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# Monophyly of *β-tubulin* and *$H^+$-ATPase* gene variants in *Glomus intraradices*: consequences for molecular evolutionary studies of AM fungal genes

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**Abstract**

Arbuscular mycorrhizal fungi (AMF) are an ecologically important group of fungi. Previous studies showed the presence of divergent copies of *β-tubulin* and V-type vacuolar *$H^+$-ATPase* genes in AMF genomes and suggested horizontal gene transfer from host plants or mycoparasites to AMF. We sequenced these genes from DNA isolated from an in vitro cultured isolate of *Glomus intraradices* that was free of any obvious contaminants. We found two highly variable *β-tubulin* sequences and variable *$H^+$-ATPase* sequences. Despite this high variation, comparison of the sequences with those in gene banks supported a glomeromycotan origin of *G. intraradices β-tubulin* and *$H^+$-ATPase* sequences. Thus, our results are in sharp contrast with the previously reported polyphyletic origin of those genes. We present evidence that some highly divergent sequences of *β-tubulin* and *$H^+$-ATPase* deposited in the databases are likely to be contaminants. We therefore reject the prediction of horizontal transfer to AMF genomes. High differences in GC content between glomeromycotan sequences and sequences grouping in other lineages are shown and we suggest they can be used as an indicator to detect such contaminants. *$H^+$-ATPase* phylogeny gave unexpected results and failed to resolve fungi as a natural group. *β-Tubulin* phylogeny supported Glomeromeromycota as sister group of the Chytridiomycota. Contrasts between our results and trees previously generated using rDNA sequences are discussed.


*Index descriptors*: Arbuscular mycorrhizal fungi; *β-tubulin*; *$H^+$-ATPase*; Contamination; Phylogeny; Monophyly; Molecular variation; Culturing system

## 1. Introduction

One of the most common symbioses in nature is formed between plants and arbuscular mycorrhizal fungi (AMF). Indeed, over 60% of vascular plants form mycorrhizal symbioses with fungi belonging to the phylum Glomeromycota (Schüssler et al., 2001). This symbiosis is also known to be ancient since fossil records show that the first land plants were colonized by AMF (Redecker et al., 2000; Remy et al., 1994). AMF play a key role in plant ecology, influencing plant nutrient acquisition (Jakobsen, 1995), and providing a protective role against plant pathogenic fungi (Nesheim and Linn, 1969; Newsham et al., 1995). More recently, AMF diversity has been shown to determine plant diversity and ecosystem productivity (Van der Heijden et al., 1998a,b).

In view of the importance of AMF, there has been considerable interest in finding regions of the genome that will allow both accurate studies of AMF phylogeny and represent markers that are variable among AMF species for identification purposes. Ribosomal DNA sequences have rapidly become the most common regions of the genome that are used in both studies on molecular diversity of AMF colonizing plant roots (Clapp et al., 1999, 2001; Daniell et al., 2001; Van Tuinen et al., 1998) and in phylogenetic studies of AMF (Redecker et al., 2000; Schwarzott et al., 2001). Ribosomal DNA sequences are highly variable even within a

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single spore (Antoniolli et al., 2000; Lloyd-McGilp et al., 1996; Rodriguez et al., 2001; Sanders et al., 1995). Therefore, problems in identifying specific AMF sequences that are useful for identification and for phylogenetic studies can arise when using such regions of the genome. Although some other AMF genes have been identified that play a key role in the plant–AMF symbiosis, such as a phosphate transporter gene (Harrison and van Buuren, 1995) and genes expressed during early developmental stages of AMF (Requena et al., 2002), these are unlikely to be useful for phylogenetics at the present time, because of the lack of information from other fungal groups.

Few other AMF genes have been looked at in detail either as tools for identification or for phylogenetics. Two genes, namely *H*$^+$*-ATPase* and *β-tubulin*, have been studied by Ferrol et al. (2000) and Rhody et al. (2003), respectively, in order to obtain information about the possible presence of intra-specific sequence variation. Highly divergent copies of ß-*tubulin* genes have already been reported for several groups of fungi (Baldauf and Palmer, 1993; Keeling, 2003), but these paralogous sequences clustered within the clade of fungi and did not show similarities with other eukaryotic lineages. The sequencing project of *Neurospora crassa* revealed the presence of a highly divergent *ENA-ATPase* sequence (Benito et al., 2002). Despite the very high molecular variability between that sequence and the other *N. crassa* paralogs, it nevertheless clustered next to a Basidiomycete and not outside the clade of fungi. In contrast, Ferrol et al. (2000) and Rhody et al. (2003) showed that sequences obtained from a single AMF species group in different major eukaryotic lineages. These studies predicted possible horizontal gene transfer from the host plant or mycoparasites to the AMF genome as a cause of this intra-specific variation but failed to provide support for their phylogenetic analysis and sound evidence against a possible contamination within their dataset.

The problems associated with finding sequences in AMF that cluster outside the glomeromycotan fungi are documented in studies of AMF rDNA. Using a phylogenetic analysis of rDNA datasets obtained from gene databases, Redecker et al. (1999) and Schüssler (1999), demonstrated that some rDNA sequences obtained from AMF were more related to Ascomycetes than to glomeromycotan species. These authors emphasized that the sequences were so close to those of known Ascomycetes that the presence of contaminant sequences in AMF should be considered. Hijri et al. (2002) later confirmed that the sequences identified by Redecker et al. (1999) as possible contaminants actually came from ascomycetes living in the cytoplasm of healthy AMF spores.

Many glomeromycotan species are exclusively cultured together with plants growing in pots. These conditions clearly allow the fungus to come into contact with other soil microorganisms and this renders the isolation of pure fungal hyphae and spores very difficult (Schüssler, 1999). In these systems spores are isolated from the growing medium by wet sieving of the soil. Therefore, there is a high probability for other fungi to be harvested and used for DNA extraction.

Some other glomeromycotan fungi can be cultured in vitro, with RiT-DNA transformed carrot roots as a host, in a two-compartment plate system (St-Arnaud et al., 1996). This system is used frequently in laboratories studying AMF genes because large quantities of clean DNA from both spores and hyphae can be obtained (Lammers et al., 2001; Ubalijoro et al., 2001). Growing the AMF on an artificial medium avoids many possible contaminations from soil microorganisms and by using a root free compartment avoids contamination by plant material. Another method to avoid contamination from soil microorganisms has been recently published in Requena et al. (2003). In this study, fungal material was cultured in direct symbiosis with the host–plant in a growing medium composed of glass-beads. Therefore, the absence of soil microorganisms, allowed the avoidance of numerous potential contaminants.

So far most of the studies reporting the presence of polyphyletic sequences in functional genes used DNA extracted from pot-cultured AMF (Ferrol et al., 2000; Lanfranco et al., 1999). Rhody et al. (2003) claimed that they also found highly divergent sequences also from in vitro cultured AMF but failed to provide sound evidence for it. Therefore doubts about the origin of some highly divergent sequences obtained from pot-cultured AMF have arisen. The aim of our study was to analyse in detail the variability present in *β-tubulin* and *H*$^+$*-ATPase* genes in the AMF *Glomus intraradices* that was cultured in vitro and to test whether there are sequences of different evolutionary origin, as previously reported. For this purpose, degenerate primers were designed using highly conserved regions of V-type vacuolar *H*$^+$*-ATPase* and *β-tubulin* genes in order to sequence the most gene variants possible including any that might have originated from other fungal and eukaryotic groups. Phylogenetic analysis with additional data from all major fungal groups and divergent eukaryotic lineages was carried out. Our results were compared to published data on variation of *H*$^+$*-ATPase* and *β-tubulin* genes in AMF. We also aimed to examine whether there are general properties of AMF genes that could be used to predict whether sequences are contaminants or not. For this we have determined the GC content of a large number of deposited *G. intraradices* total and partial gene sequences that originated from AMF growing in the in vitro system and compared it with GC content of sequences that occur outside the Glomeromycota according to phylogenetic analyses.

