Multiple Horizontal Gene Transfers of Ammonium Transporters/Ammonia Permeases from Prokaryotes to Eukaryotes: Toward a New Functional and Evolutionary Classification

Tami R. McDonald,*[,1] Fred S. Dietrich,[2] and François Lutzoni[1]

[1]Department of Biology, Duke University

[2]Department of Molecular Genetics and Microbiology, Duke University

***Corresponding author:** E-mail: trm5@duke.edu.

Associate editor: Charles Delwiche

Abstract

The proteins of the ammonium transporter/methylammonium permease/Rhesus factor family (AMT/MEP/Rh family) are responsible for the movement of ammonia or ammonium ions across the cell membrane. Although it has been established that the Rh proteins are distantly related to the other members of the family, the evolutionary history of the AMT/MEP/Rh family remains unclear. Here, we use phylogenetic analysis to infer the evolutionary history of this family of proteins across 191 genomes representing all main lineages of life and to provide a new classification of the proteins in this family. Our phylogenetic analysis suggests that what has heretofore been conceived of as a protein family with two clades (AMT/MEP and Rh) is instead a protein family with three clades (AMT, MEP, and Rh). We show that the AMT/MEP/Rh family illustrates two contrasting modes of gene transmission: The AMT family as defined here exhibits vertical gene transfer (i.e., standard parent-to-offspring inheritance), whereas the MEP family as defined here is characterized by several ancient independent horizontal gene transfers (HGTs). These ancient HGT events include a gene replacement during the early evolution of the fungi, which could be a defining trait for the kingdom Fungi, a gene gain from hyperthermophilic chemoautolithotrophic prokaryotes during the early evolution of land plants (Embryophyta), and an independent gain of this same gene in the filamentous ascomycetes (Pezizomycotina) that was subsequently lost in most lineages but retained in even distantly related lichenized fungi. This recircumscription of the ammonium transporters/ammonia permeases family into MEP and AMT families informs the debate on the mechanism of transport in these proteins and on the nature of the transported molecule because published crystal structures of proteins from the MEP and Rh clades may not be representative of the AMT clade. The clades as depicted in this phylogenetic study appear to correspond to functionally different groups, with AMTs and ammonia permeases forming two distinct and possibly monophyletic groups.

Key words: ammonium transporter, fungal and plant evolution, horizontal gene transfer, lichen symbiosis, methylammonium permease.

Research article

Introduction

Ammonium transporters/ammonia permeases (AMTPs) are membrane-spanning proteins composed of 11 highly conserved transmembrane domains that fold into a pore through which ammonia or ammonium translocates. These proteins were described in 1994 simultaneously from plants (Ninnemann et al. 1994) and from fungi (Marini et al. 1994). The plant protein was named AMT, for AMmonium Transporter, and the fungal protein was named MEP, for MEthylammonium Permease. Collectively, the homologs of these proteins have been called the AMT/MEP family, although most subsequently described proteins have been called AMT, whereas the name MEP has been adopted for only a few of the fungal AMTP homologs. Shortly afterward, Rhesus factors (Rhs) were shown to be distantly related to the AMT/MEP family (Marini, Urrestarazu, et al. 1997). The Rh proteins have 12 transmembrane domains (Gruswitz et al. 2010) and have been shown to conduct ammonia and in some cases CO_2 (Kustu and Inwood 2006; Li et al. 2007). Together, the AMT/MEP and the Rh proteins form the AMT/MEP/Rh family.

AMT/MEP/Rh genes have a complicated evolutionary history marked by duplication and larger gene family expansions (e.g., Couturier et al. 2007), loss, and horizontal gene transfer (HGT) (McDonald et al. 2010). For example, land plants have, in addition to the AMTPs first defined in 1994 (plant family AMT1), a second family of AMTPs that has been shown to be more closely related to bacteria than to other plant AMTPs (plant family AMT2, Sohlenkamp et al. 2002). Fungal AMTPs are also more closely related to prokaryotic AMTPs than to most other eukaryotic AMTPs (Monahan et al. 2002). Many lineages of eukaryotes including apicocomplexans, microsporidia, and diplomonads such as *Giardia* have no AMTPs at all in their genomes. Reconstructing the evolutionary history of the AMT/MEP/Rh family is thus not a trivial task. A large

sampling of diverse groups of organisms is essential for untangling the evolutionary intricacies of such a problem. With over 1,500 prokaryotic genomes and nearly 300 eukaryotic genomes publicly available, it is possible to assemble a large data set with the requisite phylogenetic density, that is, a sampling of all of life.

Genomically, the most extensively sampled of all groups of eukaryotes are the fungi because they have relatively small genomes compared with most plants and animals. However, key lineages of fungi are missing in public databases, notably the lecanoromycetes, a large class of lichenizing fungi. The genomes of the model lichen *Cladonia grayi*, a symbiotic system composed of the lecanoromycete fungus *C. grayi* and the green alga *Asterochloris* sp. (Trebouxiophyceae) were recently sequenced (Armaleo, Dietrich, and Lutzoni, unpublished data). Initial similarity-based searches using these fungal and algal genomes revealed sequences encoding three AMTPs from the algal partner, all of which fell into the plant AMT1 family, as expected. It also revealed sequences encoding four AMTPs from the fungal partner. Two of these fungal AMTPs showed highest similarity to other fungal AMTPs. However, the remaining two fungal AMTPs showed highest similarity to land plant (Embryophyta) AMTPs of the plant AMT2 family (absent from all green algae with sequenced genomes) and subsequently to a small assemblage of unrelated prokaryotes. No putative homologs of the plant-like AMTPs from the fungal partner were found in the genome sequence of the green algal partner, leaving open the question of how this fungus came to acquire plant-like genes.

One way in which the fungus may have acquired a plant-like gene is through HGT. HGT, sometimes called lateral gene transfer, is the process by which DNA from a donor organism is incorporated into the genome of a recipient organism of a different species (e.g., the passing of antibiotic resistance genes between unrelated bacteria). This is opposed to the transmission of genetic material from parent to offspring via reproduction, which is known as vertical gene transfer. Although HGT is rampant among prokaryotes (Gogarten et al. 2002), the extent of HGT in the evolutionary history of eukaryotes is unclear. Recent works taking advantage of the diversity and sheer number of fungal genomes available have demonstrated HGT between plants and fungi (Richards et al. 2009) and from bacteria to fungi (Marcet-Houben and Gabaldon 2010), suggesting that the fungi have been the recipients of horizontally transferred genes. Moreover, recent works have demonstrated horizontal transfer of genes encoding nitrate or ammonia/AMTs (McDonald et al. 2010), including horizontal transfers in which fungi have been the recipients and donors of horizontally transferred genes (Slot and Hibbett 2007).

Recent HGT events are relatively easy to detect. They are characterized by signatures such as the gene being present in donor lineages and in an isolated evolutionarily distant recipient lineage or lineages; features such as insertions, deletions, or intron positions that are more similar to those of distantly related organisms than to those of close relatives; or lack of introns in a eukaryotic recipient if the donor organism was prokaryotic. Ancient events are more difficult to detect because these signatures are usually lost. However, phylogenetic analyses with broad sampling can uncover ancient horizontal transfers. The signatures of these events are gene tree topologies in which well-supported monophyletic groups violate well-established common ancestry among major groups of taxa.

