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Arbuscular mycorrhizal fungi (*Glomeromycota*) harbour ancient fungal tubulin genes that resemble those of the chytrids (*Chytridiomycota*)

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Abstract

The genes encoding α- and β-tubulins have been widely sampled in most major fungal phyla and they are useful tools for fungal phylogeny. Here, we report the first isolation of α-tubulin sequences from arbuscular mycorrhizal fungi (AMF). In parallel, AMF β-tubulins were sampled and analysed to identify the presence of paralogs of this gene. The AMF α-tubulin amino acid phylogeny was congruent with the results previously reported for AMF β-tubulins and showed that AMF tubulins group together at a basal position in the fungal clade and showed high sequence similarities with members of the *Chytridiomycota*. This is in contrast with phylogenies for other regions of the AMF genome. The amount and nature of substitutions are consistent with an ancient divergence of both orthologs and paralogs of AMF tubulins. At the amino acid level, however, AMF tubulins have hardly evolved from those of the chytrids. This is remarkable given that these two groups are ancient and the monophyletic *Glomeromycota* probably diverged from basal fungal ancestors at least 500 million years ago. The specific primers we designed for the AMF tubulins, together with the high molecular variation we found among the AMF species we analysed, make AMF tubulin sequences potentially useful for AMF identification purposes.

Keywords: Arbuscular mycorrhizal fungi; α-Tubulin; β-Tubulin; Molecular evolution; *Chytridiomycota*; *Glomeromycota*; Fungal phylogeny; Microtubules

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophic root symbionts of most land plants and are ubiquitous in terrestrial ecosystems (Smith and Read, 1997). AMF benefit plants by improving nutrient acquisition (Jakobsen, 1995) and AMF species diversity influences plant diversity and ecosystem productivity (Van der Heijden et al., 1998). This fungal group has recently been erected to the status of a phylum, the *Glomeromycota* (Schüßler et al., 2001). This is based on an analysis of small subunit rDNA sequences that supports AMF as sister-group of *Ascomycota* and *Basidiomycota*. The *Glomeromycota* are an ancient group of fungi that were present in the roots of the first plants to colonize land over 500 million years ago (Remy et al., 1994a).

Due to their high ecological importance, many efforts aimed at understanding the evolution and functioning of AMF genomes have been undertaken. However, most of the AMF molecular data deposited in public databases results from amplification of ribosomal DNA regions, such as internal transcribed spacers (ITS) or small (SSU) and large subunits (LSU). The data available concerning glomeromycotan protein-coding gene sequences is very low compared to other fungal phyla. At present, less than 150 sequences encoding putative AMF proteins are deposited in GenBank (http://www.ncbi.nlm.nih.

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gov). This low number of AMF protein encoding gene sequences is due to difficulties in extraction of clean AMF DNA, especially from those species cultured on plants in pots with soil. Many sequences previously assigned to AMF species have later been found to be of other evolutionary origins and most likely stemming from contaminants. Indeed, evidence for a likely contaminant origin of deposited AMF sequences have been shown for ribosomal DNA sequences (Redecker et al., 1999; Schüßler, 1999) as well as for protein encoding genes (Corradi et al., 2004). Fortunately, the advent of "in vitro" culturing of AMF with RiT-DNA transformed roots as a host have rendered possible the isolation of higher quantities of AMF DNA as well as avoiding many contaminants (Corradi et al., 2004). Despite the many positive aspects of culturing AMF in these monoxenic cultures, however, "in vitro" cultures of many AMF species and genera are still not available and this leads to difficulties in having a broad range of glomeromycotan taxa to draw and support conclusions about the evolution of AMF genes.

Phylogenetic analyses of some AMF protein encoding genes have been recently carried out by Helgason et al. (2003). The authors isolated partial actin and elongation factor 1α sequences from four AMF species in order to detect their closest fungal relatives, with a combined phylogenetic analysis of these genes. Their study was successful in avoiding contaminants and their phylogenetic analysis showed closest relationship of AMF with a zygomycotan order, the *Mortierellales*. However, the sister-group relationship they found between AMF and *Mortierellales* was not supported by conservative phylogenetic analysis (neighbor-joining) and a Bayesian phylogenetic analysis failed to show the generally accepted evolutionary relationships between fungal groups (i.e., zygomycotan species clustering between the *Ascomycota* and the *Basidiomycota*). Furthermore, samples from the *Chytridiomycota* were not included in analyses, although they are considered as basal members of the fungi. More recently, AMF β-tubulin amino acid sequences were used in reconstructing a fungal phylogeny (Corradi et al., 2004) and an unexpected sister-group relationship between amino acid sequences of the *Glomeromycota* and the *Chytridiomycota* was found. This result was surprising since chytrids greatly differ from AMF and other fungi in their life cycle, their overall morphology and, more specifically, by the presence (in at least one stage of their life-cycle) of a flagellum. Other fungi lack the $9+2$ microtubule structure.

