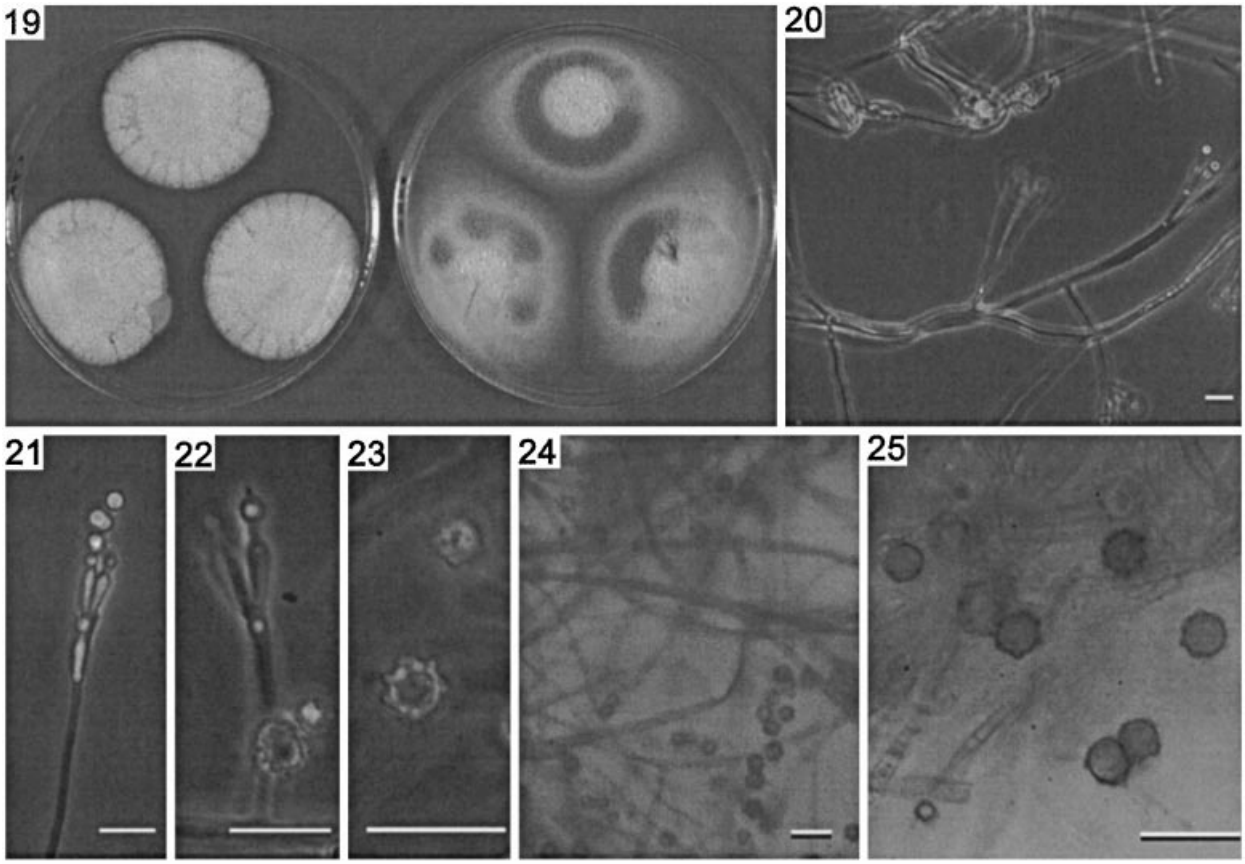
Etym.: *boreae* refers to the north wind.

Conidiophora fusca cum melaniis, crasse asperata, penicillos monoverticillatos et furcatos producentia. Pars aversa coloniae culturarum CYA fusco-viridis, fere atra. Conidia spheroidea 3.5–4 μm diam tenuiter asperata.

