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Phylogenetic analysis of the Glomeromycota by partial β-tubulin gene sequences

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Abstract The 3' end of the β-tubulin gene was amplified from 50 isolates of 45 species in Glomeromycota. The analyses included a representative selection of all families except Pacisporaceae and Geosiphonaceae. Phylogenetic analyses excluded three intron regions at the same relative positions in all species due to sequence and length polymorphisms. The β-tubulin gene phylogeny was similar to the 18S rRNA gene phylogeny at the family and species level, but it was not concordant at the order level. Species in Gigasporaceae and Glomeraceae grouped together but without statistical support. Paralogous sequences in *Glomus* species likely contributed to phylogenetic ambiguity. Trees generated using different fungal phyla as out-groups yielded a concordant topology. Family relationships within the Glomeromycota did not change regardless if the third codon position was included or excluded from the analysis. Multiple clones from three isolates of *Scutellospora heterogama* yielded divergent sequences. However, phylogenetic patterns suggested that only a single copy of the β-tubulin gene was present, with variation attributed to intraspecific sequence divergence.

Keywords Glomeromycota · Beta-tubulin · Phylogeny · Arbuscular mycorrhizal fungi

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Introduction

A study of near-full-length 18S rRNA gene (SSU) sequences has led to the current classification of arbuscular mycorrhizal fungi (AMF; Schuessler et al. 2001). The SSU gene does not provide a unifying picture because the inferred phylogeny is not congruent with morphological evolution (Morton 1990). The main conflict is the grouping of the families Acaulosporaceae and Gigasporaceae. Genes encoding 28S rRNA (LSU; da Silva et al. 2006) and large subunit of RNA polymerase II (RPB1; Redecker and Raab 2006) also suggest sister relationships between these two families, although statistical support is low. In contrast, phylogenies inferred from genes encoding α-elongation and actin proteins suggest that Acaulosporaceae and Glomeraceae are sister families (Helgason et al. 2003), but taxon sampling was low.

A phylogeny based on the β-tubulin gene region also supports sister-group relationships of Acaulosporaceae and Gigasporaceae (Corradi et al. 2004a, b). However, this analysis suffered from limited taxon sampling. Of the nine species sequenced, only three of the ten families in Glomeromycota were represented. The families Archaeosporaceae, Paraglomaceae, Geosiphonaceae, Ambisporaceae, Diversisporaceae, Entrophosporaceae, and Pacisporaceae were not evaluated. Results of these studies showed that the third codon position of the β-tubulin gene was saturated and thus was phylogenetically uninformative. Of the three families evaluated, two β-tubulin genes were measured only in species of Glomeraceae. Paralogous sequences have been known to complicate phylogenetic analysis (Redecker and Raab 2006), so greater sampling within and between species is needed for good resolution.

The aim of this study was to determine if increased taxon/isolate sampling could resolve the evolutionary

relationships within the Glomeromycota based on variation in the β-tubulin gene. Increased taxon sampling increases accuracy, improves alignment, and provides a better model evolutionary processes (Simmons and Miya 2004; Baurain et al. 2007). The third codon position also may be phylogenetically informative and show increased phylogenetic signal with increased taxonomic sampling (Simmons and Miya 2004).

Materials and methods

Fungal species

The fungal species used in this study (Table 1) were obtained from the International Culture Collection of Arbuscular and Vesicular–Arbuscular Mycorrhizal Fungi (INVAM, USA). This study did not include species of the families Geosiphonaceae and Pacisporaceae, which were not available as living cultures in INVAM.

