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Polyozellus multiplex (Thelephorales) is a species complex containing four new species

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ABSTRACT

Geographic, morphological, and internal transcribed spacer (ITS)-based molecular review of collections identified as *Polyozellus multiplex* revealed that it is a complex of five phylogenetic species. Average spore size—either less or more than 7 × 6 µm—splits the complex into a small-spored group of two (*P. multiplex* and *P. atrolazulinus*) and a large-spored group of three (*P. mariae, P. marymargaretae*, and *P. purpureoniger*). Basidiocarps of the small-spored species are somewhat smaller than the large-spored ones, are various shades of blue, dark all the way to black, with brownish tomentum only in early growth, have dark context, and have pilei that tend to flare out at the edge. The large-spored species produce somewhat larger sporocarps, have light or lighter context than the pileipelis, and usually retain some brown on the mature pileipellis, the edge of which tends to curl like a cabbage leaf. All will darken or blacken with age. The species of the *P. multiplex* complex are distributed in the northern coniferous region, with the exception of Europe. One species (*P. atrolazulinus*) is known from three regions, eastern Asia, western North America, and northeastern North America. Two species are known from two regions: *P. purpureoniger* in eastern Asia and northwestern North America and *P. multiplex* in eastern Asia and eastern North America. Two species have been documented in one region only: *P. mariae* in northeastern North America and *P. marymargaretae* in western North America. A combination of location, macromorphology, and spore size will usually differentiate the species of the complex.

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Agaricomycetes; black chanterelle; blue chanterelle; four new taxa; one new typification

INTRODUCTION

In 1899, Lucien Underwood described a new black chanterelle species, *Cantharellus multiplex* Underw., from Maine (Underwood 1899). Because its cespitose imbricate habit differed from that of other species of *Cantharellus* Adans. ex Fr., Murrill (1910) transferred it to a new genus, *Polyozellus* Murrill. Imazeki (1938) synonymized a collection from Japan, described by Lloyd as *Phyllocarbon yasudae* Lloyd (Lloyd 1921; as "*yasudai*"), with *P. multiplex* (Underw.) Murrill, supported its placement in *Polyozellus*, and subsequently moved the genus to the *Thelephora* Ehrh. ex Wild. group because, unlike species in *Cantharellus*, it had lobed nodulose basidiospores and contained thelephoric acid (Imazeki 1953). Since then, *Polyozellus* has remained a monotypic genus whose distribution has been documented across northern North America, with southward extension at higher altitudes (Appalachian Mountains, Rocky Mountains, and Cascade Range), and Asia as far as southern China (Zhang et al. 2010). Bigelow (1978) reported two collections from Maine with "larger basidiocarps" and larger basidiospores than had been described earlier but did not suggest that these were a different species.

Through his ongoing study of the Thelephorales, Kõljalg has suspected the existence of more than one species, based on examination of Asian collections with differing basidiospore sizes, supported by DNA sequence diversity in the genus. The discovery of a *Polyozellus* in the Canadian Province of Newfoundland and Labrador (NL) with an unusual brownish pileus surface and whitish flesh, unlike the entirely black species common there, prompted a reassessment of the species with a combined geographic, morphological, and phylogenetic analysis to determine whether "*P. multiplex*" is a species complex, and if so, to circumscribe the previously unrecognized species. A search for DNA sequences in GenBank and the European Molecular Biology Laboratory database revealed that all of the North American sequences came from material collected in the West. Thus, in

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addition to studying more specimens generally, review of the genus required study, including sequencing, of a representative number of collections of "*P. multiplex*" from eastern North America, where the type species was described.

MATERIALS AND METHODS

Study material.—In addition to our own collections, herbaria, individuals, and mushroom clubs were contacted for specimens, particularly from eastern North America. In total, 89 collections were studied, including examination of all *Polyozellus* collections at NY (Voitk 2017a), including the holotype of *Cantharellus multiplex*, the holotype of *Phyllocarbon yasudai* at BPI (Voitk 2015), and all collections at DBG (Voitk 2017b). Forty-four collections were used in the phylogenetic analysis (TABLE 1), including eight sequences from GenBank, one unpublished sequence from AFTOL (ID 530), and one unpublished sequence submitted by Brandon Matheny. Seventy-four collections were examined microscopically, but to ensure accuracy, macro- and microscopic descriptions in this study are based only on examination of sequence-confirmed specimens: nine collections of *P. multiplex*, eight of *P. atrolazulinus* Trudell & Kõljalg, four of *P. mariae* Voitk & Kõljalg, three of *P. marymargaretae* Beug & I. Saar, and six of *P. purpureoniger* Spirin & I. Saar. All specimens are deposited in institutional herbaria (TABLE 1), identified by codes from *Index Herbariorum* (Thiers, continuously updated).

Morphological study.—Macroscopic observations were made in situ and on fresh specimens after collection. Light microscopic observations (Zeiss 392560 with Apo 100/1.25; Vienna, Austria), were conducted at 1000× magnification, using 2% KOH. Spores were measured from squash mounts of the hymenium of exsiccatae and/or fresh specimens to 0.5-µm accuracy; reported measurements deviate from 0.5-µm increments due to calculation of calibration error for the objective. The basidiospores have complex shapes with variable size, number, and location of projections of decreasing size: lobes, lobules, and nodules (FIG. 1). These projections are not "ornamentation" in the sense of outgrowths of the cell wall. Because such irregular basidiospore structure seems to make decisions of what to exclude as "ornamentation" subjective, we follow Bigelow (1978) in measuring spores of this group from edge to edge, measuring length as the longer axis and width as the greatest distance at right angles to the long axis (FIG. 1). A minimum of 20 spores per specimen were used to calculate the average size. In reporting range, measurements of extremes seen less than 10% of the time are placed in parentheses as extraordinary. In the text, "small-spored" indicates species with average spore sizes under 7 × 6 µm, and "large-spored" indicates species with average spore sizes greater than those measurements. To reduce interobserver error, all measurements for comparative spore size were done by one author. Color designations of the form, for example, 5A3 are based on Kornerup and Wanscher (1978), and color names within quotation marks are from Ridgway (1912). Where available, location coordinates are reported as measured with a Global Positioning System (GPS) receiver, close to ±5–10 m. Where such records do not exist, an estimate is made from the description and notes. These coordinates are felt to be accurate to approximately ±1 km, and are indicated by a tilde (~).

DNA sequencing.—DNA was extracted in 10× reaction buffer B (0.8 M Tris-HCl, 0.2M $(NH_4)_2SO_4$, 0.2% *w/v* Tween-20; Solis Biodyne, Estonia) including proteinase K (0.5 mg/mL; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated at 56 C overnight. The High Pure PCR template preparation kit (Roche Applied Science, Penzberg, Germany) was used for the older specimens, following the protocol of the manufacturer. Polymerase chain reaction (PCR) amplification was performed with primers ITS0F (5′-ACTTGGTCATTTAGAGGA AGT-3′)–LB-W (5′-CTTTTCATCTTTCCCTCACGG-3′) (Tedersoo et al. 2008) or ITS0F–ITS4B (5′-CAGGAGACTTGTACACGGTCCAG-3′) (Gardes and Bruns 1993) for the ITS (internal transcribed spacer) and partial LSU (large subunit of ribosomal RNA) regions, using 5× HOT FIREPol Blend Master Mix Ready to Load (with 10 mM $MgCl_2$; Solis BioDyne) with 0.5 µM of each primer and 1–3 µL of DNA solution. PCR amplifications were tried with several primer combinations from holotype specimens, but only one relevant read of the LSU region (83 bp only from NY808298) was achieved using primers LR0R (5′-ACCCGCTGAACTTAAGC-3′) (Hopple and Vilgalys 1994)–ITS4-Tom (5′-AACTCGGACGACCAG AGGCA-3′) (Tomentella-Thelephora specific; Tedersoo et al. 2011). Further PCR amplification and purification protocols follow those described in Saar and Voitk (2015). Sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands) using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White

Table 1. Collections and ITS sequences used in this study.

