Mycorrhiza (2009) 19:501–513
DOI 10.1007/s00572-009-0248-z

ORIGINAL PAPER

Isolation and sequence analysis of a β-tubulin gene from arbuscular mycorrhizal fungi

Zola Msiska · Joseph B. Morton

Received: 30 September 2008 / Accepted: 15 April 2009 / Published online: 15 May 2009

Abstract A full-length β-tubulin gene has been cloned and sequenced from *Gigaspora gigantea* and *Glomus clarum*, two arbuscular mycorrhizal fungi (AMF) species in the phylum Glomeromyota. The gene in both species is organized into five exons and four introns. Both genes are 94.9% similar and encode a 447 amino acid protein. In comparison with other fungal groups, the amino acid sequence is most similar to that of fungi in the Chytridiomycota. The codon usage of the gene in both AMF species is broad and biased in favor of an A or a T in the third position. The four introns varied in length from 87 to 168 bp for *G. gigantea* and from 90 to 136 bp for *G. clarum*. Of all fungi in which full-length sequences have been published, only AMF do not have an intron before codon 174. The introns positioned at codons 174 and 257 in AMF match the position of different introns in β-tubulin genes of some Zygomycete, Basidiomycete, and Ascomycete fungi. The 5′ and 3′ splice site consensus sequences are similar to those found in introns of most fungi. Sequence analysis from single-strand conformation polymorphism analysis confirmed the presence of two β-tubulin gene copies in *G. clarum*, but only one copy was evident in *G. gigantea* based on Southern hybridization analysis.

Keywords Glomeromycota · Inverse PCR · Beta-tubulin · Arbuscular mycorrhizal fungi

Z. Msiska (✉) · J. B. Morton
West Virginia University,
1090 Agricultural Science Building,
Morgantown, WV 26506, USA
e-mail: zolamsiska@gmail.com

Introduction

The tubulin gene family consists of six distinct but highly conserved subfamilies, alpha-, beta-, gamma-, delta-, epsilon-, and zeta-tubulins (McKean et al. 2001). Since alpha- and beta-tubulins are the most abundant proteins in a eukaryotic cell, they have been studied most extensively. Heterodimers of these proteins are the primary constituents of microtubules, which are the main components of the cell cytoskeleton. Eukaryotic flagella, cilia, and mitotic spindles also are composed of microtubules. The β-tubulin gene is fairly conserved, with at least 60% amino acid similarity between the most distantly related lineages (Juuti et al. 2005).

Many fungal species contain only a single β-tubulin gene, but some also contain two copies (Ayliffe et al. 2001). Paralogs of the β-tubulin gene encode proteins ranging from 60% to 89% amino acid sequence similarity within a species (Mukherjee et al. 2003; Juuti et al. 2005). In some fungal species, the gene copies may be differentially expressed during sporulation and vegetative growth (Panaccione and Hanau 1990; Cruz and Edlind 1997; May et al. 1987; Buhr and Dickman 1994). Gene copies also may differ functionally, such as only one conferring fungicide resistance (Orbach et al. 1986; Panaccione et al. 1988; Jung et al. 1992; Goldman et al. 1993; Cruz and Edlind 1997). In other cases, two β-tubulin genes are functionally interchangeable, such as those of *Aspergillus nidulans* (May 1989; Weatherbee et al. 1985). Phylogenetic analysis shows that although different in sequence, the two β-tubulin paralogs of *A. nidulans* cluster in a single clade of Ascomycetes, while those of *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, *Trichoderma viride*, and *Galactomyces geotrichum* diverge sufficiently to be positioned in different clades (Keeling et al. 2000; Ayliffe et al. 2001). Copies of β-tubulin genes in *Powellomyces variabilis* and *Spizellomyces punctatus* di-

verged so rapidly that one clusters in Chytridiomycota and the other in Zygomycota (Corradi et al. 2004a). Similarly, TUB1gene of *Suillus bovinus* clusters with Basidiomycota but TUB2 gene clusters with Ascomycota (Juuti et al. 2005). Corradi et al. (2004a, b) identified two β-tubulin gene variants in four *Glomus* species: *Glomus intraradices*, *Glomus claroideum*, *Glomus diaphanum*, and *Glomus proliferum*, but paralogs were not detected in *Acaulospora laevis*, *Gigaspora margarita*, *Glomus* sp. BEG19, *Glomus geosporum*, and *Scutellospora castanea*. Unlike those of some species in Chytridiomycota and Basidiomycota, the β-tubulin paralogs of sampled arbuscular mycorrhizal fungi (AMF) all clustered within Glomeromycota and supported a sister grouping between Chytridiomycota and Glomeromycota (Corradi et al. 2004a). In contrast, the 18S rRNA (SSU) gene phylogeny places AMF as a sister group of Ascomycota and Basidiomycota clades, while actin, α-elongation, and RPB1 (large subunit of RNA polymerase II) genes group Glomeromycota with Zygomycota (Helgason et al. 2003; Redecker and Raab 2006).

Intron positions within fungal β-tubulin genes may provide some clues of evolutionary relationships among species. Intron locations generally are characteristic of fungal class and are conserved within a class, based on comparisons of β-tubulin genes of 33 fungal species from the Basidiomycota and Ascomycota (Ayliffe et al. 2001). Intron numbers in the β-tubulin gene range from none for *Saccharomyces cerevisiae* (Neff et al. 1983) to 21 for *S. bovinus* (Juuti et al. 2005).

The majority of full-length fungal β-tubulin genes/complementary DNA (cDNA) have been isolated by screening genomic libraries, cDNA libraries, or the use of rapid amplification of cDNA ends (RACE). Inverse polymerase chain reaction (PCR) is a method that rapidly amplifies unknown DNA sequences flanking a region of a known sequence (Ochman et al. 1988). Only Li and Yang (2007) used this method to obtain a full-length β-tubulin gene in fungi. None of the 13 full-length genes of AMF deposited in Genbank has been isolated using inverse PCR (Table 1). The aim of this study was to characterize β-tubulin genes from genomic DNA of the arbuscular mycorrhizal fungi *Gigaspora gigantea* and *Glomus clarum* after isolation and amplification by inverse PCR. Isolation of full-length AMF β-tubulin gene allowed comparison of intron position and evolution with other fungal phyla.

