Two new *Cryptosporiopsis* species from roots of ericaceous hosts in western North America

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Abstract: *Cryptosporiopsis* species are anamorphs of ascomycetes in the genera *Pezicula* and *Neofabraea* (*Dermataceae*). These fungi are occasionally isolated from roots of woody plants but may be difficult to identify due to absence of sporulation. Some isolates obtained from roots of ericaceous hosts had previously been linked phylogenetically to *Pezicula* and, when regrown, revealed conidiomata and conidia typical of *Cryptosporiopsis* species. Cultural and molecular data allowed for the recognition of the new species, *Cryptosporiopsis ericae* and *C. brunnea*.

Taxonomic novelties: *Cryptosporiopsis ericae* Sigler sp. nov.; *Cryptosporiopsis brunnea* Sigler sp. nov.
Key words: *Cryptosporiopsis*, ericoid endophyte, ITS region sequences, *Neofabraea*, *Pezicula*, root associated fungi.

INTRODUCTION

Cultural studies of fungi from plant roots yield large numbers of fungal isolates, many of which are difficult to identify due to absence of sporulation. However, mycobionts can often be grouped by cultural attributes suggestive of possible relationship. Three nonsporulating isolates obtained from roots of *Vaccinium ovalifolium* Sm. Mathers and *Gaultheria shallon* Pursh present one such morphotype. These isolates, sent for deposit in the University of Alberta Microfungus Collection and Herbarium (UAMH), were grown on several media and at different temperatures in attempts to induce sporulation. Sparse sporulation occurred only in one isolate, but characteristics of conidiomata and conidia were sufficient to allow identification as a *Cryptosporiopsis* Bubák & Kabát species.

Many species of *Cryptosporiopsis* are known and most are anamorphs of *Pezicula* Tul. & C. Tul. or *Neofabraea* H.S. Jacks. species (*Helotiales*) (Verkley 1999). However, three species described from roots of woody plants are exclusively anamorphic. *Cryptosporiopsis radicicola* Kowalski & Bartnik (Kowalski & Bartnik 1995) and *C. melanogena* Kowalski & Halmschlager (Kowalski *et al*. 1998) were described from roots of *Quercus* species. *Cryptosporiopsis rhizophila* Verkley & Zijlstra is known only from roots of ericaceous plants collected in the Netherlands (Verkley *et al*. 2003). Verkley *et al*. used phylogenetic analyses to provide support for their new taxon and included in their tree nuclear ribosomal internal transcribed spacer (ITS) region sequences of two isolates from *G. shallon* (AF149074 = UBC tra 288 and AF149087 = UBC tra29). The salal isolates had been shown previously to group with *Pezicula* species (Berch *et al*. 2002), and this was confirmed by Verkley *et al*. Although the salal isolates were closer to *C. radicicola* than to *C. rhizophila* from European ericaceous hosts, bootstrap support was low. Based on these data, UBCtra 288 = UAMH 10106 appeared to represent another *Cryptosporiopsis* species; however, no evidence of sporulation had been observed in this isolate when originally deposited at UAMH.

To determine whether our isolates from North American ericaceous hosts were conspecific, we obtained sequences from the ITS region and examined morphological features under different growth conditions. Also included was a *Cryptosporiopsis* sp. isolated from roots of *Vaccinium membranaceum* Dougl. ex Torr. collected in Priest Lake, Idaho.

MATERIALS AND METHODS

Morphology

Provenance of isolates is shown in Table 1. To induce sporulation, isolates were grown on oatmeal agar overlaid with a sterile cellophane membrane (OATC) (Carmichael 1961, Weitzman & Silva-Hutner 1967) and incubated at 22 º, 18 º, and 15 ºC, all in the dark. Colony morphologies were examined on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, U.S.A.) and 2 % malt extract agar (MEA 20 g Difco malt extract; 15 g agar) incubated at 15 º and 22 ºC, and on OATC incubated at 15 ºC, all in the dark. Inoculum was a 5 mm diam disc transferred from a

Table 1. *Cryptosporiopsis* isolates from roots of ericaceous hosts in western North America.

Identification	UAMH[1] #	Original number	Host and locality	Locality	Isolator and Date	Mycorrhizal status[2]	GenBank #
Cryptosporiopsis ericae	9445[T]	22 (PL3BciiE)	*Vaccinium membranaceum*[3]	U.S.A.: Idaho, Priest Lake	B. McCracken	unknown	AY540126; this study
	10348	UBCtra 1129	*Gaultheria shallon*[4]	Canada: British Columbia, northern Vancouver Is.	T.R. Allen, Oct. 1998	unknown	AY442322; this study
	10349	UBCtra 1204	*Vaccinium ovalifolium*[4]	Canada: British Columbia, northern Vancouver Is.	T.R. Allen, Oct. 1998	unknown	AY442321; this study
	10350	UBCtra 1234	*Vaccinium ovalifolium*[4]	Canada: British Columbia, northern Vancouver Is.	T.R. Allen, Oct. 1998	unknown	AY442323; this study
	not deposited	UBCtra 29	*Gaultheria shallon*	Canada: British Columbia, northern Vancouver Is.	T.R. Allen, July 1996	– (Berch *et al*. 2002)	AF149087 (Berch *et al*. 2002; Allen *et al*. 2003)
	10419[5]	UBCtra 374	culture ex root (56) of *Gaultheria shallon* in soil (Meter Trial)	Canada: British Columbia, northern Vancouver Is.	T.R. Allen, May 1998	unknown	AY853167; this study
Cryptosporiopsis brunnea	10106[T]	UBCtra 288	*Gaultheria shallon*[3]	Canada: British Columbia, north Vancouver Is.	T.R. Allen, May 1998	– (Berch *et al*. 2002)	AF149074 (Berch *et al*. 2002; Allen *et al*. 2003)

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[2]'–' indicates an isolate that did not form ericoid mycorrhizae in tests *in vitro.*

[3]Isolated from surface sterilized hair roots.

