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High-Level Molecular Diversity of Copper-Zinc Superoxide Dismutase Genes among and within Species of Arbuscular Mycorrhizal Fungi[▽]†

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In the ecologically important arbuscular mycorrhizal fungi (AMF), *Sod1* encodes a functional polypeptide that confers increased tolerance to oxidative stress and that is upregulated inside the roots during early steps of the symbiosis with host plants. It is still unclear whether its expression is directed at scavenging reactive oxygen species (ROS) produced by the host, if it plays a role in the fungus-host dialogue, or if it is a consequence of oxidative stress from the surrounding environment. All these possibilities are equally likely, and molecular variation at the *Sod1* locus can possibly have adaptive implications for one or all of the three mentioned functions. In this paper, we analyzed the diversity of the *Sod1* gene in six AMF species, as well as 14 *Glomus intraradices* isolates from a single natural population. By sequencing this locus, we identified a large amount of nucleotide and amino acid molecular diversity both among AMF species and individuals, suggesting a rapid divergence of its codons. The *Sod1* gene was monomorphic within each isolate we analyzed, and quantitative PCR strongly suggest this locus is present as a single copy in *G. intraradices*. Maximum-likelihood analyses performed using a variety of models for codon evolution indicated that a number of amino acid sites most likely evolved under the regime of positive selection among AMF species. In addition, we found that some isolates of *G. intraradices* from a natural population harbor very divergent orthologous *Sod1* sequences, and our analysis suggested that diversifying selection, rather than recombination, was responsible for the persistence of this molecular diversity within the AMF population.

Positive Darwinian selection is a potential signature of genetic conflicts. This has been demonstrated in host-pathogen interactions using molecular evidence (3, 37) and is also supported by theoretical predictions (7, 29, 34, 35). In sharp contrast, how genes evolve in a mutualistic environment remains largely unexplored. Strong selective constraints on genes that are specifically expressed during symbiotic stages are expected, and, indeed, slow rates of evolution have been predicted in conceptual models of mutualism because only weak selection pressures are expected in the host environment (8). Conversely, mutualism has been considered as a less harmonious interaction because the organisms involved have been predicted to evolve mechanisms to avoid overexploitation by their partners (20, 24). Evolution of such mechanisms in the host could create selective pressures for change in the symbiont. In the case of relatively unspecific mutualisms, a symbiont may have to maintain the ability to colonize different hosts that present different environments and have different abilities to avoid exploitation. Furthermore, competition among symbionts to colonize a host could provide selection pressures for rapid evolution of the genes involved in recognition and establishment of symbiosis. Recently, positive selection has been observed in the plant gene *NORK* that is involved in the establishment of the nitrogen-fixing mutualism with rhizobia and the arbuscular mycorrhizal symbiosis with fungi (17). It is highly pertinent to study evolutionary forces shaping the diversity of genes that are up- or downregulated in the symbiosis. This is an important and necessary step to predict their functions and their potential role between the mutualists.

The copper-zinc superoxide dismutase (Sod1) is a ubiquitous metalloprotein catalyzing the dismutation of superoxide into molecular oxygen and hydrogen peroxide. In the plant, expression of this molecule is usually one of the earliest detectable plant responses to pathogen infection and a key element of the plant defense system (5). In AMF, *Sod1* encodes a functional polypeptide that scavenges reactive oxygen species (ROS) from metabolic processes (14). Potentially, it could be environmentally induced (i.e., heavy metal contamination in soils), but this is currently unknown. Furthermore, *Sod1* has been suggested to be an essential component in the plant/fungus dialogue necessary to reach functional and structural compatibility between the partners (18, 27), but this has not been experimentally demonstrated. However, the gene was shown to be strongly expressed in the root during early stages of the plant-AMF symbiosis (27). Furthermore, evidence of its ROS-deactivating role in roots is supported by observations that hydrogen peroxide accumulates in the same tissue and that blocking of *Sod1* in mycorrhizal roots markedly reduces hydrogen peroxide accumulation. Evidence therefore suggests that the *Sod1* gene plays an important role for the fungus during establishment of symbiosis with the host plant, but its exact function remains unclear.

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AMF are putative ancient asexuals, belonging to the fungal phylum *Glomeromycota* (12, 32), that form obligate symbiotic interactions with about 80% of land plants. AMF are known as important determinants of plant biodiversity, ecosystem variability, and productivity (36), yet very little is known about the evolution of their genes and genomes. Understanding the evolutionary fate of AMF genes, as well as studying their molecular divergence within and among species, can provide useful insights about the evolution of these ecologically important symbionts (16). In this study, homologous sequences encoding a copper-zinc superoxide dismutase protein (Sod1) have been isolated from five AMF species and a collection of *Glomus intraradices* isolates that were all initiated from single spores from one agricultural field to identify the level of molecular diversity within an AMF population. Codon-based maximum-likelihood procedures and recombination detection methods have been used to identify potential selective forces acting on these genes throughout their evolutionary history.

MATERIALS AND METHODS

Origin of isolates, AMF cultivation, and DNA extraction. *G. intraradices* single-spore isolates were established from a population located in Switzerland. The 17 isolates examined originate from a small field site (90 m by 110 m) located at Hausweid, Tänikon. For details on spatial arrangement see Koch et al. (25). All isolates of *G. intraradices* (Tänikon population and within-population comparisons), isolate DAOM 181602 (interspecific comparisons), *Glomus* sp. (strain MUCL 43196), *Glomus proliferum* (MUCL 41827), *Glomus geosporum*, and *Gigaspora rosea* were clonally subcultured every 15 weeks in two-compartment plates with root-inducing transfer DNA-transformed carrot roots (29). Freshly isolated hyphae and spores of each isolate and species were separately dried overnight at 48°C and ground into a fine powder using a Retsch MM300 mixer mill (Retsch, GmbH). The DNA was extracted using a modified version of the Cenis method for fungal DNA extraction with an additional step of a 1:1 dilution with a solution of 24:1 of chloroform-isoamyl alcohol before the final precipitation to remove remaining impurities (9).