Although the studies by Corradi et al. (2004) and Helgason et al. (2003) compared AMF sequences with other fungal phyla, reasons behind their findings were poorly explored for obvious reasons since Corradi et al. (2004) mainly corrected AMF sequences of contaminant origin and Helgason et al. (2003) focused exclusively on the position of the *Glomeromycota* within the fungal clade. To study the evolution of AMF genes it is necessary to compare their features with the ones identified in other fungal relatives. Phylogenetic analyses are the first steps that can be followed by a more comprehensive comparison with other fungal phyla and by an analysis of the driving forces that act on the evolution of the genes under study. The unexpected finding by Corradi et al. (2004) that AMF β-tubulin amino acid sequences strongly resemble those of the chytrids opened-up questions about the true relationships between these two fungal phyla and about the features of the β-tubulin that may have led to this sister-grouping. Corradi et al. (2004) discussed the nesting of the *Glomeromycota* in the fungal tree as a possible consequence of ancient duplicative events of β-tubulins in the fungal clade as well as high levels of purifying selection acting on glomeromycotan and chytridiomycotan β-tubulins.

An analysis of other "tubulin-family" members might give new insights about AMF gene evolution. Tubulin genes are attractive for comparing evolution among fungi and for reconstructing the fungal phylogeny because many representatives of ascomycotan, basidiomycotan, chytridiomycotan, microsporidian, and zygomycotan α- and β-tubulin sequences exist in gene databases. Moreover, both tubulin phylogenies support general features of fungal evolution and taxonomy, for instance the separation of the *Chytridiomycota* and the *Zygomycota* (Keeling, 2003). Therefore, a parallel analysis of tubulin genes in AMF is certainly warranted, with the prerequisite that a broad range of AMF taxa is used in the analyses to support conclusions about the evolution of these genes.

In this study, we report the first identification of α-tubulin gene sequences from several genera in the *Glomeromycota*. Phylogenetic analyses, with fungal relatives, have been carried out to test their position in the fungal clade and the congruence of α-tubulin phylogeny with the previously published data on AMF β-tubulin amino acid sequences (Corradi et al., 2004). In the report by Corradi et al. (2004), representatives of major AMF genera were lacking from the analyses. Moreover, some nucleotide sites were unresolved in sequences recovered from databases and highly variable paralogs were only reported for *Glomus intraradices*. For these reasons we re-investigated the presence of orthologous and paralogous sequences of the β-tubulin gene in AMF species that belong to several genera within the *Glomeromycota* (*Glomus*, *Gigaspora*, *Scutellospora*, and *Acaulospora*).

2. Materials and methods

2.1. AMF cultivation and genomic DNA extraction

Isolates of *G. intraradices* (DAOM 181602), *Glomus diaphanum* (MUCL 43196), *Glomus claroideum* (MUCL

43205), and *Glomus proliferum* (MUCL 41827) were grown with Ri T-DNA transformed *Daucus carrota* roots. These fungi were 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. For cultures of *Glomus* sp. (BEG19), *Glomus geosporum* (BEG18), *Scutellospora castanea* (BEG1), *Gigaspora margarita* (BEG 34), and *Acaulospora laevis* (BEG13) spores were harvested by wet sieving of the soil and DNA was extracted according to Hijri et al. (1999).

2.2. Polymerase chain reaction and DNA sequencing

Two 27-mer degenerate oligonucleotides for α-tubulin designated α TubDegF (sense): 5′-GGG CCC CAG GTC GGC AAY GCN TGY TGG-3′ and α TubDegR (antisense): 5′-GGG CCC CGA GAA CTC SCC YTC YTC CAT-3′ (Keeling, 2003) were synthesized (Microsynth GmBH, Switzerland) and used for amplification of genomic DNA. This primer set amplifies approximately 95% of the α-tubulin gene. 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 $MgCl_2$, 0.1% Triton X-100, and 0.2 mg/ml BSA), 100 nM dNTP, 1 μM final concentration of each degenerate primer, and 0.5 U of *Taq* polymerase (Qbiogene). PCR was performed 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 30 s at 94 °C, annealing for 30 s at 44 °C and extension for 2 min at 72 °C, followed by a final extension for 10 min at 72 °C. The AMF β-tubulin fragments were amplified with the primers designated Deg.Tub.F (sense): 5′-GAY GTY GTB CGY AAR GAR and Deg.-Tub.R. (antisense): 5′-TTS ARY TGW CCV GGG AAA CG according to Corradi et al. (2004). When target DNA sequences were present in too few copies in the total genomic DNA extraction, a nested PCR approach was found to be useful in amplifying AMF tubulin genes. In this case, a first PCR with degenerate primers was performed according to the conditions above. PCR products were then diluted 50 times in sterile water (or TE) and used as template in a nested PCR by using the following primers sets: α. spe.F 5′-TGC CTT GAG CAC GGT ATT CAA GT-3′-α. spe.R 5′-CAT ACC AAT GGA CGA AAG CAC GT-3′ and β. Tub.F 5′-GCT GTT CTC GTT GAC CTT GA-3′ and β. Tub.R 5′-GCA AAT CCG ACC ATG AAG AA-3′ for amplification of AMF α- and β-tubulins, respectively. These primers were designed using conserved regions of AMF α- and β-tubulin sequences obtained with degenerate primers, and were shown to be useful for amplifying these genes in all AMF genera tested. The nested-PCR was performed 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 30 s at 94 °C, annealing for 30 s at 55 °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 and then 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 the manufacturer's instructions. Sequences were obtained using M13 forward and reverse primers with the Big Dye 3.1 Terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions, and separated on an ABI Prism 3100 genetic analyser (Applied Biosystems).