[4]Isolated from roots of plants collected about one meter apart in the same study site located 50°60'N, 127 °35'W.

[5]Isolate examined later, the sequence of which was compared but not included in phylogenetic analysis.

[T]ex-type culture.

colony on PDA. The incubation temperature of 15 ºC was chosen for comparison with prior studies (Verkley *et al*. 2003), but when isolates demonstrated better growth at 22 º or 18 ºC, additional observations were made on plain OAT at 22 ºC. Colony color terms are described according to Kornerup and Wanscher (1978). Microscopic mounts and measurements were made from structures on OATC in lactofuchsin (Carmichael 1955).

Direct DNA extraction, PCR amplification and sequence analysis

ITS ribosomal DNA (rDNA) sequences were determined initially for four isolates and compared with sequences on deposit in GenBank (Table 1, Fig. 1). Mycelium for DNA extraction was scraped from the surfaces of PDA agar cultures. Genomic DNA was extracted using a DNeasy® Plant Mini Kit (QIAGEN Inc., Mississauga, Ont., Canada). Total fungal DNA was amplified with primers ITS1-F (Gardes & Bruns 1993) and TW13 (sequence available through <http://plantbio.berkeley.edu/~bruns/primers.html>). PCR products were analyzed by sequencing with primers cTB6, ITS4 (White *et al*. 1990), ITS1-F and TW13, using BigDye® Terminator v.3.1 Cycle Sequencing Kit and following the manufacturer's instructions (PE Applied Biosystems, Foster City, CA, U.S.A.). Sequences were assembled and related sequences were found using BLAST searches. Sequences were aligned with ClustalX (Thompson *et al*. 1997, distributed by the authors <ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>) followed by manual adjustment with SeqApp (available through <ftp://iubio.bio.indiana.edu/molbio/seqapp/>). One additional isolate (UAMH 10419) was examined and sequenced subsequently. The sequence was aligned and compared, but not included in the phylogenetic analysis.