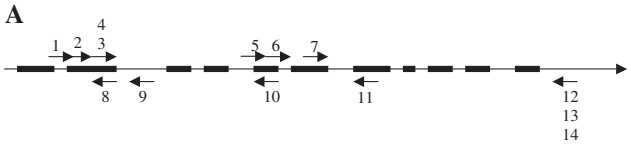
To determine if the plant-like AMTPs in the genome of the fungal partner of the lichen *C. grayi* were the result of a HGT event and to suggest a possible donor of these genes, we performed a polymerase chain reaction (PCR) survey for plant-like AMTPs in cultures of fungi from nearly all main lichen-forming lineages. We used the resulting 16 sequences in a phylogenetic analysis of the AMT/MEP/Rh gene family from lichenized and nonlichenized fungi (81 sequences) as well as 416 published AMTP sequences from diverse prokaryotes and eukaryotes. Here, we present the results of our phylogenetic analysis of the AMT/MEP/Rh family to detect HGTs in the evolutionary history of AMTPs, to estimate their relative time of occurrence within the tree of life, and to propose a new classification for AMTPs that reflects their evolutionary history and molecular function.

Materials and Methods

Cultures and DNA Extraction

Apothecia from lichen-forming fungi were washed and affixed to the lid of an inverted petri dish, allowing the spores to be shot up onto various media. Germinated ascospores were subcultured onto solid media for long-term storage or liquid media for tissue production for DNA extraction. Media used were malt extract–yeast extract (Ahmadjian 1993), nutrient medium based on Bold's basal medium, (Trembley et al. 2002), oatmeal (10 g homogenized oatmeal flakes), potato–carrot (as in Simmons 1992 except made as a 10× stock and centrifuged rather than sieved; Dyer, Crittenden, personal communication), and yeast extract plus supplements (MP catalog #4101-532). A total of 20 g/l agar was added to all solid media. DNA was extracted from cultured fungal partners of the lichens *Arthonia cinnabarina* (Arthoniomycetes), *C. cristatella*, *C. pezizaformis*, *Ramalina* sp., *Usnea strigosa* (Lecanoromycetideae, Lecanoromycetes), *Dibaeis baeomyces* (Ostropomycetideae, Lecanoromycetes), *Endocarpon* cf. *pallidulum*, *Pyrenula cruenta* (Chaetothyriomycetideae, Eurotiomycetes), *Laurera megasperma*, and *Trypethelium virens* (Dothideomycetes) and from washed whole dissected lichen thalli for *Lasallia papulosa*, *Parmotrema michauxiana*, *Stereocaulon tennesseense*, and *U. mutabilis* (Lecanoromycetes). DNA was extracted following the alkali lysis method outlined in Zolan and Pukkila (1986) as modified by Gueidan et al. (2007). Briefly, each sample was ground to a powder, resuspended in 500 μl of a 2% sodium dodecyl sulfate (SDS) extraction buffer (2% SDS, 50 mM Tris (pH 8), 10 mM EDTA, 150 mM NaCl) extracted once with phenol:choroform:isoamyl alcohol (IAA) (25:24:1), and then centrifuged at maximum speed for 5 min. The aqueous phase was removed to a new tube and extracted once with 500 μl of chloroform:IAA (24:1) and centrifuged at maximum speed for 5 min. The aqueous phase was moved to

A



B

#	Primer name	Sequence (5' – 3')
1	Cgr_PLAMT_A_F1	TGACAGCAGGTACTTTGGTCGC
2	Rama_PLANTA_F	CACAAATGGTCYATYAAYTCCG
3	FMAFYAF	TTYATGGCITTYTAYGCITT
4	FMVFYAF	TTYATGGTITTYTAYGCITT
5	Laurera_F	AGCTTGGTGTCATTGACTATTCTGG
6	Laurera_F2	GTTTTCTGGTCTGGATGGCATTG
7	WNGFNG	TGGAAYGGNTTYAAYGGNGG
8	Rama_PLANTA_R	TTYATGCCNTTYTAYGCNTT
9	Cgr_PLAMT_A_R1	GGTAACATCTGAGTCCCGAAAGCC
10	GGYVIH_R	GTGDATNACRTANCCNCC
11	FFKKPSV2	ACIGAIGGYTTYTTRAARAA
12	AVHGEE_R	YTCYTCICCRTGIACNGC
13	AVHGEE_pen	CTCCTCACCGTGNACNGC
14	AIHGEE_R	YTCYTCICCRTGDATNGC

Fig. 1. Degenerate primers and priming sites. (*A*) Placement of degenerate primers relative to coding regions for transmembrane domains of *Cladonia grayi mep1a* (MEP α clade, fig. 2). Transmembrane domains shown as thickened bars. Primers shown as numbered arrows. (*B*) Sequences of degenerate primers used to amplify "plant-like" MEP α AMTPs from lichenized fungi (supplementary table S2, Supplementary Material online).

a new tube and the DNA precipitated with 300 μl (0.6 volumes) of isopropanol and centrifuged as above. The resulting pellet was washed with 70% ethanol and then allowed to air dry before resuspension in 25 μl sterile water.

Degenerate PCR and Sequencing

A list of the degenerate primers used for this study and a depiction of the placement of these primers relative to transmembrane domains is found in figure 1. PCR was performed on a MJ Research PTC200 thermocycler or an Applied Biosystems (Foster City, CA) Veriti thermocycler using a series of two touchdown PCR programs. In the first program, an initial denaturation step for 3 min at 94°C was followed by 10 cycles of 30 s at 94°C, 30 s at 60°C with a −1°C step down at each cycle, and 60 s at 72°C, followed by 25 cycles of 30 s at 94°C, 30 s at between 50°C and 47°C, and 45 s at 72°C with a final elongation step at 72°C for 7 min. If no products were formed or if faint products were seen, a second program was implemented consisting of no initial denaturation step, 24 cycles of 30 s at 94°C, 30 s at 55°C with a −0.4°C step down at each cycle, and 60 + 2 s per cycle at 72°C, followed by 12 cycles of 30 s at 94°C, 30 s at 45°C, and 120 + 3 s per cycle at 72°C with a final elongation step at 72°C for 10 min. PCR products were visualized on a Tris-Acetate-EDTA 1% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, CA). If necessary, faint products or products with multiple bands were cloned with the TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. For each cloning reaction, we screened at least eight clones by colony PCR using T7 and M13R primers and a PCR program consisting of a 10-min initial denaturation step, followed by 25 cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C followed by a final elongation step of 7 min. PCR products were cleaned with a Montage PCR filter column (Millipore, Bilerica, MA) or with an Exo-SAP cleanup using 1 μl dilution buffer, 0.5 μl exonuclease 1, 0.5 μl Shrimp alkaline phosphatase, added to 10 μl PCR reaction and incubating on one of the aforementioned thermocyclers for 30 min at 37°C, and then 15 min at 80°C (USB, Cleveland, OH). Cleaned PCR products were sequenced in 10 μl reactions using: 1 μl primer, 3 μl purified PCR product, 0.5 μl Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; PE Applied Biosystems), 1.5 μl Big Dye buffer, and 4 μl double distilled water. Automated reaction cleanup and visualization were performed at the Duke Institute for Genome Sciences and Policy (IGSP) Genome Sequencing & Analysis Core Facility using Big Dye chemistry with an ABI 3730xl automated sequencer (PE Applied Biosystems). Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI) was used to edit sequences and assemble contigs.

