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Taxonomy of the *Rhizopogon vinicolor* species complex based on analysis of ITS sequences and microsatellite loci

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Abstract: We are re-addressing species concepts in the *Rhizopogon vinicolor* species complex (Boletales, Basidiomycota) using sequence data from the internal-transcribed spacer (ITS) region of the nuclear ribosomal repeat, as well as genotypic data from five microsatellite loci. The *R. vinicolor* species complex by our definition includes, but is not limited to, collections referred to as *R. vinicolor* Smith, *R. diabolicus* Smith, *R. ochraceisporus* Smith, *R. parvulus* Smith or *R. vesiculosus* Smith. Holo- and/or paratype material for the named species is included. Analyses of both ITS sequences and microsatellite loci separate collections of the *R. vinicolor* species complex into two distinct clades or clusters, suggestive of two biological species that subsequently are referred to as *R. vinicolor* sensu Kretzer et al and *R. vesiculosus* sensu Kretzer et al. Choice of the latter names, as well as morphological characters, are discussed.

Key words: fungal species concepts, internal transcribed spacers, microsatellite markers, *Rhizopogon*

INTRODUCTION

Rhizopogon is an ectomycorrhizal genus in the Boletales (Basidiomycota) that forms hypogeous, truffle-like fruit bodies. It is closely related to the epigeous mushroom genera *Suillus*, *Gomphidius* and *Chroogomphus* and is thought to be derived from an epigeous (possibly *Suillus*-like) ancestor through loss of complex morphological characters, such as a stipe, vertically oriented tubes and forcible spore discharge (Bruns et al 1989). Because of its reduced morphology, *Rhizopogon* is a taxonomically difficult genus and the current taxonomy has largely been shaped by the influential work of Smith and Zeller (1966). In addition to describing numerous new species, Smith and Zeller (1966) also established a detailed subgeneric classification with two subgenera (*Rhizopogonella* and *Rhizopogon*) that comprise two sections (*Rhizopogonella*, *Fibulatae*) and four sections (*Rhizopogon*, *Amylopogon*, *Fulviglebae*, *Villosuli*), respectively. Species in the subgenus *Rhizopogonella* later have been transferred to the genus *Alpova* (Trappe 1975), and what we recognize as genus *Rhizopogon* today is essentially Smith and Zeller's subgenus *Rhizopogon* plus *Alpova olivaceotinctus* (from section *Rhizopogonella*) which has been transferred back to the genus *Rhizopogon* by Grubisha et al (2001); it remains to be determined in future studies, however, if other species from *Alpova* section *Rhizopogonella* will have to be transferred back to genus *Rhizopogon* as well.

Grubisha et al (2002) recently conducted an ITS sequence-based phylogenetic study of the genus *Rhizopogon*. They found that Smith's and Zeller's (1966) section *Amylopogon* was monophyletic, section *Rhizopogon* was polyphyletic and section *Fulviglebae* likely would have been polyphyletic, if more species had been included; those species from section *Fulviglebae* that were included in the study formed a monophyletic clade nested within a paraphyletic section *Villosuli*. Based on these results, Grubisha et al (2002) have proposed a number of changes to the subgeneric classification of *Rhizopogon* that include reinstitution of an emended subgenus *Rhizopogon*, elevation of sections *Amylopogon* and *Villosuli* to subgenus level and creation of two new subgenera, *Roseoli* and *Versicolores* (for more details see Grubisha et al 2002). Those species of section *Fulviglebae* that group with former section *Villosuli* were transferred to the new subgenus *Villosuli*. Subgenus *Villosuli* is a strongly supported, monophyletic clade that will be the focus of the study presented below. Members of the subgenus *Villosuli* share a high level of host specificity

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and are known to associate only with *Pseudotsuga* spp. (Massicotte et al 1994, Molina et al 1997). Another detailed phylogenetic study of North American collections of subgenus *Amylopogon* recently has been published by Bidartondo and Bruns (2002).

In addition to subgeneric classification schemes, there has been controversy about species delineations in *Rhizopogon*. Most species currently recognized in North America date back to Zeller and Dodge (1918), Zeller (1941) and Smith and Zeller (1966), with subsequent additions by Smith (1966, 1968), Harrison and Smith (1968), Trappe and Guzmán (1971), Hosford (1975), Cázares et al (1992) and Allen et al (1999). However, many species morphologically are very similar and described differences might represent morphological and/or ontogenetic variation within biological species. A number of synonymies therefore have been proposed (Martín et al 1998). Grubisha et al (2002) also reported a number of irregularities with species delineations and identifications. Of particular interest in the context of this study was the fact that several collections of *R. vinicolor* were not monophyletic but appeared to form a paraphyletic grade. Collections of *R. diabolicus*, *R. ochraceisporus* and *R. parvulus* were derived from within the paraphyletic *R. vinicolor* grade. We collectively will refer to these taxa as the *R. vinicolor* species complex. *R. vinicolor* and *R. ochraceisporus* can be differentiated primarily by subtle color differences in the reaction of the peridium to KOH as well as by an olive-brown versus rusty-brown ("russet") gleba at maturity, according to Smith and Zeller (1966). In *R. diabolicus*, the mature gleba is "russet," as in the case of *R. ochraceisporus*, but maintains a bright rusty-cinnamon color after drying. *R. parvulus* is distinguished by some irregularly shaped spores. Finally, we also include *R. vesiculosus* in the *R. vinicolor* species complex because Smith calls it "scarcely distinguishable" from *R. vinicolor* in the dried stage; when fresh, *R. vesiculosus* is distinguished by yellow-brown inflated cells in the epicutis (Smith and Zeller 1966).

