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Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale

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Abstract

In most ectomycorrhizal (EM) community studies involving molecular identification methods there is a poor correspondence between fungi that appear dominant as sporocarps and those that appear dominant on EM roots and the species richness belowground is higher than that above ground. As a consequence, many fungi from root tip samples remain unidentified. In most studies, genetic data from multiple samples of an EM morphotype collected from various sampling locations are compared to genetic data from one to a few sporocarps of each species for identification purposes. The mismatch between above- and belowground species richness may be influenced by these different sampling efforts. To address this, intra-specific variation in the ITS region first investigated in Kårén et al. (1997) is revisited here, but at a spatial scale in which variation is expected to be low. Sporocarps were collected across a 7 km region of the Oregon Dunes National Recreation Area in western North America. ITS–RFLP data are presented for 3-18 sporocarps from each of 44 EM species in 18 genera. A total of 311 sporocarps were analyzed. Fifty-three ITS–RFLP types were observed. Of the 44 species, 38 (86% of total) yielded a single, species specific, RFLP type. No 2 species had the same RFLP type. Polymorphic ITS–RFLP types were observed in six species (14%). The following three species had two ITS-RFLP types with one type dominating: *Inocybe lacera, Laccaria proxima,* and *Rhizopogon subcaerulescens*. The following three species had three RFLP types with no type dominating: *Laccaria laccata, Lactarius deliciosus*, and *Tricholoma flavovirens*. A phylogenetic analysis of ITS sequences in *Tricholoma* revealed that two of the RFLP types in *T. flavovirens* were apparently the result of intra-specific variation, while the third RFLP type was likely a cryptic species. All the other RFLP types observed in *Tricholoma* represented unique phylogenetic species. These results suggest that ITS–RFLP data from single samples (sporocarp or EM) are robust for characterizing most of the species at this scale. However, restriction endonucleases detect a limited amount of existing nucleotide variation and thus have limited value to detect cryptic species. Therefore, additional analyses of sequence data should be added to the RFLP matching technique to identify unknown RFLP types. These data also suggest that polymorphic RFLP types within species do not adequately explain the mismatch between above- and belowground views of EM species richness.

Introduction

A great deal of knowledge about the diversity and ecology of ectomycorrhizal (EM) fungi is available from decades of study (Smith and Read, 1997). Much of the knowledge on fungal diversity resulted from field-based surveys of sporocarps. It can be argued that the presence of a fungus at a site is best assessed by its presence in its vegetative state because many fungi fruit sporadically or cryptically or both. Morphological descriptions of EM roots have provided useful data for identifying the fungi below ground (Agerer, 1987–1996; Goodman et al., 1996–1998; Ingleby et al., 1990), but most species have not been described by this method. Sporocarp surveys and morphological descriptions continue to lay a solid foundation

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from which EM fungal diversity can be assessed and this foundation increases the utility of molecular techniques in studies of EM fungal diversity and ecology.

The use of the polymerase chain reaction (PCR) has dramatically increased our ability to document the diversity of EM fungi primarily because fungi can be identified directly from vegetative structures, and genetic data is obtained rapidly enough to allow relatively large numbers of samples to be processed. The technique has been applied broadly to identify EM fungi from root tip samples (Agerer et al., 1996; Dahlberg et al., 1997; Eberhardt et al., 2000; Gardes et al., 1991; Gehring et al., 1998; Henrion et al., 1992; Mahmood et al., 1999). In most cases, a combined approach employs morphological sorting of EM root tips with molecular identification using restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the nuclear rRNA gene repeat.

Horton and Bruns (2001) review the molecular techniques applied to studies of EM communities and what has been revealed from these studies. They suggest that the RFLP typing using ITS sequences is an effective tool for identification purposes, but does have its limitations. For instance, many types remain unknown because no matching RFLP type is observed in available sporocarp databases. This may impact two of the more important findings from these studies: (1) there is a poor correspondence between fungi that appear dominant as sporocarps and those that appear dominant on EM roots; (2) species richness appears higher when analyzing root tip data than when analyzing sporocarp data (Dahlberg et al., 1997; Gardes and Bruns, 1996a; Gehring et al., 1998; Jonsson et al., 1999a,b; Kårén and Nylund, 1997; Mehmann, 1995). A number of issues likely interact to produce these patterns including differential investment in vegetative growth and sexual reproduction, and the production of cryptic sporocarps (Gardes and Bruns, 1996a). However, the pattern may also be influenced by the different sampling efforts applied above and below ground. Many studies analyze ITS–RFLPs from many EM samples of a morphological type (morphotype) and compare these to the ITS–RFLPs from a few sporocarps of each species collected at the research site. If large numbers of sporocarps are sampled for a species, then samples collected at a relatively large spatial scale are included and studies have shown that polymorphic ITS–RFLP types often occur when specimens are collected at such scales (Agerer et al., 1996; Baura et al. 1992; Eberhardt et al., 1999; Farmer and Sylvia, 1998; Gardes et al., 1991; Henrion et al., 1992; Kårén et al., 1997).

