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REGIONAL SPECIALIZATION OF *SARCODES SANGUINEA* (ERICACEAE) ON A SINGLE FUNGAL SYMBIONT FROM THE *RHIZOPOGON ELLENAE* (RHIZOPOGONACEAE) SPECIES COMPLEX[1]

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We have sampled the mycorrhizal roots of 76 snow plants (*Sarcodes sanguinea,* Monotropoideae, Ericaceae) in two areas of the Sierra Nevada of California that are ~180 km apart. To identify the fungal symbionts associated with these plants, we first analyzed restriction fragment length polymorphisms (RFLPs) of the internal transcribed spacer region (ITS) of the fungal nuclear ribosomal repeat. Fungal ITS-RFLPs were successfully produced from 57 of the 76 plants sampled, and all symbionts shared the same DNA fragment pattern. The morphology of *S. sanguinea* mycorrhizae was consistent with that expected from a *Rhizopogon* species in section *Amylopogon.* To confirm and refine this identification, a total of six fungal ITS sequences were determined from *S. sanguinea* mycorrhizae. These sequences were analyzed together with eight existing and eight newly determined ITS sequences from *Rhizopogon* section *Amylopogon.* The newly determined sequences include an ITS sequence from the fungal symbiont of pine drops (*Pterospora andromedea,* Monotropoideae, Ericaceae), a plant that was previously reported to be exclusively associated with the *Rhizopogon subcaerulescens* group. When these sequences were analyzed together, the *Sarcodes* symbionts grouped tightly with several collections of *R. ellenae* including the holotype, one collection of *R. idahoensis,* and one collection of *R. semireticulatus.* A different lineage comprised collections of *R. subgelatinosus, R. subcaerulescens,* another collection of *R. semireticulatus,* and the *Pterospora* symbiont. We conclude that *S. sanguinea* associates exclusively with a single species in the *R. ellenae* species complex throughout our sampling range. These results indicate a much higher level of specificity in *S. sanguinea* than was previously reported and confirm the emerging pattern that nonphotosynthetic, monotropoid plants generally associate very specifically with a narrow range of ectomycorrhizal fungi.

Key words: monotropoid mycorrhizae; *Pterospora andromedea*; *Rhizopogon*; *Sarcodes sanguinea*; specificity (host).

The snow plant, *Sarcodes sanguinea,* is the only member of a monospecific genus in the Monotropoideae (Ericaceae) and occurs throughout the Sierra Nevada of California as well as in southern Oregon, western Nevada, and northern Mexico (Wallace, 1975). Like other members of the Monotropoideae, it is nonphotosynthetic and must receive all of its carbon from fungal root symbionts. The roots of *S. sanguinea* are coralloid, fleshy, and surrounded by a fungal mantle except for the tips, which are uncolonized. Although the importance of fungal root symbionts for the nutrition of monotropoid plants has been recognized for a long time (e.g., Kamienski, 1881; Francke, 1934), the fungi involved are just starting to be identified through the use of molecular techniques. Cullings, Szaro, and Bruns (1996) sampled several monotropoid taxa mostly in the western United States and used partial sequence analysis as well as oligonucleotide probing of the mitochondrial large subunit rDNA to identify the fungal symbionts. The picture that emerged from these studies was that in contrast to photosynthetic mycorrhizal plants, which typically associate with large numbers of distantly related fungi, most monotropes appear to associate with specific groups of closely related ectomycorrhizal fungi: *Monotropa uniflora* was found associated with taxa in the Russulaceae, while *Pterospora andromedea* was found only in symbiosis with the *Rhizopogon subcaerulescens* species group. Recently, Lefevre, Carter, and Molina (1998) found *Allotropa virgata* to be specifically associated with *Tricholoma magnivelare.* This pattern of specificity is also observed in nonphotosynthetic orchids (Taylor and Bruns, 1997), and so it appears that specific fungal associations are the norm in nonphotosynthetic mycorrhizal plants.

Sarcodes sanguinea so far appeared to be the only exception to this pattern. Cullings, Szaro, and Bruns (1996) reported that it associated with at least three distantly related lineages of fungi within the Hymenomycetes. Furthermore, only 12 individual plants were sampled; thus the diversity of associates could have been underestimated. None of the symbionts of *S. sanguinea* was identified to species level, and only one fell within a lineage known to be ectomycorrhizal at the time of publication. For these reasons we decided to investigate the mycorrhizal associates of *S. sanguinea* more closely.