Table 1 A synthesis of full-length genes sequenced from arbuscular mycorrhizal fungi and deposited in Genbank

Gene	Species	Accession #	Gene isolation method	References
DNA binding protein	*G. intraradices*	EF488828		Flores-Gomez et al. (unpublished manuscript)
Neutral trehalase	*G. intraradices* *Glomus mosseae*	AY787134 AY787133	cDNA library, RACE	Ocon et al. (2007)
Heat shock protein 60	*G. intraradices*	DQ383980 DQ383981	cDNA library	Gadkar and Rillig (2006)
Alkaline phosphatase	*G. margarita* *G. intraradices*	AB114299 AB114298	5′-RACE, RT-PCR	Aono et al. (2004)
Glutamine synthetase	*G. mosseae* *G. intraradices*	AY360451 AJ315337	RACE	Breuninger et al. (2004)
H(+)-ATPase	*G. mosseae BEG12*	AY149918	RACE	Requena et al. (2003)
	G. mosseae	AY193825		
Glycogen branching enzyme	*G. intraradices*	AF503447		Lammers and Yair (unpublished manuscript)
Acyl-CoA dehydrogenase	*G. intraradices*	AY033936	cDNA library	Bago et al. (2002)
Phosphate transporter	*G. intraradices* *Glomus versiforme*	AF359112 U38650	cDNA library	Harrison and van Buuren (1995)
3-phosphoglycerate kinase	*G. mosseae*	AF072893	RACE	Harrier et al. (1998)
Putative cruciform DNA binding protein (Gv1)	*G. versiforme*	AF034574	cDNA library	Burleigh and Harrison 1998
Phosphatidylinositol 3-kinase (TOR2)	*G. mosseae*	AJ276633	Genomic DNA library	Requena et al. (2000)
Beta-oxidation protein (FOX2)	*G. mosseae*	AJ243538	Genomic DNA library	Requena et al. (1999)
Amino acid permease	*G. mosseae*	AY882560	cDNA library	Cappellazzo et al. (2008)
ena1 and ena2 gene; P-type II D ATPase	*G. intraradices*	AM118102 AM118103	RACE	Corradi and Sanders (2006)
Chitin synthase	*G. versiforme*	AJ009630	Genomic DNA library	Lanfranco et al. (1999)

Materials and methods

Genomic DNA extraction

Two AMF taxa were chosen as model species, one with small spores (*G. clarum*) and another with large spores (*G. gigantea*). Spores of *G. gigantea* and *G. clarum* were extracted from cultures of the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM, USA) by wet sieving followed by sucrose gradient centrifugation and then thorough washing with distilled water (Gerdemann and Nicolson 1963). Spores were checked under a dissecting microscope and approximately 40 μL of visually healthy spores were transferred to 1.5 mL Eppendorf tubes. Spores were surface-cleaned three times for 15 s by ultrasonication followed each time by a sterile distilled water rinse. Fungal DNA was released from spores using a mini-bead beater (Biospec, USA) and 0.5 mm glass beads. After spores were broken, DNA was extracted using a DNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. The amount and quality of DNA extracted was checked by migration on 1.5% agarose gel stained with ethidium bromide.

PCR amplification of the β-tubulin gene internal region

Design of primers was based on partial AMF cDNA sequences of the β-tubulin gene available at the National Center for Biotechnology Information (Franken and Requena 2001). Several primers were designed in the conserved regions using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3). PCR amplification of a partial β-tubulin gene was achieved using primers i36F (5′-ACCCAC TCCCTTGGTGGTGGT-3′) and 2iR (5′-GTGAAGACGT GGGAAAGGAAC-3′) in a reaction mixture containing 500 ng genomic DNA, 1.25 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 U Taq DNA polymerase (Promega), 3.5 mM Mg_2Cl, 7 μL 10× buffer, and 1 μM primers. PCR cyling conditions were as follows: an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, followed by a final extension step 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 the Qiagen Gel purification kit. The PCR products were cloned using a Qiagen PCR cloning kit. Positive transformants were screened further by direct colony PCR. Plasmid DNA containing the insert was purified using the Qiagen miniprep kit. The sequence of the internal region was obtained from Davis sequencing (USA). This β-tubulin gene fragment specific for *G. gigantea* was used as a probe for Southern hybridization analysis in order to determine β-tubulin gene copy number.

Southern blot analysis

Initial analysis was performed with 3 μg of genomic DNA from both species, but no bands were detected. Hence, aliquots of 10 μg of genomic DNA were digested with *Eco*RI and *Hin*dIII. A known partial sequence (600 bp) contained one *Hin*dIII cleavage site and no *Eco*RI cleavage site. Digested DNA was electrophoresed on 0.8% agarose gels and bands were transferred onto Hybond N+ membranes by capillary transfer. Hybridization was carried out with a radioactively labeled probe (32-P[dCTP]) (Decaprime™ II random priming DNA labeling kit, Ambion, Inc., USA). Prehybridization solution contained 2.5 mL 20× saline–sodium phosphate–EDTA (SSPE), 5 mL formamide, 1 mL 10% sodium dodecyl sulfate (SDS), 1 mL 50× Denhardt's solution, 400 μL ddH2O, and 100 μL Herring sperm DNA (10 ng/mL) in a total volume of 10 mL. The sperm DNA was boiled for 3 min and then added to the prehybridization solution. Prehybridization was performed at 42°C for 4 h.

Hybridization solution contained 2.5 mL 20× SSPE, 5 mL formamide, 1 mL 10% SDS, 200 μL 50× Denhardt's solution, 1.2 mL ddH2O, and 100 μL Herring sperm DNA (10 ng/mL). Hybridization was performed overnight at 42°C. The membrane was washed twice at room temperature for 15 min in a solution containing 1× SSPE and 0.1% SDS. A final wash at 50°C was performed for 15 min. Labeled DNA was detected using a phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA).

Nested PCR using conserved primers

Conserved primers with the potential to amplify paralogs of the β-tubulin gene were used in order to determine gene copy number. DNA from *G. gigantea* and *G. clarum* was first amplified using the primers Deg.Tub.F and Deg.Tub.R according to Corradi et al. (2004a). The nested PCR employed primers β.Tub.F and β.Tub.R according to Corradi et al. (2004b). Amplification products were electrophoretically separated on 1.5% agarose gels and stained with ethidium bromide. The expected bands were purified with a Qiagen PCR purification kit. PCR fragments then were cloned into a Qiagen vector according to the manufacturer's protocol. Positive transformants were selected according to the blue/white screening procedure. Transformed clones differing in sequences were determined by screening with single-strand conformation polymorphism (SSCP). From each species, 30 transformed colonies were re-amplified with primers B.Tub.F and B.Tub.R. The resultant PCR products were denatured at 95°C for 10 min and then snap cooled for 5 min. Submerged gel electrophoresis was carried out at 4°C and 200 V for 5 h. Gels were stained with silver nitrate for 20 min. Differences in the banding patterns

indicated clones with different sequences. Each unique pattern was chosen for sequencing and analysis.