Despite known difficulties with species delineations in the *R. vinicolor* species complex, we have chosen to work with *R. vinicolor* as a model taxon to study the population structure of hypogeous basidiomycetes (false-truffles). *Rhizopogon vinicolor* is a predominantly spring-fruiting species (Luoma et al 1991) that is very abundant in the Pacific Northwest. Most important, it forms morphologically distinct ectomycorrhizae (EM) on Douglas fir (*Pseudotsuga menziesii*) known as tuberculate EM (Zak 1971, Massicotte et al 1992). Tuberculate EM consists of tight clusters of ectomycorrhizal roots encased in a weft of hyphae that often is referred to as the "peridium". They lend themselves to population genetic work because they are large (up to several cm across) and are encountered more frequently than fruit bodies, making them relatively easy to sample in the field.

Spurred by our interest in population genetics of this group, we are re-addressing taxonomic issues and species delineations within the *R. vinicolor* species complex using both ITS sequences and microsatellite markers. Characterization of six polymorphic loci with trinucleotide repeat motifs from *R. vinicolor* was reported earlier (Kretzer et al 2000). Included in this study are several *Rhizopogon* spp. type collections from which DNA has not been extracted and analyzed before.

MATERIALS AND METHODS

Type collections were obtained from The University of Michigan Fungus Collection. When collections consisted of several sporocarps, one well-preserved sporocarp was chosen for DNA extraction and amplification. Tuberculate EM analyzed in this study were collected in the spring of 1998 in two plots, one located at Mary's Peak (44° 31.86′ N and 123° 32.86′ W) in the Oregon Coast Range and the other at Mill Creek (44° 12.17′ N and 122° 13.95′ W) in the Oregon Cascade Range. Plots were within 40–80- and 40–50-year-old second-growth forests, respectively, dominated by Douglas fir (*Pseudotsuga menziesii*), western hemlock (*Tsuga heterophylla*) and western red cedar (*Thuja plicata*). The plots were 10 m × 10 m, and an 11 × 11 point grid was established with 1 m point intervals. Soil cores (5 cm wide and approx. 30 cm deep) were taken at each grid point (121 cores per plot), and soils were sifted through a W. S. Tyler Company 2 mm sieve. In total, 30 independent collections of tuberculate EM were obtained, 15 from Mary's Peak and 15 from Mill Creek. Tuberculate EM collections were freeze-dried before molecular analysis and will be identified as MP1-98 (Mary's Peak) and MC1-98 (Mill Creek) samples, respectively. Finally, cultures of *R. vinicolor* T20787 and T20874 were kindly provided by Dr. Ari Jumponen (now at Kansas State University). Voucher collections of sporocarps from which these cultures were derived are housed in the Fungus Collection of the Oregon State University Herbarium (OSC#61147 and OSC#61148).

Genomic DNA was extracted from dried material, as described in Kretzer et al (2000). The ITS region (comprising ITS-I, the 5.8S rRNA gene, and ITS-II) was PCR-amplified with primers ITS1f and ITS4. When amplification of the entire region was unsuccessful, only ITS-I was amplified with primers ITS1f and ITS2. For primer sequences, see White et al (1990) and Gardes and Bruns (1993). PCR reactions contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 C), 2.5 mM $MgCl_2$, 0.1% Triton® X-100, 0.2 mM of each dNTP, 0.5 μM of each primer, 50 U/mL Taq DNA polymerase, and empirical amounts of template DNA. Temperature cycling conditions were: 2 min at 94 C, followed by 35–40 cycles of 45 s at 94 C, 30 s at 50 C, 60 s + 1 s/cycle at 72 C and a final extension of 10 min at 72 C. PCR products were di-

gested with *AluI* and *HinfI* to produce restriction fragment-length polymorphisms (RFLP's) according to Gardes and Bruns (1996). For sequencing purposes, PCR products were purified by electrophoresis on 1% agarose gels followed by extraction with the QIAquick Gel Extraction Kit (Qiagen Inc.). Nucleotide sequences were determined with a BigDye Terminator sequencing kit and an ABI 373 automated sequencer. Sequencing Analysis and SeqEd software was used to process raw data (PE Applied Biosystems).