## 2. Materials and methods

### 2.1. G. intraradices cultivation and genomic DNA extraction

A culture of *G. intraradices* (isolate DAOM 181 602) growing on RiT-DNA transformed *Daucus carrota* roots was obtained from the group of Prof. G. Bécard (University Paul Sabatier, Toulouse, France). The fungus was maintained on two-compartment plates that allowed proliferation of large amounts of hyphae and spores in a compartment that is free of roots (St-Arnaud et al., 1996). Roots that directed their growth to the fungal compartment were regularly cut to avoid contamination with plant DNA. The cultures were grown for an average period of 3 months at 25 °C before DNA extraction. Spores and hyphae were harvested by dissolving the medium in citric acid (Nagahashi and Douds, 1999). Fungal material was collected on a 22 μm sieve and used for DNA extraction. Freshly harvested mycelium was placed in a 2 ml microcentrifuge tube and disrupted using a magnetic stirrer (Cenco instruments) and a micro-stirring bar for 10 min at 1200 rpm. DNA was then extracted from the resulting suspensions using the DNeasy plant mini kit (Qiagen). DNA concentration was estimated using a fluorimeter (Hoefer DyNA Quant 200) and DNA quality was checked by migration of 150 ng DNA on a 0.6% TAE agarose gel after electrophoretic separation for 30 min at 8 V/cm.

### 2.2. Polymerase chain reaction and DNA sequencing

Two 20mer degenerate oligonucleotides for V-type vacuolar *H*$^+$*-ATPase* designated as P1 (sense): 5′-TGY WSY GAY AAR ACY GGI AC-3′ and P2 (antisense): 5′-TTV ACH CCR TCH CCI GTC AT-3′ were synthesized (Microsynth GmBH, Switzerland) and used for genomic DNA amplification (Ferrol et al., 2000). Additionally, two degenerate primers, newly designed to match highly conserved regions of fungal *β-tubulin* genes were synthesized (Microsynth GmBH, Switzerland) and named as Deg.Tub.for: 5′-GAY GTY GTB CGY AAR GAR GC-3′ and Deg.Tub.rev: 5′-TTS ARY TGW CCV GGG AAA CG-3′. *β-Tubulin* and *H*$^+$*-ATPase* specific primers were designed using the different copies for *β-tubulin* we obtained and for *H*$^+$*-ATPase* mRNA sequence of *G. intraradices* found in GenBank (AF420481). They have been designated as GiTub.spe.F: 5′-GCT GGT CCT TTT GGA CAA -3′ and GiTub.spe.R: 5′-TGC AAA TCC GAC CAT GAA GAA-3′ for *β-tubulin* and GiHA.F: 5′-TAG AGT AGC AAC AGG TGC TCC A-3′ and GiHA.R: 5′-CTT CAA TTG CCT TTG TTG ATG A-3′ for *H*$^+$*-ATPase*.

Amplifications were carried out in a final volume of 50 μl containing 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, and 0.2 mg/ml BSA), 100 nM dNTPs, 1 nM final concentration of each degenerate primer (500 nM for Tub.spe.F and Tub.spe.R), and 0.5 U of *Taq* polymerase (Qbiogene). PCR was perfomed using 40 ng of DNA as template, in an automated thermal cycler (T-gradient, Biometra) with an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 44 °C, and extension for 1 min at 72 °C, followed by a final extension for 7 min at 72 °C. Amplification products were electrophoretically separated on 1.2% agarose gels, stained with ethidium bromide and the expected bands were excised from the gel. Excised bands were purified with a Qiaquick gel extraction kit (Qiagen, GmBH). PCR fragments were then cloned into the pTZ57R/T vector (Mbi Fermentas GMBH C/O labforce AG), following manufacturer's instructions. Sequences were obtained using M13 forward and reverse primers with a Big Dye 3.0 Terminator cycle sequencing kit according to manufacturer's instructions and separated on an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster city, USA). All sequences were manually corrected for ambiguities. A total number of 96 clones have been screed for variation by SSCP for both *β-tubulin* and *H*$^+$*-ATPase* genes.

### 2.3. SSCP procedure

Clones that differed in sequence were found by screening with SSCP. Three hundred nanograms of plasmid from positive clones were double digested using 10 U of *Alu*I/*Hin*fI and *Alu*I/*Dpn*I for *β-tubulin* and *H*$^+$*-ATPase* genes, respectively, in 1× incubation buffer for restriction enzymes A (Roche) in a volume of 10 μl and incubated at 37 °C for 2 h, followed by heat inactivation of the enzymes at 70 °C for 10 min. Samples were mixed and then denatured at 95 °C for 3 min. Submerged Gel electrophoresis was carried out at 9 °C and 6 V/cm for 15 h on precast GMA Gels (Elchrom Scientific). Gels were stained with SYBR Gold for 40 min in the dark and rinsed for 20 min. Differences in the banding patterns indicated different clones. These clones were chosen for sequencing and analysis.

### 2.4. Sequence analysis

Sequences were analysed using the Vector NTI package (Informax, Oxford, UK). Sequences related to *G. intraradices β-tubulin* and *H*$^+$*-ATPase* genes were deposited in GenBank under Accession Nos. (*β-tubulin*: AY326320; AY326321; *H*$^+$*-ATPase*: AY326322–AY326329). Sequence homology with genes deposited in gene databases was conducted using BLAST (Altschul et al., 1990). Sequences were aligned using clustalX and refined by eye. The choice of highly divergent eukary-

Table 1
List of taxa used in the phylogenetic analysis of *β-tubulin* genes

| Locus | Taxa | Accession Nos. |
|---|---|---|
| *β-Tubulin* | Ascomycota | |
| | *Alternaria infectoria* | Y17083 |
| | *Emericella nidulans BenA* | M17519 |
| | *Emericella nidulans TubC* | M17520 |
| | *Erysiphe pisi* | X81961 |
| | *Neurospora crassa* | M13630 |
| | *Pneumocystis carinii* | L05466 |
| | *Truchoderma viridae 1* | Z15054 |
| | *Trichoderma viridae 2* | Z15055 |
| | *Venturia inaequalis* | M97951 |
| | Basidiomycota | |
| | *Melanpsora lini* | AF317682 |
| | *Piriformospora indica* | AJ459235 |
| | *Pleurotus sajor-caju* | AF008134 |
| | *Schyzophyllum commune* | X63372 |
| | *Suillus bovinus* | AY112730 |
| | *Uromyces fabae* | AJ311552 |
| | Zygomycota | |
| | *Basidiobolus ranarum* | AF162060 |
| | *Basidiobolus ranarum2* | AF162059 |
| | *Conidiobolus coronatus1* | AF162057 |
| | *Conidiobolus coronatus2* | AF162058 |
| | *Entomophaga maimaiga* | AF162062 |
| | *Rhyzopus microsporus* | AF162064 |
| | *Syncephalis depressa* | AF162070 |
| | *Spiromyces minutus* | AF162067 |
| | Chytridiomycota | |
| | *Blastocladiella britannica* | AY131271 |
| | *Nowakowskiella elegans* | AY138800 |
| | *Nowakowskiella haemisphaerospora* | AY138798 |
| | *Powellomyces variabilis 1* | AY138796 |
| | *Powellomyces variabilis 2* | AY138797 |
| | *Spizellomyces punctatus 1* | AF162076 |
| | *Spizellomyces punctatus 2* | AF162077 |
| | *Rhyzophlyctis rosea* | AF162078 |
| | *Rhyzophlyctis* sp. | AF138802 |
| | Glomeromycota | |
| | *Acaulospora laevis* | AF158391 |
| | *Gigaspora margarita* | AJ459237 |
| | *Gigaspora rosea* | AF158389 |
| | *Gigaspora rosea2* | U49665 |
| | *Glomus coronatum* | AF158395 |
| | *Glomus mossae* | AF158387 |
| | *Glomus mossae2* | AF159109 |
| | *Scutellospora castanea* | AF158400 |
| | Plants | |
| | *Arabidopsis thaliana* | NM122291 |
| | *Hordeum vulgare* | Y09741 |
| | *Lupinus albus* | X70181 |
| | *Orzya sativa* | AB104733 |
| | Animals | |
| | *Gallus gallus* | V00389 |
| | *Xenopus laevis* | L06232 |
| | Choanoflagellata | |
| | *Monosiga brevicollis* | AY026071 |

