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Biology of the ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of ITS sequences

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Abstract: *Rhizopogon* (Basidiomycota, Boletales) is a genus of hypogeous fungi that form ectomycorrhizal associations mostly with members of the Pinaceae. This genus comprises an estimated 100$^+$ species, with the greatest diversity found in coniferous forests of the Pacific northwestern United States. Maximum parsimony analyses of 54 nuclear ribosomal DNA internal transcribed spacer (ITS) sequences including 27 *Rhizopogon* and 10 *Suillus* species were conducted to test sectional relationships in *Rhizopogon* and examine phylogenetic relationships with the closely related epigeous genus, *Suillus*. Sequences from 10 *Rhizopogon* type collections were included in these analyses. *Rhizopogon* and *Suillus* were both monophyletic. *Rhizopogon* section *Rhizopogon* is not monophyletic and comprised two clades, one of which consisted of two well supported lineages characterized by several long insertions. *Rhizopogon* sections *Amylopogon* and *Villosuli* formed well supported groups, but certain species concepts within these sections were unresolved. Four species from section *Fulviglebae* formed a strongly supported clade within section *Villosuli*. Subgeneric taxonomic revisions are presented.

Key Words: Boletales, indels, phylogeny, Rhizopogonaceae, *Suillus*

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INTRODUCTION

Rhizopogon Fries (Basidiomycota, Rhizopogonaceae) contains more than 100 species (Martín 1996). It is ectomycorrhizal mostly with Pinaceae and its worldwide distribution correlates with natural and exotic Pinaceae forests (Molina et al 1999). Despite this cosmopolitan range, most species are found in pine (*Pinus* L.) and Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] forests of the Pacific northwestern United States (Smith 1964, Smith and Zeller 1966). *Rhizopogon* is a common ectomycorrhizal fungus in these coniferous forests and thus an important component of the forest ecosystem.

The systematics of *Rhizopogon* remains in a state of flux that dates to the early 19th century, when notes on fresh characters were scanty and only gross morphological characters were used to describe species (Lange 1956, Smith 1971, Smith and Zeller 1966). Current understanding of *Rhizopogon* taxonomy is based primarily on a landmark publication by Smith and Zeller (1966) who increased the number of described North American species from 17 to 110, and included redescribed ''European'' species found in North America. Smith and Zeller (1966) divided the genus into two subgenera, *Rhizopogonella* and *Rhizopogon*. Species in subgenus *Rhizopogonella* were subsequently moved to *Alpova* (Trappe 1975). Subgenus *Rhizopogon* was divided into four sections, *Amylopogon*, *Fulviglebae*, *Rhizopogon*, and *Villosuli*, based on macroscopic and microscopic sporocarp characters and color changes on the peridium from chemical reactions and bruising of the sporocarp (Smith 1964, Smith and Zeller 1966).

Although this work is an important contribution to the systematics of *Rhizopogon*, several unresolved issues remain regarding placement of species in sections *Fulviglebae* and *Rhizopogon*. In Smith and Zeller (1966), *R.* section *Fulviglebae* comprises twenty-two species, of which six are identified as unusual species (e.g., *R. hysterangioides*, *R. lowii*, and *R. pannosus*), including the type for the section, *R. exiguus*. Ten species in this section share morphological and ecological affinities with section *Villosuli*, e.g., *Rhizopogon vinicolor*, *R. clavitisporus*, etc., but are tied to species in section *Fulviglebae* only by possessing truncated spores (Smith and Zeller 1966, Molina and Trappe 1994, J. Trappe unpubl).

TABLE I. Taxonomic divisions in *Rhizopogon* section *Rhizopogon* based on spore width and peridium coloration as defined by Smith and Zeller (1966)

Rhizopogon section *Rhizopogon*	Spore width (μm)	Peridium coloration		
		Yellow[a]	Bruises red	Other
Subsection *Rhizopogon*	3.5–5			
Strips *Rubescens*		yes[b]	yes	
Strips *Luteolus*		yes	no	
Subsection *Angustispori*	1.6–3			
Series *Lutei*		yes		
Strips *Vulgaris*		yes	yes	
Strips *Ochraceorubens*		yes	no	
Series *Versicolores*[c]		no		yes
Strips *Subsalmonius*			no	peach-pink to salmon pink
Strips *Evadens*			yes	

[a] Yellow color refers to whether the peridium develops yellow colors during development, and should not be confused with bruising yellow.

[b] Three species in Strips *Rubescens* do not have a yellow stage.

[c] Only two of the seven strips in Series *Versicolores* are mentioned here.

Species placed in *Rhizopogon* section *Rhizopogon* lacked characters that defined the other three sections (Smith and Zeller 1966). Divisions within *Rhizopogon* section *Rhizopogon* were based on spore width and colors of the sporocarp when bruised (Smith and Zeller 1966, TABLE I). Section *Rhizopogon* contained an estimated 60 species at the writing of Smith and Zeller (1966) making it the largest section in the genus. Since section *Rhizopogon* was not based on common morphological or ecological features of these species, analysis of sequence data provides a way to test the validity of this taxonomic group. Smith and Zeller (1966) emphasized that this major taxonomic work was based on techniques available at the time and future revision was expected. A current review of the status of *Rhizopogon* taxonomy is found in Martín (1996).

Hypotheses regarding the evolutionary relationship between *Suillus* and *Rhizopogon* are not new (Malençon 1931, Heim 1971, Thiers 1971, 1984), and molecular evidence supports the hypothesis that *Suillus* and *Rhizopogon* are closely related (Bruns et al 1989). In a recent study of nuclear ribosomal large subunit (28S) DNA sequences, Grubisha et al (2001) found that *Rhizopogon* and *Suillus* were not sistergroups. *Suillus* was inferred to be more closely related to *Truncocolumella citrina* and the Gomphidiaceae than it was to *Rhizopogon*. Thus, questions remain concerning the nature of this relationship. The monophyly of these genera has been supported in previous molecular phylogenetic studies, but few have contained a large number of *Rhizopogon* and *Suillus* sequences, or multiple sequences from exemplar species from each of the four sections of *Rhizopogon* (Bruns et al 1989, Bruns and Szaro 1992, Kretzer et al 1996, Johannesson and Martín 1999, Grubisha et al 2001).

As part of a continuing series of studies into the systematics of *Rhizopogon* and related fungi, maximum parsimony analyses were performed on nucleotide data from the nuclear ribosomal DNA internal transcribed spacer regions 1 and 2 and the 5.8S subunit. The major objectives of this study were to: i) categorize infrageneric sectional relationships in *Rhizopogon*, and ii) further qualify the phylogenetic relationship between *Rhizopogon* and *Suillus*.