MATERIALS AND METHODS

Sampling—Rootballs of *S. sanguinea* were sampled by digging a hole next to a flowering plant and by carefully following the inflorescence to locate the

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TABLE 1. *Sarcodes sanguinea* rootballs sampled.

Location	Year	No. populations	No. plants	No. symbionts identified
Shaver Lake Area				
''high-density'' site[d]	1995	1	15	15[a]
	1997		11	9[b]
along McKinley grove road[e]	1996	3	13	9[a]
along roads no. 168 and 80[f]	1996	6	15	13[a]
near the USFS Dinkey Creek work station	1997	1	1	1[b]
Lake Tahoe Basin	1997	6	21	10[a] (+1[c])
Total		17	76	58 (76%)

[a] Identified by RFLP analysis after direct amplification of the fungal ITS region from *S. sanguinea* roots.

[b] Identified by RFLP analysis of fungal cultures derived from *S. sanguinea* roots.

[c] One symbiont from the Lake Tahoe populations was identified directly by sequencing of the fungal ITS region without RFLP analysis.

[d] This site is located at 119°07′50′′ W and 37°09′06′′ N. The spatial distribution of *Abies magnifica* ectomycorrhizae around *S. sanguinea* rootballs has also been studied at this site and is being reported in an accompanying paper.

[e] Between Dinkey Creek and McKinley Grove.

[f] Between Shaver Lake and Huntington Lake, and between Huntington Lake and Mono Hot Springs.

Fig. 1. Location of the two sampling areas within California. The areal distance between Lake Tahoe and Shaver Lake is ~180 km. Grey shades indicate counties where *Sarcodes sanguinea* occurs.

rootball; small pieces of roots were removed, rinsed in water, and either used for culturing or lyophilized for molecular analysis.

In the spring of 1995, 1996, and 1997, we sampled a total of 64 *S. sanguinea* rootballs for direct identification of the fungal symbiont based on polymerase chain reaction (PCR) with fungus-specific primers. The plants originated from 16 populations in two areas of the Sierra Nevada of California (Table 1, Fig. 1). Plants sampled within a population were 5–100 m apart, and populations were 5–180 km apart. Twelve additional plants were sampled in the spring of 1997 at the ''high-density site'' and at one other site (Table 1) for cultivation of the fungal symbiont from *S. sanguinea* roots (and subsequent identification of the cultured mycelium).

Cultivation of the fungal symbiont—Multiple root tips from 12 different *S. sanguinea* plants were washed, surface sterilized in 30% H_2O_2 for 10 sec, rinsed in sterile distilled water, and cut under a dissecting microscope into small pieces. Pieces of root that appeared healthy and well colonized by the symbiont were placed onto malt extract glucose agar (Stevens, 1981) that had been supplemented with 50 mg chloramphenicol, 50 mg streptomycin, 50 mg ampicillin, and 1 mg benomyl per litre. Fast growing and conidial isolates (primarily *Mucor*) were removed and discarded. After ~4 wk of growth, cultures were transferred to modified Hagem's without antibiotics.

Molecular methods—Genomic DNA was extracted from mycorrhizae, fruitbodies, or mycelium using the method of Gardes and Bruns (1993). PCR reactions contained 10 mmol/L Tris/HCl pH 8.3, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.1 mg/mL gelatin, 200 μmol/L of each of the four deoxyribonucleotide triphosphates, 0.5 μmol/L of each of two different primers, 25 U/mL *Taq* polymerase, and empirical amounts of genomic DNA. Reaction conditions were: denaturing at 94°C for 35 sec, annealing at 53°C for 55 sec, and polymerization at 72°C initially for 45 sec but increasing by 4 sec on every cycle (35 cycles total). PCR primers used were either ITS5 (general) or ITS-1f (fungus-specific) in combination with either ITS4 (general) or ITS-4b (basidiomycete-specific). For sequencing, the internal primers ITS2 and ITS3 were used as well. For primer sequences see White et al. (1990) and Gardes and Bruns (1993).

ITS-RFLPs were produced as described before (Gardes and Bruns, 1996). In brief, the internal transcribed spacer (ITS) of the nuclear ribosomal repeat was amplified by PCR and subsequently digested with the restriction enzymes *Alu*I and *Hinf*I; *Dpn*II and *Cfo*I were also used occasionally. Restriction fragments were then separated on agarose gels (1% ultrapure agarose from GibcoBRL, Grand Island, New York, and 2% NuSieve agarose from FMC BioProducts, Rockland, Maine, USA) and visualized with ethidium bromide.

