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A molecular and morphological analysis of the genus *Rhizopogon* subgenus *Villosuli* section *Villosuli* as a preface to ecological monitoring

Ryan Bubriski
Peter Kennedy[1]
Department of Biology, Lewis and Clark College, 0615 SW Palatine Hill Rd., Portland, OR 97219

Abstract: *Rhizopogon* (Boletales) represents a model genus for ecological studies of ectomycorrhizal fungi, but the identification of species in subgenus *Villosuli* section *Villosuli* has long been challenging due to variation in taxonomically informative morphological characters. Here we re-examine species concepts in this section using data from nuclear ribosomal internal transcribed spacer (ITS) and large subunit (LSU) sequences as well as spore measurements. Our phylogenetic analyses from 34 type and non-type collections of eight species, including the seven currently recognized in this section, found consistent support for only three species-level clades. Each of the clades had a significantly different combination of mean spore widths and Q ratios using digital-assisted measurements, which suggests both molecular and spore-based morphological approaches can be used to identify species in this section. Based on our analysis, we propose that only three species names be applied to future ecological studies: *R. hawkerae*, *R. parksii* and *R. villosulus*. We consider *R. subareolatus* and *R. colossus* as taxonomic synonyms of *R. hawkerae* and *R. pseudovillosulus*, *R. rogersii*, *R. villescens* and *R. zelleri* as taxonomic synonyms of *R. villosulus*.

Key words: ecology, ITS, LSU, *Rhizopogon*, spore size, taxonomy, *Villosuli*

Introduction

Rhizopogon (Basidomycota: Boletales) is a genus of truffle-forming fungi that establish ectomycorrhizal symbioses with a wide range of tree species in the family Pinaceae (Molina et al. 1999). Members of the genus are most common and abundant in the pine forests of western USA but also are native to other geographic regions and continents (Dowie et al. 2011, Mujic et al. 2013). Early studies suggested that *Rhizopogon* consisted of a limited number of species (Zeller 1941), but a detailed monograph by Smith and Zeller (1966) dramatically increased the number of *Rhizopogon* species to 117 in North America alone. Since that taxonomic revision, a number of other additional species have been described in USA and elsewhere (Smith 1966, 1968; Harrison and Smith 1968; Trappe and Guzmán 1971; Hosford 1975; Cázares et al. 1992; Allen et al. 1999; Grubisha et al. 2005; Mujic et al. 2013). Currently the genus comprises more than 250 named species (MycoBank 2013).

Due to the combination of frequent and abundant sporocarp production, spores that readily germinate in the presence of host roots, distinctive ectomycorrhizal root tip morphologies, associations with mycoheterotrophic plants and a major role in the fire-associated successional dynamics of western USA forests, *Rhizopogon* has become a model genus for ecological studies of ectomycorrhizal fungi (Molina et al. 1999, Taylor and Bruns 1999, Bidartondo and Bruns 2002, Kretzer et al. 2003, Kjøller et al. 2003, Parlade et al. 2004, Rusca et al. 2006, Bruns et al. 2009, Kennedy 2010). Those studies, however, have been predicated on clear taxonomic delineations among *Rhizopogon* species. Kretzer et al. (2003) provides an excellent example. Using ITS and microsatellite sequence data, the authors determined that *R. vinicolor*, in the subgenus *Villosuli* section *Vinicolores*, actually was composed of two nearly morphologically identical species, *R. vinicolor* and *R. vesiculosus* (see Luoma et al. (2012), for morphological distinctions between these species). A number of subsequent investigations of these two *Rhizopogon* species have shown that they differ in many ecologically significant ways, including genet size (Kretzer et al. 2005), frequency of mycorrhizal network formation (Beiler et al. 2010) and depth of soil inhabitation (Beiler et al. 2012).

Based on morphological characteristics, Smith and Zeller (1966) divided *Rhizopogon* into two subgenera: *Rhizopogonella* and *Rhizopogon*, with *Rhizopogon* made up of four sections, *Amylopogon*, *Fulviglebae*, *Rhizopogon* and *Villosuli*. Trappe (1975) subsequently transferred subgenus *Rhizopogonella* to genus *Alpova*. Using nuclear ribosomal internal transcribed spacer (ITS) region sequences, Grubisha et al. (2002) redefined the subgeneric classifications of *Rhizopogon*. Their phylogenetic analysis resulted in the

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[1] Corresponding author. E-mail: kennedyp@umn.edu
[2] Current address: Department of Plant Biology and Department of Ecology, Evolution and Behavior, University of Minnesota, 1445 Gortner Ave., St Paul, MN 55108.

