INTRODUCTION

Nitrogen is a limiting nutrient in most forest soils, usually obtained in the form of nitrate. Acquisition of nitrate is mediated by NRT2, a high affinity transporter of nitrate in prokaryotes, viridiplantae and fungi. The diversity of *nrt2* in fungi is not well characterized. We propose to analyze the molecular and functional evolution of NRT2 in mushroom-forming fungi. In preliminary studies, we have successfully amplified and sequenced putative *nrt2* homologs from several different clades of basidiomycetes in *Hebeloma* and related taxa. Phylogenetic reconstructions suggest a recent duplication in *Hebeloma nrt2* followed by functional divergence. Phylogenies inferred using *nrt2* sequences do not conflict with established phylogenies and may provide improved resolution at higher taxonomic levels.

Studies in the ascomycete *Aspergillus* revealed two *nrt2* copies with different affinities for binding nitrate, although until this point there has been no indication of functional divergence in other fungi. Fine regulation of nitrate uptake is essential because of rapid shifts in soil nitrate concentration, and mechanisms of *nrt2* regulation appear to be diverse in Basidiomycete fungi (Jargeat et al., 2003). Jargeat et al. (2003) began to characterize the structure and expression of a putative *nrt2* homolog in *Hebeloma cylindrosporum,* which (with its host, *Pinus pinaster*) is a model system for functional studies of ectomycorrhizal symbioses with tree roots. The genus *Hebeloma* is nested within a clade that also includes many decay fungi (saprotrophs). *Hebeloma* species inhabit niches with diverse and sometimes extreme nitrogen conditions, and are known as early ectomycorrhizal colonizers of disturbed landscapes. A comparative phylogenetic database is available for *Hebeloma* based on rDNA internal transcribed spacers (Aanen et al., 2000). For these reasons, *Hebeloma* is an ideal genus in which to study *nrt2* and ecological transitions from a phylogenetic perspective. The proposed research has two major goals:

1. **Characterize diversity and evolutionary origin of *nrt2* homologs in *Hebeloma* and related taxa**. The results of this research will provide insight into the evolution of an ecologically important family of nitrate transporters, and it will provide new phylogenetic markers for *Hebeloma* and other Basidiomycota.
2. **Address evidence of functional diversification in *nrt*2:** Real time RT PCR studies will enable us to suggest specific roles for alternative forms of NRT2, and address the functional evolution of the *nrt2* gene in association with ecological transitions.

BACKGROUND INFORMATION

Diversity and gene phylogeny of NRT2

<u>Nrt2 *homologs are broadly distributed*</u>. The NRT2 protein belongs to the Nitrate/Nitrite Porter Family in the Major Facilitator Superfamily of transporters involved with the transport of various solutes. The NRT2 branch of the MFS apparently diverged early, as homologs exist in prokaryotes, Viridiplantae and Fungi (Pao, et al, 1998). Within the Fungi, *nrt*2 homologs have been discovered in the ascomycetes, *Hansenula*, *Aspergillus, Gibberella, Neurospora,* and *Tuber*, and in the basidiomycetes, *Hebeloma*, *Ustilago*, and *Phanerochaete* (Perez et al., 1997; Unkles et al., 2001; Gao-Rubinelliet al., 2004; Montanini et al., 2005; Jargeat et al., 2003). *Nrt*2 has also been found in the green algae *Chlamydomonas* and *Chlorella*, in 14 genera of angiosperms, including dicots (e.g., *Aradbidopsis, Glycine*) and monocots (e.g., *Hordeum*, *Phragmites*), two genera of diatoms, and several prokaryotes (Quesada et al., 1998; Orsel et al., 2002; Vidmar et al., 2000; Hildebrandtet al., 2002; Koltermann et al., 2003; Amarasingh et al., 1998; Prosser et al., 2005; Faure-Rabasse et al., 2002; Fraisier et al., 2000; Araki et al., 2005; Collier et al., 2003; Pao, et al., 1998).