Species	COUNTRY, Province/state/region	Main fungarium[a] code[b] (collection code)[c]	Secondary fungarium[d] code	GenBank accession UNITE code
Polyozellus atrolazulinus	CANADA, BC	SMI299		HQ650735
Polyozellus atrolazulinus	CANADA, BC	WTU-F-068937 (SAT-08-256-08)	TU102999	**MF100820** **UDB023739**
Polyozellus atrolazulinus	CANADA, QC	DAOM836206 (15.09.08.av06)	TU117478	**MF100835** **UDB032206**
Polyozellus atrolazulinus	CANADA, QC	DAOM836207 (15.09.15.av01)	TU117349	**MF100828** **UDB032574**
Polyozellus atrolazulinus	CANADA, QC	DAOM836205 (15.09.08.av05)	TU117477	**MF100839** **UDB033203**
Polyozellus atrolazulinus	CANADA, NL	DAOM879656 (17.09.11.av04)	TU117559	**MG214657**[e] **UDB034063**
Polyozellus atrolazulinus	RUSSIA, Far East	TAAM013662		**MF100825** **UDB023793**
Polyozellus atrolazulinus	USA, AK	WTU-F-065677 (FTNF-10-250-21)	TU117096	**MF100822** **UDB023741**
Polyozellus atrolazulinus	USA, ME	MAINE-8680 (Homola 8680)	TU117453	**MF100832** **UDB031417**
Polyozellus atrolazulinus	USA, NM	WTU-F-068938 (SAT-06-239-02)	TU102996	**MF100818** **UDB023737**
Polyozellus atrolazulinus	USA, OR	OSC112577		EU846255
Polyozellus atrolazulinus	USA, OR	OSC97131		EU846256
Polyozellus atrolazulinus	USA, OR	WTU-F-001763**HOLOTYPE** (SAT-99-296-13)	TU102998**ISOTYPE**	**MF100819** **UDB023738**
Polyozellus atrolazulinus	USA, UT	AFTOL679		DQ911595
Polyozellus mariae	CANADA, NL	DAOM836202**HOLOTYPE** (09.09.25.av02)	TU117348**ISOTYPE** NY2859131**ISOTYPE** FH01142406**ISOTYPE**	**MF100831** **UDB032173**
Polyozellus mariae	CANADA, NL	DAOM836204 (15.09.20.av01)	TU117235	**MF100826** **UDB024772**
Polyozellus mariae	CANADA, NL	DAOM836203 (FNL-GM5-491)	TU117346	**MF100834** **UDB031374**
Polyozellus mariae	USA, ME	NY2859130 (06.09.19.av01)	TU117345	**MF100840** **UDB033282**
Polyozellus marymargaretae	USA, OR	OSC108168		EU846257
Polyozellus marymargaretae	USA, OR	OSC105070		EU846253
Polyozellus marymargaretae	USA, WA	OSC108798		EU846254
Polyozellus marymargaretae	USA, WA	WTU-F-015164 (JEL9729)	TU102995	**MF100817** **UDB023736**
Polyozellus marymargaretae	USA, WA	WTU-F-015158 (PBM2412)	TU115269	**MF100813** **UDB011122**
Polyozellus marymargaretae	USA, WA	WTU-F-068939**HOLOTYPE** (01MWB091115 15.09.11.av01)	TU117347**ISOTYPE**	**MF100841** **UDB033283**
Polyozellus marymargaretae	USA, WA	TU119561A		**MF100827** **UDB024597**
Polyozellus multiplex	CANADA, NL	DAOM836196 (10.08.23.av07)	TU115322	**MF100814** **UDB016202**
Polyozellus multiplex	CANADA, NL	DAOM836197 (10.08.23.av04)	TU115323	**MF100815** **UDB016203**
Polyozellus multiplex	CANADA, NL	DAOM836199 (FNL-TN1-408)	TU117479	**MF100837** **UDB032730**
Polyozellus multiplex	CANADA, NL	DAOM836198 (FNL-TN2-216)	TU117480	**MF100838** **UDB032731**
Polyozellus multiplex	CANADA, NL	DAOM836201 (16.09.18.av01)	TU117481	**MF100836** **UDB032207**
Polyozellus multiplex	CANADA, QC	DAOM836200 (13.08.28.av01)	TU117351	**MF100829** **UDB032575**
Polyozellus multiplex	CHINA	TU115049		**MF100812** **UDB018721**
Polyozellus multiplex	CHINA	HKAS43115		AFTOL-530[f]
Polyozellus multiplex	JAPAN	TMI22322		**MF100816** **UDB023429**
Polyozellus multiplex	USA, ME	NY2859132**EPITYPE** (12.09.20.av02)	TU117350**ISOEPITYPE**	**MF100830** **UDB032576**
Polyozellus multiplex	USA, ME	MAINE –E	TU117454	**MF100833** **UDB031421**
Polyozellus multiplex	USA, NC	TENN071088		MF686488
Polyozellus purpureoniger	RUSSIA, Far East	TAAM013653		**MF100824** **UDB023792**
Polyozellus purpureoniger	RUSSIA, Far East	H7021363 (VS4302)		**MF100843** **UDB033285**
Polyozellus purpureoniger	RUSSIA, Far East	H7021198**HOLOTYPE** (VS4351)		**MF100842** **UDB033284**
Polyozellus purpureoniger	USA, AK	WTU-F-010266 (SAT-09-246-12)	TU103000	**MF100821** **UDB023740**
Polyozellus purpureoniger	USA, AK	DAOM836208 (AKFF-157-14)		**MF100844** **UDB033290**

(*Continued*)

Table 1. (Continued).

Species	COUNTRY, Province/state/region	Main fungarium[a] code[b] (collection code)[c]	Secondary fungarium[d] code	GenBank accession UNITE code
Polyozellus purpureoniger	USA, WA	WTU-F-001788 (SAT-05-265-16)	TU117097	**MF100823 UDB023742**
Pseudotomentella mucidula[OUTGROUP]	ESTONIA	TU108047		UDB018563

Note. Codes of sequences generated for this study shown in boldface.
[a]Fungarium, where the main, or largest, portion of the collection housed.
[b]Fungarium acronym as per Index Fungariorum.
[c]Collection code given by original collector and/or private fungarium, where it was housed until transfer.
[d]Fungarium/a where duplicate or isotypic portion, usually smaller, of main collection housed.
[e]Collected after analysis finished and manuscript submitted. Not used in phylogeny, but included to extend known range.
[f]Unpublished AFTOL sequence made by Zai-Wei Ge.

et al. 1990). Sequences were inspected and assembled using Sequencher 5.2 (Gene Codes, Ann Arbor, Michigan, USA). The DNA sequences (TABLE 1) were uploaded to the PlutoF cloud database (Abarenkov et al. 2010b), including collection data; all of the information is available online from UNITE (http//unite.ut.ee; Abarenkov et al. 2010a) and GenBank. A sequence of *Pseudotomentella mucidula* was added into the data matrix as an outgroup because it is a type species of the genus that is the closest relative of *Polyozellus* inside the thelephoroid clade, based on both morphological and molecular characters (Kõljalg and Saar, unpublished data).

Phylogenetic analysis.—Alignments were performed using L-INS-i strategy as implemented in MAFFT 7.310 (Katoh and Standley 2013). Minor manual adjustments were performed with SeaView 4.6.1 (Gouy et al. 2010). Bayesian inference (BI) was performed with MrBayes 3.2.6 (Ronquist et al. 2012) for 1 million generations, with four chains, and trees sampled every 500 generations, discarding the first 100 000 generations without reaching a stable likelihood score. Maximum likelihood (ML) and rapid bootstrap (BS) analyses were run using RAxML-HPC BlackBox 8.2.9 (Stamatakis 2014), at the CIPRES Science Gateway (Miller et al. 2010; http://www.phylo.org/). Two data matrices and best ML trees were combined as one NEXUS file: (1) the 43-taxon, 723-character ITS matrix; and (2) the 25-taxon, 84-character LSU matrix were deposited in TreeBASE, accession number S21308.

Species hypothesis.—All *Polyozellus* ITS sequences were also analyzed according to the UNITE species hypothesis (SH) approach (Kõljalg et al. 2013). The SHs found will be incorporated into UNITE data and all will receive a digital object identifier (DOI) for stable communication (Kõljalg et al. 2016). They will be available for the identification of fungal ITS sequences through UNITE resources (https://unite.ut.ee/repository.php) and major pipelines such as QIIME, MOTHER, and USEARCH.

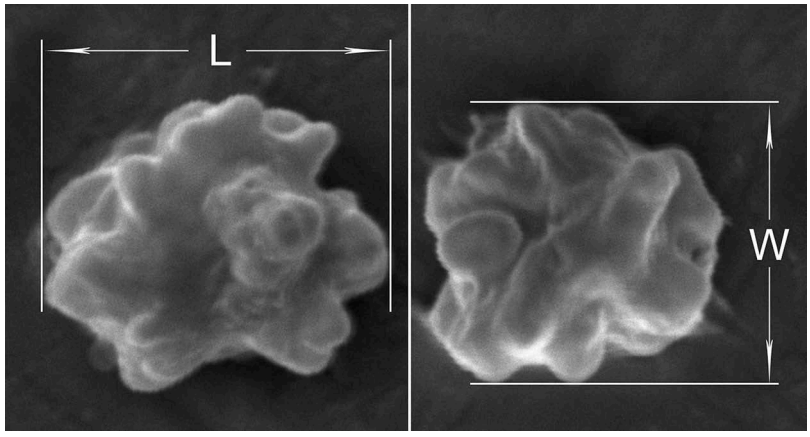

Figure 1. Scanning electron microscope images of two spores, aligned with their longer axes horizontally, from a specimen of *Polyozellus mariae* (DAOM836204), to illustrate the morphology of *Polyozellus* spores, and the method used for their measurement. Length is measured edge-to-edge along the long axis, and width at right angles to the long axis. The angular, lobed, and nodular structure of spores is similar for all species of *Polyozellus*.

Table 2. Average evolutionary divergence estimates among sequence pairs within and between species of the *Polyozellus multiplex* complex.