Preparation of DNA for inverse PCR

The program NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php) was used to determine the occurrence of restriction enzyme sites within the known DNA sequence for the restriction enzymes *Hin*dIII and *Hae*III. Restriction analysis was performed by digesting 1 μg of genomic DNA in 20 μL reactions containing 2 μL of the specific enzyme buffer (10×), 0.2 μL bovine serum albumin, and 1 μL restriction enzyme. DNA was digested overnight at 37°C and then cleaned using the MinElute Reaction Cleanup kit (Qiagen, USA). Digested DNA was circularized by self-ligation overnight at 4°C in a total volume of 50 μL. The reaction mixture contained 10 μL of clean digested DNA, 5 μL buffer, and 3 U of T4 DNA ligase (Promega, USA).

Inverse PCR

Based on a known (600 bp) DNA sequence, specific primers were designed for each of the two AMF species and a series of inverse PCR reactions were carried out using primers listed in Table 2. For *G. gigantea*, primer pairs GIGA1F/GIGA1R and GIGA2F/GIGA2R amplified circularized *Hin*dIII-digested DNA and primer pair GIGA3F/GIGA3R amplified circularized *Hae*III-digested DNA. For *G. clarum*, the primer pair BtubS/GiH4R amplified genomic DNA; primers GC1F/GC1R amplified circularized *Hin*dIII-digested DNA. PCR was done in a 50-μL volume containing 5.0-μL circularized DNA, 0.5 U Expand high fidelity DNA polymerase (Roche diagnostics), 10× PCR buffer, 3.5 mM $MgCl_2$, 1.25 mM each of dATP, dCTP, dGTP, and dTTP, and 1 μM primers. PCR conditions were as follows: initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, extension at 72°C for 3 min, and a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Bands were excised and purified using the Qiagen Gel purification kit. PCR products were cloned using a Qiagen PCR cloning kit. Positive transformants were further screened by direct colony PCR. Plasmid DNA containing the inserts was purified using the Qiagen miniprep kit. Sequences were obtained from Davis sequencing (USA). The full-length β-tubulin gene (coding and intron regions) was generated by constructing consensus sequences in BioEdit from overlapping fragments. Nucleotide and amino acid sequence homology searches were performed using NCBI databases by BLAST search. Regulatory elements in 3′ and 5′ flanking sites were predicted using software programs from Harvard University (http://mbcf.dfci.harvard.edu/cmsmbr/biotools.html).

Table 2 Primers designed for inverse PCR to isolate and amplify the β-tubulin genes of two representative glomeromycotan species from genomic DNA preparations

Primer	Sequence (5'-3')
Gigaspora gigantea	
GIGA1F	GGTTTCGGATACTGTTGTTG
GIGA1R	AGGACCGACAGAAAACGTGC
GIGA2F	GTTCCTTTCCCACGTCTTCAC
GIGA2R	AATGTACGGAAGCAAATATC
GIGA3F	GCTGCCGTGTGTGATATTCC
GIGA3R	TTTGGACAGCCAACATTTGA
Glomus clarum	
BtubS	ATGAGNGARATHGTTCACTTACAA
GiH4R	CATACCCTCACCAGTATACCAATG
GC1F	CCGCTGTTTGTGACATTCCACC
GC1R	AAAGTTCGAATGCAAATGTC

Phylogenetic analysis

Beta-tubulin protein sequences were searched and downloaded from Genbank and the Broad Institute (www.broad.mit.edu). The sequences were aligned in ClustalX excluding the highly variable carboxyl-terminus. The sequences were further edited manually in McClade before analysis with PAUP. Only exon regions were used to generate trees showing the distribution of paralogous sequences in *Glomus* species. Sequences of *G. clarum* clones were deposited in Genbank under accession numbers FJ687212–FJ687219.

Results

PCR amplification of the β-tubulin gene internal region

Approximately 26 and 10 μg of high-quality genomic DNA was extracted from a 40-μL volume of pooled *G. gigantea* and *G. clarum* spores, respectively. A 600-bp internal region of the β-tubulin gene was amplified from both species. The β-tubulin gene fragment specific to *G. gigantea* provided the probe to analyze *G. gigantea* genomic DNA by Southern blot. The gene copy number for *G. clarum* could not be determined by Southern blot because of insufficient genomic DNA.

Southern blot analysis to determine gene copy number

Upon hybridization with the 600-bp β-tubulin gene fragment specific to *G. gigantea*, two and one signals were

detected from DNA digested with *Hin*dIII and *Eco*RI, respectively. The banding pattern was as predicted from the nucleotide sequence of the partial β-tubulin gene fragment. The *Eco*RI signal was detected at less than 2 kb. The longer *Hin*dIII signal was detected at less than 2 kb while the smaller signal was detected at less than 1 kb (Fig. 1). No bands were observed except those predicted from the nucleotide sequence, even under conditions of low stringency (2× SSPE wash buffer).

Identification of two β-tubulin genes in *G. clarum* using conserved primers

Sequencing of multiple clones from *G. clarum* indicated two highly variable β-tubulin gene sequences designated BTUB1 and BTUB2. A high number of synonymous substitutions were observed within the exon regions. The two genes differed significantly in the length and sequence of introns. For example, position 1 intron of BTUB1 and BTUB2 was 136 and 207 bp in length, respectively. Position 2 intron of BTUB1 and BTUB2 was 87 and 69 bp in length, respectively. A phylogenetic analysis of both genes together with glomeromycotan β-tubulin gene sequences deposited in Genbank showed divergence consistent with a duplication event (Fig. 2). Full-length sequences were obtained only for BTUB1 using inverse PCR. SSCP screening and sequencing of multiple clones suggested that paralogs are absent in *G. gigantea*.

Inverse PCR to obtain full-length β-tubulin sequences

Inverse PCR products generated from *G. gigantea* DNA digested with *Hin*dIII were approximately 650 and 550 bp in length, while the product generated from *Hae*III digest was approximately 750 bp long. The 650-bp product included 120 bp of 5′-flanking sequence, while the 750-bp product included 330 bp of 3′ flanking sequence. Inverse PCR product generated from *G. clarum* DNA digested with *Hin*dIII was approximately 850 bp, while the product generated from primers BtubS/GiH4R was approximately 1500 bp. The *Hin*dIII fragment included more than 400 bp of the 3′ flanking sequence. The β-tubulin gene isolated from *G. gigantea* was 1,779 bp long, while the BTUB1 gene from *G. clarum* was 1,752 bp long.