<u>*Fungal* nrt*2 sequences form a clade*</u>. *Aspergillus* contains three *nrt*2 isoforms, but only one isoform has been dicovered in each of the other fungi that have been studied

(references above). *Arabidopsis* and *Hordeum* have 7-10 isoforms, but most other Viridiplantae have only one or two isoforms. It is clear that *nrt*2 has undergone multiple duplication events, some of which may have occurred prior to the divergence of certain angiosperms (Orsel et al., 2002), but the precise timing of these duplication events is not well understood. Nevertheless, the few phylogenetic analyses with both plant and fungal *nrt*2 sequences suggest that the fungal homologs have a single origin distinct from Viridiplantae sequences (Orsel et al., 2002), and this has been confirmed in our own preliminary analyses (not shown). The basic relationships of *nrt*2 in fungi are consistent with hypotheses of fungal phylogeny based on other genes (e.g., Lutzoni et al, 2004).

Structure and Function of NRT2

The NRT2 protein is structurally similar to other MFS transporters. All members of the Major Facilitator Superfamily bear key structural elements, such as 12 transmembrane helices, a putative kinase domain associated with active transport and other signature amino acid motifs whose function is not known (Hirai et al., 2003). A nitrate signature sequence, found in all nitrate transporters, but not other MFS families is found in transmembrane helices 5 and 11 in fungi (Unkles et al, 2004). A large intracellular loop between helices 6 and 7 appears to be unique to fungal NRT2 proteins, and contains one conserved putative kinase motif unlike in plants where one site is located between helices 10 and 11 and one in the C-terminal tail lacking in fungi (Forde, 2000). Mutational analyses in *Aspergillus* have revealed 2 essential, highly conserved Arginine residues, suggested to be involved in nitrate-binding (Unkles et al., 2004), while 8 cysteine residues were individually found to be unessential to transporter function (Unkles et al., 2005). More specific links between structural motifs and transporter function are lacking.
NRT2 isoforms have different affinities for nitrate and regulation of the nrt*2 gene is diversified*. In *Aspergillus nidulans* and *Chlamydomonas reinhardtii*, net nitrate influx analyses with mutants deficient in one or another nitrate transporter have shown that there are 10-fold differences in affinity for nitrate between two isoforms, although expression profiles were not distinguishable with Northern analyses (Unkles et al., 2001). Such findings indicate that each isoform functions optimally in a distinct range of environmental nitrate. There are no reports of differential nitrate affinities in other fungi. Expression of most *nrt2* genes in plants and fungi is shown to be inducible by the presence of nitrate (Unkles et al., 2001, Montanini et al., 2005, Vidmar et al., 2000 Rexach et al., 1999, Orsel et al.,2002, Koltermann et al., 2003), and different isoforms were shown to be differentially expressed in various tissues and nitrogen conditions (Orsel, et al, 2002). In both ectomycorrhizal species investigated, up-regulation was observed in response to both nitrogen starvation and nitrate feeding. (Montanini et al., 2005, Jargeat et al., 2003). Expression in *H. cylindrosporum,* however*,* was also repressed by ammonium (Jargeat et al., 2003). It is not known if this mode of regulation is unique to *H. cylindrosporum,* an inferred ammonium fungus with a reliance on more reduced nitrogen sources, or if it is a general property of *Hebeloma* and possibly other genera.
NRT2 is concentrated in mycorrhizae in Tuber**:** Studies using immunofluorescence microscopy have suggested that TbNRT2 from *Tuber borchii* is most concentrated at the interface of specialized fungal hyphae and the plant roots (Montanini et al., 2005). This suggests functional interaction between the host and symbiont with regard to *nrt2* expression. It is not known why these cells should have elevated levels NRT2. Comparable studies have not been performed in other mycorrhizal systems.