TABLE I. Collections included in this study. All species were described in Smith and Zeller (1966) with the exception of *R. Villosulus*, which was described in Zeller (1941)

Species	Collection	Type	Collection location/date	ITS accession	LSU accession
R. colossus var. *colossus*	MICH 2904	paratype	Multnomah Co., OR; unknown	KC306752	KF305545
R. colossus var. *colossus*	MICH 2932	paratype	Del Norte Co., CA; 1939	—[a]	NA
R. colossus var. nigromaculatus	MICH 5444	holotype	Yakima Co., WA; 1962	—	NA
R. hawkerae	MICH 3220	paratype	Pend Oreille Co., WA; 1964	—	NA
R. hawkerae	MICH 3250	paratype	Wasco Co., OR; 1964	—	NA
R. hawkerae	MICH 5455	holotype	Valley Co., ID; 1962	KC306757	—
R. hawkerae	OSC 117402	non-type	Lane Co., OR; 2001	KC306746	KF305538
R. hawkerae	OSC 116174	non-type	Lewis Co., OR; 2000	KC306762	KF305536
R. hawkerae	OSC 116321	non-type	Benton Co., OR; 2000	KC306745	—
R. parksii	MICH 0451	paratype	Skamania Co., WA; 1963	KC306761	KF305544
R. parksii	MICH 3706	paratype	Multnomah Co., OR; 1964	KC306756	KF305546
R. parksii	MICH 12403	isotype	Humboldt Co., CA; 1966	—	NA
R. parksii	OSC 130792	non-type	Lane Co., OR; 2003	KC306747	KF305539
R. pseudovillosulus	OSC 104676	non-type	Klamath Co., OR; 2000	KC306763	KF305535
R. pseudovillosulus	OSC 40160	non-type	Benton Co., OR; 1980	KC306764	—
R. rogersii	MICH 549	paratype	Bonner Co., ID;1964	KC306758	—
R. rogersii	OSC 44320	paratype	Bonner Co., ID;1964	—	NA
R. rogersii	OSC 58551	non-type	Thurston Co., WA; 1994	KC306748	KF305540
R. rogersii	OSC 61538	non-type	Clackamas Co., OR; 1998	KC306749	KF305534
R. subareolatus	MICH 12414	holotype	Clackamas Co., OR; 1944	KC306770	—
R. subareolatus	OSC 116323	non-type	Douglas Co., OR; 2000	KC306765	KF305537
R. subareolatus	OSC 80252	non-type	Coos Co., OR; 1999	KC306766	KF305543
R. villescens	MICH 5513	holotype	Bonner Co., ID;1964	KC306759	KF305548
R. villescens	OSC 63499	non-type	Siskiyou Co., CA 1983	KC306750	—
R. villescens	OSC 80073	non-type	Clackamas Co., OR; 1999	KC306767	KF305542
R. villosulus	MICH 5233	paratype	Idaho Co., ID;1964	—	NA
R. villosulus	MICH 5250	paratype	Bonner Co., ID;1964	KC306753	KF305547
R. villosulus	MICH 5256	paratype	Valley Co., ID; 1958	KC306751	—
R. villosulus	OSC 79870	non-type	Benton Co., OR; 2000	KC306769	—
R. villosulus	OSC 79818	non-type	Clearwater Co., ID; unknown	KC306768	KF305541
R. zelleri	MICH 5884	paratype	Adams Co., ID; 1958	KC306760	KF305549
R. zelleri	MICH 5885	paratype	Valley Co., ID; 1958	KC306754	—
R. zelleri	MICH 5886	paratype	Adams Co., ID; 1962	KC306755	KF305550
R. zelleri	MICH 12419	isotype	Multnomah Co., OR; 1944	—	NA

[a] Em dashes indicate unsuccessful sequencing and NA indicates that sequencing was not attempted.

delineation of five subgenera (*Amylopogon*, *Fulviglebae*, *Rhizopogon*, *Roseoli*, *Villosuli*), with subgenus *Villosuli* being separated into two sections, *Vinicolores* and *Villosuli*. Species in subgenus *Villosuli* differ from the other subgenera in associating exclusively with species of *Pseudotsuga*, and they are the only *Rhizopogon* species that associate with the genus *Pseudotsuga* (Grubisha et al. 2002).

Alexander Smith readily acknowledged that there was ''considerable intergradation'' among *Rhizopogon* species in the subgenus *Villosuli* section *Villosuli* and that further taxonomic work was required (Smith and Zeller 1966). Suspecting that species names were liberally applied to sect. *Villosuli* and that species names actually may reflect different developmental stages of a single species, Martín et al. (1998) attempted to characterize the relationships among species in this section. Those authors did not find any differences in the ITS region derived from type material of eight species, which supported the hypothesis that they all belonged to one species, *R. villosulus*. However, their study relied on restriction fragment length polymorphism (RFLP) analysis, which severely limited the amount of sequence variation they were able to examine.