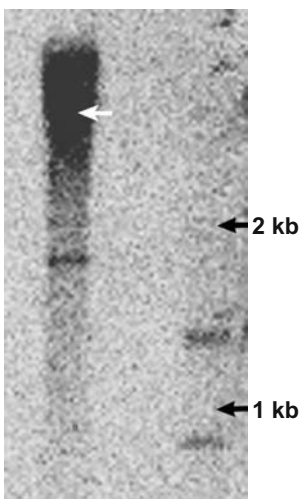

Fig. 1 Southern blot of genomic DNA from *Gigaspora gigantea*. Total genomic DNA samples (10 μg/lane) were digested with *Eco*RI—*lane 1* and *Hin*dIII–*lane 2* and separated on 0.8% agarose gel. Hybridization was performed using a partial 600-bp β-tubulin gene fragment as a probe. DNA standard sizes are marked with *black arrows*. The *white arrow* shows undigested DNA

Based on comparisons of the deduced amino acid and known β-tubulin sequences, the *G. gigantea* β-tubulin gene encoded a 447 amino acid protein with a calculated molecular mass of 50.08 kD and an isoelectric point of 4.46 (Fig. 3). The *G. clarum* BTUB1 gene also encoded a 447 amino acid protein with a calculated molecular mass of 50.16 kD and an isoelectric point of 4.41 (Fig. 4). The amino acid sequences of *G. gigantea* and *G. clarum* BTUB1 were 94% similar, differing in only 27 amino acids. The region from amino acid 427 to the C-terminus was highly variable and accounted for ten of the 27 different amino acids. A high number of synonymous substitutions were observed along the exon sequences.

Comparison of the AMF β-tubulin amino acid sequences with β-tubulin proteins from other fungi revealed that overall amino acid sequence similarity was highest with the β-tubulin gene from species in Chytridiomycota (92% to 94% in overlapping regions). Neighbor joining (NJ; Fig. 5) and 50% majority rule maximum parsimony (MP; data not shown) trees generated using nearly full-length amino acid sequences (436 amino acids in length) did not indicate a sister grouping of AMF with chytrids. Bootstrap support was highly significant in the NJ tree at the phylum level, except for the Ascomycota. Zygomycotan sequences grouped in the same clade as the highly divergent *S. bovinus btub2*, which groups within Ascomycota (Juuti et al. 2005). In contrast to the NJ tree, the five fungal phyla were monophyletic in the MP tree; however, support at the phylum level was only moderately significant.

Intron positions of AMF β-tubulin genes were inferred by comparison with genes from other fungi rather than verified by cDNA sequence analysis. Four introns were positioned after codon 174, within codons 257 and 350, and after codon 421. In *G. gigantea*, the position 1 intron was 168 bp long while position 2, 3, and 4 introns were 91, 87, and 89 bp long, respectively. In *G. clarum* BTUB1, position 1, 2, 3, and 4 introns were 136, 87, 90, and 95 bp long, respectively. All introns contained 5′ and 3′ consensus splice junctions similar to those in β-tubulin genes of other fungi that conformed to the GT-AG rule.

The G+C content of the coding and intron regions of *G. gigantea* and *G. clarum* β-tubulin genes was 35.3% and 36.6%, respectively. Introns alone had an average G+C content of 16.2% and 22% for these two fungi, respectively, while exons alone averaged a G+C content of 41.4% in both fungi. The G+C content of the 5′ and 3′ flanking

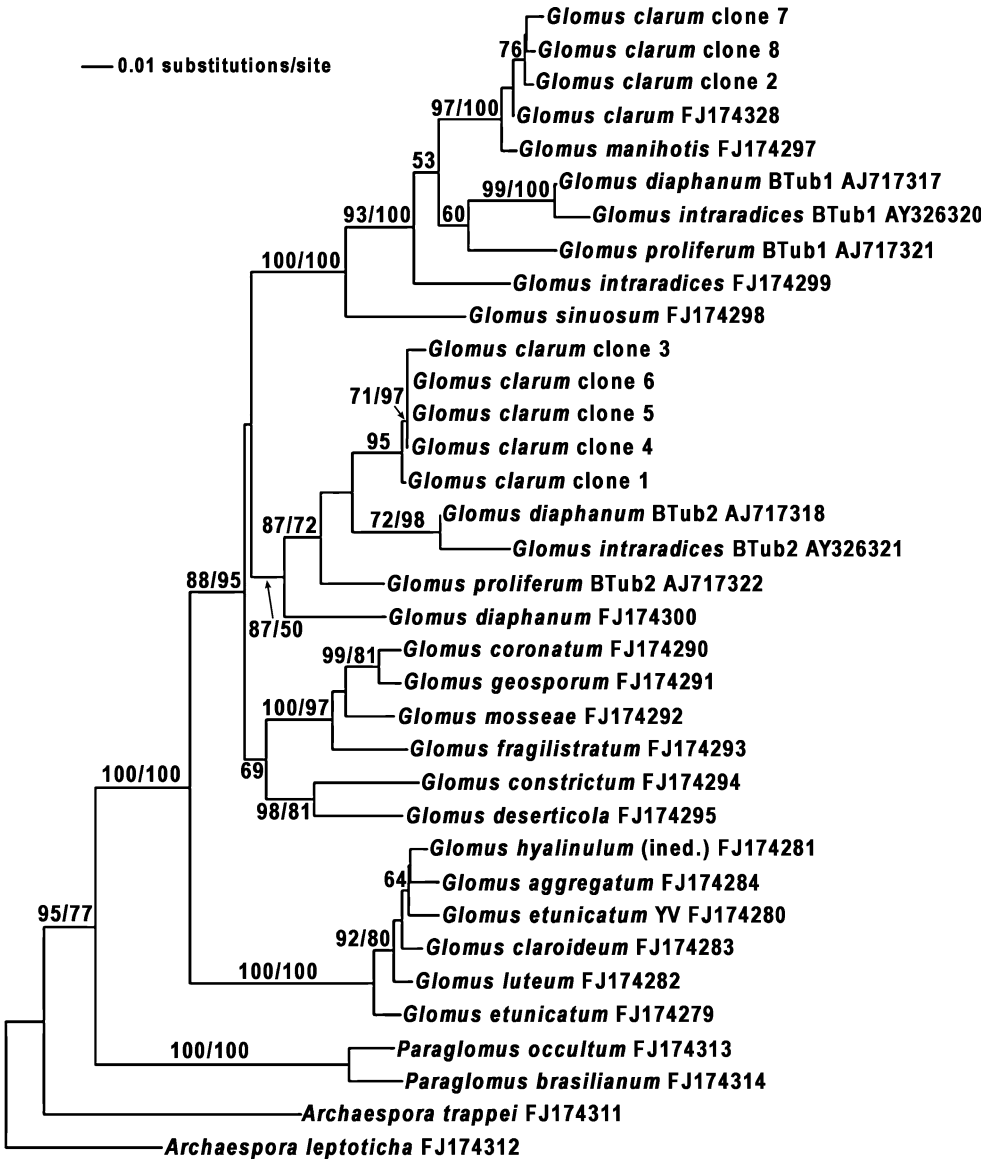

Fig. 2 Phylogeny of *Glomus* species based on partial nucleotide (626 bp) β-tubulin gene sequences showing the distribution of paralogous sequences. Numbers at nodes indicate NJ/MP analysis bootstrap values ≥50%. The tree was rooted with *Paraglomus leptoticha*

sequences were 30.2% and 24.2%, respectively, for *G. gigantea*. The G+C content at the 3′ flanking region of *G. clarum* was 21.8%.