Gene amplification and cloning. A total of four specific and slightly degenerate primers were designed based on the published *Sod1* sequence of *Gigaspora margarita* (27). Primer sequences were the following: 5′-AAAGCTATTGCAG TTCTTAG-3′ and 5′-CCCAATAACACCACAGGCAA-3′; 5′-GCAGTTITTT ASWCCTGATAA-3′ and 5′-TAACACCACAGGCAACACGA-3′.

DNA amplifications yielded one single fragment of the expected size with each pair of primers tested. The PCR products were separated on agarose gel and purified using a MinElute Gel Extraction Kit (Qiagen). The fragments were cloned with a TOPO TA Cloning Kit (Invitrogen, Inc.) following the manufacturers instructions. DNA sequences were determined using a version 3.1 Terminator cycler sequencing kit and separated on an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Sequence analyses, phylogenetic reconstruction, detection of positive selection, and recombination. Sequences were analyzed and aligned using the Vector NTI package (Informax, Oxford, United Kingdom). Chromatograms were carefully examined by eye. A phylogenetic framework was reconstructed based on all *Sod1* genes and internal transcribed spacer (ITS) sequences using the neighbor-joining and minimum-evolution methods implemented in MEGA, version 3.1 (26). The maximum-likelihood tree was computed under the WAG+G model of evolution, chosen according to the Akaike information criterion (as computed with ProtTest, version 1.4 [1]), using Phyml, version 3.0 19). Bootstrap support was calculated from 1,000 replicates. Bayesian posterior probabilities were calculated using MrBayes, version 3.1.2, (22) with priors, chain number, and temperature set to default values; the aamodelpr parameter was fixed to particular protein models of evolution chosen according to the Akaike information criterion. Two parallel Markov chains were run for 2×106 generations; every 100th tree was sampled, and the first 5×105 generations were omitted from topology and probability reconstruction.

Codon-based likelihood ratio tests (LRTs), implemented in the PAML package, were used to detect potential signatures of selection in our data. Comparing the number of nonsynonymous to synonymous substitutions per site (dN/dS) is typically used to detect selection at the molecular level. Positive selection is inferred by a dN/dS ratio (ω) exceeding 1, while purifying selection is characterized by a ω of <1, and neutrality is indicated by a ω of 1. To test hypotheses of molecular evolution, we used models that allow different ω rates at different sites: the null model M1a (assuming two site classes: sites under purifying selection and neutrally evolving sites) and the alternative model M2a (adding a third site class: sites under positive selection) (30, 38). Comparison of the log-likelihood values under the two models reveals whether the model that allows for positively selected sites fits the data set better than the null model. The LRT compares twice the difference of the log-likelihood to a χ^2 distribution with a degree of freedom equal to the difference of free parameters in the corresponding models. Naïve and empirical Bayes calculations were used to analyze in more detail the site class of positive selection to identify particular sites under positive selection. The codon-based likelihood ratio tests have been performed among the six AMF species using the phylogenetic relationships represented in Fig. S1A in the supplemental material. The *G. intraradices* sequence used in the species data set corresponds to the isolate DAOM 181602. Rearranging the positions of *G. proliferum*, *G. geosporum*, and *Glomus* sp. within the AMF phylogeny did not affect the results reported in this study. The population data sets have been analyzed using the phylogenetic relationships shown in Fig. S1B in the supplemental material. The presence of recombination has been investigated using the program RDP2 (28) and default parameters. Recombination events have been investigated using an alignment of nucleotide sequences of the *Sod1* gene from the totality of AMF species and isolates.

Real-time PCR procedures. Real-time PCR was performed on three *G. intraradices* isolates (A2, A4, and C2) to compare relative copy numbers of the *Sod1* genes. Using the *Sod1* sequences we obtained from the 14 *G. intraradices* isolates, we designed primers that amplify a 100-bp fragment and that annealed to a region conserved among all variants. The primers and probe were as follows: forward primer (5′ to 3′), CGA TTA CAG AGT TGG GAC CAC TTA; reverse primer (5′ to 3′), CGT TGG CGA TTT GGG TAA TG; probe FAM (6-carboxyfluorescein), (5′ to 3′): AGC AAC TTT ACC ATC CGG GCC AGC. The probe was labeled with FAM at the 5′ end and with Black Hole quencher 1 at the 3′ end. In the real-time PCR, the fluorescence of the probe was measured at each cycle at the annealing phase of the reaction. Real-time PCR amplification with the FAM-labeled probe was performed in 40 μl containing 1× qPCR Mastermix (Eurogentec), a 0.5 μM concentration of each primer, 0.25 μM probe, and 10 μl of DNA at different concentrations. The thermal cycling conditions comprised an initial step of 50°C for 2 min and 94°C for 10 min, followed by 45 cycles of 94°C for 15 s and 55°C for 1 min. Fluorescence data were collected using an ABI Prism 7000 Sequence Detection System (SDS; Applied Biosystems). The SDS software then generated each real-time PCR profile after multicomponent analysis by plotting the log of the change in fluorescence (delta Rn, where Rn is the normalized reporter signal) versus cycle numbers. The cycle threshold (C_T) was determined by the SDS software as the fractional cycle number. This indicates where the fluorescence crosses an arbitrary threshold intersecting the signal curves in their exponential phases. In each experiment, twofold serial dilutions of *G. intraradices* genomic DNA from the different isolates (ranging between 5,000 and 39.06 pg) were included to generate C_T values. Two independent experiments were performed, each with eight concentrations of the genomic DNA. In half of the experiments, the DNA of the *G. intraradices* isolates was obtained from two independent extractions, thus allowing the possibility of checking whether a bias occurred in the C_Ts because of DNA extraction procedures. The C_T values shown in Fig. 3 represent averages between the two independent experiments. All replicates offered very similar results and produced standard curves with regression coefficients (R^2) of >0.99. The relative copy number of the *Sod1* gene in each of the *G. intraradices* isolates could then be calculated easily by comparing the C_T values of each of the isolates for a given amount of genomic DNA. The slopes resulting from the analyses of C_T values relative to the log of the amount of genomic DNA were consistent and reproducible among isolates, and the efficiency of the quantitative PCR from genomic DNA was found to be more than 95%.