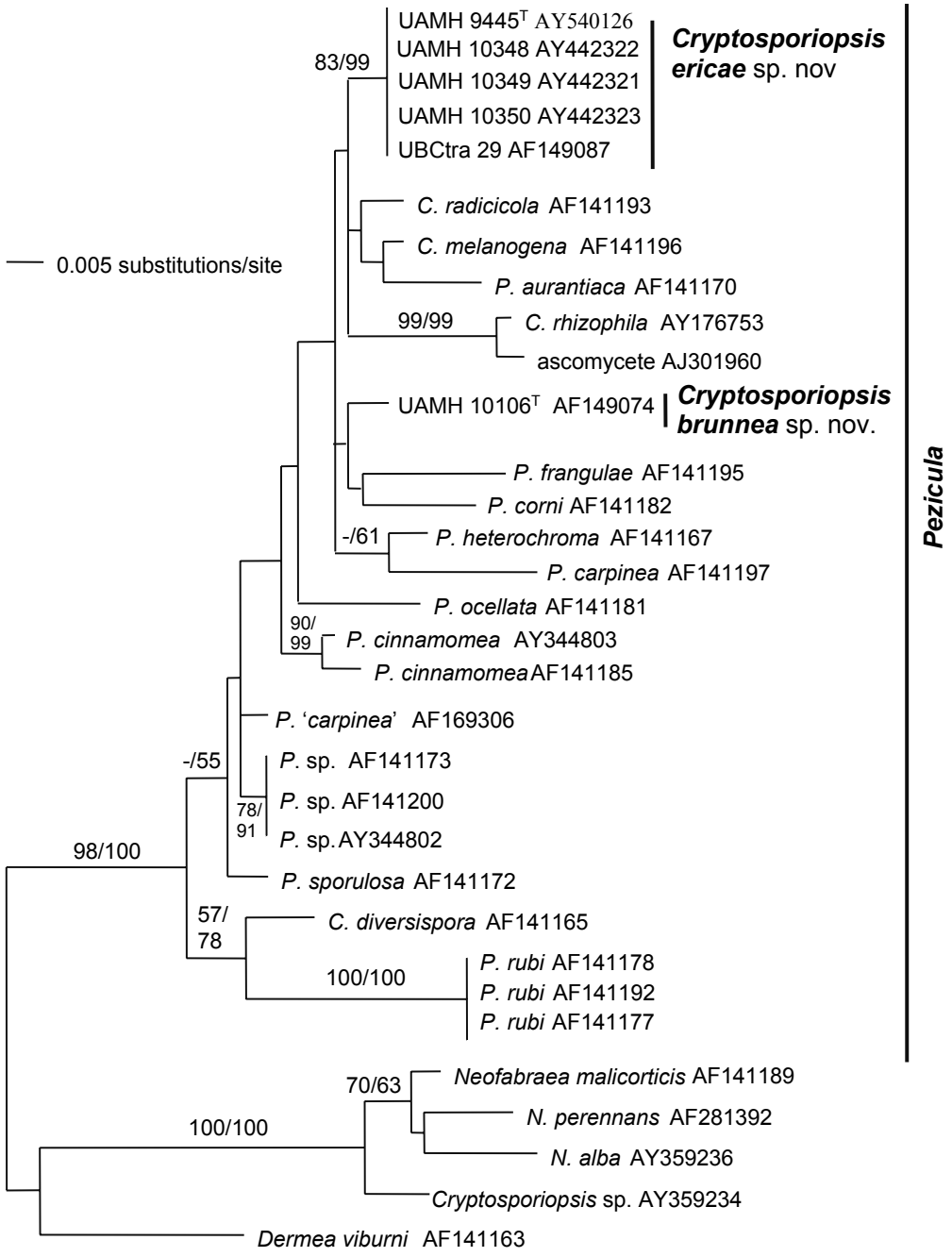


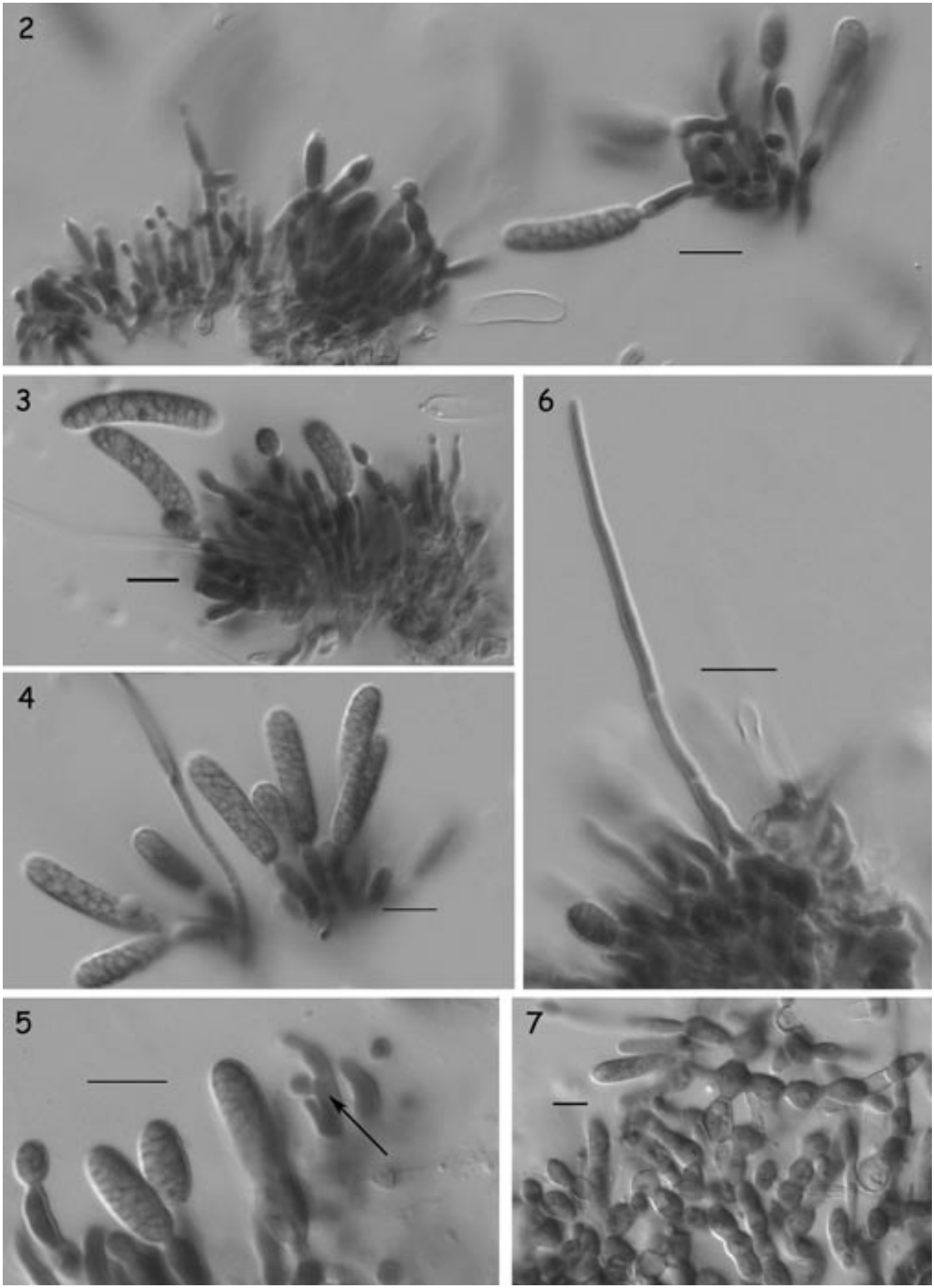
Fig. 1. Maximum likelihood tree showing the position of two new species of *Cryptosporiopsis* from ericaceous plant roots within the *Pezicula* clade. Of the pairs of numbers associated with a branch, the first is a parsimony bootstrapping percentage and the second is a minimum evolution (distance) bootstrapping percentage. Only percentages over 50 % are provided.

