*Mycologia*, 103(6), 2011, pp. 1267–1276. DOI: 10.3852/11-022


# A six-gene phylogeny reveals the evolution of mode of infection in the rice blast fungus and allied species

Ning Zhang[1]
Shuang Zhao

*Department of Plant Biology and Pathology, Foran Hall 201, Rutgers University, New Brunswick, New Jersey 08901*

Qirong Shen

*College of Resource and Environmental Sciences, Nanjing Agriculture University, Weigang 1, Nanjing, Jiangsu Province, P.R. China*

***Abstract:*** The family Magnaporthaceae contains devastating fungal cereal and grass pathogens, such as *Magnaporthe oryzae* (rice blast fungus, formerly known as *M. grisea*), *M. poae* (summer patch pathogen of turf grasses) and *Gaeumannomyces graminis* (take-all fungus of various cereals and grasses), which are popular model organisms in fungal biology and host-pathogen interaction studies. Despite their ecological and economic importance, the phylogenetic relationships among the constituent species remain ambiguous due to the lack of convincing morphological characters and paucity of molecular data for the majority of the non-model species in the family. In this study our multilocus phylogeny suggests that both *Magnaporthe* and *Gaeumannomyces* are polyphyletic genera. The phylogeny also provides insights into fungal biology and pathogenesis. *Magnaporthe oryzae* formed a basal clade, while *M. poae* and *M. rhizophila* formed another well supported clade with *G. incrustans* and *G. graminis*. The basal species infect both root and aerial parts of the plant host, while the aerial infection capacity seems to be lost in the taxa of the latter clade. The phylogeny is corroborated by evolution of the anamorphs and a cAMP-dependent protein kinase (*CPKA*) gene. *Magnaporthe oryzae* produces *Pyricularia*, while taxa in the latter clade all produce *Phialophora*-like anamorphs. *CPKA* is present in animals and many fungal lineages with various functions. In *M. oryzae CPKA* is essential for the formation of functional appressoria for leaf penetration. In root-infecting *G. graminis* var. *tritici* and *M. poae* however only non-functional *CPKA* homologous pseudogenes were found in their genomes. The study indicates that anamorphic and ecological features are more informative than the teleomorphic characters in defining monophyletic groups among these taxa.

***Key words:*** *CPKA*, *Gaeumannomyces*, Magnaporthaceae, *Magnaporthe*, root pathogen, systematics, systemic infection, taxonomy

Submitted 14 Jan 2011; accepted for publication 4 Apr 2011.
[1] Corresponding author. E-mail: zhang@aesop.rutgers.edu

## INTRODUCTION

In 1876 stem rot, a new disease of rice, was recorded in Italy and the causal fungus was described as *Sclerotium oryzae* based on its sclerotial state (Cattaneo 1876). In the same paper Cattaneo also described *Leptosphaeria salvinii*, which was finally recognized as the teleomorph of the same fungus 57 y later (Tullis 1933). Based on morphological and ontogenetic studies, Krause and Webster established a new genus, *Magnaporthe*, in the order Diaporthales to accommodate this fungus as *M. salvinii* (Krause and Webster 1972). Since then four more species have been placed in this genus based on teleomorph morphology: *M. grisea*, *M. rhizophila*, *M. poae* and *M. oryzae* (Kirk et al. 2008). All five species are widespread pathogens of cereals and grasses in the Poaceae.

The best studied *Magnaporthe* species is the blast fungus *M. oryzae* (recently segregated from *M. grisea*), which is one of the most devastating threats to food security worldwide (Couch and Kohn 2002, Ou 1985, Sesma and Osbourn 2004). This fungus is better known for its anamorph, *Pyricularia oryzae*, a paradigm for understanding fungal aerial infection. Its root-infecting capacity was not discovered until 2004 when Sesma and Osbourn first described the mechanism of root entry—a completely new facet in the life cycle of this species (Sesma and Osbourn 2004). *Magnaporthe poae* and *M. rhizophila*, in contrast, are root infecting only. The rice blast fungus enters the host leaves with a sophisticated method that increases turgor pressure in a melanized appendage (the appressorium) to force an infection peg through the leaf surface into the plant (Howard and Valent 1996).

It has been demonstrated that cyclic AMP (cAMP)-mediated signaling processes (e.g. *CPKA* gene) are essential for the formation of functional appressoria for leaf penetration in *M. oryzae* but which are not required for penetration of roots (Sesma and Osbourn 2004, Xu et al. 1997). Analysis of *CPKA* evolution among *M. oryzae* and related species may shed light on the polarity of fungal pathogenicity.