Nucleotide sequence accession numbers. AMF sequences obtained in this study have been deposited in GenBank under accession numbers EU232637 to EU232655.

RESULTS AND DISCUSSION

Genetic variability among AMF zinc-copper superoxide dismutases. To better understand the molecular evolution of the *Sod1* genes in AMF, we sequenced 360 bp of the locus (almost the entire open reading frame) from five different AMF species (in two genera), as well as the genes from 14 different *G.*

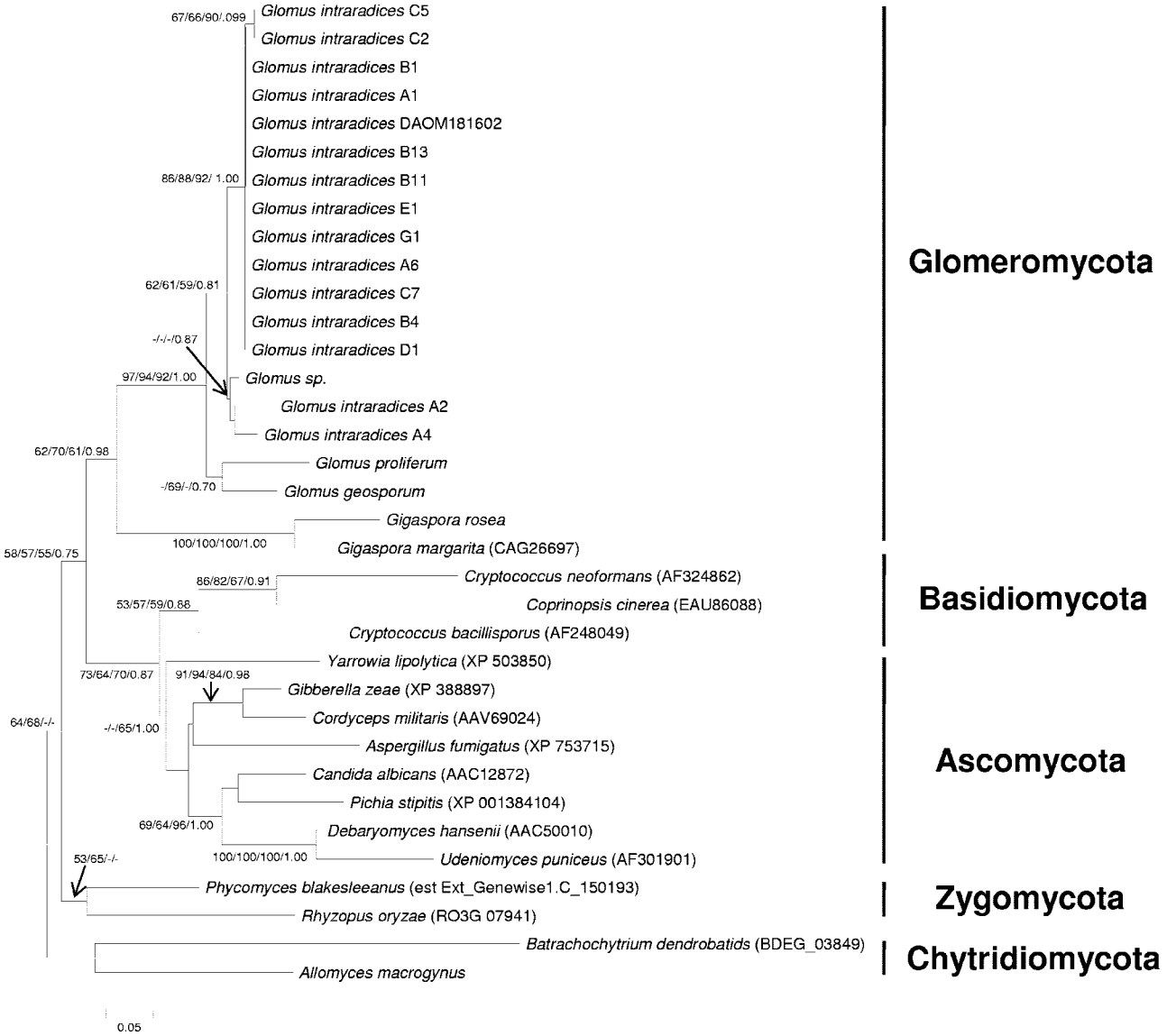

FIG. 1. Phylogenetic analysis of the amino acid sequences of the fungal Cu-Zn superoxide dismutase gene *Sod1*. *Sod1* genes from five AMF species and 14 isolates of *G. intraradices* were obtained in this study and compared with previously published sequences from the *Ascomycota*, *Basidiomycota*, and *Zygomycota* and a sequence from *Gigaspora margarita* deposited in the GenBank. Values at nodes correspond to bootstrap support (1,000 bootstrap replicates) from neighbor-joining (first), minimum evolution (second), and maximum likelihood (third) and to posterior probabilities (fourth) from Bayesian analyses. Only bootstrap supports above 50 or posterior probabilities over 0.75 are shown. Scale bar represents 0.05 substitutions per site. The sequences from *Rhizopus oryzae*, *Batrachochytrium dendrobatids*, *Phycomyces blakesleeanus*, and *Allomyces macrogynus* were obtained from http://www.broad.mit.edu/annotation/genome, http://genome.jgi-psf.org/Phybl1/Phybl1.home.html, and http://www.bch.umontreal.ca/pepdb/pepdb.html. Please note that *Sod1* sequences from isolate C2 and C5 differ only by synonymous substitutions and, therefore, share the same amino acid sequence.

intraradices isolates harvested from the same field. All the sequences obtained showed a high degree of homology to the deposited sequences encoding fungal Cu-Zn superoxide dismutases according to a tBlastx search procedure (2). A phylogeny reconstructed using amino acid sequences from a variety of fungal Cu-Zn superoxide dismutase (*Sod1*) sequences, including the AMF sequences analyzed in this study, revealed the monophyly of all major fungal phyla, including the *Glomeromycota* (Fig. 1). The relationships we have identified between the fungal phyla are consistent with the most recent phylogenetic studies of the fungal kingdom (23). Interestingly, all the phylogenetic models used resulted in a basal positioning of the chytrids and the zygomycetes within the fungal kingdom; however, their monophyletic origin was not supported by maximum-likelihood and Bayesian methodologies. Overall, statistical support for the major fungal clades was relatively low, a likely consequence of high amino acid sequence diversity within and across the major fungal phyla and the small size of the studied locus.