Phylogenetic taxon sampling

BLAST searches showed that the sequences determined in this study were more than 93 % identical to previously published sequences from the *Pezicula* clade. Verkley *et al*. (2003) and Abeln *et al*. (2000) showed that *Pezicula* species and their *Cryptosporiopsis* anamorphs form a monophyletic group nested with strong bootstrap support within *Dermea* Fr. species and *Neofabraea* species. Based on Verkley *et al*.'s analysis, we used *Dermea viburni* J.W. Groves and *Neofabraea* species as outgroups. Twenty-seven other GenBank sequences representing known species in the *Pezicula* clade were included in alignments.

Phylogenetic analysis

PAUP v.4.0b10 (Swofford 2003) was used for likelihood, parsimony and distance (minimum evolution) analyses of sequences. The sequences showed few differences and so a simple Kimura 2-parameter model was used to correct pair-wise genetic distances for multiple hits. Bootstrapping was performed using tree-bisection-reconnection branch swapping and parsimony (limiting the number of trees per replicate to 500) or using minimum evolution. Maximum likelihood and minimum evolution heuristic searches were conducted with 10 search replicates and parsimony searches were conducted with 100 search replicates.



Figs 2–7. *Cryptosporiopsis ericae* (UAMH 9445, ex-type). 2–4. Phialides in sporodochia producing macro- and microconidia. 5. Terminal and rare lateral (arrow) phialides. 6. Seta. 7. Moniliform hyphae associated with immature sporodochium. Bars = 10 µm.

RESULTS

Five *Cryptosporiopsis* isolates from ericaceous plant roots from western North America (UAMH 9445, 10348, 10349, 10350 and UBCtra 29) had identical sequences and formed a monophyletic group with 83 % support from parsimony bootstrapping and 99 % bootstrap support from minimum evolution bootstrapping (Fig. 1). The sequence of UAMH 10419, examined subsequently, was identical to members of this group. UAMH 10106, from an ericaceous plant root from western North America, differed from members of the first group by 1.1 %. Although isolate UAMH 10106 clustered with the other five isolates in a minimum evolution analysis and in 12 % of the parsimony trees (not illustrated), the group of six isolates never received bootstrap support and it did not appear in maximum likelihood (Fig. 1) trees. Within the genus *Pezicula*, the ITS sequences of different species were between 93 % and 99 % identical. Our isolates differed from others in the *Pezicula* clade by between 1.3 % and 4.9 %. The six isolates from North American *Ericea*e differed from the next most similar taxa, *C. radicicola* and *C. melanogena*, by 1.3 %.

Within *Pezicula*, the phylogeny was not well resolved and few clades received more than 50 % bootstrap support. This low resolution, the many, nearly equal trees, and the high sequence similarities suggested that the ITS regions used here were not variable enough to reconstruct relationships among species. In 100 search replicates, 763 equally parsimonious trees (207 steps long) were found. The equally parsimonious trees differed in placement of species in the *Pezicula* clade, where branches received less than 50 % parsimony bootstrap support. Although a maximum likelihood heuristic search found a single most likely tree (log likelihood = –2035) PAUP detected 5 other "islands"of almost equally likely trees. The two different sequences from isolates from ericaceous plant roots from western North America clustered differently in different trees. Neither grouped closely with any other known species.

Based on the molecular data and morphological features, the isolates are described here as the new species *C. ericae* and *C. brunnea*. Although isolates of *C. ericae* had identical sequences, they demonstrated some cultural differences. Three from British Columbia (UAMH 10348 to 10350) demonstrated no or scant sporulation in repeated attempts; UAMH 10349 sporulated only once on OATC at 22 ºC producing sparse conidiomata with macroconidia only. In contrast, UAMH 9445 and UAMH 10419 sporulated profusely on OATC at all incubation temperatures with production of both macroconidia and microconidia. Colonies of UAMH 10348 to 10350 were identical and greyish yellow (blond) to light brown centrally on PDA (Figs 18–19). Colonies on PDA often developed feathery to mottled pale greyish brown patches centrally as shown in Fig. 16 for UAMH 9445. Similar patchy coloration occurred on other media, in age. UAMH 10106, selected as type of *C. brunnea*, was nonsporulating on deposit, but it sporulated profusely on OATC on recovery from preserved stock material.

Taxonomy

Cryptosporiopsis ericae Sigler **sp. nov.**, MycoBank MB500253, Figs 2–11; 16–21.