*Gaeumannomyces* is another grass-associated genus that had been placed in the Diaporthales. The type species, the take-all fungus *G. graminis*, originally was described as *Rhaphidophora graminis* (Saccardo 1875) and later placed in *Gaeumannomyces* in the Diaporthales based on developmental studies of the teleomorph (von Arx and Olivier 1952). The diagnostic feature that distinguishes *Magnaporthe* and *Gaeumannomyces* is the ascospore shape, fusiform in *Magnaporthe* vs. filiform in *Gaeumannomyces* (TABLE I). In addition ascospores of *Magnaporthe* species are often versicolorous. Currently there are 13 species in *Gaeumannomyces*, all of which are root colonizing (occasionally crown infecting). Albeit variable, the anamorphs are all *Phialophora*-like (FIG. 1).

The Magnaporthaceae was established in 1994 for a group of grass-associated fungi centered on *Magnaporthe* (Cannon 1994). The family is characterized by nonstromatic black perithecia, usually with long hairy necks, persistent asci and elongate fusiform or filiform ascospores, and usually necrotrophic pathogenicity on grasses. The anamorphs are hyphomycetous and variable but can be categorized as two types, *Pyricularia*-like or *Phialophora*-like (FIG. 1). Originally described with six genera and about 20 species, the Magnaporthaceae currently contains 13 genera and > 100 species. Many of the newly added taxa are endophytic or apparently saprotrophic on non-gramineous hosts (TABLE I) (Kirk et al. 2008). In addition a number of sequences from uncultured Magnaporthaceae obtained in soil explorations by various researchers have been deposited in GenBank (http://www.ncbi.nlm.nih.gov), suggesting cryptic diversity of the Magnaporthaceae.

Mycologists traditionally have placed considerable weight on ascospore morphology (e.g. ascospore shape, size, pigmentation, wall ornamentation) in delimiting ascomycete genera. The Magnaporthaceae are no exception. However some molecular phylogenetic studies suggest that this generic concept does not always reflect the evolutionary history. For instance in the Melanosporales genera delimited based on ascospore shape and ornamentation are not monophyletic, likely due to convergent evolution (Zhang and Blackwell 2002). In the Sordariales ascomal wall morphology was found to be a better character than ascospore morphology in defining genera (Miller and Huhndorf 2005). In the Hypocreales asexual states seem to be more informative in defining monophyletic taxa because the sexual states tend to be more conserved (Chaverri et al. 2003, 2008). Molecular phylogenetic studies of the Magnaporthaceae based on single or few genes often have yielded contradictory and poorly supported trees (Huhndorf et al. 2008, Thongkantha et al. 2009, Zhang and Blackwell 2001). A stable phylogeny is needed to test the generic concept in the Magnaporthaceae.

In this study we sampled *M. salvinii*, the type species of the Magnaporthaceae, and several other representative species of *Magnaporthe* and *Gaeumannomyces*. We sequenced six loci that have been used widely in fungal phylogenetic studies: the largest subunit of RNA polymerase II gene (*RPB1*), translation elongation factor 1-alpha gene (*TEF1*), a DNA replication licensing factor gene (*MCM7*), the internal transcribed spacer of the rRNA genes (ITS), 18S rRNA gene (SSU) and 28S rRNA gene (LSU) (James et al. 2006, Peterson et al. 2010, Zhang et al. 2006). The objectives of this study were (i) to test the monophyly of the genera *Magnaporthe* and *Gaeumannomyces* based on multilocus phylogenetic analyses, (ii) to understand the evolution of ecological and morphological characteristics of these species and (iii) to identify phylogenetically informative characters at the generic level.

## MATERIALS AND METHODS

*Fungal isolation and culture conditions.*—The isolate number, source and host of 24 fungal isolates used in this study are provided (TABLE II). Fungal cultures were derived from single conidia or ascospores produced on host tissue. When fruiting structures were absent, the roots of infected plants were surface disinfected with a 5 min rinse of 75% ethanol, followed by 1% hypochlorite solution for 5 min and a final rinse in sterile water (Arnold and Lutzoni 2007, Marquez et al. 2010). Disinfected roots were cut transversely in 5 mm long fragments and placed on the *Magnaporthe* and *Gaeumannomyces* selective medium (Juhnke et al. 1984). Cultures were purified further by the hyphal-tipping method and transferred to potato dextrose agar (PDA, Difco). Host infection was conducted for the strains that did not produce perithecia on PDA, following the protocol of Holden and Hornby (1981). Fungal identification was based on morphology of the reproductive structures and confirmed by DNA sequences. Fungi were grown on PDA at room temperature 2 wk before DNA extraction. The genome sequences of *G. graminis* var. *tritici* strain R3 111a, *M. poae* ATCC 64411, *M. oryzae* 70-15 and the outgroup taxon *Cryphonectria parasitica* (FIG. 1 in boldface) are available publically. Sequences of the six loci for these four model organisms were downloaded from GenBank and Broad Institute databases and included in the phylogenetic analysis.