The ITS region is expected to show intraspecific variation across large scales because (1) it evolves rapidly, and (2) populations of a species can be reproductively isolated at large scales. Kårén et al. (1997) reported that intra-specific variation was not a factor at a local scale, but only sampled a few sporocarps for each species from herbarium specimens. A broader genetic sampling of sporocarps from a research site will provide a more reliable analysis as to whether ITS polymorphisms are a factor at a local scale. These data will provide additional support for the use of variation in the ITS region for species richness estimates in below ground studies.

The primary objective here is to investigate the use of ITS–RFLP data to rapidly assess the diversity of EM fungi from multiple sporocarps collected at a scale of approximately 10 km^2. Because there is relatively little sequence information in an RFLP pattern, close matches between patterns cannot be satisfactorily analyzed even though they may indicate a high degree of genetic similarity (Bruns et al., 1991; Horton and Bruns, 2001). A secondary objective then is to use a phylogenetic approach using ITS sequence data as a follow up to RFLP analysis in order to clarify species delimitations in *Tricholoma*.

Methods

Site description

The research was conducted at the Oregon Dunes National Recreation Area on the western coast of North America. The coastal sand dune EM plant community includes established forests of *Picea sitchensis, Pseudotsuga menziesii, Tsuga heterophylla*, and on the edges, *Pinus contorta.* Primary succession is continuously occurring in the area as a result of shifting sands covering and killing established forests and exposing uncolonized areas suitable for plant establishment. Primary successional EM plant communities include *P. contorta, Artctostaphylos uva-ursi* and *Salix hookeriana*, with scattered *P. sitchensis* and *P. menziesii* occurring as well. The focus in the current work is on areas dominated by *P. contorta,* but fungi potentially specific to other hosts were also collected. Within this ecosystem, four replicate sites were chosen, each site having similar plant community

characteristics. Each replicate site was approximately 500 m × 500 m. The four sites were spread across approximately 7 km of the National Recreation Area.

Sporocarp collections

Sampling of sporocarps occurred weekly in the fall of 1999 and 2000, starting the last week of September and ending the second week of December (the September collection date was omitted in 2000). A time-based sampling approach was employed. Three individuals collected for 45 min at three sites during the peak fruiting periods, with the three sites selected randomly from the four possible sites each week. A total of 21 visits were made over the 2 fall seasons. Epigeous and hypogeous fungi were collected simultaneously during the trips. Hypogeous fungi were located by evidence of fresh mammal digs in the sand.

Data are presented only for species that could be confidently identified based on a morphological concept and for those species that were collected in at least two of the four replicate sites. Sporocarps were studied fresh when possible and were placed on a food dehydrator within 1 week of removal from the field. Macroscopic and microscopic characters were used to identify the fungi and available keys in the OSU mycology collection were consulted (Oregon State University, J. Trappe). Experts in the taxonomy of several difficult genera were consulted (J. Ammirati, M. Seidl, B. Methany, Thom O'Dell for *Cortinarius*; B. Methany for *Inocybe*; K. Shanks for *Tricholoma*; D. Luoma, M. Castellano, E. Cázares, R. Molina for *Rhizopogon*). Many sporocarps could not be identified to the species level because taxonomic treatments are incomplete in general, and because novel species were potentially collected. A large number of *Cortinarius* sporocarps were omitted from this analysis because of uncertain species delimitations. Other species from a variety of genera are not included here because they were collected infrequently.