In this study we revisited the taxonomic designations of *Rhizopogon* species in subgenus *Villosuli* sect. *Villosuli* as a preface to studying their ecology in the native *Pseudotsuga menziesii* forests of western USA as well as other geographic areas where this host species has been introduced. We used an approach similar to Kretzer et al. (2003), in which our goal was to assess

more thoroughly the genetic variation among species designated in this section. We focused on the seven species identified by Grubisha et al. (2002) as belonging to section *Villosuli* as well as *R. pseudovillosulus* (due to its morphological similarity with *R. villosulus*). To assess the validity of species concepts in this section, we generated rDNA ITS and ribosomal large subunit (LSU) sequences from type and non-type collections and assessed their phylogenetic relatedness with maximum likelihood analyses. We also measured the spore size characteristics for the majority of the collections and assessed how they correlated with sequence-based results.

MATERIALS AND METHODS

Collections.—We obtained 34 type and non–type dried sporocarp collections from the Oregon State University (OSC) and University of Michigan herbariums for molecular and morphological analysis (TABLE I). The collections were made 1939–2003, with the median collection age being 1964.

DNA extraction, PCR amplification and sequencing.—DNA extractions on all collections initially were carried out using the REDE Extract-N-Amp tissue kit following manufacturer's instructions (Sigma-Aldrich, St Louis, Missouri). Due to successful amplification of only the more recent collections with this method (data not shown), we re-extracted DNA from all type collections with the DNeasy Plant Mini Kit following manufacturer's instructions (QIAGEN, Venlo, the Netherlands). Before using the DNeasy method, 15 mg tissue from each type collection and four glass beads were placed into individual 1.5 mL tubes and shaken in a bead beater (Spex SamplePrep, Metuchen, New Jersey) 15–20 min until the samples were ground to a powder. Because collections were made as early as 1939, we applied three different polymerase chain reaction (PCR) kits to attempt to achieve maximum amplification success: MasterAmp (Illumina Inc., San Diego, California), PureTaq Ready-To-Go Beads (GE Healthcare, Cleveland, Ohio) and REDExtract-N-Amp (Sigma-Aldrich, St Louis, Missouri). To amplify the ITS region, we first used the primer pair ITS1F (5′–CTTGGTCATTTAGAGGAAGTAA–3′ and ITS4 (5′ –TCCTCCGGCTTATTGATATGC–3′) on all samples. If PCR from that primer set failed, we used the primer pairs ITS1F and ITS2 (5′–GCTGCGTTCTTCATCGATGC –3′) or ITS-3 (5′–GCATCGATGAAGAACGCAGC–3′) and ITS4 to amplify the ITS1 and ITS2 regions individually. Thermo-cycler settings were: (i) 94 C for 2 min, (ii) 58 C for 1 min, (iii) 72 C for 1 min, return to step one 34 times, (iv) 72 C for 10 min.

To supplement the results generated from the ITS sequence data, which has been the primary gene region for species-level *Rhizopogon* taxonomy (Grubisha et al. 2002, Kretzer et al. 2003, Grubisha et al. 2005, Martin and Garcia 2009), we targeted four additional genes: transcription elongation factor 1 alpha (*tef*-1α), ribosome polymerase subunit 1 (RPB1), ribosome polymerase subunit 2 (RPB2) and ribosomal large subunit (LSU). To amplify *tef*-1α, we used these primer pairs: EF1-526 and 1567R, EFdf and EF1-2218R, and 983F and 1953R (see Matheny et al. 2007 for all primer sequences). To amplify RPB1, we used the primer pair gRPB1-A and fRPB1-C (5′–CNGCDATNTCRTTRTCCATRTA–3′) (Matheny et al. 2002). To amplify RPB2, we used the primer pair fRPB2–5F (5′–GAYGAYMGWGATCAYTTYGG–3′) and bRPB2-7R2 (5′–ACYTGRTTRTGRTCNGGRAANGG–3′) (Matheny et al. 2007).

For congruency between the analyses, we only attempted to amplify LSU DNA from collections for which we successfully generated ITS sequences. We used the primer pair LROR (5′–CCGCTGAACTTAAGCATATCAATA–3′) and LR-F (5′–CGATCGATTTGCACGTCAGA–3′), with the same PCR reagent combinations for the ITS sequencing. Thermo-cycler settings were: i. 94 C for 3 min, ii. 95 C for 1 min iii. 58 C for 1 min, iv. 72 C for 1 min, return to step two 34 times, v. 72 C for 7 min. We checked ITS and LSU PCR reactions for success on 1.5% agarose gels stained with SYBRSafe (Life Technologies, Grand Island, New York). All successful reactions were purified with Exo-SAP IT (Affymetrix, Cleveland, Ohio) following manufacturer's instructions and sent for sequencing in both 5–3′ and 3–5′ directions at the University of Arizona Genetics Core Center.

Sequence editing.—We analyzed sequence chromatograms using with Sequencher 5.1 (Gene Codes Corp., Ann Arbor, Michigan). We first assembled sequences from the same collection and then made any manual corrections based on the partner sequence with the highest clarity. Any sequences without a successful partner sequence were edited by visually assessing chromatogram peak accuracy, with the standard base ambiguity code to define ambiguous peaks.