MATERIALS AND METHODS

Fungal specimens.—Species representing the sections *Amylopogon*, *Fulviglebae*, *Rhizopogon*, and *Villosuli* of the genus *Rhizopogon* were selected for phylogenetic analysis of nucleotide data (TABLE II). Forty collections used for DNA extraction were from the University of Michigan Herbarium (MICH) and the Mycological Collection of the Oregon State University Herbarium (OSC). Pieces of ten of these were donated from type collections by MICH. Specimens of *Alpova trappei*, *Boletus edulis*, and *Chalciporus piperatus* were also included (TABLE II). GenBank numbers are given in TABLE II for sequences from *Chroogomphus vinicolor*, *Gomphidius glutinosus*, *Rhizopogon subcaerulescens*, 10 *Suillus* spp., and *Truncocolumella citrina*.

Nucleic acid extraction, polymerase chain reaction, and DNA sequencing.—Nucleic acid extraction, PCR amplification, quantification, purification, sequencing, and alignment of sequences were previously described (Grubisha et al 2001). Primer pairs ITS-5 and ITS-4, ITS-5 and ITS-2, ITS-4 and ITS-3 (White et al 1990) and ITS1-F and ITS4-B (Gardes and Bruns 1993) were used for amplification of the ITS rDNA. The ITS1 and ITS2 spacer regions and 5.8S subunit

were sequenced with combinations of the primers listed above.

Choice of outgroup.—Complete ITS sequences were obtained from *Alpova trappei*, *Boletus edulis*, and *Chalciporus* (*Boletus*) *piperatus* for use as an outgroup. Using only *Alpova trappei* as the outgroup, ambiguous and difficult alignment regions were excluded from the *A. trappei* sequence and replaced with missing character states (-) while ingroup characters were retained (Nixon and Carpenter 1993). Once the polarity of the topology was determined, the most basal taxa were designated the outgroup and the *Alpova trappei* sequence was removed from the data set used for phylogenetic analyses.

Phylogenetic analysis.—Using *Chroogomphus* and *Gomphidius* as an outgroup, an alignment of 892 nucleotide bases representing the ITS1, ITS2, and 5.8S subunit was analyzed. Alignment gaps were treated as follows: 1) ALL SET—all characters were included and gaps treated as missing data; 2) CULLED SET—multiple-base insertion/deletion events (indels) and areas of ambiguous alignment were excluded, remaining gaps treated as missing data; 3) I-GAP SET—a new character "I" was inserted to indels, ambiguous areas deleted, and remaining gaps treated as missing data; and 4) BINARY SET—indels were excluded and re-coded as presence/absence (0,1) in the data matrix at the end of the alignment, remaining single-base gaps treated as missing, and ambiguous areas of the alignment were excluded. The alignment is available in TreeBASE as S689. Maximum parsimony analyses were performed using PAUP* version 4.0 (Swofford 1999). Uninformative characters were excluded from all phylogenetic analyses. One hundred heuristic searches were conducted with random sequence addition and tree bisection-reconnection (TBR) branch-swapping algorithms, collapsing zero-length branches and saving all minimal length trees (MulTrees). To measure relative support for the resulting clades, 1000 bootstrap replications (Felsenstein 1985) were performed only on phylogenetically informative characters with the following parameters: 10 random sequence additions, TBR, and MulTrees off. Because the alignment revealed several indels that did not align across all species and may have resulted in loss of resolution within sections, unrooted branch and bound searches from section-specific alignments of sections *Amylopogon*, *Rhizopogon*, and *Villosuli* were performed. Bootstrap analyses were conducted as described above, with MulTrees option in effect.

RESULTS

Choice of outgroup.—We wanted to use an outgroup that was outside the suilloid group and obtained ITS sequences of *Alpova trappei*, *Boletus edulis*, and *Chalciporus* (*Boletus*) *piperatus*. However, sequences from these species were highly divergent and simply too difficult to align with the ingroup. Introduction of excessive and ambiguous alignment gaps was necessary and lead to problems in homology assessment. The 5.8S region was the only area that aligned with confidence across all species and since there were only 33 phylogenetically informative characters in the 5.8S region it was considered an unsuitable region to base the root of the tree. An unrooted tree is presented in FIG. 1. The placement of the root with *Alpova trappei* as the outgroup is indicated. When analyses were run with *A. trappei* as the outgroup, there was only bootstrap support for the sections within *Rhizopogon* and for *Suillus* as monophyletic. To test infrageneric relationships in *Rhizopogon*, further analyses were conducted using *Chroogomphus* and *Gomphidius* as the outgroup, which were basal in the *Alpova*-rooted tree.

Parsimony analyses.—No major differences in tree topology could be inferred from the four indel treatments. Bootstrap values varied slightly, but remained essentially unchanged, except for the CULLED SET (treatment 2), when all ambiguous areas and large indels were removed. In this case somewhat lower bootstrap values were recovered. The highest bootstrap support was observed in the ALL SET. Results from the four analyses are summarized in TABLE III. One of the most parsimonious trees from the maximum parsimony analyses of the CULLED SET is presented in FIG. 2 [consistency index (CI) = 0.525, retention index (RI) = 0.752, rescaled consistency index (RC) = 0.416]. Bootstrap values greater than 70% are indicated above the respective internode. *Rhizopogon* and *Suillus* formed well supported monophyletic clades (FIG. 2). *Rhizopogon* section *Rhizopogon* was not monophyletic and formed two well supported groups, one comprising two distinct lineages (*Rhizopogon* section *Rhizopogon* clades A, B, and C; FIG. 2). *Rhizopogon* section *Amylopogon* is monophyletic and well supported by bootstrap analysis. Section *Villosuli* is paraphyletic because species sampled from *R.* section *Fulviglebae* formed a well supported group within the section *Villosuli* clade. Because of large indels, typically found in the ITS1 region, some loss of resolution occurred within the sections due to an alignment of 54 sequences across 5 genera. Unrooted trees from analyses of section-specific alignments of sections *Amylopogon*, *Rhizopogon*, and *Villosuli* are shown in FIG. 3.

DISCUSSION

Phylogenetic relationship between Rhizopogon *and* Suillus.—*Suillus* and *Rhizopogon* both form monophyletic clades with bootstrap values of 96 and 78, respectively, although the sister-group relationship was not supported by bootstrap analyses. *Suillus* species in this study associate with a variety of conifer hosts as indicated by Kretzer et al (1996). Although previous