Molecular approaches

DNA extraction, PCR, and RFLP analysis followed Gardes and Bruns (1996b). The ITS region of the rDNA was amplified using the primer pair ITS–1f and ITS–4b. RFLP patterns were generated with *Hinf*I, *Alu*I, and *Dpn*II. RFLP data were analyzed using Gene Profiler (Scanalytics). These data are not reported here for brevity, but are available from the author. An RFLP type is defined as a composite of data from the three

Table 1. Tricholoma voucher numbers and Genbank accession numbers for ITS sequences

Tricholoma species	Voucher number[a]	Genbank accession number
T. atroviolaceum		AF319432
T. caligatum		AF204813
Tricholoma sp. 2	trh1237	AF458443
T. equestre		AJ236081
T. flavovirens		AB036895
T. flavovirens		AF349689
T. flavovirens6	trh545	AF458449
T. flavovirens6	trh894	AF458450
T. flavovirens6	trh901	AF458451
T. flavovirens7	trh546	AF458452
T. flavovirens7	trh1000	AF458453
T. flavovirens7	trh1023	AF458454
T. flavovirens8	trh670	AF458455
T. flavovirens8	trh652	AF458456
T. focale	trh909	AF462638
T. focale	trh597	AF462639
T. focale		AF319437
T. imbricatum		AF319426
T. imbricatum	trh895	AF462636
T. imbricatum	trh912	AF462637
T. intermedium		AF319434
T. intermedium		AF319434
T. japonicum		AF204810
T. luteomaculosum	trh914	AF458446
T. luteomaculosum	trh1033	AF458447
T. luteomaculosum	trh1187	AF458448
T. magnivelare	trh905	AF458441
T. magnivelare	trh906	AF458442
T. magnivelare		AF309541
T. matsutake		AF309538
T. matsutake		AF204868
T. muricata	trh610	AF458438
T. muricata	trh815	AF458439
T. muricata	trh820	AF458440
T. mutabile	trh916	AF458444
T. mutabile	trh1184	AF458445
T. myomyces		AF319428
T. myomyces		AF319428
T. pardinum		AF319427
T. portentosum		AF241517
T. portentosum		AF241517
T. saponaceum		AF319429
Tricholoma sp. 1	trh567	AF462640
Tricholoma sp. 1	trh883	AF462641
Tricholoma sp. 1	trh899	AF462642
T. ustale		AF204812
T. ustale	trh884	AF458435
T. ustale	trh902	AF458436
T. ustale	trh885	AF458437
T. venenatum		AF319433
T. vernaticum		AF319424

[a] Voucher collection located at SUNY-College of Environmental Science and Forestry Herbarium.

restriction digests and types were considered identical if they matched with all three endonucleases.

Sequence variation in the ITS region was analyzed for 25 putative species in *Tricholoma,* eight of which were collected at the site. The genus *Tricholoma* was chosen because there was a relatively high number of species found at the site and a fairly high number of sequences was available in web databases such as GenBank and EMBL. A phylogenetic analysis was employed to identify unclear species delimitations in the following two cases. RFLP analysis revealed three ITS types in *T. flavovirens*, but it was unclear how much variation in the ITS sequence was represented by these three types. In addition, *Tricholoma* sp. 1 could not be identified with a taxonomic key of western *Tricholoma* species (Shanks, 1997); this may be an unknown species sharing some morphological features with both *T. caligatum* and *T. focale,* but lacking the spicy odor of the former and the orange, viscid cap of the latter (Kris Shanks, pers. comm.). The ITS region of two to three sporocarps of each *Tricholoma* RFLP type from the study area was sequenced. Additional sequences were downloaded from GenBank, selected to increase the diversity of *Tricholoma* spp. covered (Table 1). All available *T. flavovirens* and *T. equestre* sequences are included. While some interesting trends are discussed, the results are not meant to represent a complete phylogenetic analysis of *Tricholoma*. The alignment is available from the author.

PCR products for sequencing reactions were generated with the primers ITS–1f and ITS–4b (Gardes and Bruns, 1996b). The sequencing reactions were conducted on the resultant PCR product with internal primers ITS–2, ITS–4, and ITS–3 (White et al., 1990). Sequences were determined using an ABI Model 373 DNA sequencer (Perkin-Elmer Corporation). DNA sequencing Analysis (version 2.01) and Sequence Navigator software were used to process the raw data. Sequences were aligned by visual estimation using a matrix created in PAUP 4.0b5c (Swofford, 2001). Phylogenetic analyses were conducted with PAUP 4.0b5c using the heuristic search option. Bootstrap values were generated using the fast-bootstrap option and represent 10,000 replicates.

Results

A total of 311 sporocarps in 18 genera and 44 species were analyzed, with a range of 3 – 18 sporocarps per species. Most of the species were collected in both years of the study. Fifty-three ITS–RFLP types were observed (Table 2). Of the 44 species, 38 (86% of total) yielded a single RFLP type for all sporocarps.