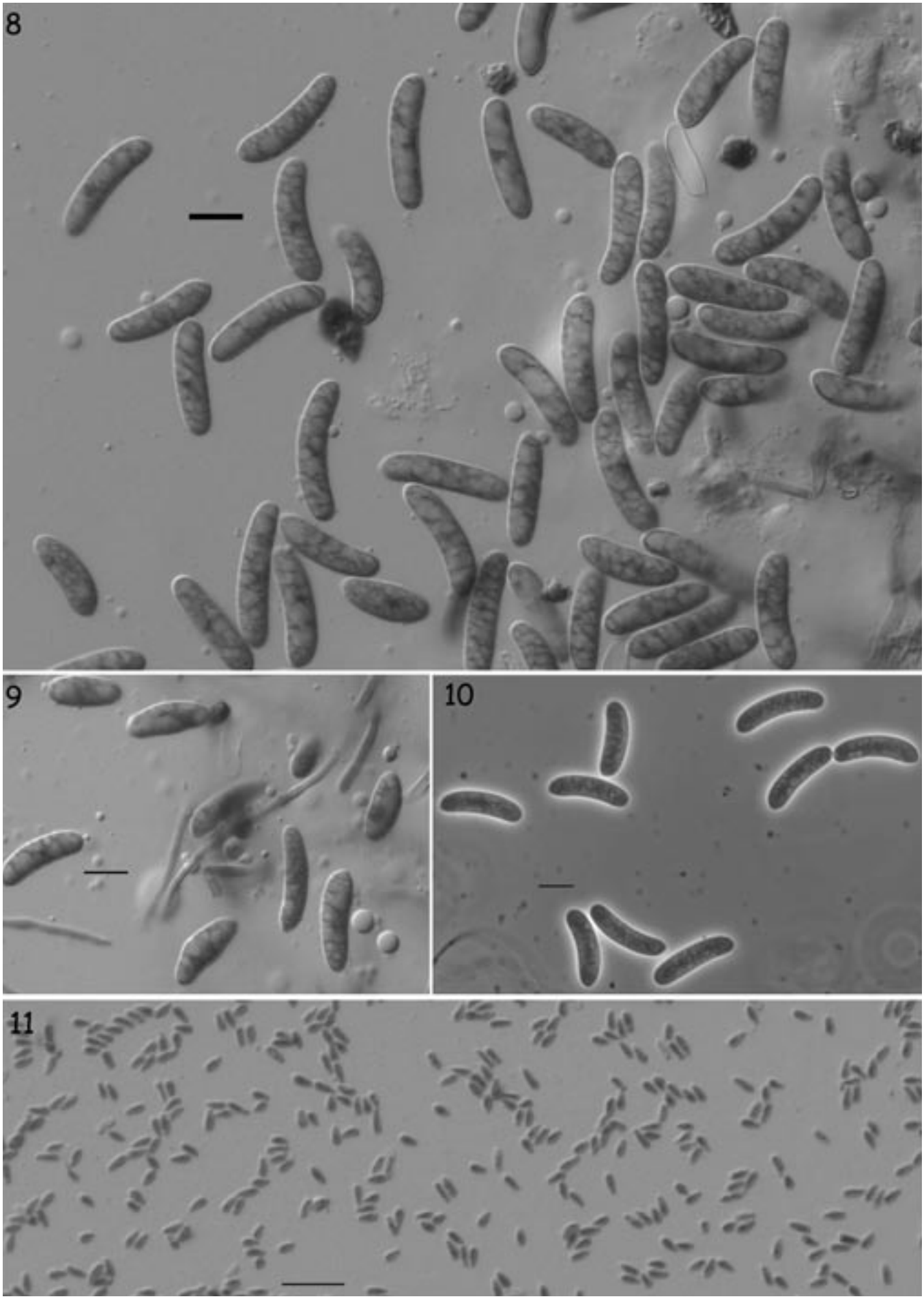
Etymology: associated with ericaceous hosts

Cellulae conidiogenae phialides formantes in conidiomatibus sporodochiis vel hyphis aggregatis. Conidia in massa alba, deinde pallide aureo-flava vel rubro-brunnea. Macroconidia cylindrica, curvata, in apice rotunda, ad basim exigue truncata, aseptata, levia, hyalina, deinde aurea et guttulata. Macroconidia (18.5) 20–30 × 5.5–7 µm ad 22 °C et 26–33 × 5.5–7.5 µm ad 18 °C. Microconidia aseptata, hyalina, oblonga, 2.5–4 × 1–2 µm. Setae sparsae, chlamydosporae absunt. Teleomorphosis ignota.