Genome Sequencing

Cladonia grayi strain *Cgr/DA2myc/ss* was isolated from fungal ascospores (Armaleo et al. 2011). The unicellular green alga *Asterochloris* sp. *Cgr/DA1pho* was isolated from soredia (Armaleo and May 2009). A total of 5 μg of DNA was submitted to the Duke IGSP Genome Sequencing & Analysis Core Facility and sequenced with pyrosequencing (454) technology. The genome data used in this work were obtained through the *C. grayi* genome project in progress at Duke University and currently housed at http://genome.jgi-psf.org/Clagr2/Clagr2.home.html and http://genome.jgi-psf.org/Astpho1/Astpho1.home.html.

Database Mining

All publicly available eukaryotic genomes as of 10 November 2009 in Genbank (http://www.ncbi.nlm.nih.gov/nuccore), the Broad Institute (http://www.broadinstitute.org), and the Department of Energy Joint Genome Initiative (http://www.jgi.doe.gov) were searched for AMTPs. Additional AMTPs from the *Cyanidioschyzon merolae* genome project (http://merolae.biol.s.u-tokyo.ac.jp) and *Galdieria sulphuraria* genome project (http://genomics.msu.edu/galdieria) were also included. As AMTPs are present in multiple copies in most genomes (e.g., 4 or 5 in most fungi, 5 or more in most plants, 8 in *Clamydomonas reinhardii*, and up to 15 as in *Populus trichocarpa*), it was necessary to choose one or a few genomes from each major group of interest to maximize the phylogenetic coverage of the analysis while maintaining a manageable number of genes. We used all the AMTPs from each of the selected genomes. Whether a gene was an AMTP was determined using annotation, if available, Blast similarity of greater than ~70% similarity to one of the query AMTPs:

C. grayi mep1a, *C. grayi mep2* (a high-affinity fungal AMTP, Marini, Soussi-Boudekou, et al. 1997; Lorenz and Heitman 1998; Montanini et al. 2002; Javelle et al. 2003; Lopez-Pedrosa et al. 2006; Rutherford et al. 2008), *C. grayi mep3* (a low-affinity fungal AMTP, Javelle et al. 2006), *Arabidopsis thaliana AMT2*, or *Oryza sativa AMT1;1* and by the presence of highly conserved AMTP motifs (FMAFYAF and variants, transmembrane (TM) domain 2; FQFAAIT and variants, TM domain 3; WxWGGG and variants, TM domain 4; GGYVIH or FAGGxxxH, TM domain 5; WNGFNG and variants, TM domain 7; and AIHGEE and variants, cytosolic tail). If structural or experimental data had been published for one or more of the transporters, we chose that genome over other genomes in the major group of interest. Some groups of interest, like the conifers, do not have completed genome projects. To represent this group and others without completed genome projects, we chose expressed sequence tag data and therefore do not have a full representation of the AMTPs in the genome. In addition, we used all sequences published in Huang and Peng (2005). All publicly available prokaryotic genomes in Genbank were also searched and selected as above, endeavoring to include at least one representative of each major phylum. Genomes in the analysis are listed in supplementary table S1 and fig. S1, Supplementary Material online.

To determine the phylogenetic extent of the "plant-like" AMTPs within the fungi, we searched all fungal genomes available (86 as of 11 October 2009) through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) as well as the whole-genome shotgun reads (40 additional fungi) using TBlastX and BlastP. We searched the fungal genomes at the Saccharomyces Genome Database site (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl, 2 additional fungi), the Department of Energy Joint Genome Institute (http://genome.jgi-psf.org, 23 additional fungi), and the Broad institute (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html, 3 additional fungi) using BlastX and TBlastX. All AMTPs from fungal genomes containing plant-like AMTPs (four genomes) were included in the analysis.

Phylogenetic Methods

Manual alignments were performed using MacClade 4.08 (Maddison and Maddison 2005). Ambiguously aligned regions and introns were delimited manually and excluded from phylogenetic analyses. Alignments are available through TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11394). Models of molecular evolution were selected using the Akaike Information Criterion implemented in jModeltest (Guindon and Gascuel 2003; Posada 2008) or Mr Modeltest 2.3 (Nylander 2004). Phylogenetic relationships and confidence values were inferred using a maximum likelihood approach at the nucleotide level. Maximum likelihood analysis at the nucleotide level used a general time reversible model with a gamma parameter and a proportion of invariable sites (GTR GAMMAI = GTR + Γ + I). The program RAxML-VI-HPC (Stamatakis et al. 2005) was used for the maximum likelihood search for the most likely tree. The same program using the same settings was used for the bootstrap (BS) analysis with 1,000 BS replicates. The consensus tree was calculated and visualized using the majority rule consensus tree command in PAUP 4.0d701 (Swofford 2002).

Results and Discussion

Overview of the Phylogenetic Tree

A phylogenetic tree of the AMT/MEP/Rh family resulting from a maximum likelihood analysis at the nucleotide level of 513 sequences is presented in figure 2 and supplementary figure S1 (Supplementary Material online). In this tree, two main monophyletic groups [eukaryotic ammonium transporter (AMT-Euk) and MEP clades] and one basal grade (MEP grade) are evident. The Rh group is very distinct from the MEP and AMT family and has been defined previously (Huang and Peng 2005). We use the Rh family sequences to root our AMTP phylogeny. Of interest is the placement of some of the eukaryotic sequences within the tree.

Although the name "AMT" is usually applied to all AMTP sequences not in the Rh family, here, we restrict the term AMT to the sequences arising from node AMT (fig. 2). The AMT genes found in eukaryotes form a monophyletic group, referred to here as the AMT-Euk clade (corresponding to the AMT1 clade of McDonald et al. 2010). The AMT-Euk clade represents regular vertical transfer of AMTPs into eukaryotes. Notably, although AMTPs from animals, land plants, green algae, red algae, slime molds, chromalveolates, and excavates are found in the AMT-Euk clade, fungal AMTPs are entirely missing from the AMT-Euk clade. Instead, they are found exclusively in the MEP clade (fig. 2 and supplementary fig. S1, Supplementary Material online).

Although the name "MEP" is currently applied to AMTPs from only some fungi, here, we expand that usage to include all sequences arising from the well-supported MEP node supporting the MEP clade (corresponding to the AMT2 clade of McDonald et al. 2010; fig. 2). The MEP clade may be further subdivided into the plant-like MEP clade (MEP α) of putative archeal origin, the prokaryotic MEP clade (MEP β), and the fungal MEP clade (MEP γ) of prokaryotic origin, discussed separately below. The MEP clade is a largely prokaryotic clade with unrelated groups of eukaryotes interspersed within it. Each eukaryotic lineage within the MEP clade therefore represents one or more HGT events, which will be discussed in greater detail below, in chronological order from the earliest event to the latest event.