The following sequences from GenBank were included in the phylogenetic analyses: *Mycosphaerella pini* (AF044975), *Cercospora beticola* (AY856373), *Neurospora crassa* (M13630), *Melampsora lini* (AF317682), *Uromyces fabae* (AJ311552), *Mortierella verticillata* (AF162072, AF162071), *Basidiobolus ranarum* (AF162060, AF162059), *Mucor racemosus* (AY937402), *Micromucor ramannianus* (AF162073), *Conidiobolus coronatus* (AF162058, AF162057), *Blastocladiella britannica* (AY131271), *Blastocladiella emersonii* (EF064248), *Rhizophydium sphaerotheca* (AY944848), *Rhizophlyctis rosea* (AF162078), and *Sus scrofa* (NM_001113696). Fungal species in the Zygomycota, Chytridiomycota, Basidiomycota, and Ascomycota contain highly divergent copies of the β-tubulin gene. The fungal species included in this study are some of the most conserved representatives of each fungal phylum.

DNA extraction

DNA was extracted from single spores. Each spore was crushed in 9 μL of 10× polymerase chain reaction (PCR) buffer using a sterile dissecting needle, heated for 5 min at 94°C, and then centrifuged at 14,500 rpm for 2 min. The supernatant containing the DNA was carefully removed by pipetting and then transferred to a PCR tube and stored at −20°C until use.

Three-step nested PCR

A three-step nested PCR approach (Dar et al. 2005) was needed in order to generate enough PCR products for sequencing from single spores of some species, such as those in *Glomus* and *Acaulospora*. The first round of PCR amplification employed the primer pair C2F (5'-GCTGTTCTCGTTGACCTTGA-3'; Corradi et al., 2004a, b) and FBtub4R (5'-GCCTCAGTRAAYTCCATYTCRTCCAT-3'; Einax and Voigt 2003). Newly designed primers IB36F (5'-CACTCNCTNGGNGGTGGNAC-3') and GiH4R (5'-CATACCCTCACCAGTATACCAATG-3') were used for the second round of PCR, while the third round of PCR amplification employed the primers FSP (5'-GARTAYCCNGAYMGNATGATG-3') and GiH3R (5'-TGGTGGAATATCACACACGGCAGC-3'). PCR was performed in 50-μL volume containing 6.0-μL single-spore DNA, 1.0 U Taq DNA polymerase (Promega, USA), 1.0 μL 10× PCR buffer, 3.5 mM $MgCl_2$, 1.25 mM each of dATP, dCTP, dGTP, and dTTP, and 1-μM primers. The amplification conditions were as follows: an initial denaturation at 94°C for 2 min, 30 cycles at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Bands were excised and purified using a Qiagen Gel purification kit. PCR products were cloned using a Qiagen PCR cloning kit. The blue/white color screening technique was used to select for transformant colonies. Positive transformants were screened further by direct colony PCR. Positive colonies were inoculated into 5 mL of liquid LB media containing ampicillin and grown overnight at 37°C. Plasmid DNA containing the insert was purified from the liquid cultures using a Qiagen miniprep kit. Plasmids were sequenced once with T7 primer using the Applied Biosystems Big Dye Terminator V3.0 sequencing chemistry. Sequences and their corresponding chromatographs obtained from Davis Sequencing (USA) were checked in BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) for any sequence errors. Given that there were no frameshift mutations or stop codons observed within exon sequences, taq error rate was considered to be very low in these regions (between 1×10^{-4} and 2×10^{-5}). However, intron regions of some species have tracts of As and Ts which could have contributed to a higher error rate (1.5×10^{-2}; Shinde et al. 2003). Davis sequencing reports a sequencing error rate of 1% (personal communication). Nucleotide homology searches were performed on the National Center for Biotechnology Information databases by BLAST search. Sequences were deposited in GenBank under accession numbers FJ174268–FJ174326.

To discover any putative paralogous sequences, multiple clones of three *Scutellospora heterogama* isolates (WV859, BR155A, and SN722) were characterized by restriction fragment length polymorphisms (RFLP). Thirty positive colonies from each isolate were used as template for PCR reaction. RFLP was performed on the resultant PCR