Phylogenetic analysis.—For the ITS sequences, we created two separate alignments, one with sequences containing the full ITS gene region (i.e. ITS1, 5.8S, ITS2) and one with sequences containing only ITS2. We did this because we could obtain only an ITS2 sequence for the holotype collection of *R. subareolatus* (see below). For both ITS alignments, in addition to the sequence data generated for this study, we included 9 sequences of species in section *Villosuli* from Grubisha et al. (2002). Similarly, we supplemented our LSU dataset with three additional sequences from Grubisha et al. (2001). We created ITS and LSU alignments with the MUSCLE algorithm (Edgar 2004) in the MEGA 5.0. We visually checked each alignment for accuracy and deleted all positions that contained an unknown nucleotide in one of the sequences. In addition we trimmed each alignment so that all sequences had the same total number of bases. The three alignments can be accessed at TreeBASE.org (submission ID 14437).

For phylogenetic reconstructions, we examined each dataset (i.e. full ITS, ITS-only and LSU) with maximum likelihood (ML) algorithms. For those analyses we used the Jukes-Cantor model of evolution, which was determined to be the best model for this dataset in MEGA. To root the tree, we included an ITS sequence of *R. vinicolor* shown to be within the subgenus *Villosuli* but outside section *Villosuli* (Grubisha et al. 2002). In all three ML analyses, we used 1000 bootstrap replications to assess support for the resulting clades.