2.3. Sequence analysis

Sequences were analysed using the Vector NTI package (Informax, Oxford, UK). Sequence homology with genes deposited in databases was conducted using BLAST (Altschul et al., 1990). Sequences were aligned using clustalW (Thompson et al., 1994) and refined by eye. As the amino acid sequences of tubulins are highly conserved in both their sequence and their length, no significant gaps existed in the data. Amino acid sequences were corrected for site-to-site variation according to a gamma distribution with the α shape parameter that was previously estimated for fungal tubulins (α parameter = 0.75 and 0.44 for α- and β-tubulin, respectively) (Keeling, 2003). Such parameters are realistic since AMF tubulin genes did not vary in their rate of variation from those of the chytids. A Poisson correction of amino acid sequences led to the same tree topology with similar support of the main nodes. To allow a comprehensive analysis of a wide range of fungal amino acid sequences, all analyses were restricted to animals, a choanoflagellate and fungi. AMF nucleotide sequences were analysed

phylogenetically with chytridiomycotan sequences as outgroups in order to test for their true evolutionary origin with a K2P model. Neighbor-joining and minimum evolution (Kumar, 1996) genetic distances were calculated using MEGA2 (Kumar et al., 2002) for both nucleotide and amino acid sequence data. Phylogenetic tree topologies were assessed by 1000 bootstrap replicates. Accession numbers of taxa used in the phylogenetic analyses are provided in the tree topologies. Twenty glomeromycotan sequences were identified in this study and deposited in public databases under Accession Nos. AJ717308–AJ717327. Alignments used in phylogenetic analyses are available in the EMBL gene database under the following Accession Nos. ALIGN_000725, ALIGN000726, ALIGN000727, and ALIGN000728. Uncorrected, as well as maximum likelihood distances for saturation plots were calculated with PAUP* (Swofford, 2002).

3. Results and discussion

3.1. Identification and phylogeny of α-tubulin genes from the Glomeromycota

For all the AMF species we investigated, the PCR with α-tubulin degenerate primers yielded a fragment within the range of 1.5–1.6 kb in length, and when a nested PCR approach was used, the fragment had a length within the range of 1.4–1.5 kb. Sequences of these fragments showed highest similarities with α-tubulin genes deposited in databases and showed that between 80 and 95% of the α-tubulin coding region (depending on the primer set) was successfully amplified from these AMF species. Even though ten positive clones for each species were sequenced on both strands, no evidence for the presence of paralogs was found. Their alignment with cDNA of diverse fungal origin deposited in EMBL or GenBank showed the presence of a single intron in all AMF species. The position of the single intron did not differ among AMF species and only slight differences in the length of introns were observed between AMF genera. Molecular divergence between the introns at homologous positions in the AMF was very high and for this reason could not be aligned with confidence. The substitutions along the coding sequences of the AMF α-tubulin genes were mostly synonymous (>95%) with around 30% of the coding region being variable in the second exon (1132 bp).