Typus: **Canada**: *Northwest Territories*: near Norman Wells, isolated from petroleum contaminated soil, 1975, *D. Westlake* (BPI 841395 – holotypus, dried colonies of NRRL 31002 grown for 7 d on CYA and MEA).

Colonies on CYA growing moderately well (Fig. 1, left side), attaining 30–33 mm diam, composed of a dense, low mat of hyphae. Colonies velutinous, wrinkled, radially sulcate and centrally sunken, with a 2–3 mm white fringe around the colony and deep margins. No exudate produced. Colony centre grey coloured (near mineral grey), the colour of the peripheral third of the colony more bluish-grey (near Puritan grey R-XLVII). Colony reverse Andover green (R-XLVII) centrally with the peripheral third of the colony coloured near Vetiver green (R-XLVII). No soluble pigments produced. No sclerotia or ascomata found in 7 d cultures.

Colonies on MEA growing moderately well (Fig. 1, right side), attaining 35–36 mm diam, composed of a low, loose mat of conidiophores arising from basal hyphae. Colonies velutinous and plane, sporulating densely in the central 3/4 of the colony, with sporulation diminishing to none at the periphery. Colonies dark greyish-blue green (near Deep Medici blue R-XLVIII) centrally, less intense near the margins but not changing hue. No exudate or soluble pigments produced. Colony reverse very dark green (near Lincoln or Leaf green R-



Figs 19–26. *Penicillium pullum* (holotype). **Fig. 19.** CYA (left) and MEA (right) cultures, incubated 12 d at 25 °C. **Fig. 20.** Aerial hyphae with short monoverticillate conidiophores. **Fig. 21.** Long conidiophore arising from basal hyphae, with smooth wall. **Fig. 22.** Short conidiophore and penicillus from aerial hypha. **Fig. 23.** Typical spinose conidium. **Fig. 24.** Mass of vegetative hyphae and conidia with darkly melanized walls. **Fig. 25.** Spinose conidia, showing the darkly melanized walls. Figs 20–23, phase contrast illumination, Figs 24–25, Koeller illumination. Bar = 10 μm.

XLI). No sclerotia or ascomata were found in 7 d cultures.