Species	*P. atrolazulinus*	*P. mariae*	*P. marymargaretae*	*P. multiplex*	*P. purpureoniger*
P. atrolazulinus	0.001				
P. mariae	0.102	0.000			
P. marymargaretae	0.097	0.011	0.003		
P. multiplex	0.104	0.120	0.117	0.003	
P. purpureoniger	0.094	0.033	0.033	0.110	0.006

Evolutionary divergence.—To estimate evolutionary divergence between sequence pairs, base substitutions per site between sequences were analyzed using the Tamura 3-parameter model (Tamura 1992) for the 43 full nucleotide sequences used in this study. After removing ambiguous positions, 723 aligned positions remained in the final data set. These were subjected to evolutionary analyses with MEGA7.0.14 (Kumar 2016), considering each species clade as a "group." TABLE 2 shows the average distances between individuals within each species, as well as between the species groups in the *P. multiplex* complex.

RESULTS

DNA sequencing.—We obtained partial LSU sequences from the holotype of *P. multiplex* and full ITS sequences from 33 additional specimens, including 17 from northeastern North America—the first sequences from the same region as the type collection of *P. multiplex*. Details of these specimens, as well as sequences used from GenBank and one unpublished sequence, are shown in TABLE 1.

Phylogenetic analysis.—The ML and BI analyses of the above sequences and an additional 10 from GenBank and

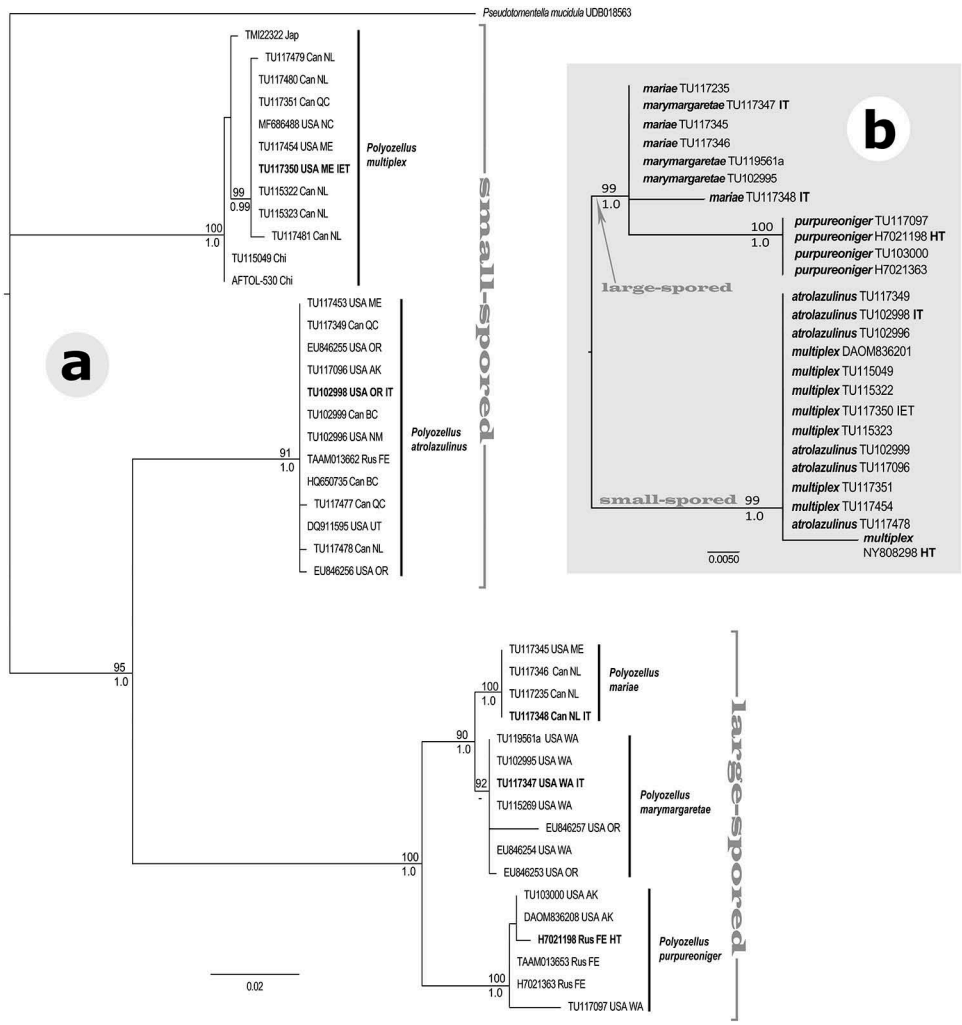

Figure 2. a. Best tree from the ML analysis of the ITS data set. Bootstrap values ≥80% and posterior probabilities ≥0.95 (from the BI analysis, which returned essentially the same tree topology as ML) are shown above and below branches, respectively. b. Reduced tree, showing placement of the 83-bp segment recovered from the holotype of *P. multiplex* (gray panel).

the unpublished sequence each returned five species-level clades within the group, with tree topology being essentially the same in both; FIG. 2a depicts the ML results and 2b the attempted placement of the holotype of *P. multiplex*. One clade is represented by a large number of small-spored specimens of *P. multiplex* sensu stricto from the forested part of northeastern North America from NL to North Carolina, as well as collections from China and Japan. A second clade consists of small-spored specimens from a broad geographic range and represents the new species *P. atrolazulinus*. It appears to be most common in the Pacific Northwest, from southern Alaska to Oregon, and in the Rocky Mountains as far south as New Mexico. It also occurs in eastern Asia, and northeastern North America, where it has been documented from Maine, Québec, and NL. The third clade comprises large-spored fruiting bodies from NL to Maine and represents the new species *P. mariae*. The fourth clade, sister to the third, includes large-spored specimens from the Cascade and Olympic mountain ranges in Oregon and Washington and represents the new species *P. marymargaretae*. The fifth clade consists of large-spored specimens extending from eastern Russia, across the Bering Strait to Alaska and Washington. This represents the new species *P. purpureoniger*.

Species hypothesis.—Calculation of the UNITE species hypotheses (SHs) for the 43 ITS sequences of *Polyozellus* specimens used in this study (TABLE 1) revealed four distinct groups at the 3% threshold: *P. multiplex, P. atrolazulinus, P. mariae*, and *P. purpureoniger* (FIG. 3). *Polyozellus marymargaretae* matches *P. mariae* at 3% and separates into a genetically distinct entity at similarity threshold values of 1% and less. *Polyozellus multiplex* collections from eastern Asia diverge from North American collections to form their own genetic group at the 0.5% threshold.

Evolutionary divergence.—Estimation of evolutionary divergence showed average intraspecific distances between collections to vary from 0.0% to 0.6%, whereas average interspecific distances varied from 1.1% to 12% (TABLE 2). The lowest average difference (1.1%) was observed between *P. mariae* and *P. marymargaretae*. TABLE 2 shows the average distances between individuals within each species, as well as between the species groups in the *P. multiplex* complex.

Morphology.—Mature fruiting bodies of each species may be differentiated by a combination of color,

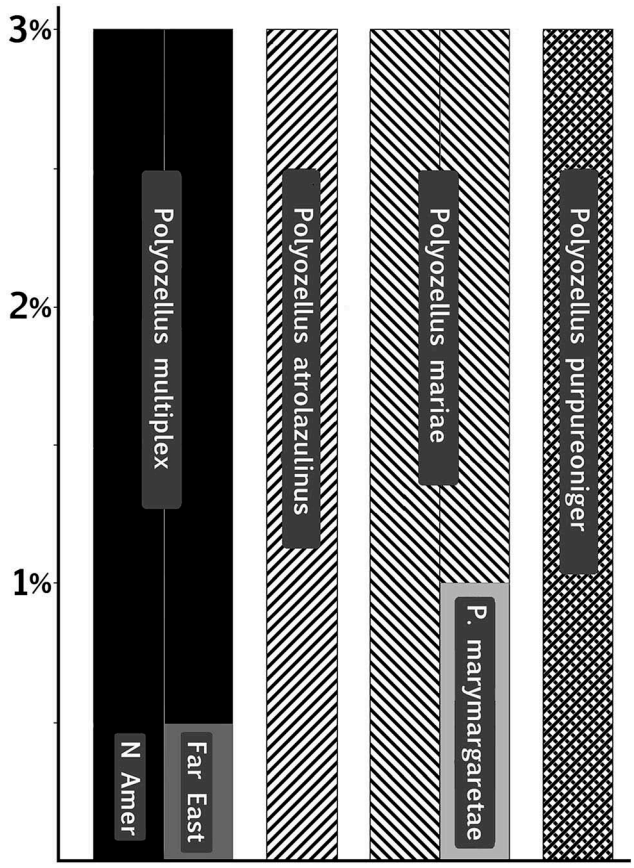

Figure 3. Diagrammatic representation of the UNITE species hypothesis (SH) alignments for *Polyozellus* ITS sequences at 3% and less base pair similarity (*y*-axis). Each column (*x*-axis) design indicates a distinct SH pattern.

texture, and pattern of the upper pileus surface, but because of great variation and overlap, differentiation by macroscopic appearance alone is not always possible. Basidiomes of all species change markedly during development (FIGS. 4, 5). All are woolly to hirsute in early development, and beyond maturity most become blackish with a smooth pileipellis. Although they readily divide into two small-spored species and three large-spored species (FIG. 6), overlapping spore sizes within each group make it impossible or very difficult to use this character as a further differentiating feature most of the time.

