Diversification of NRT2 and the Origin of Its Fungal Homolog

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We investigated the origin and diversification of the high-affinity nitrate transporter NRT2 in fungi and other eukaryotes using Bayesian and maximum parsimony methods. To assess the higher-level relationships and origins of NRT2 in eukaryotes, we analyzed 200 amino acid sequences from the Nitrate/Nitrite Porter (NNP) Family (to which NRT2 belongs), including 55 fungal, 41 viridiplantae (green plants), 11 heterokonts (stramenopiles), and 87 bacterial sequences. To assess evolution of NRT2 within fungi and other eukaryotes, we analyzed 116 amino acid sequences of NRT2 from 58 fungi, 40 viridiplantae (green plants), 1 rhodophyte, and 5 heterokonts, rooted with 12 bacterial sequences. Our results support a single origin of eukaryotic NRT2 from 1 of several clades of mostly proteobacterial NNP transporters. The phylogeny of bacterial NNP transporters does not directly correspond with bacterial taxonomy, apparently due to ancient duplications and/or horizontal gene transfer events. The distribution of NRT2 in the eukaryotes is patchy, but the NRT2 phylogeny nonetheless supports the monophyly of major groups such as viridiplantae, flowering plants, monocots, and eudicots, as well as fungi, ascomycetes, basidiomycetes, and agaric mushrooms. At least 1 secondary origin of eukaryotic NRT2 via horizontal transfer to the fungi is suggested, possibly from a heterokont donor. Our analyses also suggest that there has been a horizontal transfer of *nrt2* from a basidiomycete fungus to an ascomycete fungus and reveal a duplication of *nrt2* in the ectomycorrhizal mushroom genus, *Hebeloma*.

Introduction

Nitrogen is a limiting nutrient in most forest soils (Fernandez, Simmons, and Briggs 2000) that can be obtained in the form of nitrate by organisms equipped with 1 of the nitrate assimilation pathways. One such pathway involves nitrate uptake by NRT2, a high affinity nitrate transporter with homologs previously identified in bacteria, viridiplantae, heterokonts (including diatoms and oomycetes, but not yet kelp), and fungi. NRT2 belongs to the Nitrate/Nitrite Porter family (NNP) of the Major Facilitator Superfamily (MFS), characterized by 12 transmembrane helical motifs (fig. 1*A*), 1 broader MFS motif between the second and third transmembrane helices (G-x-x-x-D-x-x-G-x-R, Forde 2000) and an NNP signature motif located in the fifth transmembrane helix (G-W/L-G-N-M/A-G, Jargeat et al. 2003). Fungal sequences also contain a large intracellular loop of unknown function between the sixth and seventh helix (Forde 2000; Jargeat et al. 2003).

Key words: nitrate transporter, *Hebeloma*, horizontal gene transfer, gene duplication, ectomycorrhizae.

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Within fungi, *nrt*2 homologs have been discovered in diverse lineages of Ascomycota (*Hansenula, Aspergillus, Gibberella, Neurospora,* and *Tuber*) and Basidiomycota (*Hebeloma, Ustilago,* and *Phanerochaete*) (Perez et al. 1997; Unkles et al. 2001; Jargeat et al. 2003; Gao-Rubinelli and Marzluf 2004; Montanini et al. 2006). *Nrt*2 has also been found in the green algae (in viridiplantae) *Chlamydomonas reinhardtii* and *Chlorella sorokiniana,* bryophytes, 14 genera of angiosperms, including eudicots (e.g., *Aradbidopsis thaliana, Glycine max*) and monocots (e.g., *Hordeum vulgare, Phragmites australis*), 2 genera of diatoms, and several bacteria (Amarasingh et al. 1998; Pao, Paulsen, and Saier 1998; Quesada, Hidalgo, and Fernandez 1998; Fraisier et al. 2000; Vidmar et al. 2000; Faure-Rabasse et al. 2002; Hildebrandt, Schmelzer, and Bothe 2002; Orsel, Krapp, and Daniel-Vedele 2002; Collier et al. 2003; Koltermann et al. 2003; Araki et al. 2005; Prosser et al. 2006). Hundreds of prokaryotic sequences that are similar to *nrt2* but are of unknown function are also available on GenBank. Phylogenetic analyses of homologous *nrt2* genes have been limited, especially within the fungi where diversity is not well understood (Orsel, Krapp, and Daniel-Vedele 2002; Montanini et al. 2006). The NNP family phylogeny has been explored more deeply in plants (Forde 2000) and also more broadly to include representatives of the known diversity (Pao, Paulsen, and Saier 1998). While Pao, Paulsen, and Saier (1998) discussed distinct prokaryotic and eukaryotic clades, they did not critically address the specific origin of eukaryotic *nrt2* sequences. Duplications have apparently led to novel functions in the NNP family (Pao, Paulsen, and Saier 1998) and in plant NRT2 (Orsel, Krapp, and Daniel-Vedele 2002; Little et al. 2005). Two NRT2 isozymes in the mitosporic fungus *Aspergillus nidulans* were found to display different affinities for nitrate binding and to thereby facilitate ecological plasticity (Unkles et al. 2001).

Interest in fungal NRT2 has increased with recent discoveries of these transporters in 2 ectomycorrhizal fungi, which form symbiotic associations, generally with roots of vascular plants, and appear to benefit the nitrogen nutrition of the host (Chalot et al. 2002). The transporters were found in the basidiomycete *Hebeloma cylindrosporum* (Jargeat et al. 2003), a model system for nutritional processes in ectomycorrhizal associations (Marmeisse et al. 2004), and the ascomycete *Tuber borchii* (Montanini et al. 2006), which forms economically important truffles.

Our investigations into NRT2 evolution in the fungi have focused on the euagaric (mushroom forming) genus, *Hebeloma*. Certain members of this ectomycorrhizal genus are adapted to high-nitrogen niches, such as mole latrines, decayed animal carcasses (Sagara 1995; Suzuki et al. 2003), and anthropogenic ammonium gradients (Lilleskov et al. 2002), from which nitrogen can be delivered to the host plant. A clear understanding of *Hebeloma* phylogeny for evolutionary analyses of these ecological characters is not yet available (Aanen et al. 2000; Boyle et al. 2006). Future analyses of *nrt2* nucleotide sequences may improve