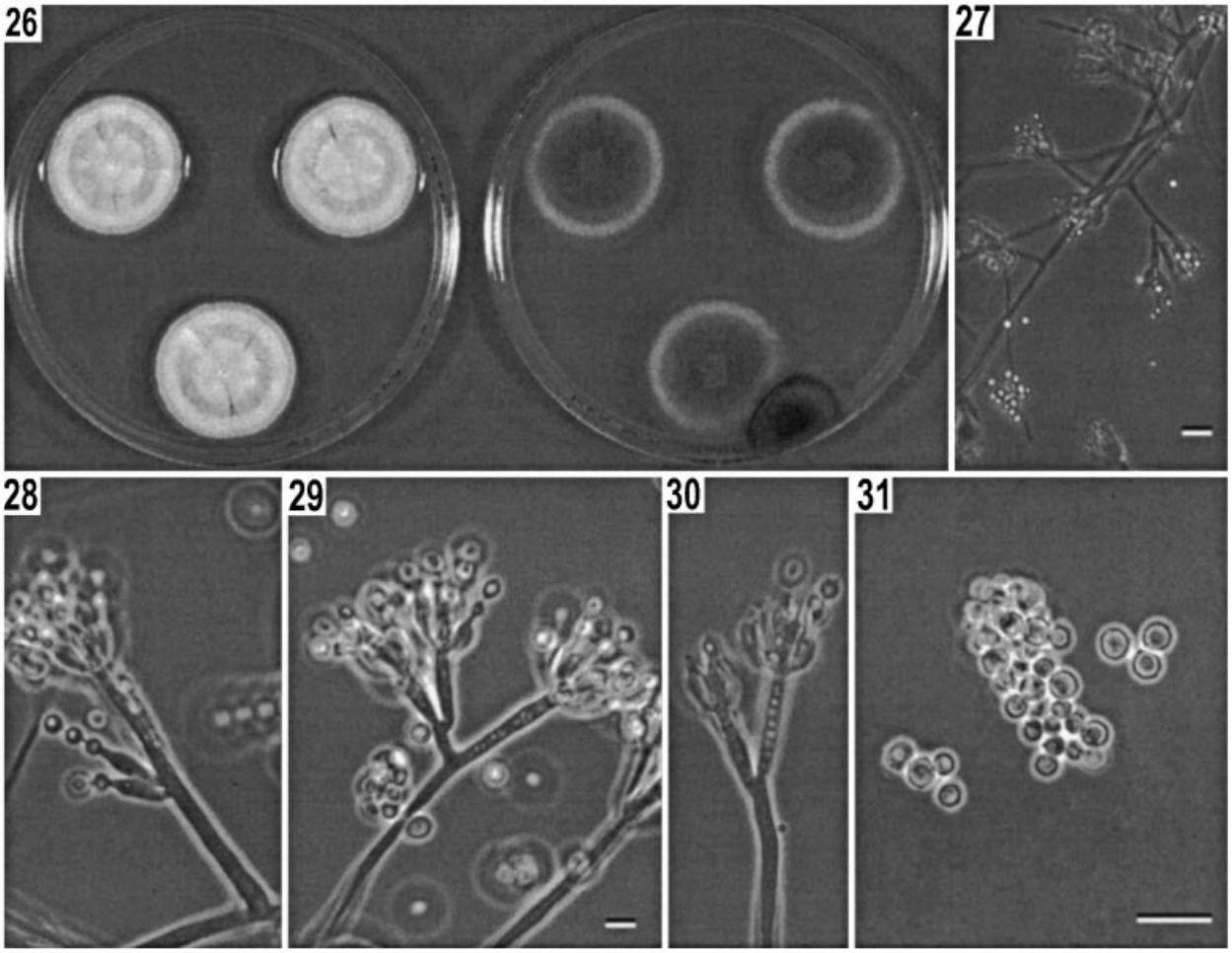
Incubation on G25N agar produced micro-colonies 2–3 mm diam. No growth or germination of conidia was observed when cultures were incubated at either 5 ° or 37 ° on CYA.

Conidiophores 100–200 × 3–4 μm, darkly melanized (Figs 2, 5), arising directly from basal hyphae and conspicuously roughened (Figs 8, 10), especially near the apex. Most conidiophores furcate (Figs 4, 6) bearing 2–3 metulae (conidiogenous supporting cells) that measure 10–15 × 2.5–3.5 μm. Phialides (conidiogenous cells) 7–9 × 2–2.5 μm, ampulliform (Figs 4, 6, 8, 10), and present as whorls of 5–8 units apically on the metulae. *Conidia* spheroidal 3.5–4 μm diam and finely roughened (Figs 7, 9). Some of the conidiophores are relatively short (40–60 μm) and monoverticillate (Fig. 3), but the phialides and conidia produced on them are the same size as those produced on furcate penicilli.

The isolates representing *P. boreae* were initially identified as *Thysanophora* sp. on the basis of the melanized conidiophore, and phialides arranged in whorls at the apices of broad metulae; however, they do not produce the sympodial proliferation of subapical metulae and phialides that are typically present in *Thysanophora penicillioides* (Kendrick 1961, Peterson & Sigler 2002). The complex, coarsely roughened conidiophore and the deep green to blackish green of the CYA colony reverse is reminiscent of *P. roquefortii*, but numerous other characters including the melanized conidiophore and the reduced penicillus structure of *P. boreae* distinguish these two species. *P. boreae* resembles *P. fagi*, *P. cordubense*, *P. mali*, and *P. farinosum* on the basis of the gross conidiophore morphology. Each of these species can be distinguished from *P. boreae* on the basis of colony colour, growth rates, the non-melanized conidiophore, and conidium size and shape. On the basis of ITS DNA sequences, *P. mali* is a probable synonym of *P. echinulatum*, *P. cordubense* is a probable synonym of *P. polonicum*, and *P. farinosum* is a probable synonym of *P. crustosum*. All are phylogenetically distinct from the *E. stolkiae* clade. *P. fagi* is closely related to *P. velutinum* (Fig. 32). *P. boreae* is known only from soil of the northern boreal forest areas of Canada.