PCR conditions for the amplification of microsatellite loci as well as primer sequences have been reported before (Kretzer et al 2000). Sizing of PCR products was performed by acrylamide gel electrophoresis on an ABI 377 automated sequencer using the "GS500 Tamra" internal size standard. Band sizes were estimated with GeneScan software (PE Applied Biosystems). Because the mobility of DNA fragments is influenced by base composition as well as the fluorescent dyes used, size units translate only roughly into numbers of basepairs and commonly include fractions of a unit. Nevertheless, they are known to be highly reproducible with standard deviations for fragments in the 200–300 bp range commonly ≤0.15 (e.g., Haberl and Tautz 1999). Alleles therefore were scored as different whenever a break in the continuous distribution of allele measurements was detected. In some cases, that meant that allele sizes might differ by as little as 0.5 size units. Small differences of this kind can be explained by single basepair insertions and/or substitutions that are known to occur in the microsatellite flanking regions (e.g., Ortí et al 1997).

Phylogenetic analysis of ITS sequences was conducted in PAUP* (Sinauer Associates Inc.). Alignment of ITS sequences was performed manually with the PAUP* editor and a color font. Sequences immediately adjacent to the priming sites of the sequencing primers were of poor quality in some of the sequences, and the respective areas from the 18S, 5.8S and 28S genes were excluded from the analysis. Four additional nucleotide positions were excluded because they appeared to be polymorphic, not only between taxa but also within individual collections as indicated by double peaks in the sequencing chromatograms. Transitions and transversions were weighted equally (=1 step). Alignment gaps were treated as missing data, but parsimony-informative alignment gaps were recoded by changing one character per alignment gap to a new state "I" (for "indel"). The new state "I" was introduced in the same position, when alignment gaps were identical in size and position and in different positions otherwise. Change from any nucleotide to an "I" was weighted as one step. Parsimony analysis was performed using the heuristic search option with 10 random sequence additions. Bootstrap analysis was based on 10 000 replicates using the fast stepwise addition option. The dataset minus the excluded positions was deposited at TreeBase.

Microsatellite data were analyzed by neighbor-joining (NJ) analysis of genotypic distances. Allele frequency-based analyses were not possible because of the relatively small number of type collections available for many of the species analyzed here and because the group is taxonomically so difficult that existing species concepts can be tested reliably only on type material. We used a simple allele sharing distance, which is [1 − (no. of shared alleles/2 × no. of loci compared)] (Bowcock et al 1994). Distances based on stepwise mutation models were not suitable for our dataset, because at some loci alleles sizes were not strictly spaced in multiples of three nucleotides as would be expected for loci with trinucleotide repeats under a stepwise mutation model. As already was discussed, irregular spacing can be caused by small insertions/deletions and/or substitutions in the microsatellite flanking regions, and was particularly pronounced when collections belonging to different species were analyzed together.

RESULTS

A total of 14 new ITS sequences were determined in this study and are listed in TABLE I with their GenBank accession numbers. Most new sequences were derived from holo- and paratype material designated by Alexander Smith (Smith and Zeller 1966) and made available to us by The University of Michigan Fungus Collection. Collection AHS68673, the holotype for *R. parvulus*, was not available to us for DNA extraction because of small collection size, and DNA extraction and amplification from collection AHS65267, the holotype for *R. ochraceisporus*, was unsuccessful. All tuberculate EM collections were screened before sequencing by ITS-RFLP's, using the PCR primers ITS1f and ITS4 and the restriction enzymes *AluI* and *HinfI*. Two different fungal ITS-RFLP patterns were observed with the restriction enzyme *AluI*, and one exemplary collection of each pattern was chosen for sequencing (for details on band sizes see discussion).

ITS sequences determined in this study were aligned and analyzed together with 18 sequences from *Rhizopogon* subgenus *Villosuli* that were determined in a previous study (Grubisha et al 2002). The complete ITS dataset included 32 taxa and 542 characters, of which 47 characters were parsimony informative. Parsimony analysis resulted in 100 most-parsimonious trees that were 81 steps long. One of the most-parsimonious trees is shown in FIG. 1A, and branches supported by all 100 trees are highlighted in bold lines. Numbers above branches indicate support from bootstrap analysis. Collections of the *R. vinicolor* species complex fall into two well-resolved and strongly supported clades that further on we will refer to as *R. vinicolor* sensu Kretzer et al and *R. vesiculosus* sensu Kretzer et al (for choice of clade names, see discussion); there was no resolution between collections within either clade due to little or no sequence variation. Both *R. vinicolor* sensu Kretzer et al and *R. vesiculosus* sensu Kretzer et al clades include exemplary sequences from tuberculate EM, indicating that taxa in both clades form

TABLE I. Collections from the *R. vinicolor* species complex studied by either ITS sequence or microsatellite analysis or both. Additional ITS sequences from *Rhizopogon* subgenus *Villosuli* were taken from Grubisha et al. (2002)