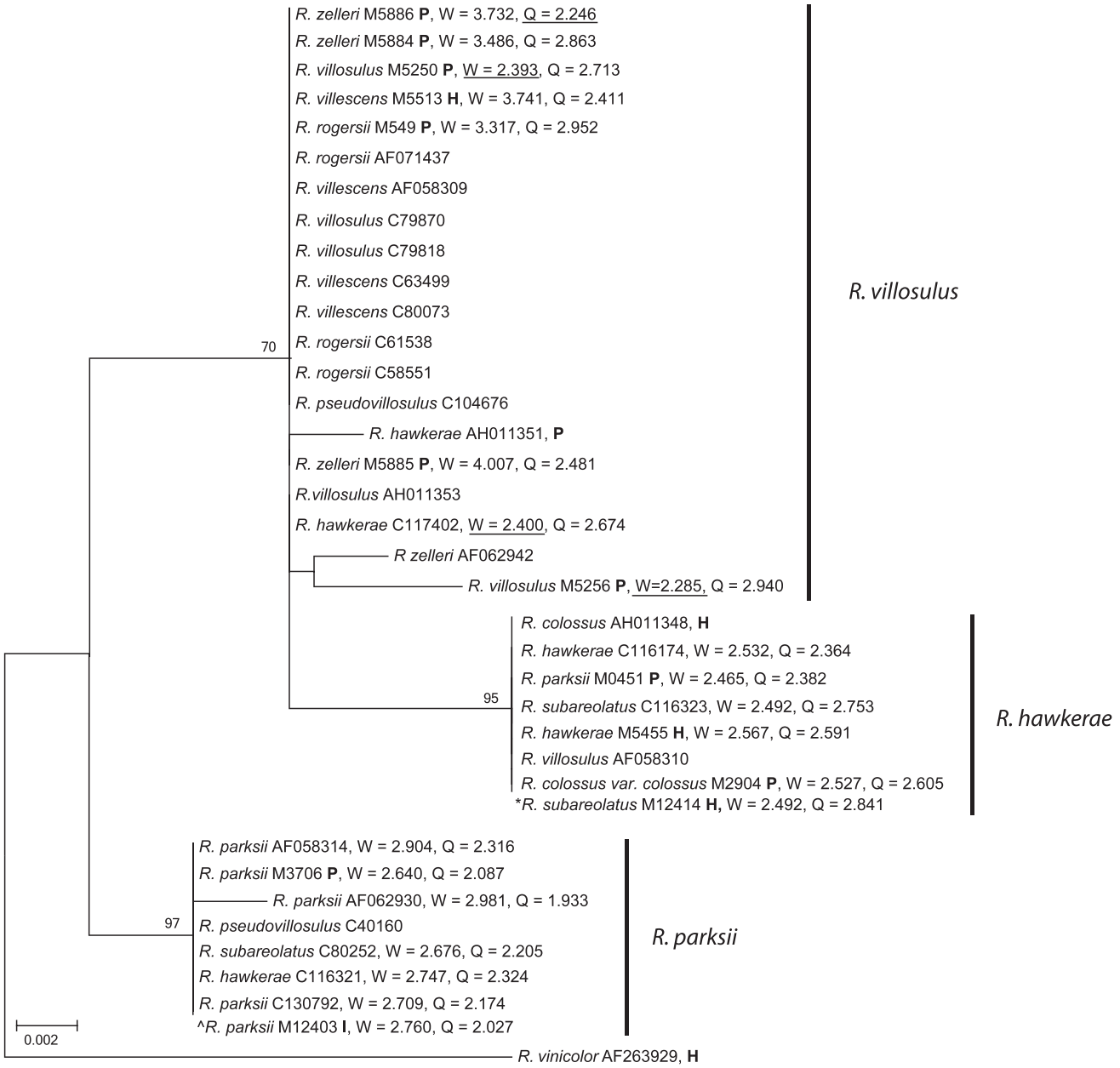

FIG. 1. A maximum likelihood (ML) phylogenetic reconstruction of the *Rhizopogon* subgenus *Villosuli* section *Villosuli* based on full ITS region (i.e. ITS1, 5.8S, ITS2) sequences from 34 collections. The dataset consisted of 412 positions and included 31 phylogenetically informative characters. Numbers above branches indicate consensus bootstrap support (%), with only values greater that 60 presented. Collections are labeled with collection numbers preceded by either M or C, for University of Michigan and Oregon State University herbariums, respectively. Previously determined sequences are labeled with accession numbers instead of collection numbers. H or P follows holotype and paratype collection numbers, respectively. Mean spore widths and Q values are presented for individual collections based on 50 spore measurements per collection. The tree was rooted with a previously determined ITS sequence of the *R. vinicolor* holotype collection. Collections marked with * indicates placement based on ITS2-only analysis and ^ indicates placement based on spore size data.

Morphological analysis.—To assess how spore size characteristics correlated with the results of the sequence analyses, we measured the width and length of 50 spores from 6–8 collections within each major ITS clade. Hand sections of gleba tissue from each collection were mounted on slides with 5% KOH solution and examined using a compound light microscope. Three pictures per collection were taken at 1000× when each field of view contained ~ 30–70 spores. We then uploaded these pictures, along with a picture of a slide micrometer, to the freeware program ImageJ (http://rsb.info.nih.gov/ij/). We used a slide micrometer to quantify the number of pixels per micron and used this