Two putative polyadenylation signals typical of higher eukaryotes (AATAAA) were present at positions 1,957 and 2,087 bp in the 3′ flanking sequence of *G. gigantea* (Fig. 3) and at positions 1,962 and 1,992 bp of the G. *clarum* 3′ flanking sequence (Fig. 4). Putative TATA-like elements at −112 bp and a putative transcription start site at −83 bp were present in the 5′ flanking region of the *G. gigantea* gene. However, no corresponding CAAT box sequence was found.

Discussion

Most full-length AMF cDNAs have been characterized by RACE or by screening cDNA libraries (Table 1). In this study, inverse PCR provided a simple way to sequence the full-length β-tubulin gene (coding and intron regions) and its flanking sequences from two widely recognized AMF species, *G. gigantea* and *G. clarum*. As only 13 full-length genes have been isolated from AM fungi to date, inverse PCR offers a tool for rapid isolation of other AMF genes provided that partial sequences are available to design specific primers. The inverse PCR method also is advantageous in that the use of overlapping regions ensures that no contaminants in the sample are sequenced. This technique also can be used to isolate full-length cDNAs provided that RNA instead of genomic DNA is used as the starting material (Huang et al. 2003).

The β-tubulin gene was highly conserved, encoding a 447 amino acid polypeptide in both AMF and most other fungi (Genbank, www.ncbi.nlm.nih.gov). The highly conserved glycine-rich sequence, GGGTGAG, was present

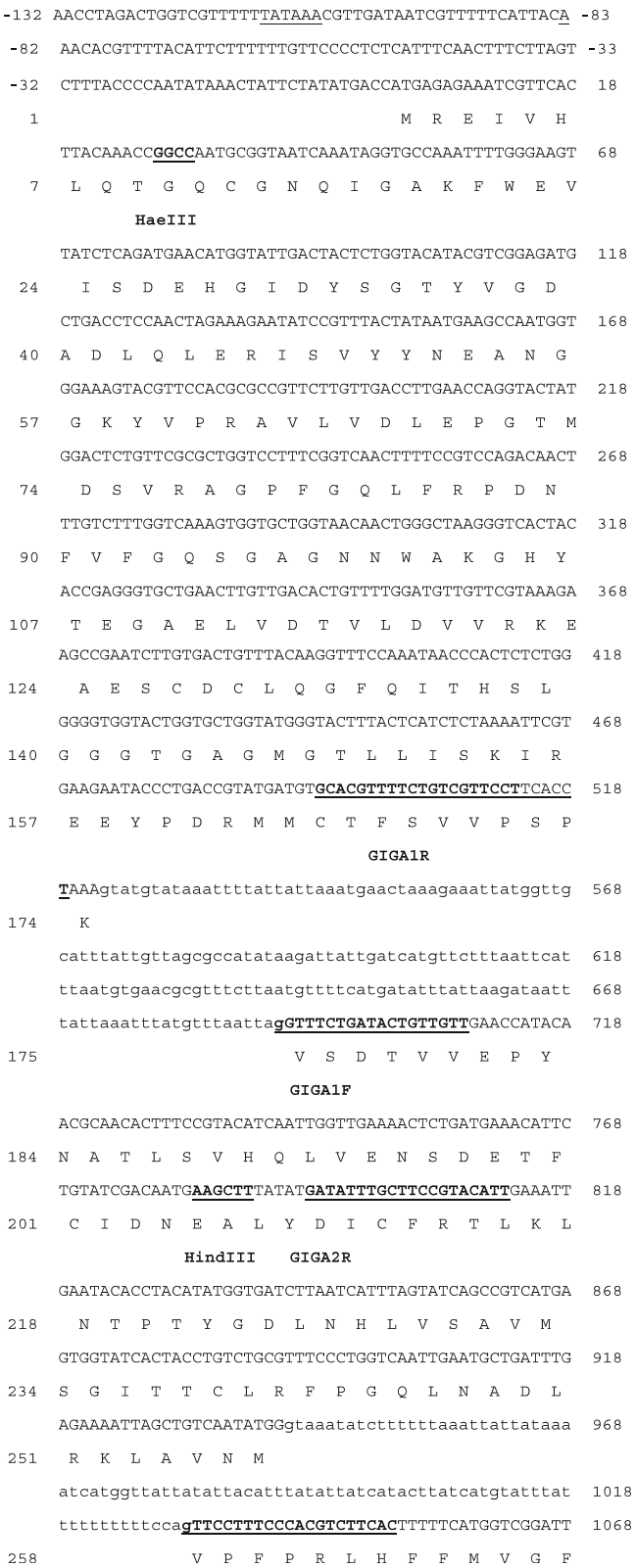

Fig. 3 Nucleotide and deduced amino acid sequence of the *Gigaspora gigantea* β-tubulin gene. Intron regions are in *lower case*, restriction enzyme recognition sites and primer sequences are in *bold and underlined*. Putative transcriptional control signals are *underlined*. Number of nucleotides and amino acids are displayed in the *right and left margins*, respectively

Fig. 3 (continued)

from residues 140 to 146 in AMF β-tubulin genes. This peptide forms part of the GTP-binding site by interacting with the nucleotide's phosphates (Nogales et al. 1998), A potential Mg^{2+} binding site (EALY; Farr and Sternlicht