A phylogenetic analysis of fungal α-tubulin amino acid sequences was carried out (377 amino acids in length) (Fig. 1). The alignment is deposited in the EMBL gene database under Accession No. ALIGN000725. The overall tree topology was congruent with Keeling (2003). Phylogenetic analysis showed the presence of the six major fungal groups: *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Glomeromycota*, *Microsporidia*, and *Zygomycota*. Many features of the currently accepted fungal phylogeny were supported. For instance, the sister-group relationship between *Ascomycota* and *Basidiomycota* and the separation of *Chytridiomycota* from *Zygomycota* were supported. In all analyses the *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Glomeromycota* formed monophyletic groups. Also consistent with Keeling (2003), the *Zygomycota* were paraphyletic. *Microsporidia* grouped together with members of zoopagales and entomophtorales. Consistent with AMF phylogenies based on SSU rDNA, elongation factor 1α- and β-tubulin (Corradi et al., 2004; Helgason et al., 2003; Schüßler et al., 2001), the α-tubulins from the *Glomeromycota* clustered together and were monophyletic. However, only consistent with Corradi et al. (2004), the analysis of fungal α-tubulin sequences also supported a sister-group relationship between *Glomeromycota* and *Chytridiomycota* with relatively high bootstrap values. The α-tubulin genes from AMF and chytrids are, therefore, similar and tend to segregate from other fungal lineages. The amino acid sequence similarity between the AMF and chytrids is so high that the sister-grouping between these fungal phyla is supported even more than the monophyly of each of the two phyla. This feature could have suggested a possible contamination of our AMF dataset with some chytridiomycotan sequences. However, some of the AMF species we used were cultured "in vitro" where contamination is unlikely. Furthermore, a phylogeny based on the nucleotide sequences (Accession No. ALIGN000726) shows that the *Glomeromycota* form a strongly supported clade that is separate from the *Chytridiomycota* (Fig. 2). Within the *Glomeromycota* the main families proposed by Schüßler et al. (2001) are supported.

To determine if the relationship between the glomeromycotan and chytridiomycotan sequences was only reflected at the amino acid level, we carried out a phylogenetic analysis including all major fungal phyla by using α-tubulin nucleotide sequences (1105 bp; Accession No. ALIGN000728) (data not shown). When all codon positions were used to infer the phylogeny, the saturation at the third codon positions in fungal tubulins led to a polyphyly of fungal phyla on a large scale. However, by excluding the third codon position the phylogeny was fully congruent with the tree topology recovered from amino acid sequences. The only exception was that sequences from *Candida albicans* (U38534) and *Saccharomyces cerevisiae* (M28429) grouped outside the ascomycotan clade with microsporidian species. The most likely explanation for this unusual nesting is a "long-branch attraction" artefact since all these sequences show much longer branches compared to other fungal taxa included in the analysis. Moreover, when *Microsporidia* are excluded from the analysis, the *Ascomycota* are shown to be monophyletic. Consistent with the phylogeny based on amino acid sequences, the α-tubulin nucleotide sequences from *Glomeromycota* and

