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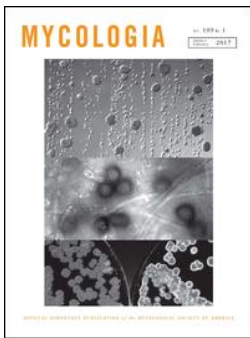**113** PUBLICATIONS   **5,259** CITATIONS

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A revision of the Alpova diplophloeus complex in North America

Jeremy Hayward, Samuel G. Tourtellot & Thomas R. Horton

A revision of the *Alpova diplophloeus* complex in North America

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Abstract: *Alpova diplophloeus* (Boletales, Paxillaceae) is the only currently recognized *Alpova* in North America with a brownish peridium, large gleba chambers and which forms ectomycorrhizas with *Alnus*. However, *A. diplophloeus* as currently circumscribed is a polyphyletic species, with at least three distinct genetic entities. Using a combination of molecular and morphological characters, we examined the type collections of *A. diplophloeus*, as well as species synonymized with it, including *A. cinnamomeus* and *Rhizopogon parvisporus*. We also examined several other collections of *A. diplophloeus* complex basidiomata. We describe *A. diplophloeus* sensu stricto; we also resurrect *A. cinnamomeus*, synonymized with *R. parvisporus* and describe a new species, *A. concolor*, from the complex.

Key words: barcode gap analysis, hypogeous fungi

INTRODUCTION

Alpova diplophloeus (Zeller & C.W. Dodge) Trappe & A.H. Sm. originally was described (as *Rhizopogon diplophloeus* Zeller & C.W. Dodge) from a collection made in the San Juan Islands, Washington (Zeller and Dodge 1918). Dodge (1931) subsequently described the genus *Alpova* to hold *A. cinnamomeus* C.W. Dodge, from specimens collected on Isle Royale, Michigan. Trappe (1975) transferred *R. diplophloeus* into *Alpova* and recognized *A. cinnamomeus*, as well as another recently described taxon, *R. parvisporus* Bowerman, as synonyms.

Recently a number of genetically distinct entities identified as *Alpova* "diplophloeus" have appeared in phylogenies (Rochet et al. 2011, Moreau et al. 2011). Moreau et al. (2011) highlighted the need for a better taxonomic understanding of *A. diplophloeus* for the biogeography and ecology of the genus as a whole. Because *A. diplophloeus* continues to be a well studied organism in the study of ectomycorrhizal anatomy and function, taxonomic confusion poses significant

practical difficulties not only for systematists but also for ecologists and physiologists (Godbout and Fortin 1983, Massicotte et al. 1986–1989, Becerra et al. 2009).

The purpose of this study is to re-examine the delimitation of *Alpova diplophloeus* using molecular and morphological evidence. We studied the type specimens of *A. diplophloeus* (Zeller & C.W. Dodge) Trappe & A.H. Sm., *A. cinnamomeus* and *Rhizopogon parvisporus* as well as several other collections of *A. diplophloeus* complex basidiomata. Based on morphological, barcode gap-based and phylogenetic criteria, we report one resurrection, a synonymization, and a new species from the *A. diplophloeus* complex. We discuss these results in light of the ectomycorrhizal specificity and global diversity of *Alpova* species.

MATERIALS AND METHODS

The type specimen of *Alpova diplophloeus* and accompanying annotation by the collector were made available by U.S. National Fungal Collection. Specimens of *Alpova concolor* and accompanying annotations were made available by the Oregon State University Herbarium and the University of British Columbia Herbarium. The holotype of *Alpova cinnamomeus* and accompanying annotations were made available by the University of Michigan Herbarium. Fresh characters are as observed by the authors for *Alpova diplophloeus* and *A. cinnamomeus*; fresh characters for *A. concolor* are drawn from the annotations of T. O'Dell, M. Madsen and an anonymous member of the Pacific Northwest Key Council, while fresh characters for *Alpova* sp. are drawn from the annotations of J. Trappe. Colors follow Ridgeway (1912). All spore measurements are given as (minimum value)–1st quartile-*mean*–3rd quartile-(maximum value).

Microtomy and microscopy.—We embedded basidiome fragments in LR White (London Resin Company, London, UK) as follows: We rehydrated tissue in 3% KOH for 6–12 h, then dehydrated with a graded ethanol series 20–100% in 10% increments with 15 min per increment. We infiltrated tissues with resin for approximately 12 h at 4 C, changed the resin once and then cured at 65 C for 24–36 h. We sectioned samples to 320 nm thickness with a glass knife (Latta-Hartmann type). We transferred sections to glass slides and either stained with toluidine blue or left sections unstained. We captured images with a Nikon Eclipse E600 phase contrast microscope with attached SPOT camera (Diagnostic Instruments Inc., Sterling Heights, Michigan). Observations of non-embedded spores in 3% KOH and hand-cut peridial sections (tangential and radial) were made with a Nikon Eclipse E200 microscope. We measured peridia thickness of each collection section in five locations at

400× magnification and measured 30 spores at 1000× magnification.