*DNA extraction, PCR amplification and sequencing.*—Mycelium (approximately 100 mg wet weight) was scraped off each fungal culture on PDA and the genomic DNA was extracted with an UltraClean Soil DNA Isolation Kit (MoBio, California) following the manufacturer's protocol. PCR was performed with Promega GoTaq and buffer system (Promega, Wisconsin) in an Applied Biosystems 2620

TABLE I. Ecological, biological, morphological and other features of the current constituent genera of the Magnaporthaceae[a]

| Genus (teleomorph)[b] | #Species | Host | Associated Host Part | Distribution | Nutrition | Anamorph | Ascospore |
|---|---|---|---|---|---|---|---|
| *Buergenerula* | 6 | Cyperaceae, Poaceae, Typhaceae | Leaf | Europe, N. America | Necrotroph | *Pyricularia*-like | Clavate |
| *Ceratosphaerella* | 2 | Various | Wood | Tropics | Saprotroph | *Didymobotryum*-like | Fusiform |
| *Ceratosphaeria* | 11 | Various | Decaying wood | Widespread | Saprotroph | *Harpophora*-like | Fusiform |
| *Clasterosphaeria* | 2 | Cyperaceae | Leaf | Malaysia | Unknown | *Clasterosporium* | Fusiform |
| *Clavatisporella* | 1 | Banana | Senescent leaf | Indonesia | Saprotroph | Unknown | Clavate |
| *Gaeumannomyces* | 13 | Poaceae | Root | Widespread | Necrotroph | *Harpophora* | Filiform |
| *Herbampulla* | 1 | Cyperaceae | Leaf | Austria | Unknown | Unknown | Fusiform |
| *Juncigena* | 1 | Juncaceae | Submerged leaf | N. America | Unknown | *Cirrenalia* | Fusiform/cylindrical |
| *Magnaporthe* | 5 | Poaceae | Leaf, root and stem | Widespread | Necrotroph | *Pyricularia*-like | Fusiform |
| *Muraeriata* | 2 | Unidentified | Bark/wood | Tropics | Saprotroph | Unknown | Fusiform |
| *Omnidemptus* | 1 | Poaceae | Leaf | Australia | Unknown | *Mycoleptodiscus* | Fusiform |
| *Ophioceras* | 37 | Unidentified | Submerged wood | Widespread | Saprotroph | Unknown | Filiform |
| *Pseudohalonectria* | 14 | Unidentified | Submerged wood | Widespread | Saprotroph | *Harpophora*-like | Cylindrical/filiform |

[a] Taxonomy is based on the Dictionary of The Fungi (Kirk et al. 2008) and recently published new names (Huhndorf et al. 2008, Yuan et al. 2010).

[b] There are approximately 40 asexual species in the Magnaporthaceae with unknown teleomorphs that are not listed due to space limitations. The majority of them belong to the anamorphic genera *Phialophora*, *Pyricularia* and *Harpophora*. Some are symbiotic with their gramineous hosts (Ulrich et al. 2000, Yuan et al. 2010).

thermo cycler, following the manufacturer's protocols. PCR cycling conditions for ITS, *TEF1*, *MCM7*, SSU and LSU consisted of an initial denaturation step at 95 C for 2 min, 35 cycles of 95 C for 1 min, 57 C for 1 min, 72 C for 1 min, and a final extension at 72 C for 10 min. For *RPB1* the cycling conditions were the same except for a ramp rate of 0.2 C/s from the 57 C annealing temperature to the 72 C extension temperature. Primers used in this study are included (TABLE III). Sequencing was conducted by Genewiz (South Plainfield, New Jersey).

*CPKA gene.*—The *M. oryzae CPKA* sequence was downloaded from GenBank and queried with BLASTp against the genome and transcript databases of *M. poae* and *G. graminis* var. *tritici* (http://www.broadinstitute.org).