TABLE II. Species included in this study

Species	Voucher number[a]	Geographic location	Herbarium[b]	GenBank[c]
Alpova trappei Fogel	JMT 16394	California, USA	OSC	AF074920
Boletus edulus Bull. : Fr.	LCG 184	Oregon, USA	OSC	AF074921
Chalciporus piperatus (Bull. : Fr.) J. Bataille	LCG 185	Oregon, USA	OSC	AF074922
Choogomphus vinicolor (Peck) Miller				L54095
Gomphidius glutinosus (Schaeff. : Fr.) Fr.				L54114
R. burlinghamii A. H. Smith	JMT 17882	California, USA	OSC	AF058303
R. colossus A. H. Smith	AHS 49480 (HOLOTYPE)	Oregon, USA	MICH	AF071441 AF071442
R. diabolicus A. H. Smith	AHS 68424 (PARATYPE)	Washington, USA	MICH	AF071444 AF071443
R. ellenae A. H. Smith	AHS 66137 (HOLOTYPE)	Idaho, USA	MICH	AF071445 AF071446
R. ellenae A. H. Smith	JMT 17476	Oregon, USA	OSC	AF058311
R. evadens A. H. Smith	AHS 65484 (HOLOTYPE)	Oregon, USA	MICH	AF062927
R. evadens A. H. Smith	JMT 16402	California, USA	OSC	AF058312
R. evadens A. H. Smith	JMT 12321	California, USA	OSC	AF062932
R. fuscorubens A. H. Smith	JMT 17446	South Carolina, USA	OSC	AF058313
R. hawkerae A. H. Smith	AHS 68417 (PARATYPE)	Washington, USA	MICH	AF071447 AF071448
R. luteolus Fr.	JMT 22516	Uppsala, Sweden	OSC	AF062936
R. occidentalis Zeller & Dodge	JMT 17564	Oregon, USA	OSC	AF058305
R. occidentalis Zeller & Dodge	LCG 211	California, USA	OSC	AF062939
R. ochraceisporus A. H. Smith	AHS 65963 (PARATYPE)	Idaho, USA	MICH	AF071439
R. ochraceisporus A. H. Smith	JMT 17944	Oregon, USA	OSC	AF058306
R. ochraceisporus A. H. Smith	JMT 17916	Oregon, USA	OSC	AF062935
R. ochraceorubens A. H. Smith	AHS 59643 (HOLOTYPE)	Idaho, USA	MICH	AF062928
R. ochraceorubens A. H. Smith	JMT 19192 (TOPOTYPE)	Idaho, USA	OSC	AF071440
R. parksii A. H. Smith	JMT 17679	Oregon, USA	OSC	AF062930
R. parksii A. H. Smith	JMT 19446	Oregon, USA	OSC	AF058314
R. parvulus A. H. Smith	AHS 68364 (PARATYPE)	Idaho, USA	MICH	AF071449 AF071450
R. rogersii A. H. Smith	JMT 17228	Oregon, USA	OSC	AF071437
R. roseolus Corda	JMT 8227	California, USA	OSC	AF058315
R. semireticulatus A. H. Smith	JMT 7899	Oregon, USA	OSC	AF058307
R. semireticulatus A. H. Smith	JMT 17562	Oregon, USA	OSC	AF062940
R. sp. nov.	JMT 17466	Oregon, USA	OSC	AF071438
R. subcaerulescens A. H. Smith				M91613
R. subgelatinosus A. H. Smith	JMT 7624	Oregon, USA	OSC	AF062937
R. subpurpurascens A. H. Smith	AHS 65669 (PARATYPE)	Idaho, USA	MICH	AF062929
R. subpurpurascens A. H. Smith	JMT 19168	Idaho, USA	OSC	AF058308
R. subsalmonius A. H. Smith	JMT 17218	Oregon, USA	OSC	AF062938
R. succosus A. H. Smith	JMT 19321	West Virginia, USA	OSC	AF062933
R. villescens A. H. Smith	JMT 17681	Oregon, USA	OSC	AF058309
R. villosulus Zeller	AHS 59143	Idaho, USA	MICH	AF071451 AF071452

[a] LCG, Lisa C. Grubisha, AHS, Alexander H. Smith; JMT, James M. Trappe.

[b] MICH, Herbarium of the University of Michigan; OSC, Mycological Collection of the Oregon State University Herbarium.

[c] When one GenBank number is given, it is for the sequence of the entire ITS region, ITS 1, ITS 2, and 5.8S subunit. When two GenBank numbers are given, one is for the ITS 1 and partial 5.8S subunit sequence, and the second is for the sequence for the ITS 2 region and partial 5.8S subunit. Species listed only by GenBank number were not sequenced in this study.

TABLE II. Continued

Species	Voucher number[a]	Geographic location	Herbarium[b]	GenBank[c]
R. villosulus Zeller	JMT 19466	Washington, USA	OSC	AF058310
R. vinicolor A. H. Smith	JMT 17899	Oregon, USA	OSC	AF058316
R. vinicolor A. H. Smith	JMT 19383	Oregon, USA	OSC	AF058304
R. vinicolor A. H. Smith	JMT 20787	Idaho, USA	OSC	AF062941
R. vulgaris (Vitt.) M. Lange	JMT 19154	Oregon, USA	OSC	AF062934
R. vulgaris (Vitt.) M. Lange	JMT 17998	California, USA	OSC	AF062931
R. zelleri A. H. Smith	JMT 12974	New Mexico, USA	OSC	AF062942
Suillus americanus (Peck) Snell				L54103
S. brevipes (Peck) Kuntze				L54111
S. caerulescens Smith & Thiers				L54096
S. cavipes (Opat.) Smith & Thiers				L54085
S. grevillei (Klotzsch) Singer				M91614
S. granulatus (Fries) Kuntze				L54113
S. luteus (Fries) Gray				L54100
S. lakei (Murrill) Smith & Thiers				L54086
S. sinuspaulianus (Pomerleau & Smith) Dick & Snell				L54078
S. tomentosus (Kauffmann) Singer, Snell & Dick				L54106
Truncocolumella citrina Zeller				L54097

studies have shown that *Suillus* and *Rhizopogon* are closely related, the monophyly of these two respective genera was uncertain due to limited species sampling or because the choice of loci was less variable than the ITS region (Bruns and Szaro 1992, Bruns et al 1998). We attempted to include enough species from both genera to represent the range of conifer associates. Our results provide further evidence for *Suillus* and *Rhizopogon* as monophyletic genera, but their exact relationship to other taxa of the suilloid radiation remains unclear. Presently a good outgroup for the suilloid group has not been identified. We have found that it is difficult to align sequences from taxa outside of the suilloid group of the Boletales when using the nrDNA ITS region. Grubisha et al (2001) found that *Suillus* and the Gomphidiaceae were sister groups, not *Suillus* and *Rhizopogon*. These results were not corroborated here when choice of outgroup rooting was determined by an *Alpova trappei* sequence (FIG. 1). Previous studies have shown *Truncocolumella citrina* to be more closely related to *Suillus* than to *Rhizopogon* (Grubisha et al 2001, Kretzer and Bruns 1999). In this study, *Truncocolumella citrina* did not group within or sister to *Suillus*. The polarity of the relationship between *Rhizopogon*, *Suillus*, *Truncocolumella citrina*, and the Gomphidiaceae requires further examination. Identification of a suitable outgroup outside the suilloid radiation in the Boletales is needed in future studies investigating relationships within the suilloid clade.