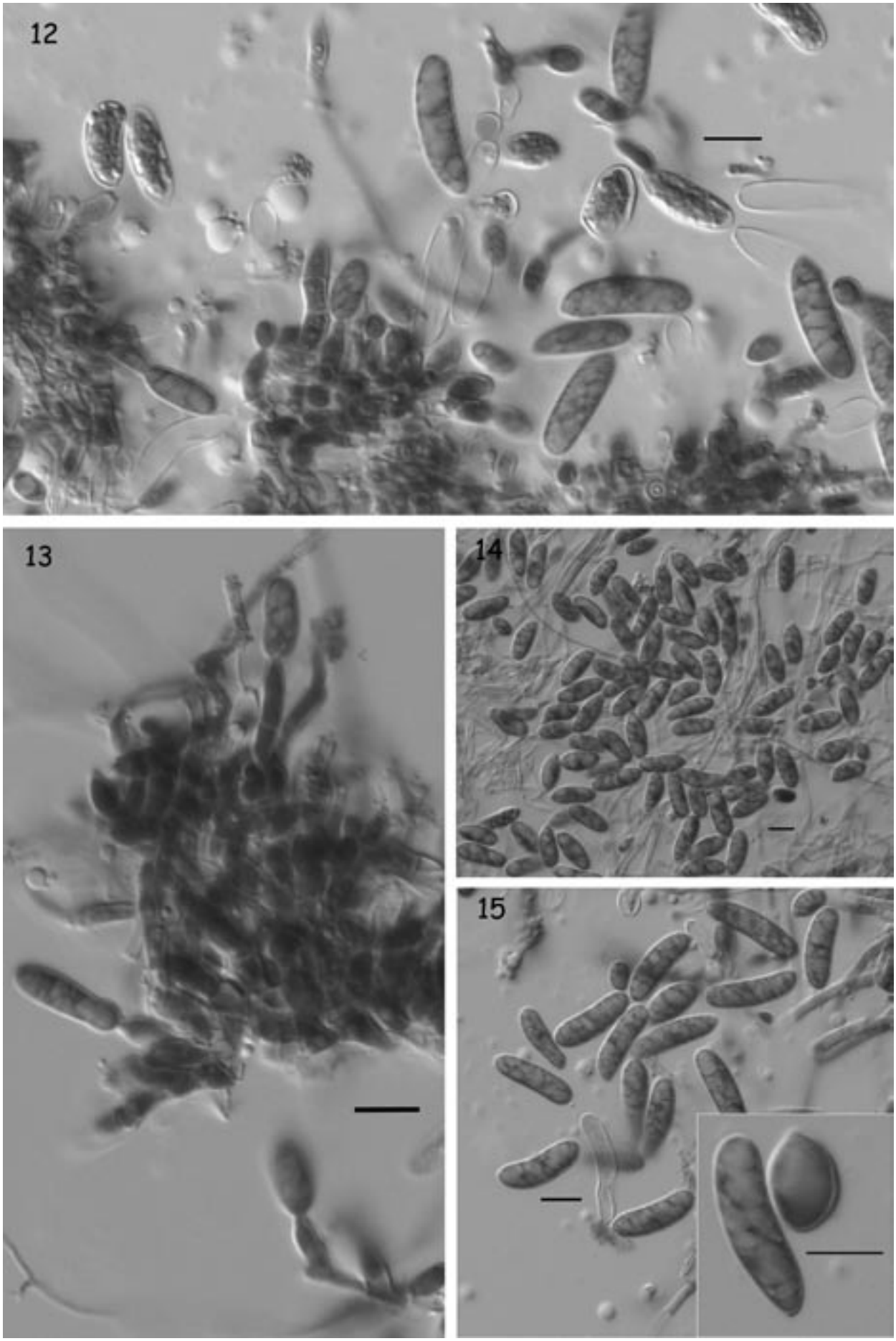
Typus: **U.S.A.**: Idaho, Priest Lake, ex radicis *Vaccinium membranaceum*, B. McCracken (22; PL3BciiE). Colonia exsiccata et cultura viva, UAMH 9445.

Conidiogenous cells are phialides measuring 10.5–14 × 3–4 µm and borne terminally, rarely laterally, on simple conidiophores formed in hemispherical sporodochial conidiomata, or sometimes in raised synnemata-like tufts. Hyphae associated with young conidiomata are often moniliform and hyaline. In older cultures conidiomata are associated with aggregations of dark brown hyphae. Conidial masses are white initially, becoming pale to golden yellow to reddish brown in age. Macroconidia are cylindrical, slightly curved, rarely almost straight, rounded at the apex and minutely truncate at the base, nonseptate, smooth, hyaline, becoming golden yellow and guttulate in age. Macroconidia measure (18.5) 20–30 × 5.5–7 µm at 22 ºC and 26–33 × 5.5–7.5 µm at 18 ºC. Microconidia are borne on phialides (8–11.5 × 1.5–3 µm) produced in the same conidiomata, nonseptate, hyaline, oblong, 2.5–4 × 1–2 µm. Seta-like hyphae very sparse; chlamydospores not observed. Teleomorph unknown.

Colonies on PDA at 22 ºC after 14 d were almost 8.5 cm diam, flat, smooth, felty, greyish yellow (blond) to greyish orange on the obverse (4C3 to 6A/B3) and reverse (4C6 to 5B3); in 21 d, becoming mottled grayish brown (6D3) to light brown (6DE8) centrally, reverse darkening to brownish orange or greyish brown (6C6–8F3). Pale brown exudate droplets occurred in the centre and the medium became discolored dark brownish orange in age. Growth on PDA at 15 ºC was similar but colonies were slower growing (7.5 cm diam after 21 d) and paler. On 2 % MEA colonies were slow



Figs 8–11. *Cryptosporiopsis ericae* (UAMH 9445 except Fig. 10, UAMH 10349). 8–10. Curved macroconidia with minutely truncate base, phase contrast. 11. Microconidia. Bars = 10 µm.