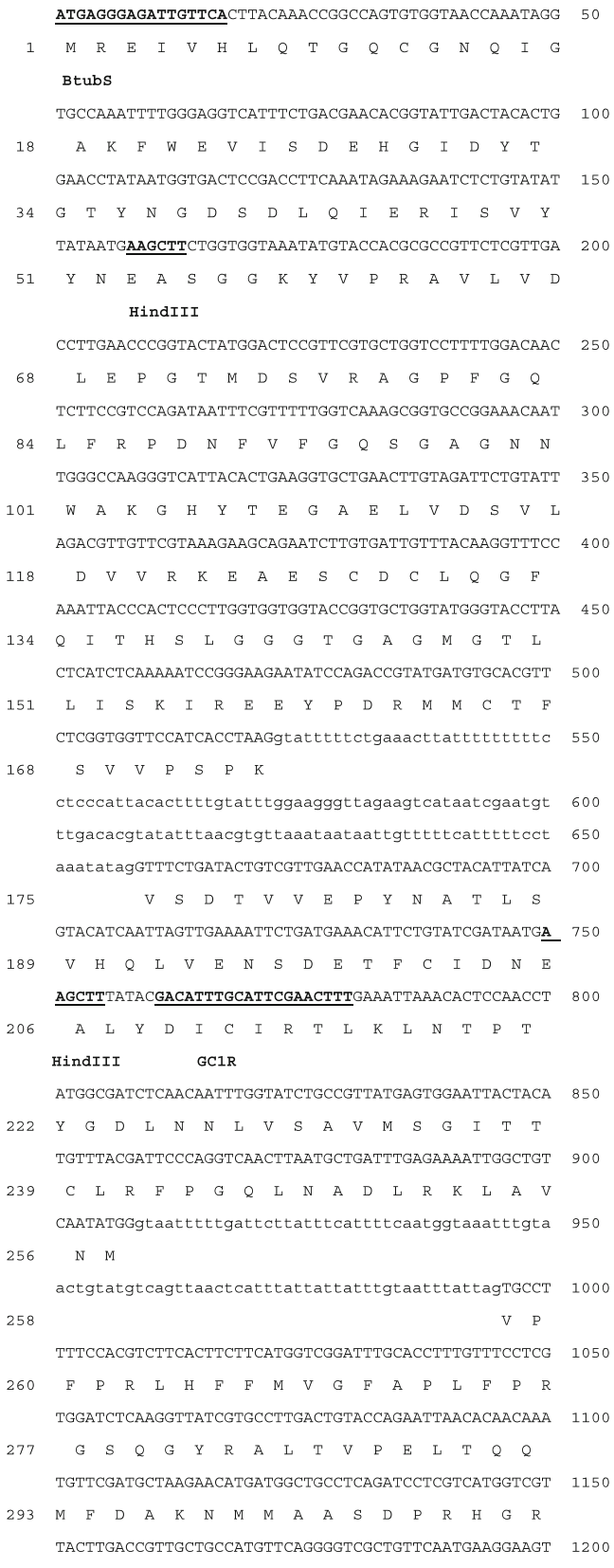

Fig. 4 Nucleotide and deduced amino acid sequence of the *Glomus clarum* β-tubulin gene (BTUB1). Intron regions are in *lower case*, restriction enzyme recognition sites and primer sequences are in *bold and underlined*. Putative transcriptional control signals are *underlined*. Number of nucleotides and amino acids are displayed in the *right and left margins*, respectively

1992) was identified between amino acids 205 and 208. The β-tubulin gene in AMF and other fungi encode an N-terminal tetrapeptide MREI involved in the autoregulation of β-tubulin expression in mammalian cells (Cleveland and Theodorakis 1994). Point mutations at codons 6 (His to Tyr/Gln), 50 (Tyr to Asn/Ser), 134 (Gln to His/Lys), 165 (Ala to Val), 167 (Phe to Leu/Tyr), 198 (Glu to Gly/Ala/Lys/Gln/Asp/Val), 200 (Phe to Tyr), 240 (Leu to Phe), 241 (Arg to His), and 257 (Met to Leu) have conferred benzimidazole resistance in the β-tubulin gene of more than 16 fungal species (Goldman et al. 1993; Cruz and Edlind 1997; Ma et al. 2005). None of these point mutations were found in AMF β-tubulin genes, which is consistent with previous results showing that benomyl reduces mycorrhizal infection (Fitter and Nichols 1988), inhibits spore germination (Chiocchio et al. 2000), and can have long-term inhibition of colonization depending on host and fungal species, soil type, and the formulation and concentration of the applied benomyl (Spokes et al. 2006).

The region from amino acid 427 to C-terminus of the AMF polypeptide was highly variable, as observed in all other fungal β-tubulin genes in Genbank. The highly variable carboxyl terminal, which was rich in glutamic acid residues, is important for binding of microtubule-associated proteins MAP2 and MAPT (Cross et al. 1994).

The full-length AMF amino acid sequences were in agreement with partial sequences (201 amino acids) reported by Corradi et al. (2004a, b) and corroborated the conclusion by these authors that the amino acid sequence of glomeromycotan β-tubulin genes was most similar to those of chytridiomycotan fungi. Such close similarity could be a result of the maintenance of the ancestral state due to functional and/or structural constraints on amino acid sequences, even after extensive species divergence.

Phylogenetic trees generated in this study using longer amino acid sequences do not show a sister group relationship between AMF and chytrids. This is in contrast to the study done by Corradi et al. (2004b) in which shorter sequences were used. The positioning of AMF within the fungal tree using beta-tubulin genes was not clear, as bootstrap support for the clade clustering AMF with Zygomycetes/Basidiomycetes/Ascomycetes was not significant. The branching position of Zygomycota was inconsistent, changing with different analysis performed in this study. This is not surprising, as similar results have been reported by Keeling et al. (2000).

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310   Y  L  T  V  A  A  M  F  R  G  R  C  S  M  K  E  V
      TGATGATCAAATGTTATCCGTTCAAACAAAGAACAGTTCATACTTTGTTG  1250
327    D  D  Q  M  L  S  V  Q  T  K  N  S  S  Y  F  V
      AATGGATCCCCAATAACGTCAAAAgtaagcataaatataaaatatatatg  1300
343  E  W  I  P  N  N  V  K
      tatgcgttgaaaattttccattgcctatttaatttttctaactcggtcata  1350
      cgctttaattatagCCGCTGTTTGTGACATTCCACCAAAAGGTCTTAAGA  1400
351               T  A  V  C  D  I  P  P  K  G  L  K
                  GC1F
      TGTCAGTTACTTTCATTGGTAATTCGACATCTATTCAGGAACTTTTCAAA  1450
363  M  S  V  T  F  I  G  N  S  T  S  I  Q  E  L  F  K
      CGTATCAGCGATCAATACACTGCTATGTTTAGACGTAAGGCCTTCTTGCA  1500
380   R  I  S  D  Q  Y  T  A  M  F  R  R  K  A  F  L  H