*Phylogenetic analyses.*—Sequences of each gene were aligned with Clustal X (Thompson et al. 1997) and manually optimized. Maximum likelihood (ML), maximum parsimony (MP) and Bayesian analyses (BI) were conducted for the combined dataset and for each individual gene. jModeltest (Posada and Buckley 2004, Posada and Crandall 1998) was used to select the best evolutionary model for the ML and BI analyses. MP and ML analyses were performed with PAUP* 4.0b10 (Swofford 2000). MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) was used for BI. Bayesian analysis with 10 000 000 generations was conducted in four chains, with sampling every 100 generations. The independent analyses with 1 000 000 generations from different starting trees were performed to confirm the adequate log-likelihood convergence and mixing of chains (Zhang et al. 2006). Nodal supports were assessed by nonparametric bootstrapping with 500 replicates. Ecological, morphological and biological characteristics were mapped on the phylogeny to infer the evolutionary events. Genealogical concordance of the three individual datasets was evaluated with the Wilcoxon signed-ranks Templeton test implemented in PAUP*4.0b10 (Swofford 2000), with 90% bootstrap consensus trees used as constraints. The Shimodaira and Hasegawa test (Shimodaira and Hasegawa 2001) was conducted to examine the monophyly of the current generic concept of *Magnaporthe* and *Gaeumannomyces*.

## RESULTS

*DNA sequence data.*—A total of 6447 nucleotide characters were obtained for the selected taxa, among which 1050 (16.3%) were parsimony informative (TABLE III). Gaps and ambiguously aligned regions were excluded in the phylogenetic analyses. DNA sequences generated in this study were deposited in GenBank (for accession numbers see TABLE II). The concatenated combined alignment was submitted to TreeBASE with the study number S11292.

*Multilocus phylogenetic analyses.*—The Wilcoxon signed ranks test results indicated that the six sequence datasets were congruent with $P$ values greater than 0.05. The TrN + I + G model was selected by the jModeltest and was used for ML and