Figs 12–15. *Cryptosporiopsis brunnea* (UAMH 10106, ex-type). 12– 13. Phialides in sporodochia producing macroconidia. 14. Macroconidia on OATC at 22 ºC. 15. Macroconidia on OATC at 18 ºC. Insert showing macroconidium with truncate base and smaller ovoid conidium. Bars = 10 µm.

growing (4–4.5 cm at 22 ºC; 2.5–3 cm at 15 ºC after 14 d), flat, pale whitish with thin aerial mycelium, reverse uncolored except UAMH 9445 which demonstrated strong brown pigmentation (6E8). On OATC after 21 d at 15 ºC, colonies were 8.5 cm, thin, pale whitish to patchy brownish orange; sporulation occurred only in UAMH 9445. On OATC at 22 ºC, sporulation was sparse in UAMH 10348 and profuse in UAMH 9445 with conidiomata forming within 14 d more heavily on the cellophane membrane than on the plain medium. In age, growth on the cellophane membrane became dark brown and sporodochia were associated with dark brown stromatic hyphae enmeshed in brownish pigment. No sporulation occurred on PDA or MEA. Cellulolytic.

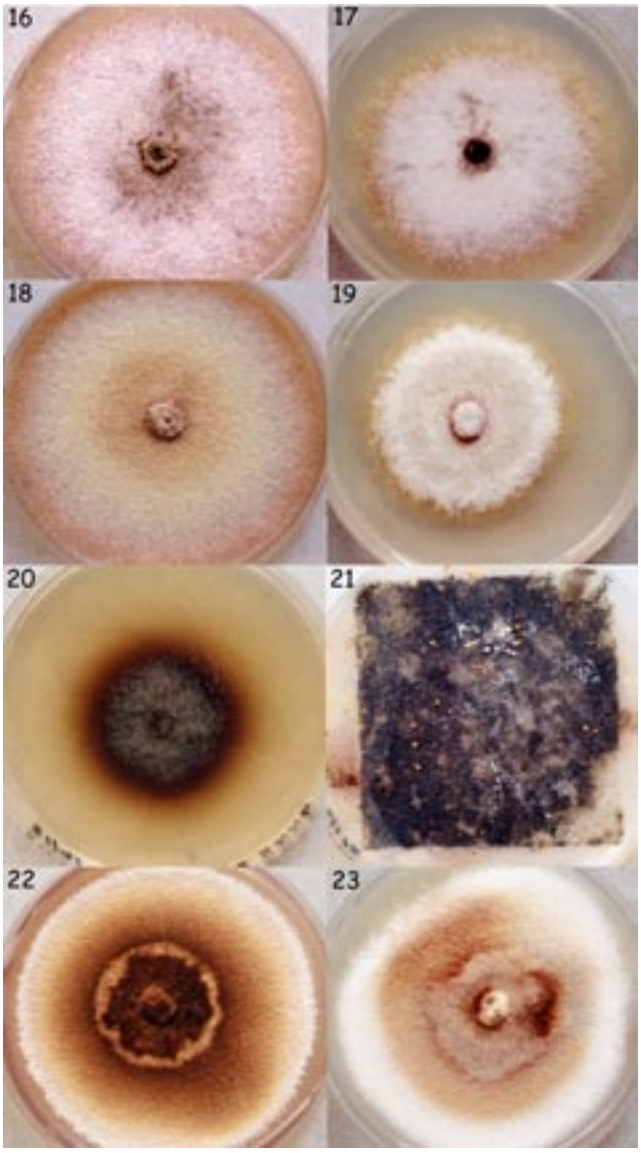
Cryptosporiopsis brunnea Sigler **sp. nov.**, MycoBank MB500254, Figs 12–15; 22–23.

Cellulae conidiogenae phialides formantes in conidiomatibus sporodochiis. Conidia in massa pallide-flava ad aureo-flava. Macroconidia late ellipsoidea, latera inaequalia - latus alterum directum vel exigue convexum, alterum curvatum, in apice rotunda, ad basim exigue truncata, aseptata, levia, hyalina, deinde flava et guttulata. Conidia 14.5–25 × 6–8 µm ad 18 °C (15–22 × 6–8 µm ad 22 °C). Microconidia typica absunt; aliquot conidia minora 7–10 × 5–6 µm, ovoidea, apiculata. Chlamydosporae et setae absunt. Teleomorphosis ignota.

Typus: Canada: British Columbia, northern Vancouver Is. (50° 60' N, 127° 35' W), ex radicis *Gaultheria shallon*, T. Allen (UBCtra 288). Colonia exsiccata et cultura viva, UAMH 10106.

Conidiogenous cells are phialides measuring 8.5–12 by 3–5 µm and borne on simple conidiophores formed in hemispherical conidiomata. Conidial masses are pale to golden yellow. Macroconidia are broadly ellipsoidal, inequilateral with one side straight or slightly convex and the other curved, rounded at the apex and narrowly truncate at the base with apiculus slightly off centre, nonseptate, smooth, hyaline, becoming yellow and guttulate in age. Conidia on OATC measure 14.5–25 × 6–8 µm at 18 ºC (15–22 × 6–8 µm at 22 ºC). Typical microconidia were not observed, but some conidia are smaller, ovoid, truncate at the base, measuring 7–10 × 5–6 µm. Chlamydospores and setae were not observed. Teleomorph is unknown.