Table 1 Fungal isolates from INVAM used for phylogenetic analyses of AMF

Species	Isolate code	Origin, contributor	Accession #	Family affiliation
[1]*S. heterogama*	BR155A	Brazil, Ming-Tie Lin	FJ174319-22	Gigasporaceae
[2]*S. heterogama*	WV859	USA, Joyce Spain	FJ174323-6	
[3]*S. heterogama*	SN722	Singapore, Isabelle Louis	FJ174315-8	
[4]*S. verrucosa*	VA105B	USA, Chris Walker	FJ174270	
[5]*S. gregaria*	NC210	USA, Jim Bever	FJ174271	
[6]*S. persica*	MA461A	USA, Rick Koske	FJ174272	
[7]*S. fulgida*	NC303A	USA, Sidney Sturmer	FJ174273	
[8]*S. pellucida*	FL704	USA, US Sugar Corporation	FJ174269	
[9]*S. calospora*	NC128	USA, Peggy Shultz Bever	FJ174268	
[10]*G. gigantea*	MA401C	USA, Sidney Sturmer	FJ174276	
[11]*G. albida*	BR601	Brazil, Margarida Mendonca	FJ174275	
[12]*G. margarita*	BR444	Brazil, Norm Schenck	FJ174278	
[13]*G. rosea*	UT102	USA, Chris Walker	FJ174274	
[14]*G. decipiens*	AU102	Australia, Chris Gazey	FJ174277	
[15]*A. laevis*	AU211	Australia, Chris Gazey	FJ174308	Acaulosporaceae
[16]*A. scrobiculata*	AU303	Australia, Chris Gazey	FJ174307	
[17]*A. foveata*	CR401	USA, Catherine Lovelock	FJ174304	
[18]*A. lacunosa*	NH102	USA, Dolly Watson	FJ174305	
[19]*A. koskei*	WV736	USA, Michael Dant	FJ174309	
[20]*A. dilata*	WV204	USA, Joseph Morton	FJ174301	
[21]*A. mellea*	CL697	Colombia, Ewald Sieverding	FJ174303	
[22]*A. morrowiae*	HA725	Hawaii, Rick Koske	FJ174302	
[23]*E. kentinensis*	TW111	Taiwan, Chi-Guang Wu	FJ174306	
[24]*E. colombiana*	NB104C	Namibia, Carole Klopatek	FJ174310	
[25]*G. spurcum*	AZ420A	USA, Jean Stutz	FJ174287	Diversisporaceae
[26]*G. eburneum*	AZ414	USA, Jean Stutz	FJ174288	
[27]*G. trimurales*	BR608	Brazil, Sidney Sturmer	FJ174289	
[28]*G. versiforme*	BEG47	USA, John Dodd	FJ174285-6	
[29]*G. clarum*	WV234	USA, Jeff Ning	FJ174296	Glomeraceae
[30]*G. manihotis*	FL879	USA, David Sylvia	FJ174297	(*Glomus* Grp A)
[31]*G. intraradices*	NB102C	Namibia, Carole Klopatek	FJ174299	
[32]*G. sinuosum*	MD126	USA, Dolly Watson	FJ174298	
[33]*G. diaphanum*	WV579	USA, Joseph Morton	FJ174300	
[34]*G. geosporum*	MD215	USA, Pat Milner	FJ174291	
[35]*G. coronatum*	AU202	Australia, Chris Gazey	FJ174290	
[36]*G. fragilistratum*	DN988	Denmark, J.P. Skou	FJ174293	
[37]*G. mosseae*	JA205C	Japan, Kazutaka Akashi	FJ174292	
[38]*G. deserticola*	NC302A	USA, Sidney Sturmer	FJ174295	
[39]*G. constrictum*	FL328	USA, David Sylvia	FJ174294	
[40]*G. hyalinulum* (ined.)	FL707G	USA, US Sugar Corporation	FJ174281	Glomeraceae
[41]*G. claroideum*	SE105B	Senegal, Mark Decousso	FJ174283	(*Glomus* Grp B)
[42]*G. luteum*	ON201A	Canada, unknown	FJ174282	
[43]*G. aggregatum*	FL711	USA, US Sugar Corporation	FJ174284	
[44]*G. etunicatum*	NB103B	Namibia, Carole Klopatek	FJ174279	
[45]*G. etunicatum*YV	BR232C	Brazil, Leonor Maia	FJ174280	
[46]*Ar. leptoticha*	CR312	Costa Rica, Laura Aldrich-Wolfe	FJ174312	Ambisporaceae
[48]*Ar. trappei*	MG103	Madagascar, Erica Styger	FJ174311	Archaeosporaceae
[49]*P. brasilianum*	WV215A	USA, Kelly Heldreth	FJ174314	Paraglomeraceae
[50]*P. occultum*	HA771	Hawaii, Rick Koske	FJ174313	