Systematics and Ecology of *Hebeloma*

Hebeloma *(Cortinariaceae, Agaricales) is diverse and its phylogeny is not well resolved.* *Hebeloma* forms mycorrhizal associations with a range of angiosperms and conifers (Aanen et al., 2001). Species of *Hebeloma* produce agaricoid badidiocarps (i.e. gilled mushrooms) with white to yellow or red-brown pigmentation, serrated gill edges, club-

shaped cheilocystidia (sterile cells on gill edges), and smooth or ornamented almond-shaped spores that are dull-brown (and in exception, reddish) in deposit. Some *Hebeloma* fruit-bodies have a veil, and others do not (Smith et al., 1983). *Hebeloma* species are dispersed throughout the world's temperate regions (Aanen et al., 2000). Murrill (1917) described 49 species of *Hebeloma* in North America, some of which have been subsequently transferred to other genera. Smith (1984) revised *Hebeloma* in the Great Lakes Region of North America and included several new species. There is ongoing taxonomic work to determine the number of *Hebeloma* species and genus boundaries in Europe (U. Eberhardt, Pers. Comm.) and Asia (Yang et al., 2005). Many ecologically notable collections have been made in Japan and Australia (Suzuki et al., 2003). There have been no molecular systematics studies of *Hebeloma* in North America.

The broadest molecular study of *Hebeloma* used nuclear ribosomal gene sequences to show support for 11 minor clades and one major clade. The few parsimony-informative characters in the internal transcribed spacers of ribosomal DNA provide little support for higher-level relationships among these 11 clades (Aanen et al., 2000). We suggest that the recent shift to mycorrhizal status in *Hebeloma* has facilitated a rapid radiation of species that contributes to the poor phylogenetic resolution within the group and morphologically cryptic species found by Aanen et al. (2000). A recent study using nuclear rDNA by Yang et al. (2005) suggested that the genus *Anamika* may be nested within *Hebeloma*, although the monophyly of *Hebeloma* could not be rejected.

Hebeloma *species are known as early mycorrhizal colonizers and nitrophilic species are reported from multiple clades.* *Hebeloma* mycorrhizae and fruit bodies are among the first fungi identified in post-fire succession and inorganic nitrogen deposition (Lilleskov et al., 2002). Sagara (1995) suggested that *H. radicosum* is involved in an inorganic nitrogen "cleaning symbiosis" with mole latrines. Reports of *Hebeloma* in high-nitrogen habitats come from multiple clades (Suzuki et al., 2003), although no published reports of nitrophilic species from the *H. crustuliniforme/H. helodes clade* (Aanen et al., 2000) are known to the authors. It is not known whether possession of additional copies of *nrt2* in this clade is related to the apparent lack of nitrophilic species.

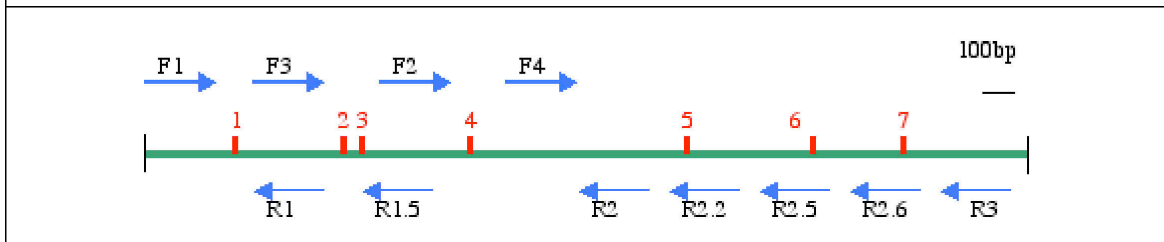
Hebeloma cylindrosporum *has become a model system for the study of nutritional dynamics in mycorrhizal fungi*. *H. cylindrosporum* and its host, *Pinus pinaster* are readily grown in aseptic dual culture, producing mycorrhizae in 14 days of inoculation of the host roots with fungal hyphae (Marmeisse et al., 2004, Wong and Fortin, 1989). *Hebeloma* can also be grown in isolated culture, which is an unusual property for ecotomycorrhizal fungi (Hutchinson, 1991). Consequently, *H. cylindrosporum* (with its host, *Pinus pinaster*) has become a model system for functional studies in ectomycorrhizal symbiosis. Results from such studies, however, may not always be directly applicable to other species because *H. cylindrosporum*, like a number of *Hebeloma* species, occupies extreme nitrogen niches. Ammonium fungi, such as *H. cylindrosporum* may lack a finely tuned nitrate transport system due to an inferred reliance on ammonium for nitrogen nutrition. The lack of alternate forms of NRT2 in *H. cylindrosporum* and its unique regulation could be more an indication of its nitrogen niche than taxonomic position. *Laccaria* and *Coprinopsis,* one mycorrhizal and one saprotrophic genus in the Agaricales have a diversity of nitrogen niches (Suzuki et al., 2003), making them excellent candidates for comparative studies of nitrogen metabolism.