Molecular methods.—We extracted DNA with a modified glassmilk protocol as in Hayward and Horton (2012) but using 6 m guanidium hydrochloride (QIAGEN buffer PB; QIAGEN Inc., Valencia, California) instead of NaI as the chaotropic salt necessary for DNA to bind to silica. We amplified the internal transcribed spacer (ITS) region from DNA extracts with ITS1f and NLB4 primers (Gardes and Bruns 1993, Martin and Rygiewicz 2005). We amplified highly fragmented DNA that could not be amplified with these primers using a semi-nested design and amplifying ITS1 (NSI1 and ITS2 followed by ITS1f and ITS2; White et al. 1990, Martin and Rygiewicz 2005) and ITS2 regions (ITS3 and NLB4 followed by ITS3 and ITS4b; White et al. 1990, Gardes and Bruns 1993, Martin and Rygiewicz 2005). We amplified a segment of the glyceraldehyde-3-phosphate dehydrogenase (GPD) with primers CTK052 and CTK032R (Kreuzinger 1996). The GPD gene region was sequenced by Moreau et al. (2011), providing a collection of reference sequences that can be downloaded from NCBI's GenBank database. PCR conditions for all reactions were: 94 C for 3 min; 35 cycles at 94 C for 35 s, 53 C for 35 s; 72 C for 45 s and adding 2 s per cycle; followed by 72 C for 10 min but with annealing temperature 56 C for GPD reactions. We submitted amplicons for bidirectional sequencing with PCR primers on an ABI 3730xl sequencer. All sequences generated in this study were uploaded to GenBank under accession numbers KF835988–KF836005.

Data analysis.—We calculated statistics relating to spore measurements in R 2.15 (R Development Core Team 2012).

Barcode gap analysis.—We computed barcode gaps for the ITS region and GPD gene segment. Rochet et al. (2011) amplified these gene regions from several isolates of the well characterized species *Alpova alpestris* P.-A. Moreau & F. Rich. and *A. corsicus* P.-A. Moreau & F. Rich.; we used these sequences to compute barcode gaps. We aligned sequences with MUSCLE (Edgar 2004) with minor corrections by eye in Seaview 4 (Gouy et al. 2010). We used Mothur (Schloss et al. 2009) to determine pairwise dissimilarity between sequences in the alignments, treating indels of any length as a single event and not counting end gaps. We computed summary statistics for distances between *A. alpestris* isolates, *A. corsicus* isolates and interspecific distances. We compared these to genetic distances between ITS and GPD sequences generated in this study. Where multiple exemplar sequences were available, we selected one at random for inclusion in barcode gap analysis.

Phylogeny.—To demonstrate the placement of the known species of *Alpova*, we created a phylogeny with the ITS and GPD sequences generated here, as well as those generated by Nouhra et al. (2005), Moreau et al. (2011) and Moreau et al. (2013). We used Beauti 1.7.1 (Drummond et al. 2012) to generate xml files for analysis in BEAST 1.7.1. We used the GTR + I + G nucleotide model with a lognormal relaxed clock and a Yule process prior. We did not tune parameters; we let the chain run for 30 000 000 generations and discarded the first 10 000 000 as burn-in.

To investigate the range of compatible plant hosts of *Alpova* species, we used the ITS sequences generated here as query sequences against the NCBI GenBank and UNITE databases. We downloaded closely related sequences with host plant annotations and used barcode gaps and placement in the ITS phylogram as above to confirm species identities.

RESULTS

A line drawing depicts the peridial structure and spore morphology of the *Alpova* species described here (FIG. 1). Spore morphology was of relatively little use in delimiting the *Alpova* species described here; while *A. diplophloeus* could be differentiated from the other North American species, *A. concolor* and *A. cinnnamomeus* did not differ significantly in spore size or shape (TABLE I, FIG. 4). Peridial structure proved consistent within species and variable between species, providing a more useful delimiting character. We sequenced the internal transcribed spacer regions as well as 650 bp of the GPD gene, providing total coverage of approximately 1300 bp per species. The results of the barcode gap analyses are shown (TABLE II). Both ITS and GPD sequences yielded usable barcode gaps, allowing differentiation of species. A phylogeny of *Alpova* species generated from ITS and GPD sequences is illustrated (FIG. 2).

In the UNITE and NCBI GenBank databases, environmental sequences (deriving from ectomycorrhizal root tips) representing *Alpova concolor, A. diplophloeus, A. alpestris, A. cinnamomeus, A.* sp. and one undescribed taxon were present, as confirmed with barcode gap analysis and ITS phylogenetic placement. Geographic and host ranges for these taxa deriving from these data, published accounts and annotations are provided (TABLE III). An ITS phylogram showing the placement of these sequences is included (FIG. 3).

TAXONOMY

I. *Alpova* C.W. Dodge *Ann. Missouri Bot. Gard. 18*:461. 1931

I. 1. *Alpova concolor* Hayward, sp. nov.   FIG. 1a, b
MycoBank MB808068

Etymology: from the similarly dark rusty brown peridium and gleba when dried.