Accession numbers of sequences are provided.

otes in our phylogenetic analysis prevented us from using nucleotide sequences because the alignment was not reliable enough. Therefore, we used alignments of amino acid sequences. Only sequences related to exons were used in the phylogenetic analysis. Trees were constructed by neighbor-joining and minimum evolution (Kumar, 1996) methods using MEGA2 (Kumar et al., 2002). Genetic distances for protein sequences of *β-tubulin* and *$H^+$-ATPase* genes were estimated using the Poisson corrections. Phylogenetic tree topologies were assessed by 1000 bootstrap replicates. Only GiHA5 to GiHA8 clones were translated into amino acid sequences for our phylogenetic analysis of *$H^+$-ATPase* genes because they covered a longer region of the gene and were, therefore, more useful for phylogenetic analysis. We obtained data from gene libraries on *β-tubulin* and *$H^+$-ATPase* genes from AMF (Ferrol et al., 2000; Rhody et al., 2003) and from other different fungal groups (Ascomycetes, Zygomycetes, Basidiomycetes, and Chytridiomycetes). Some divergent eukaryotic lineages were also obtained from databases and added to the dataset for phylogenetic analysis. The absence of *$H^+$-ATPase* genes from Zygomycetes in GenBank prevented us from using them in the phylogenetic analysis. Accession numbers of taxa used in this study are provided in Tables 1 and 2 for *β-tubulin* and *$H^+$-ATPase* genes, respectively.

Table 2
List of taxa used in the phylogenetic analysis of *$H^+$-ATPase* genes

| *$H^+$-ATPase* | Ascomycota | |
|---|---|---|
| | *Candida albicans* | AF002134 |
| | *Kluveromyces lactis* | P49380 |
| | *Neurospora crassa* | P07038 |
| | *Pichia angusta* | AF109913 |
| | *Pneumocystis carinii* | U65004 |
| | *Saccharomyces cerevisiae* | P05030 |
| | *Schizosaccharomyces pombe* | P09627 |
| | *Zygosaccharomyces rouxii* | P24545 |
| | Basidiomycota | |
| | *Cryptococcus neoformans* | AF077766 |
| | *Uromyces fabae* | AJ003067 |
| | *Ustilago maydis* | AJ315590 |
| | Glomeromycota | |
| | *Glomus mossae* | AY193825 |
| | *Glomus intraradices* | AF420481 |
| | Plants | |
| | *Arabidopsis thaliana* | NM114131 |
| | *Lycopersicum esculentum* | AY178911 |
| | *Medicago trunculata* | AJ132892 |
| | *Nicotiana plumbaginifolia* | AF156684 |
| | *Orzya sativa* | AJ440417 |
| | *Zea mays* | X85805 |
| | Oomycota | |
| | *Phytophtora infestans* | AY344962 |

Accession numbers of sequences are provided.

### 2.5. Analysis of GC content in the G. intraradices genome

Since little is known about GC content of the *G. intraradices* genome, an extensive analysis of the GC content of 51 total and partial gene sequences, corresponding to 37,399 bp of its genome was carried out by screening the *NCBI* gene library. Analysed sequences were exclusively obtained from DNA isolated from in vitro cultured *G. intraradices* isolates. Putative protein names, accession

Table 3
AT and GC content of sequences of putative genes obtained from in vitro cultured *G. intraradices*

| *G. intraradices* putative proteins | Accession Nos. | Sequence length | AT (%) | GC (%) |
|---|---|---|---|---|
| Actin related protein | BI246180 | 768 | 61.98 | 38.02 |
| Adenosylcyteinase | BE603835 | 489 | 66.67 | 31.29 |
| α-3-Tubulin | BI246191 | 550 | 56.00 | 44.00 |
| α-Glucan branching enzyme | BE604014 | 492 | 63.41 | 36.38 |
| Arsenite transloctaing ATPase | BI246187 | 621 | 67.31 | 32.53 |
| β-Actin | BI246188 | 759 | 57.18 | 42.82 |
| β-Tubulin | BI246179 | 810 | 58.89 | 40.25 |
| β-Tubulin | BE603903 | 681 | 57.86 | 34.65 |
| β-Tubulin | BE603888 | 228 | 59.65 | 39.04 |
| cAMP dependent protein kinase | BE603750 | 1103 | 67.09 | 32.55 |
| CDC25 protein | BI246183 | 760 | 64.47 | 35.39 |
| Cell-wall protein | BE603846 | 647 | 50.23 | 49.15 |
| Cullin | BE603928 | 481 | 68.61 | 30.15 |
| Cysteine synthase | BI246184 | 817 | 58.38 | 41.49 |
| Cytochrome P450 family 4 | BE603996 | 643 | 68.58 | 31.10 |
| Dynein | BI246192 | 556 | 56.83 | 43.35 |
| Dynein | BE603784 | 507 | 70.41 | 27.81 |
| Dynein | BE604013 | 1062 | 57.53 | 41.90 |
| EF1 α | BE603839 | 543 | 61.14 | 35.73 |
| Glutamine-fructose-6-phosphate transaminase | BE603749 | 708 | 62.71 | 37.29 |
| Glyoxal oxydase precursor | BI246189 | 787 | 57.18 | 42.82 |
| Glycil-tRNA synthase | BE604032 | 422 | 63.03 | 35.78 |
| Glycogen synthase | BE603748 | 695 | 61.73 | 38.27 |
| GTP-binding nuclear protein | BE603881 | 752 | 61.44 | 38.30 |
| $H^+$-ATPase | AF420481 | 1561 | 66.11 | 33.89 |
| Histone 3A | BE604019 | 813 | 63.35 | 34.44 |
| Histidine kinase | BE603964 | 200 | 66.00 | 27.00 |
| Homing endonuclease | BI246193 | 785 | 52.10 | 47.90 |
| Hsp70 | BE603837 | 450 | 65.78 | 34.00 |
| Hsp83 | BE603993 | 602 | 62.96 | 36.88 |
| Hsp83 | BE603992 | 817 | 65.61 | 33.78 |
| IF3A translation initiation protein | BE603820 | 805 | 61.24 | 38.63 |
| Linoleate diol synthase | BI246194 | 730 | 62.05 | 37.81 |
| Malate synthase | BE603747 | 1826 | 65.77 | 34.23 |
| MAT-3 protein (mating type protein) | BE603853 | 508 | 61.61 | 37.99 |
| Methionine adenosyiltransferase | BI246182 | 570 | 57.19 | 42.81 |
| Myeloblastosys oncogene | BI246185 | 663 | 60.33 | 39.52 |
| NDT80 protein | BI246190 | 757 | 58.65 | 40.55 |
| Neutral theralase | BI246186 | 777 | 66.80 | 33.20 |
| Phosphate transporter | AF359112 | 1665 | 61.50 | 38.50 |
| Profilin | BE603794 | 552 | 61.59 | 36.96 |
| Protein kinase | BE603797 | 879 | 62.46 | 35.38 |
| Rad15 helicase | BE604029 | 650 | 67.85 | 32.15 |
| Rad32 | BE604008 | 886 | 65.69 | 34.31 |
| Ribosomal protein P10 | BE603982 | 624 | 58.49 | 41.35 |
| Spermidine synthase | BE603977 | 409 | 69.44 | 28.61 |
| Sterol-*O*-acyltransferase | BE604005 | 776 | 72.94 | 25.26 |
| STE12-α protein | BE603782 | 694 | 64.27 | 35.01 |
| Transcription factor | BE603970 | 797 | 62.99 | 36.89 |
| Tripeptidyl peptidase II | BE603867 | 408 | 64.95 | 34.80 |
| Ubiquitin conjugating enzyme | BE603773 | 497 | 71.23 | 26.96 |
| Wall protein | BE603957 | 817 | 56.30 | 43.45 |
| Total | | 37,399 | 62.57 | 36.58 |