Examination of infrageneric relationships in Rhizopogon.—Although many sectional relationships as defined by Smith (Smith 1964, Smith and Zeller 1966) are well supported, many lower taxonomic groupings, e.g., subsections, series, stirps, are polyphyletic. Section *Amylopogon* is strongly supported as monophyletic with a bootstrap value of 99. Section *Rhizopogon* is not monophyletic and forms three well supported clades with high bootstrap values of 100, 93, and 95 (clades A, B, C; FIG. 2). The type of the genus, *R. luteolus*, is present in the *Rhizopogon* section *Rhizopogon* clade A. Section *Villosuli* is well supported, but the species sampled from section *Fulviglebae* are found within section *Villosuli*, and form a strongly supported group with a bootstrap value of 99. Although *Rhizopogon* section *Rhizopogon* clades A and B appear to form a sister-group to species sampled from the other sections in *Rhizopogon*, which form another monophyletic group, bootstrap support for this placement is moderate at best. *Rhizopogon* sections *Amylopogon*, *Rhizopogon* clade C, and *Villosuli* are well supported as separate groups and distinct from the *Rhizopogon* section *Rhizopogon* clade A and B, but the relationships among these groups are not resolved.

Section Rhizopogon. Smith and Zeller (1966) divided *Rhizopogon* section *Rhizopogon* into two subsections, *Angustispori* and *Rhizopogon*, two series and 11 stirps. We sampled 15 sequences from 12 species representing both subsections. The subsections are sep-

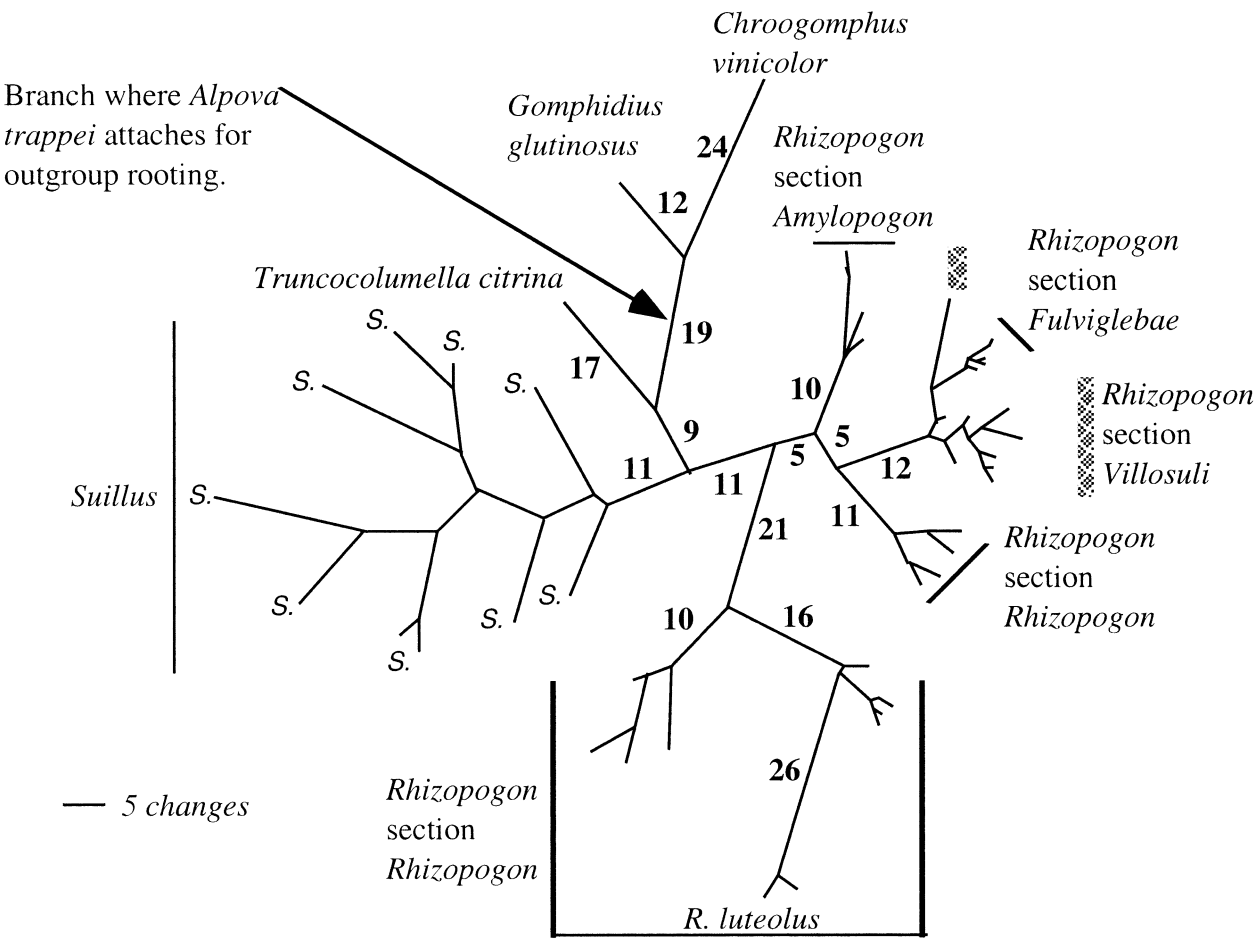

FIG. 1. Unrooted tree based on the CULLED SET analyses to show outgroup rooting with *Alpova trappei*. *Rhizopogon luteolus* is the type species for the genus *Rhizopogon*. Placement of species in sections of genus *Rhizopogon* is according to Smith and Zeller (1966).

TABLE III. Results from maximum parsimony analysis of four insertion-deletion (indel) coding strategies

Analysis treatment[a]	Indel coding			Most parsimonious trees					
	Gaps as missing	"I" inserted to indel	Presence/absence (0, 1)	Number of characters[b]	Number	Length	CI	RI	RC
1	yes	no	no	279	6	879	0.522	0.763	0.398
2	yes	no	no	189	360	585	0.525	0.792	0.416
3	yes	yes	no	209	8	622	0.537	0.809	0.434
4	yes	no	yes	196	204	580	0.545	0.814	0.444

[a] Different treatments of indels (see text for further discussion): 1 = All set: all characters states were included, even ambiguous areas of the alignment, all gaps scored as missing data; 2 = Culled set: ambiguous areas of alignment and large inserts were excluded, gaps treated as missing data; 3 = Indel "I" set: ambiguous areas of alignment excluded, character "I" inserted into gaps; 4 = Binary coded set: ambiguous areas of alignment excluded; large gaps excluded and coded as presence/absence.

[b] Number of parsimony informative characters included in the analysis.