Amino acid comparisons among all the AMF species and isolates revealed a substantially low average sequence similarity of 73% among the analyzed species. This suggested that

TABLE 1. Pairwise distances among representatives of the fungal Sod1 amino acid sequences analyzed in this study

Organism	Pairwise distance relative to *Glomus intraradices* DAOM 181602[a]																									
Glomus intraradices isolates																										
A1	0.0000																									
A2	0.0770	0.0770																								
A4	0.0572	0.0572	0.0670																							
A6	0.0000	0.0000	0.0770	0.0572																						
B1	0.0000	0.0000	0.0770	0.0572	0.0000																					
B4	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000																				
B11	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000	0.0000																			
B13	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000	0.0000	0.0000																		
C2	0.0093	0.0093	0.0870	0.0670	0.0093	0.0093	0.0093	0.0093	0.0093																	
C5	0.0093	0.0093	0.0870	0.0670	0.0093	0.0093	0.0093	0.0093	0.0093	0.0000																
C7	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000	0.0000	0.0000	0.0000	0.0093	0.0093															
D1	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000	0.0000	0.0000	0.0000	0.0093	0.0093	0.0000														
E1	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000	0.0000	0.0000	0.0000	0.0093	0.0093	0.0000	0.0000													
G1	0.0000	0.0000	0.0770	0.0572	0.0000	0.0000	0.0000	0.0000	0.0000	0.0093	0.0093	0.0000	0.0000	0.0000												
Glomus sp. strain MUCL 43196	0.0187	0.0187	0.0572	0.0377	0.0187	0.0187	0.0187	0.0187	0.0187	0.0282	0.0282	0.0187	0.0187	0.0187	0.0187											
Glomus geosporum	0.1074	0.1074	0.1388	0.0972	0.1074	0.1074	0.1074	0.1074	0.1074	0.1178	0.1178	0.1074	0.1074	0.1074	0.1074	0.0972										
Glomus proliferum	0.1603	0.1603	0.1935	0.1603	0.1603	0.1603	0.1603	0.1603	0.1603	0.1713	0.1713	0.1603	0.1603	0.1603	0.1603	0.1713	0.1495									
Gigaspora rosea	0.4195	0.4195	0.4774	0.4480	0.4195	0.4195	0.4195	0.4195	0.4195	0.4336	0.4336	0.4195	0.4195	0.4195	0.4195	0.4480	0.4480	0.4195								
Gigaspora margarita	0.3514	0.3514	0.4055	0.3781	0.3514	0.3514	0.3514	0.3514	0.3514	0.3646	0.3646	0.3514	0.3514	0.3514	0.3514	0.3781	0.3917	0.3781	0.1283							
Batrachochytrium dendrobatids	0.7118	0.7118	0.6931	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.7118	0.6931	0.7503	0.8109	0.8535	0.8755						
Allomyces macrogynus	0.4626	0.4626	0.4774	0.4774	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.5232	0.6568	0.5878	0.6568					
Phycomyces blakesleeanus	0.3254	0.3254	0.3781	0.3383	0.3254	0.3254	0.3254	0.3254	0.3254	0.3383	0.3383	0.3254	0.3254	0.3254	0.3254	0.3254	0.3646	0.3781	0.4925	0.4480	0.7309	0.3917				
Rhyzopus oryzae	0.4480	0.4480	0.4774	0.4925	0.4480	0.4480	0.4480	0.4480	0.4480	0.4336	0.4336	0.4480	0.4480	0.4480	0.4480	0.4774	0.5077	0.4774	0.6046	0.5550	0.7309	0.5077	0.3383			
Coprinopsis cinerea	0.6748	0.6748	0.6391	0.6568	0.6748	0.6748	0.6748	0.6748	0.6748	0.6748	0.6748	0.6748	0.6748	0.6748	0.6748	0.6568	0.6568	0.6931	0.7503	0.6931	0.7503	0.6391	0.6391	0.6568		
Cryptococcus bacillisporus	0.4480	0.4480	0.4626	0.4195	0.4480	0.4480	0.4480	0.4480	0.4480	0.4626	0.4626	0.4480	0.4480	0.4480	0.4480	0.4195	0.4336	0.4925	0.4774	0.4336	0.8109	0.5550	0.4055	0.5077	0	
Cryptococcus neoformans	0.5713	0.5713	0.6046	0.5713	0.5713	0.5713	0.5713	0.5713	0.5713	0.5878	0.5878	0.5713	0.5713	0.5713	0.5713	0.5550	0.6046	0.6391	0.6391	0.6568	0.8755	0.7309	0.5232	0.6217	0	
Candida albicans	0.3646	0.3646	0.4336	0.3917	0.3646	0.3646	0.3646	0.3646	0.3646	0.3781	0.3781	0.3646	0.3646	0.3646	0.3646	0.3781	0.4774	0.4626	0.5878	0.5390	0.8320	0.5713	0.4195	0.5550	0	
Aspergillus fumigatus	0.4626	0.4626	0.4774	0.4626	0.4626	0.4626	0.4626	0.4626	0.4626	0.4774	0.4774	0.4626	0.4626	0.4626	0.4626	0.4626	0.5077	0.5232	0.6391	0.5878	0.7903	0.5713	0.4195	0.5713	0	
Debaryomyces hansenii	0.4055	0.4055	0.4055	0.4055	0.4055	0.4055	0.4055	0.4055	0.4055	0.4195	0.4195	0.4055	0.4055	0.4055	0.4055	0.3917	0.4626	0.5077	0.6046	0.5713	0.7503	0.6046	0.4774	0.5390	0	
Pichia stipitis	0.4336	0.4336	0.4925	0.4626	0.4336	0.4336	0.4336	0.4336	0.4336	0.4480	0.4480	0.4336	0.4336	0.4336	0.4336	0.4626	0.5390	0.5232	0.5232	0.4925	0.8979	0.6046	0.4925	0.4925	0	

[a] Distances were calculated using a Poisson correction (amino acid) included in the MEGA software, version 3.