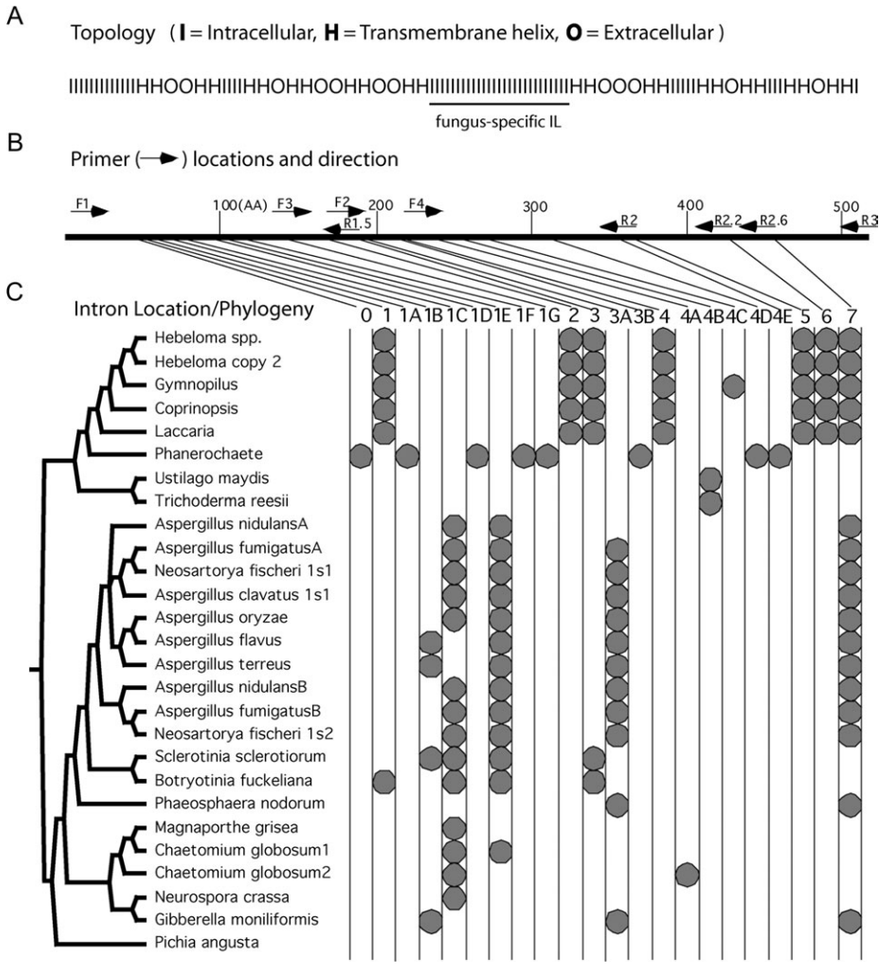

FIG. 1.—Structural motifs (*A*), primer positions (*B*), and intron position/phylogeny (*C*) relative to the 519-amino acid *H. cylindrosporum* NRT2 (Jargeat et al. 2003). The fungus-specific intracellular loop is underlined. Sequences included in this figure cover at least the region including introns 0–7. Phylogenetic framework is based on maximum parsimony analyses of amino acid alignment. Primer direction is indicated by an arrow and intron presence indicated by a shaded circle. Lines connect intron number to approximate location in gene translation. Location of additional structural features can be found in referenced literature (Jargeat et al. 2003).

resolution in *Hebeloma* phylogeny and address the question of a selective influence of nitrate in these transitions. Here, we present phylogenetic analyses of new NRT2 amino acid sequences from *Hebeloma* and other fungi, as well as published sequences from diverse eukaryotes and prokaryotes, which raise provocative questions about the evolution of the nitrate acquisition apparatus in fungi: Is fungal *nrt2* secondarily derived from other eukaryotic sequences? Has high affinity nitrate transport been acquired horizontally within the fungi?

Materials and Methods

DNA Extraction

We sampled cultures and fruiting bodies of 10 species of Basidiomycota from the genera *Hebeloma, Gymnopilus,* and *Laccaria,* which were identified with reference to Smith (1984) and Hansen, Knudsen, and Dissing (1992). DNA was extracted from fungal cultures grown on MEA at 25°C and from fruiting bodies that were collected in the field and dried, using standard mini-prep or maxi-prep procedures (http://www.clarku.edu/faculty/dhibbett/HibbettLab.protocols.htm). The DNA extracted by the maxi-prep method was further purified with a GENECLEAN kit (Bio101 Systems Products, Qbiogene, Vista, CA).

Degenerate PCR and Sequencing

We designed degenerate primers (fig. 1*B*; Primers 5′ to 3′:F1 ggygcrccraarttyaartgg, F2 ggnggngcnacnttygcnathatg, F3 acnttygtnccntgycargcntgg, F4 aycayccngcnggnaartgg, R1.5 ytgraanarnrwngtcatdatngcg, HR2 gaggaccccaaaataaccgc, R2.2 agctgcgcccatgattagacc, R2.6 ngcraarttngcnccrttncc, R3 nswdatracncccatdatcc) based on the *Hebeloma cylindrosporum* NRT2 sequence (Jargeat et al. 2003). We performed PCR on a MJR Research PTC200 thermocycler. The program used a 2-min initial denaturation step at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at a temperature from 55°C to 45°C (depending on primers and success), 90 s at 72°C, and a final

elongation step at 72°C for 10 min. PCR products were screened by agarose gel electrophoresis and cleaned with Pellet Paint NF coprecipitant (Novagen, San Diego, CA). Some products required gel purification to separate multiple bands, and we purified those products using a GENECLEAN kit (Bio101 Systems Products, Qbiogene, Vista, CA). We cloned all products into the TA or TOPO TA cloning kit (Invitrogen, Carlsbad, CA). For each cloning reaction, we screened at least 10 positive clones by PCR product size (using M13 primers) on an agarose gel, and sequenced 3–5 positive clones with full, bidirectional coverage on either an ABI 377 or 3700 automated DNA sequencer using ABI Prism Terminator BigDye ver1.1 or 3.1 (Applied Biosystems, Foster City, CA). Sequences were edited, and contigs were assembled using Sequencher version 4.1.2 (Gene Codes Corporation, Ann Arbor, MI, 1991-2000).

Database Searches for *nrt2* Homologs

We used the tBlastn program (Altschul et al. 1997) with *Aspergillus nidulans* and *Hebeloma cylindrosporum* translated *nrt2* sequences as queries against all public fungal genome projects and trace archives (as of June 2006), selecting sequences with greater than 50% similarity to the query at the amino acid level. We obtained 19 unique, putative *nrt2* homologs from fungal genome projects of 15 species from 11 genera, including *Laccaria bicolor* (http://mycor.nancy.inra.fr/ectomycorrhizadb/), *Coprinopsis cinerea* (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html), *Phanerochaete chrysosporium* (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html), *Aspergillus terreus* (http://www.broad.mit.edu/annotation/genome/aspergillus_terreus/Home.html), *Aspergillus oryzae* (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao), *Aspergillus flavus* (http://www.aspergillusflavus.org/genomics/), *Neosartorya fischeri* (http://www.tigr.org/), *Botryotinia fuckeliana* (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html), *Sclerotinia sclerotiorum* (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html), *Phaeosphaera nodorum* (http://www.broad.mit.edu/annotation/genome/stagonospora_nodorum/Home.html), *Gibberella zeae* (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html), *Chaetomium globosum* (http://www.broad.mit.edu/annotation/genome/chaetomium_globosum/Home.html), *Magnaporthe grisea* (http://www.broad.mit.edu/annotation/fungi/magnaporthe/), and *Trichoderma reesei* (http://gsphere.lanl.gov/trire1/trire1.home.html). An additional homolog was obtained from the rust fungus, *Leucosporidium scottii* EST database (https://fungalgenomics.concordia.ca/fungi/Lsco.php). We searched *Glomus intraradices* (http://darwin.nmsu.edu/~fungi/), *Rhizopus oryzae* (Zygomycota, http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html), and *Batrachochytrium dendrobatis* (Chytridiomycota, http://www.broad.mit.edu/annotation/genome/batrachochytrium_dendrobatidis) data in GenBank and elsewhere. Additionally, we obtained sequences from *Galdieria sulphuraria* (http://genomics.msu.edu/galdieria), *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp/), *Phytophthora ramorum* (http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html), and *Phytophthora sojae* (http://genome.jgi-psf.org/Physo1_1/Physo1_1.home.html) genome projects. We searched the Taxonomically Broad EST Database (TbestDB, http://tbestdb.bcm.umontreal.ca/). We also searched for hypothetical proteins from environmental sequences in the Sargasso Sea Marine Microbial Community genome project, and we searched GenBank for sequences annotated as eukaryotic and prokaryotic nitrate/nitrite transporter sequences (supplementary table 1) from the MFS. Sequences from these latter sources were included if they shared >40% (in an NNP family alignment, see below) or >50% (in a Eukaryotic NRT2 alignment) amino acid sequence similarity and possessed (when sequences were complete) 12 transmembrane helices (inferred by HMMTOP 2.0, Tusnády and Simon, 2001, http://www.enzim.hu/hmmtop/) and NNP and MFS signature sequences. Sequences with lower similarity to the query were initially considered when found; however we determined by reciprocal Blast that these generally fell into other subfamilies within the MFS, lacked NNP family signature sequences, and were not alignable with NNP family sequences. The size of most retained sequences ranged from 60% to 100% of the estimated complete protein sequence.

Alignment

We inferred spliceosomal intron (fig. 1*C*) boundaries with reference to existing amino acid sequences from Basidiomycota (Jargeat et al. 2003) and Ascomycota (Unkles et al. 1991) and translated the exons with the EXPASY Translate Tool (http://www.Expasy.org). A set of sequences representative of plant and fungal diversity was aligned with Clustal X (Thompson, Plewniak, and Poch 1999) and adjusted manually in MacClade v. 4.07 (Maddison and Maddison 2001). New sequences were added manually to the existing alignment. Prokaryotic sequences were analyzed for transmembrane helix topology (Tusnády and Simon 2001) to aid alignment with eukaryotic sequences, and conserved NNP and MFS signature motifs in the fifth and eleventh transmembrane domains (Forde 2000) were used as anchor positions for alignment of diverse prokaryotic clades. Ambiguously aligned positions were excluded from phylogenetic analyses.