Penicillium canariense S. W. Peterson & Sigler, **sp. nov.** (Figs 11–18)

Etym.: *canariense* refers to the Canary Islands.



Figs 26–31. *Penicillium subarcticum* (holotype). **Fig. 26.** Colonies grown 7 d on CYA (left) and MEA (right) at 25 °C. **Fig. 27.** Hyphae producing monoverticillate penicilli on short conidiophores. **Fig. 28.** Conidiophore with an apical whorl of phialides and also several subapical phialides growing directly from the monoverticillate penicillus. **Figs 29–30.** Furcate conidiophores. **Fig. 31.** Aggregation of (sub)spheroidal conidia with slight surface roughening. Bar Fig. 27 = 20 μm, Figs 28, 31 (bar in Fig. 31) = 10 μm, Figs 29, 30 (bar in Fig. 29) = 10 μm.

Conidiophora levia, fusca cum melaniis, penicillos monoverticillatos et furcatos vel in apice vel sub apice producentia. Pars aversa coloniae culturarum CYA fusco-viridis ad griseo-olivacea. *Conidia spheroidea* (2–)2.5–3 μm diam, levia.

Typus: **Spain**: *Canary Islands*: isol. ex soil, A. T. Martinez, IJFM a-543 (BPI 841396 – holotypus, dried colonies of NRRL 31003 grown for 7 d on CYA and MEA).

Colonies on CYA growing moderately well, attaining 26–28 mm diam, umbonate, 3–5 mm deep, central 2/3 funiculose and granular appearing, peripheral 1/3 radially sulcate and velutinous (Figs 11–12). No exudate or soluble pigments produced. Central colony area olive grey (R-XI), and the periphery white but tinged with court grey (R-XLVII). Colony reverse near capucine buff (R-III) centrally, the outer 1/3 near artemisia green (R-XLVII).

Colonies on MEA growing moderately well, attaining 32–33 diam, plane, thin and velutinous, with broad margin 4–5 mm wide (Figs 11, 13). Sporulation is dense with no exudate or soluble pigments apparent. Colonies glaucous to porcelain blue (R-XXXIV). Colony reverse deep green near slate olive (R-XLVII).

Conidiophores (Figs 14–15) arising from basal hyphae, measuring 150–300 × 3–4 μm, melanized, smooth, mostly producing regular, furcate penicilli composed of 3–4 metulae (9–12 × 3–3.5 μm) and whorls of 5–8 ampulliform phialides (6–9 × 2–2.5 μm) on each metula (Figs 16, 18). *Conidia* spheroidal, smooth to very finely roughened measuring (2–)2.5–3 μm (Fig. 18). Mature conidiophores often with monoverticillate and furcate penicilli produced subapically (Fig. 17).

Incubation on G25N agar produced micro-colonies 2–3 mm diam. No growth or germination of conidia observed when cultures were incubated at either 5 ° or 37 ° on CYA.