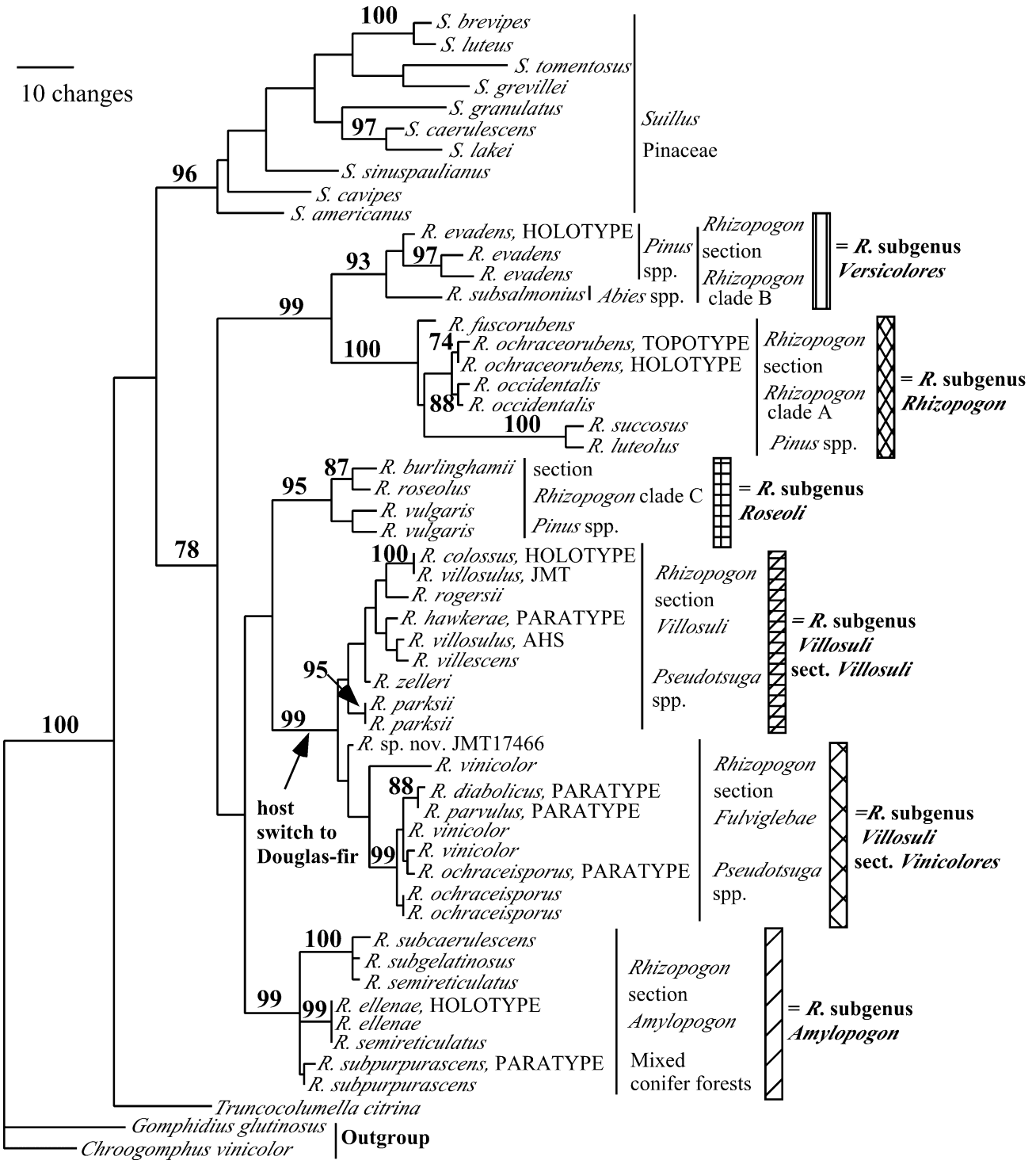

FIG. 2. One of 360 equally parsimonious trees of 585 steps based on ITS1, ITS2, and 5.8S subunit nrDNA sequences resulting from the CULLED SET of analyses. Bootstrap values are indicated at the respective internode. CI = 0.512, RI = 0.801. Placement of species in sections of genus *Rhizopogon* is according to Smith and Zeller (1966). Sections appear to be associated with either pines or Douglas-fir (*Pseudotsuga menziesii*), with the exception of subgenus *Amylopogon* that is associated with a broad range of hosts. Host information is based on collection and ecological data, and pure culture synthesis studies (Molina et al 1999). Proposed taxonomic revisions at the subgeneric level, and sectional level in subgenus *Villosuli*, are provided in bold print next to sectional classification of Smith and Zeller (1966).

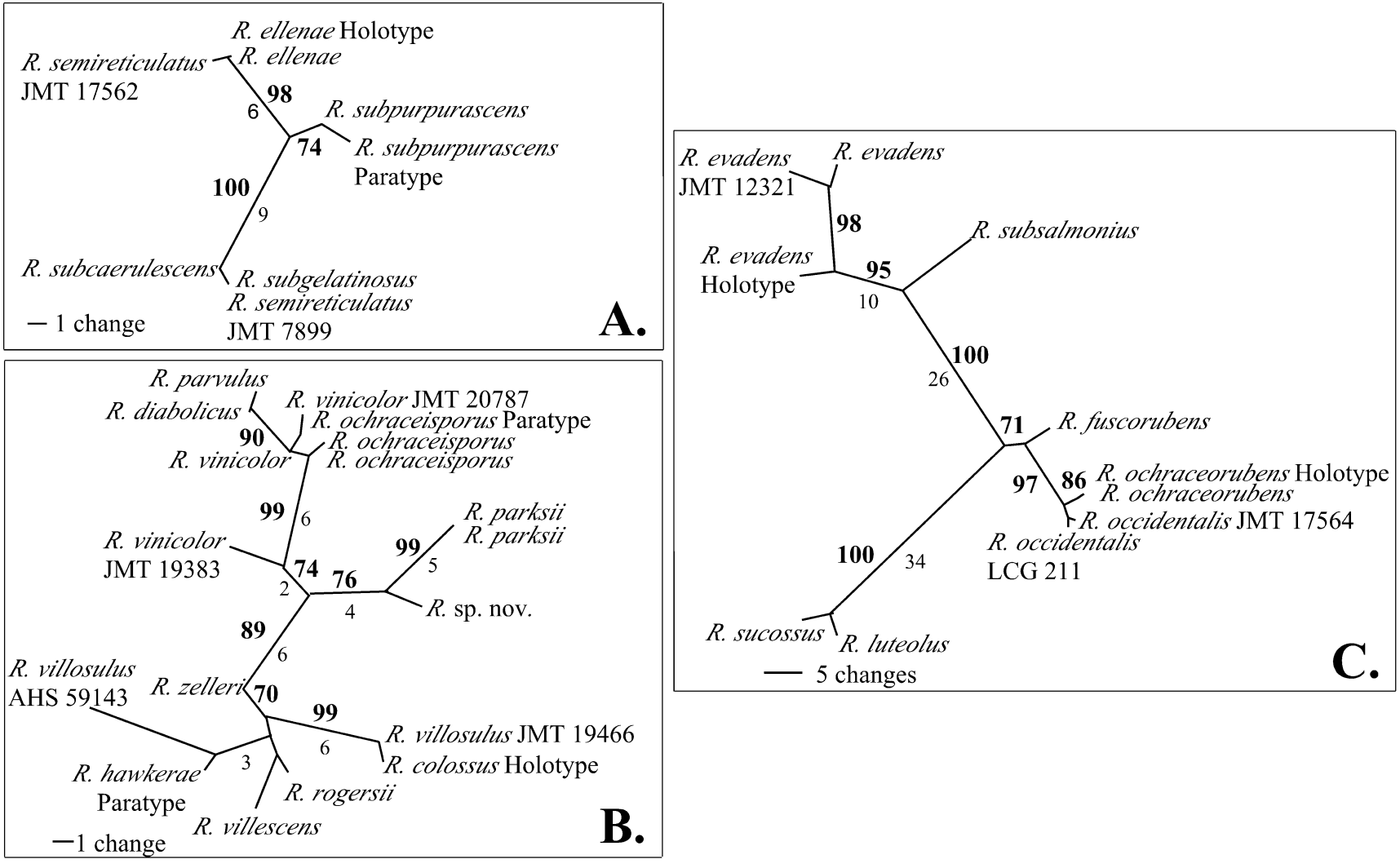

FIG. 3. Unrooted section-specific trees resulting from branch and bound searches based on alignments of *Rhizopogon* sequences from A. section *Amylopogon*, B. sections *Fulviglebae* and *Villosuli*, and C. section *Rhizopogon*. Placement of species in sections of genus *Rhizopogon* is according to Smith and Zeller (1966). Bootstrap values are in bold noted above the respective internode. The number of changes is in smaller font below internodes.