PCR products were prepared for sequencing using a QIAquick PCR purification kit (QIAGEN, Valencia, California, USA). Nucleotide sequences were determined by the cyclic reaction termination method using fluorescence labeled dideoxyribonucleotide triphosphates. Sequencing reactions and the processing of reaction products were performed following instructions for the ABI PRISM® Dye Terminator Cycle Sequencing Core Kit (PE Applied Biosystems, Foster City, California, USA). Electrophoresis and data collection were done on an ABI Model 377 DNA Sequencer (Perkin-Elmer Corporation). DNA Sequencing Analysis (version 2.1.2) and Sequence Navigator (version 1.0.1) were used for processing the raw data.

Phylogenetic analysis—Phylogenetic analyses were performed in PAUP*. ITS sequences were aligned manually using the PAUP editor and a color font. Sequences immediately adjacent to the priming sites of the sequencing primers were of low quality in some of the sequences, and the respective areas from the 18S, 5.8S and 28S genes were excluded from the analysis. Within the areas included in the active data set, most single-basepair alignment gaps were parsimony uninformative and were treated as missing data. Presence or absence of one parsimony informative single-bp alignment gap and several 2–4 bp alignment gaps was weighted equal to one substitution (=1 step). Technically, this was done by replacing one dash per alignment gap with a new character state ''I.'' The longest alignment gap observed was 6 bp long, and was coded for by inserting the character state ''I'' twice, giving it a total weight of two steps. This recoding method attempts to conserve some of the information provided by alignment gaps, without weighting the insertion or deletion of every single nucleotide equal to a substitution. This downweighting of the alignment gaps seems justified, because several of the larger insertions (deletions) were found to represent duplications (losses) of two and four nucleotide motifs likely stemming from single insertion (deletion) events. The alignment with the recoded gaps can be viewed at http://ajbsupp.botany.org/v87/kretzer.txt.

Most parsimonious trees were retrieved through ten heuristic searches with random sequence addition. Bootstrap values are based on 500 replicates with five random sequence additions each.

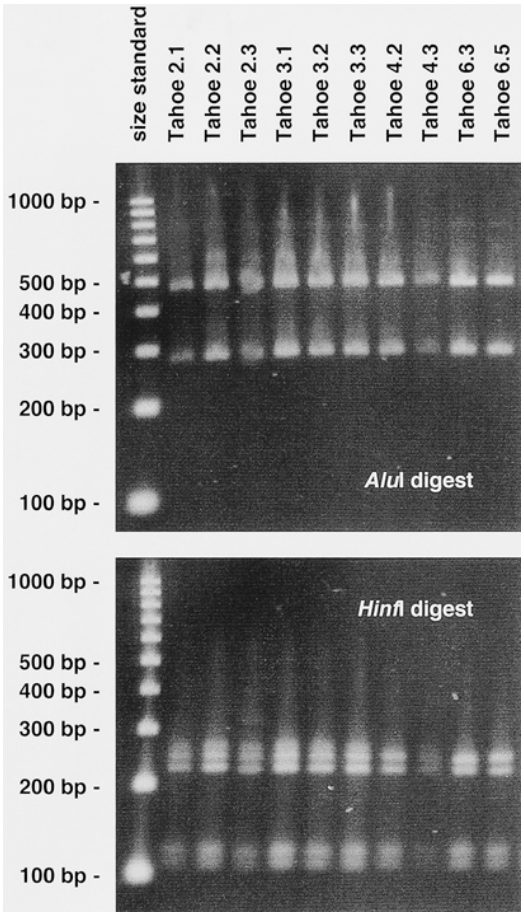

Fig. 2. Fungal ITS-RFLPs produced from *Sarcodes sanguinea* mycorrhizae with the PCR primers ITS-1f and ITS4 and the restriction enzymes *Alu*I and *Hinf*I. Only a small selection of samples are shown here; they represent the ten symbionts from the Lake Tahoe area for which we successfully produced ITS-RFLPs.

RESULTS

Although all *S. sanguinea* plants were sampled at comparable stages of flower development (= before seed production), the root samples appeared to fall into two distinct age classes. About 75% of the root samples appeared fresh and lush and were covered with a dense white mycelium except for the tips, which in *Sarcodes sanguinea* usually grow beyond the sheathing mantle. About 25% of the root samples, however, appeared old, and the mantle was reduced to a few pink patches leaving the roots with an overall brown appearance. Roots in the latter category generally failed to yield PCR amplification products, but occasionally allowed for culturing of the fungal symbiont.