Distribution and ecology.—Species of the complex were documented primarily from the northern coniferous forest, with extensions to temporate montane or coastal regions, of North America and Asia, but not Europe (FIG. 7). *Polyozellus atrolazulinus* was documented throughout much of the range, *P. purpureoniger* from eastern Asia and northwestern North America, *P. multiplex* from

Figure 4. Small-spored species of the *Polyozellus multiplex* complex. A–C. *Polyozellus multiplex*. A. Mature specimen (TN2-216). B. Postmature specimen, showing blue cast that makes it difficult to distinguish from *P. atrolazulinus* (09.09.25.av01). C. Very young specimen, showing brown woolly hairs, not often seen with this species (16.09.18.av01). Photos: Henry Mann (A–C). D–F. *Polyozellus atrolazulinus*. D. Mature specimen showing obvious blue color (SAT-99-296-13). E. Immature specimen, showing brown pileal hairs, which persist much longer for this species (SAT-8-296-08). F. Mature specimen, more black than blue, making differentiation from *P. multiplex* difficult (15.09.08.av01). Photo: Jacques Landry. Bars: A, B = 10 cm; C = 3 cm; D, F = 5 cm; E = 15 cm.

eastern Asia and northeastern North America, and *P. mariae* and *P. marymargaretae* from northeastern and northwestern North America, respectively. All species seem to share the same ecological niche, growing as ectomycorrhizal associates of conifers. In one monitored area of NL, three species fruited in the same forest within hundreds of meters of each other. Combining macroscopic characters, spore size, and

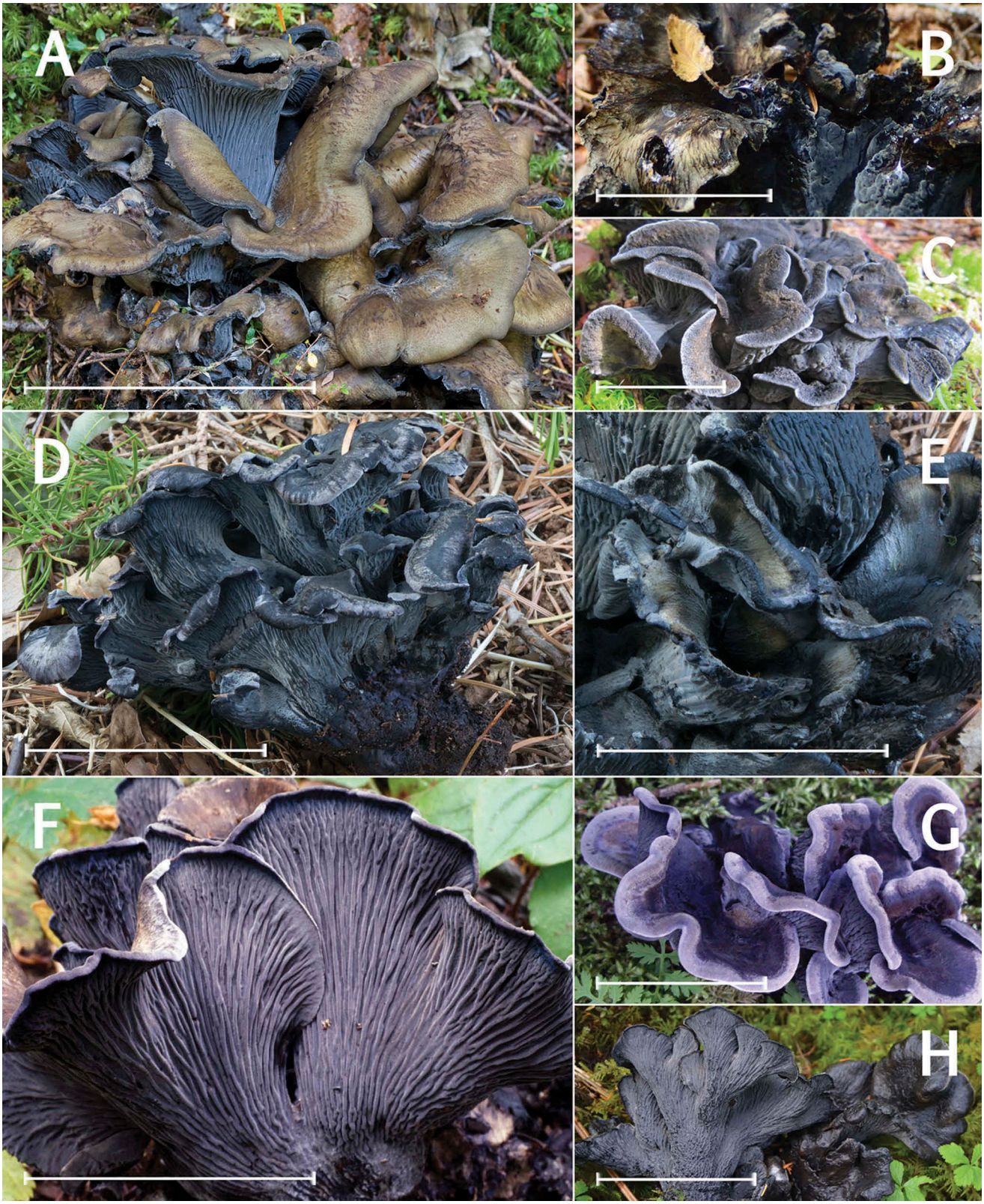

Figure 5. Large-spored species of the *Polyozellus multiplex* complex. A–C. *Polyozellus mariae*. A. Mature specimen (09.09.25.av02). B. Postmature specimen: even when the pileal fibrils and hymenium have turned black, the pileal base color remains tan and the flesh (not shown here) whitish (15.09.20.av01). C. Young specimen, showing brown woolly hairs (15.09.20.av01). D–E. *Polyozellus marymargaretae*. D. Mature specimen showing typical dark blue-violet color (01MWB091115). E. Older fruiting body from same collection, showing development of brownish matte pileal surface. Appearance remains distinct from its sister species, *P. mariae*. F–H. *Polyozellus purpureoniger*. F. Mature specimen with purplish hymenium and matte, brown hymenial surface (SAT-05-265-16). G. Young specimen showing florid purple coloration and lighter edge (VS4351). H. Older specimen, hymenium blackish purple and pileus blackish brown (AKFF-157-14). Photo: Alissa Allen. Scale bars: 5A = 15cm; 5B = 5 cm; 5C = 3.5 cm; 5D and E =10 cm; 5F =13 cm; 5G = 4 cm; 5H =15 cm.

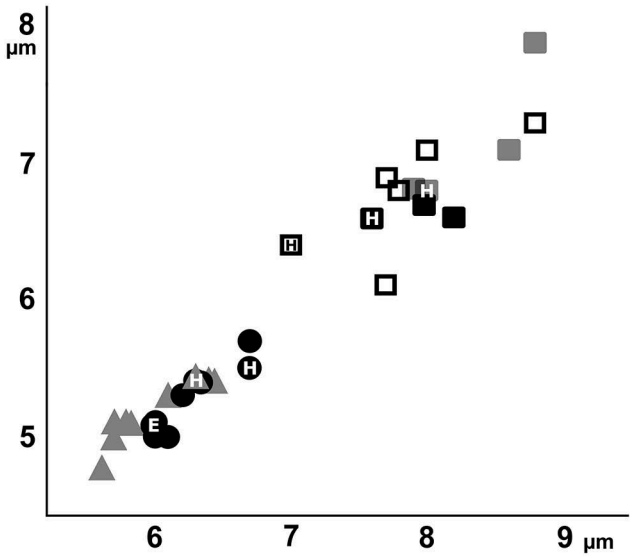

Figure 6. Average basidiospore size (minimum, 20 spores) of all sequenced *Polyozellus* collections. Black circles = *P. multiplex*; gray triangles = *P. atrolazulinus*; gray squares = *P. mariae*; open squares = *P. purpureoniger*; black squares = *P. marymargaretae*. H denotes holotype and E denotes epitype.

geographic location allows correct identification most of the time (see Key).

Type species typification.—The holotype for *P. multiplex* yielded no sequences from the ITS location and only an 83-bp segment of LSU. This allowed it to be placed among the small-spored species (FIG. 2b) but lacked the markers to determine in which of the two clades it belonged. The decision became one of judgment of maximal likelihood, estimated from the following observations:

(i) One clade was more common in the region than the other.
(ii) The protolog description of *P. multiplex* fitted better with the commoner clade.
(iii) The photo published in the protolog also fitted better with the macroscopic appearance of specimens from that clade.
(iv) The average spore size of the holotype of *P. multiplex* fell in the range for the commoner clade, but outside that of the hitherto measured range for the less common clade (FIG. 6).