arated by spore width and whether or not the peridium stains red and/or yellow at some stage of development (TABLE I). Our results show that spore width, the presence or absence of yellow in the peridium, or the pink-red staining reaction are not phylogenetically informative at the sectional (subgeneric, see taxonomic revision below) level, inasmuch as these characters occur in both *Rhizopogon* section *Rhizopogon* clades A and C. However, the absence of yellow does seem to be important for *Rhizopogon* clade B. Combined with other characters, the type of peridium appears to be a meaningful phylogenetic character, although this was not included in Smith's keys, e.g., a peridium of interwoven hyphal strands (*Rhizopogon* clade A) or interwoven hyphae (*Rhizopogon* clades B, C). Subsections *Angustispori* and *Rhizopogon* appear to be polyphyletic based on our results.

Rhizopogon section *Rhizopogon* clade A comprises *R. fuscorubens*, *R. luteolus*, *R. occidentalis*, *R. ochraceorubens*, and *R. succosus*. *Rhizopogon succosus* and *R. luteolus* share several morphological characters but are distinct species (Miller 1986, Hosford and Trappe 1988). Based on peridium coloration, microscopic characters, and the glass-hard consistency of the dried gleba, Miller (1986) suggested that a better placement of *R. succosus* is in stirps *Luteolus*. These observations are supported by the data presented here. The relationship between these two species is supported by a bootstrap of 100 in these analyses. In addition to being morphologically similar, they share similar long insertions in the ITS1 sequences and are both associated with *Pinus* spp.

The two holotypes from MICH that were sampled from *Rhizopogon* section *Rhizopogon*, *R. ochraceorubens* and *R. evadens*, are from subsection *Angustispori*, series *Lutei* and *Versicolores* respectively. *Rhizopogon ochraceorubens* and *R. fuscorubens* are closely related and placed in stirps *Ochraceorubens*. Smith indicates that the major difference between these two is that the rhizomorphs on the peridium of *R. fuscorubens* dry black and the peridium dries yellow, whereas the rhizomorphs on the peridium of *R. ochraceorubens* do not dry black and the peridium dries red. When rehydrated in KOH, the sectioned peridium is bright red for both species and very prominent in the holotype specimen. *Rhizopogon occidentalis*, originally placed in stirps *Rubescens*, appears to be closely related to both *R. ochraceorubens* and *R. fuscorubens*, although the sectioned peridium lacks the bright red reaction to KOH. All three species fruit in association with pines and generally form ectomycorrhizae only with pines in pure culture syntheses (Molina and Trappe 1982, 1994). *Rhizopogon occidentalis* will form mycorrhizae with *Arctostaphylos* and *Arbutus* spp. if pines are present as the primary host (Molina et al 1997).

Two species were sampled from series *Versicolores*, *R. subsalmonius* and *R. evadens*, and belong to stirps *Subsalmonius* and *Evadens* respectively. These species form *Rhizopogon* section *Rhizopogon* clade B (FIG. 2). *Rhizopogon subsalmonius* does not stain red when cut. *Rhizopogon evadens* stains red, but the peridium is white and lacks yellow coloration. The peridium does not stain bright red when sections are treated with KOH. Both have a peridum of interwoven hyphae and lack yellow coloration/staining. *Rhizopogon subsalmonius* is found with *Abies* spp. while *R. evadens* is associated with *Pinus* spp. These species form a clade distinct from *Rhizopogon* section *Rhizopogon* clade A.

Rhizopogon burlinghamii, *R. roseolus*, and *R. vulgaris* form *Rhizopogon* section *Rhizopogon* clade C. Smith and Zeller (1966) placed *R. vulgaris* in subsection *Angustispori*, stirps *Vulgaris* because it has narrow spores, stains red, and is yellow at some point during its development. Smith recognized the similarity of species in stirps *Vulgaris* with those in stirps *Rubescens* and mentions that stirps *Vulgaris* is a continuation of stirps *Rubescens* into the narrow spored species. Smith's descriptions of *R. roseolus* and *R. vulgaris* included in Smith and Zeller (1966) are based on examinations of North American collections. These two species were originally described from Europe in the nineteenth century (Smith and Zeller 1966). This study supports the close relationship of these species, sensu A. H. Smith. *Rhizopogon roseolus (rubescens)*, *R. vulgaris*, and *R. burlinghamii*, form a distinct clade (B) separate from the other species sampled from section *Rhizopogon* (clades A and B). These species also lack several large indels present in species found in the other section *Rhizopogon* clades. These three species all associate with *Pinus* spp. These results and the morphological similarities of these species support their separation from *Rhizopogon* section *Rhizopogon*.

Section Amylopogon. Section *Amylopogon* is monophyletic and forms a well-supported clade with a bootstrap value of 99. The HOLOTYPE of *R. ellenae* and a PARATYPE of *R. subpurpurascens* were sampled. Martín (1996) moved *R. ellenae* to section *Rhizopogon* because dried specimens did not have amyloid spores. In our results, the holotype of *R. ellenae* is found in the strongly supported section *Amylopogon* clade. Amyloid spores seem to be an important character for taxonomic and phylogenetic studies in *Rhizopogon*. The fact that this character may not be detected in dried herbarium specimens may lead to misidentifications and should be considered in future studies of herbarium specimens of section *Amy-*

lopogon. Smith and Zeller (1966) stated that although not all species in section *Amylopogon* have amyloid spores, all *Rhizopogon* species with amyloid spores are placed in this section. Section *Amylopogon* is supported by anatomy, the olive to green, blue, pink, or red reaction of the peridium to KOH, and, when present, amyloid spores. Species in section *Amylopogon* are the most broad-ranging in the genus in terms of mycorrhizal hosts, but they typically occur in conifer forests with pines and true firs (*Abies* Mill). *Rhizopogon subcaerulescens* forms ectomycorrhizae with Douglas-fir in laboratory conditions (Massicotte et al 1994).