We were able to directly amplify fungal ITS regions from 48 out of 64 *S. sanguinea* plants (Table 1). Amplification success and failure were strongly correlated with the presence or absence of a well-developed mantle as described above. Since the primer pair ITS-1f/ITS4 amplifies a shorter fragment than ITS-1f/ITS-4b, it was often successful when the latter failed. When the obtained PCR products were digested with the restriction enzymes *Alu*I or *Hinf*I, identical banding patterns were obtained across all samples, a selection of which is shown in Fig. 2.

Ten of the 12 *S. sanguinea* plants from which isolations were attempted yielded cultures of a relatively slow-growing, nonsporulating, white fungus that developed yellowish-brown coloration in the center of the plate over time. The other two plants yielded no fungal cultures other than fast-growing sporulating types, which were considered to be contaminants. ITS-RFLPs derived from the slow-growing cultures were identical to those derived directly from *S. sanguinea* roots (data not shown).

Similarity of the fungal ITS-RFLPs from *S. sanguinea* mycorrhizae to those obtained previously from *Rhizopogon subcaerulescens* (data not shown) as well as morphological characteristics (white mantle staining violaceous with age; occasional presence of rhizomorphs) led us to believe that the *S. sanguinea* root symbiont might be a taxon in *Rhizopogon* section *Amylopogon.* To address this hypothesis, we determined the nucleotide sequences of the fungal ITS region from four different *S. sanguinea* rootballs by direct sequencing of PCR-products obtained with the fungus-specific primer pairs ITS1f/4b or ITS1f/4. In addition, we also sequenced the ITS region from two fungal cultures that had been isolated from two different *S. sanguinea* plants (for details see Materials and Methods and Table 2). These sequences were aligned with previously published sequences from a wide range of *Rhizopogon* species (Grubisha, 1998) and proved to be most similar to the ITS sequences of *Rhizopogon ellenae* AHS66137 and T17476 (data not shown). *Rhizopogon ellenae* is a species within the monophyletic section *Amylopogon* (Grubisha, 1998). To increase the resolution within this section, we sequenced the ITS region from eight additional taxa given in Table 2. Collections with ''SNF'' collection numbers are *Amylopogon* fruitbodies collected in 1994 and 1995 in the ''Sierra National Forest'' of California (southeast of Shaver Lake). To determine genetic diversity among those collections, we first produced ITS-RFLPs with the restriction enzymes *Hinf*I, *Alu*I and *Cfo*I, and subsequently sequenced one representative of every RFLP type. Collections with ''HDT'' collection numbers were obtained from the Harry Thiers Herbarium at San Francisco State University. Finally, we also sequenced the ITS region from one fungal symbiont of *Pterospora andromedea.* The complete dataset has been posted at the following website: http://ajbsupp.botany.org/v87/kretzer.txt. It comprises 22 taxa from section *Amylopogon* and 539 characters, of which 26 were parsimony informative.

A heuristic search with ten random sequence additions resulted in 16 most parsimonious trees that were 57 steps long. One of the trees is shown in Fig. 3, and internal branches that were present in all 16 trees are highlighted in boldface. Four lineages were resolved by the current analysis within section *Amylopogon*: (1) lineage 1 comprises collections of *R. semireticulatus, R. subgelatinosus, R. subcaerulescens,* and the *Pterospora* symbiont; (2) lineage 2 comprises collections of *R. ellenae* (including the holotype), *R. idahoensis,* a different collection of *R. semireticulatus,* and all of the *Sarcodes* symbionts; this lineage will be referred to as the ''*R. ellenae* species complex''; (3) lineage 3 is made up by two collections of *R. subpurpurascens,* one of which is a paratype; and finally (4) an unidentified collection forms a fourth lineage.

DISCUSSION

About 25% of the *S. sanguinea* roots sampled appeared old and failed to produce PCR products of the fungal ITS region

TABLE 2. ITS sequences analyzed in this study.