products that included intron regions. Clones from a single spore of each isolate were restricted with the enzymes *Dde*I and *Nde*I according to the manufacturer's protocol (Promega, USA). These enzymes were chosen after analysis of three β-tubulin sequences of *S. heterogama* isolates sequenced previously. Restriction digest products were separated on 1.5% agarose gel by electrophoresis for 2 h at 70 V. Gels were stained with ethidium bromide and photographed under UV light. Polymorphic bands from each RFLP profile were sequenced for each isolate.

Phylogenetic analysis

Nucleotide sequences were translated into amino acids and then aligned based on these amino acid sequences in MacClade (Maddison and Maddison 2005). Intron sequences were excluded because sequence and length polymorphisms prevented definitive alignments. Distance analyses by neighbor joining (NJ) were obtained by the Kimura two-parameter. Maximum parsimony (MP) trees were obtained in phylogenetic analysis using parsimony (Swofford 1998). Gaps were treated as missing data. Because saturation of the third codon position has been reported for the β-tubulin gene (Corradi et al. 2004a, b), analyses excluding this position also were performed to test for improved resolution of a phylogeny. To date, the sister group to the Glomeromycota remains a subject of debate (Redecker and Raab 2006). Therefore, phylogenetic relations within AMF was investigated using alternative outgroups consisting of representative species of Zygomycota, Basidiomycota/Ascomycota, and Chytridiomycota. Node stability in the trees was assessed by 1,000 bootstrap replications. All branches with a 70% or higher bootstrap support (BS) were considered well supported.

Results

A three-step nested PCR procedure successfully amplified a partial β-tubulin gene fragment (861–1,014 bp) from single spores of selected AMF species (Table 1). This fragment was sequenced from 45 AMF species in INVAM. Sequences showed highest similarities with those of the Glomeromycotan β-tubulin gene deposited in GenBank (AJ717317–AJ717327). In addition to exon regions, this fragment also contained three introns of variable lengths located at the same positions in all AMF species. Position 1 intron ranged in length from 89 to 207 bp while position 2 and 3 introns ranged in length from 68 to 146 and 74 to 120 bp, respectively. Microsatellite sequences were identified at the 3' end of position 1 and 2 introns of some species. The trinucleotide sequence (TTA)5 was identified at the end of position 2 intron of *Scutellospora pellucida* and *Acaulospora scrobiculata*. *Glomus sinuosum* contained a dinucleotide (TA)10 at the end of position 2 intron. *Glomus versiforme* and *Glomus trimurales* contained the microsatellite repeat sequence (TTTA)4 and (TTTA)9, respectively, at the end of position 1 intron. Microsatellite sequences also were detected in *S. heterogama* (see below).

Exon sequences could be aligned unambiguously since no insertions or deletions were present. Translation of all sequences into amino acids revealed no stop codons. Complete alignment of the β-tubulin gene exon nucleotide sequences consisted of 603 bp.

The MP analysis generated 105 most parsimonious trees. A 50%-majority rule consensus tree did not resolve relationships at the order level. However, the topologies of MP trees at the family and species level were congruent with the NJ tree even when fungi from different phyla were used as out-groups. MP bootstrap (MPBS) support levels were similar to those obtained with the NJ tree. An NJ tree with zygomycotan species as the out-group and all codons employed is shown in Fig. 1. All 45 AMF species resolved a highly supported monophyletic group (BS=99%; MPBS=99%). The β-tubulin gene separated AMF species into six families. All families except Glomeraceae were statistically supported at 98–100%. Bootstrap support for Glomeraceae was not significant (60%; MPBS=60%), although support for *Glomus* groups A and B was significant (98% and 100%, respectively; MPBS=87% and 100%, respectively). *Glomus* group A included *Glomus diaphanum* and *Glomus* group B included *Glomus aggregatum* and *Glomus hyalinulum* (ined.), none of which had been analyzed in these groups previously. *G. trimurales* also was placed in the family Diversisporaceae. As with the SSU phylogeny (Schuessler