Penicillium canariense is known only from the ex-type culture, obtained from soil collected in the Canary Islands. It resembles the other species described here in having melanized conidiophores, but it can be distinguished from *P. boreae* by the smooth stipe, smaller, smooth conidia and dark green to olive, rather than nearly black, colony reverse colour. *P. pullum* produces primarily monoverticillate penicilli while *P. canariense* produces primarily furcate penicilli. *P. citrinum* is

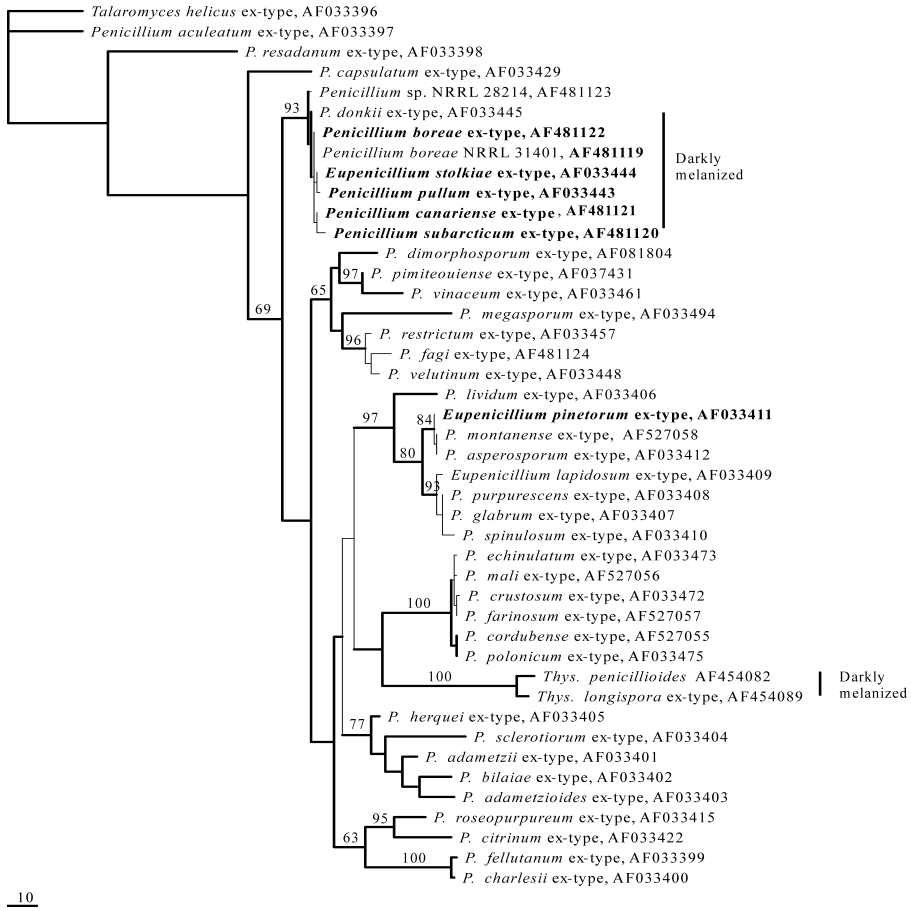


Fig. 32. Parsimony tree based on ITS-LSU (ID) data set representing the wide phylogenetic breadth of the genus *Penicillium* in the *Eupenicillium* lineage. Thick lines represent the strict consensus of the equally parsimonious trees. Numbers above the internodes are bootstrap percentages based on bootstrap analysis using maximum parsimony criterion and 1000 bootstrap samples. Two distinct groups of species that possess darkly melanized conidiophores are shown in this tree. Sequences were derived from ex-type cultures except *T. penicillioides* where no ex-type cultures exist. Alignments in TREEbase no. M1211.

somewhat similar, but grows more rapidly and has hyaline conidiophores.

Penicillium pullum S. W. Peterson & Sigler, **sp. nov.** (Figs 19–25)

Etym.: *pullum*, Latin, refers to dark-coloured, blackish or greyish-black objects, here to the darkly melanized conidiophores.

Conidiophora levia cum melaniis, penicillos monoverticillatos producentia, phialides ampulliformes vel duo vel tres ferentia. Conidia 3.5–4.5 μm diam, valide spinosa.