Taxon	Collection ID[a]	Collection site	Accession[b]
Rhizopogon ellenae	AHS66137 (holotype)	Valley Co., Idaho	Grubisha, 1998
R. ellenae	T17476	Josephine Co., OR	Grubisha, 1998
R. ellenae	HDT46517	Sierra Co., CA	GBAN-AF224470, GBAN-AF224471
R. idahoensis	HDT30140	Sierra Co., CA	GBAN-AF224472
R. subcaerulescens	F2882	Colorado	Baura et al., 1992
R. subgelatinosus	T7624	Hood River Co., OR	Grubisha, 1998
R. semireticulatus	T7899	Josephine Co., OR	Grubisha, 1998
R. semireticulatus	T17562	Josephine Co., OR	Grubisha, 1998
R. subpurpurascens	AHS65669 (paratype)	Custer Co., Idaho	Grubisha, 1998
R. subpurpurascens	T19168	Bonner Co., Idaho	Grubisha, 1998
Rhizopogon sp.	SNF210B	Sierra National Forest, CA	GBAN-AF224475, GBAN-AF224476
Rhizopogon sp.	SNF32	Sierra National Forest, CA	GBAN-AF224480, GBAN-AF224481
Rhizopogon sp.	SNF38	Sierra National Forest, CA	GBAN-AF224473, GBAN-AF224474
Rhizopogon sp.	SNF260	Sierra National Forest, CA	GBAN-AF224477
Rhizopogon sp.	SNF306	Sierra National Forest, CA	GBAN-AF224478, GBAN-AF224479
Sarcodes symbiont	Shaver8	Shaver Lake Area, CA	GBAN-AF224489
Sarcodes symbiont	Shaver13	Shaver Lake Area, CA	GBAN-AF224276
Sarcodes symbiont	Tahoe5.2	Lake Tahoe Basin, CA	GBAN-AF224486
Sarcodes symbiont	Tahoe2.2	Lake Tahoe Basin, CA	GBAN-AF224487
Pterospora symbiont	—	Sierra National Forest, CA	GBAN-AF224488
Culture (*Sarcodes* HD1)	355	Shaver Lake Area, CA	GBAN-AF224482, GBAN-AF224483
Culture (*Sarcodes* HD3)	361	Shaver Lake Area, CA	GBAN-AF224484, GBAN-AF224485

[a] ''F'' collections were made by Dr. R. Fogel, ''T'' collections by Dr. J. Trappe, ''HDT'' collections by Dr. H. Thiers, ''AHS'' collections by Dr. A. Smith, ''SNF'' collections are located at the last author's laboratory in Berkeley, ''HD'' collections are from the ''high-density site'' (see Table 1).

[b] The prefix GBAN- has been added to link the online version of *American Journal of Botany* to GenBank but is not part of the actual accession number. In the case of single accession numbers, the whole ITS region (consisting of both internal transcribed spacers plus the 5.8S rRNA gene) were submitted as one continuous sequence; in the case of two accession numbers, the sequences of spacers 1 and 2 were submitted individually.

(see Table 1). Luoma (1996, and personal communication) has monitored a population of *S. sanguinea* over 17 yr and reports that most plants flowered only once during this period. However, in a certain number of cases (varying from 0 to 35% between years and averaging 12%) an inflorescence will appear in the very same location as in the previous year. We hypothesize that a small percentage of plants flower in two subsequent years and that the roots of those plants have an older appearance in the second year. Because those plants failed to yield PCR products, direct molecular evidence is missing to support the fact that they were colonized by the same fungal species as were the younger roots. Two other lines of evidence, however, suggest that the same fungus was present. (1) Remnant patches of a fungal mantle present on the aging roots did exhibit the pinkish to purplish color characteristic of aging mycorrhizae formed by section *Amylopogon.* (2) In a few cases we were able to culture the fungal symbiont from such aging roots, and it exhibited the same cultural and ITS-RFLP characteristics as did all the other symbionts. We conclude that all *S. sanguinea* plants that we sampled were associated with the same mycorrhizal fungus that is a species in the *R. ellenae* species complex.

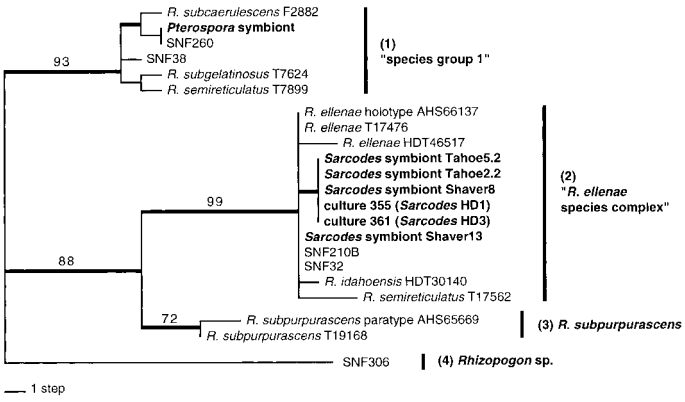

Fig. 3. One of 16 most parsimonious trees (57 steps long). Branches drawn in boldfaced lines are supported by all 16 most parsimonious trees. Numbers indicate support for individual branches from bootstrap analysis; only bootstrap values >70 are given. The tree is unrooted.