From the above observations, we concluded that most likely *P. multiplex* belongs in the commoner clade, leaving the other small-spored clade as the new species *P. atrolazulinus*. Because the holotype for *P. multiplex* no longer yielded adequate DNA, a recent collection from the same region that yielded a successful ITS sequence is herein named an epitype for *Polyozellus multiplex*. We are satisfied that the epitype conforms with the description for *P. multiplex* and its microscopic morphology matches that of the holotype.

TAXONOMY

Polyozellus multiplex (Underw.) Murrill, North Am Flora 9:171. 1910. FIG. 4A–C
MycoBank MB124246, MBT377783

Typification: USA. MAINE: Mt. Desert Island, Seal Harbor. One of two basidiocarps in sandy soil, associated

Figure 7. World distribution of the *Polyozellus multiplex* complex. Only sequence-confirmed collections for which location known are shown. Not all collections seen because of overlap. The complex is found in the boreal belt, excluding Europe, with extensions into temperate regions at higher altitude. Black circles = *P. multiplex*; gray triangles = *P. atrolazulinus*; gray squares = *P. mariae*; black solid squares = *P. marymargaretae*; black open squares = *P. atropurpureus*.

with conifers, Aug 1898, leg. *Elizabeth W. Woodworth* (holotype NY Fung-677, bar codes 808296, 808297, 808298, 808300!). UNITE species hypothesis: SH215633.07FU. **Epitype** of *Polyozellus multiplex*, here designated: USA. MAINE: Sagadahoc County, near Elliottsville (45.3625°N, 69.3980°W, 236 m), single, under conifers, 20 Sep 2012, *Michaeline Mulvey* (NY2859132; **isoepitype** TU117350). GenBank/UNITE accessions: MB100830/UDB032576.

≡ *Cantharellus multiplex* Underw. (basionym), Bull Torrey Bot Club 26:254. 1899.

≡ *Craterellus multiplex* (Underw.) Shope, Mycologia 30:373. 1938.

≡ *Thelephora multiplex* (Underw.) Kawam., Icones of Japn Fungi 6:638. 1954.

= *Phyllocarbon yasudae* Lloyd [as "yasudai"], Mycol Writings 6(Letter 65):1066. 1920 [1921] (holotype, BPI723399!). MycoBank MB282241.

Basidiocarp 5–18+ cm high and 5–25 cm diam, imbricately foliose. Pilei multiple, complex, flabelliform to spathulate, sometimes infundibuliform, up to 7 cm long and 2–6 cm wide, tapering into stipe; surface tomentose in active growth, becoming matte, then smooth and shiny with irregular longitudinal ribbing, black to very dark purple, with variable bluish overtones, not concentrically zonate; margin slightly incurved, then flaring out, matte to tomentose, light gray to gray-white, turning darker at maturity. Hymenium composed of irregular, longitudinal, sinuous forking and anastomosing, decurrent folds, dark to light bluish gray. Stipe 2–5 cm long and 5–18 mm thick, tapering upwards, several often fused, forming a 4–9 cm diam subterranean base, solid, fibrous, matte to shiny, outer or lower surface covered with decurrent hymenium to near base, dark purple to black. Context soft, brittle, black to very dark purple throughout; odor chemical to fruity. Spore deposit white. Basidiocarps relatively resistant to decay and invertebrate damage (except by slugs) and last over a month in the field in good condition.

Basidiospores (240 spores, 12 basidiocarps, 9 collections) edge-to-edge, including nodules 4.8–7.7(–8.7) × 3.9–7.2 µm, average 6.3 × 5.3; Q = 1.0–1.5, average 1.2; subglobose to broadly elliptical, angular, lobed, with multiple nodules 0.5–1.5 µm high, and a prominent apiculus; hyaline, inamyloid, content homogeneous. Basidia 50–95 × 6–11 µm, 4-spored, clavate. Cystidia 3–5 µm wide, filiform, straight to irregularly sinuous, nodulose, irregularly cylindrical, equal or with subclavate apices, not extending beyond basidia. Hymenial hyphae irregular, nodulose, interwoven, with a dark bluish black incrusting pigment in the walls; produces a blue-greenish solution in KOH. Clamp connections in all tissues, but not at all septa.

Ecology and distribution: Fruits singly or in small groups on (often sandy) soil and conifer duff among mosses or fruticose lichens, under conifers, particularly *Abies, Picea*, and *Pinus* (the last with *Quercus*). One presumed individual has been observed to fruit in the same site 7 of the last 10 y. Recurrent annual fruiting is uncommon, although some specimens fruit on the same site for several years, sometimes more than 10. Relatively uncommon, in 1 of 14 y of monitoring, the species was found much more commonly and in locations where it had not been seen before or since. Fruits from the end of Aug to the beginning of Oct, most plentiful Sep. Documented in eastern North America from NL to North Carolina and in eastern Asia as far as southern China.

Notes: Polyozellus multiplex is the type species for the genus and, until now, has been applied to all collections of *Polyozellus*. It differs from *P. mariae, P. purpureoniger*, and *P. marymargaretae* by smaller basidiospores and a shiny black pileus surface. It is not as zonate or as blue as *P. atrolazulinus*, but variation of each is so wide that color alone may not always differentiate them. Phylogenetic analysis, UNITE SH, and evolutionary divergence calculation all show it to be genetically distinct from these other species. So far, it is not known from western North America. It is edible, but reports about taste vary. Like the other species, it is collected by craftspeople for dyeing natural fibers.

Other specimens examined: CANADA. NEWFOUNDLAND AND LABRADOR: Gros Morne National Park, trail to Stanleyville (49.4622°N, 57.7656°W, 58 m), on soil in moss under *Abies balsamea* and *Picea glauca*, 25 Sep 2009, *Maria Voitk, 09.09.25.av01* (DAOM836195, TU117352); Gros Morne National Park, trail to Stanleyville (49.4622°N, 57.7656°W, 58 m), on soil in moss under *Abies balsamea* and *Picea glauca*, 23 Aug 2010, *Maria Voitk, 10.08.23.av04* (DAOM836197, TU115323), and *Maria Voitk, 10.08.23.av07* (DAOM836196, TU115322); Pasadena, Ski Park trail (49.0033°N, 57.5931°W, 38 m), on soil among mosses under *Abies balsamea* and *Picea glauca*, 16 Sep 2016, *Henry Mann, AV coll. 16.09.18.av01* (DAOM836201, TU117481); Terra Nova National Park, Blue Pond trail (48.5908°N, 53.9383°W, 49 m), on soil in moss under *Picea glauca*, 29 Sep 2012, *Helen Spencer, FNL coll. TN1-408* (DAOM836199, TU117479); Terra Nova Town (48.5017°N, 54.1811°W, 100 m), on soil among fruticose lichens under *Picea glauca*, 27 Sep 2012, *Andrus Voitk, FNL coll. TN2-216* (DAOM836198, TU117480); QUEBEC: Saint-Léon-de-Labreque, Lac Saint-Jean (48.6733°N, 71.4853°W, 155 m), on soil among mosses under *Abies balsamea* and *Picea glauca*, 28 Aug 2013, *Guylaine Gagnon, AV coll. 13.08.28.av01*

(DAOM836200, TU117351). USA. MAINE: Penobscot County, Howland Experimental Forest (44.5339°N, 68.6497°W, 61 m), on soil in mixed conifer forest, 1999, *Bryan Dail* (Maine-E, TU117454); NORTH CAROLINA: Macon County, Highlands (35.0533°N, 83.1528°W, 800 m), on soil in mixed *Pinus-Quercus* forest, *Dolville & G. Bozdog, AMC091612* (TENN071088).

Polyozellus atrolazulinus Trudell & Kõljalg, sp. nov. FIG. 4 D–F

MycoBank MB821996

Typification: USA. OREGON: Willamette National Forest, Crescent Mountain trail (~44.4°N, ~122.1°W, ~1200 m), scattered on ground in mixed conifer forest with Douglas-fir (*Pseudotsuga menziesii*), Engelmann spruce (*Picea engelmannii*), western hemlock (*Tsuga heterophylla*), and western red cedar (*Thuja plicata*), 23 Oct 1999, *Steve Trudell, SAT-99-296-13* (**holotype** WTU-F-001763). **Isotype** TU102998. GenBank/UNITE accessions: MF100819/UDB023738. UNITE species hypothesis: SH028342.07FU.

Etymology: Atrolazulinus, "dark blue" in Latin, in reference to the typical color of the young basidiocarp.

Misapplied name: P. multiplex auct.

Diagnosis: A member of the genus *Polyozellus* by virtue of its macromorphology, pigmentation, subglobose nodulose hyaline spores, and ITS sequence. Differs from all other species in the genus by its ITS sequence phylogenetic analysis, UNITE SH, and evolutionary divergence calculation. Differs from *P. multiplex* principally by its blue to dark blue versus purplish black pileus surface, and a greater tendency for the pileus to be zonate; from *P. mariae* by its dark blue versus brownish coloration; from both *P. multiplex* and *P. mariae* by its prevalence in western North America; from *P. purpureoniger* by its bluish versus purplish coloration; and from *P. mariae, P. purpureoniger*, and *P. marymargaretae* by its smaller spores.