Distinguished from other *Alpova* sp. by the combination of a structurally well differentiated two-layered peridium and the presence of dermatocystidia. Apparently forming ectomycorrhizas with *Alnus rubra* Bong. and *Alnus rhombifolia* Nutt. Holotype: O'Dell 3824, OSC 65696, collected Siuslaw National Forest, Oregon, Oct 1996.
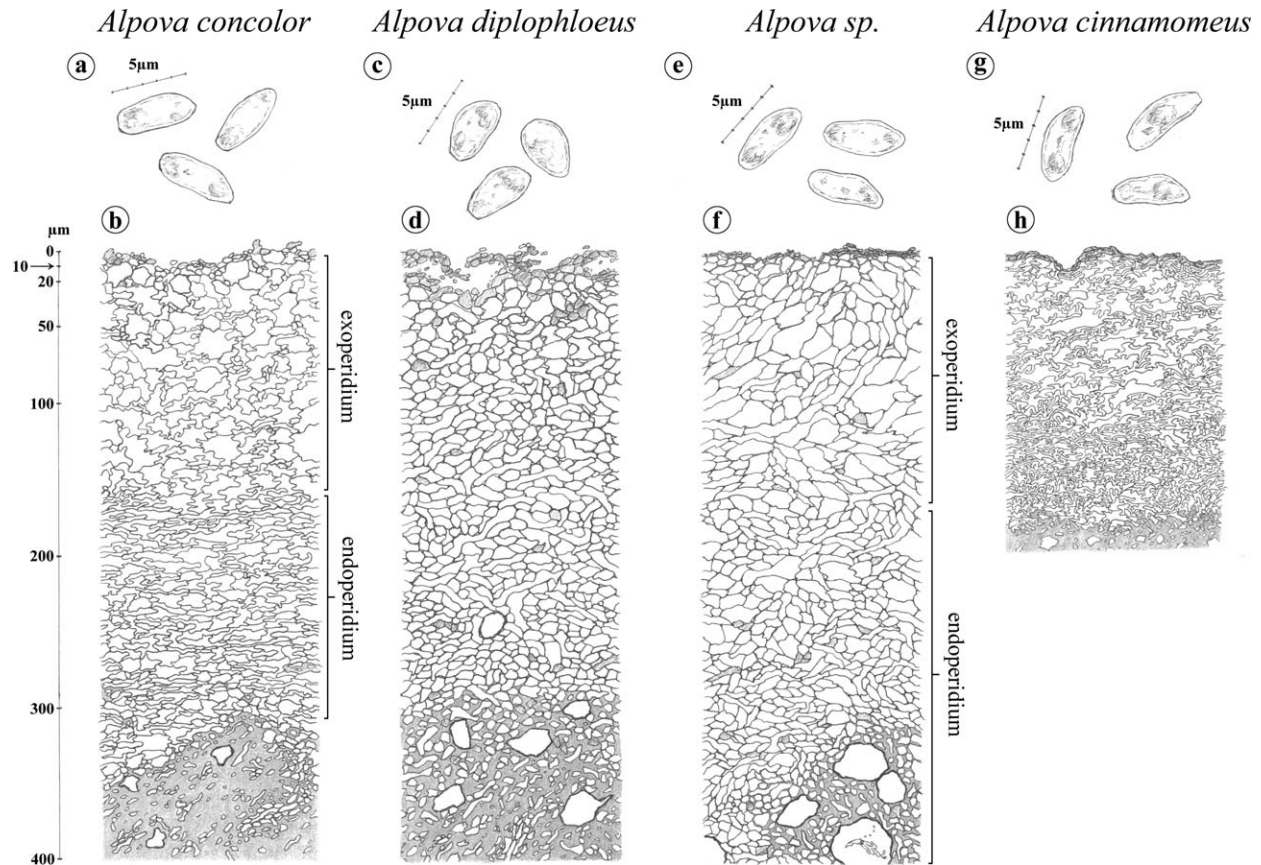
FIG. 1. Line drawings showing cross sections through the peridium of the four *Alpova* species described here, sectioned to 320 nm thickness and projected to the same scale. Sections drawn were stained with Toluidine blue. A. *A. concolor*. B. *A. diplophloeus*. C. *A.* sp. D. *A. cinnamomeus*. Spores were mounted in 3% KOH and observed at 1000× magnification.

Basidiomata primarily subhypogeous to erumpent, gregarious, irregularly subglobose, 11–23 mm diam. Odor, flavor none. Peridium amber brown to brownish olive, oxidizing to cinnamon-rufous where exposed to air or when bruised, drying cinnamon-rufous or slightly darker; smooth to slightly irregular, without apparent rhizomorphs. Gleba solid, gelatinous, with glebal chambers embedded in meandering stroma. Glebal chambers 0.3–0.4 × 0.4–0.5 mm; burnt umber; darkening slightly with exposure to air, composed of hardly distinct, gelatinized hyphae with occasional interspersed buffer cells, 1.5–3.2 μm. Stroma off-white at first, darkening with exposure to air to burnt sienna, 60–120 μm wide, composed of gelatinized and hardly distinct hyphae bordering on each side a layer of pseudoparenchymatous hyphae, these inflated hyphae 5–10 μm (mean 7.5 μm) diam.

Peridium two-layered, tough, remaining amber-brown to brownish olive even with exposure to air but drying dark brown, approximately 500 μm thick when fresh, 300–400 μm thick when revived in KOH, consisting of loosely woven (i.e. with visible gaps between hyphae in hand-cut scalp sections) exoperidium 20–55 μm thick, with pigmented hyphae 5.5–9 μm diam and frequent much larger hyphae 12–15 μm diam, and endoperidium 350–390 μm thick with abundant thick-walled inflated cells 8–20 μm diam but with smaller elements also present, inflated cells somewhat collapsed upon drying and remaining so even when revived in KOH. Fibrils dark, consisting of darkly pigmented hyphae 4–10 μm diam, to a depth of 20 μm, but absent from many areas. Dermatocystidia rare, 6.5–9.0 × 12.0–22.5 μm. Clamp connections evident.

Spores (4.0)–5.0–*5.40*–6.05–(6.0) × (1.5)–2.0–*2.1*–2.1–(3.0) μm, oblong to allantoid, thin walled, biguttulate when suspended in 3% KOH. In Melzer's reagent empire yellow to buff yellow in mass. In trypan blue hyaline to buff yellow in mass.