Broadly speaking, this gene tree follows the expectations for vertical gene transfer; a eukaryotic clade (AMT-Euk) arises from a grade of prokaryotic sequences (MEP grade) at the base of the AMTP tree. However, punctuating this vertical evolution are several recent and at least three ancient transfers from prokaryotes into diverse eukaryotic lineages, all found in the MEP clade. Thus, although all the eukaryotic sequences in the AMT-Euk clade represent

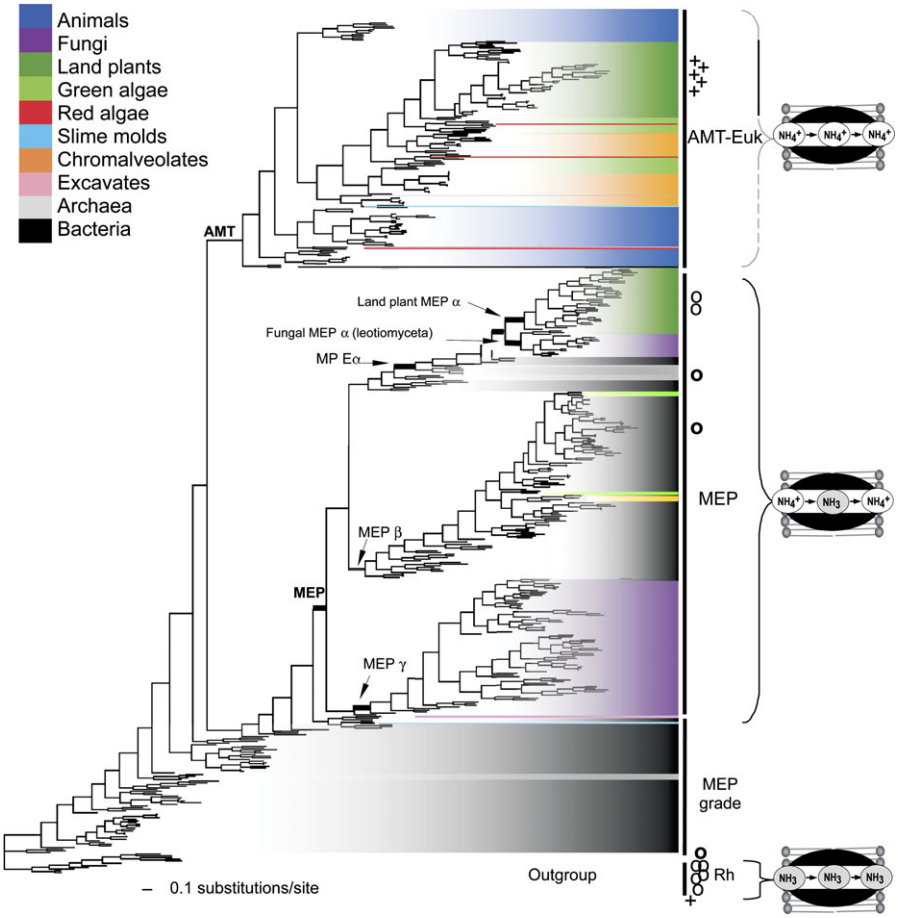


Fig. 2. Evolution of AMTPs. Maximum likelihood analysis of 513 AMTP genes shows the presence of a eukaryotic clade (AMT-Euk) representing vertical gene transfer of AMTPs into eukaryotes and a predominantly prokaryotic clade (MEP) in which eukaryotic lineages demonstrate HGT. Thickened lines show ≥70% BS support for nodes discussed in the text. More detailed information about this phylogenetic tree, including all supported nodes, is shown in supplementary fig. S1, Supplementary Material online. Proposed transport mechanisms are schematically represented to the right of each clade. + = electropositive transport demonstrated by electrophysiology; O = electroneutral transport demonstrated by electrophysiology; and O = electroneutral transport demonstrated by crystal structure.

vertical evolution, all the eukaryotic monophyletic groups in the MEP clade result from HGTs.

One Horizontal Transfer during the Early Evolution of the Fungi Creates the MEP γ Clade

Fungi are the only major group of eukaryotes entirely missing AMTPs from the AMT-Euk clade. Fungal sequences are instead found in the MEP clade. Every fungus sampled so far has multiple AMTPs in the fungal MEP γ clade (supplementary fig. S1, Supplementary Material online). Most have three to four copies, indicating that the MEP γ gene family has expanded through gene duplication. Because all fungi lack an AMT-Euk gene resulting from vertical inheritance and all fungi have at least one copy of the MEP γ gene, we interpret this phenomenon as a gene replacement event. Fungal ancestors, like other eukaryotes, must have originally had the AMT-Euk gene in their genomes. They then acquired the MEP γ gene near the time of the divergence of the fungi and lost the native AMT-Euk gene. Therefore, the replacement of an AMT-Euk gene by a MEP γ gene could be a defining trait of the kingdom Fungi.

Horizontal Transfers from Hyperthermophilic Chemoautolithotrophic Prokaryotes into the Pezizomycotina (Leotiomyceta) and Land Plants (Embryophyta) Characterize the MEP α Clade

Fungal AMTP genes are also found in the MEP α clade. This clade comprises a well-supported group of MEP genes from land plants (excluding green algae) sister to a well-supported group of genes from a subgroup of filamentous ascomycete fungi within the Pezizomycotina (referred to as the leotiomyceta; Schoch et al. 2009), where most of the Ascomycota species richness as well as all known lichen-forming ascomycetes are concentrated (fig. 2; supplementary fig. S1, Supplementary Material online). This sister relationship between the MEP genes found in the leotiomyceta and those found in land plants explains the initial similarity search results in which these fungal MEP genes appeared to be most similar to AMTPs from land plants. These leotiomyceta-specific MEP α sequences are very distinct from the sequences in the fungal MEP γ clade and have a separate evolutionary origin. The same is true for the land plant MEP α

Table 1. Summary of Characteristics Uniting Prokaryotic Members of the MEP α Clade (fig. 2; supplementary fig. S1, Supplementary Material online) of Ammonia Permeases.

Organism	Electron Donor	pH Optimum	Temperature Optimum (°C)	Carbon Source	Guanine-Cytosine Content (%)
Bacteria					
Acidimicrobium ferrooxidans[a]	Iron	2	45–50	A/H	68.3
Acidithiobacillus caldus[b]	Sulfur	2.0–2.5	45–50	A	61.4[c]
A. ferrooxidans[b]	Iron and sulfur	1.5–2	30–35	A	58–59
Leptospirillum rubarum[d]	Iron	1.1	41	A	55[e]
Archaea					
Caldivirga maquilingensis[f]	Sulfur	3.7–4.2	85	H	43.1
Ferroplasma acidarmanus[g]	Iron	1.2	42	H	36.8
Picrophilus torridus[h,i]	Iron	0.7	60	H	36
Sulfolobus tokodaii[j]	Sulfur	2.5–3	75–80	H/A	32.8
S. solfataricus[j,k]	Sulfur	2–4	80	H/A	35.79[l]

NOTE.—All are acidothermophilic chemolithotrophs or chemoautolithotrophs isolated from volcanic hotsprings, acid mine seeps, and similar extreme environments. Carbon source: A = autotroph; H = heterotroph.