Putative proteins are listed alphabetically. Accession numbers in GenBank are provided.

numbers and relative GC content are described in Table 3.

## 3. Results

### 3.1. Identification of two highly variable β-tubulin genes in G. intraradices

Two highly variable *β-tubulin* sequences were obtained from *G. intraradices* that had been cultivated with RiT-DNA transformed roots. The two sequences designated *GiβTub1* and *GiβTub2* were 1078 and 1100 bp in length, respectively. Intra-lineage genetic variation was present as a low number of single substitutions. Alignment of the sequences showed an overall similarity of 73%. Comparison with sequences present in databases showed that they both encode a putative *β-tubulin* protein, and thus, can be considered as *β-tubulin* gene variants, rather than two sequences belonging to different members of the tubulin gene family (e.g., α, *β*, and *γ-tubulin*). A comparison of both variants with a glomeromycotan *β-tubulin* mRNA sequence deposited in GenBank allowed us to identify two introns in both sequences, with a high number of indel mutations. One hundred and ten substitutions were observed along the exon sequences of *GiβTub1* and *GiβTub2*. *GiβTub1* and *GiβTub2* show an elevated transition versus transversion ratio of 3.29. The high number of substitutions would be unlikely to greatly affect the protein homology, as reflected by the very low dN/dS ratio ($\omega = 0.071$). The average GC content for *GiβTub1* and *GiβTub2* was 38 and 39%, respectively, including both exon and intron sequences. Introns alone showed an average GC content of 24 and 27% for *GiβTub1* and *GiβTub2*, respectively.

### 3.2. Identification of a single $H^+$-ATPase gene lineage in G. intraradices

PCR with highly degenerate primers yielded two distinct amplifications of about 1.2 kb and 800 bp. Sequencing of the 1.2 kb fragments and comparison with databases showed similarities to *ENA-ATPase* gene sequences deposited in GenBank (58% of homology with the Zygomycete fungus, *Blakeslea trispora*). No homologies of the 1.2 kb PCR products with a putative *$H^+$-ATPase* were found. The sequenced fragment of 800 bp showed highest similarities with the subunit A of V-type vacuolar *$H^+$-ATPase* sequences from *G. intraradices* (AF420481) and *Glomus mossae* (AY193825); both of which had been obtained from monoxenic fungal cultures. Comparison with mRNA sequences of AMF and other fungal species showed that the *$H^+$-ATPase* sequences corresponded to a 790 bp region that did not include any intron. Among eight different *$H^+$-ATPase* clones, an overall number of 11 variable sites were detected. Four of them led to a change in the amino acid sequence, leading to a "Replacement versus Synonymous" substitution ratio ($\ll 1$) that is lower than the ratio that would be expected by *Taq* polymerase errors (ratio of 3:1) (Bracho et al., 1998; Dunning et al., 1988; Ennis et al., 1990). These sequences have been designated GiHA1 to GiHA8 and deposited in GenBank under the following Accession Nos.: AY326322–AY326329.

### 3.3. Evolution of β-tubulin and $H^+$-ATPase gene variants

The *β-tubulin* tree topology showed the presence of five major fungal groups: Basidiomycetes, Ascomycetes, Zygomycetes, Chytridiomycetes, and Glomeromycota (Fig. 1). Other eukaryotes (Plants, Oomycetes, and Animals) diverged greatly from fungi. In all analyses the Ascomycetes, Basidiomycetes, Chytridiomycetes, Glomeromycota, and the fungi as a whole formed supported monophyletic groups. A Glomeromycota cluster is supported by a high bootstrap value and contains exclusively sequences related to Glomeromycotan species. Paralogous sequences were recovered from databases and phylogenetically analysed. Their grouping always supported a fungal origin. Paralogs of Ascomycota did not diverge from the Ascomycotan clade. Chytridiomycotan *β-tubulin* paralogs clustered with Zygomycotan sequences. In this case our results are not completely congruent with Keeling (2003). Differences in the clustering may be attributed to the shorter length of sequences analysed in our study. The *β-tubulin* tree topology supports Chytridiomycota as the sister group of Glomeromycota. The phylogenetic analysis suggest a fungal origin of Glomeromycotan sequences but is in contrast with results obtained with Ribosomal DNA sequences (Schüssler et al., 2001) suggesting Ascomycetes and Basidiomycetes as being sister groups of Glomeromycota. Even though the exon sequences of Gi*βTub1* and Gi*βTub2* in *G. intraradices* differed by 110 substitutions, both types clearly grouped with glomeromycotan sequences rather than with other eukaryotic lineages. The Glomeromycota cluster shows some unusual features such as the clustering of *Gigaspora rosea* (AF158389) with *G. mossae* and our *G. intraradices* sequences with *Gigaspora margarita*. One of the causes for this grouping could be due to the use of amino acid sequences in our phylogenetic analysis. Therefore, the high number of synonymous substitutions that may occur in the nucleotide sequences, and which could be informative, are no longer present. This may lead to species positions that do not reflect the real evolutionary steps within a lineage.

Some glomeromycotan sequences obtained from the databases (*Acaulospora laevis*, AF158391; *Scutellospora castanea*, AF158400; *G. mossae*, AF159109 and *G. rosea*, U49665) showed higher similarities with a highly divergent eukaryotic lineage, the Oomycetes (Stramenopiles).