These findings are in conflict with a previous report by Cullings, Szaro, and Bruns (1996) who found *S. sanguinea* associated with fungi from at least three different lineages of Hymenomycetes. There are two possible reasons for this discrepancy: (1) Cullings, Szaro, and Bruns (1996) included samples from a somewhat broader geographic range in the Sierra Nevada of California and also sampled at least some remote and unmanaged habitats (K. Cullings, personal communication), while all our samples were taken in two areas of the southern and central Sierra Nevada within a few hundred metres of roads. Geographic mosaics are a common feature of many parasites; often what appears to be a generalist at a broad geographic scale is composed of several geographically defined host-specific populations (Thompson, 1994). (2) Cullings, Szaro, and Bruns (1996) may have overestimated diversity through PCR chimeras or may have inadvertently amplified saprobic fungi associated with older roots. In either case, however, *S. sanguinea* can no longer be considered the generalist that it earlier appeared to be (Cullings, Szaro, and Bruns, 1996). Instead, it appears restricted to associations with a single species of *Rhizopogon* within at least two large areas of its range and most of its habitats. This removes *S. sanguinea* as the only current exception to the emerging pattern that nonphotosynthetic mycorrhizal plants exhibit high levels of specificity. It also changes the way we view the evolution of spe-

cialization in the Monotropoideae. Initially it appeared that there was a gradual shift toward specialization (Cullings, Szaro, and Bruns, 1996); now there is no clear evidence for a member of the Monotropoideae that is a true generalist.

ITS sequences collected and analyzed in this study have greatly refined our understanding of species groups within section *Amylopogon.* We have therefore revisited the *P. andromedea* symbiont that Cullings, Szaro, and Bruns (1996) identified as a species in the ''*R. subcaerulescens* group'' based on sequence analysis and oligonucleotide probing of the mitochondrial large subunit rDNA. Cullings, Szaro, and Bruns (1996) also report having sequenced the ITS region of six *P. andromedea* symbionts, but only two partial ITS sequences have been published in Cullings (1993). We therefore sequenced the complete ITS region of one *P. andromedea* symbiont and included it in the current analysis. Our sequence, as well as Cullings' two partial sequences (data not shown), groups tightly with species group 1 in the current analysis and is clearly distinct from the ''*R. ellenae* species complex'' to which the *Sarcodes* symbiont belongs (Fig. 3). However, because Cullings, Szaro, and Bruns (1996) report RFLP variation as well as 0.3–2% sequence variation in the ITS region of *P. andromedea* symbionts, complete ITS sequences should be generated from the symbionts of more *P. andromedea* plants.

Although our work has contributed to a better understanding of species groups in *Rhizopogon* section *Amylopogon,* taxonomy of the group remains unsettling. Current taxonomy of *Rhizopogon* is based mostly on the morphological species concepts of Smith and Zeller (1966), which often turn out to be in conflict with genetic species concepts. From other studies we know for example that different paratype collections of *Rhizopogon vinicolor* Smith represent two very distinct genetic species (Kretzer, unpublished data). On the other hand, several collections representing different species according to the current morphological species concepts are often indistinguishable by ITS sequence data. The latter is also true for several collections in what we call the ''*Rhizopogon ellenae* species complex.'' We have chosen that name, because *R. ellenae* is the only holotype sequence currently known to fall within this group of taxa that may represent one or more reproductively isolated species.

In conclusion, this study has shown that *S. sanguinea* specializes at least regionally on a single fungal symbiont within the *R. ellenae* species complex. Furthermore, based on the analysis of fungal ITS sequences from three *P. andromedea* plants (two partial sequences are not being shown), the emerging picture is that the two monotropoid species *P. andromedea* and *S. sanguinea* specialize on two closely related but distinct *Rhizopogon* species from section *Amylopogon.* Since both monotropoid plant species often co-occur within metres of each other, specificity of their fungal associations is apparently not governed by habitat or local availability but rather by direct plant–fungus interaction.

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