PROGRESS TO DATE

Summary of preliminary results: We have generated *nrt*2 sequences from four genera of the Agaricales using degenerate primer PCR. We discovered a putative second locus in *Hebeloma helodes* that has a sister-group relationship with all other *nrt2* sequences in *Hebeloma*. Phylogenetic analyses suggest that *nrt*2 sequences provide improved resolution and support for higher-level relationships in *Hebeloma*.

We have successfully amplified and sequenced nrt2 *from 3 genera*. We designed degenerate and specific primers based on published sequences of fungal *nrt2*. We have successfully amplified and generated sequences of 1.4-2kb *nrt2*-homologous fragments from genomic DNA of all major clades of *Hebeloma*. Using these same primers, we have also obtained a homologous sequence from the related genus, *Gymnopilus*. We retrieved and assembled homologous sequences from the *Laccaria bicolor* and *Coprinopsis cinerea* (Agaricales) genome databases, and designed new degenerate primers based on the consensus of all fungal sequences, along with primers targeted to specific genera. We then successfully amplified and generated sequences of 1.2kb products from several *Laccaria* species. All homobasidiomycete sequences are readily alignable and share the same 7 putative intron splicing sites (Figure 1).

Figure 1. Map of 1.5 kb Open Reading Frame of *nrt2* in homobasidiomycetes. Splice sites and Primer binding sites are indicated. Primer locations and splice sites not to scale.