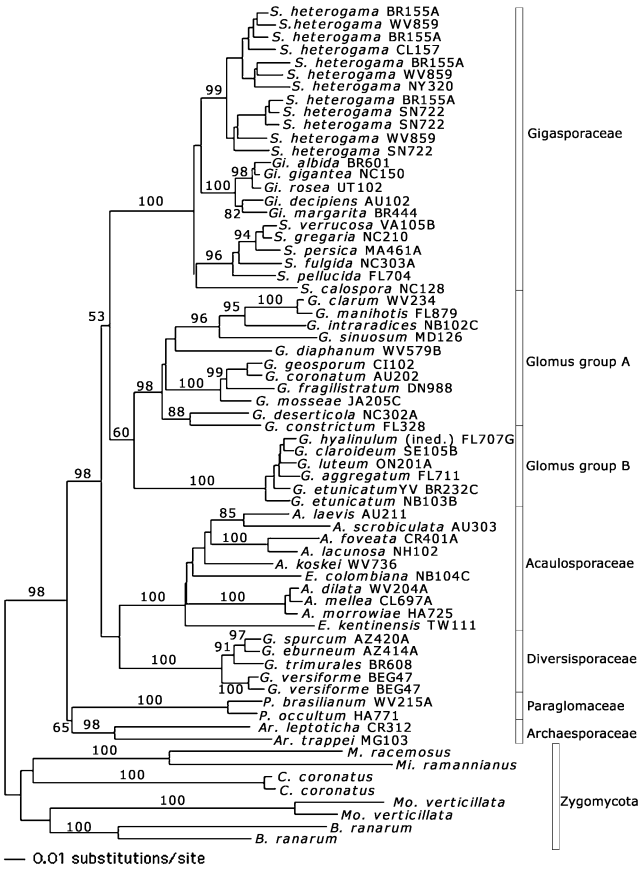

Fig. 1 A neighbor-joining tree generated using all codon positions of the β-tubulin gene. Numbers at nodes indicate bootstrap values ≥50%. Statistically significant clades are assigned numerical designations as indicated. Families are labeled to the right of the tree. The tree was rooted with species in Zygomycota

et al. 2001), Acaulosporaceae and Diversisporaceae grouped as sister families but with no bootstrap support (MPBS= 60%). Within Acaulosporaceae, *Entrophosphora* species appeared to be polyphyletic. *Gigaspora* species grouped with isolates of *S. heterogama* isolates, although with no bootstrap support. Archaeosporaceae was positioned as a basal group in Glomeromycota. This β-tubulin phylogeny differed from other Glomeromycotan phylogenies (SSU, LSU, and RPB1) in that Glomeraceae grouped with Gigasporaceae, although poorly supported (BS=53%). Bootstrap support at the order level was not significant.

When the third codon position was included in the analysis, trees generated polyphyletic fungal phyla. However, all AMF families still were grouped as described above. Exclusion of the third codon position resulted in highly supported monophyletic Glomeromycotan, Basiodiomycotan, and Ascomycotan clades. The Chytridiomycota was weakly supported while Zygomycota was unresolved (Fig. 2).