Section Fulviglebae. The four species sampled from section *Fulviglebae* (*R. diabolicus*, *R. ochraceisporus*, *R. parvulus*, and *R. vinicolor*) were selected because they shared some peridial characters with section *Villosuli* and, as with the *Villosuli*, are associated with Douglas-fir. They form a well supported clade with a bootstrap value of 99, that is placed within section *Villosuli*. Although *Rhizopogon parvulus* and *R. diabolicus* are closely related species, both morphologically (Smith and Zeller 1966) and based on our data, their relationship to *R. vinicolor* and *R. ochraceisporus* is unclear and currently under investigation (A. Kretzer pers comm).

Species in stirps *Vinicolor* (e.g., *R. diabolicus*, *R. parvulus*, and *R. vinicolor*) and *R. ochraceisporus* (stirps *Thaxteri*) in section *Fulviglebae* are morphologically similar. Although Smith and Zeller (1966) mention that within stirps *Vinicolor* there is a trend towards brown-walled hyphae in the peridium, a characteristic of species in section *Villosuli*, descriptions of brown-walled hyphae are not included in species descriptions for stirps *Vinicolor*. The species in stirps *Vinicolor* and *R. ochraceisporus* also associate with Douglas-fir. *Rhizopogon vinicolor* and *R. ochraceisporus* may be ontogenetic stages of a single species, because except for glebal color, these two species are very similar morphologically.

Section Villosuli. Smith (1964) recognized twenty-one species of *Rhizopogon* in section *Villosuli*. These are separated from the other three sections by having brown-walled hyphae that form a distinct epicutis in the peridium and nontruncate, nonamyloid spores. Based on the findings presented here, *R. colossus*, *R. villosulus*, *R. rogersii*, *R. hawkerae*, and *R. villescens* could be a single species that shows variation, or several very closely related species. Martín et al (1998) synonomized *R. colossus* var. *colossus*, *R. hawkerae*, *R. parksii*, *R. reticulatus*, *R. subareolatus*, and *R. villosulus* to *R. villosulus* based on the lack of polymorphic bands in Restriction Fragment Length Polymorphism (RFLP) analyses of ITS rDNA. However, their findings do not entirely agree with those presented here. The two vouchers of *Rhizopogon parksii* always group as a pair and are distinct from the *R. colossus*, *R. hawkerae*, and *R. villosulus* in these analyses. In addition, some years after publication by Smith and Zeller (1966), Smith concluded from additional collecting that *R. colossus* was a developmental stage of *R. villosulus* (pers comm to J. M. Trappe), and we agree based on morphological and molecular evidence. In order to address this question of conspecificity, the two *R. villosulus* vouchers included in this study were re-examined macroscopically and microscopically. *Rhizopogon villosulus* AHS 59143 does not entirely match with the descriptive features. It lacks flagellate hyphae or any suggestion of pink blush, so it also does not fit *R. hawkerae*, and microscopically the best match is with *R. viridis*. Although we have some doubts about the identity of *R. villosulus* AHS 59143, we feel quite certain of the identification of *R. villosulus* JMT 19466. This exemplifies the need for additional critical studies of the species in this section.

Host specificity and evolution.—Rhizopogon spp. show a great deal of host specificity with members of the Pinaceae (Smith 1964, Smith and Zeller 1966, Molina et al 1992). Smith and Zeller (1966) noted that the greatest species diversity occurs in the coniferous forests of the Pacific Northwest of the United States; however, *Pseudotsuga* forests in Asia and Mexico have not been extensively searched. In general, sections of *Rhizopogon* show a certain degree of specificity for particular genera of Pinaceae and some species show specificity with either *Pinus* spp. or Douglas-fir (Molina et al 1999). For several *Rhizopogon* species host specificity was supported by pure culture synthesis (Molina and Trappe 1982, 1994) and spore inoculation studies (Massicotte et al 1994, Molina et al 1997). These ecological data offer further support to Smith's sectional hypotheses (Smith 1964, Smith and Zeller 1966) (Fig. 4). Molina and Trappe (1994) and Molina et al (1999) suggest that because of its diversity and quantity of Pinaceae hosts, the Pacific Northwestern United States has been a major area for the evolution and speciation of *Rhizopogon* and their conifer hosts.

Evolutionary relationships at the generic level of the Pinaceae are not strongly supported in phylogenetic studies (Prager et al 1976, Price et al 1987, Chaw et al 1997, Stefanovic et al 1998). Hart's (1987) cladistic analysis of morphological characters includes the genera *Larix* Adans., *Pseudotsuga*, *Pinus*, *Abies*, *Picea* A. Dietr., and *Tsuga* Carr., but provides no measure of support for the resulting clades. In that study, *Pinus* appeared to be the ancestral host genus, while the pairs *Pseudotsuga*/*Larix* and *Abies*/

Tsuga formed a sister group. Based on comparison to *Suillus* (Kretzer et al 1996), it appears that *Rhizopogon* clades A, B, and C have retained the plesimorphic association with *Pinus*. Conversely, the monophyly of the *Rhizopogon* associates of *Pseudotsuga* (*R.* section *Villosuli* and the isolates sampled from *R.* section *Fulviglebae*) suggests a single origin of the *Pseudotsuga* mycorrhizal association within *Rhizopogon*.

TAXONOMY

We propose revision at the subgeneric levels within *Rhizopogon*. Detailed examination of species-level taxonomic relationships is beyond the scope of this study and is reserved for a future publication. Species sampled from this study are listed below in the proposed revisions. The disposition of species not included in this study must await reexamination of the types to insure accuracy of their placement.

Rhizopogon Fries in Symb. Gast. 1:5. 1817.

Type: *Rhizopogon luteolus* Fr.

Rhizopogon subgen. Rhizopogon sensu A. H. Smith emend. Grubisha & Trappe

Peridium of interwoven hyphal strands, not producing a green to olive, blue or black reaction to KOH, the strands yellow to red, reddish brown or black, lacking brown-walled hyphae on the surface of peridium or rhizomorphs, the pigments in KOH mounts not blue. *Gleba* not reacting to Melzer's reagent in shades of gray to purple, blue or black. Spores neither truncate nor amyloid. Forming mycorrhizae with *Pinus* spp. Type species: *Rhizopogon luteolus* Fr.

Commentary. Subgenus *Rhizopogon* forms a cohesive group of species with peridia formed of interwoven, cable-like mycelial strands and rhizomorphs but with nonamyloid spores. Species from this study are: *R. fuscorubens*, *R. luteolus*, *R. occidentalis*, *R. ochraceorubens*, and *R. succosus*.

Rhizopogon subgen. Amylopogon (A. H. Smith) Grubisha & Trappe, stat. nov. Basionym: *Rhizopogon* subgen. *Rhizopogon* sect. *Amylopogon* A. H. Smith, Mich. Botanist 3:17. 1964.