While no two species had the same RFLP type, polymorphic ITS–RFLP types were observed in six species (14%). Three species yielded two ITS–RFLP types, with one type dominating: *Inocybe lacera, Laccaria proxima,* and *Rhizopogon subcaerulescens*. Three other species yielded three RFLP types with no type dominating: *Laccaria laccata, Lactarius deliciosus*, and *Tricholoma flavovirens*. In most of these instances, the RFLP variation is the result of variation in only one of the three endonucleases, suggesting limited variation in the ITS sequences. The exceptions were in *Laccaria proxima, L. laccata* and one of the *T. flavovirens* types.

Phylogenetic analysis of *Tricholoma* ITS sequences revealed that all 3 ITS–RFLP types observed in *T. flavovirens* clustered in one clade together with the *T. flavovirens* and *T. equestre* sequences from GenBank. Among these however, three distinct lineages could be distinguished that were separated by 17–20 steps on the tree shown in Figure 1. ITS sequence variation between these lineages was 3.8 to 6.2% in pairwise comparisons (Table 3, Figure 2). Two of the RFLP types from the Oregon dunes, types 6 and 7, show very little sequence variation and are likely to be conspecific. The third *T. flavovirens,* type 8, proved to cluster with the others, but was divergent enough to occur on its own branch supported by a high bootstrap value. The divergence seen between the *T. flavovirens* sequences (and *T. equestre*) is greater than that seen for the *T. caligatum, T. matsutake,* and *T. magnivelare* group.

Although *Tricholoma* sp. 1 has some morphological features similar to both *T. caligatum* and *T. focale,* the phylogenetic analysis clearly does not support a close relationship to *T. caligatum*. The region in the tree with *T. focale* and *Tricholoma* sp. 1 has low overall support, but at present, the unknown species seems most likely to belong to the *T. focale* species group.

Discussion

Unique, species specific ITS–RFLP types were observed in 38 out of 44 species. Six morphologically defined species (14%) yielded multiple ITS–RFLP types. These numbers are very similar to those reported in a larger scale study by Kårén et al. (1997).

Table 2. ITS–RFLP variation in sporocarps of ectomycorrhizal species

	Species	ID[a]	# of plots	# of sporocarps sampled	# of RFLP types
1	*Amanita muscaria*	trh	4	7	1
2	*Bankera fuligneo-alba*	trh	4	10	1
3	*Boletopsis subsquamosus*	trh	3	41	
4	*Boletus edulis*	trh	4	5	1
5	*Boletus piperatus*	trh	2	3	1
6	*Boletus subtomentosus*	trh	4	6	1
7	*Chroogomphus rutilus*	trh	4	6	1
8	*Chroogomphus vinicolor*	trh	3	5	1
9	*Cortinarius aurantiobasis* complex	ja	4	9	1
10	*Cortinarius aureifolius*	pbm	3	7	1
11	*Cortinarius muscigenus*	ms	4	8	1
12	*Cortinarius semisanguineus*	ja	4	6	1
13	*Hydnellum scrobiculatum*	trh	4	6	1
14	*Inocybe lacera*	pbm	4	18	2 (17:1)[b]
15	*Inocybe sambucina*	pbm	2	4	1
16	*Laccaria bicolor*	trh	2	3	1
17	*Laccaria laccata* var. *pallidifolia*	trh	4	11	3 (6:4:1)[b]
18	*Laccaria proxima*	trh	4	9	2 (6:3)[b]
19	*Lactarius deliciosus*	trh	4	10	3 (4:4:2)[b]
20	*Lactarius rufus*	trh	4	7	1
21	*Leccinum manzanitae*	trh	4	6	1
22	*Phellodon melaleucus*	trh	4	9	1
23	*Phellodon niger*	trh	3	6	1
24	*Rhizopogon fuscorubens*	rm	3	3	1
25	*Rhizopogon occidentalis*	dl	4	5	1
26	*Rhizopogon subcaerulescens*	ec	3	4	2 (3:1)[b]
27	*Rhizopogon vulgaris*	mc	4	11	1
28	*Russula cascadensis*	trh	4	5	1
29	*Russula cremoricolor*	trh	3	4	1
30	*Russula pectinoides*	trh	4	5	1
31	*Sarcodon scabrosus*	trh	4	13	1
32	*Suillus albidipes*	trh	2	4	1
33	*Suillus brevipes*	trh	4	7	1
34	*Suillus tomentosus*	trh	4	6	1
35	*Suillus umbonatus*	trh	4	8	1
36	*Thelephora americana*	trh	4	8	1
37	*Tricholoma flavovirens*	trh	4	13	3 (6:4:3)[b]
38	*Tricholoma focale*	trh	3	8	1
39	*Tricholoma imbricatum*	kms	3	4	1
40	*Tricholoma luteomaculosum*	kms	2	2	1
41	*Tricholoma magnivelare*	trh	4	5	1
42	*Tricholoma mutabile*	kms	4	10	1
43	*Tricholoma* sp. 1	kms	3	9	1
44	*Tricholoma ustale*	kms	4	12	1