[a] Clark and Norris (1996).
[b] Kelly and Wood (2000).
[c] Valdes et al. (2009).
[d] Goltsman et al. (2009).
[e] Calculated from data available on 14 October 2010 at http://ww.ebi.ac.uk/ena/data/view/Taxon:419542&portal=con_release&page=1.
[f] Itoh et al. (1999).
[g] Dopson et al. (2004).
[h] Schleper et al. (1995).
[i] Futterer et al. (2004).
[j] Brock et al. (1972).
[k] She et al. (2001).
[l] http://microbes.ucsc.edu/cgi-bin/hgGateway?db=sulSol1accessed on 14 October 2010.

sequences compared with their AMT-Euk plant counterparts. At the base of the MEP α clade is a grade of phylogenetically unrelated prokaryotes that are united by ecology: They are all hyperacidophilic, thermophilic, chemolithotrophic, and chemoautolithotrophic bacteria and Archaea (table 1) isolated from volcanic hotsprings and similar extreme environments (Clark and Norris 1996; Schleper et al. 1996; Itoh et al. 1999; Suzuki et al. 2002; Goltsman et al. 2009). Most closely related to the land plant and fungal MEP α are AMTPs from a small lineage of gamma proteobacteria (*Acidithiobacillus*) known from acid mine drainages (Kelly and Wood 2000; Valdes et al. 2009). The rest of the proteobacterial sequences, including the bulk of the gamma-proteobacterial sequences, fall elsewhere in the tree (supplementary fig. S1 and table S1, Supplementary Material online). Likewise, *Leptospirillum rubarum* (Nitrospirae) and *Acidimicrobium ferrooxidans* (Actinobacteria) are unusual iron-oxidizing representatives of their phyla with AMTPs in this clade. More distantly related is a paraphyletic group of hyperacidophilic thermotolerant Archaea including *Caldivirga*, *Ferroplasma*, *Picrophilus*, and *Sulfolobus*. Except for the fungi, which were not extensively sampled in their work, this clade was also recovered by McDonald et al. (2010).

Because the early diverging diversity in this well-supported clade is archeal, we suggest that land plants and filamentous ascomycetes in the leotiomyceta each inherited, independently, an archeal-type AMTP. Although the original source of the gene is likely archeal, the actual donor organism may have been a eubacterium. We suggest this because the gamma-proteobacterial sequences share a most recent common ancestor with the eukaryotic sequences, suggesting that they are more closely related to the sequences of the donor than are the archeal sequences.

Recent Transfers into Eukaryotes Occur during the Evolution of the MEP β Gene Family

Other eukaryotic sequences are found in the bacterial MEP β clade. MEP sequences from the chromalveolate *Phytophthora infestans*, an oomycete, which is the causative agent of potato late blight, cluster together without support with one cyanobacterium (*Gloeobacter violaceus*) and *Deinococcus radiodurans* as a sister clade to the actinobacteria (supplementary fig. S1, Supplementary Material online). All other chromalveolates, including diatoms and other stramenopiles, which are close relatives of oomycetes, are absent from the MEP β clade, indicating a relatively recent horizontal transfer of the MEP β gene from bacteria into the oomycetes. A HGT of AMTPs between fungi and oomycetes has been suggested elsewhere (McDonald et al. 2010). However, the more extensive prokaryotic sampling in this analysis allows us to exclude that possibility as the oomycetes clearly fall inside the bacterial MEP β clade and far from the well-supported MEP fungal clades within the MEP α and γ clades. Therefore, our results do not support a horizontal transfer scenario between fungi and oomycetes. Rather, the relationship previously detected likely reflects the separate bacterial origins of fungal and oomycete MEPs.

Shown here as a sister group without support to the aforementioned clade of oomycetes, actinobacteria, and intervening sequences are the Mamielallean green algae *Ostreococcus* and *Micromonas*, suggesting a separate horizontal transfer of a MEP β AMTP from a bacterial donor to these green algae. One additional horizontal transfer event is indicated by the placement of another *Ostreococcus* sequence, which arises from a group of proteobacteria. These horizontal transfers of AMTPs into the Mamielalles were also detected by McDonald et al.

(2010), who had the complete complement of AMTPs from Mamielallean genomes in their analyses.

Because there is no support at the deeper nodes of the bacterial clade (MEP β), we cannot detect HGT events between groups of prokaryotes. We recover proteobacteria, actinobacteria, and firmicutes clades but have no support for the relationships between these groups. Likewise, we recover a basal grade containing more proteobacterial and cyanobacterial sequences, as well as Bacteriodes, Themotoga, Green sulfur bacteria, and Purple bacteria, with no support for the relationships between these groups. Although eubacterial phyla are recovered, the relationships between the phyla cannot be resolved.

Multiple Independent HGTs into Restricted Lineages of Eukaryotes in the MEP Grade

Most unusual is a small but well-supported clade containing AMTP sequences from *Trypanosoma cruzi* (excavate), *Naegleria gruberi* (excavate), *Dictyostelium discoideum* (slime mold), and the phylogenetically unrelated prokaryotes *Ureaplasma urealyticum* (Tenericutes, Mycoplasmatales), *Enterococcus faecium* (Firmicutes), and *Methanosarcina* spp. (Archaea, Euryarchaeota). The lack of a copy of this AMTP in related lineages (including *T. brucei*) suggests that each of the eukaryotic lineages gained this AMTP recently and independently. Among the prokaryotic members of this clade, *Methanosarcina* in particular has experienced horizontal transfer of AMTP genes from multiple sources as shown in this study. However, it is difficult to find a characteristic that unites all the organisms in this group. Many are pathogens or commensals of animals. The association of eukaryotic and prokaryotic symbionts together in the gastrointestinal tract, urinary tract, or blood stream of an animal host may have facilitated horizontal transfer. By extending the MEP clade one node deeper into the AMTP phylogeny (supplementary fig. S1, Supplementary Material online), the MEP clade would remain monophyletic and encompass four MEP subclades instead of three. However, because of our low sampling for this fourth MEP subclade and its uncertain phylogenetic placement within the MEP grade, we decided to keep it as part of the MEP grade.

In summation, the eukaryotic MEP genes are not most closely related to each other. Eukaryotic MEP clades have arisen through at least nine putative separate HGT events (fig. 3) from at least three lineages of bacteria and from hyperthermoacidophilic Archaea (supplementary fig. S1, Supplementary Material online). Our data show no evidence of HGT from eukaryotes to prokaryotes or from eukaryotes to eukaryotes.

Fungal MEP α Gene Preferentially Retained in Lichens

This project was initiated by the discovery of two plant-like AMTP genes (MEP α) in the newly sequenced genome of the lichen-forming ascomycete *C. grayi*. To determine the distribution of these plant-like MEP α genes among fungi, we queried all publicly available fungal genomes. In addition, we attempted to amplify by degenerate PCR the fungal MEP α gene from 15 cultures of lichenizing fungi from three classes of Pezizomycotina (supplementary table S2, Supplementary Material online). Although most lichens are found in one large class of Ascomycota (Lecanoromycetes), lichens are also found in four other fungal classes that include nonlichenized fungi (Lutzoni et al. 2001, 2004; James et al. 2006; Schoch et al. 2009). We found the MEP α gene in 9 of 15 lichens sampled from Lecanoromycetes, Eurotiomycetes, and Dothidiomycetes (supplementary table S2, Supplementary Material online). These three classes of Ascomycota belong to the leotiomyceta (fig. 3). As more lichen genomes accumulate, we should be able to refine our primers and identify this gene in additional lichenized fungal genomes.