To assess for paralogous sequences, 90 clones of three geographically distant isolates of *S. heterogama* were

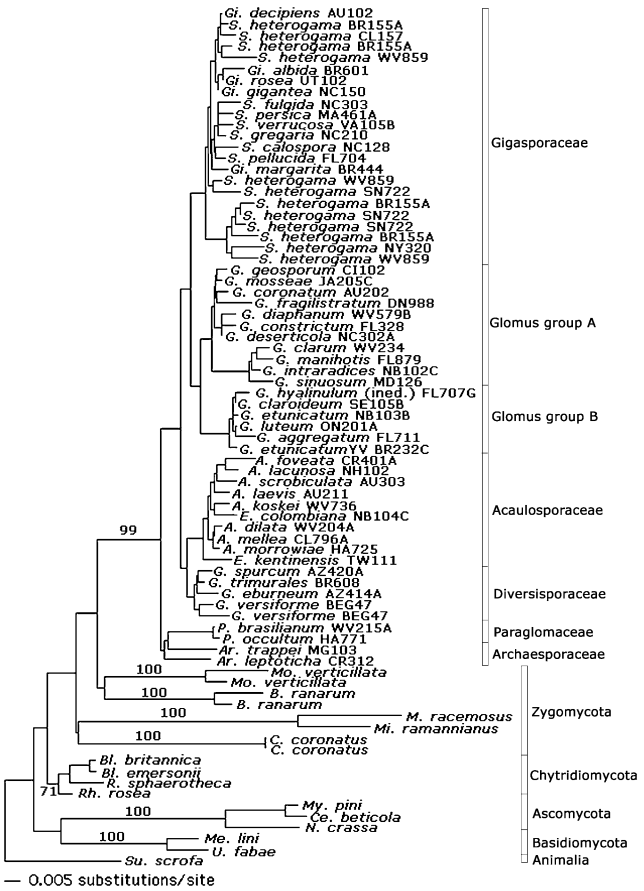

Fig. 2 A neighbor-joining tree generated using only the first and second codon positions of the β-tubulin gene. Numbers at nodes indicate bootstrap values ≥50%. Statistically significant clades are assigned numerical designations as indicated. Families are labeled to the right of the tree. The tree was rooted with *Sus scrofa*

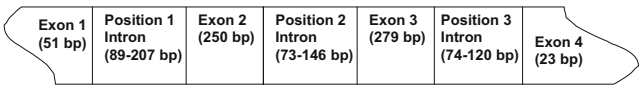

Fig. 3 Illustration of a β-tubulin gene fragment amplified. The exon regions (exons 1, 2, 3, and 4) used for phylogenetic analysis collectively were 603 bp long and intron regions were variable between species

analyzed. All β-tubulin PCR fragments were approximately 940 bp in size (Fig. 3). Restriction of fragments resulted in three and two RFLP patterns from *Dde*I and *Nde*I enzymes in isolates BR155A and WV859, respectively (Fig. 4). Isolate SN722 produced two and three RFLP patterns from *Dde*I and *Nde*I enzymes, respectively. A total of 12 sequences were obtained from distinct RFLP patterns of each isolate. RFLP sequences obtained from divergent bands using each enzyme matched profiles observed on agarose gels. All 12 sequences clustered into a single clade with high bootstrap support (BS=99%). None segregated in the phylogenetic tree to indicate a duplication event. Sequence types were grouped into two subclades, one with seven members and the other with five (Fig. 1). Sequence identity of the exon region ranged from 94% to 99%. Variation among sequences derived from 72 substitutions occurring at all three codon positions, with some leading to amino acid changes.

Comparison of sequences from multiple clones of individual isolates revealed intraisolate heterogeneity. Three β-tubulin sequence types from isolate SN722 clustered in a subclade while one clustered with the other two isolates. Isolates WV859 and BR155A sequence types followed a similar pattern, where three sequence types clustered in one subclade while the other clustered with SN722. All 12 sequences were unique, suggesting that more β-tubulin sequence types may be found in other isolates of *S. heterogama*.