Specimens examined: Benton county, Oregon: OSC 65696 (Holotype of *Alpova concolor*), British Columbia: UBC F14673

Comments: The structurally distinct two-layered peridium and existence in the Pacific Northwest of the United States causes us to suspect that Zeller and Dodge included this species in their concept of *Alpova diplophloeus* even though the holotype of

Table I. Spore lengths, widths and quotients (length/width) for the holotype specimens examined here, based on 50 measurements/collection

Species	*Alpova diplophloeus*	*A. cinnamomeus*	*A. concolor*
Spore length (μm)	(4.0)–5.13–*5.67*–6.0–(7.0)	(4.0)–5.0–*5.15*–5.18–(7.0)	(4.0)–5.0–*5.33*–6.0–(6.0)
Length group	**A**	**B**	**B**
Spore width (μm)	(2.0)–2.0–*2.40*–2.5–(3.0)	(1.8)–2.0–*2.17*–2.2–(3.0)	(1.5)–2.0–*2.1*–2.1–(3.0)
Width group	**A**	**B**	**B**
Quotient	(1.6)–2.4–*2.43*–3.0–(3.5)	(1.5)–2.1–*2.41*–2.68–(3.3)	(1.6)–2.4–*2.57*–2.96–(3.3)
Quotient group	**A**	**A**	**A**

Measurements are presented as (minimum), first quartile, *mean*, 3rd quartile, (maximum). Groups refer to Tukey's honest significant difference test groupings as a post hoc test following an ANOVA.

Alpova diplophloeus does not belong to this species. At least among surveys that have deposited sequences in international sequence repositories, this species appears to be more common than *A. diplophloeus* sensu stricto.

I. 2. *Alpova cinnamomeus* C.W. Dodge *Ann. Missouri Bot. Gard.* 18:461. 1931

= *Rhizopogon parvisporus* Bowerman in Bowerman and Groves, Canadian Journal of Botany 40:239–240, 1962.

MycoBank MB214141   Fig. 1e–g

Distinct from other *Alpova* species by virtue of the combination of a peridium that revives poorly and is easily separated from the gleba when revived, relatively large gleba chambers, frequently biguttulate spores, and large buffer cells. Apparently forming ectomycorrhizal associations with *Alnus alnobetula* (Ehrh.) K. Koch (and perhaps others; see *Comments* below). Holotype: C.A. Brown Fp 73, MICH 4961, found on Isle Royale, Michigan, Jul 1930.

Basidiomate primarily subhypogeous, in groups of 3–12 individuals; irregularly globose, 6–35 × 8–40 mm at maturity. Odor, flavor none. Peridium smooth with shallow sharply demarcated round depressions; at maturity amber brown to brownish olive, with irregular darker rufous areas, darkening somewhat when bruised, the depressions paler; experidium occasionally cracking to reveal subcutical tissue; peridial layers burnt sienna in cross section. Rhizomorphs concolorous with peridium or slightly darker, appressed at the basidioma base and scattered on its sides. Gleba solid, gelatinous, with chambers 0.26–0.70 × 0.28–0.74 mm, filled with gelatinous material, embedded in meandering off-white stroma, producing a marbled appearance, the contents darkening when exposed or aged; glebal stroma composed of a layer of gelatinized hyphae surrounding on each side a pseudoparenchymatous layer with inflated cells 6–10 μm (mean 7.9 μm) diam. Gleba chambers composed of gelatinized, hardly distinct hyphae with sparse buffer cells, 4–7 μm diam. KOH on surface quickly rufous, darkening to Sudan brown. Drying overall bone-hard or waxy-cheesy.

Peridium two-layered, 300–450 μm in total with pigmentation to a depth of 45–125 μm, composed of darkly pigmented fibrils consisting of close, tightly woven hyphae 5–7 μm in diam, 14–25 μm thick; exoperidium lightly pigmented, 20–50 μm thick, composed of abundant inflated cells 25–30 μm across supported with uninflated hyphae 5–8 μm diam; and endoperidium 80–110 μm thick, composed of unpigmented hyphae 5–9 μm diam with occasional inflated hyphae to 25 μm; peridium as a whole easily separable from gleba when revived in KOH. Clamp connections conspicuous in peridium.

Spores (4.0)–5.0–*5.10*–5.15–(7.0) μm × (1.8)–2.0–*2.12*–2.17–(3.0) μm, oblong to allantoid, thin-walled, smooth, typically biguttulate when suspended in KOH but some spores with one or three oil droplets. In Melzer's reagent empire yellow to buff yellow in mass. In trypan blue hyaline to buff yellow in mass.

Specimens examined: Isle Royale, Michigan: MICH 4961 (host unknown); Labrador, Canada: Voitk Z5, Hayward AL4 (putative host *Alnus alnobetula*); Voikt K8, Hayward AL7 (putative host *Alnus alnobetula*); Voitk A5, Hayward AL9 (putative host *Alnus alnobetula*); Newfoundland, Canada: Mann 01, Hayward AL11; Labrador, Canada: DAOM 45792 (Holotype of *Rhizopogon parvisporus*, host unknown).