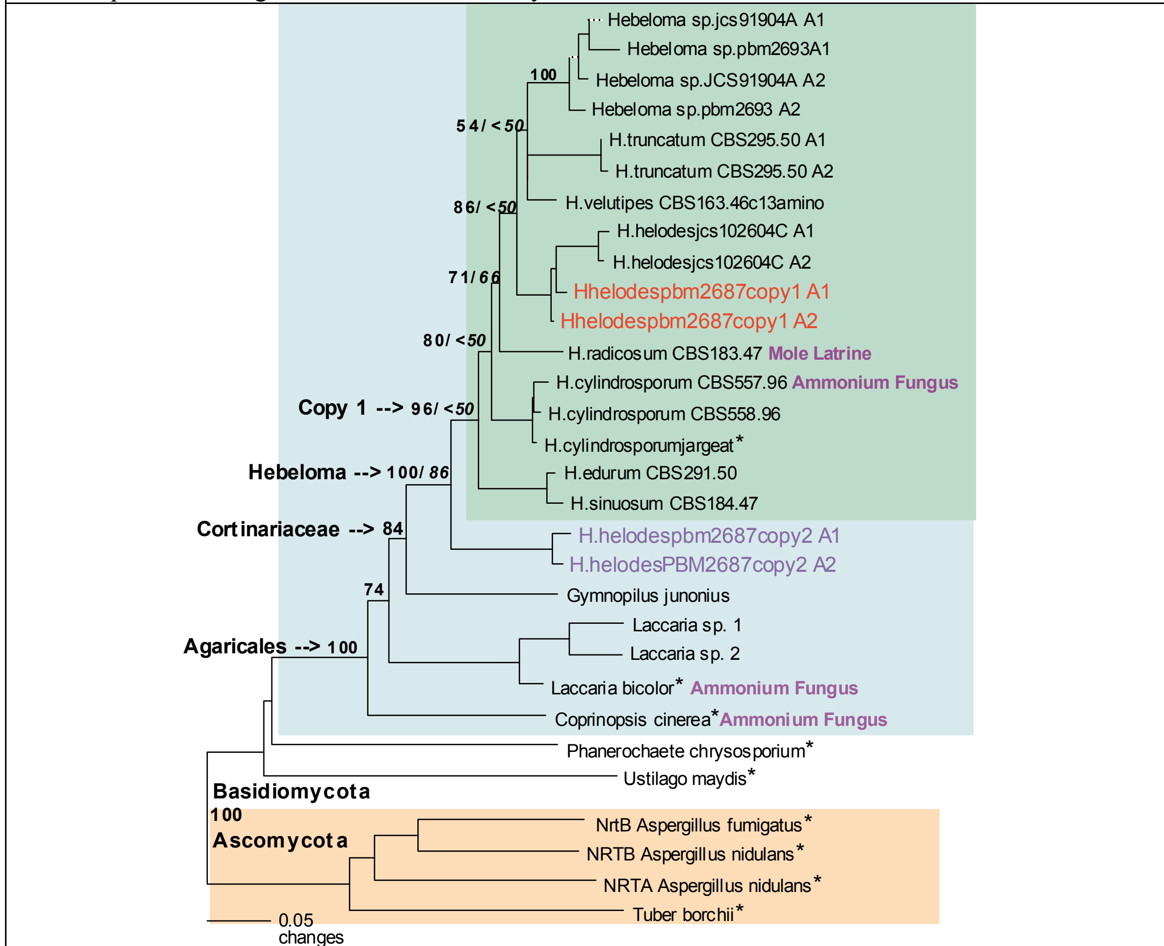


We discovered a putative second locus encoding nrt2 in Hebeloma helodes. We cloned portions of two distinct *nrt2* homologs (80% ORF identity) from *H. helodes*, using 2 different pairs of degenerate primers (F1/R2 and F1/R3, figure 1). We refer to homologs most similar to other *Hebeloma* sequences as *Hhnrt2* and the divergent copy as *Hhnrt2.2*. These sequences are easily distinguished by the difference in hydrophobic residues adjoining the putative site of nitrate binding in the inferred amino acid sequence (Unkles et al., 2001), and differences in other regions that are well-conserved in other *Hebeloma* sequences. We expect that a more thorough sampling will rule out the possibility of hybridization or horizontal transmission as the origin of the second copy. The presence of a second copy in *H. helodes* with a non-random pattern of substitution raises the possibility of functional specialization or differential regulation, as has been demonstrated in *Aspergillus* and *Chlamydomonas*.

We are able to use nrt2 *nucleotide and amino acid sequences to improve support for higher-level nodes in* Hebelom*a and to and to confirm higher level relationships in the fungi* We have constructed a data set that includes *nrt2* from prokaryotes, Viridiplantae and fungi that are alignable in the first half of the predicted open reading frame (ORF), but only the fungal data are presented here. We analyzed alignments of 60-90% of *nrt2* ORF in the fungi to improve resolution in the backbone of *Hebeloma* phylogeny. Figure 2. is a neighbor joining tree with bootstrap values from 5000 replicates using inferred amino acid sequences. Similar trees are obtained using maximum parsimony. Support values clearly suggest that *nrt2* is monophyletic in the basidiomycetes (100%), in the Agaricales (100%), and in the Cortinariaceae (89%). The inferred duplication occurred within *Hebeloma* (100%), suggesting that this was a recent, not ancient event. The relationship between *Phanerochaete* and *Ustilago* is not well resolved (54%), suggesting a limit to the phylogenetic utility of *nrt2*, although a broader sampling may improve resolution.

Figure 2. NRT2 provides stronger support for many nodes in the backbone of *Hebeloma* phylogeny than rDNA. Neighbor joining analysis of inferred amino acid sequences. The inner, green box delineates copy 1 in *Hebeloma*, the outer, aqua box delineates euagaric mushrooms and the lower, gold box

delineates Ascomycota. Bold support values are NRT2 bootstrap percentages and italic support values are rDNA bootstrap percentages (from Aanen et al., 2000) for respective nodes. Duplicates denoted by A1,A2 are presumed alleles. Sequences marked with asterisks were obtained from published sources. All other sequences were generated in our laboratory.



PROJECT DESIGN

Overview: This project has two major goals:
Goal 1. To characterize the diversity and evolutionary origin of NRT2 homologs in *Hebeloma* and related taxa and to test the phylogenetic utility of the gene.
Goal 2. To address evidence of functional diversification between putatively duplicated *nrt*2 loci, and between *nrt*2 copies in fungal lineages with alternate ecologies.