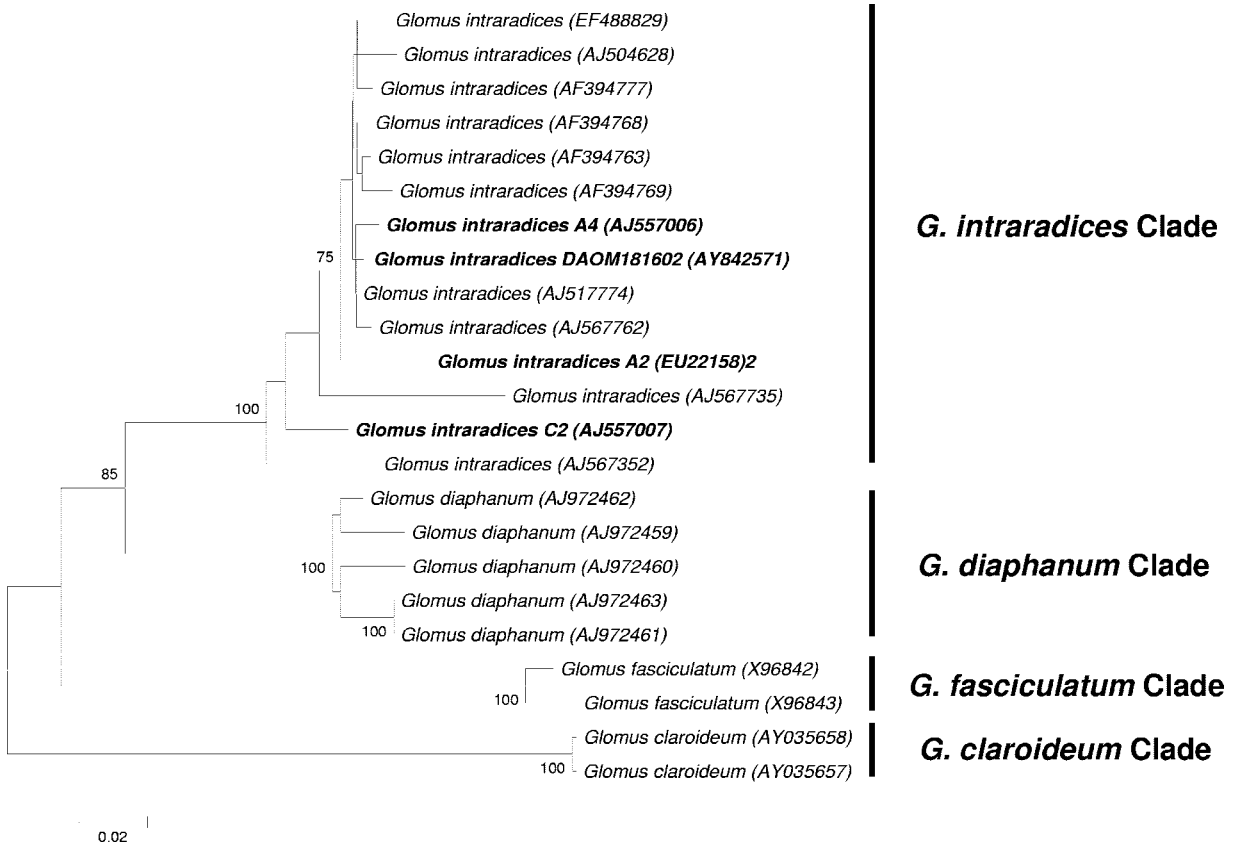

FIG. 2. Consensus of neighbor-joining (uncorrected *P* distance) analyses is shown for ITS sequences from *G. intraradices* isolates analyzed in this study and harboring the most divergent *Sod1* sequences, as well as from *Glomus* sp. (strain MUCL 43196), *G. fasciculatum*, and *Glomus claroideum* (defined as the outgroup). Numbers on branches indicate clade support in the percentage of 1,000 bootstrap replicates (only clade support that is >75% is shown), using the neighbor-joining analysis. Sequences are identified by their GenBank accession numbers. Sequences in bold were recovered from isolates used in this study. Scale bar represents 0.02 substitutions per site. Our analyses suggest that the isolate MUCL 43196 likely corresponds to an isolate from *G. intraradices*.

these sequences might have evolved either under neutrality or positive selective constraints for some time. High sequence variation was also observed among 14 *G. intraradices* isolates harvested from one field. A total of five different sequences of *Sod1* were obtained from 14 isolates of *G. intraradices*. Sequences from three of the isolates (A2, A4, and C2) accounted for most of the sequence variation, suggesting that the *Sod1* locus diverged significantly in these isolates, a feature that is unexpected for individuals from a single population (Table 1).