Peridium of usually white or, often at the surface, brown, interwoven hyphal strands with extracellular pigment deposits that in KOH mounts show pink to olive or blue pigments that form orange to red or brown pigment globules in Melzer's reagent; peridium mostly becoming dark brown to black when dried. Spores hyaline to weakly or strongly amyloid (gray, blue or purple) in Melzer's reagent mounts, if hyaline or weakly amyloid, then fresh gleba reacting to a drop of Melzer's reagent by turning gray to purple or black. Forming mycorrhizae with various genera of the Pinaceae. Type species: *Rhizopogon subpurpurascens* A. H. Smith

Commentary. Subgen. *Amylopogon* remains as originally described as a section by Smith (1964). Smith did not mention the striking peridial structure characteristic of the group: strongly interwoven, cable-like hyphal strands. Some species, e.g., *R. rudus* A. H. Smith, seem more closely related to subgen. *Villosuli*, and preliminary sequence data supports this relationship (M. Bidartondo pers comm). Species in this study included in *R.* subgenus *Amylopogon* are: *R. ellenae*, *R. semireticulatus*, *R. subcaerulescens*, *R. subgelatinosus*, and *R. subpurpurascens*.

Rhizopogon subgen. Roseoli Grubisha & Trappe, subgen. nov.

Peridium hyphis intertextis, mox lutescens vel luteobrunescens, noxis rubescens, in KOH non viridescens, olivascens, cyanescens vel nigrescens, sine hyphis brunneis in paginis peridiorum vel rhizomorphorum. Gleba solutione Melzeri non canescens, purpurascens, cyanescens vel nigrescens. Sporae truncatae vel non truncatae, nonamyloideae.

Peridium of interwoven hyphae, becoming yellow to yellowish brown early in development, often staining pink to salmon or red where cut or bruised, not producing a green to olive, blue or black reaction to KOH, lacking brown-walled hyphae on the surface of peridium or rhizomorphs, lacking blue pigments in KOH mounts. *Gleba* not reacting to Melzer's reagent in shades of gray to purple, blue or black. Spores truncate or not, not amyloid. Type species here designated: *Rhizopogon roseolus* Corda.

Rhizopogon subgen. Roseoli sect. Roseoli

As in subgen. *Roseoli* except spores not truncate. Type species here designated: *Rhizopogon roseolus* Corda.

Species in this study in sect. *Roseoli* are: *R. burlinghamii*, *R. roseolus*, and *R. vulgaris*.

Rhizopogon subgen. Roseoli sect. Fulviglebi A. H. Smith emend. Grubisha & Trappe

As in subgen. *Roseoli* except spores truncate. Type species; *Rhizopogon exiguus* Zeller.

Commentary. Subgenus *Roseoli* includes species placed by Smith and Zeller (1966) in stirps *Rubescens* and *Vulgaris*. Based on mophological, ecological, and sequence data, species in stirps *Vinicolores* (*R. diabolicus*, *R. parvulus*, *R. vinicolor*, etc.), *R. ochraceosporus*, *R. clavitisporus* and *R. subclavitisporus* placed by Smith in his section *Fulviglebae* are reassigned to *Rhizopogon* subgen. *Villosuli* sect. *Vinicolores* (this study, Smith and Zeller 1966, Molina and Trappe 1994). The remaining species from Smith's descriptions of section *Fulviglebae* appear to fit in our subgenus *Ro-*

seoli, so we are transferring the rest of section *Fulviglebi*, including the type for section *Fulviglebae R. exiguus*, as we have emended it to subgenus *Roseoli*. As more data on these species accrue, further species reassignments will likely be appropriate.

Rhizopogon subgen. Versicolores (A. H. Smith) Grubisha & Trappe stat. nov. Basionym: *Rhizopogon* subgen. *Rhizopogon* sect. *Rhizopogon* subsect. *Angustispori* ser. *Versicolores* A. H. Smith in Smith & Zeller, Mem. New York Bot Gard. 14 (2): 141. 1966.

Peridium lacking yellow colors in all stages of development, of interwoven hyphae rather than hyphal strands, in some species staining pink to red where bruised or cut. Type species: *Rhizopogon evadens* A. H. Smith.

Commentary. Subgenus *Versicolores* is phylogenetically close to subgenus *Rhizopogon* but differs strikingly from the latter in peridial structure. Morphologically it rather more closely resembles subgenus *Roseoli*, but lacks the yellow color of the latter. The molecular data indicate that this difference in peridial coloration is phylogenetically meaningful. Species in this study included in subgen. *Versicolores* are: *R. evadens* and *R. subsalmonius*.

Rhizopogon subgen. Villosuli (A. H. Smith) Grubisha & Trappe, stat. nov. Basionym: *Rhizopogon* subgen. *Rhizopogon* sect. *Villosuli* A. H. Smith, Mich. Botanist 3: 17, 1964.

With brown-walled, often versiform or flagellate hyphae thinly to thickly covering the surface of the peridium or rhizomorphs; inner peridium and adjacent gleba often with black granules in H_2O mounts, these dissolving into a green to olive pigment in KOH mounts. Spores truncate or not. Forming mycorrhizae with *Pseudotsuga* spp. Type species: *Rhizopogon villosulus* Zeller.

Rhizopogon subgen. Villosuli sect. Villosuli A. H. Smith

Peridial and rhizomorph surfaces covered thinly to thickly with brown-walled, often versiform or flagellate hyphae; spores not truncate. Type species: *Rhizopogon villosulus* Zeller

Species from this study included in subgen. *Villosuli* sect. *Villosuli*: *R. colossus*, *R. hawkerae*, *R. parksii*, *R. villescens*, *R. villosulus*, and *R. zelleri*.

Rhizopogon subgen. Villosuli sect. Vinicolores Grubisha & Trappe, sect. nov.

[≡ *Rhizopogon* subgen. *Rhizopogon* sect. *Fulviglebae* subsect. *Fulviglebae* stirps *Vinicolor* A. H. Smith nom. nud., Mem. NY Bot. Gard. 14(2):50.]

A sectione Villosuli sporis truncatis vel subtruncatis et hyphis brunneis paucis vel nullis in pagina peridii differt.

Differing from Section *Villosuli* by the truncate to subtruncate spores and few or no brown hyphae on the spore surface (but such hyphae on surface of rhizomorphs). Type species: *Rhizopogon vinicolor* A. H. Smith

Commentary. The brown-walled hyphae on surfaces of peridia and/or rhizomorphs plus the evidently obligate mycorrhizal association with *Pseudotsuga* spp. distinguish subgen. *Villosuli* from the other subgenera of the genus, and the nrDNA ITS sequence data confirm its cohesiveness, once the related species from Smith's original sect. *Fulviglebae* are included. Species from this study that are transferred from *Rhizopogon* section *Fulviglebae* Smith to subgen. *Villosuli* sect. *Vinicolores* are: *R. vinicolor*, *R. diabolicus*, *R. ochraceisporus*, and *R. parvulus*. The remaining species from section *Fulviglebae* stirps *Vinicolores* (*R. inquinatus*, *R. olivaceofuscus*, *R. subcinnamomeus*, and *R. vesiculosus*) and stirps *Clavitisporus* (*R. clavitisporus* and *R. subclavitisporus*) (Smith and Zeller 1966), are also transferred to subgen. *Villosuli* sect. *Vinicolores* based on the morphological and ecological evidence mentioned above.

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