[a] Initials of individual who identified the species: trh = T.R. Horton, kms = K. M. Shanks, dl = D. Luoma, ja = J. Ammarati, mc = M. Castellano, ms = M. Seidl, pbm = P.B. Matheny, rm = Randy Molina, ec = Efrén Cázares.
[b] Number of collections with each RFLP type.

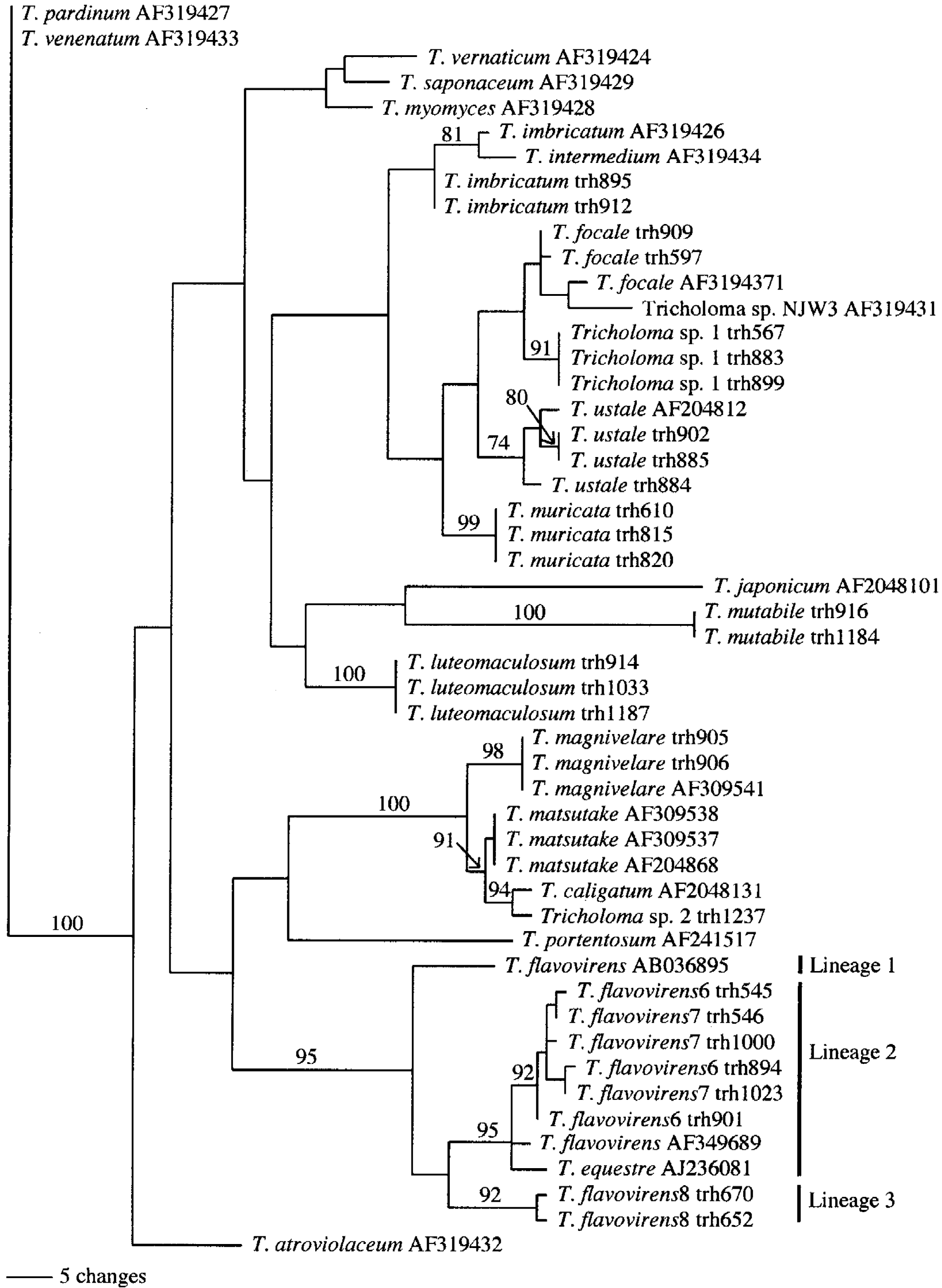

Figure 1. One of 3432 most parsimonious trees for ITS sequence data from *Tricholoma.* Areas that were difficult to align and the entire 5.8s gene were omitted from the analysis. A total of 431 characters were used in the data set, with 241 characters being