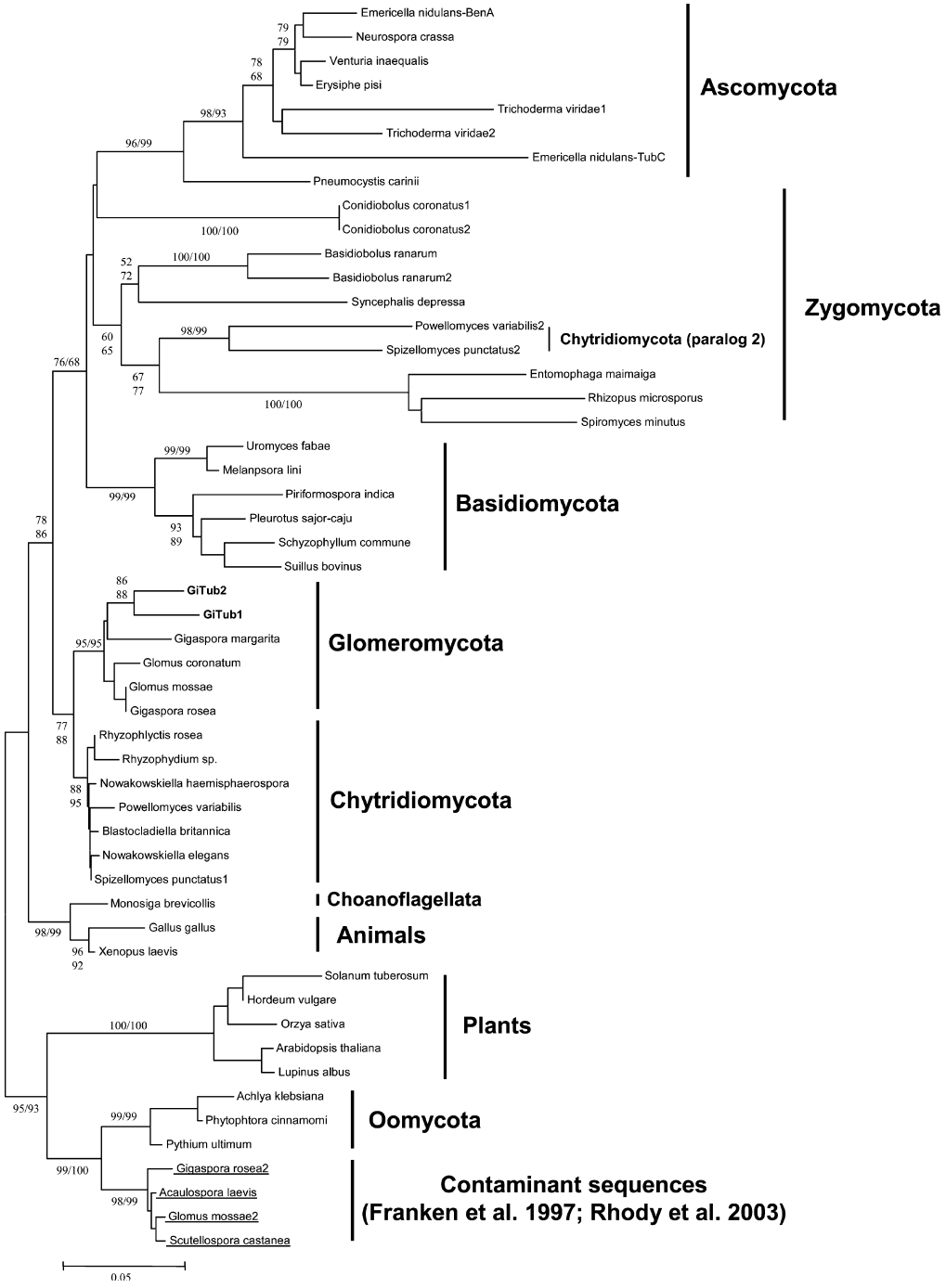


Fig. 1. Phylogenetic analysis of *β-tubulin* amino acid sequences. *β-Tubulin* gene variants obtained from *G. intraradices* (bold) were compared with previously published glomeromycotan *β-tubulin* sequences (Franken et al., 1997; Rhody et al., 2003) and with other sequences belonging to the Zygomycetes, Ascomycetes, Basidiomycetes, Stramenopiles, Plants, and Animals. Previously published AMF sequences grouping outside the fungal clade are underlined. Numbers at nodes correspond to bootstrap support from neighbor-joining (top/left) and minimum evolution (bottom/right) genetic distances.

Phylogenetic analysis of $H^+$-*ATPase* amino acid sequences resolved four major evolutionary lineages, corresponding to three major fungal groups (Glomeromycota, Ascomycetes, and Basidiomycetes) and a cluster containing all sequences related to plants (Fig. 2). All major clusters are supported by high bootstrap values. The $H^+$-*ATPase* variants from this study (GiHA1-4) clustered together with a sequence from *G. intraradices* deposited in GenBank (AF420481). The sequence from *G. mossae* (GmHA5, AJ133843), formerly defined as related to the plant lineage (Ferrol et al., 2000) and another sequence from *Glomus mossae*