Typus: **USA**: *Texas*: near Austin, isol. ex soil, 1930, *M. B. Morrow* (BPI 841398 – holotypus, a dried colony of NRRL 721 grown 7 d on MEA).

Colonies on CYA attaining 21–25 mm diam, low, plane, and lanose in texture, cream coloured. After 12 d growth the outer 1/3 of the colony showed radial sulcations and the colony was mealy (Fig. 19, left). After 16 d, the colonies took on a wet appearance. Conidiophores not seen in the CYA cultures of this isolate.

Colonies on MEA attaining 20–25 mm diam, low, plane and mealy in texture. Colony creamy white. Reverse slightly more brown than cream white. No exudate or soluble pigments present. After 12 d growth, the colonies had attained 55–60 mm diam, with a loose overgrowth of floccose hyphae that produced abundant conidiophores and conidia (Fig. 19, right). Colony in sporulating areas dark olive-grey (R-LI) to dusky grey-green (R-LII), and grey in the colony reverse beneath the sporulating regions.

Conidiophores arising as a very thin lawn (Figs 20–22) directly from the agar surface, 100–200 × 2–2.5 μm diam, smooth walled, non-vesiculate, pigmented brown, and bearing 2–3 ampulliform phialides (6–9 × 2–2.5 μm) apically (Fig. 22). *Conidia* (Figs 23–25) spheroidal, 3.5–4.5 μm diam, initially hyaline and finely spinulose, maturing to darkly pigmented (Figs 24–25) and very roughly spinulose.

Penicillium pullum is based on the culture (NRRL 721) used by Raper & Thom (1949), Ramirez (1982), and Peterson (2000) to represent *P. fuscum*. However, Stolk & Samson (1983) neotypified *P. fuscum* (syn. *Citromyces fuscus*) using dried material of CBS 295.62 (equivalent to NRRL 3008). This proposal was based on their assessment that Sopp's species was identical with the anamorph of *Eupenicillium pinetorum*; the

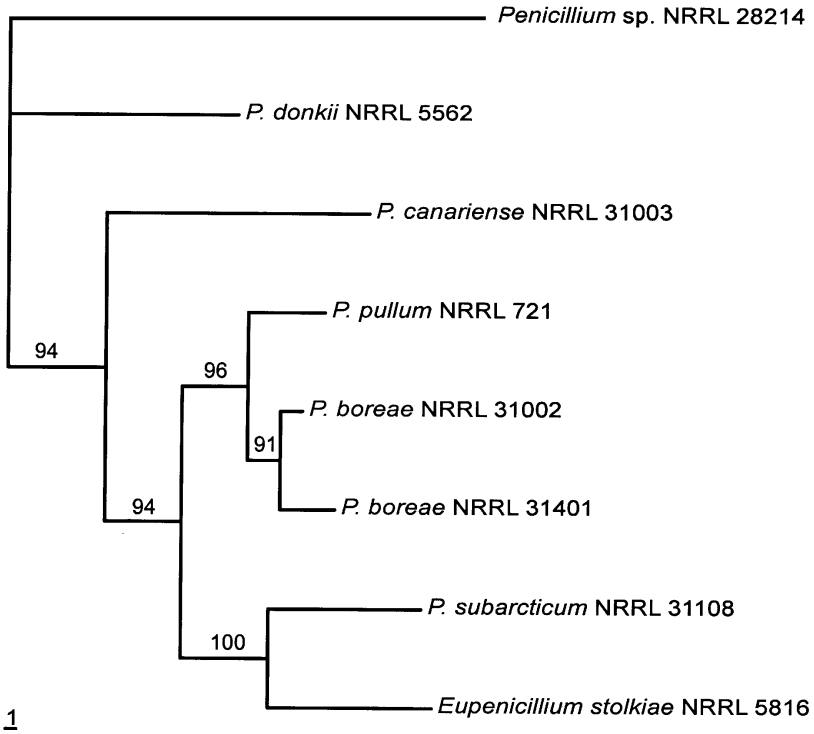


Fig. 33. Single most parsimonious tree based on combined ID, calmodulin and β-tubulin data showing the relationship of the species. Bootstrapping was performed using maximum parsimony and 1000 replicate samples. DNA sequences are deposited in GenBank as: ID region AF033443–AF033445, AF481119–AF481124; calmodulin region AF481133–AF481141; β-tubulin region AF481125–AF481132. Alignments in TREEbase no. M1212.