This surprisingly high molecular diversity warranted independent controls to ensure that it was not caused by an erroneous species assignment and to possibly rule out paralogous origin of the *Sod1* sequences from our population. These controls were performed as follows. All isolates of *G. intraradices* used in this study were identified through their ITS sequences, and their monophyly was confirmed by using the closest related species, *Glomus diaphanum* and *Glomus fasciculatum* (Fig. 2) (15). In parallel, the putative orthology of the *Sod1* sequences and the absence of intraindividual variation were determined using two approaches. First, we screened and sequenced 32 clones of the *Sod1* gene in every single isolate used in this study, using two independent sets of primers (see Materials and Methods); no sequence variation was found within isolates. Based on the sequencing results, we were very confident that the molecular diversity we identified was representative of variation among isolates and that only a single sequence variant was present in each isolate. The absence of intraindividual variation also suggested that the divergent *Sod1* sequences did not arise through gene duplication (i.e., absence of paralogy). However, as cloning techniques are prone to randomly missing certain sequences and do not provide information about *Sod1* copy numbers, we performed a real-time PCR experiment using an additional set of primers (see Materials and Methods) targeting a highly conserved region of the *Sod1* gene in three of the most divergent isolates used in this study (Fig. 3). A significant difference in standard curves (one C_T difference corresponding to a twofold increase in gene copy number) would have suggested a difference in copy number for the *Sod1* gene among the isolates (11, 13). The real-time PCR procedure showed very similar standard curves for all three isolates, suggesting that all *G. intraradices* isolates share the same copy number for the *Sod1* gene.

Potential occurrence of positive selection on AMF *Sod1* genes. Considering the amount of sequence variation we found among AMF species for the *Sod1* locus, we tested whether this locus might have evolved either under neutrality or under the

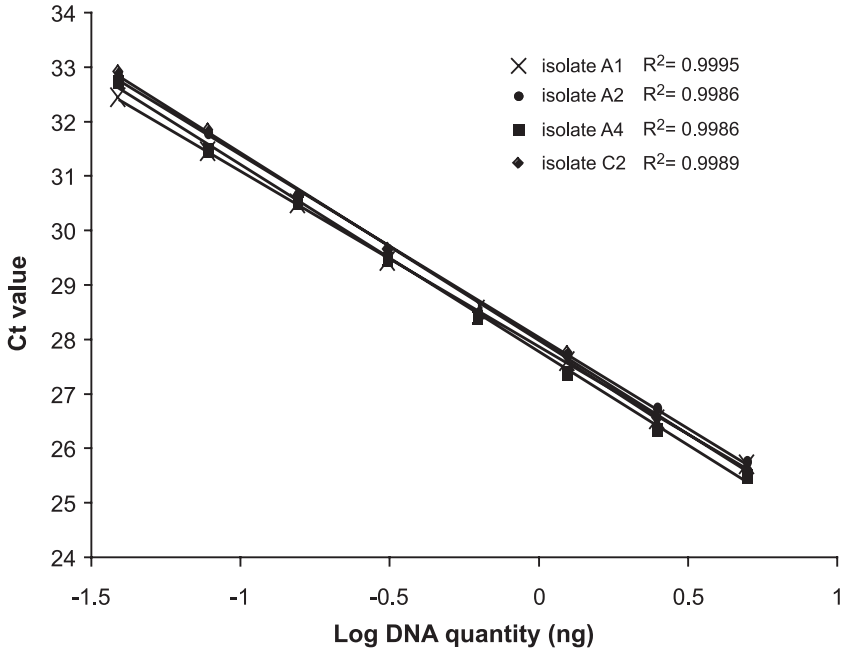

FIG. 3. Results of real-time quantitative PCR showing linear regressions of the C_T values and the log concentration of the *G. intraradices* isolate A1, A2, A4, and C2 genomic DNA.

regime of positive selection. To test this, we analyzed the *Sod1* sequences from the six different AMF species analyzed in this study using different models of codon evolution implemented in the PAML software package (30).

The results from the maximum-likelihood analysis of codon evolution showed that positive selection potentially acted at specific codons throughout the evolution of these AMF species. Indeed, all models that allowed for sites with a ω (*dN*/*dS*) greater than 1 (M1, M2, and M8), that is, the models of positive selection, fitted the *Sod1* data significantly better than the corresponding neutral models (M0, M3, and M7) (Table 2). More specifically, the ω estimates, averaged over all sites and all branches, ranged from 0.5 to 0.61 among selection models. Models M1a and M7 gave lower estimates as they do not account for sites under positive selection. An LRT comparing model M1a (nearly neutral) to M2a (positive) indicated that the selection models fitted better ($P = 0.009$) (Table 3). The LRT comparing the model M7 (β, neutral) with M8 (β, selection) also rejected the null model ($P = 0.004$). Most sites in the fragment examined have apparently evolved either under purifying selection (M2a, 69%; M8, 92%) or neutrality (M2a, 26%;

TABLE 2. Site-specific models for positive selection[a]

Model and comparison (type)	$-\ln L$[b]	ω[c]	Parameter(s)[d]
Within the *G. intraradices* population			
M0 (one ratio)	−562.07	0.898	None
M1a (nearly neutral)	−561.32	0.557	$\rho_0 = 0.443$ ($\rho_1 = 0.557$), $\omega_0 = 0.000$
M2a (positives election)	−558.00	1.938	$\rho_0 = 0.951$, $\rho_1 = 0.000$ ($\rho_2 = 0.049$), $\omega_0 = 0.476$, $\omega_2 = 30.241$
M3 (discrete)	−558.00	1.938	$\rho_0 = 0.000$, $\rho_1 = 0.951$ ($\rho_2 = 0.049$), $\omega_0 = 0.000$, $\omega_1 = 0.477$, $\omega_2 = 30.242$
M7 (β, neutral)	−561.35	0.500	$\rho = 0.005$, $q = 0.005$
M8 (β, positive selection)	−558.00	1.938	$\rho_0 = 0.951$ ($\rho_1 = 0.049$), $\rho = 90.348$, $q = 99.000$, $\omega = 30.249$
M8A (β, ω = 1)	−561.32	0.557	$\rho_0 = 0.443$ ($\rho_1 = 0.557$), $\rho = 0.005$, $q = 2.033$
Among species			
M0 (one ratio)	−1,165.38	0.201	None
M1a (nearly neutral)	−1,123.63	0.324	$\rho_0 = 0.697$ ($\rho_1 = 0.303$), $\omega_0 = 0.031$
M2a (positive selection)	−1,119.78	0.609	$\rho_0 = 0.680$, $\rho_1 = 0.275$ ($\rho_2 = 0.045$), $\omega_0 = 0.032$, $\omega_2 = 6.977$
M3 (Discrete)	−1,117.85	0.475	$\rho_0 = 0.538$, $\rho_1 = 0.371$ ($\rho_2 = 0.091$), $\omega_0 = 0.008$, $\omega_1 = 0.367$, $\omega_2 = 3.670$
M7 (β, neutral)	−1,123.49	0.271	$\rho = 0.144$, $q = 0.387$
M8 (β, positive selection)	−1,118.00	0.495	$\rho_0 = 0.928$ ($\rho_1 = 0.072$), $\rho = 0.197$, $q = 0.786$, $\omega = 4.291$
M8A (β, ω= 1)	−1,122.20	0.281	$\rho_0 = 0.766$ ($\rho_1 = 0.234$), $\rho = 0.308$, $q = 4.359$