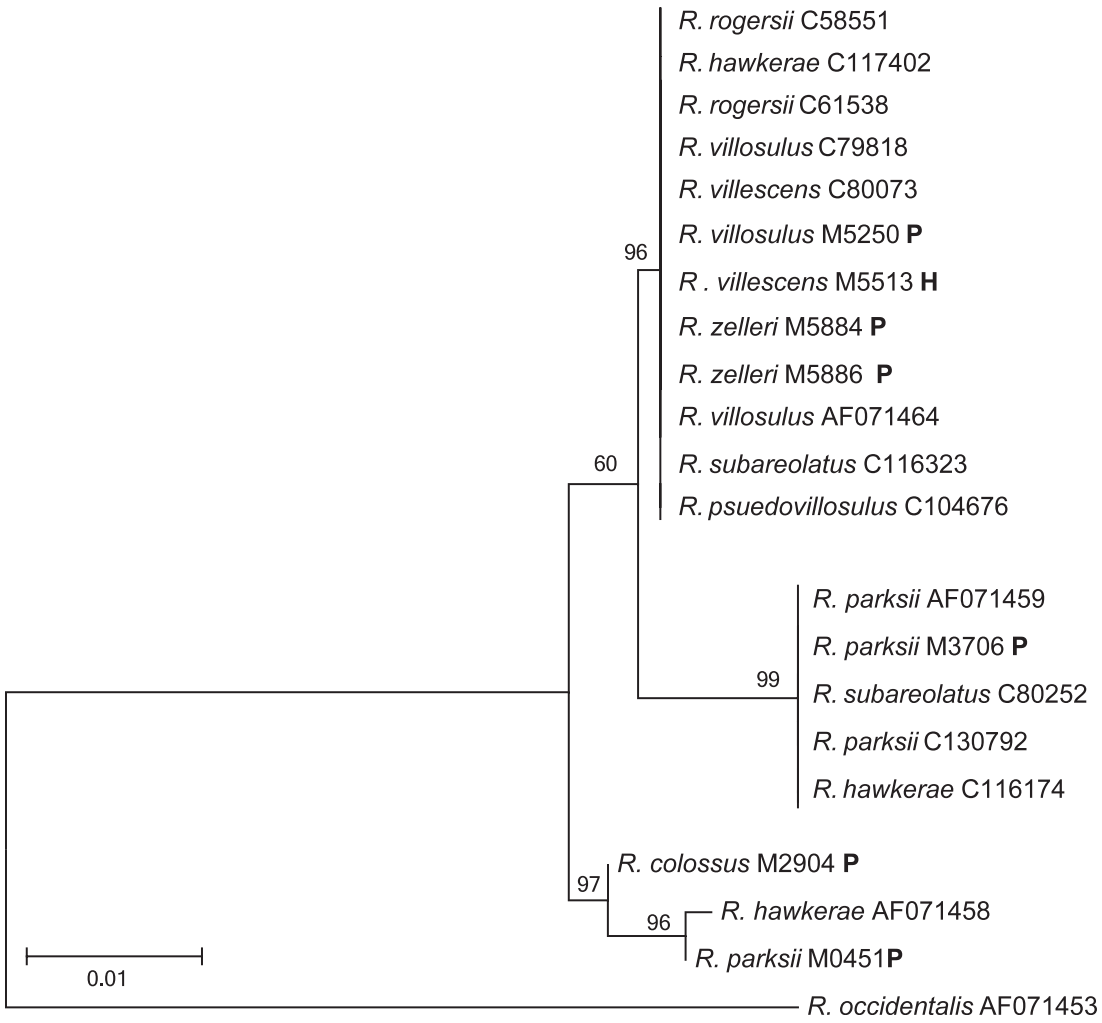

FIG. 2. A maximum likelihood (ML) phylogenetic reconstruction of the *Rhizopogon* subgenus *Villosuli* section *Villosuli* based on LSU gene sequences from 21 collections. The dataset consisted of 680 positions and included 15 parsimony informative characters. Numbers above branches indicate consensus bootstrap support (%), with only values greater that 60 presented. H or P follows holotype and paratype collection numbers, respectively. The tree was rooted with a previously determined LSU sequence of a *R. occidentalis* collection.

scale to measure spore dimensions from the collection images. Because this program allows measurements at the nanometer scale, whereas compound microscopy allows measurements only at the micrometer scale, we carried out two types of analyses. The first used the 50 exact values determined with ImageJ (i.e. analysis based on the nanometer scale). The second involved rounding the same 50 values to the nearest micrometer (i.e. analysis based on the micrometer scale). For both analyses we tested for differences in mean spore widths and length:width (Q) ratios between the clades determined from the phylogenetic analysis with one-way ANOVAs in SPSS 20 (IBM, Armonk, New York). Specific differences between the clades means were determined with post-hoc Tukey tests.

RESULTS

We successfully obtained 26 new ITS sequences, including sequences for 12 holo- and paratype collections (TABLE I), from the 34 collections from which sequencing was attempted (TABLE I). For eight of the type collections, we were not able to obtain an ITS sequence despite attempts with three different PCR systems. While we obtained the full ITS region (ITS 1, 5.8S, ITS 2) for 25 out of 26 collections, we were able only to determine the sequence of the ITS2 region for collection MICH 12414, the *R. subareolatus* holotype.

The phylogenetic analyses examining both the full ITS and ITS2-only regions indicated the presence of three clades, which each contained collections involving multiple species names (full ITS: FIG. 1, ITS2 only: SUPPLEMENTARY FIG. 1). We found that the clade containing the holotype collections of both *R. hawkaerae* and *R. colossus* as well as *R. subareolatus* in the ITS2-only analysis was strongly supported in

TABLE II. Spore-size characteristics for the species-level clades determined in the phylogenetic analyses

	R. hawkerae	*R. parksii*	*R. villosulus*
Exact measurement			
Mean width (μm)	2.466 a	2.774 b	3.170 c
Standard error (μm)	0.013	0.012	0.037
Standard deviation	0.235	0.229	0.742
Clade designation (μm)	< 3	< 3	> 3
Mean Q	2.550 a	2.157 b	2.660 c
Standard error	0.019	0.014	0.018
Standard deviation	0.344	0.266	0.361
Clade designation	> 2.35	< 2.35	> 2.35
Collections conforming to Clade designations	6/6	7/7	4/8
Rounded measurement			
Mean width (μm)	2.460 a	2.880 b	3.100 c
Standard error (μm)	0.029	0.017	0.043
Standard deviation	0.505	0.324	0.866
Clade designation (μm)	< 3	< 3	> 3
Mean Q	2.610 a	2.080 b	2.670 a
Standard error	0.028	0.014	0.023
Standard deviation	0.488	0.271	0.478
Clade designation	> 2.35	< 2.35	> 2.35
Collections conforming to clade designations	4/6	6/7	4/8

both datasets (full ITS bootstrap: 97; ITS2-only bootstrap: 90). A second clade containing the majority of the *R. parksii* collections also had strong bootstrap support in the full ITS analysis (90) but considerably lower support in ITS2-only analysis (63). Both ITS datasets showed moderate support for the most diverse clade, which included the holotype collection of *R. villescens*, two paratypes of *R. villosulus* and one paratype of *R. zelleri*, *R. rogersii* and *R. hawkarae* respectively (full ITS bootstrap: 70; ITS2-only bootstrap: 66). The relative positioning of the three clades also differed between the full ITS and ITS2-only datasets. In the full ITS analysis the clade containing the majority of *R. parksii* collections was basal to the other two clades, while in the ITS2-only analysis the clade containing the *R. hawkerae*, *R. colossus* and *R. subareolatus* holotypes was basal. The ML analysis of the LSU dataset was highly congruent with those based on ITS sequences, with evidence for the same strongly supported three species-level clades (> 95 bootstrap support for each clade) (FIG. 2). The relative clade positioning from the LSU analysis matched that of the ITS2-only analysis.