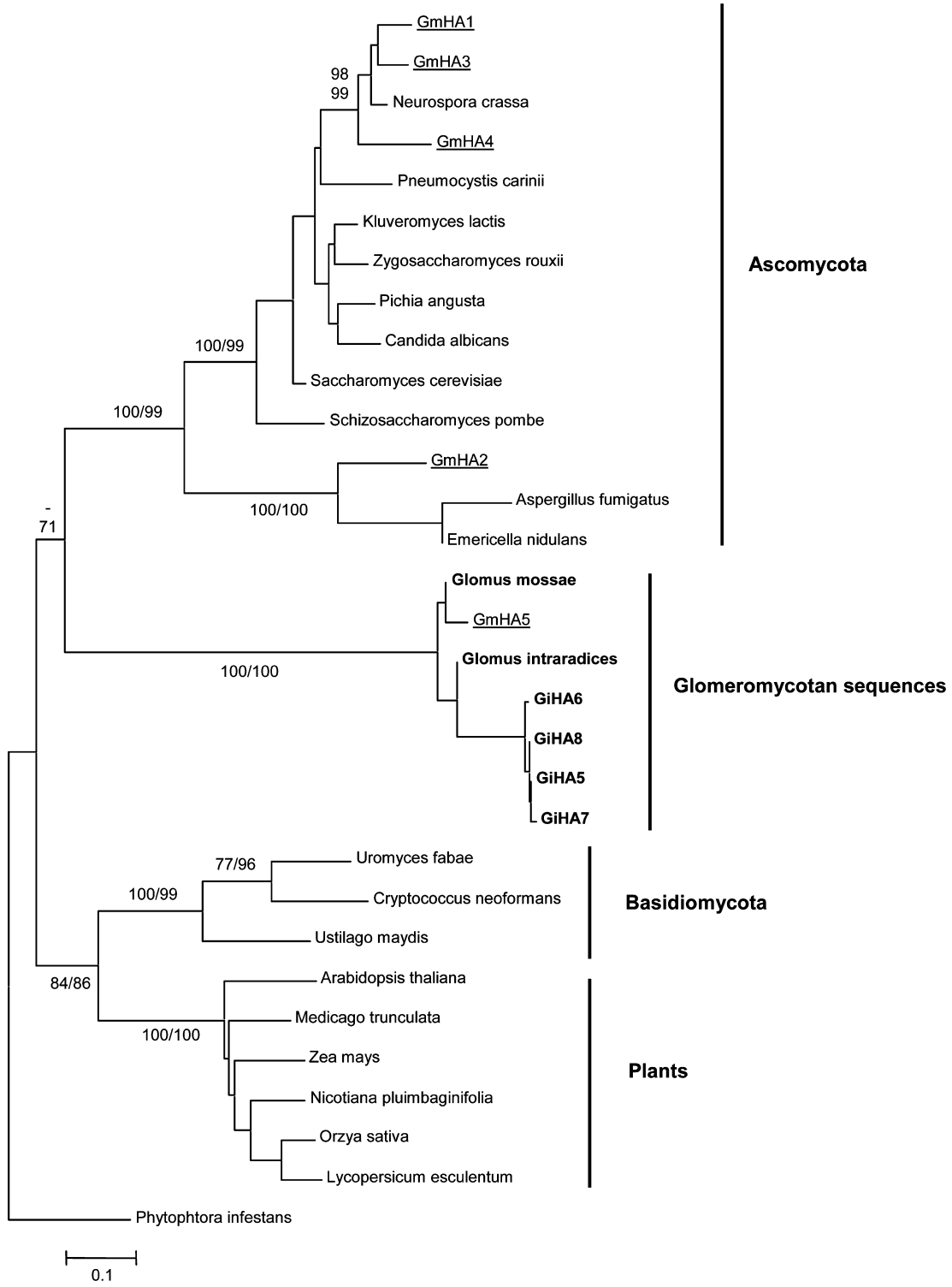


Fig. 2. Phylogenetic analysis of *H*$^+$*-ATPase* amino acid sequences. Amino acid sequences of *H*$^+$*-ATPase* genes that were not obtained from pot cultures are shown in bold. Previously published *H*$^+$*-ATPase* sequences from *G. mossae* (*GmHA1–5*, underlined), Plants, Ascomycetes, and Basidiomycetes were used in the phylogenetic analysis. An Oomycete (*Phytophtora infestans*) was added as an outgroup. Numbers at nodes correspond to bootstrap support from neighbor-joining (top/left) and minimum evolution (bottom/right) genetic distances.

deposited in GenBank (AY193825) (Requena et al., 2003) grouped as a sister group of *G. intraradices* sequences. All other previously studied *H*$^+$*-ATPase* variants from *G. mossae* (GmHA1–4; AJ133939–AJ133842) in the databases grouped with Ascomycetes. A single clade only corresponding to only Basidiomycetes is

represented within the phylogenetic analysis. Unexpectedly the tree topology supported Basidiomycetes as a sister group of plants and tree topology do not resolve the fungi as a natural group.

### 3.4. GC content of putative coding regions in G. intraradices

Analysis of 51 total and partial gene sequences deposited in GenBank, that were obtained from *G. intraradices* growing in the in vitro system (Lammers et al., 2001), showed that out of 37,399 nucleotides only 36% were G or C. This is a particularly low value compared with many other fungi. GC content of exons of the *GiβTub1*, *GiβTub2*, and *GiHA* genes were 42, 43, and 36%, respectively. The *β-tubulin* and *$H^+$-ATPase* sequences that were previously reported to cluster outside the Glomeromycota had an average GC content of 55 and 57%, respectively.

## 4. Discussion

### 4.1. Evolution of AMF β-tubulin genes

From an in vitro culture of *G. intraradices*, we obtained highly variable *β-tubulin* gene variants. Both sequences grouped in a single clade containing only sequences related to the phylum Glomeromycota (Fig. 1). This clade groups next to the Chytrids and support a fungal origin of these sequences. None of the *β-tubulin* fragments showed strong similarities with Ascomycetes, Basidiomycetes or other eukaryotes such as Stramenopiles or plants. A phylogenetic analysis of glomeromycotan *β-tubulin* sequences that are present in public databases showed that, all published AMF sequences that clustered next to the Oomycetes were obtained from pot-cultured AMF. In the report by Rhody et al. (2003), the presence of glomeromycotan sequences grouping with Ascomycetes has been discussed as a clear evidence of contamination. In contrast, when glomeromycotan sequences grouped with Oomycetes in their phylogenetic analysis, the authors predicted that this was due to horizontal gene transfer between the host-plant and the AMF genome because of their long-term co-evolution. Our phylogenetic analysis clearly demonstrates that these divergent sequences did not group within plants or Oomycetes and that they are likely to be considered as an unknown eukaryotic group that is highly divergent from the fungi. Analysis of these *β-tubulin* sequences highlights the correlation between their culture origin and their evolutionary history: (i) The two sequences of *G. rosea* we used in our analysis were obtained in two different studies. One sequence clustering within the Glomeromycota (AF158389) was obtained from in vitro cultured spores of *G. rosea* (Rhody et al., 2003), while the sequence that clustered outside the Glomeromycota (U49665) originated from pot-cultured material (Franken et al., 1997); (ii) Rhody et al. (2003) found three *β-tubulin* sequences from *G. intraradices* deposited in databases. One originated from a pot-culture and grouped, in their analysis, within Oomycetes; the two others originated from an in vitro culture of *G. intraradices* and clustered within the Glomeromycota.

The alignment of the putative AMF sequences that clustered outside the Glomeromycota provided clear evidence that predicted gene transfer from Stramenopiles or plants to AMF genomes is unlikely. These sequences were found in four different genera (*Acaulospora*, AF158391; *Scutellospora*, AF158400; *Glomus*, AF159109, and *Gigaspora*, U49665) that diverged from each other hundreds of millions of years ago (Simon et al., 1993). However, they showed almost no variation among their 800 bp length, compared with a very high amount of substitutions within the other evolutionary lineages. Since these sequences were used to predict horizontal gene transfer, the lack of variation among them would mean that the transfer would have had to occur from the same host plant to four different genera at the same time and recently enough to avoid the presence of synonymous substitutions. We argue it is more likely that those sequences originated from the same contaminant of unknown eukaryotic origin and that the very low number of substitutions between these four genera is likely to be due to *Taq* polymerase errors. We did not obtain highly divergent sequences from in vitro cultured *G. intraradices* as Rhody et al. (2003) reported. We demonstrate that although high molecular variation is present in AMF *β-tubulin* genes, this variation has arisen within AMF and does not appear to be a result of horizontal transfer.

### 4.2. $H^+$-ATPase gene family and phylogenetic tree resolution

The *$H^+$-ATPase* tree topology showed unexpected features (Fig. 2). *$H^+$-ATPase* amino acid sequences from Basidiomycetes clustered with high bootstrap values as a sister group of plants. These sequences have been independently obtained by three different authors. The amino acid sequences are conserved between the three basidiomycetes and show large enough differences to distinguish them from plants. Therefore, we would reject horizontal transfer or contamination from plant material as a cause of this unusual nesting. In fact, amino acid alignment showed that fungal specific amino acid motifs are totally absent among the sequences that we used for the phylogenetic analysis. In contrast, sequences are more conserved within each fungal class and within the plant lineage. In our opinion, strong positive selection (i.e., accelerated evolution of certain regions of the protein) between each evolutionary lineage is a

probable prediction for the absence of fungal specific amino acid motifs and for the absence of a well-resolved fungal clade for *H⁺-ATPase*. Another prediction could be that *H⁺-ATPase* sequences from Basidiomycota represent paralogous sequences that have arisen from ancient gene duplication and, subsequently maintained by selection in the Basidiomycota, with the simultaneous loss of the corresponding ancestral gene. Glomeromycota and Ascomycetes share some amino acid motifs that segregate them from the plant-Basidiomycetes cluster. The fact that *Pneumocystis carinii* is not basal in the phylogenetic tree could be attributed to the same phenomenon of positive selection for certain amino acid motifs within the analysed protein sequence.

Our analysis of *H⁺-ATPase* genes was carried out to obtain information about the origin of the five different isoforms reported by Ferrol et al. (2000). We show that intra-lineage variation is low and that the different fungal taxa and the plants group together with high bootstrap values. Therefore, even if phylogenetic analysis shows unexpected features regarding the position of the Basidiomycetes, it is still informative enough to assign an origin to the *H⁺-ATPase* isoforms reported by Ferrol et al. (2000). Based on the fungal taxonomic diversity that was included in our phylogenetic analysis, *GiHA* clones and all the deposited glomeromycotan *H⁺-ATPase* sequences that were not obtained from pot cultures clustered together outside the clade of Ascomycetes. Our findings are in contrast with results obtained by Ferrol et al. (2000) and we present evidence against horizontal transfer of the GmHA5 sequence from plants to the AMF genome. The GmHA5 sequence groups within the cluster of glomeromycotan sequences that were not obtained from pot cultures. The original nesting of GmHA5 within plants is probably due to the low number of fungal taxa that was used in the phylogenetic analysis of Ferrol et al. (2000). The poor resolution of their tree is further highlighted by the clustering within plants of the only non-Ascomycete fungus, *Uromyces fabae* (Basiomycota). In our analysis, this species clustered with other Basidiomycetes, as expected.