[a] Analysis was preformed using Codeml and site-specific models to test for positive selection in the AMF *Sod1* genes.
[b] $-\ln L$, log likelihood.
[c] For ω, average estimates exceed 1 within the population of *G. intraradices* under the selection models, indicating positive selection. Among species, the estimated average ω ranges between 0.5 and 0.6 but does not exceed 1.
[d] ρ_0, ρ_1, and ρ_2 are the proportions of sites drawn from three discrete classes of ω. ρ and q are parameters of the β distribution.

TABLE 3. LRTs for positive selection[a]

Models compared[b]	$2\Delta\ln L$[c]	df[d]	*P* value	Positively selected sites[e]
Among AMF				
M0 vs M3	95.06	4	0.001	6 G*, 15 S, 55 F*, 58 T *, 63 K, 79 P**, 88 D**, 91 K
M1a vs M2a	11.56	3	0.009	6G, 55F, 58T, 79P**, 88D**
M7 vs M8	10.98	2	0.004	6G, 15S, 55F, 58T, 63K, 79P*, 88D*
M8 vs. M8A	8.40	1	0.004	
Within the *G. intraradices* population				
M0a vs M3	8.14	4	<0.09	6G*,18G*,58F
M1a vs M2a	6.64	2	0.036	6G*,18G*,58F
M7 vs M8	6.70	2	0.035	3 I, 6 G*, 14 E, 18 G*, 30 A, 38 I, 46 N, 49 T, 58 F, 61 T, 108 I
M8 vs M8A	6.64	1	0.01	

[a] LRTs of different models of ω variation among codons.
[b] Selection models were preferred in all comparisons at the species level. In within-population comparisons, selection models M2a and M8 were preferential.
[c] Twice the difference of the log likelihoods between two models.
[d] df, degrees of freedom.
[e] Sites possibly affected by events of positive selection, identified with a posterior probability of over 50%. Sites at a high level of significance are shown in bold. *, >95%; **, >99%.

M8, 8%) (Table 2). The discrete model associated 10% of the sites under positive selection. Similarly, M2a and M8 indicated positive selection at a small subset of sites (4.7% and 7.7%) located within the fragment analyzed (Table 2). In more detailed analyses, a Bayesian approach identified seven sites affected by positive selection within the AMF lineage under model M8 and five under model M2a (Table 3). Overall, five amino acid sites were identified as potentially affected by positive selection when all sites that allowed for the presence of positive selection were used in the analysis (5G, 55F, 58T, 79P, and 88D). Sites 79P and 93D were predicted to be subject to positive selection at the 95% confidence level using all models.

In the Cu-Zn superoxide dismutase protein, the binding of copper and zinc ions requires the presence of histidine amino acids at specific conserved sites, and, as expected, these motifs did not show significant signs of positive selection. Apparently, the motifs potentially subject to positive selection are spread throughout the coding sequence, and none have been identified within the conserved internal catalytic cleft. Although the posterior probability for these mentioned sites to be under positive selective constraints was relatively high, we could not match any of these with a known function for the Sod1 protein. The significance of our tests might denote a functional importance for this rapid variation at the amino acid level, especially for the amino acids 55F and 58T, as these positions are located, respectively, 1 and 4 amino acids away from the to the catalytic cleft and have a 93% posterior probability (under model M3) of being shaped by events of positive selection.

Molecular diversity within a population of *G. intraradices*: recombination or diversifying selection? Likelihood methods for detecting positive selection can be inaccurate if significant recombination has occurred among the sequences (4). The *G. intraradices* isolates analyzed in this study have been the subject of a multilocus genotyping study based on newly developed simple sequence repeat and nuclear gene intron markers (15). No events of recombination have been found between the isolates A2, A4, C2, and C5 using sequence data that was generated from these markers (14a). These isolates are the same ones that showed the highest molecular divergence in our study. To further test whether recombination played a significant role in shaping the diversity of the *Sod1* sequences identified in our population, we performed an analysis to detect possible recombination events within the *G. intraradices* population (28). This analysis failed to identify any recombination events (data not shown), suggesting that other evolutionary forces played an important role in shaping the molecular diversity among the sequences.

To identify other possible evolutionary forces shaping the diversity at the *G. intraradices Sod1* locus, we applied the same likelihood procedures reported in the previous section to the 14 *G. intraradices* isolates (which were initiated as single-spore cultures) harvested from one field. Consistent with what we identified among AMF species, we found that selection models applied to our population explained the data significantly better than neutral models ($P < 0.05$) (Tables 2 and 3). Although an LRT comparison between the discrete model M1a with M3 was only marginally significant ($P = 0.09$), the more stringent comparisons using M2, M8, and M8A (supplementary category ω fixed at 1) models were significant ($P = 0.036$) (Table 3). Importantly, models accounting for positively selected sites produced very similar parameter estimates and associated the same proportion (4.9%) of amino acids to the supplementary category of positively selected sites. Testing alternative tree topologies to take into account the phylogenetic incertitude did not alter the outcome of our analysis. Overall, sites at positions 6 and 18 (6G and 18G) were predicted to be under positive selection by all models at a high level of significance. Importantly, amino acids 6G and 58F were commonly identified as being under positive selection at both the population and species levels (Table 3).