By contrast, only 4 of the 151 nonlichen fungal genomes searched had the MEP α gene, 2 in a small subgroup of *Penicillium* (Eurotiomycetidae; only those with *Talaromyces* telomorphs) (also detected by McDonald et al. 2010) and 2 in a subgroup of *Fusarium*. The *Penicillium* members are in the Eurotiomycetes, a class with lichenized members that retain the gene. The *Fusarium* members are in the class Sordariomycetes, which is entirely nonlichenized but share a common ancestor with three lichen-rich groups (Lecanoromycetes, Lichinomycetes, and Eurotiomycetes) as part of the leotiomyceta (fig. 3).

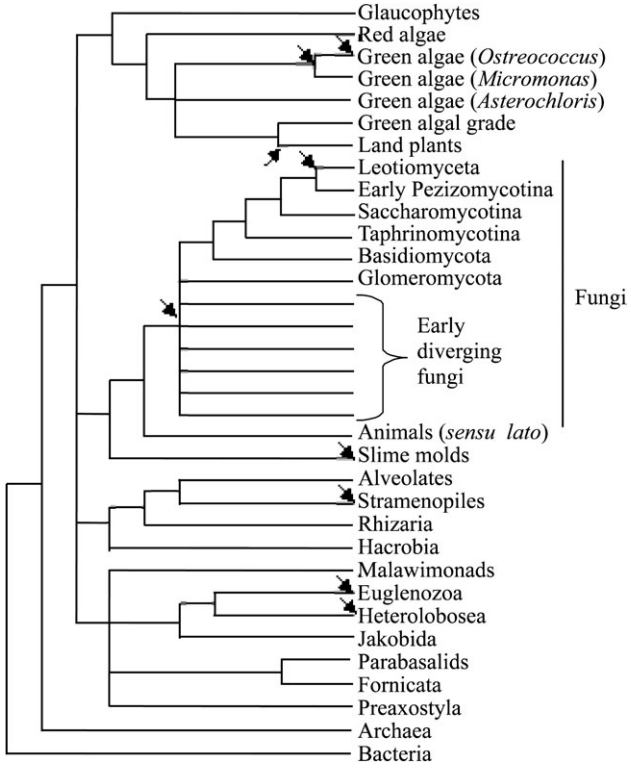


Fig. 3. Schematic representation of the tree of life (organismal phylogeny), for taxa of interest for this study, showing the phylogenetic placement (arrows) of the proposed HGT events during the evolution of eukaryotes, derived from our inferred AMTP gene tree (fig. 2; supplementary fig. S1, Supplementary Material online). Classification and tree adapted from Hibbett et al. (2007), Schoch et al. (2009), Keeling et al. (2009).

We interpret this distribution as a HGT into filamentous ascomycetes (Pezizomycotina), after the divergence of the Pezizomycetes but before the radiation of the leotiomyceta (James et al. 2006; Schoch et al. 2009). This was followed by a subsequent loss of the gene in almost all nonlichenized lineages, whereas the gene was retained and in fact duplicated in lichenized lineages of Lecanoromycetes, Eurotiomycetes, and Dothidiomycetes (supplementary fig. S1 and table S2, Supplementary Material online).

Because only land plants and a relatively late-evolving group of fungi have the MEP α gene, we rule out the possibility of one transfer to the base of eukaryotes and subsequent losses by intervening eukaryotic clades, which would require at least 14 separate events. Instead, we interpret this distribution as the result of two separate horizontal transfers, one to the ancestor of the land plants and one to an ancestral lineage of the leotiomyceta, by the same or similar prokaryotic donors. This scenario explains the sister relationship between MEP α genes from land plants (Embryophyta) and a group of filamentous ascomycetes (leotiomyceta). Within leotiomyceta, the gene was then lost in most lineages but retained by many lichenized lineages, no matter how distantly related.

Dating the Transfers

The MEP α gene appears to have entered the fungal lineage after the divergence of the Saccharomycotina from the Pezizomycotina ($\approx$773 Ma) but before the radiation of the leotiomyceta ($\approx$673 Ma, Blair 2009), suggesting a window of about 100 My for this horizontal transfer event into the Pezizomycotina. The MEP α gene appears to have entered the plant lineage after the divergence of the land plants from the various chlorophyte algae $\approx$936 Ma (Bhattacharya et al. 2009) and before the diversification of extant land plants, $\approx$440–550 Ma.

Why this particular MEP gene should be retained in land plants and in lichenizing Ascomycota is unclear. But the fact that both groups rely on photosynthesis for carbon, colonized land at about the same time or in close succession when sources of nitrogen might have been limiting, and gained the AMT more or less contemporaneously suggests that this gene may be advantageous under these conditions. For example, it may allow a balanced efficiency in nitrogen transport between algal and fungal partners forming lichen symbioses and between land plants and their fungal partners forming mycorrhizal symbioses. It could be argued that if one of the partners was more efficient in transporting nitrogen, it might have prevented the establishment and maintenance of these symbioses and the subsequent diversification of the lichen-forming Ascomycota and land plants. Therefore, this gene might have been one in a suite of preadaptations to lichenization of ascomycetes, or a copy of the MEP transporter might have eased the transition to land for fungi and plants. Functional studies are required to explore this and other hypotheses.

Functional Differences of AMT-Euk, MEP, and Rh Transporters?

Although it was originally thought that all proteins in the AMT/MEP/Rh family were ion channels transporting ammonium, the publication of the crystal structure from *Escherichia coli* (Khademi et al. 2004; Zheng et al. 2004) and the archean *Archaeoglobus fulgidus* (Andrade et al. 2005) challenged this view. These crystal structures suggested that at least some of the proteins are modified gas channels conducting ammonia (NH_3). Although there is some debate (Lamoureux et al. 2007, 2010), the current model for the mechanism of action of these modified gas channels is that the ammonium proton is stripped at the entrance to the pore, whereupon ammonia moves through the channel and is reprotonated at the cytoplasmic side (Winkler 2006; Li et al. 2006; Yang et al. 2007). These results have been extrapolated to the whole AMT/MEP/Rh family (Winkler 2006). However, because both of these proteins fall into the MEP family (the *A. fulgidus* protein belongs to the MEP α clade and the *E. coli* protein belongs to the MEP β clade), it is unlikely that these structures represent the whole AMT/MEP/Rh family. In fact, the crystal structure of the *Nitrosomonas europaea* Rh protein (Li et al. 2007) suggests that Rh proteins are gas channels for ammonia (NH_3) and/or CO_2; no deprotonation and reprotonoation are involved (fig. 2). Although no crystal structure is available for any protein in the AMT-Euk clade, some proteins in this clade, notably from plants, have been identified as bona fide AMTs (fig. 2) using electrophysiology experiments (Ludewig et al. 2002; Mayer et al. 2006). Work on plant family AMT1 genes, which fall into the AMT-Euk clade, demonstrates that transport in this group is electropositive, meaning either that ammonium is transported or that ammonia and a proton are cotransported (Mayer and Ludewig 2006).

We raise the possibility that each of these evolutionarily ancient families of AMTPs functions in a unique manner: MEP proteins strip the proton and transport ammonia, Rh proteins transport ammonia and/or CO_2 without deprotonation and reprotonation, and AMT-Euk proteins transport ammonium or cotransport ammonia and a proton (fig. 2). Another interpretation could be that, after land plants acquired a MEP AMTP, the native AMT-Euk copy was freed to neofunctionalize into a true AMT, such that AMTs are restricted to a subset of AMT-Euk lineages related to plants. Electrophysiological experiments on members of the AMT-Euk clade that are distantly related to plants are needed to explore the mode of action of these proteins (fig. 2).