Collection	No.	Type	Origin	ITS accession
R. diabolicus	AHS68404	paratype	Pend Oreille Co., WA	AF366386
R. diabolicus	AHS68424	paratype	Pend Oreille Co., WA	Grubisha et al., 2002
R. diabolicus	AHS68489	paratype	Bonner Co., ID	AF366387
R. diabolicus	AHS70305	holotype	Idaho Co., ID	AF366388*
R. ochraceisporus	AHS58928	paratype	Idaho Co., ID	AF366389
R. ochraceisporus	AHS65963	paratype	Adams Co., ID	Grubisha et al., 2002
R. ochraceisporus	AHS68395	paratype	Pend Oreille Co., WA	AF366390*
R. ochraceisporus	T17916	—	OR	Grubisha et al., 2002
R. ochraceisporus	T17944	—	OR	Grubisha et al., 2002
R. parvulus	AHS68364	paratype	Bonner Co., ID	Grubisha et al., 2002
R. vesiculosus	AHS68040	holotype	Bonner Co., ID	AF366391
R. vesiculosus	AHS68041	paratype	Bonner Co., ID	AF366392
R. vinicolor	Tru2195	holotype	Boise Co., ID	AF263929
R. vinicolor	AHS68071	paratype	Bonner Co., ID	AF418268
R. vinicolor	AHS68092	paratype	Pend Oreille Co., WA	AF263930
R. vinicolor	AHS68177	paratype	Bonner Co., ID	—
R. vinicolor	AHS68179	paratype	Bonner Co., ID	AF263931
R. vinicolor	AHS68189	paratype	Bonner Co., ID	—
R. vinicolor	AHS68227	paratype	Pend Oreille Co., WA	—
R. vinicolor	AHS68576	paratype	Bonner Co., ID	—
R. vinicolor	AHS68579	paratype	Bonner Co., ID	—
R. vinicolor	AHS68595	paratype	Bonner Co., ID	—
R. vinicolor	AHS68602	paratype	Bonner Co., ID	AF263932
R. vinicolor	AHS68690	paratype	Bonner Co., ID	—
R. vinicolor	AHS68705	paratype	Bonner Co., ID	—
R. vinicolor	T17899	—	Benton Co., OR	Grubisha et al., 2002
R. vinicolor	T19383	—	Coos Co., OR	Grubisha et al., 2002
R. vinicolor	T20787	—	Adams Co., ID	Grubisha et al., 2002
R. vinicolor	T20874	—	Bonner Co., ID	—
Tuberculate EM	MC1-98-H9 = type I	—	Lane Co., OR	AF263938
Tuberculate EM	MC1-98-B0 = type II	—	Lane Co., OR	AF263939
Tuberculate EM	MC1-98-XX	—	Lane Co., OR	—
Tuberculate EM	MP1-98-XX	—	Benton Co., OR	—

* Only the ITS1 region was successfully amplified and sequenced; see methods.

ectomycorrhizae with tuberculate morphology on Douglas fir.

We subsequently have scored microsatellite alleles at five loci for 22 sporocarp voucher collections from the *R. vinicolor* species complex (again mostly type material, see FIG. 1B and TABLE I) and 30 collections of tuberculate EM from two plots described in the methods. Characterization of six polymorphic microsatellite loci from *R. vinicolor* T20787 was reported previously (Kretzer et al 2000). PCR primers developed for five of the six loci also were found to amplify fragments of expected size from collections of *R. vesiculosus* sensu Kretzer et al; those were the primers for loci Rv02, Rv15, Rv25, Rv46 and Rv53, which consequently were used in this study. Microsatellite markers revealed that the 30 collections of tuberculate EM made in two 10 m by 10 m plots represented a total of 11 different multilocus genotypes (= genets); six were genets of *R. vinicolor* sensu Kretzer et al, and five were genets of *R. vesiculosus* sensu Kretzer et al as indicated by ITS-RFLP analysis conducted earlier (see first paragraph of the results section). Details on the size and distribution of genets across both plots will be published elsewhere. To avoid redundancy, only one representative tuberculate EM sample of each genet was included in the cluster analysis presented below; in addition, one genet was omitted from the analysis because of missing data at one locus.

Calculation of multilocus pairwise distances (see methods) and neighbor-joining analysis resulted in the phenogram shown in FIG. 1B. It separates collections into two distinct clusters, which correspond to *R. vinicolor* sensu Kretzer et al and *R. vesiculosus* sensu Kretzer et al clades in FIG. 1A. Within the two clusters, however, again there is no clear evidence for