Comments: A. cinnamomeus, the type species for the genus, is apparently a northern species. Trappe (1975) synonymized it with *A. diplophloeus*, with which it shares many macroscopic and microscopic features; however, the poorly reviving, easily separable peridium is a distinctive macroscopic feature. The type collection is from Isle Royale, Michigan, where two species of *Alnus* Mill. are recorded: *A. crispa* (Aiton) Pursh, a member of *Alnus* subgenus *Alnobetula* Peterm., and *A. incana* (L.) Moench, a member of *Alnus* subgenus *Alnus* Endl. (Cooper 1913, Chen and Li 2004). While the host for the holotype collection is not known, all other basidiomata of

TABLE II. Barcode gap analyses for ITS and GPD sequences

A.[a]

	ITS			GPD		
	A. alpestris intraspecific	*A. corsicus* intraspecific	Interspecific	*A. alpestris* intraspecific	*A. corsicus* intraspecific	Interspecific
Maximum	0.032	0.0693	0.1359	0.0149	0.0111	0.06814
Minimum	0	0.002	0.0693	0.0029	0	0.0486
Mean	0.008225	0.0467	0.1021	0.0099	0.0068	0.05818
95% CI	(0, 0.019)	(0.004, 0.068)	(0.070, 0.134)	(0.003, 0.014)	(0.006, 0.011)	(0.041, 0.067)

B.[b]

	A. concolor	*A. cinnamomeus*	*A. diplophloeus*	*A. alpestris*	*A. corsicus*	*A. komoviana*	*A. austroalnicola*	*A.* sp.
A. concolor	—	0.05469	0.04677	0.05477	0.112	0.07985	0.06182	0.05754
A. cinnamomeus	0.04706	—	0.04362	0.05938	0.1225	0.07156	0.06434	0.06568
A. diplophloeus	0.0463	0.04074	—	0.01948	0.1111	0.07664	0.04984	0.05797
A. alpestris	0.04636	0.03565	0.01667	—	0.1427	0.0765	0.083	0.08245
A. corsicus	0.04383	0.0453	0.04806	0.05397	—	0.1	0.09415	0.07386
A. komoviana	0.06341	0.06555	0.06667	0.06602	0.06477	—	0.07078	0.07273
A. austroalnicola	*	*	*	*	*	*	—	0.04
A. sp.	0.0495	0.05184	0.04806	0.04714	0.006601	0.07096	*	—

[a] Summary statistics for distances calculated between reference sequences of *Alpova corsicus* (six ITS sequences; five GPD sequences) and *A. alpestris* (14 ITS sequences; four GPD sequences). Sequences are from Moreau et al. (2011).

[b] Distances calculated for exemplar sequences from known species of *Alpova*. Upper triangle distances reflect ITS sequences; lower triangle distances reflect GPD sequences. GPD sequence data is not available for *A. austroalnicola*. Sequences are from this study, Moreau et al. (2011) and Moreau et al. (2013).

A. cinnamomeus for which putative hosts are known associate with members of *Alnus* subgenus *Alnobetula*.

I. 3. *Alpova diplophloeus* (Zeller & C.W. Dodge) Trappe & A.H. Sm. FIG. 1c–d
MycoBank MC308484

Distinct from other *Alpova* species by virtue of a structurally poorly differentiated peridium with variation in the peridium only in pigmentation, and wide spores (some > 3.5 μm wide at widest point). Apparently forming ectomycorrhizas with *Alnus rubra*, *Alnus incana* and *Alnus rhombifolia*. Holotype: BPI 737239, collected in Friday Harbor, Washington, Jul 1917.

Basidiomata 10–50 mm, emergent, subhypogeous or even epigeous, in scattered groups, irregularly globose with lobes or folds at maturity, smooth with or without folds or ridges to 0.5 cm deep or tall, at maturity cinnamon-rufous, with irregular darker rufous areas, lighter in folds, darkening substantially when bruised and drying deep brownish drab to black. Peridial layers deep brownish drab in cross section. Appressed rhizomorphs darker than peridium. Gleba solid when fresh but sometimes drying with abundant air pockets (or a single large air pocket), gleba chambers 0.8–2.4 mm wide, filled with gelatinous material cinnamon but darkening at maturity, embedded in meandering off-white stroma, producing a marbled appearance, the contents darkening when exposed and drying dark brown to black, hard and waxy when dry. Gleba stroma composed of a layer of hardly distinct, gelatinized hyphae surrounding on each side a layer of pseudo-parenchymatous tissue with inflated cells 3–7 μm (mean 4.5 μm) diam. Odor not apparent. KOH on surface quickly rufous, darkening deep brownish drab. Drying overall deep brownish drab, bone-hard.

Peridium one-layered, 180–300 μm thick in total when revived in KOH, fibrils composed of darkly pigmented hyphae 6–10 μm diam, and lightly pigmented or unpigmented peridium 180–290 μm thick, with pigmentation largely restricted to the region adjacent to the exterior to a depth of up to 30 μm, composed largely of inflated cells 20–35 × 50–60 μm but with uninflated hyphae 3–6 μm diam, with darkly pigmented innate-appressed fibrils to a depth of up to 20 μm, consisting of hyphae 4–8 μm diam. Clamp connections abundant.

Spores (4.0)–5.2–*5.70*–6.0–(7.0) μm × (2.0)–2.1–*2.45*–2.5–(3.0) μm, oblong to broadly allantoid, thick walled, smooth, with one or very rarely two oil drops
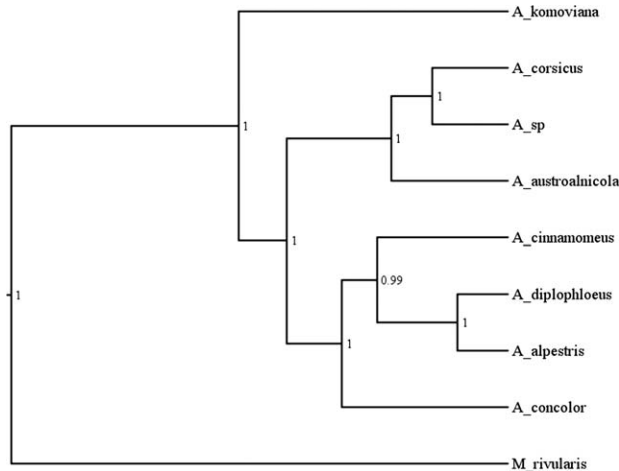
FIG. 2. Bayesian phylogeny of the known species of *Alpova*, outgrouped with *Melanogaster rivularis* P.-A. Moreau & F. Rich., based on ITS and GPD sequences for all species except *A. austroalnicola*, for which no GPD sequence is available. Branch support scores reflect posterior probabilities. *Alpova* species from the New World are largely monophyletic, with the exception of the European *Alpova alpestris*, which is included in an otherwise Nearctic clade.