Basidiocarps 6–15+ cm high and 10–20+ cm diam, with multiple stipes and pilei. Pileus flabelliform to spathulate, sometimes infundibuliform, 3–10 × 2–8 × 0.1–0.5 cm, attenuated into the stipe; surface distinctly to indistinctly concentrically zoned with alternate bands of tomentum that disappear with age, surface then matte to glabrous, somewhat roughened, and faintly zonate, often radially ridged, dark purplish blue to deep or grayish blue, with moderate to light purplish blue zones (fresh colors generally within range 20–21/C–E/4–8, including "Berlin blue," "deep dull violaceous blue," "grayish violaceous blue," "dark dull violet blue," "slate violet," and "deep violet plumbeous"), soon violaceous black, then black in age; margin incurved when young, wavy or lobed in age, pubescent at first, later matte; pale purplish blue to whitish. Hymenium composed of sinuous folds or ridges, frequently forking and anastomosing, and at times forming a reticulate or almost poroid surface, sometimes nearly smooth over large areas or, more often, smooth near pileus margin; light to moderate grayish purplish blue (near 21A7). Spore deposit white. Stipe 3–5 × 0.5–2 cm, often irregularly compound, multiple stipes fused, converging to a common subterranean base, solid slightly roughened, upper portion covered with hymenium, dark purplish blue. Context soft brittle dark purplish blue, dark blackish green to olivaceous with KOH, azonate, odor nondistinctive or faintly pungent, taste mild. Fruiting bodies relatively resistant to decay and invertebrate damage (except by slugs) and can last over a month in the field in good condition.

Basidiospores (191 spores, 10 basidiocarps, 8 collections) including nodules 4.8–7.7 × 3.9–6.7 µm, average 6.1 × 5.3; Q = 1.0–1.7, average 1.2; subglobose to broadly elliptical, with multiple nodules 0.5–1 µm high and prominent apiculus; hyaline, inamyloid, content homogeneous. Basidia 30–70 × 5–10 µm, 4-spored, clavate. Cystidia 3–7 µm diam, filiform, straight to irregularly sinuous, irregularly cylindrical, tips even or subclavate, not extending beyond basidia. Hymenial hyphae irregular, nodulose, interwoven, with a dark bluish black incrusting pigment in the walls; produces a bluish-green solution in KOH. Clamp connections in all tissues, but not at all septa.

Ecology and distribution: In western North America, fruits on soil and conifer duff, often among mosses, under *Picea* (most commonly) and *Abies*, mainly Aug through Oct, depending in part on latitude and elevation, with earlier fruiting in the north and at high elevations. Widespread in montane areas of western North America and at lower elevations in northern areas such as southeast Alaska, generally not common, but can be abundant locally; documented from spruce-fir forests in northeastern North America as far as NL, and the Kuril Islands in eastern Asia.

Notes: Brilliant blue specimens are dramatic and unlikely to be confused with other species, but less colorful, or older, darkened, specimens can be difficult to differentiate from *P. multiplex*, which is so far not reported in western North America. Its smaller basidiospore size allows it to be distinguished from the other species known from western North America (*P. marymargaretae* and *P. purpureoniger*). *Polyozellus atrolazulinus* is edible, but opinions vary as to its desirability. Whether this is due to differences in personal preference or to differences in edibility among the previously unrecognized species is unknown. It can be used as a source of natural

dyes for fibers such as wool and silk, yielding violet, greens, and blues in a high-pH solution.

Other specimens examined: CANADA. BRITISH COLUMBIA: Manning Provincial Park, Lightning Lake trail (~49.1°N, ~120.8°W, ~1270 m), on soil in mixed conifer forest with Douglas-fir (*Pseudotsuga menziesii*), Engelmann spruce (*Picea engelmannii*), hemlock (*Tsuga heterophylla/mertensiana*), lodgepole pine (*Pinus contorta*), black cottonwood (*Populus trichocarpa*), and western redcedar (*Thuja plicata*), 12 Sep 2008, *Steve Trudell, SAT-08-256-08* (WTU-F-068937, TU102999); NEWFOUNDLAND AND LABRADOR: Gros Morne National Park, trail to Stanleyville (49.4644°N, 57.7758°W, 48 m), on soil in mixed conifer forest with *Abies* and *Picea*, 11 Sep 2017, *Maria Voitk, AV coll. 17.09.11.av04* (DAOM879656, TU117559); QUEBEC: Anticosti Island (49.5494°N, 62.9556°W, 180 m), on soil in conifer forest, 8 Sep 2015, *Herman Lambert, Anticosti 118* (DAOM836205, TU117477); Anticosti Island (49.5494°N, 62.9556°W, 180 m), on soil in conifer forest, 8 Sep 2015, *Jacques Landry* (DAOM836206, TU117478); Alma (48.5242°N, 71.5689°W, 128 m), on soil in moss under *Abies* and *Picea*, 15 Sep 2015, *Rachelle Simard, AV coll. 15.09.15.av01* (DAOM836207, TU117349). USA. ALASKA: Tongass National Forest, Petersburg Ranger District, Mitkof Island, Green's Camp (~56.5°N, ~132.7°W, ~5 m), clustered on ground in moss under Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*), 7 Sep 2010, *Steve Trudell, FNTF-10-250-21* (WTU-F-065677, TU117096); MAINE: Site unknown, 9 Sep 1986, *Richard Homola, Homola 8680* (MAINE-8680, TU117453); NEW MEXICO: Santa Fe National Forest, in fir-spruce (*Abies lasiocarpa, Picea engelmannii*) forest (~38.8°N, ~105.7°W, ~2700 m), 27 Aug 2006, New Mexico Mycological Society foray, collector unknown, *SAT-06-239-02* (WTU-F-068938, TU102996).

Polyozellus mariae Voitk & Kõljalg, sp. nov.

FIG. 5A–C

MycoBank MB821997

Typification: CANADA. NEWFOUNDLAND AND LABRADOR: Gros Morne National Park, trail to Stanleyville (49.4622°N, 57.7656°W, 58 m), on soil in moss under *Abies balsamea* and *Picea glauca*, 25 Sep 2009, *Maria Voitk, 09.09.25.av02* (**holotype** DAOM836202). **Isotypes** TU117348, NY2859131, FH01142406. GenBank/UNITE accessions: MF100831/UDB032173. UNITE species hypothesis: SH188416.07FU.

Etymology: Mariae pays tribute to Maria Voitk who, on finding the specimen designated herein as the holotype, called attention to its being different from previous collections of "*P. multiplex*," and thus provided the impetus for this study.

Misapplied name: P. multiplex auct.

Diagnosis: A member of the genus *Polyozellus* by virtue of its macromorphology, pigmentation, subglobose nodulose hyaline spores, and ITS sequence. It differs from *P. multiplex* and *P. atrolazulinus* by its brownish versus dark coloration, pale context, larger spores, restriction to eastern North America, and ITS sequence. It differs from *P. purpureoniger* and *P. marymargaretae* by its lack of blue or purple tones, pale context, and eastern North America distribution. Differs from all other species in the genus by its ITS sequence phylogenetic analysis, UNITE SH, and evolutionary divergence calculation; although these differences with *P. marymargaretae* are relatively small, they, together with significant differences in distribution and macromporphology, serve to separate these two species.

Basidiocarp 8–35+ high and 12–40+ cm diam, imbricately foliose. Pilei multiple complex flabelliform to spathulate, sometimes funnel-shaped, up to 18 cm long and 5–15 cm wide, tapering into a stipe arising from a 5–12 cm diam base; surface markedly downy during active growth, becoming matte and squamulose, with blackish longitudinal fibrils over a tan to light olive-brown base, which retains its tan-brown color in age that remains in the dried specimen; edge turned under, with a narrow, whitish-gray to light violet-blue margin in growth that becomes involute and darkened with age. Hymenium composed of irregular longitudinal sinuous forking and anastomosing decurrent folds, violet-gray to black in age. Stipe 3–7 cm long and 7–20 mm wide, widening upwards, several often fused to form a common subterranean base, solid, fibrous, scaly, outer surface covered with hymenium, inner surface black to dark brown. Context soft brittle cream to whitish—cross-section reveals distinct brown pileipellis above and black hymenium below the whitish context—white color persists in the dried specimen; odor mildly sweetish or unremarkable. Spore deposit white. Basidiomes relatively immune to decay or invertebrate damage (except by slugs) and last over a month in the field in good condition.

Basidiospores (170 spores, 8 basidiocarps, 5 collections) edge-to-edge, including nodules, 6.7–10.6 × 5.3–9.6 µm, average 8.3 × 7.0; Q = 1.0–1.4(–1.5), average 1.2; subglobose to broadly elliptical angular, lobed with multiple nodules 0.5–2 µm high, apiculus not well seen in most cases; hyaline, homogeneous, inamyloid. Basidia 70–115 × 8–12 µm, 4-spored, clavate. Cystidia 4-7 µm wide, segmented, filiform, straight to irregularly

sinuous, nodulose, irregularly cylindrical, tips even or subclavate, not extending beyond basidia. Hymenial hyphae irregular, nodulose, interwoven, with a dark bluish black incrusting pigment in the walls; produces a greenish solution in KOH. Clamp connections in all tissues, but not at all septa.