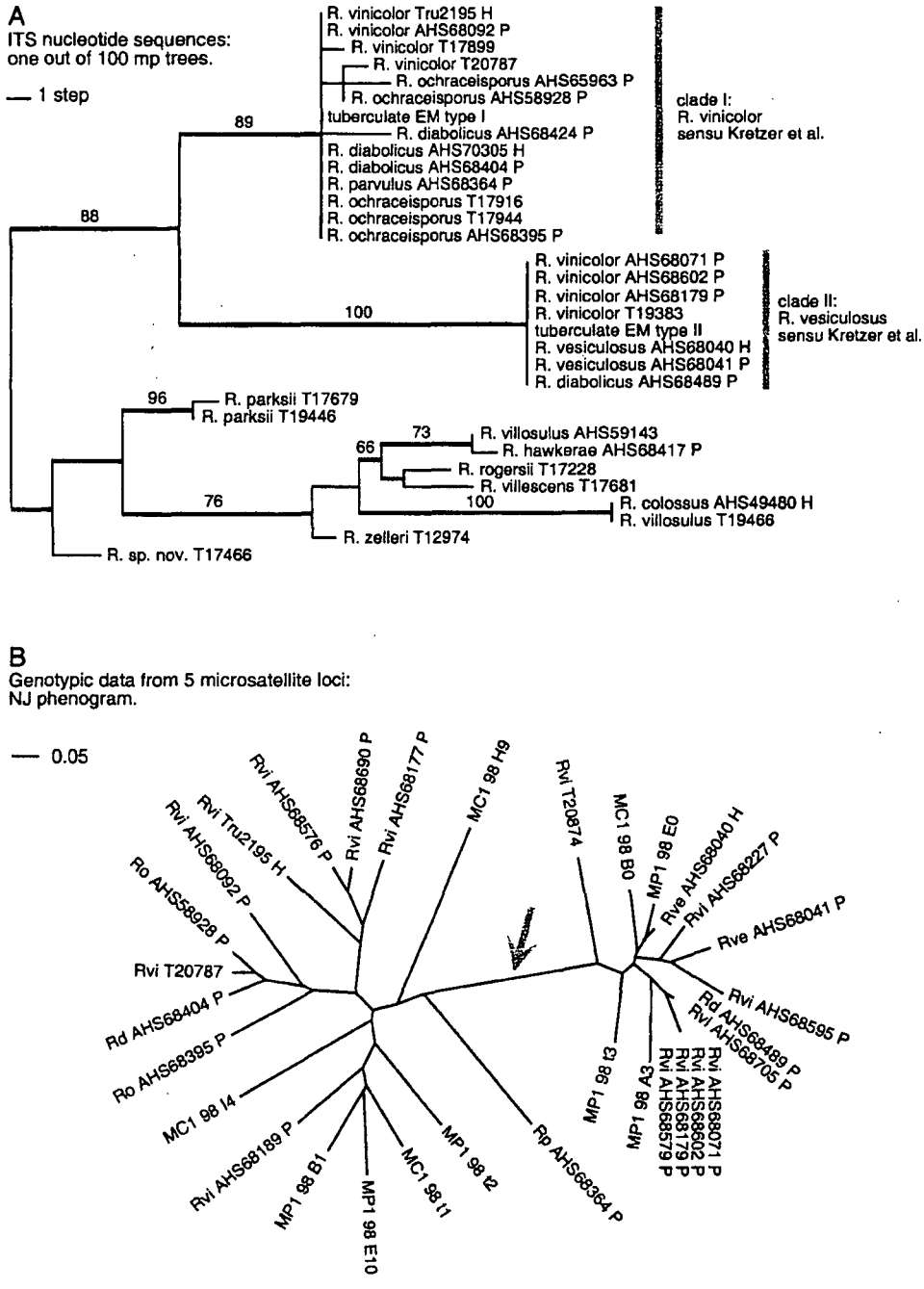

FIG. 1. (A) One of 100 most-parsimonious (mp) trees obtained from nucleotide sequences of the internal-transcribed spacer region. Branches that are supported by all 100 mp trees are highlighted in bold; numbers above branches indicate support from bootstrap analysis. The tree is unrooted. (B) Neighbor-joining phenogram based on genotypic data from five microsatellite loci. Abbreviations are: Rd = *R. diabolicus*, Ro = *R. ochraceisporus*, Rp = *R. parvulus*, Rve = *R. vesiculosus*, Rvi = *R. vinicolor*, H = holotype, P = paratype. MC1 and MP1 samples are from tuberculate mycorrhizae; see Methods. Arrow indicates branch separating *R. vinicolor* sensu Kretzer et al and *R. vesiculosus* sensu Kretzer et al.

any sort of sub-clustering that would correlate with current taxonomy or otherwise indicate the presence of multiple biological species. Internal branch lengths are longer in the *R. vinicolor* sensu Kretzer et al cluster than in the *R. vesiculosus* sensu Kretzer et al cluster. This simply reflects the fact that the microsatellite markers originally were developed for *R. vinicolor*, and it is not uncommon for microsatellite markers to be less polymorphic in nontarget species than in target species (e.g., FitzSimmons et al 1995).