      TTGGTATACAGGAGAGGGTATGGATGAAATGGAATTCACTGAAGCTGAAT  1550
397    W  Y  T  G  E  G  M  D  E  M  E  F  T  E  A  E
       GiH4R
      CTAACATGAATGATTTGGTATCTGAgtaagttatttttttcttcttttacc  1600
413  S  N  M  N  D  L  V  S  E
      aattaaatgtaataagtttttatagaatttttttaaaaaaattaacaata  1650
      attatattttattattatagATATCAACAATATCAAGAAGCCACTGTAGA  1700
422                      Y  Q  Q  Y  Q  E  A  T  V  E
      AGAAGAAGAAGGCGAATACGAAGAAGAAGAATTAGAGCAAGATGCAGAAT  1750
431    E  E  E  G  E  Y  E  E  E  E  L  E  Q  D  A  E
      AATTTCATTTTTTTTTTACCATAATTACGCATTCATATTTCAAAACATCA  1800
      TTCTATTTTTTTCAATTTTCTATTTTTTTTTTTTTCTTTTTTTTCAATCATT  1850
      TTAAAATAGATTTTTATTAAGTTAAAAATAATCAATTTTACATTTGAATA  1900
      CAATGTATTATTCTTTTAGAATTAACTTTTTACTATTGGAAAAATTAGCA  1950
      AAGTAACGTGTAATAAAAAAAAAAGGTTTATTTTTTTTTAAAATAAAATT  2000
ATGTAACACTTGTATTGGTTATAATTCATGTTGTACACAAATATTGTTTA  2050
ATGATAGATTCAGCGGGTAGCAACAATAATGTTGCGTCTTTAAACTCCTT  2100
AATCCACGAATTGCGCCTTCCATTTAGGAATTAATTTATAAATCAAATCT  2150
TTGTCTTTAAATCTTGTTTCAATTGAAAGGACATTTTTTTTTATGGACTC  2199
```

Fig. 4 (continued)

The location of the first intron in the β-tubulin gene of the two AMF species (at codon 174) differed strikingly from all other fungal phyla from which full sequences have been published. None of the chytridiomycotan sequences in Genbank or in the database at the Broad Institute shared any similar intron positions with AMF. In Zygomycota, an intron is present at codon 174 (Table 3), but its location in relation to other introns in the β-tubulin gene is unknown because full-length sequences are not available for comparison. It is plausible that some species in Zygomycota also have the first intron located at codon 174. In Ascomycete and Basidiomycete fungi characterized thus far (Ayliffe et al. 2001), the first intron is located within the first 15 amino acids (Table 3). The second intron occurring at codon 257 in AMF β-tubulin gene corresponds to a sixth intron in the Basidiomycete *S. bovinus* and S*chizophyllum commune* and a fifth intron in the Ascomycete *Mycosphaerella pini*. Several Zygomycete species also have an intron at this position (Table 3). Introns at codons 350 and 421 were unique to *G. gigantea*, *G. clarum*, and most likely other AMF species.

Comparisons of partial and full-length β-tubulin sequences indicated that intron positions are conserved across the 50 AMF species studied to date (Corradi et al. 2004a, b; Msiska and Morton 2009). Intron positions seem to be conserved at the order level in glomeromycotan fungi whereas they are more conserved at the class level in Basidiomycota and Ascomycota (Ayliffe et al. 2001). The degree of conservation of introns within Zygomycota and Chytridiomycota could not be assessed, as only one full-length sequence containing intron regions is available for each phylum.

Ayliffe et al. (2001) suggest that intron positions within fungal β-tubulin genes may reflect evolutionary relationships between species. None of the intron positions within Chytridiomycotan sequences deposited in Genbank/Broad Institute appear similar to intron positions in AMF (Table 3). For now, intron positioning suggests that AMF are more closely related to the Basidiomycota, Ascomycota, and Zygomycota. According to the "intron late" theory, spliceosomal introns emerged only in eukaryotes and have been inserted into protein-coding genes continuously throughout their evolutionary history (Koonin 2006). Therefore, the last shared ancestor of the Zygomycota, Glomeromycota, Ascomycota, and Basidiomycota may have possessed introns at codons 174 and 257 of the β-tubulin gene. All four introns of AMF β-tubulin gene contained conserved 5′ and 3′ splice signals similar to those of other fungi.

The low G+C content of the β-tubulin genes calculated in this study agrees with that reported for Glomeromycotan partial β-tubulin gene sequences (Corradi et al. 2004b). The low G+C content of the AMF β-tubulin genes is reflected in codon usage, wherein 68.5% of all codons end in either an A or a T. In most fungal β-tubulin genes, pyrimidines occur more frequently than purines in the third codon position. In *Neurospora crassa* and *A. nidulans* β-tubulin genes, adenine generally is excluded from the third codon position (May et al. 1987; Orbach et al. 1986). A comparison of all AMF cloned full-length genes shows a preference for an A or T in the wobble position, except for genes encoding amino acid permease, chitin synthase, and 3-phosphoglycerate kinase. The genes encoding amino acid permease, chitin synthase, and 3-phosphoglycerate kinase are high in G+C content

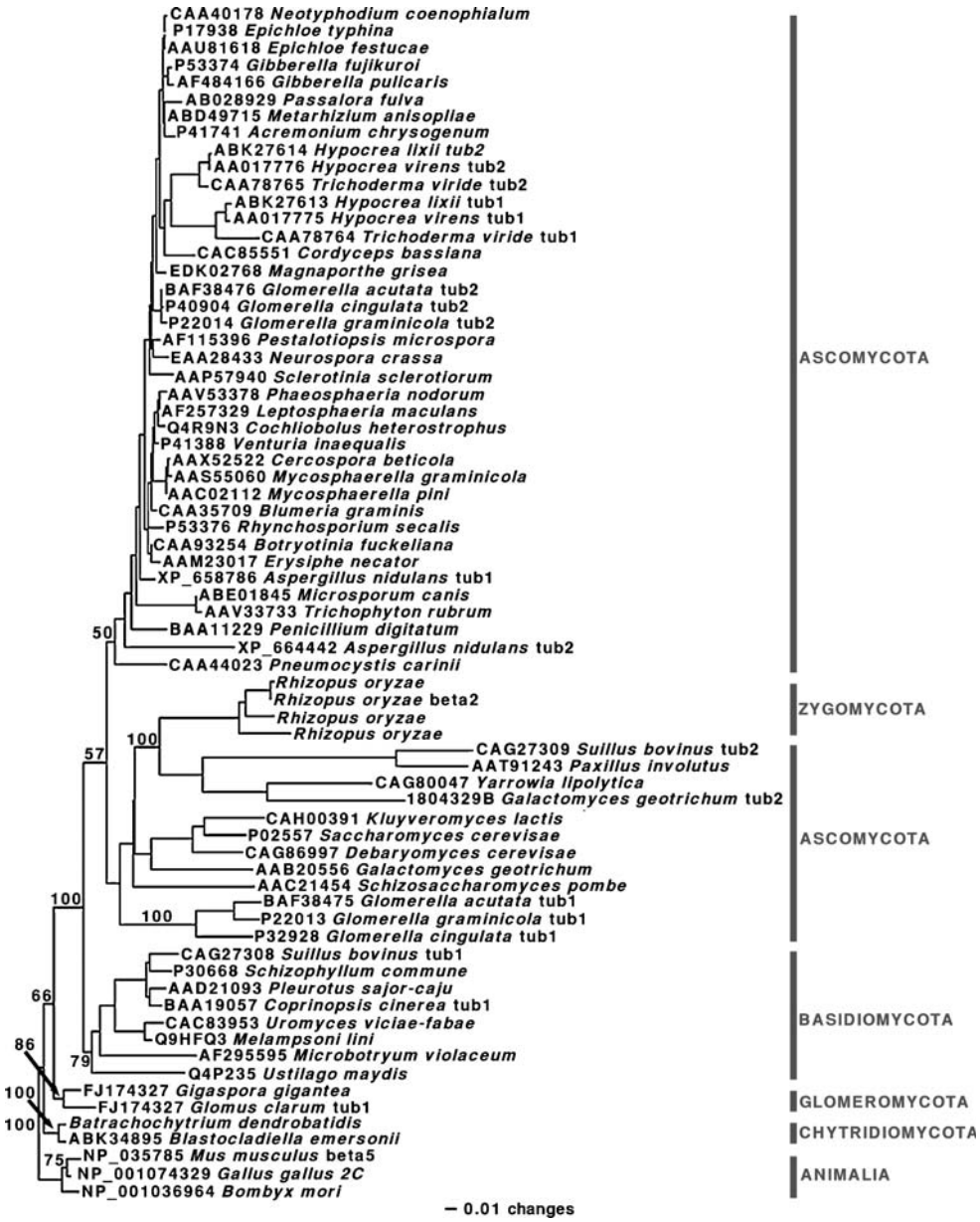

Fig. 5 Fungal phylogeny based on nearly full-length amino acid sequences (436 amino acids in length). *Numbers at nodes* indicate bootstrap values ≥50%. Phyla are labeled to the right of the tree. The tree was rooted with Animalia

(55.5%, 52%, and 48.9%, respectively). For all other AMF genes surveyed in Genbank, the G+C content varies from 35% to 42% (Hosny et al. 1997).

The β-tubulin gene nucleotide sequences of both *G. gigantea* and *G. clarum* contain the consensus sequence AATAAA in the 3′ flanking sequence, which may be involved in 3′ end polyadenylation of precursor messenger RNA (mRNA). Only one AMF gene (FOX2) has been sequenced which contained the polyadenylation signal (Requena et al. 1999). The *G. gigantea* β-tubulin gene included the consensus TATAAA sequence 29 bp upstream of the putative transcription start site as predicted by a computer program. Promoters of genes that transcribe relatively large amounts of mRNA have a TATA sequence about 30 bp upstream from the transcription start site, as well as one or more promoter elements further upstream (Gilbert 2006). The *G. gigantea* β-tubulin gene did not have the corresponding CCAAT-box in the promoter region due to a truncated sequence. Only four AMF genes have been isolated which contain sequences upstream of the start codon (Requena et al. 1999, 2000; Lanfranco et al. 1999; Harrier 2001). For *G. mosseae* beta-oxidation protein (FOX2) and 3-phosphoglycerate kinase genes, several TATA sequences were found upstream of the start codon. In contrast, the *Glomus versiforme* chitin synthase gene had only one TATA sequence 65 bp upstream from the start codon.