constant, 50 characters being variable and not parsimony informative, and 140 characters being variable and parsimony informative. Numbers on branches refer to bootstrap values $\geq$70 from 10,000 replications using the fast bootstrap option.

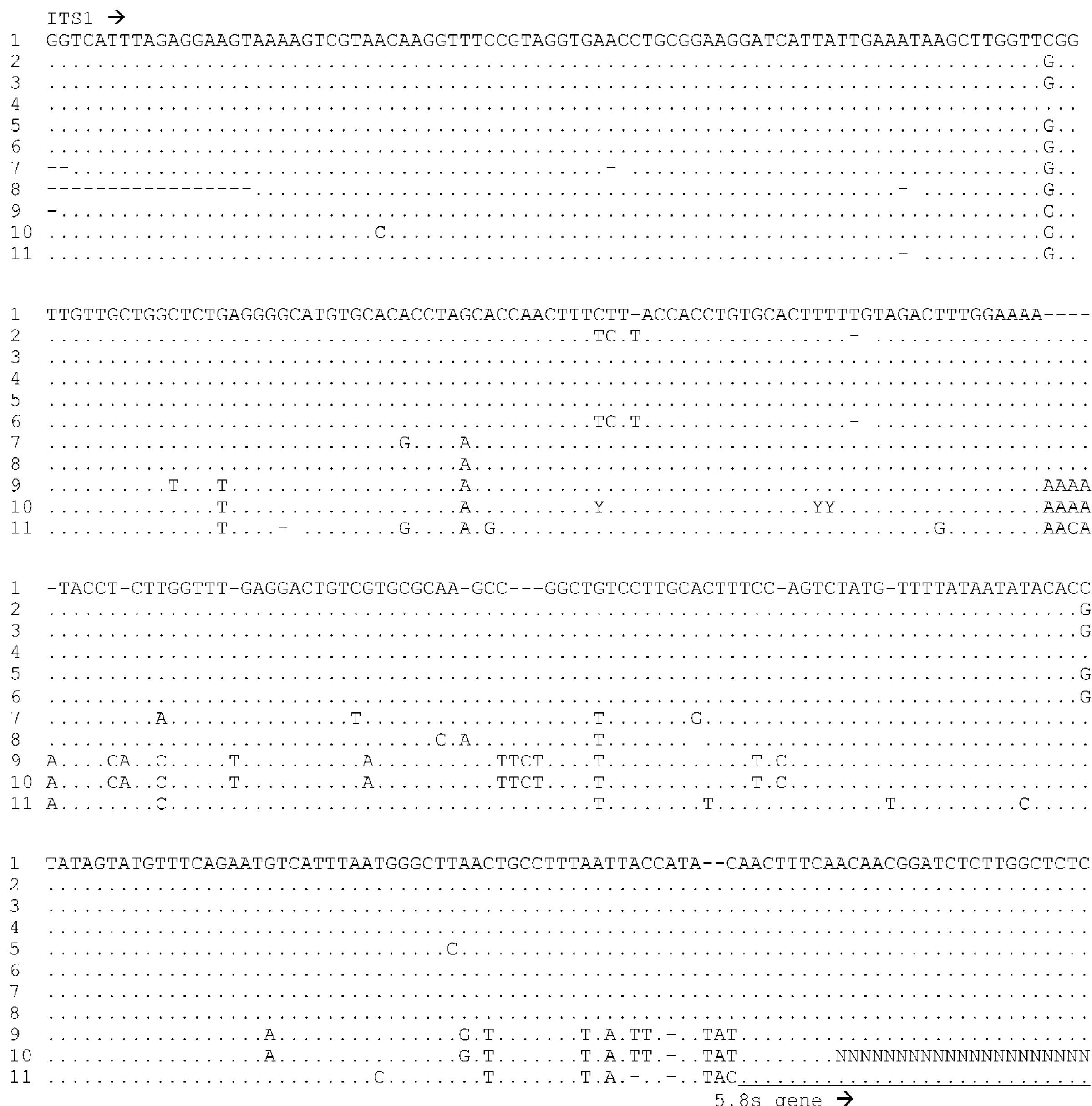

Figure 2. ITS (ITS1, 5.8s, ITS2) sequences for 11 sporocarps of *Tricholoma flavovirens* shown in Figure 1 and Table 3. The location of the 5.8s gene was identified following (Baura et al., 1992). Gaps introduced for alignment are indicated (–). Unknown nucleotides are indicated (N).