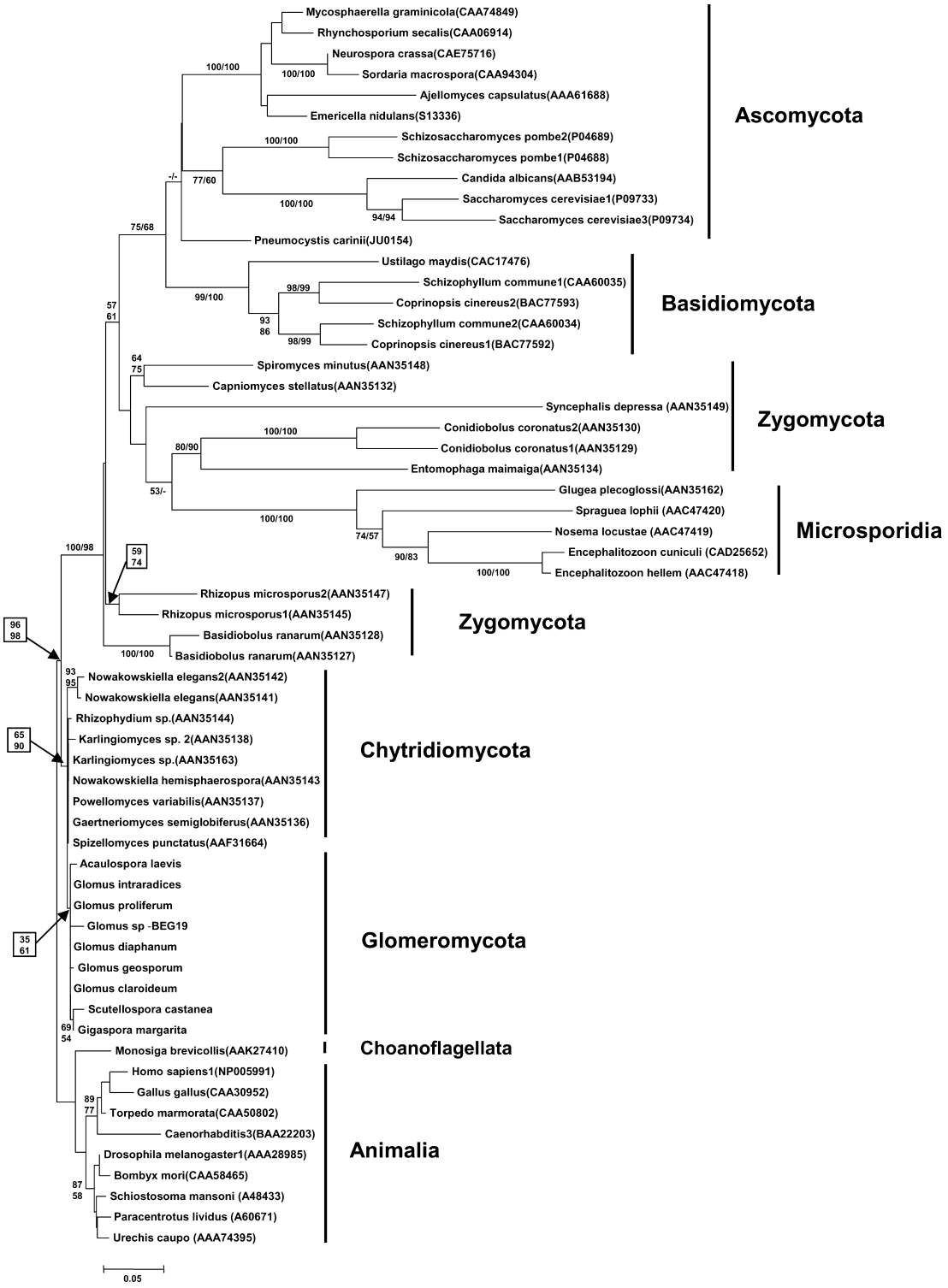

Fig. 1. Phylogenetic analysis of fungal α-tubulin amino acid sequences (377 amino acids in length). α-Tubulin genes obtained from several arbuscular mycorrhizal fungi were compared with previously published sequences from the *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Microsporidia*, *Zygomycota*, *Animalia*, and *Choanoflagellata*. Numbers at nodes correspond to bootstrap support from neighbor-joining (top/left) and minimum evolution (bottom/right) analyses. Scale bar represents 0.05 substitutions per site.

Chytridiomycota grouped at a basal position in the fungal clade and clustered together as a sister-group with relatively high bootstrap supports of 78 and 97% for neighbour-joining and minimum evolution analyses, respectively. Monophyly of glomeromycotan sequences was supported by bootstrap values of 98 and 96% for neighbour-joining and minimum evolution analyses, respectively.

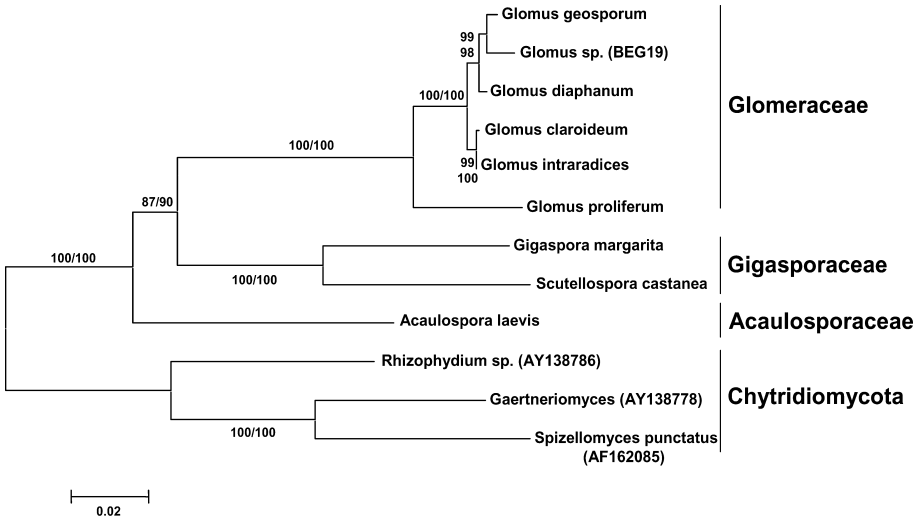

Fig. 2. Phylogenetic analysis of glomeromycotan α-tubulin nucleotide sequences (1132 bp). α-Tubulin sequences obtained from arbuscular mycorrhizal fungi are compared with previously published sequences belonging to the *Chytridiomycota*. Numbers at nodes correspond to bootstrap support from neighbor-joining (top/left) and minimum evolution (bottom/right) analyses. Scale bar represents 0.02 substitutions per site.

3.2. Identification of β-tubulin genes in the Glomeromycota

Two highly variable sequences were reported as being present in the genome of *G. intraradices* (Corradi et al., 2004). These paralogous sequences had not previously been identified in other species, have been shown to be monophyletic within *G. intraradices* and to be highly conserved at the amino acid level. However, in this study, the identification of β-tubulin sequences in other AMF genera allowed us to carry out a more comprehensive analysis of duplication events that have occurred during AMF evolution.