Ecology and distribution: Fruits singly or in small groups in conifer woods among moss and conifer duff, preferring sandy soil, under *Picea* and *Abies*. Recurrent fruiting in the same site has not been observed, except for one individual that has been observed to fruit in the same site five consecutive years. Fruiting from the middle of Aug to the middle of Oct, most plentiful Sep. Found in the same habitat and at the same time as *P. multiplex*, but much less common.

Notes: This is the only large-spored species in eastern North America, easily differentiated from the two small-spored ones by its larger size, light brown to tan pileipellis, and whitish flesh.

Other specimens examined: CANADA. NEWFOUNDLAND AND LABRADOR: Gros Morne National Park, Trout River campground (49.4592°N, 58.1241°W, 36 m), on soil in moss under *Abies balsamea* and *Picea glauca*, 4 Sep 2005, *Karole Pittman, GM5-491* (DAOM836203, TU117346); Humber Village, trail to Mt. Ignoble (48.9986°N, 57.7603°W, 206 m), on soil in moss under *Abies balsamea* and *Picea glauca*, 20 Sep 2015, *Maria Voitk, 15.09.20av01* (DAOM836204, TU117235). USA. MAINE: Sagadahoc County, Boudoin (44.1068°N, 69.9429°W), on sandy soil under white pine and hemlock, 19 Sep 2006, *Michaeline Mulvey* (NY2859130, TU117345).

Polyozellus marymargaretae Beug & I. Saar, sp. nov. FIG. 5 D–E

MycoBank MB821998

Typification: USA. WASHINGTON: Skamania County, Gifford Pinchot National Forest, Big Tire Junction (46.0984°N, 121.7221°W, 1080 m), on soil in moss under old growth *Abies grandis, Pseudostuga menziesii*, and *Picea engelmannii*, 11 Sep 2015, *Michael Beug, 01MWB091115* (**holotype** WTU-F-068939). **Isotype** TU117347. GenBank/UNITE accessions: MF100841/UDB033283. UNITE species hypothesis: SH094227.07FU.

Etymology: Marymargaretae pays homage to Mary Margaret "Maggie" Rogers, mushroom lover and lifelong resident of Washington and Oregon where this species is found. Maggie was cofounder of *Mushroom: The Journal of Wild Mushrooming* and is a lover of dye mushrooms.

Misapplied name: *P. multiplex* auct.

Diagnosis: A member of the genus *Polyozellus* by virtue of its macromorphology, pigmentation, subglobose nodulose hyaline spores, and ITS sequence. Differs from *P. multiplex* and *P. atrolazulinus* by its larger basidiospores and ITS sequence. Differs from *P. mariae* by its bluish versus brownish pileus coloration, deep bluish versus pallid flesh, and western versus eastern North American distribution, and from *P. purpureoniger* by its absence of purple tones. Differs from all other species in the genus by its ITS sequence phylogenetic analysis, UNITE SH, and evolutionary divergence calculation; although the differences with *P. mariae* are relatively small, they, together with significant differences in distribution and macromporphology, serve to separate these two species.

Basidiocarp 8–20+ high and 8–20+ cm diam, imbricately foliose. Pilei initially thumb-like, soon flat or depressed, and finally multiple complex flabelliform to spathulate, sometimes funnel-shaped, up to 10 cm long and 4–12 cm wide, tapering into a stipe arising from a 5–10 cm diam base; upper surface slightly downy during active growth, becoming matte, with finely fibrillose "straw yellow" hairs over a light blue (23A5) to dark blue (20D8) to whitish faintly banded surface, which turns deep blue-black (20F8) in age; edge turned under, whitish blue (23A5) margin when young, with blue (20D8), white, and "straw yellow" regions that becomes involute and darkened with age. Hymenium composed of irregular longitudinal sinuous forking and anastomosing decurrent folds, with a light blue (22B7) downy covering when young, turning blue-gray (20F8) to black in age. Stipe 3–8 cm long and 7–20 mm wide, widening upwards, several often fused to form a common subterranean base, solid fibrous scaly; surface lavender-blue (19B–C7) and dark blue (20D–F8) to black. Context soft, brittle, deep blue (20E–F8); odor mildly sweetish or unremarkable. Spore deposit white. Basidiomes relatively immune to decay or invertebrate damage and last over a month in the field in good condition.

Basidiospores (56 spores, 3 basidiocarps, 3 collections) including nodules 5.8–9.6 × 4.8–7.7 µm, average 7.9 × 6.7; Q = 1–1.5, average Q = 1.2; subglobose to broadly elliptical, with multiple nodules 0.5–1 µm high and prominent apiculus; hyaline, inamyloid, homogeneous. Basidia 30–70 × 5–9 µm, 4-spored, clavate. Cystidia 2–7 µm diam, filiform, straight to irregularly sinuous, irregularly cylindrical, tips even or subclavate, not extending beyond basidia. Hymenial hyphae irregular, nodulose, interwoven, with a dark bluish black incrusting pigment in the walls; produces a bluish-green solution in KOH. Clamp connections in all tissues, but not at all septa.

Ecology and distribution: To date, *P. marymargaretae* is known only from the Oregon and Washington Cascade Mountain Range and from the Olympic Mountain Range in Washington. It is uncommon, although locally abundant in three small, unusually moist old-growth mixed conifer patches in Skamania County, Washington, where it appears most years. It fruits with the first fall rains in late Aug to Sep through early Nov when snow falls.

Notes: To date, *Polyozellus marymargaretae* has been recorded only from Washington and Oregon. It is distinguished from two other *Polyozellus* species known to occur in the region by its light blue coloration when young (with olive-buff streaks and hints of lavender in the cap) and absence of both purple and violet colors. As a dye mushroom, it produces beautiful soft greens, blues, and violets ("lumière green," "elm green," "Biscay green," "robin's egg blue," "Indian purple," and "blackish brown."

Other specimens examined: USA. WASHINGTON: Skamania County, Gifford Pinchot National Forest, Steamboat Research Natural Area (~46.1°N, ~121.8° W, ~1200 m), on soil in mixed conifer forest with fir, spruce, and huckleberry, 15 Oct 1997, *Jan Lindgren, JEL-9729* (WTU-F-015164, TU102995); Clallam County, Olympic National Park, Deer Lake trail (~48.0°N, ~123.8°W, ~610 m), on soil under *Abies, Pseudotsuga, Tsuga*, 22 Sep 2005, *Brandon Matheny, PBM 2412* (WTU-F-015158, TU115269).

Polyozellus purpureoniger Spirin & I. Saar, sp. nov. FIG. 5F–H

MycoBank MB821999

Typification: RUSSIA. KHABAROVSK REGION: Solnechnyi District, Razlivnoi (51.0694°N 135.7030°E, 872 m), in a spruce forest, 24 Aug 2011, *Viacheslav Spirin, VS4351* (**holotype** H-7021198). GenBank/UNITE accessions: MF100842/UDB033284. UNITE species hypothesis: SH490341.07FU.

Etymology: Purpureoniger (Latin) refers to the purple-black color that distinguishes this species from the others in the genus.

Misapplied name: P. multiplex auct.

Diagnosis: A member of the genus *Polyozellus* by virtue of its macromorphology, pigmentation, subglobose nodulose hyaline spores, and ITS sequence. Differs from *P. multiplex* and *P. atrolazulinus* by its larger fruiting bodies, upper surface of the pileus purple in youth and brown in maturity, involute margin, lighter context, and larger spores. Differs from the large-spored *P. mariae* by its western North American/eastern Russian versus eastern North American distribution and from *P. mariae* and *P. marymargaretae* by its purple color and darker pileus. Differs from the latter also by its lighter context. Differs from all other species in the genus by ITS sequence phylogenetic analysis, UNITE SH, and evolutionary divergence calculation.

Basidiocarp 5–20+ high and 5–30+ cm diam, imbricately foliose. Pilei multiple, complex, flabelliform to spathulate, uncommonly funnel-shaped, up to 15 cm long and 5–15 cm wide, tapering into a stipe arising from a 2–10 cm diam base; surface markedly downy during active growth, zonate royal purple with a lighter edge, becoming matte and dry, streaked brown, darkening markedly with age but maintaining the lighter edge, eventually shiny dark brown to blackish; edge turned under, lighter during growth, darkening only at extreme senescence. Hymenium composed of irregular, longitudinal, sinuous, forking and anastomosing, decurrent folds, dark purple, becoming purplish gray to dark gray. Stipe 3–8 cm long and 7–20 mm wide, widening upwards, several, often fused, all cespitose, forming a common subterranean base, solid, outer surface covered with hymenium, inner surface dark brown. Context soft, brittle, straw to light gray; odor mildly sweetish or unremarkable. Spore deposit white. Basidiomes relatively immune to decay or invertebrate damage (except by slugs) and last over a month in the field in good condition.