In Melzer's reagent, empire yellow to buff yellow in mass. In trypan blue hyaline to buff yellow in mass.

Specimens examined: Friday Harbor, Washington: BPI737239 (Holotype of *Alpova diplophloeus*, putative host *Alnus rhombifolia*); Telluride, Colorado: Hayward A4 (putative host *Alnus incana*); Verona, NY: Hayward A7 (putative host *Alnus incana*).

Comments: Structurally *Alpova diplophloeus* has single-layered peridium, but its pigmentation is darkest near the basidiocarp surface, while hyphae near the gleba are hyaline, giving the impression of differentiation. *A. diplophloeus* is similar to *A. alpestris* and apparently is closely related to that species. The

putative hosts of *A. diplophloeus* (*Alnus rubra,Alnus rhombifolia* and *Alnus incana*) are members of *Alnus* subgenus *Alnus.*

I. 4 *Alpova* sp. FIG. 1e, f

Apparently distinct from other *Alpova* species by virtue of the thick, structurally differentiated two-layered peridium and spores frequently with two or more oil drops. Found in conjunction with *Alnus alnobetula*, according to Trappe,. Because *A. alnobetula* sensu stricto is absent from Sweden (Jalas and Suominen 1976), we suggest that the species associates with another species in *Alnus* subgenus *Alnobetula.*

Basidiomata drying to approximately 5 × 10 mm, irregularly subglobose or oblong, lobed and folded, appearing to the naked eye slightly felt-like, orange-rufous to rufous, with adherent sand and vegetation particles. Peridium chocolate in cross section. Rhizomorphs not apparent. Gleba drying solid, with deep brownish drab glebal chambers, 0.7–1.3 × 0.9–1.5 μm, embedded in meandering off-white stroma; the contents apparently darkening when exposed and drying black, bone-hard when dry. Glebal stroma 85–125 μm wide, composed of layers of gelatinized hyphae surrounding on each side a pseudoparenchymatous layer with inflated cells 5–12 μm (mean 8.1 μm) diam. Glebal chambers composed of gelatinized, hardly distinct hyphae with sparse buffer cells, 4.8–8 μm diam. KOH on surface quickly rufous, darkening to deep brownish drab. Drying overall rufous, bone-hard.

Peridium two-layered. Exoperidium consisting of loosely woven (visible gaps between hyphae in hand-cut scalp sections) hyphae 25–35 μm thick; endoperidium predominantly of substantially collapsed hyphae upon drying, 140–180 μm thick when dry; 180–250 μm thick when revived in KOH; inflated cells in

TABLE III. Names, known ectomycorrhizal hosts and known distributions of *Alpova* species

Alpova species	Hosts	Regions known	Sources
Alpova diplophloeus	*Alnus rhombifolia, A. incana*	North America	This study
A. concolor	*Alnus rubra, Alnus rhombifolia*	Western North America	This study
A. cinnamomeus	*Alnus viridis*	Northern North America	This study
A. alpestris	*Alnus alnobetula*	Europe	Moreau et al. 2011, Moreau et al. 2013
A. corsicus	*Alnus cordata, Alnus glutinosa*	Corsica	Moreau et al. 2011, Moreau et al. 2013
A. austroalnicola	*Alnus acuminata*	South America	Nouhra et al. 2005
A. komoviana	*Alnus incana*	Europe	Moreau et al. 2013
A. sp	*Alnus incana*	Northern Europe	This study, Moreau et al. 2011
A. sp2 (unknown)[a]	*Alnus maximowicii*	Japan	Environmental sequences (exemplar: GB HE979127)

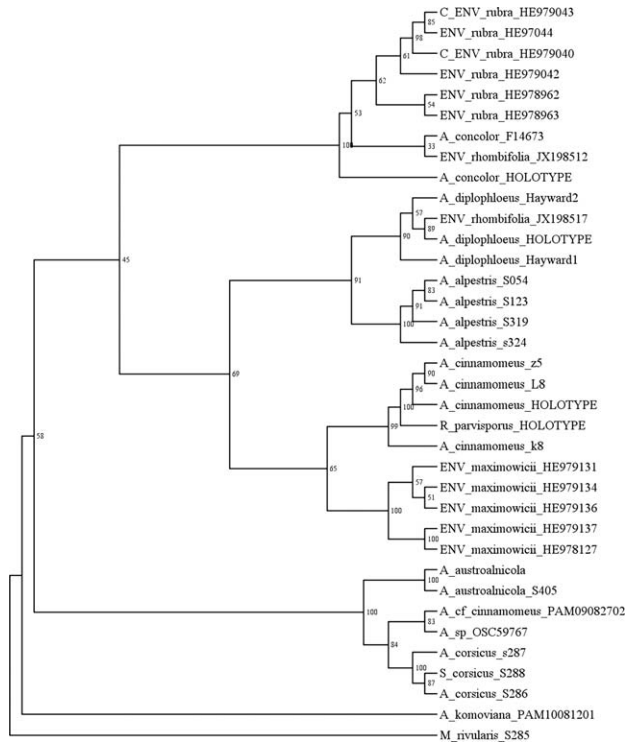[a] Two unnamed species, one based only on environmental sequence data, are included (see also FIG. 3).