Phylogenetic Analyses

We constructed 2 separate NRT2 alignments for phylogenetic analyses at different taxonomic scales, including (1) prokaryotes and eukaryotes (the NNP family alignment) and (2) eukaryotes only, rooted with closely related prokaryotes inferred from the larger analysis (Eukaryotic NRT2 alignment).

NNP Family Alignment

The NNP family alignment contained 200 amino acid sequences, including 55 fungal, 41 viridiplantae, 11 heterokont, and 87 bacterial sequences. We conducted a Bayesian analysis in MrBayes 3.1 (Huelsenbeck and

Table 1
Sequences Generated As Part of This Study

Species[a]	Accession #	Primers	#AA	Taxonomy	Source[b]
Gymnopilus junonius C4	EF520283	Nrt2f1/nrt2r3	468	B Agaricales; Cortinariaceae	JCS102604A
Hebeloma cylindrosporum C1	EF520276	Nrt2f1/nrt2r3	484	B Agaricales; Cortinariaceae	CBS558.96
Hebeloma cylindrosporum C2	EEF520278	Nrt2f1/Hcnrt2r2	347	B Agaricales; Cortinariaceae	CBS557.96
Hebeloma cylindrosporum C3	EF520277	Nrt2f1/nrt2r3	484	B Agaricales; Cortinariaceae	CBS558.96
Hebeloma edurum C1	EF520259	Nrt2f1/Hcnrt2r2	348	B Agaricales; Cortinariaceae	CBS291.50
Hebeloma helodes (Copy1)C2	EF520268	Nrt2f1/Hcnrt2r2	366	B Agaricales; Cortinariaceae	PBM 2687
Hebeloma helodes (Copy1)C7	EF520267	Nrt2f1/Hcnrt2r2	366	B Agaricales; Cortinariaceae	PBM 2687
Hebeloma helodes (Copy2) C1	EF520269	Nrt2f1/nrt2r3	480	B Agaricales; Cortinariaceae	PBM 2687
Hebeloma helodes (Copy2) C4	EF520270	Nrt2f1/nrt2r3	480	B Agaricales; Cortinariaceae	PBM 2687
Hebeloma helodes C1	EF520265	Nrt2f2/nrt2r3	309	B Agaricales; Cortinariaceae	JCS102604C
Hebeloma helodes C2	EF520266	Nrt2f2/nrt2r3	309	B Agaricales; Cortinariaceae	JCS102604C
Hebeloma radicosum C1	EF520275	Nrt2f1/Hcnrt2r2	344	B Agaricales; Cortinariaceae	CBS183.47
Hebeloma sinuosum C3	EF520260	Nrt2f1/Hcnrt2r2	361	B Agaricales; Cortinariaceae	CBS184.47
Hebeloma sp. C2	EF520261	Nrt2f3/nrt2r3	227	B Agaricales; Cortinariaceae	PBM2693
Hebeloma sp. C3	EF520262	Nrt2f3/nrt2r3	227	B Agaricales; Cortinariaceae	PBM2693
Hebeloma sp. C4	EF520264	Nrt2f1/Hcnrt2r2	367	B Agaricales; Cortinariaceae	JCS91904A
Hebeloma sp.C2	EF520263	Nrt2f1/Hcnrt2r2	347	B Agaricales; Cortinariaceae	JCS91904A
Hebeloma truncatum C1	EF520272	Nrt2f2/nrt3r3	308	B Agaricales; Cortinariaceae	CBS295.50
Hebeloma truncatum C2	EF520273	Nrt2f2/nrt2r3	308	B Agaricales; Cortinariaceae	CBS295.50
Hebeloma truncatum C3	EF520274	Nrt2f2/nrt2r3	308	B Agaricales; Cortinariaceae	CBS295.50
Hebeloma velutipesc13	EF520271	Nrt2f1/Hcnrt2r2	346	B Agaricales; Cortinariaceae	CBS163.46
Laccaria sp. C1	EF520281	Nrt2f2/nrt2r2.6	244	B Agaricales;Tricholomataceae	SK05034
Laccaria sp. C4	EF520282	Nrt2f2/nrt2r2.6	244	B Agaricales;Tricholomataceae	SK05034
Laccaria sp. C6	EF520279	Nrt2f2/nrt2r2.6	245	B Agaricales;Tricholomataceae	SK05030
Laccaria sp. C7	EF520280	Nrt2f2/nrt2r2.6	194	B Agaricales;Tricholomataceae	SK05030

[a] C1, C2, etc. denote clones corresponding to alternate alleles; copy1 and copy2 denote paralogous sequences in *Hebeloma helodes*. Alleles were designated by less than 10, mainly silent nucleotide differences between clones from 1 collection/culture. Paralogs were designated by significant differences in the length and inferred structural motifs of the translated sequences of clones. Paralogs were suspected when 4 unique sequences were found in a diploid collection/culture, or 2 unique sequences were found in a haploid genome project.

[b] CBS numbers represent cultures obtained from the Central Bureau voor Schimmelcultures. JCS, PBM, and SK numbers represent fruit bodies collected in the field.

Ronquist 2001) using mixed protein models for 1 million generations sampling every 100 generations. Likelihood tree scores of 2 independent runs were plotted to estimate the point of convergence to a stable likelihood. Trees from both runs were combined, and Bayesian posterior probabilities were calculated by computing a 50% majority rule consensus of 10,000 trees remaining after 5,002 trees were removed as the burnin. We conducted an equally weighted maximum parsimony bootstrap analysis in Paup* 4.0 (Swofford 2002) using a heuristic search, with TBR branch swapping and 1,000 stepwise addition replicates, saving 10 trees per replicate. Trees were rooted with a divergent clade of bacterial nitrate/nitrite transporter/extruder sequences from the Proteobacteria, Actinobacteria, and Deionococcus-Thermus groups. Clades that received greater than 0.95 Bayesian posterior probabilities (BPP) or 50% bootstrap support (MPB) were considered to have significant support.

Eukaryotic NRT2 Alignment

The eukaryote alignment contained 116 amino acid sequences including 58 from fungi, 40 from green plants and green algae, 1 from rhodophytes, 5 from heterokonts, and 12 bacterial sequences that were included for rooting purposes. We conducted a maximum parsimony analysis with 500 random addition sequence replicates, saving 10 trees per replicate, swapping branches via TBR on best trees. A Bayesian analysis and a maximum parsimony bootstrap analysis were conducted as described above. We also conducted parsimony analyses under constraints, which forced heterokont sequences to be monophyletic or forced heterokonts to form a clade with green plants (no other topological features were specified). Differences in parsimony scores for the resulting topologies were evaluated with the Kishino-Hasegawa test.