Proposed research in detail:
Goal 1. To characterize the diversity and evolutionary origin of NRT2 homologs in *Hebeloma* and related taxa and to test the phylogenetic utility of the gene. We propose to amplify and sequence *nrt2* from the major clades within the genera *Hebeloma*, *Laccaria* and *Coprinopsis*. We will use this database of sequences to address three questions regarding patterns of sequence variation and gene phylogeny.

Question 1A: *What is the sequence variation among nrt2 homologs?* Using techniques described above (see **Progress to Date**), we will expand our sampling of *nrt2* in *Hebeloma*, *Laccaria* and *Coprinopsis* to include a number of species from extreme nitrogen niches. These sequences will build and improve the phylogenies of the genera,

and also provide a framework for testing our hypothesis that nitrogen availability and form contribute to ecological isolation and species radiation in soil fungi.

Sampling: Table 1 displays the taxa to be sampled. *Hebeloma* taxa have been selected to thoroughly cover all clades detailed by the nuclear rDNA sequences of Aanen et al (2000). This will allow us to confirm species identity and investigate the higher level relationships in *Hebeloma*. All reported nitrophilic fungi to which we have access have been included for ecological transition analysis. Local collections are currently in our herbarium and culture collection. *Nrt2* sequences from *Laccaria* and *Coprinopsis* will be obtained from ongoing genome projects, as well as local collections of these genera, and will provide appropriate outgroups for phylogenetic analyses focused on *Hebeloma* (Figure W). In addition, *nrt2* sequences from species of *Laccaria* and *Coprinopsis* from different nitrogen niches will provide a useful comparison to *nrt2* sequences from ecologically diverse *Hebeloma* species.

Analysis: We will perform alignments and sequence analyses to address the level of DNA and amino acid sequence variation among the taxa in the dataset and identify regions of conservation associated directly with transporter function.

Table 1. Species to be sampled for *nrt2* phylogeny.

***Hebeloma* and related Cortinariaceae:** *H.helodes* clade IId (Aanen, 2000) *(1,2,3); H. cavipes* (1,3); *H. tomentosum* (1,2,3); *H. mesophaeum* (1,3,4);*H. cf affine* (1,3); *H. radicosum* (1,3,4);*H. cylindrosporum* (1,2,2*,3);*H. truncatum (1,2,3);H. edurum* (1,2,3); *H. velutipes* (1,2,3); *H. crustuliniforme* (1,2,3);*H. sarcophyllum* (1,3); *H. spoliatum* (3,4); *H. luchuense* (4); *H. bulbiferum*;*H. sinapizans*; *H. circinans*; *H. radicosoides* (3,4);*H. aminophilum* (3,4); *H.vinosophyllum* (3,4);*H. syrjense* (3,4);*Anamika angustulamellata* (1,3);*Gymnopilus junonius* (1,2,3).
***Laccaria* and *Coprinopsis*:** *L. amethystina* (1,2,3,4); *L. ochropurpurea* (1,3); *L. bicolor* (1,2*,3,4);*L. laccata* (1,2,3);*Coprinopsis cinerea* (1,2*,3,4); *Coprinopsis atramentaria* (1,3); *Coprinopsis lagopus* (1,3); *Coprinopsis phlyctidiospora* (3,4).
Key to availability of sequences and materials: 1 = ITS sequence already generated; 2 = *nrt*2 sequence already generated; 2* = *nrt*2 sequence available from published sources or genome projects; 3 = cultures, DNAs, and/or collections on hand; 4 = occurs in extreme nitrogen habit. ITS sequences of Aanen et al. (2000) are not listed.

Question 1B: *How many copies of nrt2 are present in* Hebeloma, *and how did they originate?*

Determimation of copy number in other major clades of *Hebeloma.* We will perform Southern hybridizations under low stringency with a combination of fluorescent probes specific to both copies in genomic DNA digested separately with 3 endonucleases that do not cut in the known probe region. Probes will be synthesized with the PCR DIG labeling system (Roche). Hybridized probes will be detected with anti-DIG visualized on X-ray film. Separate blots will be conducted under high stringency for each specific probe. These studies will be conducted with representative species from 6 major clades of *Hebeloma*. Genomic DNA for these analyses will be obtained from dried fruit bodies and living cultures.