Selection on superoxide dismutases in fungal mutualists and pathogens. AMF have sometimes been shown to harbor different alleles of one locus among the different genomes present in one isolate, but such intraindividual variability was not identified at the *Sod1* locus. Although it could be important for the AMF to have copies with various functions for the *Sod1* gene, the absence of such allelic variation is not surprising and has also been reported for other adaptively important genes, such as the plasma membrane-type II ATPases (13).

Rapid evolution of *Sod1* has already been reported in pathogenic fungi (10). For example, the Sod1 enzymes from different varieties of the human pathogen *Cryptococcus neoformans* and *Aspergillus* sp. show substantial biochemical and physical differences (14, 21). In *Aspergillus* species, amino acid variation in the N-terminal domain of Cu-Zn dismutases has been shown to increase thermostability. This possibly affects their virulence as increased thermostability and activity have been reported in the most virulent strains with the potential to develop aspergillosis (21). While the role of the *Sod1* genes in defense systems in plants, immune system in animals, and the pathogenesis of some fungi has been well characterized, their molecular variability has rarely been discussed in the context of adaptive evolution.

Our results are consistent with a rapid evolution of the *Sod1* gene, which has been shown to be specifically upregulated during mycorrhizal symbiosis and upon exposure to root exudates. However, its specific role during the establishment of the mycorrhizal symbiosis is still unclear. For instance, it is not known whether its expression is directly related to the plant-fungus dialogue or whether it is in response to a change in environmental conditions dictated by the plant defense system (i.e., expression required to counteract the ROS released by the host). Rapid evolution of genes in a plant-fungal interaction might also be an indication of arms races, but such arms races are usually seen in host-pathogen interactions (6) and not expected to evolve in a mutualisitic symbiosis. Thus, an arms race scenario, although possible in the case of the *Sod1* gene, seems unlikely considering the generalist nature of the AMF. From complementation studies in yeast, it has been demonstrated that the AMF *Sod1* encodes an active polypeptide that effectively scavenges ROS (27). Moreover, this almost certainly occurs in AMF-colonized roots because suppression of *Sod1* activity reduces build-up of peroxidase in the roots (27). It seems evident, therefore, that one important role for AMF *Sod1* is the scavenging of ROS. This adaptive scenario implies that AMF are facing environmental situations similar to pathogens and, therefore, need to counteract the same plant defense barriers upon establishment of the mycorrhizal symbiosis. In this case, positive selection on the copper-zinc superoxide dismutase protein is better explained as a direct consequence of adaptation directed toward scavenging ROS produced by the host.

In parallel, Sod1 proteins have also been suggested as playing an important role in the fungal-plant molecular dialogue (18, 27) although conclusive evidence has not been provided for this. In this communication function of the Sod1 proteins, the rapid evolution of their amino acids could be related to the fact that tightly coevolved mutualisms are constantly exposed to nonmutualistic cheaters or parasites. As *Sod1* plays a key role for pathogens to avoid plant immune systems (production of ROS), the AMF might need to slightly change their biochemical properties accordingly to avoid their proteins' being recognized as of pathogen origin. An alternate explanation could be related to the poor specificity of AMF species toward their host. High rates of protein evolution facilitating host interactions could render AMF species more broad in their host range or allow them to rapidly colonize hosts in the presence of competitors.

Diversifying selection in an AMF population? The molecular evidence we have gathered in this study points toward an orthologous origin of the *Sod1* sequences we identified within the *G. intraradices* population. Although it is true that we cannot conclusively reject a paralogous origin for a few of these, the absence of AMF isolates and species harboring more than one *Sod1* sequence and the impossibility to absolutely reject the monophyly of our *G. intraradices* sequences are both additional lines of evidence that make this latter evolutionary scenario far less parsimonious than the one suggesting an accumulation of mutations among orthologous sequences.

Intraspecific sequence polymorphism for a given gene has most often evolved as a consequence of random mutations accumulated by genetic drift. In this case, amino acid replacements are considered as neutral, with no effect or weak effects on protein function. An important departure from neutrality is observed when recombination produces molecular diversity or when positive selection fixes advantageous mutations. In the latter case, selective sweeps of favorable mutations lead to an excess of nonsynonymous over synonymous substitutions between species.

In this study, the molecular diversity identified among spores of *G. intraradices* is unlikely a consequence of recombination as other studies and our own analyses failed to identify recombination events. However, even if recombination did account for part of the intraspecific variation, this would not have significant consequences on our codon-based likelihood analyses for two independent reasons. First, rare events of recombination have been demonstrated as not interfering with the detection of positive selection (4). Second, LRT comparisons using the more realistic model M7 against M8 were shown to be robust to false positives due to recombination. This is particularly the case if the model M7 allows the positive selection pressure to vary between 0 and 1 (not accounting for positive selection) and if the alternative model M8 allows an additional discrete class with a dN/dS that could be estimated to be >1 (and thus account for positive selection) (4). In this study, the LRT comparison between these two models was the most significant one. This suggests that even under rare recombination in the population, diversifying selection is certainly a likely explanation for the diversity observed at the *Sod1* locus.

Within a population, directional selection is thought to shift allele frequency in favor of a newly derived mutation through a fitness advantage to certain individuals over others. Signals of positive selection are, therefore, rarely detectable at the population level. However, a changing environment, with variable selective pressures over time (i.e., interactions with multiple symbiotic partners), could lead to balanced polymorphism that is maintained within a single population. This feature is known for genes involved in plant defense systems, mating systems in fungi and plants, or immune response in animals, where a high level of polymorphism is favored and maintained through selection (31). Thus, rapid protein evolution and balancing selection at the locus *Sod1* may offer evolutionary advantages for some isolates compared to others in a heterogeneous environment, resulting in the maintenance of high protein divergence within the population.

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