Results

NRT2 Sequences

We obtained 27 unique partial NRT2 sequences, ranging between 194 and 484 amino acids in length, from *Gymnopilus, Hebeloma,* and *Laccaria,* including 2 divergent sequences obtained from *Hebeloma helodes* (tables 1 and 2). Sequences obtained from genome projects and whole genome shotgun sequences were generally complete with a length of approximately 500 amino acids (table 2). We obtained multiple sequences for individual strains of Ascomycota from genome projects. We recovered *nrt2* homologs from all complete filamentous ascomycete and basidiomycete genomes listed in table 2, but not from *Cryptococcus* (Basidiomycota), *Rhizopus* (Zygomycota), *Glomus* (Glomeromycota), or most Saccharomycotina (Ascomycota) genomes/EST databases, with *Pichia angusta* as the exception. The amino acid sequence with greatest similarity to the query retrieved from *Rhizopus oryzae* was 45% similar to the second half of the query, and 50% similar to a monocarboxylate transporter from *Aspergillus oryzae* (GenBank accession XM_715677). The amino acid sequence with greatest similarity to the query from

Table 2
Sequences Obtained from Genome/EST Project Databases

Database[a]/species	#AA	Taxonomy[b]	Scaffold/contig/WGS[c]	Position
Aspergillus clavatus GB	507	A Eurotiomycetes; Trichocomaceae	wgs AAKD02000001	1340031–1338251
Aspergillus flavus seq 1NRRL3357 GB	503	A Eurotiomycetes; Trichocomaceae	wgs AAIH01004625	1618198–1616461
Aspergillus flavus seq 2	173(inc)	A Eurotiomycetes; Trichocomaceae	wgs AAIH01001138	4778–5405
Aspergillus flavus seq 3	262(inc)	A Eurotiomycetes; Trichocomaceae	wgs AAIH01004625	2–833
Aspergillus terreus NIH2624 Broad	509	A Eurotiomycetes; Trichocomaceae	Superctg 1	1355266–1357038
Bigelowiella natans TbestDB seq 1	223(inc)	Chlorarachniophyceae	BNL00000086	N/A
Bigelowiella natans TbestDB seq 2	225(inc)	Chlorarachniophyceae	BNL00000067	N/A
Botryotinia fuckeliana (Botrytis cinerea) Broad	498	A Helotiales; Sclerotiniaceae	Superctg 1.1	905398–907229
Chaetomium globosum Locus 1 CBS148.51 Broad	513	A Sordariales; Chaetomiaceae	Sc. 4	4104513–4106208
Chaetomium globosum Locus 2	519	A Sordariales; Chaetomiaceae	Sc. 5	222519–220855
Coprinus cinereus Broad	506	B Agaricales; Psathyrellaceae	Con. 309	91685–93687
Cyanidioschyzon merolae	568	Rhodophyta	Superctg 190	
Galdieria sulphuraria MSU	384	Rhodophyta	Ctg.1002	128062- 129382
Gibberella moniliformis7600 Broad	529	A Hypocreales; Nectriaceae	chromosome 1 cont3.11	357954–356156
Heterocapsa triquetra TbestDB	535	Chlorarachniophyceae	HTL00001520	N/A
Isochrysis galbana TbestDB	273(inc)	Chlorarachniophyceae	ISL00000982	N/A
Laccaria bicolor v1.0 JGI	503	B Agaricales;Tricholomataceae	Sc. 41	205060–203174
Leucosporidium scottii ATCC 90774 FEADB	309	Urediniomycetes	EST 14056	N/A
Magnaporthe grisea 70-15 Broad	533	A Sordariomycetes incertae sedis; Magnaporthaceae	ctg 5.72	205230–206915
Mesostigma viride TBestDB	172	Viridiplantae; Streptophyta	MVL00001572	N/A
Neosartorya fischeri locus 1 seq. 1 NRRL-181 GB	507	A Eurotiomycetes; Trichocomaceae	wgs AAKE03000002	134428–1547952
Neosartorya fischeri Locus 1 seq 2	212(inc)	A Eurotiomycetes; Trichocomaceae	wgs AAKE03000002	134428–133657
Neosartorya fischeri Locus 2	495	A Eurotiomycetes; Trichocomaceae	wgs AAKE02000002	Unavailable
Phaeosphaera nodorum SN15 GB	554	A Pleosporales; Phaeosphaeriaceae	wgs AAGI01000277	49689–51447
Phanerochaete chrysosporium v2.0 JGI	581	B Aphyllophorales; Corticiaceae	Scaffold 7	1566918–1569137
Phytophthora ramorum v1.1 JGI seq 1	550	Heterokonts	Scaffold 19	under const.
Phytophthora ramorum v1.1 JGI seq 2	429	Heterokonts	Scaffold 19	under const.
Phytophthora ramorum v1.1 JGI seq 3	417	Heterokonts	Scaffold 19	under const.
Phytophthora ramorum v1.1 JGI seq 4	507	Heterokonts	Scaffold 19	under const.
Phytophthora ramorum v1.1 JGI seq 5	501	Heterokonts	Scaffold 19	under const.
Phytophthora sojae v1.1 JGI seq. 1	571	Heterokonts	Scaffold 87	under const.
Phytophthora sojae v1.1 JGI seq. 2	387	Heterokonts	Scaffold 87	under const.
Phytophthora sojae v1.1 JGI seq. 3	549	Heterokonts	Scaffold 6	under const.
Sclerotinia sclerotiorum 1980 Broad	539	A Helotiales; Sclerotiniaceae	ctg 1.582	6528–8373
Trichoderma reesei QM9414 JGI	476	A Hypocreales; mitosporic Hypocreales	ctg. 1179	25551–24011

[a] GB = GenBank; Broad = The Broad Institute Fungal Genome Initiative (http://www.broad.mit.edu/annotation/); JGI = Joint Genome Institute Eukaryotic Genomics (http://genome.jgi-psf.org/); MSU = Michigan State University Galdieria Database (http://genomics.msu.edu/galdieria); FEADB = Fungal EST Annotation Database (https://fungalgenomics.concordia.ca/feadb/search.php); TbestDB = Taxonomically Broad EST Database (http://tbestdb.bcm.umontreal.ca/searches/login.php).

[b] GenBank Taxonomy (A = Ascomycota; B = Basidiomycota).

[c] Locus and position are relative to database.

Batrachochytrium dendrobatis (November 2006) was 55% similar to approximately 150 amino acids from the second half of the query and 50% similar to a mammalian monoamine transporter (GenBank accession XM_001100696). All fungal sequences contained the fungus-specific large intracellular loop (Forde 2000; Jargeat et al. 2003).

Spliceosomal Intron Positions in the Fungi

We inferred 22 intron positions (fig. 1*C*) in our analyses of fungal *nrt2* sequences, all of which began with ''gt-'' and ended with ''-ag.'' We assigned the introns identified by Jargeat et al. (2003) the names intron 1 through intron 7 to represent introns at positions 5886–6006, 6385–6445, 6456–6513, 6619–6672, 7147–7220, 7400–7474, and 7576–7636 in the nucleotide sequence of the nitrate assimilation gene cluster in *Hebeloma cylindrosporum* (GenBank accession AJ238664 , Jargeat et al. 2003). We named additional introns according to their position in the gene relative to these sites (fig. 1*C*).

In general, closely related fungi have similar intron patterns (fig. 1*C*). For example, all members of the

euagarics clade (*Hebeloma, Gymnopilus, Coprinopsis, Laccaria*) share introns 1, 2, 3, 4, 5, 6, and 7. *Gymnopilus* also displays a potential eighth position (4C) between introns 4 and 5. In contrast, the basidiomycete *Phanerochaete,* a member of the Polyporales, has no intron positions in common with the euagarics, or the corn smut basidiomycete *Ustilago maydis,* which has only 1 intron, here labeled 4B. It is therefore significant that *Ustilago maydis* and *Trichoderma reesei* (asexual Ascomycota) have an identical pattern of introns, which supports a basidiomycetous origin of the *T. reesei* sequence (see below).

Phylogenetic Analyses

NNP Family Alignment

The amino acid alignment of prokaryotic and eukaryotic NNP family sequences was 1,983 positions long. Unambiguously aligned positions numbered 1,156, and 911 of these positions were parsimony informative. Alignment length was inflated by the presence of clade-specific extended N- and C-terminal domains that were excluded from analyses and by small regions that could be aligned within, but not between major clades. The average likelihood score for credible trees from both Bayesian analyses was −153798.09. Cyanobacteria sequences (98% MPB, 1.0 BPP) and a clade of predominantly actinobacteria sequences (75% MPB, 1.0 BPP) including sequences from the nitrogen-fixing *Frankia* sp. and the nitrogen-fixing alphaproteobacterium, *Bradyrhizobium japonicum,* were each supported as monophyletic (fig. 2). Analyses also supported multiple distinct clades of proteobacterial proteins containing alpha-, beta-, gamma-, and delta- proteobacteria. Also supported by our analyses were 2 lineages of gamma proteobacteria sequences that form a clade with the cyanobacteria (100% MPB, 1.0 BPP). A eukaryotic clade including viridiplantae and other photosynthetic eukaryote, heterokont and fungal sequences received strong support from Bayesian analysis (1.0 BPP) and weak support from parsimony bootstrap analysis (59% MPB). A bacterial sister group to the eukaryotic sequences, including several beta and gamma proteobacteria including *Burkholderia* species and *Cytophaga hutchinsonii* and the alpha proteobacterium, *Roseobacter,* received support from maximum parsimony bootstrap analysis (98% MPB), while a less inclusive sister group including *Burkbolderia spp*. (beta proteobacteria) received strong support from Bayesian analysis (1.0 BPP) and did not receive maximum parsimony bootstrap support.