FIG. 3. Uncorrected distance tree (P distances) generated with the BioNJ algorithm based on ITS sequences of known species of *Alpova* as well as unidentified environmental sequences annotated with host information. Generic abbreviations: A = *Alpova*, M = *Melanogaster*, R = *Rhizopogon*. Environmental sequences are named according to the scheme: ENV_*Alnus* host species_GenBank accession number. Other sequences are named following their published strain numbers or type status. Branch support scores reflect posterior probabilities. Sources: Nouhra et al. 2005, Moreau et al. 2011, Moreau et al. 2013, Roy et al. 2013, Polme et al. 2013, unpubl GenBank sequences this study.

FIG. 4. Photomicrographs of exoperidial details and spore morphologies of *Alpova* species. A. Exoperidium of *Alpova concolor*, microtome section stained in toluidine blue and photographed at 400× magnification, showing single dermatocystidium. B. Spores of *Alpova cinnamomeus* in 3% KOH, 1000× magnification. C. Spores of *Alpova* sp. D. Spores of *Alpova diplophloeus*. E. Spores of *Alpova concolor*. Bars: A = 10 μm, B–E = 1 μm.

subcutis 25–35 μm diam but frequently radially collapsed to 9–12 μm. Clamp connections present but difficult to discern and may not be present at all septae.

Spores (5.0)–5.5–*5.8*–6.0–(6.5) μm × 1.8–2.0–*2.23*–2.5–(3) μm, oblong to broadly allantoid, thick walled, smooth, biguttulate, these guttulations sometimes giving the appearance of two false septae. In Melzer's reagent empire yellow to buff yellow in mass. In trypan blue hyaline to buff yellow in mass.

Specimens examined: Lek Sands Parish, Sweden: OSC65696 (host not known).

Comments: The specimen examined here is similar to the description of Alpova cf. cinnamomeus PAM09082702 given by Moreau et al. (2011), who commented on the specimen's genetic similarity to that studied here. Those authors suggest that the specimen they examined probably represents a novel taxon. Our obse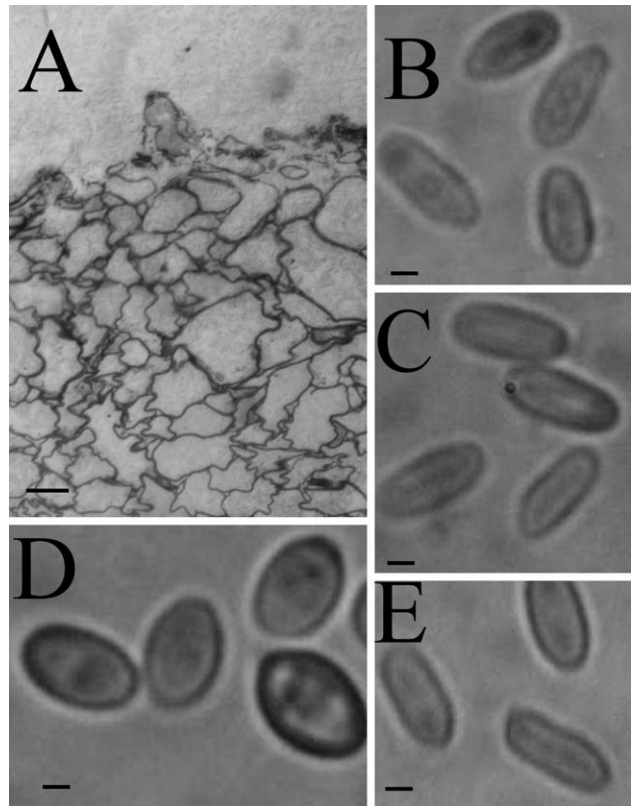rvations of this specimen (OSC 59767) suggest that they were correct. However, without morphological characters described from more specimens, we are hesitant to formally describe the species.

KEY TO *ALPOVA* IN NORTH AMERICA

1. Peridium structurally single-layered, with wide spores (more than 2.2 μm wide) . . . . . . *Alpova diplophloeus*
1'. Peridium with two structurally distinct layers, spores on average less than 2.2 μm wide 2
2. Peridia cells collapsing fully upon drying; peridium separating easily from gleba; apparently northern distribution . . . . *Alpova cinnamomeus*
2'. Peridia cells not collapsing entirely upon drying; peridium not easily separable . . . . . *Alpova concolor*

DISCUSSION

Barcode gap analysis of North American species strongly supports the differentiation of *Alpova*

diplophloeus, *A. cinnamomeus* and *A. concolor*. The differentiation of the European *A. alpestris*, *A. corsicus* and *A.* sp. are strongly supported also; however, *A. alpestris* and *A. diplophloeus* differ by approximately 1.9% in ITS sequence and < 1.7% in GPD sequence. Interspecific differences are expected to be 2–3 times larger in each gene region (TABLE II; Schoch et al. 2012). However, sequences generated here and represented on GenBank of each species are reciprocally monophyletic (data not shown). Consequently we suggest that *A. alpestris* and *A. diplophloeus* represent recently separated lineages. The species immediately basal to *A. alpestris* are all North American, leading us to hypothesize that *A. alpestris* may represent colonization of the Old World by an otherwise New World lineage; however, further phylogeographic analysis is needed to support this suggestion.