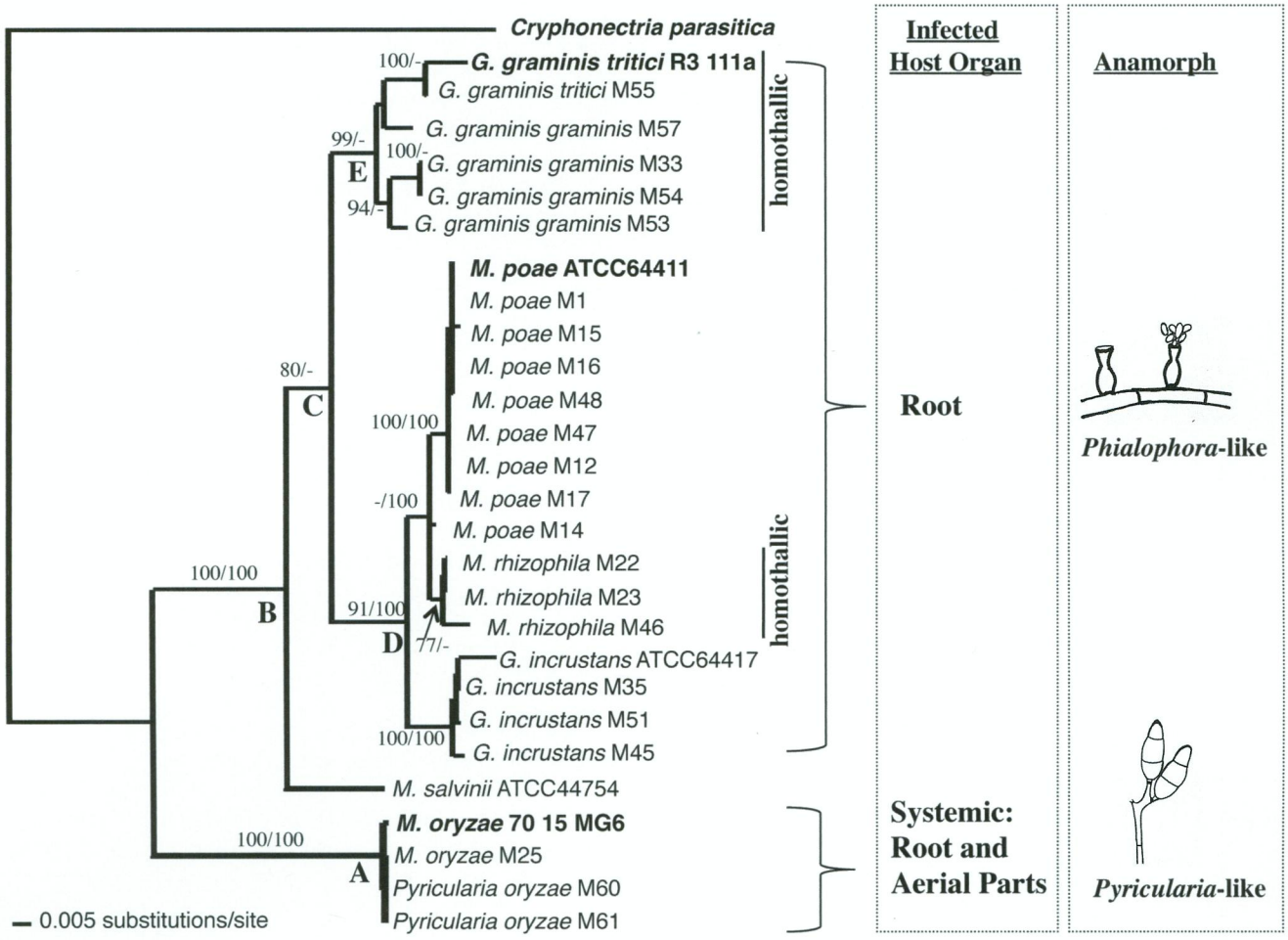


FIG. 1. Maximum likelihood tree showing relationships of *Magnaporthe oryzae* and allied species based on the combined six-gene sequences (*RPB1*, *TEF1*, *MCM7*, ITS, SSU and LSU). Nodes with support values > 75% are labeled for the MP and BI analyses respectively. – indicates a support value less than 75%. Five clades are named A–E for the convenience of discussion. Homothallic species are labeled and the unlabeled species are heterothallic. *Cryphonectria parasitica* was used as outgroup. Sequences of the strains in boldface were obtained from public genome databases. All other data were generated in this study.

BI analyses. The ML tree was based on the six-gene combined dataset, which was identical to the MP tree topology (FIG. 1). The BI tree topology was similar except for the placement of *G. graminis* and *M. salvinii* within Clade B. The monophyly of *G. graminis* was not supported by BI, with *G. graminis* var. *tritici* strain R3 111a alone forming a basal lineage in Clade B instead of *M. salvinii*. Nonetheless all three analyses (ML, MP and BI) supported that the ingroup taxa separated into two major clades, A and B (FIG. 1). Clade A consisted of *M. oryzae* and its *Pyricularia* anamorph, which are able to infect both aerial parts and root of the host. Clade B included *M. salvinii*, *M. poae*, *M. rhizophila*, *G. incrustans* and two varieties of *G. graminis*. Taxa in Clade B produce *Phialophora*-like anamorphs and are root infecting, except for *M. salvinii*. Constrained analysis with Shimodaira and Hasegawa test rejected the monophyly of *Magnaporthe* and *Gaeumannomyces* with *P* values less than 0.01. Within Clade B, *M. poae*, *M. rhizophila* and *G. incrustans* formed a well supported monophyletic group (Clade D). The close relationship between *M. rhizophila* and *M. poae* was highly supported by BI but had less than 75% bootstrap support in MP.

*Single-gene phylogenetic analysis with MP, ML and BI.*—A MP tree based on SSU did not resolve the relationship of the ingroup taxa. Clades A and D were supported in all the other five single-gene MP trees with 75% or higher bootstrap value. Clade B was supported by ITS, *MCM7*, *RPB1* and *TEF1* trees. Clade E was supported by LSU, ITS and *RPB1*. ML and BI analyses for single genes yielded similar results as the MP analysis. Clade C was well supported only in the combined analysis (TABLE III).

TABLE II. Species name, isolate number, alternative number, source, host and GenBank accession numbers of the fungi in this study