Eukaryote Alignment (NRT2 Phylogeny)

The eukaryote NRT2 amino acid alignment was 1,079 positions long, of which we included 741 unambiguous positions. Parsimony informative characters numbered 622. Maximum parsimony analysis resulted in 26,804 most parsimonious trees with a score of 7,302. The average likelihood score for credible trees from both Bayesian analyses was −39432.766. Results of these analyses are presented (fig. 3). Viridiplantae received strong support (100% MPB, 1.0 BPP), and heterokonts + fungi received strong support from Bayesian analysis (BPP 1.0), but not by maximum parsimony bootstrap analysis. Plants, fungi, diatoms, and Phytophthora (oomycetes) all received strong support in the Bayesian analysis (1.0 BPP) and maximum parsimony (100% MPB). The heterokonts were resolved as paraphyletic, with the fungi nested within the clade. The Kishino-Hasegawa test did not detect a significant difference between the optimal (unconstrained) topology and topologies that forced heterokonts to be monophyletic or sister to green plants. Three well-supported clades in the viridiplantae include mosses, represented by 5 sequences from *Physcomitrella* (87% MPB, 1.0 BPP), dicots (68% MPB, .97 BPP), including Brassicales, Papillionoideae, and Euasterids, and monocots, represented by the Poaceae (98% MPB, 1.0 BPP).

NRT2 Phylogeny within Fungi

Within the Fungi, Ascomycota (90% MPB, 1.0 BPP) and Basidiomycota (73% MPB, 1.0 BPP) NRT2 sequences were strongly supported as monophyletic. The 1 exception was the *Trichoderma reesei* (Ascomycota) sequence, which formed a clade (100% MPB, 1.0 BPP) with *Ustilago maydis* (Basidiomycota). Within Ascomycota, our analyses recovered the Sordariomycetes (90% MPB, 1.0 BPP), *Aspergillus*/*Neosartorya* (94% MPB, 1.0 BPP), and Helotiales (100% MPB, 1.0 BPP) as monophyletic, with the exception of the aforementioned *Trichoderma* sequence, which did not form a clade with other Sordariomycetes, contrary to expectation based on organismal phylogeny. The Pezizomycetes and Dothideomycetes each had only 1 representative species (*Tuber borchii* and *Phaeosphaeria nodorum,* respectively), and both received moderate support for monophyly with Eurotiomycetes (represented by *Aspergillus* and *Neosartorya*) and Leotiomycetes (represented by *Botryotinia* and *Sclerotinia*).

Basidiomycota NRT2 phylogeny included 4 genera of euagarics, 1 polypore, 1 pucciniomycete (*Leucosporidium scottii*), and 1 ustilaginomycete (*Ustilago maydis*). Agaricales (euagarics clade) received strong support for monophyly (99% MPB, 1.0 BPP). A *Hebeloma* clade (96% MPB, 1.0 BPP) and a *Laccaria* clade (100% MPB, 1.0 BPP) also received support. *Hebeloma helodes, H. tomentosum-like, H. velutipes, H. radicosum,* and *H.truncatum* formed a clade that was poorly supported by maximum parsimony bootstrap analysis but well supported by Bayesian analysis (52% MPB, 1.0 BPP) that excluded *H. edurum* and *H. cylindrosporum.*

Discussion

Where resolved, the NRT2 phylogeny in eukaryotes generally tracks accepted organismal relationships, but the NNP phylogeny in prokaryotes conflicts with accepted taxonomy. These findings suggest a more complex history involving ancient duplications and/or horizontal gene transfer. Below, we first discuss relationships of the entire NNP family across prokaryotes and eukaryotes, then consider evolution of NRT2 in fungi and other eukaryotes.

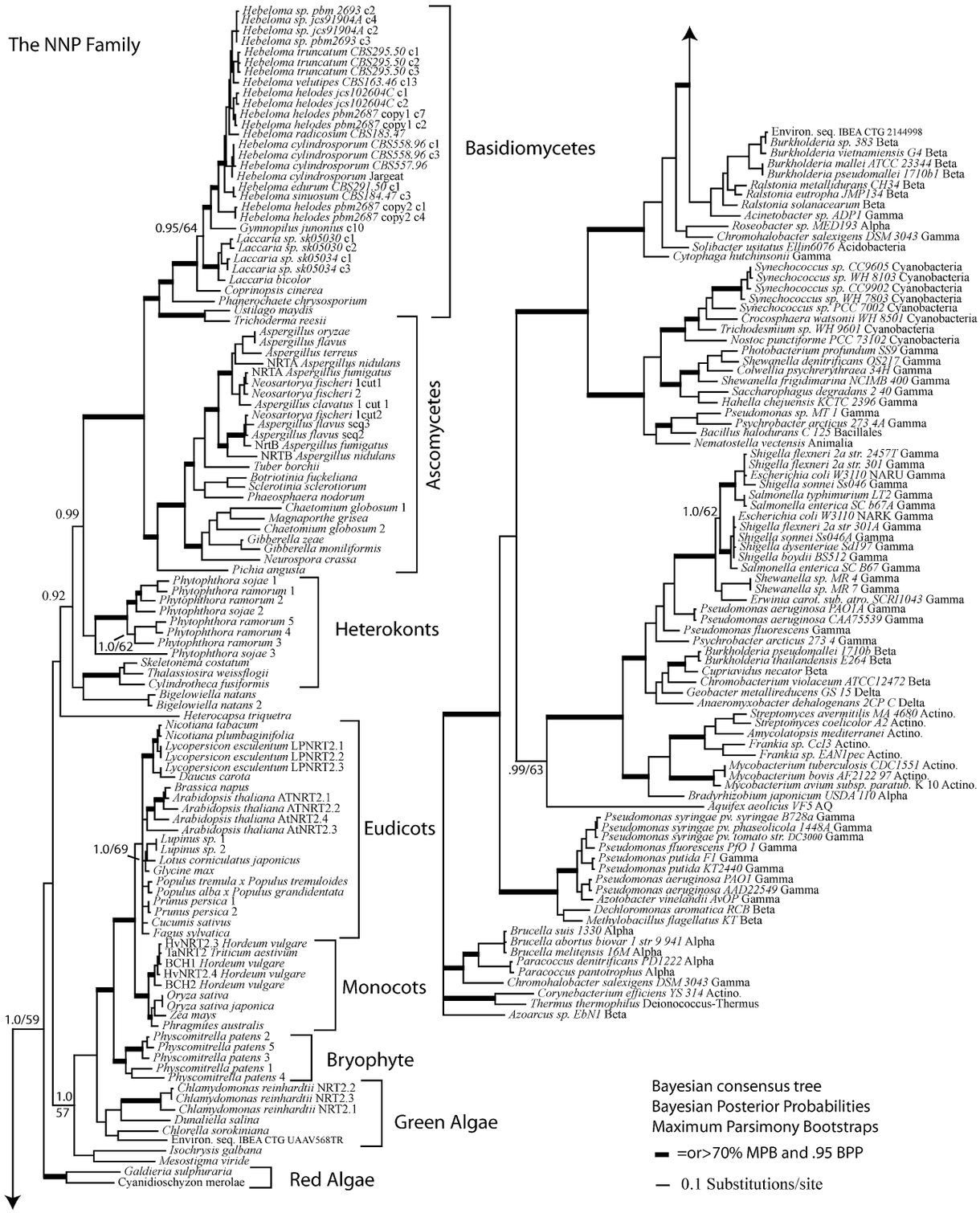

FIG. 2.—Bayesian analysis of NNP family amino acid alignment. Support values for selected nodes are indicated by Bayesian Posterior Probabilities (BPP). Darkened nodes receive greater than 70% maximum parsimony bootstrap support and .95 BPP. Support is not indicated for most terminal bifurcations.