Analysis: We will use hybridization patterns in the three endonuclease digests to determine the number of *nrt2*-related loci in each genome, and to determine which copies are present in each species. Determination of copy number will allow us to reconstruct the positions of gains and losses of *nrt2* homologs in *Hebeloma* phylogeny, and address the ecological significance of *nrt2* copy number.

Determination of copy location in the genome. We will perform inverse PCR (Triglia et al., 1988) by digesting genomic DNA with multiple endonucleases that do not cut within the known region of each gene copy. Potential enzymes may already be identified by our previous Southern hybridizations. Digests producing positive DNA fragments of appropriate size will be identified by an additional Southern hybridization. We will circularize fragments by ligation and perform PCR with pairs of copy-specific internal primers directed outward. The resulting products will be cloned into the TA

cloning vector (Invitrogen) to isolate alleles, and sequences generated to obtain the flanking region of each putative locus in *H. helodes.*

Analysis: Flanking regions will be compared with the paralogous copy and with databases of published sequences to determine whether the putative copies share a locus or occupy different loci in the genome. The presence of different flanking genes and unalignable regulatory sequences will suggest that these are independent loci, which we suspect to be the case based on the level of sequence divergence and the existence of two allelic forms of each copy.

Question 1C: *What is the phylogeny of* nrt*2 in* Hebeloma *and can we develop an improved species phylogeny using* nrt*2 combined with rDNA? Nrt2* and rDNA nucleotide (and amino acid where applicable) sequences will be aligned with Clustal X and adjusted manually using MacClade 4 (Maddison and Maddison, 2001). We will use tests of congruence (e.g. Shimodaira and Hasegawa, 1999) to assess conflict between the *nrt2* and rDNA gene phylogenies. If possible, we will combine the data sets, and develop a hypothesis of species phylogeny using distance and parsimony methods in PAUP 4.0. Confidence will be determined by distance and parsimony bootstrapping using PAUP*4.0 (Swofford, 2002) and Bayesian posterior probabilities using Mr.Bayes v 3.1.1 (Huelsenbeck and Ronquist, 2001) We will obtain new rDNA sequences if necessary to complement *nrt*2 sequences and improve support in the species phylogeny.

Analysis: We will use tree reconciliation methods (Page, 2001) to address the number and order of *nrt2* origins and losses in *Hebeloma.* We expect a single duplication of *nrt2* basal to *Hebeloma* and multiple losses corresponding to transition to reduced nitrogen niche. We will use parsimony-based ancestral state reconstruction methods to infer the origins and losses of specific domains and domain qualities (i.e. calculated hydrophobicity around the putative binding domain) for correlation analysis with species ecology. We expect multiple gains of specific motifs linked to substrate binding based on current sequence data. We will conduct constraint analyses to test the monophyly of *Hebeloma.* We expect to confirm *Anamika* as sister to *Hebeloma*.

Goal 2. To address evidence of functional diversification between putatively duplicated nrt2 loci, and between nrt2 copies in fungal lineages with alternate ecologies. We will compare expression profiles of each copy of *nrt*2 in *H. helodes* to determine if there is evidence of functional differentiation at the regulatory level. We expect expression to differ based on form and availability of nitrogen in the environment because functional divergence is linked to ecological plasticity (Unkles et al., 2001).We will assess the mode of *nrt*2 regulation as character states to be mapped onto the organismal phylogeny of *Hebeloma* and outgroups along with ecological niche. This will enable us to expose evidence of the selective pressure of nitrogen form and availability on ecological diversification in soil fungi. We expect that a species' *nrt*2 expression profile is correlated with nitrogen niche, but not always with phylogeny or carbon niche.

Question 2A. *Does* H. helodes *grow in dual culture like* H.cylindrosporum*?* We will develop a protocol based on previous work (Wong et al., 1989, Marmeisse et al., 2004) to grow *H. helodes* in dual petri dish culture with its host, *Quercus sp*., in order to control for the effect of symbiosis on expression patterns. Roots of aseptically germinated *Quercus* seedlings (acorns found near fruiting *H. helodes*) will be placed in petri dishes slit for the protrusion of the shoot. Culture medium will be inoculated with dikaryotic clones of *H. helodes* mycelium.and incubated under light/dark cycle for 14 days to establish mycorrhizae. These dual cultures will be used for expression analysis under an array of nitrogen treatments.