| Species | Isolate number | Alternative number[a] | Source | Host | ITS | LSU | SSU | *TEF1* | *MCM7* | *RPB1* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Gaeumannomyces graminis* var. *graminis* | M33 | GgFL199 | Florida | *Stenotaphrum secundatum* | JF710374 | JF414896 | JF414871 | JF710411 | JF710392 | JF710442 |
| *Gaeumannomyces graminis* var. *graminis* | M53 | GggFL64 | Florida | unknown | JF414847 | JF414897 | JF414872 | JF710418 | JF710393 | JF710443 |
| *Gaeumannomyces graminis* var. *graminis* | M54 | GggFL199 | Florida | unknown | JF414848 | JF414898 | JF414873 | JF710419 | JF710394 | JF710444 |
| *Gaeumannomyces graminis* var. *graminis* | M57 | GggFL | Florida | *Stenotaphrum secundatum* | JF414849 | JF414899 | JF414874 | JF710421 | JF710396 | JF710446 |
| *Gaeumannomyces graminis* var. *tritici* | M55 | Ggt568 | Montana | *Triticum* sp. | JF414850 | JF414900 | JF414875 | JF710420 | JF710395 | JF710445 |
| *Gaeumannomyces incrustans* | M51 | GI111 | Kansas | *Zoysia matrella* | JF414846 | JF414895 | JF414870 | JF710417 | JF710389 | JF710440 |
| *Gaeumannomyces incrustans* | M26 | ATCC64417, GIKAN | Kansas | *Cynodon* sp. | JF414842 | JF414891 | JF414866 | JF710409 | JF710385 | JF710435 |
| *Gaeumannomyces incrustans* | M35 | FF9 | unknown | unknown | JF414843 | JF414892 | JF414867 | JF710412 | JF710386 | JF710437 |
| *Gaeumannomyces incrustans* | M45 | G.incrustans1 | New Jersey | *Poa pratensis* | JF414844 | JF414893 | JF414868 | JF710413 | JF710387 | JF710438 |
| *Magnaporthe oryzae* | M25 | IG1, Pg rough | unknown | *Oryza sativa* | JF414839 | JF414888 | JF414863 | JF710422 | JF710397 | JF710449 |
| *Pyricularia oryzae* | M60 | 223S | New Jersey | *Festuca arundinacea* | JF414840 | JF414889 | JF414864 | JF710423 | JF710398 | JF710447 |
| *Pyricularia oryzae* | M61 | 223T | New Jersey | *Festuca arundinacea* | JF414841 | JF414890 | JF414865 | JF710424 | JF710399 | JF710448 |
| *Magnaporthe poae* | M1 | WilA-1 | New Jersey | unknown | JF414827 | JF414876 | JF414851 | JF710400 | JF710375 | JF710425 |
| *Magnaporthe poae* | M12 | StdA-7 | Pennsylvania | *Poa annua* | JF414828 | JF414877 | JF414852 | JF710401 | JF710376 | JF710426 |
| *Magnaporthe poae* | M14 | M.poaeJ8F.KR | unknown | unknown | JF414829 | JF414878 | JF414853 | JF710402 | JF710377 | JF710427 |
| *Magnaporthe poae* | M15 | OTA-3 | Pennsylvania | *Poa annua* | JF414830 | JF414879 | JF414854 | JF710403 | JF710378 | JF710428 |
| *Magnaporthe poae* | M16 | OTA-5 | Pennsylvania | unknown | JF414831 | JF414880 | JF414855 | JF710404 | JF710379 | JF710429 |
| *Magnaporthe poae* | M17 | RID A-7 | New Jersey | *Poa annua* | JF414832 | JF414881 | JF414856 | JF710405 | JF710380 | JF710430 |
| *Magnaporthe poae* | M47 | | New Jersey | *Poa pratensis* | JF414836 | JF414885 | JF414860 | JF710415 | JF710390 | JF710433 |
| *Magnaporthe poae* | M48 | | New Jersey | *Poa pratensis* | JF414837 | JF414886 | JF414861 | JF710416 | JF710391 | JF710434 |
| *Magnaporthe rhizophila* | M22 | PRR1-4756 | unknown | unknown | JF414833 | JF414882 | JF414857 | JF710407 | JF710383 | JF710431 |
| *Magnaporthe rhizophila* | M23 | | unknown | *Poa pratensis* | JF414834 | JF414846 | JF414858 | JF710408 | JF710384 | JF710432 |
| *Magnaporthe rhizophila* | M46 | s2 | unknown | *Poa pratensis* | JF414845 | JF414894 | JF414869 | JF710414 | JF710388 | JF710439 |
| *Magnaporthe salvinii* | M21 | ATCC44754 | Japan | *Oryza sativa* | JF414838 | JF414887 | JF414862 | JF710406 | JF710382 | JF710441 |

[a] ATCC = American Type Culture Collection, Manassas, Virginia.