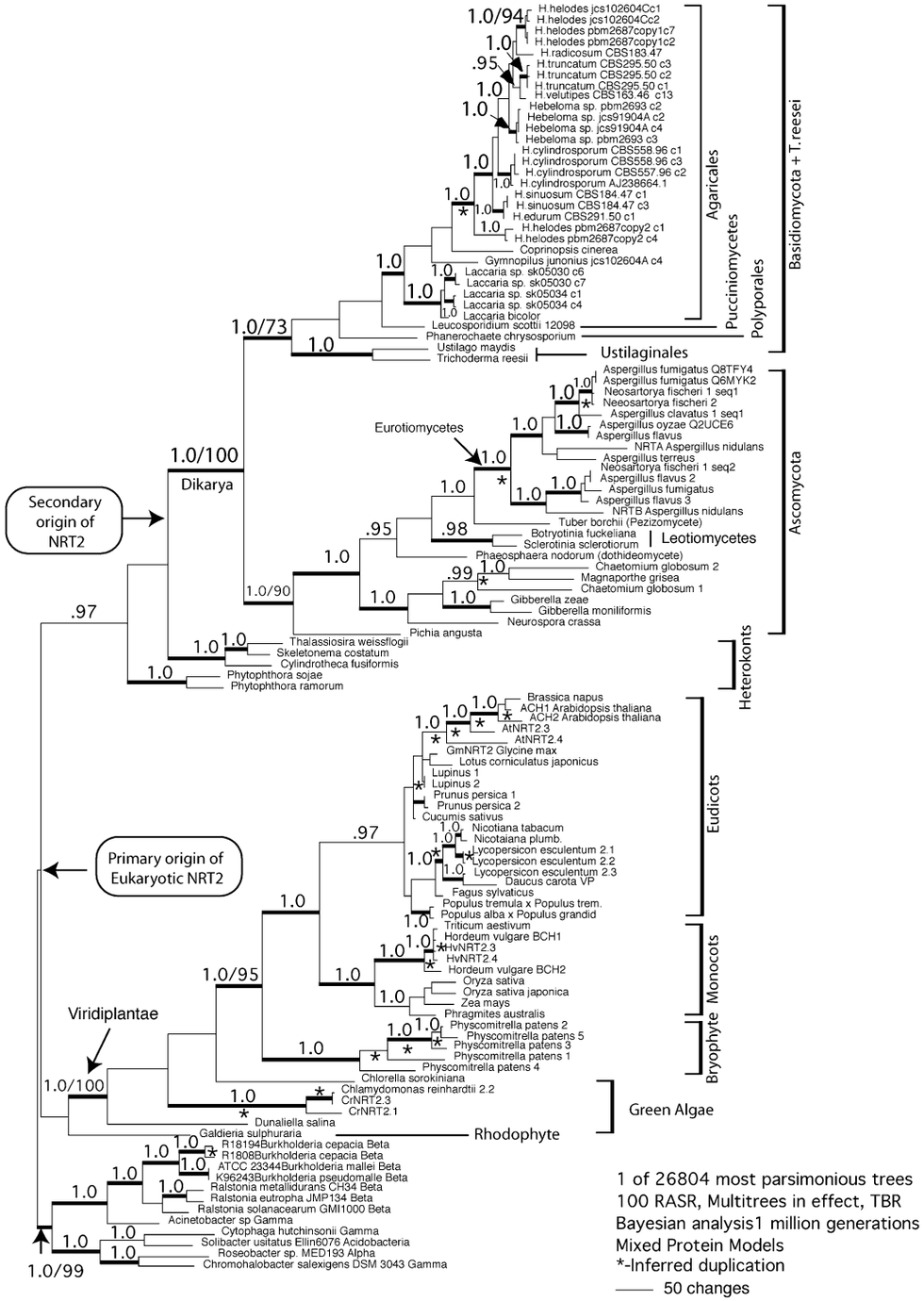

FIG. 3.—Maximum Parsimony analysis of the eukaryotic NRT2 amino acid alignment. Bold lines indicate nodes that receive >70% support by maximum parsimony bootstraps. Support values are indicated for Bayesian posterior probabilities and selected maximum parsimony bootstrap percentages (BPP/MPB). Inferred duplications are denoted by an asterisk. We indicate primary and secondary origins according to the favored hypothesis presented in this paper (fig. 4-*1*).

NNP Phylogeny

Ancient NNP family divergence events are apparent in the prokaryotes, leading to well-supported clusters of nitrate transport–associated proteins that are not necessarily restricted to specific clades of bacteria. Proteobacterial sequences represent the majority of the apparent diversity of these transporters. Bayesian and maximum parsimony analyses (fig. 2) support a single bacterial origin of the eukaryotic NRT2 protein, with the closest prokaryotic relatives in a well-supported clade of nitrate transporters including the alpha proteobacterium, *Roseobacter*. This is consistent with

an endosymbiotic transfer of *nrt2* from the mitochondrion (of alpha-proteobacterial lineage) to the nucleus, or with a more recent transfer from endoproteobacteria, such as Burkholderia spp. The only marginally similar Archaea sequence available, from *Haloarcula marismortui* (AY596297), shared 38% sequence similarity at the amino acid level, and consequently we have no evidence of a nuclear origin of the gene. Our analysis is consistent in overall phylogenetic topology with Forde's (2000) analysis of nitrate transporters in plants, and also with Pao, Paulsen, and Saier's (1998) analysis of the Nitrate Nitrite Porter Family, although we excluded *Mycoplasma* sequences due to high divergence and ambiguous alignment. Highly similar sequences are notably absent from fungi outside the Ascomycota and Basidiomycota (together the Dikarya clade) and animal genome databases. This suggests either that the early lineages of opisthokonts (animals, choanoflagellates, microsporidia and fungi) lacked *nrt2,* which was independently acquired in the common ancestor of Ascomycota and Basidiomycota, or that there were at least 7 losses in the opisthokonts according to a recent molecular phylogeny of this clade (James et al. 2006). We discuss fungal origins in more detail below.

NRT2 Phylogeny in Photosynthetic Eukaryotes

NRT2 phylogeny in viridiplantae (fig. 3) tracks accepted organismal phylogeny. On a broad basis, our analyses support plants as monophyletic, while green algae form a paraphyletic group from which the plants are derived. Mosses (represented by *Physcomitrella*) receive good support to be sister to vascular plants, and the division of monocots and eudicots also receives strong support. These results are consistent with morphological and molecular taxonomy in the plants (Palmer, Soltis, and Chase 2004). Within the grass clade (99% MPB, 1.0 BPP) in our dataset, *Oryza* received weak support as monophyletic with *Zea* and *Phragmites* (64% MPB, 1.0 BPP), which is in conflict with the suggestion of a BEP (Bambusoideae, Ehrhartoideae, and Pooideae) clade (Gaut 2002) including rice, oats, barley, and wheat inferred from certain chloroplast genes. The placement of *Daucus* within a strongly supported clade of Solanaceae (99% MPB, 1.0 BPP) is consistent with the Asterid clade of dicots (Hilu et al. 2003). While maximum parsimony did not support deep relationships between green plants, rhodophytes, heterokonts, and fungi, Bayesian analysis suggests that certain heterokonts, represented by the oomycete lineage, *Phytophthora,* may be sister to the fungi (0.97 BPP), causing heterokonts to be paraphyletic. Improved sampling of rhodophyte and heterokont NRT2 may improve support for deep relationships in the eukaryotes.

The Origins of Eukaryotic and Fungal NRT2

Our analyses suggest a heterokont (diatoms + oomycetes) origin of fungal nrt2 (fig. 3). Within the fungi, NRT2 appears to track currently accepted organismal phylogeny, with 1 exception, discussed below, which suggests horizontal gene transfer.