Using exact spore size measurements (i.e. nanometer scale), we observed significant differences in both mean widths ($F_{2,1047} = 178.71$, $P < 0.001$) and Q ratios ($F_{2,1047} = 251.77$, $P < 0.001$) across the three clades identified in the phylogenetic analyses. Post-hoc tests found significant differences among all three individual clades for both width and Q ($P < 0.05$). (No differences were observed when these analyses were run with and without the *R. subareolatus* holotype collection M12414 and the *R. parksii* isotype collection M12403.) While most of the collections within each clade matched that clade's spore size designations (TABLE II), four collections in the more diverse third clade fell slightly outside the designated sizes (FIG. 1, TABLE II). Using the rounded size measurements (i.e. micrometer scale), we again observed significantly different mean widths across all three clades ($F_{2,1047} = 91.517$, $P < 0.001$). For Q values, however, we found that the clade containing the majority of the *R. parksii* collections was significantly different from the other two clades ($F_{2,1047} = 211.623$, $P < 0.001$) but that the difference between those latter clades was not significant ($P > 0.05$). When values were rounded, we also found that nearly twice as many collections (seven vs. four) did not conform to the clade-level designations (TABLE II).

TAXONOMY

Rhizopogon hawkerae A.H. Sm. and Zeller, Mem NY Bot Gard 14:83. 1966 [as *Rh. hawkeri*]
= *Rhizopogon subareolatus* A.H. Sm. and Zeller, Mem NY Bot Gard 14: 81. 1966.
= *Rhizopogon colossus* A.H. Sm. and Zeller, Mem NY Bot. Gard 14:85. 1966.

Rhizopogon villosulus Zeller, Mycologia 33:196. 1941.
= *Rhizopogon pseudovillosulus* A.H. Sm. and Zeller, Mem. NY Bot. Gdn 14 (2): 81. 1966.

= *Rhizopogon rogersii* A.H. Sm. and Zeller, Mem NY Bot Gard 14:81. 1966.
= *Rhizopogon villescens* A.H. Sm. and Zeller, Mem NY Bot Gard 14: 81. 1966.
= *Rhizopogon zelleri* A.H. Sm. and Zeller, Mem NY Bot Gard 14: 81. 1966.

DISCUSSION

Our results indicate synonymization of multiple species names in section *Villosuli* of the *Rhizopogon* subgenus *Villosuli* is warranted. Analyses of both the ITS and LSU gene regions produced similar phylogenies containing three clades. Unfortunately, our efforts to amplify *tef*-1α, RPB1 and RPB2 genes were largely unsuccessful (< 10% of the collections generated positive PCR product). In contrast, PCR success for the LSU gene was comparable to ITS. Although we recognize that these two ribosomal subunit gene regions are physically linked and therefore do not provide completely independent assessments of genetic history, prior taxonomic work on *Rhizopogon* has involved both regions and resulted in some differences in phylogenetic topologies (Grubisha et al. 2001, Grubisha et al. 2002). As such, we thought that using both gene regions provided a more robust assessment of species concepts in this section compared to an analysis based solely on ITS data.

While bootstrap support values varied among the clades and gene regions, they were generally strong (i.e. > 80), suggesting the analyses represented accurate reconstructions of phylogenetic history. Coupled with the results from phylogenetic analysis, the differences in spore sizes across three clades reinforced support that only three species among the eight assessed species should be recognized. Accordingly we propose taxonomy that currently recognizes three species in section *Villosuli* (sensu Grubisha et al. 2002): *R. hawkerae*, *R. parksii* and *R. villosulus*. We treat *R. subareolatus* and *R. colossus* as taxonomic synonyms of *R. hawkerae* and *R. pseudovillosulus*, *R. rogersii*, *R. villescens* and *R. zelleri* as taxonomic synonyms of *R. villosulus*.

Our nomenclatural proposals reflect the phylogenetic and spore size results as well as botanical naming rules and frequency in herbarium records. In the case of the *R. hawkerae* clade, sequences from the holotype collections of *R. colossus*, *R. hawkerae* and *R. subaerolatus*, all of which were described by Smith and Zeller (1966), grouped in the same strongly supported ITS2-only and LSU clade. OSC herbarium records, which represent the primary repository for collections of this subgenus section, indicate that *R. colossus* is a name rarely applied, and while *R. hawkerae* and *R. subareolatus* are both more common, the former is used ~ 40% more often (SUPPLEMENTARY FIG. 2). Accordingly we have chosen to retain *R. hawkerae* as the name for this clade. The spore sizes of the *R. subareolatus* holotype collection M12414 and the *R. hawkerae* holotype collection M5455 also are similar and both agree with the group designations of this clade, further supporting the conclusion that these names are synonymous.