Basidiospores (55 spores, 3 basidiocarps, 3 collections) including nodules 6.7–9.6 × 5.8–7.7 µm, average 8.0 × 6.9; average Q = 1.2 (excluding nodules 5.8–8.2 × 4.8–6.3 µm, average 6.8 × 5.6; average Q = 1.2); subglobose to broadly elliptical, lobed, with multiple nodules 0.5–2 µm high, apiculus present; hyaline, content homogeneous, inamyloid. Basidia 6.5–10.5 × 50–85 µm; 4-spored, with some 2-spored, clavate. Cystidia 3-6 µm wide, filiform, straight to irregularly sinuous, nodulose, irregularly cylindrical, tips even or tapering, not extending beyond basidia. Hyphae irregular, nodulose, interwoven, with a dark bluish black incrusting pigment in the walls; produces a bluish-green solution in KOH. Clamp connections in all tissues, but not at all septa.

Ecology and distribution: Fruits on both sides of the Bering Strait, recorded from the Khabarovsk Region of eastern Russia and Alaska and Washington in the USA.

Notes: Polyozellus purpureoniger is the only large-spored species in Asia, and one of two on the west coast of North America, where it can be distinguished from *P. marymargaretae* by its purple colors. This distinction becomes difficult once it has darkened toward black in the postmature stage. Like the other species, this is also a popular dye mushroom.

Other specimens examined: RUSSIA. KHABAROVSK REGION: Solnechnyi District,

Razlivnoi (51.0911°N 135.7240°E, 926 m), in a spruce forest, 22 Aug 2011, *Viacheslav Spirin, VS4302* (H7021363); SAKHALIN OBLAST: Yuzhno-Kurilsk District, Kuril Islands, Goryachi Plyazh (43.9948°N, 145.8014°E), under *Abies sachalinensis*, 30 Sep 1960, *Erast Parmasto* (TAAM013653). USA. ALASKA: Chugach National Forest, Cordova Ranger District, McKinley Lake trail (~60.5°N, ~145.2°W, ~22 m), on soil among mosses under Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*), 3 Sep 2009, *Steve Trudell, SAT-09-246-12* (WTU-F-010266, TU103000); Chugach National Forest, Cordova Ranger District, Pipeline Lakes trail (~60.5°N, ~145.3° W, ~20 m), on soil among mosses under Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*), 31 Aug 2014, *Noah Siegel, AKFF-157-14* (DAOM836208); WASHINGTON: Skagit County, North Cascades National Park, Easy Pass trailhead (~48.6°N, ~120.8°W, ~1130 m), on soil in mixed conifer forest with Douglas-fir (*Pseudotsuga menziesii*), silver fir (*Abies amabilis*), Engelmann spruce (*Picea engelmannii*), hemlock (*Tsuga heterophylla/mertensiana*), western redcedar (*Thuja plicata*), and red alder (*Alnus rubra*), 22 Sep 2005, *Steve Trudell, SAT-05-265-16* (WTU-F-001788, TU117097).

DISCUSSION

Our analyses indicate that what once was considered a single species, *P. multiplex,* actually represents a complex of five phylogenetic species, forming a monophyletic clade in the Thelephorales. Similar examples of previously unrecognized diversity from North America include *Amanita* "*muscaria*" (L.) Lam. (Geml et al. 2006, 2008), *Armillaria* "*mellea*" (Vahl) P. Kumm. (Anderson and Stasovski 1992), *Cantharellus* "*cibarius*" Fr. (Buyck et al. 2016), *Helvella* "*lacunosa*" Afzel. (Nguyen et al. 2013), "*Lepiota rachodes*" (Vittad.) Quél. (Vellinga 2003), and *Tricholoma* "*magnivelare*" (Peck) Redhead (Trudell et al. 2017).

Spore size separates the five species into a small-spored group of two and a large-spored group of three. Among the three large-spored species, *P. mariae* is readily identified, being the only large-spored species recorded from eastern North America. In Asia, the only reported large-spored species is *P. purpureoniger*. But in western North America, where the latter coexists with *P. marymargaretae*, separating the two may require molecular studies, if their respective purple and blue colors have darkened toward black. The average spore sizes of the three large-spored species overlap widely, and we have not examined enough specimens to make confident statements about the delimitation of the ranges for each. Similarly, differentiating between the two small-spored species, *P. multiplex* and *P. atrolazulinus*, is easy in western North America, where *P. multiplex* is not known. Where they coexist, morphological differences between species are readily apparent when large populations are studied, but for individual fruiting bodies, occasionally molecular studies may be required. The distinction is easy when *P. atrolazulinus* is bright blue, but if it has darkened, differentiating a very dark blue specimen from a blackish species with dark bluish tones can be problematic (compare FIG. 4B and F). Average spore sizes overlap over most of their combined range (FIG. 6), so that this character may help in those few that fall outside the range of the other at either extreme, but more sampling than done in this study will be required to define the full extent of these ranges with certainty.

These considerations became important in deciding which small-spored clade was represented by the holotype for *Cantharellus multiplex*, given that nuclear studies were unable to resolve the question. As outlined, identifying a single fresh fruiting body by morphological criteria can be difficult; doing so with certainty with a specimen dried 120 years ago is impossible. An estimate of highest likelihood based on circumstantial observations formed the basis of our decision. Although most likely in our opinion, our decision is admittedly arbitrary, and it is conceivable that future investigators, armed with better tools, may find the less likely choice to be correct.

UNITE SH analysis showed *P. mariae* and *P. marymargaretae* as separate genetic entities only at and below the 1% threshold, and evolutionary divergence analysis showed their mean difference to be 1.1%. This relatively small difference may bring their validity as distinct species into question. However, both taxa have significant differences in distribution (FIG. 7) and macromorphology (FIG. 5), as well as being well supported on phylogenetic analysis of the ITS region (FIG. 2a). Therefore, we have no hesitation to consider them separate species but describe *P. mariae* first, so that should greater differentiating thresholds be deemed necessary to separate species, *P. mariae* will have priority.

In the phylogenetic analysis, North American specimens form a well-supported subgrouping within the *P. multiplex* clade, somewhat apart from specimens from eastern Asia. The Asian sequences form a separate SH at the 0.5% threshold of the UNITE SH analysis. Such geographic variation is not surprising, given the distance between the Asian and eastern North American populations, separated by the Great Plains and Bering Strait on one side and Atlantic Ocean and Eurasia on

the other. Whether the Asian population of *P. multiplex* merits recognition as a separate species will require further study, including comparison of fresh material and analysis of additional DNA loci. This study used ITS only, but other loci have been recorded for *P. marymargaretae*, including three different copies of the translation elongation factor 1-α gene (*TEF1α*; Matheny et al. 2007, as *P. multiplex*), generally assumed to be present as a single copy.

There appears to be little difference in the general habitat in which the species occur—conifer forests, usually including spruce, often fir, and occasionally pine, at montane elevations in the southerly portions of the genus range and progressively lower elevations, down to sea level, farther north. All of the species are probably ectomycorrhizal based on their ecological occurrence and close relationship to other ectomycorrhizal taxa, such as *Hydnellum* P. Karst., *Pseudotomentella* Svrcek, *Sarcodon* Quél. ex P. Karst., *Thelephora*, and *Tomentella* Pers. ex Pat. We report species distributions based solely on sequence-confirmed identification. The occurrence of multiple species in the same general area, such as *P. multiplex-mariae-atrolazulinus* in eastern North America (and especially the very small area known in NL) and *P. atrolazulinus-purpureoniger-marymargaretae* in the Cascade Range in Washington, presents interesting ecological questions that cannot be resolved with current information. Hopefully, the recognition that there is more than one species of *Polyozellus* will provide an incentive to future collectors to record detailed macromorphological and habitat information, so that these sympatric occurrences can be explained.

PROVISIONAL KEY TO SPECIES OF THE *POLYOZELLUS MULTIPLEX* COMPLEX

This identification key represents our best effort at this time. None of the authors is familiar with all of the species, making comparison somewhat difficult. Our familiarity with some species is not exhaustive, and we may have overlooked some differentiating characters. We expect that the revised species concepts in the genus will lead to the discovery and report of more details of the morphology and overall distribution and ecology of this complex.

1. Spores average >7 µm long 2
1′. Spores average <7 µm long 4
2. Pileipellis brownish, context whitish, known from northeastern North America .....*P. mariae*
2′. Not as above (although brownish fibrils may be present and/or context may be somewhat light-colored) 3
3. Basidiocarps with bluish coloration, especially when young, known from montane forests of Washington and Oregon .....*P. marymargaretae*
3′. Basidiocarps with purplish coloration, known from montane and lower elevation forests of northwestern North America and eastern Asia..... *P. purpureoniger*
4. Basidiocarps with bright bluish coloration, at least when young, pilei often zonate, occurs widely in western North America, also in northeastern North America and eastern Asia.....*P. atrolazulinus*
4′. Basidiocarps black, lacking bright bluish coloration, pilei usually not zonate, occurs in eastern North America and eastern Asia.....*P. multiplex*

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