Intron sequence and size also varied among clones of each *S. heterogama* isolate. Position 1 intron ranged in length from 159 to 173 bp, while position 2 and 3 introns were 85–97 and 82–84 bp long, respectively. Alignment of position 1 and 2 introns of *S. heterogama* was difficult due to insertions/deletions that accounted for length differences. Nevertheless, all three intron regions grouped isolates into two subclades. However, the subclades generated by intron sequences were not identical to those generated by exons. Two microsatellite sequence repeat types, dinucleotide TA and trinucleotide TTA, were discovered at the 3' end of position 2 intron. Three clones from isolate BR155A and one clone from WV859 contained a (TA)6 microsatellite. A (TA) 9 microsatellite was present in a clone of isolate SN722 while a (TA)5 and (TA)7 microsatellite repeats were present in two clones of isolate WV859. The second microsatellite type (TTA)4 was found in three clones of isolate SN722 and one clone each of isolates WV859 and BR155A.

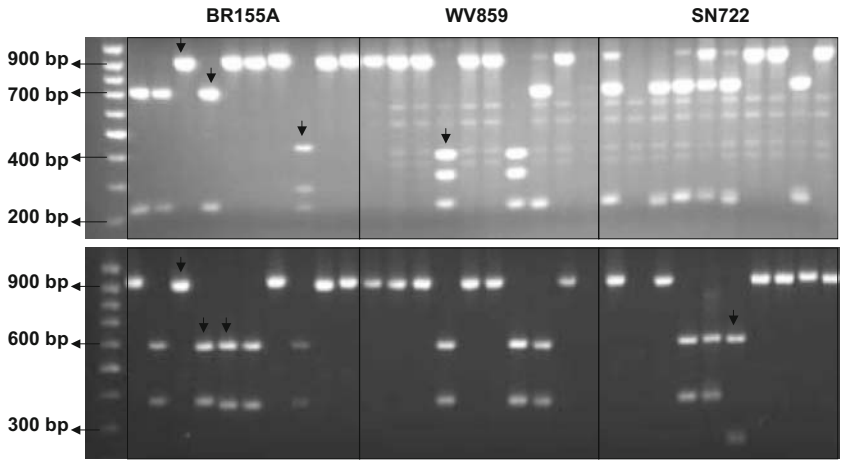

Fig. 4 Characterization of clonal variation in three *S. heterogama* isolates. RFLP patterns from 30 representatives of 90 typed clones. The *top photo* shows clones restricted with *Dde*I and the *bottom photo* shows clones restricted with *Nde*I. *Lane 1*=100-bp DNA ladder, *lanes 2–11*=BR155A, *lanes 12–21*=WV859, *lanes 22–30*=SN722. Profile differences are marked with an *arrow*. Faint bands are of bacterial DNA

Discussion

Amplification of a region of the β-tubulin gene from single spores of AMF species was achieved for the first time using a three-step nested PCR approach. The most common approach for amplifying low-copy genes from AMF species is to use large numbers of AMF spores (Kuhn et al. 2001; Corradi et al. 2004a; Sanders 2004). Other approaches, such as yellow-tip PCR, also have been used to amplify single-copy genes from AMF spores (Helgason et al. 2003).

Corradi et al. (2004a) report that the third codon position of the β-tubulin gene is mutationally saturated in the Glomeromycota and thus phylogenetically uninformative. With increased taxon sampling, this position still contained phylogenetic signal. The six families, as proposed by the SSU phylogeny, were recognized with inclusion of the third codon position. Within families, different taxa recognized by the SSU gene also were resolved. The third codon position appeared to be useful for resolving family and species-level relationships in Glomeromycota. Other studies also have shown the usefulness of the mutationally saturated third codon position in constructing phylogenies (Yang et al. 2007).

With or without the third codon position, the β-tubulin gene phylogeny reflects the SSU phylogeny at the family and species level, but it is not concordant at the order level. In the SSU phylogeny, Diversisporaceae, Acaulosporaceae, Pacisporaceae, and Gigasporaceae form a clade identified as the order Diversiporales. The β-tubulin phylogeny, in contrast, groups the Glomeraceae and Gigasporaceae in a clade separate from the Diversisporaceae/Acaulosporaceae clade.