TABLE III. Primer sequence, length and number of parsimony informative characters of the six loci in this study

| Locus | Primers | Primer sequence | Number of characters | Number and percentage of parsimony informative characters | Supported nodes [a] |
|---|---|---|---|---|---|
| SSU | SR1R, SR7 (Vilgalys and Hester 1990) | TACCTGGTTGATQCTGCCAGT, GTTCAACTACGAGCTTTTTAA | 1108 | 157, 14.2% | — |
| LSU | LS1, LR5 (Rehner and Samuels 1995) | GTACCCGCTGAACTTAAGC, TCCTGAGGGAAACTTCG | 1097 | 45, 4.1% | A,D,E |
| ITS | ITS5, ITS4 (White et al. 1990) | GGAAGTAAAAGTCGTAACAAGG, TCCTCCGCTTATTGATATGC | 650 | 119, 18.3% | A,B,D,E |
| *MCM7* | MCM7-709, MCM7-1348 (Schmitt et al. 2009) | ACIMGIGTITCVGAYGTHAARCC, GAYTTDGCIACICCIGGRTCWCCCAT | 1092 | 182, 16.7% | A,B,D |
| *RPB1* | RPB1-Ac (Matheny et al. 2002), RPB1-Cr (Castlebury et al. 2004) | GARTGYCCDGGDCAYTTYGG, CCNGCDATNTCRTTRTCCATRTA | 1445 | 298, 20.6% | A,B,D,E |
| *TEF1* | EF1-983F (Carbone and Kohn 1999), EF1-2218R (Rehner and Buckley 2005) | GCYCCYGGHCAYCGTGAYTT, ATGACACCRACRGCRACRGTYTGYAT | 1055 | 249, 23.6% | A,B,D |
| Combined | | | 6447 | 1050, 16.3% | A,B,C,D,E |

[a] Nodes (FIG. 1) are those that had a 75% or higher bootstrap value based on the parsimony analysis.

*CPKA gene.*—BLAST queries showed that the *M. oryzae CPKA* homolog is present in the genome sequences of the root-infecting *M. poae* and *G. graminis* but not in their transcript databases. There are early terminations in the *M. poae* and *G. graminis CPKA* homologs (FIG. 2).

## DISCUSSION

In recent times tremendous progress has been made in the genomics and population genetics of the model organisms in the Magnaporthaceae (Aguileta et al. 2009, Couch et al. 2005, Meng et al. 2009, Xu et al. 2007). The *M. oryzae* genome project was completed by the International Rice Blast Genome Consortium and the Broad Institute (http://www.broadinstitute.org) (Dean et al. 2005). The genomes of *M. poae* and *G. graminis* var. *tritici* have been sequenced and are currently in the process of gene annotation. However the phylogenetic relationships of this group of organisms, in particular at generic, familial and ordinal ranks, remain unsettled (Huhndorf et al. 2008, Kirk et al. 2008, Thongkantha et al. 2009, Zhang et al. 2006).

In systematic biology phylogenetic analysis of DNA or protein sequence data currently is considered the most powerful tool to elucidate evolutionary relationships. However analysis of single or few loci often yields conflicting phylogenies. The unstable systematic framework limits our understanding of the evolution of the biological and ecological characteristics of this important group of organisms. A robust systematic framework that stands the test of time should be based on genetic information at the genomic scale, which includes various independently evolving regions. It was estimated that at least 20 unlinked genes or 8000 randomly selected orthologous nucleotides are required to reach this goal (Rokas et al. 2003).

We generated a six-gene sequence database of more than 6000 nucleotides for 27 strains of *Magnaporthe* and *Gaeumannomyces* species that yielded a better supported phylogeny than single-gene trees for the selected taxa. Our six-gene phylogeny suggests that the current generic concepts of *Magnaporthe* (versicolorous, elongate fusiform ascospore) and *Gaeumannomyces* (hyaline filiform ascospore) are polyphyletic. For example *M. poae* and *M. rhizophila* were more closely related to *Gaeumannomyces* species than *M. oryzae* or *M. salvinii*. Based on ascospore morphology, the root-infecting species *M. poae* and *M. rhizophila* clearly belong to *Magnaporthe*. However Cannon (1994) already had hypothesized that the two species might be more closely related to *Gaeumannomyces* because they produce *Phialophora*-like anamorphs instead of the sympodial *Pyricularia*. This is also in line with recent findings for several other groups of fungi, in

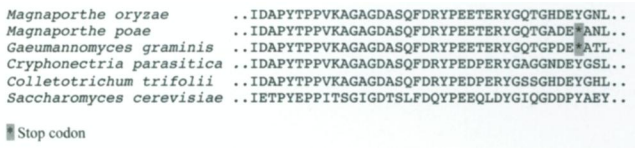


FIG. 2. Partial alignment of the cAMP-dependent protein kinase catalytic subunit (*CPKA*) gene.

which asexual states and ascomal wall morphology seem to be more informative for defining monophyletic genera than ascospore morphology (Chaverri et al. 2003, 2008; Miller and Huhndorf 2005; Zhang and Blackwell 2002).