Vizzini et al. (2010), Moreau et al. (2011) and Rochet et al. (2011) have noted some confusion in the delimitation of the genus *Alpova*: neither *A. rubescens* (Vittad.) Trappe nor *A. trappei* Fogel form a monophyletic group with other *Alpova* species. *A. rubescens* did not consistently form a monophyletic group other *Alpova* species in the analysis of Vizzini et al. (2010) and Moreau et al. (2013); it may require a new genus. Vizzini et al. (2010) and Moreau et al. (2013) both also suggest that *A. trappei* may be more closely related to *Melanogaster* Corda.

Peridia structure has been of primary importance in delimiting *Alpova* species. Zeller and Dodge (1918) described the peridium of *A. diplophloeus* (as *Rhizopogon diplophloeus*) as two-layered, with both layers more than 100 μm thick. Since Trappe (1975), it has been common for *Alpova* species to be described as possessing a two-layered peridium. However, what is meant by ''double-layered'' has always been unclear. Zeller and Dodge (1918) distinguished between the outer and inner layers on the basis of three characters: the pigmentation of the hyphae, which they describe as dark in the outer layer and light in the inner layer, inflated hyphae, which they describe as present in the outer layer and absent from the inner layer, and the texture of the hyphae, which they describe as loosely interwoven in the outer layer and tightly interwoven in the inner layer. Throughout the species of *Alpova* these traits do not always overlap. For example, Smith and Zeller (1966) described the holotype of *A. diplophloeus* (again as a *Rhizopogon*) as having a thick, single-layered peridium but with a gradation in intensity of pigmentation from outside to inside; the description presented here confirms this. Conversely Moreau et al. (2011) describe *A. alpestris* as having a double-layered peridium, with the outer layer 20 μm thick,

and an entirely yellow inner layer. The question of peridial layers is complicated by the presence of what Zeller and Dodge (1918) termed innate-appressed fibrils, described as being darkly pigmented and less than 20 μm thick in *Alpova diplophloeus*, Zeller (1939) suggested that these fibrils may originate with the deliquescence of some of the hyphae in the outermost peridial layers early in the development of basidiomata.

The only known *Alpova* species in North America with fibrils, a structurally distinct endoperidium and exoperidium, and a color shift from exterior to interior, is the one described here as *A. concolor*. *A. concolor* apparently overlaps in range with *A. diplophloeus* and is macromorphologically similar. Based on the description of Zeller and Dodge (1918), we suspect that this species was included in the concept of *A. diplophloeus*, and the peridium of that species was described from a collection of *A. concolor*. *A. concolor* has a layer of dark, appressed-innate fibrils like those described by Zeller and Dodge (1918), up to 20 μm thick. We suggest that taxonomists who have described *Alpova* species with double-layered peridia consisting of a thin (i.e. less than 100 μm) exoperidium and thick endoperidium (e.g. Moreau et al. 2011) probably described the characters termed ''fibrils'' by Zeller and Dodge (1918) as exoperidium or epicutis. However, illustrations by Zeller and Dodge agree with those of later authors, including Moreau et al. (2011), demonstrating that any disagreements are strictly terminological. If fibrils are taken to constitute a separate layer of the peridium, as opposed to ornamentation of the exoperidium (as we describe them here), *Alpova concolor*, *Alpova cinnamomeus* and *Alpova* sp. would have a three-layered peridium while *Alpova diplophloeus* would have a two-layered peridium. Our interpretation, which we think reflects the terminology used by Zeller and Dodge (1918), suggests that the holotype of *Alpova diplophloeus* belongs to the only North American *Alpova* species so far described without a double-layered peridium. The naming of a single-layered species for its supposed double-layered peridium is not a correctable error.

Based on available publications and deposited sequences, nine phylogenetically distinct clades of *Alpova* are apparent (FIG. 3) seven of these correspond to recognized species of *Alpova*: *A. cinnamomeus*, *A. concolor*, *A. alpestris*, *A. austroalnicola* L.S. Domínguez, *A. corsicus*, *A. komovianus* Perić & P.-A. Moreau and *A. diplophloeus*. The remaining two clusters, one Japanese and one European (the latter including *Alpova* sp. as described here) probably represent unnamed species. Of the nine potential species of *Alpova* only one, *A. austroalnicola*, is

indigenous to the southern hemisphere (Nouhra et al. 2005); the remainder are Holarctic. Trappe (1975) referred to a number of other *Alpova* species. While some of these have since been referred to *Melanogaster*, *Rhizopogon* Fr. or other genera (e.g. *Rhizopogon alexsmithii* [Trappe] Vizzini & Zotti), a number of reportedly distinct *Alpova* species remain to be investigated.

Of the seven named species of *Alpova*, all are apparently specific at the host subgenus level or below; *A. diplophloeus,* for example, apparently associates with both *Alnus rhombifolia* and *Alnus incana*, both members of *Alnus* subgenus *Alnus* (Chen and Li 2004; TABLE III). This narrow specificity (and in particular specificity at the subgenus level) is common among *Alnus*-associating ectomycorrhizal fungi (Horton et al. 2013, Polme et al. 2013, Roy et al. 2013). Of note, no *Alpova* species are reported associating with any of the three to four species of *Alnus* subgenus *Clethropsis* (Spach) Regel. Determining whether no *Alpovas* in fact do associate with these species should be a priority for future research on *Alpova*.

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