In this study, highly variable β-tubulin paralogs showing highest similarities to the two β-tubulin variants previously obtained from *G. intraradices* (Corradi et al., 2004) were newly identified in three other AMF species (*G. claroideum*, *G. diaphanum*, and *G. proliferum*). In *A. laevis*, *G. margarita*, *Glomus* sp. (BEG19), *G. geosporum*, and *S. castanea* no evidence for the presence of paralogs was found. All AMF β-tubulin sequences show the presence of two introns that are highly divergent between genera and paralogs. As for the α-tubulins, even though a high number of substitutions was encountered between either exonic sequences of orthologs (around 70% similarity in the coding region we analysed, 626 bp) and paralogs (70% similarity), the vast majority of these were synonymous (>90%), suggesting high levels of purifying selection acting on AMF β-tubulin genes.

The nested PCR approach did not allow us to obtain a fragment that is long enough and informative enough to infer a supported fungal phylogeny, as only an alignment of 210 amino acids was possible. The phylogeny of β-tubulin, based on amino acid sequences, show general fungal features described in Keeling (2003), as well as the sister-grouping of AMF with the chytrids but bootstrap support of the main nodes was low (data not shown). A phylogeny based on nucleotidic sequences of the β-tubulin coding region (626 bp) supported the monophyly of the AMF sequences (Accession No. ALIGN000727). These results are also consistent with the taxonomy of the families within the *Glomeromycota* proposed by Schüßler et al. (2001), with the segregation of the Acaulosporaceae and Gigasporaceae from the Glomeraceae (Fig. 3). The newly identified paralogous sequences were not found to be monophyletic within each species but rather to segregate in the phylogenetic tree in relation with a duplicative event that, according to the sequences we obtained and analysed, occurred after the divergence of Acaulosporaceae, Gigasporaceae and even after divergence of some *Glomus* species such as *Glomus sp.* (BEG19), *G. geosporum*, and *Glomus mosseae*. The support for the duplicative event is relatively high and the introns of *G. geosporum* and *Glomus* sp. (BEG19) do not show strong homology with any introns of the two paralogs. This leaves little doubt about the position of the duplicative event in the AMF phylogeny, but the presence of an independent and, as yet, unrevealed duplicative event cannot be ruled out.

3.3. Saturation analysis

Both α-tubulin and β-tubulin sequences showed high levels of synonymous substitution. Therefore, we tested the presence of saturation at the third position of the codons, where the large majority of substitutions occur and which may account for the unresolved and contrasting positions of some taxa in the phylogenies (Figs. 4A and B). To test this, we plotted all pairwise distances (*p*-dis-

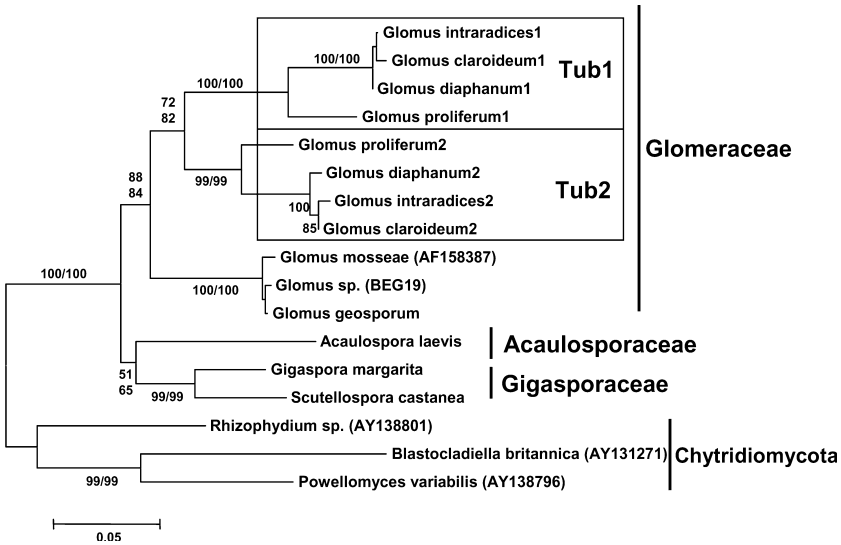

Fig. 3. Phylogenetic analysis of glomeromycotan β-tubulin nucleotide sequences (626 bp). β-Tubulin sequences identified in arbuscular mycorrhizal fungi are compared with previously published sequences belonging to the *Chytridiomycota*. When paralogous sequences were recovered, these were numbered according to their clustering in the phylogeny. Numbers at nodes correspond to bootstrap support from neighbor-joining (top/left) and minimum evolution (bottom/right) analyses. Scale bar represents 0.05 substitutions per site.