Code allows neotypification when all type material is destroyed or lost, as was the case with Sopp's material. As shown in the phylogenetic tree (Fig. 32), *P. pullum* represented by isolate NRRL 721 is clearly distinguished from *E. pinetorum* represented by the ex-type culture NRRL 3008 on the basis of the ID DNA sequences. Since the name *P. fuscum* has been considered as the appropriate name for the anamorph of *E. pinetorum*, a new name is required for isolate NRRL 721.

NRRL 721 shows some irregularities in its colony morphology from that described by Raper & Thom (1949) and Ramirez (1982); however, the description by Ramirez was based on two strains, one of which (CBS 147.45) is now identified as *P. velutinum*. Our description reflects some of the changes in cultural morphology that have arisen during the course of many transfers, but the description of Raper & Thom (1949) might provide additional details applicable to freshly obtained isolates of *P. pullum*.

P. pullum resembles *P. asperosporum*, *P. montanense*, and *P. megasporum* in the production of large, spinose conidia from primarily monoverticillate conidiophores. *P. asperosporum* and *P. montanense* were at one time considered synonyms of *P. fuscum* (Stolk & Samson 1983), but are now treated as separate species (Peterson 2000). *P. asperosporum* is acidophilic (Ramirez 1982), has larger conidia, and produces a brick-red colony reverse. *P. montanense* barely grows on Czapek's agar and produces a much larger penicillus (Ramirez 1982). *P. megasporum* produces much larger conidia, a soluble orange pigment on CYA and has conidiophores with multiple septations. Phylogenetically, each species is distinct from *P. pullum* (Fig. 32).

Penicillium subarcticum S. W. Peterson & Sigler, **sp. nov.** (Figs 26–31)

Etym.: *subarcticum* refers to the subarctic region of the Northwest Territories where the species was isolated.

Conidiophora brevia leviaque cum melaniis, penicillos monoverticillatos et furcatos producentia. Conidia tenuiter asperata 3–4 μm diam. Pars aversa coloniae pallide flavo-brunnea. Incrementum in agaro G25N abest.

Typus: **Canada**: *Northwest Territories*: near Norman Wells, isol. ex soil, 1975, *D. Westlake NWF 146* (BPI 841397 *holotypus*, dried colonies of NRRL 31108 grown 7 d on CYA and MEA).

Colonies on CYA (Fig. 26, left) *ca* 26 mm diam, composed of loose white lanose hyphae, occasionally forming funicles centrally, radially sulcate and crateriform. Sporulation very slight. Colony reverse Chamois (R-XXX). No soluble pigments or exudate formed.

Colonies on MEA (Fig. 26, right) *ca* 35 mm diam, plane, velutinous, near gnaphalium green (R-XLVII) centrally ranging to slate-olive (R-XLVIII) near the periphery. Colony reverse deep greyish olive (R-XLVI). No soluble pigments or exudate produced.

Conidiophores (Figs 27–30) mostly short, simple 25–60 (–100) × 2–2.5 μm, lacking apical knob, bearing whorls of 4–8 (–12) phialides (5–)7–10 × 2–3 μm, pro-

ducing slightly roughened, (sub)spheroidal conidia 3–4 μm diam. Scattered instances of sessile phialides arising directly from the conidiophores (Fig. 28). Furcate penicilli (Figs 29–30) also produced but less commonly than the monoverticillate penicilli. Phialides and conidia produced on either penicillus type were indistinguishable.