DISCUSSION

Both the ITS sequences and the microsatellite loci analyzed in this study separated collections of the *R. vinicolor* species complex into two distinct clades or clusters indicative of two biological species. These findings are in taxonomic conflict with the five or more species names applied to this group. All species in question were described by Alexander Smith (Smith and Zeller 1966), who intended to document all observed morphological diversity and to establish narrow species concepts, potentially including ontogenetic or environmental variants as discrete species (Smith and Thiers 1971). To address this possibility, a re-evaluation of the main morphological characters used by Smith to separate the species is warranted. A key character in differentiating *R. vinicolor* sensu Smith and *R. ochraceisporus* is the color reaction of the peridium to KOH. The differences, however, not only are subtle but also are influenced by the developmental stage. *Rhizopogon vinicolor* sensu Smith and *R. ochraceisporus* also differ in the color of the gleba at maturity, which is "dark olive-brown" in the former and "rusty cinnamon-brown" (="russet") in the latter. Color characteristics of immature fruit bodies are given in much detail for *R. vinicolor* but are absent from the description of *R. ochraceisporus*. We think that *R. ochraceisporus* constitutes a mature color variant of *R. vinicolor*. Similarly, *R. diabolicus* is characterized by a "russet" gleba at maturity that retains a "bright rusty cinnamon" color when dried. We examined type material for both *R. diabolicus* (AHS70305 = holo, AHS68404 = para) and *R. ochraceisporus* (AHS65267 = holo, AHS68395 = para) and found the color of the dried glebas comparable if not identical. *R. diabolicus* AHS68489 (=paratype) did not have a "rusty cinnamon" gleba, an observation that is consistent with its placement within the *R. vesiculosus* group by both ITS sequence and microsatellite data (FIG. 1). Mature *R. diabolicus* sporocarps strongly resemble *R. ochraceisporus* ("ochraceous" to "rusty brown" peridium staining "rusty brown" in KOH and dark "olive" in $FeSO_4$, "russet" gleba), according to Smith's description, while immature sporocarps bear much resemblance to *R. vinicolor*, including the white peridium with "vinaceous" flushes, the "lilaceous" or "vinaceous" reaction to KOH and the weak reaction to $FeSO_4$. Finally, spores of all three species (described by predominant size and extremes) are comparable in size (approx. 6.5–9.0 × 3.0–4.5 μm) (Smith and Zeller 1966). In conclusion, we believe that *R. vinicolor*, *R. ochraceisporus* and *R. diabolicus* are synonyms, an interpretation that also is supported by neighbor-joining analysis of multilocus genotypic distances (FIG. 1B). None of the three species names has priority over the others, and we therefore chose *R. vinicolor* (sensu Kretzer et al) for this group, because it currently is the most widely used name and therefore provides the greatest taxonomic continuity.

The taxonomic status of *R. parvulus* is more difficult to interpret, largely because only a single collection (AHS68364 = paratype) was available to us for DNA extraction. This collection clusters tightly with *R. vinicolor* sensu Kretzer et al in the ITS tree, a finding that is consistent with most morphological characters (Smith and Zeller 1966). In the microsatellite-based phenogram, however, it falls just slightly outside the *R. vinicolor* sensu Kretzer et al cluster. It is different from *R. vinicolor* sensu Smith primarily in that it has slightly larger (8–10 × 4–5 μm) and often irregularly shaped spores. Even if *R. parvulus* should constitute an independent species or hybrid, it is unlikely to confound future population genetic work because it is very rare.

In both the ITS tree and the microsatellite phenogram, a large number of apparently misidentified *R. vinicolor* collections group strongly and distinctly with *R. diabolicus* AHS68489 (discussed above) and with two collections of *R. vesiculosus*, including the holotype (AHS68040). Because again there is no indication for sub-clustering within this group from either dataset, we shall refer to it collectively as *R. vesiculosus* sensu Kretzer et al (FIG. 1A). As noted by Smith and Zeller (1966), *R. vesiculosus* is "scarcely distinguishable" from *R. vinicolor* in the dried stage. This high degree of similarity might explain the large number of misidentified collections, not only among Smith's own "*vinicolor*" collections but also among OSC herbarium material (data not shown). When fresh, the color of the mature gleba distinguishes both species, an observation that is consistent with our own (see below). Unfortunately, we have not been able to observe another diagnostic character for *R. vesiculosus* that Smith and Zeller (1966) describe as "yellow-brown (fresh) inflated cells" found in the epicutis that are "similar in size and shape to those found in many species of section *Villosuli*". Although these cells readily can be observed in dried material from section *Villosuli*, Smith reports that they collapse in *R. vesiculosus* upon drying and then are "not so readily demonstrable" thus making use of this character difficult to test. We have not undertaken microscopic studies of this character in fresh material but have been unable to observe the inflated cells either in Smith's collections or in our own dried collections. In addition, *R. vesiculosus* sensu Kretzer et al differs from Smith's description of *R. vesiculosus* in having a wider range of spore lengths (approx. (5)

6–9 (10) μm versus 6–6.5 μm) and in growing under Douglas fir rather than lodgepole pine.