Eukaryotic organismal phylogeny remains poorly resolved in deeper nodes (Baldauf 2003; Keeling et al. 2005). The phylogeny of Cavalier-Smith (2002; adapted in fig. 4) suggests that the chromalveolate (heterokonts + alveolates) clade is the sister group of Plantae (rhodophytes + green plants), and that the opisthokonts (fungi, animals, and choanoflagellates) form a separate clade. The chromalveolate + Plantae clade has received weak support from molecular analyses (Steenkamp, Wright, and Baldauf 2006). The chromalveolate clade has received some support from multigene phylogenies (e.g. Harper, Waanders, and Keeling 2005; Steenkamp, Wright, and Baldauf 2006), and the opisthokonts form a strongly supported clade that is distinct from plants and heterokonts (Steenkamp, Wright, and Baldauf 2006). The most parsimonious explanation (fig. 4-*1*) for the occurrence of *nrt2* under this topology requires 2 gains of *nrt2* (in Dikarya and chromalveolate + Plantae) and 1 loss (in alveolates). We leave the alveolate dinoflagellate *Heterocapsa triquetra* EST sequence out of this discussion, because its placement is not resolved in the NNP phylogeny, and also because it would is uncertain whether its *nrt2* sequence is of host or plastid origin. To assume a single eukaryotic origin under this topology (fig. 4-*2*) would require at least 8 losses (1 in alveolates and 7 in the opisthokont clade). It is equally parsimonious to infer vertical inheritance of *nrt2* in the heterokonts as to infer secondary origin from another source. To not assume a chromalveolate + Plantae clade might require a less parsimonious reconstruction of *nrt2* origins if the sister of either clade lacked *nrt2,* thereby implying additional losses. However, the topology could be explained less parsimoniously in this case by acquisition of *nrt2* from a rhodophyte plastid that was subsequently lost in oomycetes (Andersson and Roger 2002; Nozaki et al. 2004). A recent phylogeny of glutamine synthetase II (GSII), a protein involved in nitrogen assimilation with a more universal eukaryotic distribution, supported the opisthokont and heterokont + Plantae clades (Robertson and Tartar 2006), but is also consistent with a GSII transfer to the heterokonts from the red algal endosymbiont.

Based on our survey of genome and EST data and sequences in GenBank, *nrt2* appears to be absent from non-photosynthetic and parasitic Alveolata and most major clades of Opisthokonts, other than the Dikarya. These observations, coupled with the eukaryote phylogeny illustrated in fig. 4, suggest 5 hypotheses that could explain the present distribution of *nrt2* in the eukaryotes:

1. NRT2 was acquired once in the eukaryotes, in a common ancestor of the Chromalveolata + Plantae. There was at least 1 loss of NRT2, on the lineage leading to Alveolata, and 1 horizontal transfer event, from the heterokonts to Dikarya (Fungi). This scenario requires 1 origin in eukaryotes, 1 loss, and 1 horizontal transfer (3 events).
2. NRT2 was acquired once in eukaryotes, in the lineage leading to the Plantae, followed by horizontal transfer to the heterokonts, and then to the Diakrya. This scenario requires 1 origin in eukaryotes and 2 horizontal transfers (3 events).

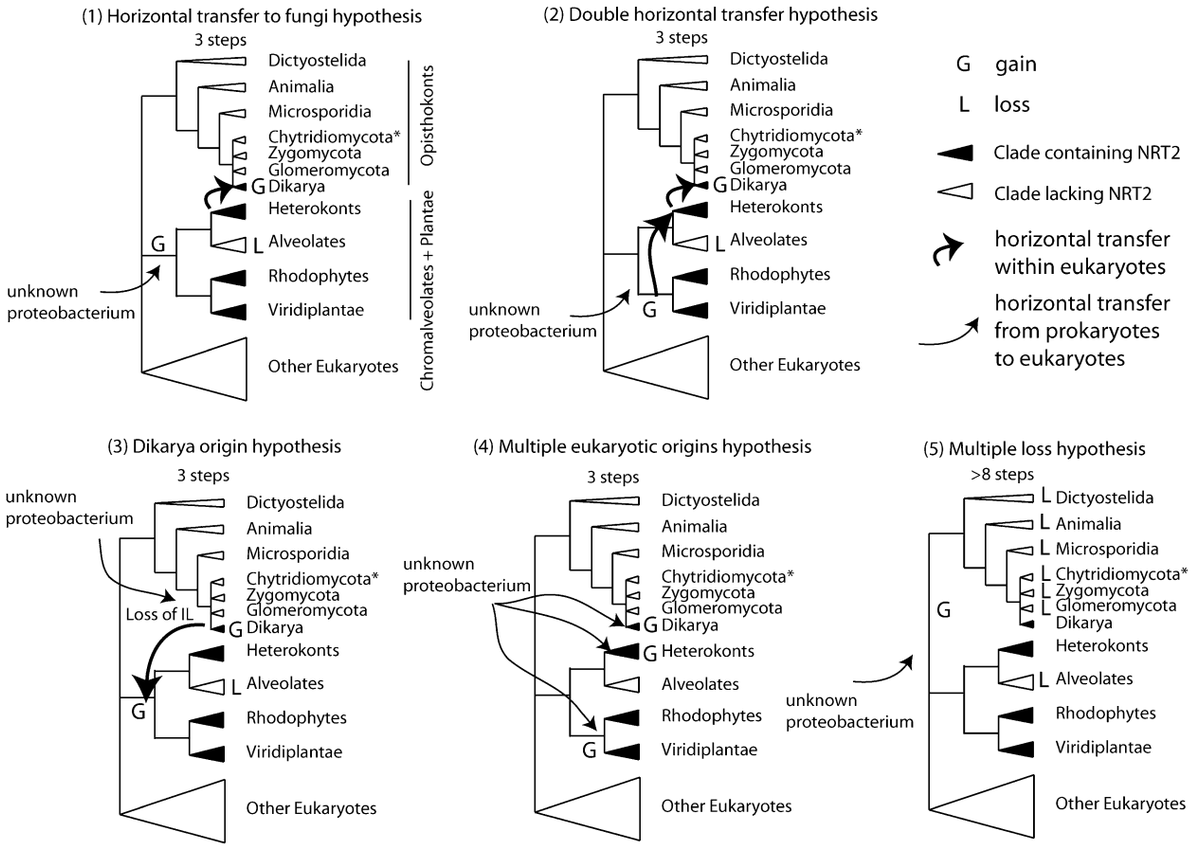

FIG. 4.—Five hypotheses explaining the observed distribution of NRT2 homologs in eukaryotes. The topology of the cladogram is based on Cavalier-Smith (2002). *Chytridiomycota is a polyphyletic group. IL refers to the fungus-specific intracellular loop.

3. NRT2 was acquired once in the common ancestor of the Dikarya, with 1 horizontal transfer to the common ancestor of Chromalveolata and Plantae, and 1 loss in the Alveolata. This scenario also requires 1 origin in eukaryotes, 1 loss, and 1 horizontal transfer (3 events).
4. NRT2 was acquired independently 3 times within eukaryotes, with no losses or horizontal transfers (3 events). Both plants and fungi are known to harbor intracellular proteobacteria related to taxa shown in the eukaryotic phylogeny (Coenye and Vandamme 2003; Bertaux et al. 2005; Artursson, Finlay, and Janson 2006). In this scenario, a certain level of convergent modification to the sequences in the eukaryotic hosts, or failure to sample the relevant proteobacterial sequences would be required to explain the support for more similar eukaryotic sequences.
5. NRT2 was acquired once in the lineage leading to the common ancestor of Fungi, Plantae, and Chromalveolata, with multiple losses within eukaryotes. Major environmental events could have provided substantial selective pressure to favor the loss of the ability to assimilate nitrate in favor of assimilation of more highly reduced forms of nitrogen. This scenario requires 1 origin in eukaryotes and at least 8 losses (at least 9 events). The lineages in which we found *nrt2* homologs are osmotrophic (except for the mixotrophic chlorarachniophyte, *Bigelowiella natans*), whereas the lineages in which we did not are phagotrophic (with the exception of certain fungi). It is possible that the loss of *nrt2* coincided with a transition to phagotrophy in some lineages (animals, alveolates, etc.) Alternatively, the gain of *nrt2,* and other osmotrophy-related sequences may have coincided with the transition to osmotrophy in the fungi.