P. subarcticum did not grow at 5 ° or 37 ° on CYA nor at 25 ° on G25N agar.

Penicillium subarcticum is known only from the type isolate, made from petroleum contaminated soil. Taxonomically this species resembles the other monoverticillate *Penicillium* species in the *P. restrictum* group, *P. restrictum*, *P. dimorphosporum*, *P. roseopurpureum*, *P. vinaceum*, *P. capsulatum*, and *P. resedanum*, but phylogenetically it is in the *E. stolkiae* clade. Using the dichotomous key in the monograph of Pitt (1980), this isolate would key out as *P. restrictum*. *P. subarcticum* is distinguished from *P. restrictum* by the melanized conidiophores, by greater growth on MEA (35 *vs* 15–25 mm) and by growth on G25N (no growth *vs* 11–14 mm). *P. pimiteouiense* is somewhat similar, but it typically makes very petite conidiophores with whorls of 2–3 phialides on each penicillus, and has smaller conidia (2–3 μm diam) compared with those of *P. subarcticum* (3–4 μm diam).

Phylogenetically, *P. subarcticum* is in the *Eupenicillium stolkiae* clade along with *P. donkii*, *P. boreae*, *P. canariense* and *P. pullum* (Fig. 32). The evidence from the combined data set (Fig. 33) depicts the relationship of the species.

DISCUSSION

The melanization of hyphae and conidiophores as taxonomic criteria in hyphomycetes has long been considered to be of debatable value (Barron 1968, Carmichael *et al*. 1980). The melanized or darkly pigmented conidiophores of *Penicillium pullum*, *P. boreae*, *P. canariense* and *P. subarcticum* are outside the usual generic concept of *Penicillium* (Raper & Thom 1949). Three of the described species were initially identified as belonging to *Thysanophora* which encompasses species having brown pigmented conidiophores and forming penicilli resembling those of *Penicillium*. However, none of them demonstrated the sympodial proliferation of subapical metulae and phialides present in *Thysanophora penicillioides*, the type species (Kendrick 1961). Although pigmentation of some *Thysanophora* species approaches black, colour was not found to be a phylogenetic character of value in separating members of this genus from those of *Penicillium* (Iwamoto *et al*. 2002, Peterson & Sigler 2002). Similar findings were obtained in molecular studies by Haugland *et al*. (2001) who showed that hyaline and darkly pigmented species were appropriately placed in *Stachybotrys*. Within the *Eupenicillum* lineage, melanized conidiophores occur in two separate clades. One includes species assigned to *Thysanophora* (Iwamoto *et al*. 2002, Peterson & Sigler 2002; Fig. 32). The current study demonstrates a second lineage centered around *Eupenicillium stolkiae*, a species that also has been described with conidiophores that may be hyaline to definitely brown (Stolk & Samson 1983). Two unclassified *Thysanophora*-like isolates were shown to form a third lineage closer to *Hamigera* species (Peterson & Sigler 2002).

The phylogenetic tree presented in Fig. 32 is not fully resolved. Many of the branching points in the tree diagram have less than 70 % bootstrap support and must be viewed as equivocal, but some of the clades are strongly supported in the bootstrap analysis, including the *E. stolkiae* clade that contains the new species. The intron regions of the β-tubulin and calmodulin genes are very informative and have low homoplasy indices as does the ID region over this narrow phylogenetic range. While each locus produced multiple most parsimonious trees, the homogeneity partition test shows that the data are compatible. When the data are combined (Fig. 33) a single most parsimonious tree results that is strongly supported by bootstrap analysis. Each of the new species has a well supported and distinct evolutionary history in the combined data tree. Three of the four new species are represented by single isolates making it difficult to form a species description that will encompass intra-specific variation. However, the differences between these species and the previously described species are clear and sufficient to merit the descriptions of these new species.

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