The species that yielded multiple RFLP types were not clustered in a single genus, but occurred in 5 of the 18 genera: *Laccaria* (2), *Lactarius* (1), *Inocybe* (1), *Rhizopogon* (1), and *Tricholoma* (1). In at least three genera there appears to be a higher probability of observing intraspecific RFLP polymorphisms: *Laccaria* spp. (seen here and in Gardes et al., 1991; Henrion et al., 1992), *Cortinarius* spp. (seen in Kårén et al., 1997), and *Inocybe* spp. (seen here). Note that despite the high probability for intraspecific RFLP polymorphisms in these latter three genera, they are less frequently encountered in EM samples than species of Thelephoraceae, Russulaceae and resupinate fungi not belonging to Thelephoraceae based on sequence analyses of unknown RFLP types (for references see Horton and Bruns, 2001).

RFLP data alone do not adequately quantify the nucleotide variation in the ITS to draw conclusions

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1  GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG
2  ......................................................................................
3  ......................................................................................
4  ......................................................................................
5  ......................................................................................
6  ......................................................................................
7  ......................................................................................
8  ......................................................................................
9  ......................................................................................
10 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
11 ......................................................................................
                                                                                 ITS2→

1  CTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATAAAATTCTCAACCTTTTCAATCTTTTATTGGTTGAATTAGGTTTGG
2  ......................................................................................
3  ......................................................................................
4  ......................................................................................
5  ......................................................................................
6  ......................................................................................
7  ......................................................................................
8  ......................................................................................
9  ........................................G...................................G....C....
10 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
11 ........................................G........................................C....

1  ATGTGGGAGTCTTTTGCTGGCTTCTTT----AAAGAGGTCGGCTCTCCTTAAATTTATTAGCAGGA-CCTTTGTGGCCCTCTC-AG
2  ......................................................................................
3  ......................................................................................
4  .........G............................................................................
5  .........G............................................................................
6  .........G............................................................................
7  ...............................-..........................................T...........
8  ...............................-..................CCC.....................T...........
9  ...............................-..............................G..GA.......T......---..
10 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
11 ..A........................TAGA...AG.A..A.....................G...........T....C.CTC..

1  C-GTTAGTGTGATAATTATCTACGCTATTTGCTGAA-GGCAACTCATAAAATAGGGTTTC-CAGCTTCTAATCGTCTCTTGTAGAC
2  ....................................NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
3  ....................................NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
4  ......................................................................................
5  ........................................................NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
6  .......................................................NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
7  ....................................A-................................................
8  -T..................................A.C...............................................
9  TGC...........G............CC.......AT......G.A....A....G..TA...........T.............
10 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
11 CT..................................A-.......-C.....G...G..TA................T........

1  AATTTATATGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
2  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
3  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
4  .........................................................................
5  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
6  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
7  .................................................NNNNNNNNNNNNNNNNNNNNNNNN
8  .........................NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
9  .........................NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
10 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
11 .........................................................................
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Figure 2. (continued.)

about species delimitations. A number of confusing relationships in the genus *Tricholoma* remained unclear after the RFLP analysis but were clarified with the addition of sequence comparisons and phylogenetic ana-

Table 3. Percent pairwise distances between taxa. Data are mean character differences (adjusted for missing data), representing ITS–1, 5.8s, and ITS–2 sequences. Percent differences range from 0.14 to 2.13% within clades and 3.85 – 6.25% across clades (see Figure 1)