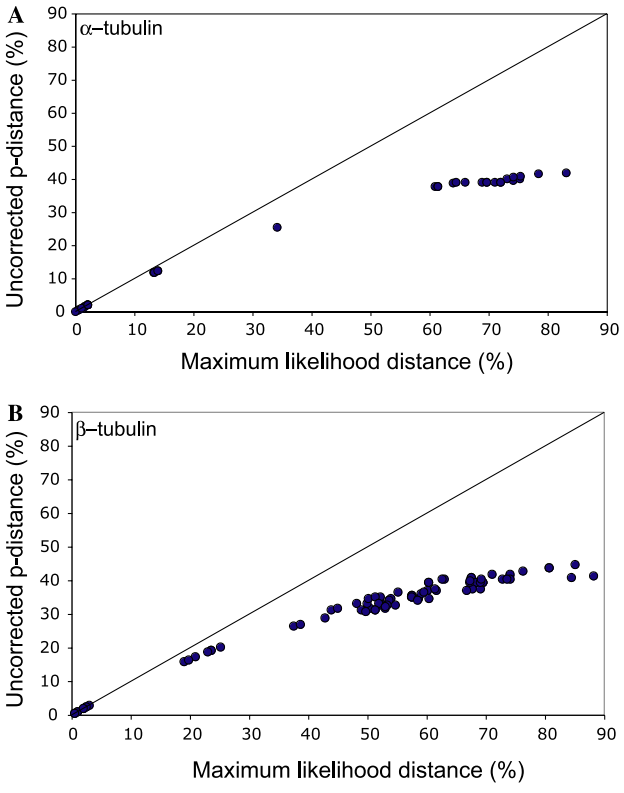

Fig. 4. Saturation plots of uncorrected distances versus maximum likelihood distances. Each dot represents a comparison of a pair of sequences. (A) AMF α-tubulin sequences (3rd codon position). (B) AMF β-tubulin sequences (3rd codon position).

tances) against maximum likelihood distances in third position of the codons. As suspected, third positions of codons are highly saturated in both α- and β-tubulin, partly due to the mutational bias towards synonymous sites. Both plots show a saturation that starts between 10 and 20%. This result highlights potential problems that could arise by using these two genes for reconstructing the AMF phylogeny, and explains the unresolved and contrasting positions of some AMF taxa between the two phylogenies. No clear levels of saturation have been observed in the first and second codon positions among AMF species (data not shown). However, while the 1st and 2nd codon positions were informative for resolving the position of the *Glomeromycota* in the fungal phylogeny, these were not informative for resolving among genera and species positions within a phylogeny of the *Glomeromycota* due to lack of substitutions.

3.4. Levels of tubulin gene conservation in the Glomeromycota

The α- and β-tubulin fungal phylogeny, based on amino acid sequences, provides evidence for the basal position of AMF tubulins in the fungal tree. According to these results, AMF tubulin sequences may represent ancient fungal amino acid sequences that have not evolved as in other fungal phyla. By observing the patterns of nucleotide substitutions occurring in coding regions between AMF species, we observed that substitutions were mostly synonymous. Additionally, the introns at homologous positions were so divergent that reliable alignments of them were not possible between different AMF genera. These observations together with the proposed age of divergence of the *Glomeromycota* (Redecker et al., 2000) and the *Chytridiomycota* (Remy et al., 1994b) confirm that these represent ancient fungal amino

acid sequences that did not evolve at the amino acid level due to strong purifying selection.

Glomeromycotan tubulins differ from those of other fungal phyla in that both orthologs and paralogs are highly conserved at the amino acid level. This result was not only unexpected but newly described for fungal tubulins. Indeed, in other fungal phyla tubulin conservation was found to be present only between orthologs or between paralogs. For instance, chytrid orthologs were shown to be under same evolutionary constraints as AMF, but when paralogous sequences were recovered, these were shown to be highly divergent (Keeling, 2003). An opposite phenomenon is observed in other fungal phyla (*Zygomycota*, *Ascomycota*, and *Basidiomycota*) where paralogs are conserved but orthologs are more divergent. AMF tubulins are also shown to be unique as a group in relation to the absence of introns' gain or losses. Indeed, gains or losses of introns have not been detected in both α- and β-tubulin. The conservation of intron position in AMF tubulins is striking considering that these species are thought to have diverged more than 500 million years ago (Redecker et al., 2000). Although conservation of intron position is common for fungal β-tubulins, the maintenance of a single intron position in AMF α-tubulins is in contrast to other fungal phyla, where gain and/or losses of introns have been shown to be either frequent (i.e., in the *Chytridiomycota*, Keeling, 2003) or at least to have occurred between species in all other main fungal lineages.