Colonies on PDA at 22 ºC after 14 d were 6.5 cm diam, flat, smooth, felty to slightly woolly, slightly zonate, centrally dark brown (6–7E/F 6–8) , mid-zone lighter brown (6–7D6–7) with fine droplets of reddish-brown exudate, margin wide and white, reverse mid to dark brown (6–7E/F 4–8); by 21–28 d attaining 8.5 cm diam and developing copious brown exudate some of which diffused into the agar. Colonies on PDA at 15 ºC were slower growing (5.5 cm diam after 14 d) and paler brown on both obverse and reverse; no exudate or diffusible pigments developed within 21 d. On 2 % MEA colonies were slow growing (3.3 cm at 22 ºC; 2.8 cm at 15 ºC after 14 d), flat with thin aerial mycelium, centrally pale brown. Colonies on OATC after 21 d at 15 ºC were 8.5 cm, very thin, pale whitish, nonsporulating. On OATC at 22 ºC, conidiomata developed profusely within 14 d, forming more heavily on the cellophane membrane, and appeared yellow to golden yellow and wet; conidiomata were absent on plain OAT after 21 d. No sporulation occurred on PDA or MEA. Cellulolytic.



Figs 16–23. Colonies of *Cryptosporiopsis ericae*. 16–19. On PDA after 21 d. 16. UAMH 9445 at 22 ºC. 17. At 15 ºC. 18. UAMH 10349 at 22 ºC. 19. At 15 ºC. 20. UAMH 9445 on 2% MEA after 27 d at 15 ºC. 21. UAMH 9445 on OATC after 35 d at 22 ºC. Note white and yellow areas are masses of conidia; dark mycelium occurs on cellophane membrane. 22–23. Colonies of *Cryptosporiopsis brunnea* (UAMH 10106) on PDA after 21 d. 22. At 22 ºC. 23. At 15 ºC.

DISCUSSION

In Europe, *C. radicicola* and *C. rhizophila* appear to be common root-inhabiting fungi of *Quercus robur* and ericaceous plants, respectively (Kowalski & Bartnik 1995, Verkley *et al*. 2003). In contrast, *C. brunnea* and C. *ericae* appear to be uncommon in roots of western North American ericoid plants as judged by their infrequent recovery in culture by isolation from surface-sterilized roots and by the inability to detect them in direct PCR amplification from roots (Berch *et al*. 2002; Allen *et al*. 2003). Allen *et al*. showed that the ITS2 sequence derived from the isolate selected here as ex-type of *C. brunnea* grouped with those from two salal root cultures in a clade with *Pezicula ocellata*. Our results (Fig. 1) agree with those of Verkley *et al*. (2003) that indicate that *C. brunnea* and the salal isolate 'UBCtra 29' are as close to *C. radicicola* (Verkley *et al*. 2003) as to the ericoid associated *C. rhizophila*. UBCtra 29 is no longer available for cultural study but its sequence was identical to those of the isolates described here as C. *ericae*.

Verkley has placed emphasis on colonies on oatmeal agar, on size and shape of macroconidia and on presence of chlamydospores and setae as useful characters in distinguishing among root-associated species of *Cryptosporiopsis* (Verkley 1999; Verkley *et al*. 2003). The use of OAT at 15 ºC has been recommended, but this regime induced sporulation in only 4 of 9 isolates of *C. rhizophila*. Similarly, only 2 of 4 isolates of *C. ericae* produced conidia, but both *C. ericae* and *C. brunnea* sporulated better at 18 º or 22 ºC and on OATC. The cellophane membrane was found to be beneficial in promoting sporulation, but it reduced expression of colonial pigmentation. *Cryptosporiopsis ericae* is most similar to *C. radicicola* both phylogenetically and morphologically. The conidia are similar in shape and size (22–35 × 6–7.5 µm for *C. radicicola* vs. 20–33 ×5.5–7.5 µm for *C. ericae*). *Cryptosporiopsis radicicola* differs in producing chlamydospores and abundant setae and in sporulating on both PDA and MEA frequently from solitary phialides borne on the vegetative mycelium, occasionally in sporodochia. Cultures of *C. ericae* did not sporulate on either medium, and conidiogenous cells were produced only in conidiomata. Also, chlamydospores were lacking and setae were very sparse. Chlamydospores are absent in both *C. brunnea* and *C. rhizophila* and conidia are similar in size and shape (14.5–25 × 6–8 µm for *C. brunnea* vs.16–25 × 6–7.5 µm for *C. rhizophila*), but *C. brunnea* lacks both microconidia and setae.

Although both *C. ericae* and *C. brunnea* have been obtained only from surface-sterilized roots of ericaceous plants, little is known about their role in roots. No mycorrhizal resynthesis was seen in experiments done with salal and with the ex-type of *C. brunnea* (Allen *et al*. 2003) or *C. ericae* UBCtra 29 (Berch *et al*., 2002). Two British Columbia isolates of *C. ericae* were obtained from roots of *Vaccinium ovalifolium* collected about 1 metre from salal. They were very slow to grow out in primary cultures, appearing only after other fungi had been subcultured (Allen unpubl. data). They were not detected among DNA extracted for cloning experiments (Allen *et al*. 2003). A third isolate (10348 = UBCtra 1129) was obtained from a salal sample not used in the cloning experiment. No additional information is available on the isolate from huckleberry (*Vaccinium membranaceum* Dougl. ex Torr.) that was received for identification (McCracken 1999).

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