The artifactual grouping together of the Glomeraceae and Gigasporaceae in this study may be due to the inclusion of paralogous sequences. Absence of Pacisporaceae and Geosiphonaceae also may have distorted the analysis. However, a "single"-copy gene, alpha-tubulin, also grouped Glomeraceae and Gigasporaceae with high bootstrap support (Corradi et al. 2004b). Phylogenies inferred from other single-copy protein-encoding genes (α-elongation and actin) are in agreement with the morphological phylogeny (Morton 1990), in that the Acaulosporaceae and Glomeraceae are sister families (Helgason et al. 2003). However, only a small number of taxa were analyzed, so interfamily relationships in Glomeromycota remains unresolved.

The first and second codon positions were most informative for resolving the position of the different fungal phyla, as concluded by Corradi et al. (2004a). With an increase in taxa sampled, these positions also were informative in resolving family relationships within Glomeromycota. However, species relationships within Gigasporaceae could not be resolved.

An increase in number of taxa coupled with the exclusion of the third codon position grouped Zygomycota and Glomeromycota as sister clades but without bootstrap support. In contrast, Corradi et al. (2004a, b) identified Chytridiomycota as a sister group using the same gene. Zygomycota is hypothesized to be a sister clade of Glomeromycota based on phylogenies of the protein-encoding genes RPB1, elongation factor, and actin (Redecker and Raab 2006; Helgason et al. 2003). In contrast, the SSU rRNA gene phylogeny suggests the Basidiomycota/Ascomycota clade is a sister clade to Glomeromycota (Schuessler et al. 2001).

In *Glomus* species, at least two copies of the β-tubulin gene are present. These copies share 86% sequence identity in exon regions and are polyphyletic (Corradi et al. 2004a, b). Analysis of multiple clones from three isolates of *S. heterogama* suggested that only a single copy of the β-tubulin gene is present in this species. Sequences shared 94–99% identity, indicating a monophyletic evolution. Clearly, sequence variation observed in *S. heterogama* isolates was due to intraspecific variation. No evidence for the presence of paralogs has been found in two other species in Gigasporaceae namely, *Gigaspora gigantea* (Msiska, in preparation) and *Scutellospora castanea* (Corradi et al. 2004a, b).

Intraspecific sequence variability of AMF is well known for rRNA genes (Sanders et al. 1995, 1996; Lloyd-Macgilp et al. 1996; Clapp et al. 1999, 2001; Lanfranco et al. 2001; Walker et al. 2004; da Silva et al. 2006). Sequence variability in protein-encoding genes in AMF species also has been reported (Kuhn et al. 2001; Helgason et al. 2003; Pawlowska and Taylor 2004). In this study, intraisolate variability of the β-tubulin gene in *S. heterogama* also was characterized. Many substitutions occurred in the coding region and resulted in some amino acid changes similar to those in the *BiP* gene (Kuhn et al. 2001). These results indicated that at least some variation is not selectively neutral, so different functional variants of the β-tubulin gene may exist in different isolates of a species. Since only one copy of the β-tubulin gene was present in *S. heterogama*, sequence variation in single spores could be attributed to variation among nuclei. This hypothesis would be supported if AMF are heterokaryotic (Kuhn et al. 2001). Since β-tubulin intron sequences are highly variable between species and genera as well as within a species, these regions could contribute useful markers in genotyping populations within a species.

In conclusion, the β-tubulin gene has the same power as the 18S and 25S rRNA genes to resolve phylogenetic relationships among AMF at the family and species level. However, due to paralogous sequences in some genera, the β-tubulin gene can produce trees with poor resolution and poor support at the order level. More work needs to be done to determine the number of β-tubulin gene copies in species of other groups and whether that number is genus specific. Inclusion of taxa from other families such as the Pacisporaceae and Geosiphonaceae could further enhance resolution of the β-tubulin phylogeny.

To date, only multicopy genes have been used to construct an AMF phylogeny from a broad range and number of species. Single-copy gene phylogenies with all families of the Glomeromycota represented need to be constructed and compared with those of multicopy genes. As AMF are economically and ecologically important organisms, a robust phylogenetic framework that encompasses different genes as well as more traditional characters is essential for producing an informative and stable classification.

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