The reasons behind the high levels of AMF tubulin conservation could be highlighted by comparisons with functional analyses of these proteins and characterization of microtubules in AMF. As far as we know, only a couple of studies reported the identification of microtubules in AMF. These studies focused on *G. intraradices* in symbiosis with tomato roots (Timonen et al., 2001) and on monoxenic cultures of *G. mosseae* (Aström et al., 1994) (see also Timonen and Peterson, 2002 for a review). Both studies were in agreement that there was a striking lack of AMF microtubule differentiation between external hyphae and arbuscules in both orientation and organisation. AMF microtubules were shown to be oriented in longitudinal filaments and bundles and, in contrast to other fungi, no "netlike" or other specialized arrangements have been observed as in other fungi, including ectomycorrhizal fungi (Timonen et al., 1993). Additionally, microtubules were often found in association with nuclei and are, therefore, likely to have a predominant role in nuclear movement. Considering the very high homology we have found among AMF tubulins, it is likely that the function and the formation of undifferentiated microtubules do not differ significantly in members of the *Glomeromycota*. The tubulins' conservation is possibly related to the coenocytic and multinucleate state of AMF, and may be explained in part by the very low morphological diversification among all members of this phylum. It may also be interesting to speculate about the relationship between the ancient status of AMF tubulins and the lack of differentiation of AMF microtubules, making them putative fungal microtubule ancestors, and possible models for functional analysis of ancient fungal tubulins.

3.5. Is the *Glomeromycota* a sister-group of the *Chytridiomycota*?

There are many differences between the morphogenesis and/or life cycles of the AMF and the chytrids. Moreover, at present, considering the lack of data concerning the functions of microtubules in these two taxa, it remains unclear why AMF and chytrids have shared these similar, and probably ancient fungal tubulin amino acid sequences, for what could have been more than 500 hundred million years. In the fungal tree, the positioning of AMF can possibly be biased by obvious amino acid rate heterogeneity between *Glomeromycota*, *Chytridiomycota* and the other fungal phyla. Indeed, the fact that AMF tubulins strongly resemble those of the chytrids is probably caused by very high levels of purifying selection acting on these genes in both of these phyla. In this case, finding the similar reason that lead to a same selection in these groups would be an interesting evolutionary case-study. Considering the high selective bias among fungal tubulins, we would expect other protein coding genes showing more homogeneous rates of protein evolution, to be potentially more useful for the positioning of the *Glomeromycota* within the fungal clade.

However, it is noteworthy that the sister-grouping of AMF and chytrids has some implication about previous predictions on tubulin evolution and, more specifically, on recent findings about the position of *Microsporidia* in the fungal clade (Keeling, 2003). Indeed, it has been predicted that the loss of flagella may result in an acceleration of the tubulin evolution in the fungal clade. This was supported by recent analyses of fungal tubulin and by the positioning of *Microsporidia* within the *Zygomycota* (Keeling, 2003). However, given that AMF lack flagella, the result that AMF tubulins have hardly evolved at the amino acid level implies that the prediction stated above is not valid for all fungal clades.

It is fitting to end on a cautionary note regarding the use of AMF tubulins in reconstructing the AMF phylogeny. Although the main families (*Glomeraceae*, *Acaulosporaceae*, and *Gigasporaceae*) were supported by analyses of coding regions, contrasting positions of *G. geosporum* and *Glomus* sp. (BEG19) were found in α- and β-tubulin phylogenies (Figs. 2 and 3). Moreover, *A. laevis* was nested differently according to which gene we analysed. In the first case, since the same sequences were recovered independently in two DNA extractions and both taxa cluster together in each phylogeny, we would not consider the contrasting positioning of the

two *Glomus* species as a consequence of cross-contamination. A probable prediction for these results could be the rapid saturation of third codon position that has been shown for AMF tubulins (Figs. 4A and B), as well as different rates of evolution of these genes. Additionally, the identification of only a single α- and β-tubulin sequence in some AMF species is not conclusive evidence that independent duplicative events did not occur within the *Glomeromycota*, leading to species positions that may not reflect true evolutionary steps within this fungal phylum. These evolutionary features, taken together, may account for the contrasting phylogenies based on nucleotide sequences and for these reasons, we would not consider these genes as useful candidates for reconstructing the AMF phylogeny. Finally, it should be noted that we successfully designed primers that specifically amplify glomeromycotan sequences through a nested PCR approach. Even when fungal DNA was isolated from a few spores harvested from pot cultures, all the sequences we recovered were found to be of glomeromycotan origin. Therefore, by considering the molecular variation we found between species of this phylum, and the lack of significant intra-specific variation, we would consider these primer sets as potentially very useful tools for species identification in this ecologically important group of fungi.

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