The other *H⁺-ATPase* sequences obtained by Ferrol et al. (2000), GmHA1–4 (AJ133839–AJ133842), grouped within Ascomycetes. The interpretation of Ferrol et al. (2000) was that GmHA1–4 represented four different isoforms of *H⁺-ATPase* in the genome of *G. mossae*. In our phylogenetic tree, GmHA1–4 still cluster with high support within the clade of Ascomycetes. Alignment of sequences shows that GmHA1, 3, and 4 are very similar to *N. crassa* and GmHA2 to *Emericella nidulans*. Given that previous studies have verified the presence of Ascomycetes in AMF cultured in pots (Hijri et al., 2002; Redecker et al., 1999; Schüssler, 1999), it is more likely that GmHA1–4 are ascomycete sequences from additional fungi present in the pot culture. Therefore, we suggest that only GmHA5 should be considered as an *H⁺-ATPase* sequence from *G. mossae*. In the present study, and in other studies using AMF cultured in the in vitro system, no sequences have been found that clustered with ascomycetes.

### 4.3. GC content and evolutionary lineages of AMF functional genes

The *β-tubulin* and *H⁺-ATPase* sequences we obtained from in vitro cultured *G. intraradices* and from the databases were analysed with respect to their GC content. With respect to the *H⁺-ATPase* gene phylogeny, all the glomeromycotan sequences obtained from monoxenic cultures show an average GC content of 40%. For the *β-tubulin* gene tree, the sequences grouping in the Glomeromycota cluster and obtained from databases have an average GC content of 44%.

In contrast, *H⁺-ATPase* and *β-tubulin* gene sequences obtained from databases that clustered in other phylogenetic lineages showed significantly higher GC content. In the case of *β-tubulin* genes, their average GC content reached 55–56%. Similarly, *H⁺-ATPase* sequences from *G. mossae* (GmHA1–4) showed average GC values of between 53 and 60%.

In order to obtain additional information about the average GC content of AMF, we analysed a dataset of deposited putative coding sequences (Lammers et al., 2001). All these sequences were obtained from *G. intraradices* growing in the in vitro culture system. Our analysis of these sequences resulted in an average GC content of only 37% (SE ± 5%) in the coding regions of *G. intraradices* (51 putative coding regions analysed, representing about 40 kb of the *G. intraradices* genome). It appears that GC content is a useful tool to distinguish AMF sequences from those that have arisen from possible contaminants present in pot cultures. Moreover, studies conducted by Hosny et al. (1997) on the whole genome of nine AMF species, demonstrated that AMF genomes are characterized by a very low GC content ranging from 30 to 35%, from either coding or non-coding regions. Although the GC content cannot give a definitive answer whether a sequence is of AMF origin or from a potential contaminant, it can be a useful indicator that should alert the researcher to a potential contaminant if the GC content is higher than that normally observed for AMF genomes.

### 4.4. Functional genes and AMF phylogeny

Previous phylogenetic analysis of AMF species have been carried out using ribosomal DNA sequences. These analyses show that AMF species group together but separately from other true fungi. Consequently, Schüssler et al. (2001) proposed a new phylum, the Glomeromycota. Ribosomal genes are present in AMF

genomes as multicopy genes. These sequences have been shown to be highly variable within a single AMF spore, and this therefore presents problems in the identification of specific molecular markers to use in a phylogenetic analysis. The very high intra-sporal molecular variation might be one of the causes for the unclear evolutionary position of many AMF species, and therefore, discussions about the utility of rDNA sequences for providing a reliable AMF phylogeny have arisen. We show that *β-tubulin* and *$H^+$-ATPase* sequences are likely to be monophyletic within a single AMF species. For both these genes, the phylogenetic representation showed that glomeromycotan sequences represent a highly supported group. Our *β-tubulin* tree topology is congruent with a previously published phylogeny of *β-tubulin* fungal sequences (Keeling, 2003) with the exception that the paralogs of Chytridiomycetes clustered within the Zygomycota in our phylogenetic analysis. One of the most intriguing characteristics of the *β-tubulin* tree is the relationship between Glomeromycota and Chytridiomycetes. This result is in contrast with data from Schüssler et al. (2001) that supported Basidiomycetes and Ascomycetes as sister groups of Glomeromycota. This unexpected cluster (Chytridiomycota–Glomeromycota) highlights the need of further phylogenetic analyses in order to determine the closest living relatives to the Glomeromycota. An analysis of 18S rDNA sequences (Schüssler et al., 2001) showed that some species of Mortierellales tend to cluster with Chytridiomycetes. The clade grouping Chytridiomycetes and Glomeromycota is at the edge of being strongly significant and it seems therefore important to add Mortierellales sequences in further studies in order to detect the evolutionary relationships between these fungal groups. Unfortunately, only two sequences from *Mortierella verticillata* are available in the database and cover a different region of the gene to that which we analysed. The loss of most informative sites resulted in the clustering of *M. verticillata* with Zygomycetes without any changes in the tree topology. Nevertheless, analysis of these two sequences showed that the 100 amino acid overlap with the sequences used in our analysis do not show any hints that *Mortierella* groups with Chytrids. A prediction for the grouping of Glomeromycota and Chytridiomycetes could be that at the amino acid level not enough variation has arisen between the two taxa because of very strong selective constraints. These constraints could have acted to maintain highly homologous *β-tubulin* sequences within and between these lineages. This is reflected in the tree genetic distances, where species in other fungal groups show higher genetic distances if compared with the clade grouping Chytrids and Glomeromycota.

Several authors found the presence of paralogous sequences in fungi (Baldauf and Palmer, 1993; Keeling, 2003). Evidence of ancient fungal paralogs of *β-tubulin* could also contribute to the sister group relationship between Chytrids and Glomeromycota. Considering these findings it appears to be likely that more, as yet unrevealed paralogous sequences could be also present in the Glomeromycota. If the presence of *β-tubulin* paralogs is shown to be a general rule within the fungi, major impacts on the *β-tubulin* fungal phylogeny are likely to appear. We think that a multigene approach with the use of a more complete set of fungal taxa (i.e., by adding Mortierellales) will be necessary in studies related to the phylogeny of Glomeromycota, especially if gene sequences do not show signs of ancient duplication that could bias the evolutionary relationships within the fungi.

The *$H^+$-ATPase* phylogeny showed unexpected features and did not resolve fungi as monophyletic. Given the high inter-lineage genetic variation we do not consider the *$H^+$-ATPase* sequences as useful tools for studying the evolutionary positions of the different fungal taxa. At the intra-lineage level, the Ascomycete clade seems to have one separate group (*Emericella/Aspergillus*). Considering the large number of sequences found in *G. intraradices*, their genetic distance with the *G. intraradices* sequence recovered from database and the relatively short branch separating them from *G. mossae*, we would not expect that *$H^+$-ATPase* sequences would be very useful for species identification.

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## References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Antoniolli, Z.I., Schachtmann, D.P., Ophel-Keller, K., Smith, S.E., 2000. Variation in rDNA ITS sequences in *Glomus mossae* and *Gigaspora margarita* spores from a permanent pasture. Mycol. Res. 104, 708–715.