Nomenclatural Notes

From the moment of publication in 1994, both MEP (Marini et al. 1994) and AMT (Ninnemann et al. 1994) have been used to name functionally characterized AMTPs. Because the name AMT was applied to a sequence that falls into the eukaryotic clade (AMT-Euk, fig. 2) and because MEP was applied to a sequence falling into the other clade (MEP, fig. 2), we have named our clades for these "founding" sequences. This clade nomenclature emphasizes not only the phylogenetic distinctness of the gene families but also the functional differences found so far between

characterized members of each clade: Bona fide AMTs showing electropositive transport all fall in the AMT clade and ammonia permeases showing electroneutral transport fall into the MEP clade. Already in the molecular literature, the designation AMT/MEP/Rh is in wide use. This designation corresponds well to the functional classes already recognized and also proposed here.

Supplementary Material

Supplementary fig. S1 and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournalsonline.org/).

Acknowledgments

We would like to thank Duke IGSP, especially Huntington Willard and Gregory Wray, for funding support for the *C. grayi* and *Asterochloris* sp. genome projects and Daniele Armaleo for his contribution to the original discovery of MEP α in *C. grayi*. We are also very grateful to D. Armaleo for his guidance and critical reading of this manuscript. We would like to thank Charles Hall and Erik McDonald for additional critical comments on the manuscript and Jolanta Miadlikowska for assistance with graphics software. This project was also funded in part by a subcontract (112442) to D. Armaleo, F.D., and F.L. as part of the Pacific Northwest National Laboratory foundational scientific focus area under the Department of Energy Biological and Environmental Research's genomic sciences program in collaboration with Scott Baker and Jon Magnuson. This work would not have been possible without the Duke Shared Cluster Resource and outstanding services provided by John Pormann and Tom Milledge.

References

Ahmadjian V. 1993. The lichen symbiosis. New York: John Wiley.

Andrade SL, Dickmanns A, Ficner R, Einsle O. 2005. Crystal structure of the archaeal ammonium transporter Amt-1 from *Archaeoglobus fulgidus*. *Proc Natl Acad Sci U S A*. 102:14994–14999.

Armaleo D, May S. 2009. Sizing the fungal and algal genomes of the lichen *Cladonia grayi* through quantitative PCR. *Symbiosis* 49:43–51.

Armaleo D, Sun X, Culberson C. 2011. Insights from the first putative biosynthetic gene cluster for a lichen depside and depsidone. *Mycologia* 103; 0; 10-335v1-10-335.

Bhattacharya D, Yoon HS, Hedges SB, Hackett JD. 2009 Eukaryotes (Eukaryota). In: Hedges SB, Kumar S, editors. The timetree of life. New York: Oxford University Press. p 116–120.

Blair JE. 2009. Fungi. In: Hedges SB, Kumar S, editors. The timetree of Life. New York: Oxford University Press. p 215–219.

Brock TD, Brock KM, Belly RT, Weiss RL. 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol*. 84:54–68.

Clark DA, Norris PR. 1996. *Acidimicrobium ferrooxidans* gen. nov., sp. nov.: mixed-culture ferrous iron oxidation with *Sulfobacillus* species. *Microbiology* 142:785–790.

Couturier J, Montanini B, Martin F, Brun A, Blaudez D, Chalot M. 2007. The expanded family of ammonium transporters in the perennial poplar plant. *New Phytol*. 174:137–150.

Dopson M, Baker-Austin C, Hind A, Bowman JP, Bond PL. 2004. Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments. *Appl Environ Microbiol*. 70:2079–2088.

Futterer O, Angelov A, Liesegang H, Gottschalk G, Schleper C, Schepers B, Dock C, Antranikian G, Liebl W. 2004. Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proc Natl Acad Sci U S A*. 101:9091–9096.

Gogarten JP, Doolittle WF, Lawrence JG. 2002. Prokaryotic evolution in light of gene transfer. *Mol Biol Evol*. 19:2226–2238.

Goltsman DS, Denef VJ, Singer SW, et al. (17 co-authors). 2009. Community genomic and proteomic analyses of chemoautotrophic iron-oxidizing "*Leptospirillum rubarum*" (Group II) and "*Leptospirillum ferrodiazotrophum*" (Group III) bacteria in acid mine drainage biofilms. *Appl Environ Microbiol*. 75:4599–4615.

Gruswitz F, Chaudhary S, Ho JD, Schlessinger A, Pezeshki B, Ho CM, Sali A, Westhoff CM, Stroud RM. 2010. Function of human Rh based on structure of RhCG at 2.1 A. *Proc. Natl Acad Sci U S A*. 107:9638–9643.

Gueidan C, Roux C, Lutzoni F. 2007. Using a multigene phylogenetic analysis to assess generic delineation and character evolution in *Verrucariaceae* (Verrucariales, Ascomycota). *Mycol Res*. 111:1145–1168.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. 52:696–704.

Hibbett DS, Binder M, Bischoff JF, et al. (67 co-authors). 2007. A higher-level phylogenetic classification of the Fungi. *Mycol Res*. 111:509–547.

Huang CH, Peng J. 2005. Evolutionary conservation and diversification of Rh family genes and proteins. *Proc Natl Acad Sci U S A*. 102:15512–15517.

Itoh T, Suzuki K, Sanchez PC, Nakase T. 1999. *Caldivirga maquilingensis* gen. nov., sp. nov., a new genus of rod-shaped crenarchaeote isolated from a hot spring in the Philippines. *Int J Syst Bacteriol*. 49(Pt 3):1157–1163.

James TY, Kauff F, Schoch CL, et al. (70 co-authors). 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 443:818–822.

Javelle A, Andre B, Marini AM, Chalot M. 2003. High-affinity ammonium transporters and nitrogen sensing in mycorrhizas. *Trends Microbiol*. 11:53–55.

Javelle A, Lupo D, Zheng L, Li XD, Winkler FK, Merrick M. 2006. An unusual twin-his arrangement in the pore of ammonia channels is essential for substrate conductance. *J Biol Chem*. 281:39492–39498.

Keeling P, Leander BS, Simpson A. 2009. Eukaryotes. Eukaryota, Organisms with nucleated cells. Version 28 October 2009. Available from: http://tolweb.org/Eukaryotes/3/2009.10.28. In The Tree of Life Web Project. [cited 2011 May 27]. Available from: http://tolweb.org.

Kelly DP, Wood AP. 2000. Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *Int J Syst Evol Microbiol*. 50(Pt 2):511–516.

Khademi S, O'Connell J 3rd, Remis J, Robles-Colmenares Y, Miercke LJ, Stroud RM. 2004. Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 A. *Science* 305:1587–1594.

Kustu S, Inwood W. 2006. Biological gas channels for NH3 and CO2: evidence that Rh (Rhesus) proteins are CO2 channels. *Transfus Clin Biol*. 13:103–110.

Lamoureux G, Javelle A, Baday S, Wang S, Berneche S. 2010. Transport mechanisms in the ammonium transporter family. *Transfus Clin Biol*. 17:168–175.