The six-gene tree also sheds light on the evolution of ecological and pathogenic features in the Magnaporthaceae. In the basal clade the rice blast fungus *M. oryzae* has both root and aerial infection capacity (Sesma and Osbourn 2004). The type species, *M. salvinii*, causes rice stem infection. Its root-colonizing capability is unknown and needs careful investigation. The rest of the ingroup taxa are all root infecting or root colonizing. They produce *Phialophora*-like anamorphs, which are thought to be spermatial at least under some circumstances (Walker 1980), whereas the *Pyricularia* conidia certainly have aerial disseminative function. Therefore this phylogeny indicates that the aerial infection ability was lost in the root-associated clade. Genome data of *M. oryzae*, *M. poae* and *G. graminis* (http://www.broadinstitute.org) made it possible to test this evolutionary hypothesis.

Studies have shown that the *CPKA* gene (cAMP-dependent protein kinase) is involved in aerial infection of the plant in *M. oryzae* and *Colletotrichum trifolii* (Xu et al. 1997, Yang and Dickman 1999), asexual differentiation in *Neurospora crassa* (Banno et al. 2005) and virulence and conidiation in *Verticillium dahliae* (Tzima et al. 2010). The biological role of *CPKA* in most of other fungal species is still unknown. However the presence of *CPKA* in animals and many fungal lineages (e.g. *Homo sapiens*, *Drosophila melanogaster*, *Cryphonectria parasitica* and *Saccharomyces cereviciae*) indicates that it is an ancient gene with diversified functions (Jurick et al. 2004). Early termination in the *CPKA* homologs in the genomes of root-infecting *M. poae* and *G. graminis* indicates that they are non-functional pseudogenes, while in *M. oryzae CPKA* is functional and essential for the formation of leaf-penetrating appressoria. Because gaining a new function from a pseudogene is rare, compared to loss of function due to a premature stop codon, the *CPKA* genomic data support our evolutionary hypothesis that systemic infection was ancestral and the aerial infection capability was lost in the clade (Clade C) containing *Gaeumannomyces graminis*, *G. incrustans*, *M. poae* and *M. rhizophila*.

The phylogeny also elucidates the reproductive evolution of the magnaporthaceous fungi. An important evolutionary question is whether heterothallism or homothallism represents the ancestral state. A suggestion that homothallism was evolved from heterothallism in the Sordariomycetes was based on a population genetics model (Nauta and Hoekstra 1992). The phylogeny presented here suggests that the homothallic *M. rhizophila* and *G. graminis* were derived from heterothallic common ancestors.

In addition the multigene sequence data generated in this study also will help elucidate the phylogenetic position of the Magnaporthaceae. The Magnaporthaceae historically was placed in various orders in the Ascomycota due to a lack of convincing morphological and developmental characters. These species have been considered members of the Diaporthales (Krause and Webster 1972), Phyllachorales (Barr 1977) and Xylariales (Barr 1977). A new single-family order, Magnaporthales, recently was established for this unique group of fungi (Thongkantha et al. 2009). Molecular phylogenetic studies indicated that this group of fungi belongs to the subclass Sordariomycetidae (Sordariomycetes, Ascomycota). However consensus regarding further phylogenetic affinities has not yet been reached (Huhndorf et al. 2008, Thongkantha et al. 2009, Zhang and Blackwell 2001, Zhang et al. 2006). Poor taxon sampling and lack of multilocus sequence data from non-model organisms in the Magnaporthaceae likely explain the conflicting tree topologies. Data generated in this study will help resolve the familial and ordinal relationships of the Magnaporthaceae.

In conclusion the multigene phylogenetic analyses presented here strongly supports that systemic infection is a plesiomorphy of the *Magnaporthe* and *Gaeumannomyces* species. The aerial infection capacity seems to have been lost in *Gaeumannomyces* species, *M. poae* and *M. rhizophila*. The current generic concepts of *Magnaporthe* and *Gaeumannomyces* are polyphyletic. Ecological features and asexual states are more informative in defining monophyletic clades among these taxa than the teleomorphic characters. Taxonomic revisions should be conducted in a future phylogenetic analysis with more inclusive taxon sampling to reflect the natural evolutionary relationships of this evolutionary and economically important group of fungi.

## ACKNOWLEDGMENTS

We thank Drs B. B. Clarke and E. Njambere for providing fungal strains. We also thank Dr M. Blackwell and two anonymous reviewers for suggestions and corrections to improve the manuscript. This work was supported partially by a scholarship from the China Scholarship Council to S.

Zhao and by startup funds from the Rutgers Center for Turfgrass Science and New Jersey Agricultural Experiment Station to N. Zhang.

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