Analysis: We will determine the success of dual culture by light microscopy of cross-sections of ectomycorrhizal structures in the culture medium. Identity of mycorrhizal symbiont will be confirmed by rDNA sequences.

Question 2B *Is there evidence of functional divergence between alternative* nrt*2 loci?* We will develop primers specific to each of the putative loci of *nrt2* in *Hebeloma*.

We have designed candidate primers for amplicons of 250 and 300bp (78% sequence identity) for *Hhnrt2* and *Hhnrt2.2* respectively in order to identify transcripts and confirm copy distinction by melting curve. Primers will be designed around splice sites and genomic DNA contamination will be assessed by PCR amplification and gel visualization. Relative levels of expression under different nitrogen conditions will be determined by a two step process. The first step will involve first strand synthesis of cDNA using mixed oligo-dT and random hexamer primers from hyphae and mycorrhizae grown in culture. The second step will be real time PCR amplification of the targeted gene copies and nuclear large subunit rDNA and actin controls quantitated by fluorometric intensity of SYBR Green flurorophore (Qiagen) in an MX3000p thermal cycler (Stratagene). Standard curves will be generated for each primer pair to determine amplification efficiency.

<u>Analysis</u>: Relative changes in steady state gene expression under different nitrogen conditions will be determined by the $\Delta\Delta Ct$ method (Livak and Schmittgen, 2001) with actin and rDNA controls. Alternate controls will be assessed independently. This will enable us to test the hypothesis that the genes are under differential regulation, in addition to providing evidence of the specific role of each putative isoform. Additionally, we will test our hypothesis that mycorrhization leads to constitutive functioning of both genes because of excessive demand for nitrogen. This same experiment can be conducted on a species in a different nitrogen niche if putative alternative loci are discovered.

Question 2C: *Is there evidence of functional diversification among orthologs in lineages with different ecologies?* Primers targeted to the universal copy of *nrt2* will generate a 250bp amplicon in real time PCR (as described above) of mRNA from cultures of fungi from selected carbon/nitrogen niches on different nitrogen substrates. Taxa to be sampled: *Hebeloma cylindrosporum, H. helodes, H. vinosophyllum, H.velutipes, Laccaria bicolor, L. laccata, Coprinopsis cinerea, C. micaceous.*

<u>Analysis:</u> We will calculate relative expression levels of *nrt2* from each niche as described above. The following characters will be mapped onto a species phylogeny and used to reconstruct ancestral states: 1. repression by ammonium 2. induction by nitrate 3. up-regulation during starvation. Ancestral state reconstruction showing multiple origins of nitrophilic habit and associated regulation patterns affirm our hypothesis that mode of *nrt2* regulation is correlated with nitrogen niche due to selective pressures, but not always with phylogeny or carbon niche.

Timetable for completion of the work

Jun. 2006-Dec. 2006: PCR, inverse PCR, cloning, Southern hybridization, and sequence analysis.

Jan. 2007- Dec. 2007: Dual culture and real time RT PCR analyses.

Jan. 2008-May 2008: Preparation of dissertation and manuscripts.

Scientific significance of the proposed research

Intellectual merit: This project will use a protein coding gene to complement ribosomal DNA in systematics of homobasidiomycetes. This will be the first detailed phylogenetic study of *nrt2* conducted within and between genera of Fungi. The results of this work will be of general interest to ecologists and evolutionary biologists, specifically as it pertains to ecologically significant nitrate transporters across ecological niches.

Broader impacts: The results of this research will be relevant to soil science, forest science, and agriculture, because human-generated nitrogen is implicated in the decline in biodiversity and ecosystem function. By understanding how nitrogen has driven evolution of extant species, we will be in a better position to assess how certain pollutants may shape future ecosystems. Finally, this project will enable the doctoral studies of the Co-PI, and will engage undergraduate researchers.