Table 3 Comparison of positions of introns in a representative range of fungal β-tubulin genes

Species	Accession #	Intron position																						
		1	4	9	11	12	15	17	21	27	29	35	36	49	50	53	58	62	86	87	90	104	108	
Glomeromycota																								
Gigaspora gigantea	FJ174327	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Glomus clarum	FJ174328	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Basidiomycota																								
Schizophyllum commune	X63372	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
Cryptococcus neoformans	AE017343	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	
Suillus bovinus	AJ698040	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
Coprinus cineria	AB000116	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
Ascomycota																								
Mycosphaerella pini	AF044975	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	
Zygomycota																								
Rhizopus oryzae		0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
Smittium simuli	AY944829	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Smittium commune	AY944828	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Conidiobolus coronatus	AF162058	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Absidia spinosa	AY944781	N	N	N	N	N	N	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	
Dissophora decumbens	AY944836	N	N	N	N	N	N	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	
Absidia parricida	AY944780	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
Chytridiomycota																								
Batrachochytrium dendrobatidis		0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	
Monoblepahris polymorpha	AY944851	N	N	N	N	N	N	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	
Powellomyces variabilis	AY138796	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Powellomyces variabilis	AY138797	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Rhizophlyctis rosea	AF162078	N	N	N	N	N	N	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	
Spizellomyces punctatus	AF162077	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Spizellomyces punctatus	AF162076	N	N	N	N	N	N	N	N	1	0	0	0	0	0	0	0	0	0	1	0	0	0	

Species	Intron position																							
	121	132	154	162	174	181	182	198	241	246	251	257	285	308	312	348	350	352	354	360	407	408	421	429
Glomeromycota																								
Gigaspora gigantea	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0
Glomus clarum	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0
Basidiomycota																								
Schizophyllum commune	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
Cryptococcus neoformans	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
Suillus bovinus	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
Coprinus cineria	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1
Ascomycota																								
Mycosphaerella pini	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Zygomycota																								
Rhizopus oryzae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Smittium simuli	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	N	N	N	N
Smittium commune	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	N	N	N	N
Conidiobolus coronatus	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	N	N	N	N
Absidia spinosa	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	N	N	N	N
Dissophora decumbens	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	N	N	N	N
Absidia parricida	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	N	N	N	N
Chytridiomycota																								
Batrachochytrium dendrobatidis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Monoblepahris polymorpha	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	N	N	N	N
Powellomyces variabilis	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	N	N	N	N
Powellomyces variabilis	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	N	N	N	N
Rhizophlyctis rosea	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	N	N	N	N
Spizellomyces punctatus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	N	N	N	N
Spizellomyces punctatus	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	N	N	N	N

Intron positions are defined by codon number according to Ayliffe et al. (2001). The number designating each intron position refers to the codon that is either immediately 5′ of the intron or interrupted by the intron. Presence of an intron = 1, absence of an intron = 0, missing data = N

Corradi et al. (2004b) found only a single copy of the β-tubulin gene in *G. margarita* and two copies in three *Glomus* species. A similar result was obtained in this study: only one gene was found in *G. gigantea* but two genes were found in *G. clarum*. The Glomeromycota are no different from other fungi in that some species have a single β-tubulin gene and others have evolved two copies. A mechanism for duplication of genes in clonally reproducing species, such as AMF, has not been elucidated experimentally. Three possible hypotheses are (1) unequal crossover of sister chromatids during mitosis, (2) chromosome duplication resulting from non-disjunction of sister chromatids (aneuploidy), or (3) whole genome duplication or polyploidy (Comai 2005).

Acknowledgments The authors wish to thank Bill Wheeler for helping with the manipulation and processing of AMF cultures. We also thank Dr. Jed Doelling for reviewing the manuscript. We are grateful to one reviewer for providing an additional source of information for full-length β-tubulin sequences of the Chytridiomycota and Zygomycota. Funding was provided by a Fulbright scholarship to Zola Msiska and National Science Foundation grants DBI0650735 and DEB0649341 to Joseph Morton.

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