Based on our extensive sampling of spring-fruiting *Rhizopogon* species under Douglas fir around Mary's Peak in the Oregon Coast Range, we find that the most useful characters for differentiating sporocarps of *R. vinicolor* sensu Kretzer et al and *R. vesiculosus* sensu Kretzer et al are the colors of the fresh peridium and gleba. However, not all stages of development are equally distinctive and more than one developmental stage often is needed to unambiguously assign material to either species: Both species begin with a white peridium that bruises pinkish-red (Smith's "vinaceous"), but only *R. vinicolor* sensu Kretzer et al develops vivid yellow patches during early maturity. At maturity, both species are light yellowish brown (Smith's "ochraceous") and turn various shades of brown from handling, the shades typically reflecting the color of the mature gleba underneath (see below). In *R. vinicolor* sensu Kretzer et al, the gleba develops from white when immature through pale yellow and pale greenish yellow-brown, to dark greenish brown (Smith's "olive-brown") or brown or more rarely reddish brown (Smith's "rusty cinnamon-brown" or "russet"). On the other hand, the gleba of *R. vesiculosus* sensu Kretzer et al appears to develop from white to greenish brown (a stage that apparently is short and was not noted in the type description) to dark blackish-brown (dominant stage). Finally, we find that tuberculate EM of both species also differ somewhat in morphology. The weft of darkly pigmented hyphae that encases the clustered ectomycorrhizae is fluffy in *R. vinicolor* and attached to the ectomycorrhizae in such a way that it molds to the ectomycorrhizae and cannot be peeled back readily. In *R. vesiculosus*, it is appressed but detached from the ectomycorrhizae such that it can be peeled back in large patches. Although a more detailed and formalized description of morphological differences at the EM level would be desirable, it was not the goal of this study.

Ultimately, sporocarps and tuberculate EM of both species most readily and reliably can be differentiated by ITS-RFLP's with the restriction enzyme *Alu*I. When PCR primers ITS1f and ITS4 are used, a single undigested band of size 743 bp characterizes *R. vesiculosus* sensu Kretzer et al while three bands of sizes 419 bp, 224 bp and 97 bp are most typical of *R. vinicolor* sensu Kretzer et al (a C to T transition is occasionally observed in one of the restriction sites of *R. vinicolor* and results in only two bands of sizes 516 bp and 224 bp). Exact band sizes have been deduced from nucleotide sequences.

Our data support the conclusion that, within our sampling range (Oregon, Washington and Idaho), the *R. vinicolor* species complex is composed of two sympatrically distributed, phylogenetic species (indicated by ITS sequence analyses), which correlate with biological species (indicated by microsatellite genotypic distances). In future population genetic work, both species readily can be differentiated from either reproductive or vegetative structures using ITS-RFLP's as described.

From a taxonomic point of view, we have shown that, in *Rhizopogon* subgenus *Villosuli*, paratype material in many cases cannot be relied on to represent the holotype. That puts us in a difficult position for making taxonomic changes with respect to *R. ochraceisporus* because DNA from the holotype was not successfully amplified. Two lines of evidence, however, lead us to believe that *R. vinicolor* and *R. ochraceisporus* should be regarded as synonyms: (i) Morphological characters as discussed above do not provide strong evidence against synonymy. In particular, we believe that morphology strongly supports synonymy of *R. ochraceisporus* and *R. diabolicus* (see above); the latter in turn is supported by our molecular data to be synonymous with *R. vinicolor*. (ii) The three paratype collections of *R. ochraceisporus* analyzed here actually did cluster within the same clade, suggesting that paratype material for this particular species might be more consistent than other species. In the next section, we therefore formally propose synonymization of *R. vinicolor*, *R. ochraceisporus* and *R. diabolicus*. Although we believe that these changes best reflect the current state of knowledge, this study does not claim to be an exhaustive treatment of the *R. vinicolor* species complex. The study was guided primarily by our desire to clarify species delineations in the Pacific Northwest, which is the geographic center of our ongoing population genetic work, and, through incorporation of type material, to provide valid names for the two species identified. The selection of taxa to be included in this study was based largely on pre-existing molecular evidence that suggested particularly close affiliations of these taxa with *R. vinicolor* (Grubisha et al 2002). A future, more comprehensive study should include vinicolor-like collections from a wider geographic range, as well as type material from other morphologically similar species, such as *R. cinnamomeus* Harrison and Smith, *R. subcinnamomeus* Smith, *R. olivaceofuscus* Smith, *R. pachyspora* Hosford and others.

TAXONOMY

Rhizopogon vinicolor A. H. Smith.

Basionym: *Rhizopogon vinicolor* A. H. Smith. In Smith and Zeller, 1966: *Mem. N. Y. Bot. Gard.* 14 (2): 67–69.