Hypotheses 1–4 each require 3 events (horizontal transfers or gene losses), whereas hypothesis 5 is by far the least parsimonious scenario. Hypotheses 1–3 each suggest a single origin of *nrt2* in the eukaryotes, which is consistent with the monophyly of eukaryotic *nrt2* sequences. Hypotheses 1 and 2 are most consistent with the phylogeny of *nrt2* in eukaryotes, which suggests that fungal sequences are nested within heterokont sequences. A further argument against hypothesis 3 (origin within Dikarya) is that the intercellular loop that is unique to Fungi would have to be lost prior to the transfer from Fungi to the common ancestor of Chromalveolata + Plantae, which would imply a reduced probability of maintaining folding kinetics and pore formation after excision of the internal sequence; it is simpler to infer that this unique sequence element evolved once within the Fungi and has not been lost. By this reasoning, hypotheses 1 and 2 are equally plausible scenarios. Thus, we infer that there was a single origin of NRT2 in the Dikarya, and that it was derived from heterokonts via horizontal transfer.

The gain of a high-affinity nitrate transporter in Dikarya could have conferred selective advantage to certain fungi in an environment with increased nitrification due to elevating atmospheric oxygen. It has been convincingly argued that the accumulation of oxygen in the neoproterozoic (Kennedy et al. 2006) contributed to an explosion of metabolic complexity that is independent of organismal phylogeny (Falkowski 2006; Raymond and Segrè 2006). Molecular clock analyses of nuclear proteins and ribosomal genes suggest that the divergence of Dikarya from other fungi occurred during (Douzery et al. 2004; Berney and Pawlowski 2006) or before (Heckman et al. 2001; Hedges et al. 2004; Padovan et al. 2005) this period of massive oxygen accumulation and may correspond to a fungus-plant colonization of land (Heckman et al. 2001). It is in Dikarya as well that we find the greatest fungal diversity of symbioses with oxygen-producing autotrophs, and we observe ~98% of the known diversity of filamentous fungi in ascomycetes and basidiomycetes (James et al. 2006). The fact that glomalean fungal symbionts of plants utilize nitrate as well (Govindarajulu et al. 2005), apparently without this particular transporter, could argue for the selective advantage of utilizing the oxidized form of nitrogen in an oxygen-rich environment. Fungi appear to have colonized dry land more than once (James et al. 2006), perhaps facilitated by acquisition of novel metabolic traits from bacterial symbionts. It is possible that another nitrate transporter is active in *Glomus;* however, in searches of the *Glomus* EST database (data not shown) we were unable to find a homolog of the formate-nitrate transporter (FNT), another known conduit of nitrate. *Glomus intraradices* prefers ammonium nitrogen to nitrate (Toussaint, St-Arnaud, and Charest 2004), and an ammonium transporter has recently been characterized (López-Pedrosa et al. 2006). We were also able to recover a single homolog of fungal amino acid transporters (AMT).

Fungi are particularly versatile in the acquisition of nitrogen from the environment. They express genes for uptake of inorganic (nitrate and ammonium) and organic (urea, amino acids, methylammonium, and peptides) forms of nitrogen (Marzluf 1997; Divon and Fluhr 2007). A diversity of nitrogen acquisition strategies appears to apply to pathogenic and mutalistic (mycorrhizal and lichen-forming) fungi alike (Hawkins, Johansen, and George 2000; Chalot et al. 2002; Dahlman, Persson, and Palmqvist 2004; Divon and Fluhr 2007), and a search of the genome of the wood-rotting fungus *Phanerochaete chrysosporium* genome project (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html) reveals nitrate, ammonium, amino acid, and peptide transporter homologs (data not shown). Plants and green algae, in contrast, devote substantially more regulation to nitrate and ammonium transporters of differential affinities and possibly subfunctions (Glass et al. 2002; Orsel, Krapp, and Daniel-Vedele 2002; Forde and Cole 2003; Couturier et al. 2007), suggesting they are more specialized on these inorganic forms of nitrogen. Soil nitrogen makeup is highly dynamic and subject to patchiness (Steltzer and Bowman 1998) and seasonal variation in nitrification (Gosz and White 1986). In most soils, nitrogen is a limiting nutrient (Fernandez, Simmons, and Briggs 2000), so a diversity of uptake mechanisms may make fungi more competitive as nitrogen pools shift with temperature and moisture variation. Nitrate assimilation in fungi is highly regulated and repressed by the presence of more readily utilized forms of nitrogen such as ammonium (Marzluf 1997; Jargeat et al. 2003). That the acquisition of nitrate should be so widespread in Dikarya suggests it is at times favorable to invest the additional energy to reduce nitrate to ammonium. For example, lichens have been shown to absorb nitrate leached from their host trees during winter precipitation (Levia 2002).

Fungal NRT2 sequences in our sample form 2 well-supported clades under multiple analyses that correspond to the Ascomycota and Basidiomycota with the exception of the well-supported placement of the Sordariomycete, *Trichoderma reesei,* NRT2 with *Ustilago maydis,* near the root of the Basidiomycota. *Trichoderma,* the asexual phase of the genus *Hypocrea* (Samuels 2006) is well supported to be in the Sordariomycetes (James et al. 2006). NRT2 from all other Sordariomycetes cluster together with strong support within Ascomycota. Thus, the placement of *T. reesei* suggests horizontal transmission of *nrt2* from Basidiomycota to Ascomycota. Within Ascomycota, our analyses have recovered strong support for Eurotiomycetes, Sordariomycetes, and Leotiomycetes with a limited sample according to the clades described in Lutzoni et al. (2004). Within the Basidiomycota, this analysis provided little support for higher-level relationships, although there is strong support for a single origin of NRT2 in the Agaricales and in the 2 Agaricales genera represented by more than 1 taxon, *Laccaria* and *Hebeloma.*

Gene Duplications

Gene duplications are a source of evolutionary novelty (Zhang 2003). The diversification of *nrt2* in fungi is not surprising considering the example of plants where diversification of this gene has led to divergent function (Orsel, Krapp, and Daniel-Vedele 2002; Little et al. 2005). *Nrt2* paralogs in *Aspergillus nidulans* were shown to code for proteins of differential affinity for nitrate (Unkles et al. 2001). The NRT2 phylogeny we present here suggests at least 3 duplications have occurred in the fungi (fig. 3). One duplication is supported to have occurred prior to diversification of *Aspergillus,* with 4 species maintaining both paralogs. *Aspergillus flavus* may contain an additional paralog; however these are 2 incomplete sequences that do not overlap, and so appear to be the same gene based on phylogenetic proximity. Montanini et al. (2006) did not report paralogous forms in *Tuber,* which is sister to *Aspergillus* in our analysis; however this could be due to gene loss or failure to detect, and consequently we cannot rule out a more ancient duplication. The other Ascomycete duplication suggested by the phylogeny appears prior to the diversification of the Sordariomycetes, with 2 distinct copies found in *Chaetomium globosum;* however there are currently no sequences from additional species to confirm that this is the point of duplication.

The duplication of *nrt2* that we have discovered in *Hebeloma helodes* is the first such report in mycorrhizal fungi and in the basidiomycetes. Amino acid analyses place the second copy as sister to the remaining *Hebeloma*

sequences. However, we recovered no paralogous forms in other *Hebeloma* species as would be expected with an early duplication. Furthermore, Jargeat et al. (2003) suggested that there is only 1 copy in *H. cylindrosporum*. We could have failed to detect additional paralogs with our methods and should confirm these results with Southern blots to determine copy number in other *Hebeloma*. Due to the possibility of differential rates of evolution between paralogs due to selection, we cannot rule out a more recent duplication of *nrt2* in *Hebeloma,* and preliminary nucleotide analyses may suggest this is the case (Slot, unpublished data). Analyses of an expanded dataset of *nrt2* nucleotides and expression patterns will attempt to improve our understanding of *Hebeloma* phylogeny and address functional divergence and lineage sorting of nitrate transporter isoforms in *Hebeloma*.

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