The nomenclatural designation of the *R. parksii* clade was complicated by the fact that the holotype collection of *R. parksii* was not available for study. We did try to sequence an *R. parksii* isotype (collection M12403) but were unsuccessful in obtaining any sequence data from that collection. Collection M0451, one of two *R. parksii* paratypes, grouped in the *R. hawkerae* clade, while the other, collection M3706, grouped with all other collections originally designated as *R. parksii* in the *R. parksii* clade (Grubisha et al. 2002). Collection M0451, however, had a mean spore width and Q value that agreed with its placement in the *R. hawkerae* clade, suggesting that that collection had been misidentified as *R. parksii*. A similar issue was observed by Kretzer et al. (2003), who found that different paratype collections of *R. vinicolor* fell into two distinct ITS-based clades. Although we were unable to obtain ITS sequences for the *R. parksii* isotype, spore size data from that collection agreed with the group designations of the *R. parksii* clade. Moreover, ITS sequences of all the collections that grouped in the *R. parksii* clade contained a conspicuous insertion of 10 bp that differentiated these sequences from others we analyzed. Because no other type collections belonged to this clade, we applied the name *R. parksii*.

For the most diverse clade, which included collections from six of the eight species examined, the only holotype sequence belonged to *R. villescens*. Although this typically would take priority under botanical naming rules, both of the *R. villosulus* paratypes also grouped in this clade. The latter species was described by Zeller in 1941, whereas *R. villescens* was described in the monograph by Smith and Zeller (1966) (as was *R. rogersii* and *R. zelleri*, which also had paratypes in this clade). Given the age of the *R. villosulus* holotype collection (1939) and our lack of success in obtaining a sequence from an *R. colossus* collection from the same year, it seems unlikely that the *R. villosulus* holotype will be included in any molecular phylogenetic analyses. Despite not having its holotype data, we think that *R. villosulus* is most appropriate name for this clade based on the sequence data we obtained. An additional rationale for conserving *R. villosulus* as the name for this clade is that the epithet *villosulus*

has been applied 10 times more commonly than *R. villescens* (as well as *R. rogersii* and *R. zelleri*) based on OSC herbarium records (SUPPLEMENTARY FIG. 2). We note that both *R. villosulus* paratype collections were the only ones in this clade that did not have mean spore widths consistent with the clade designations (TABLE II). However, the Q values were consistent with the clade designations. Therefore we suggest that researchers should use the combination of spore width and length to morphologically identify collections to this clade.

Martín et al. (1998) synonymized *R. colossus var. colossus*, *R. hawkerae*, *R. luteolus*, *R. parksii*, *R. reticulatus*, *R. subareaolatus*, *R. rocabrunae* and *R. villosulus* based on the lack of sequence variation observed via RFLP analysis. Of interest, the 10 bp insertion in the ITS1 region of all the *R. parksii* collections (sequence = TTGGATTCGT) would not be cut by any of the restriction enzymes used by Martín et al. (1998) (NEB enzyme finder analysis). This likely explains why those authors were unable to detect *R. parksii* as a distinct species. The 10-bp insertion also accounts for the changes in position between the *R. hawkerae* and *R. parksii* clades in the analysis of the full ITS versus ITS2-only datasets. Aside from that insertion, differences in the remaining ITS region between the *R. parksii* and *R. villosulus* clades were minimal whereas differences between the *R. hawkerae* and *R. villosulus* clades were present throughout ITS1 and ITS2 regions but nearly always as single nucleotide polymorphisms. This subtle sequence variation also might explain why Martin et al. (1998) were not able to distinguish *R. hawkerae* and *R. villosulus*. The topology of LSU ML analysis was consistent with that of the ITS2-only analysis, but, given the lack inconsistency across the three datasets we analyzed, it is clear that additional data from other gene regions will be key to determining exactly how these three species in section *Villosuli* are related to one another.

Our reduction in species names for this section contradicts the richness generated by Smith and Zeller (1966). While the spore sizes we observed and the clade designations we assigned agree with the original descriptions of the three species retained, most species in section *Villosuli* were differentiated by Smith and Zeller (1966) via differing hyphal structure of the peridium and peridia and subcutis staining. Because our analysis was carried out with dry material only, we could not evaluate characters such as the bluish fuscous staining that Smith described as characteristic of the subcutis of fresh *R. villescens* sporocarps or the blue to violaceous peridia staining of fresh *R. parksii* sporocarps (Smith and Zeller 1966). Since that publication, however, confidence in consistency of staining characters to differentiate species within this section has diminished (J. Trappe pers comm). Instead, we consider many of the characters described by Smith and Zeller (1966) to represent ontogenetic variation among individuals of the same species rather than stable traits distinguishing different species. In addition to the eight species examined here, a handful of infrequently collected species designated by Smith and Zeller (1966) as members of subgenus *Villosuli* (*R. brunneifibrillosus*, *R. fragrans*, *R. gilkyae*, *R. umbrinoviolascens*, *R. sepelibilis*, *R. mutabilis*, *R. ponderosus*, *R. viridis*) remain to be analyzed with sequence data (SUPPLEMENTARY FIG. 2). Future taxonomic work will be important in determining whether they are in fact valid names and, if so, to which section of subgenus *Villosuli* they belong.

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