Synonyms: *Rhizopogon ochraceisporus* A. H. Smith. In

Smith and Zeller, 1966: *Mem. N. Y. Bot. Gard.* 14 (2): 62. *Rhizopogon diabolicus* A. H. Smith. In Smith and Zeller, 1966: *Mem. N. Y. Bot. Gard.* 14 (2): 64–65.

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LITERATURE CITED

Allen MF, Trappe JM, Horton TR. 1999. NATS truffle and truffle-like fungi 8: *Rhizopogon mengei* sp. nov. (Boletaceae, Basidiomycota). Mycotaxon 70:149–152.

Bidartondo MI, Bruns TD. 2002. Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. Mol Ecol 11:557–569.

Bowcock AM, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd JR, Cavalli-Sforza LL. 1994. High resolution of human evolutionary trees with polymorphic microsatellites. Nature 368:455–457.

Bruns TD, Fogel R, White TJ, Palmer JD. 1989. Accelerated evolution of a false-truffle from a mushroom ancestor. Nature 339:140–142.

Cázares E, García J, Castillo J, Trappe JM. 1992. Hypogeous fungi from northern Mexico. Mycologia 84:341–359.

FitzSimmons NN, Moritz C, Moore SS. 1995. Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. Mol Biol Evol 12:432–440.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118.

———, ———. 1996. ITS-RFLP matching for identification of fungi. Meth Mol Biol 50:177–186.

Grubisha LC, Trappe JM, Molina R, Spatafora JW. 2001. Biology of the ectomycorrhizal genus *Rhizopogon*. V. Phylogenetic relationships in the Boletales inferred from LSU rDNA sequences. Mycologia 93:82–89.

———, ———, ———, ———. 2002. Biology of the ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of internal transcribed spacer sequences. Mycologia 94:607–619.

Haberl M, Tautz D. 1999. Comparative allele sizing can produce inaccurate allele size differences for microsatellites. Mol Ecol 8:1347–1349.

Harrison KA, Smith AH. 1968. Some new species and distribution records of *Rhizopogon* in North America. Can J Bot 46:881–899.

Hosford DR. 1975. Taxonomic studies on the genus *Rhizopogon* I. Two new species from the Pacific Northwest. Beih Nova Hedwigia 51:163–169.

Kretzer AM, Molina R, Spatafora JW. 2000. Microsatellite markers for the ectomycorrhizal basidiomycete *Rhizopogon vinicolor*. Mol Ecol 9:1190–1191.

Luoma DL, Frenkel RE, Trappe JM. 1991. Fruiting of hypogeous fungi in Oregon Douglas fir forests: seasonal and habitat variation. Mycologia 83:335–353.

Martín MP, Högberg N, Nylund J-E. 1998. Molecular analysis confirms morphological reclassification of *Rhizopogon*. Mycol Res 102:855–858.

Massicotte HB, Melville LH, Li CY, Peterson RL. 1992. Structural aspects of Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] tuberculate ectomycorrhizae. Trees 6: 137–146.

———, Molina R, Luoma DL, Smith JE. 1994. Biology of the ectomycorrhizal genus *Rhizopogon*: II. Patterns of host-fungus specificity following spore inoculation of diverse hosts grown in monoculture and dual culture. New Phytol 126:677–690.

Molina R, Smith JE, McKay D, Melville LH. 1997. Biology of the ectomycorrhizal genus *Rhizopogon*. III. Influence of co-cultured conifer species on mycorrhizal specificity with the arbutoid hosts *Arctostaphylos uva-ursi* and *Arbutus menziesii*. New Phytol 137:519–528.

Ortí G, Pearse DE, Avise JC. 1997. Phylogenetic assessment of length variation at a microsatellite locus. Proc Natl Acad Sci USA 94:10745–10749.

Smith AH. 1966. New and noteworthy higher fungi from Michigan. Mich Bot 5:18–25.

———. 1968. Further studies on *Rhizopogon*. I. J Elisha Mitchell Sci Soc 84:274–280.

———, Thiers HD. 1971. The Boletes of Michigan. Ann Arbor: University of Michigan Press. 428 p.

———, Zeller SM. 1966. A preliminary account of the North American species of *Rhizopogon*. Mem N Y Bot Gard 14:1–178.

Trappe JM. 1975. A revision of the genus *Alpova* with notes on *Rhizopogon* and the Melanogastraceae. Beih Nova Hedwigia 51:279–309.

———, Guzmán G. 1971. Notes on some hypogeous fungi from Mexico. Mycologia 63:317–332.

White TJ, Bruns TD, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols; a guide to methods and applications. San Diego: Academic Press. p 315–322.

Zak B. 1971. Characterization and classification of mycorrhizas of Douglas fir. II *Pseudotsuga menziesii* + *Rhizopogon vinicolor*. Can J Bot 49:1048–1079.

Zeller SM. 1941. Further notes on fungi. Mycologia 33:196–214.

———, Dodge CW. 1918. *Rhizopogon* in North America. Ann Mo Bot Gard 5:1–36.