Lamoureux G, Klein ML, Berneche S. 2007. A stable water chain in the hydrophobic pore of the AmtB ammonium transporter. *Biophys J*. 92:L82–L84.

Li X, Jayachandran S, Nguyen HH, Chan MK. 2007. Structure of the *Nitrosomonas europaea* Rh protein. *Proc Natl Acad Sci U S A*. 104:19279–19284.

Li X, Lupo D, Zheng L, Winkler F. 2006. Structural and functional insights into the AmtB/Mep/Rh protein family. *Transfus Clin Biol*. 13:165–169.

Lopez-Pedrosa A, Gonzalez-Guerrero M, Valderas A, Azcon-Aguilar C, Ferrol N. 2006. GintAMT1 encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices*. *Fungal Genet Biol*. 43:102–110.

Lorenz MC, Heitman J. 1998. The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *EMBO J*. 17:1236–1247.

Ludewig U, von Wiren N, Frommer WB. 2002. Uniport of NH_4^+ by the root hair plasma membrane ammonium transporter LeAMT1;1. *J Biol Chem*. 277:13548–13555.

Lutzoni F, Kauff F, Cox JC, et al. (44 co-authors). 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *Am J Bot*. 91:1446–1480.

Lutzoni F, Pagel M, Reeb V. 2001. Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 411:937–940.

Maddison DR, Maddison WP. 2005. MacClade 4. Sunderland (MA): Sinauer Associates.

Marcet-Houben M, Gabaldon T. 2010. Acquisition of prokaryotic genes by fungal genomes. *Trends Genet*. 26:5–8.

Marini AM, Soussi-Boudekou S, Vissers S, Andre B. 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 17:4282–4293.

Marini AM, Urrestarazu A, Beauwens R, Andre B. 1997. The Rh (rhesus) blood group polypeptides are related to NH_4^+ transporters. *Trends Biochem Sci*. 22:460–461.

Marini AM, Vissers S, Urrestarazu S, Andre B. 1994. Cloning and expression of the MEP1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae*. *EMBO J*. 13(15):3456–3463.

Mayer M, Dynowski M, Ludewig U. 2006. Ammonium ion transport by the AMT/Rh homologue LeAMT1;1. *Biochem J*. 396:431–437.

Mayer M, Ludewig U. 2006. Role of AMT1;1 in NH_4^+ acquisition in Arabidopsis thaliana. *Plant Biol (Stuttg)*. 8:522–528.

McDonald SM, Plant JN, Worden AZ. 2010. The mixed lineage nature of nitrogen transport and assimilation in marine eukaryotic phytoplankton: a case study of *Micromonas*. *Mol Biol Evol*. 27:2268–2283.

Monahan BJ, Fraser JA, Hynes MJ, Davis MA. 2002. Isolation and characterization of two ammonium permease genes, *meaA* and *mepA*, from *Aspergillus nidulans*. *Eukaryot Cell*. 1:85–94.

Montanini B, Moretto N, Soragni E, Percudani R, Ottonello S. 2002. A high-affinity ammonium transporter from the mycorrhizal ascomycete *Tuber borchii*. *Fungal Genet Biol*. 36:22–34.

Ninnemann O, Jauniaux JC, Frommer WB. 1994. Identification of a high affinity NH_4^+ transporter from plants. *EMBO J*. 13(15):3464–3471.

Nylander JAA. 2004. MrModeltest v2. Program distributed by the author. Uppsala (Sweden): Evolutionary Biology Centre, Uppsala University.

Posada D. 2008. jModelTest: phylogenetic model averaging. *Mol Biol Evol*. 25:1253–1256.

Richards TA, Soanes DM, Foster PG, Leonard G, Thornton CR, Talbot NJ. 2009. Phylogenomic analysis demonstrates a pattern of rare and ancient horizontal gene transfer between plants and fungi. *Plant Cell*. 21:1897–1911.

Rutherford JC, Lin X, Nielsen K, Heitman J. 2008. Amt2 permease is required to induce ammonium-responsive invasive growth and mating in Cryptococcus neoformans. *Eukaryot Cell*. 7:237–246.

Schleper C, Puehler G, Holz I, Gambacorta A, Janekovic D, Santarius U, Klenk HP, Zillig W. 1995. *Picrophilus* gen. nov., fam. nov.: a novel aerobic, heterotrophic, thermoacidophilic genus and family comprising archaea capable of growth around pH 0. *J Bacteriol*. 177:7050–7059.

Schleper C, Puhler G, Klenk HP, Zillig W. 1996. *Picrophilus oshimae* and *Picrophilus torridus* fam. nov., gen. nov., sp. nov., two species of hyperacidophilic, thermophilic, heterotrophic, aerobic archaea. *Int J Syst Bacteriol*. 46:814–816.

Schoch CL, Sung GH, Lopez-Giraldez F, et al. (64 co-authors). 2009. The Ascomycota tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Syst Biol*. 58:224–239.

She Q, Singh RK, Confalonieri F, et al. (31 co-authors). 2001. The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *Proc Natl Acad Sci U S A*. 98:7835–7840.

Simmons EG. 1992. Alternaria taxonomy: current status, viewpoint, challenge. In: Chelkowski J, Visconti A, editors. *Alternaria*: biology, plant diseases and metabolites. New York: Elsevier. p 1–36.

Slot JC, Hibbett DS. 2007. Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study. *PLoS One*. 2(10):e1097.

Sohlenkamp C, Wood CC, Roeb GW, Udvardi MK. 2002. Characterization of Arabidopsis AtAMT2, a high-affinity ammonium transporter of the plasma membrane. *Plant Physiol*. 130:1788–1796.

Stamatakis A, Ludwig T, Meier H. 2005. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics* 21:456–463.

Suzuki T, Iwasaki T, Uzawa T, Hara K, Nemoto N, Kon T, Ueki T, Yamagishi A, Oshima T. 2002. *Sulfolobus tokodaii* sp. nov. (f. *Sulfolobus* sp. strain 7), a new member of the genus *Sulfolobus* isolated from Beppu Hot Springs, Japan. *Extremophiles* 6:39–44.

Swofford DL. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sunderland (MA): Sinauer Associates.

Trembley ML, Ringli C, Honegger R. 2002. Morphological and molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. *Mycol Res*. 106(7):768–776.

Valdes J, Quatrini R, Hallberg K, Dopson M, Valenzuela PD, Holmes DS. 2009. Draft genome sequence of the extremely acidophilic bacterium *Acidithiobacillus caldus* ATCC 51756 reveals metabolic versatility in the genus *Acidithiobacillus*. *J Bacteriol*. 191:5877–5878.

Winkler FK. 2006. Amt/MEP/Rh proteins conduct ammonia. *Pflugers Arch*. 451:701–707.

Yang H, Xu Y, Zhu W, Chen K, Jiang H. 2007. Detailed mechanism for AmtB conducting NH4+/NH3: molecular dynamics simulations. *Biophys J*. 92:877–885.

Zheng L, Kostrewa D, Berneche S, Winkler FK, Li XD. 2004. The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. *Proc Natl Acad Sci U S A*. 101:17090–17095.

Zolan ME, Pukkila PJ. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. *Mol Cell Biol*. 6:195–200.