Baldauf, S.L., Palmer, J.D., 1993. Animals and fungi are each other closest relatives-congruent evidence from multiple proteins. PNAS 90, 11558–11562.

Benito, B., Garciadeblās, B., Rodríguez-Navarro, A., 2002. Potassium- or sodium-efflux ATPase, a key enzyme in the evolution of fungi. Microbiology SGM 148, 933–941.

Bracho, M.A., Moya, A., Barrio, E., 1998. Contribution of Taq polymerase-induced errors to the estimation of RNA virus diversity. J. Gen. Virol. 79, 2921–2928.

Clapp, J.P., Fitter, A.H., Young, J.P.W., 1999. Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizial fungus, *Scutellospora* sp.. Mol. Ecol. 8, 915–921.

Clapp, J.P., Rodriguez, A., Dodd, J.C., 2001. Inter- and Intra-isolate rDNA large subunit variation in *Glomus coronatum* spores. New Phytol. 149, 539–554.

Daniell, T.J., Husband, R., Fitter, A.H., Young, J.P.W., 2001. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microb. Ecol. 36, 203–209.

Dunning, A.M., Talmud, P., Humphries, S.E., 1988. Errors in the polymerase chain reaction. Nucleic Acids Res. 16, 10393.

Ennis, P.D., Zemmour, J., Russel, D.S., Parham, P., 1990. Rapid cloning of HLA-A, B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. PNAS 87, 2833–2837.

Ferrol, N., Barea, J.M., Azcón-Aguilar, C., 2000. The plasma membrane *$H^+$-ATPase* gene family in the arbuscular mycorrhizal fungus *Glomus mossae*. Curr. Genet. 37, 112–118.

Franken, P., Lapopin, L., MeyerGauen, G., Gianinazzi-Pearson, V., 1997. RNA accumulation and genes expressed in spores of the arbuscular mycorrhizal fungus, *Gigaspora rosea*. Mycologia 89, 293–297.

Harrison, M.J., van Buuren, M.L., 1995. A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378, 626–629.

Hijri, M., Redecker, M.D., Petetot, J.A.M.C., Voigt, K., Wostemeyer, J., Sanders, I.R., 2002. Identification and isolation of two ascomycetes fungi from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea*. Appl. Environ. Microb. 68 (9), 4567–4573.

Hosny, M., deBarros, J.P.P., Gianinazzi-Pearson, V., Dulieu, H., 1997. Base composition of DNA from glomeromycotan fungi: high amounts of methylated cytosine. Fungal Genet. Biol. 22 (2), 103–111.

Jakobsen, I., 1995. Transport of phosphorus and carbon in VA mycorrhizas. In: Varma, A., Hock, B. (Eds.), Mycorrhiza. Springer, Berlin, Germany, pp. 297–324.

Keeling, P.J., 2003. Congruent evidence from alpha-tubulin and beta-tubulin gene phylogenies for a zygomycete origin of microsporidia. Fungal Genet. Biol. 38, 298–309.

Kumar, S., 1996. Stepwise algorithm for finding minimum evolution trees. Mol. Biol. Evol. 13, 584–593.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2002. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.

Lammers, P.J., Jun, J., Abubaker, J., Arreola, R., Gopalan, A., Bago, B., Hernandez-Sebastia, C., Allen, J.W., Douds, D.D., Pfeffer, P.E., Shachar-Hill, M., 2001. The glyoxylate cycle in an arbuscular mycorrhizal fungus: carbon flux and gene expression. Plant Physiol. 127, 1287–1298.

Lanfranco, L., Garnero, L., Bonfante, P., 1999. Chitin synthase genes in the arbuscular mycorrhizal fungus *Glomus versiforme*: full sequence of a gene encoding a class IV chitin synthase. FEMS Microb. Lett. 170, 59–67.

Lloyd-McGilp, S.A., Chambers, S.M., Dodd, J.C., Fitter, A.H., Walker, C., Young, J.P.W., 1996. Diversity of the ribosomal internal transcribed spacers within and among isolates of *G. mossae* and related fungi. New Phytol. 133, 103–111.

Nagahashi, G., Douds, D.D., 1999. Rapid and sensitive bioassay to study signals between root exudates and arbuscular mycorrhizal fungi. Biotech. Tech. 13, 893–897.

Nesheim, O.N., Linn, M.B., 1969. Deleterious effect of certain fungitoxicants on the formation of mycorrhizae on corn by *Endogone fasciculate* and corn root development. Phytopathology 59, 297–300.

Newsham, K.K., Fitter, A.H., Watkinson, A.R., 1995. Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. J. Ecol. 83, 991–1000.

Redecker, D., Kodner, R., Graham, L.E., 2000. Glomalean fungi from the Ordovician. Science 289, 1920–1921.

Redecker, D., Hijri, M., Dulieu, H., Sanders, I.R., 1999. Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are from ascomycete origin. Fungal Genet. Biol. 28, 238–244.

Remy, W., Taylor, T.N., Hass, H., Herp, H., 1994. Four-hundred-million year-old vesicular-arbuscular mycorrhizae. PNAS 91, 11841–11843.

Requena, N., Breuninger, M., Franken, P., Ocon, A., 2003. Symbiotic status, phosphate, and sucrose regulate the expression of two plasma membrane $H^+$-ATPase genes from the mycorrhizal fungus *Glomus mosseae*. Plant Physiol. 132, 1540–1549.

Requena, N., Mann, P., Hampp, R., Franken, P., 2002. Early developmentally regulated genes in the arbuscular mycorrhizal fungus *Glomus mosseae*: identification of GmGIN1, a novel gene with homology to the C-terminus of metazoan hedgehog proteins. Plant Soil 244, 129–139.

Rhody, D., Stommel, M., Roeder, C., Mann, P., Franken, P., 2003. Differential RNA accumulation of two *β-tubulin* genes in arbuscular mycorrhizal fungi. Mycorrhiza 13, 137–142.

Rodriguez, A., Dougall, T., Dodd, J.C., Clapp, J.P., 2001. The large subunit ribosomal RNA genes of *Entrophospora infrequens* comprise sequences related to two different glomalean species. New Phytol. 152, 159–167.

Sanders, I.R., Alt, M., Groppe, K., Boller, T., Wiemken, A., 1995. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol. 130, 419–427.

Schüssler, A., 1999. Glomales SSU rDNA gene diversity. New Phytol. 144, 205–207.

Schüssler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol. Res. 105, 1413–1421.

Schwarzott, D., Walker, C., Schüssler, A., 2001. *Glomus*, the largest genus of arbuscular mycorrhizal fungi, is nonmonophyletic. Mol. Phylogenet. Evol. 21, 190–197.

Simon, L., Bousquet, J., Lévesque, R.C., Lalonde, M., 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular plants. Nature 363, 67–69.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, B., Fortin, J.A., 1996. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol. Res. 100, 328–332.

Ubalijoro, E., Hamel, C., McClung, C.R., Smith, D.L., 2001. Detection of chitin synthase class I and II type sequences in six different arbuscular mycorrhizal fungi and gene expression in *Glomus intraradices*. Mycol. Res. 105, 470–476.

Van der Heijden, M.G.A., Boller, T., Wiemken, A., Sanders, I.R., 1998a. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79, 2082–2091.

Van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998b. Mycorrhizal fungal diversity determines plant diversity, ecosystem variability and productivity. Nature 396, 69–72.

Van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., Gianinazzi-Pearson, V., 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7, 879–887.