	1	2	3	4	5	6	7	8	9	10	11
1. *T. flavovirens*, rflp 6, trh545	–	–	–	–	–	–	–	–	–	–	–
2. *T. flavovirens*, rflp 6, trh894	0.65	–	–	–	–	–	–	–	–	–	–
3. *T. flavovirens*, rflp 6, trh901	0.33	0.33	–	–	–	–	–	–	–	–	
4. *T. flavovirens*, rflp 7, trh546	0.14	0.82	0.49	–	–	–	–	–	–	–	–
5. *T. flavovirens*, rflp 7, trh1000	0.63	0.65	0.33	0.47	–	–	–	–	–	–	–
6. *T. flavovirens*, rflp 7, trh1023	0.79	0.16	0.49	0.63	0.48	–	–	–	–	–	–
7. *T. flavovirens* AF349689	0.99	1.48	1.15	1.13	1.43	1.60	–	–	–	–	–
8. *T. equestre* AJ236081	1.50	1.85	1.52	1.65	1.96	2.13	1.35	–	–	–	–
9. *T. flavovirens*, rflp 8, trh652	5.07	5.10	4.76	5.21	5.57	5.75	4.69	5.71	–	–	–
10. *T. flavovirens*, rflp 8, trh670	5.90	6.25	5.90	5.90	6.23	6.25	5.63	5.92	0.63	–	–
11. *T. flavovirens* AB036895	4.25	4.60	4.26	4.39	4.78	4.96	3.85	4.97	5.03	5.18	–

lysis. For instance, *Tricholoma* sp. 1 is more closely related to *T. focale* than *T. caligatum,* despite having some morphological features common to both species. In addition, the phylogenetic analysis provided insights into the level of variation represented by the three RFLP types in *T. flavovirens. Tricholoma flavovirens* type 8 is most likely a unique species that at present is morphologically indistinguishable from the other collections under this name. Although *T. flavovirens* is the name used in North America for this fungus with its distinctive lemon-yellow lamellae and stipe (Bessette et al., 1997; Shanks, 1997), there is some confusion regarding whether this name is a synonym of *T. equestre* (Breitenbach and Kränzlin, 1991; Moser, 1983). It is interesting that the one *T. equestre* sequence available in GenBank is within *T. flavovirens* lineage 2 (Figure 1). Whether *T. flavovirens* lineage 2 is synonomous with *T. equestre* cannot be assessed in the present analysis. However, these data strongly suggest that *T. flavovirens* and *T. equestre* group represent a species complex requiring more taxonomic attention. This point is particularly relevant in light of a recent report of poisoning from ingestion of *T. equestre,* an otherwise choice edible fungus (Bedry et al., 2001).

Evidence for multiple biological species within a morphologically defined species has been shown in *Cortinarius rotundisporus* and *Hebeloma* spp. (Aanen et al., 2000; Sawyer et al., 1999). While a phylogenetic analysis was not conducted on any other polymorphic taxa here, direct sequence comparisons suggest multiple species were recovered in *Lactarius deliciosus* and *Inocybe lacera* (data not shown). That multiple species are being discovered in some of these taxa should not come as a surprise. For example, multiple varieties of *Lactarius deliciosus* have been described in western North American (Hesler and Smith, 1979; Methven, 1997), and Bessette et al. (1997) suggest the name is misapplied in North America. If ITS variation observed in some taxa is actually a reflection of cryptic species under one morphological concept, then the ITS–RFLP typing method becomes even more robust. These results highlight that even where polymorphisms are observed, inaccurate biological species concepts may be at least as important as intraspecific variation. This highlights the fact that many EM groups require more taxonomic attention including Cortinariaceae, *Rhizopogon*, Russulaceae, and Thelephorales.

While some of the poor correspondence between above- and belowground data appears to be a function of intraspecific variation even at a local scale, these and other data suggest a greater contribution from other factors including differential investment in vegetative growth and sexual reproduction, and the production of cryptic sporocarps (Gardes and Bruns, 1996a). Sporocarp production for most species is sporadic at best, and it can take years to approach a complete species list at a site (Luoma, 1991; O'Dell et al., 1999), yet many of the species observed fruiting over a 10 year period are likely to occur belowground in a vegetative state during this time (unless the site was recently disturbed or undergoing primary succession). This suggests that even with an increased sampling effort of EM sporocarps, many EM types

will remain unidentified with the sporocarp RFLP matching approach alone. ITS–RFLP patterns continue to be a cost effective and highly useful tool for grouping EM fungal species, especially from root tip samples. However, ITS–RFLP data provide limited taxonomic information. Sequence databases, including aligned sequences, are available for a number of DNA regions that are useful at various taxonomic levels (see Horton and Bruns, 2001). As ITS sequences are deposited in GenBank or EMBL, the region becomes increasingly useful for identifying unknown EM types at the species or species-group level. Direct sequencing of the ITS or other region followed by blast searching in GenBank or a phylogenetic analysis is highly recommended to increase the taxonomic information of unknown RFLP types.

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