

Phylogenetic relatedness of the M2 double-stranded RNA in *Rhizoctonia* fungi

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Abstract: Isolates from closely related fungi in the *Rhizoctonia* species complex were examined for the occurrence of the M2 double-stranded RNA (dsRNA) by amplifying a conserved 1000 nucleotide region of the dsRNA with reverse transcription PCR. The M2 dsRNA was detected in representative isolates belonging to three anastomosis groups (AG) of *R. solani* (AG-1-IA, AG-4 and AG-6; teleomorph = *Thanatephorus*) and four AGs of binucleate *Rhizoctonia* (AG-A, AG-F, AG-R and AG-U; teleomorph = *Ceratobasidium*). Amplified PCR products from the 3' region of the M2 dsRNA from a representative sample of 12 isolates from eight different AGs were sequenced and subjected to parsimony analysis and coalescent simulations to infer ancestral lineages and to reconstruct the ancestral history of haplotypes. Seven dsRNA haplotypes were inferred from the sample of 12 isolates. One haplotype was composed of only isolates of *Ceratobasidium* belonging to different AGs. The rooted gene genealogies from coalescent simulations suggested that the ancestral M2 dsRNA haplotype most likely evolved in *Thanatephorus* (anamorph = *R. solani* AG-1-IA) and has been acquired recently by isolates of *Ceratobasidium*. Reconstruction of the ancestral history of haplotypes with a parsimony-based approach that assumes both mutation and recombination suggested that four haplotypes recombined before coalescing to their most recent common ancestor, while three haplotypes coalesced without recombination in the recent past. There was no unique association of haplotype within a specific AG of either *Ceratobasidium* or *Thanatephorus* to support co-evolution of the M2 dsRNA within the fungal host. To our knowledge this is the

first report of a dsRNA occurring in *Ceratobasidium* that also is present in *Thanatephorus*.

Key words: anastomosis groups, binucleate *Rhizoctonia*, *Ceratobasidium*, mycoviruses, *Thanatephorus*

INTRODUCTION

Double-stranded RNA (dsRNA) virus and virus-like elements commonly occur in most anastomosis groups (AG) of the soil fungus *Rhizoctonia solani* Kühn (Zanzinger et al 1984, Bharathan and Tavantzis 1990, Kousik et al 1994, Robinson and Deacon 2002, Bharathan et al 2005). In *R. solani* dsRNA have been the subject of scientific investigation because of their observed effect on the pathogenic activity of this soilborne plant pathogen (Castanho and Butler 1978, Finkler et al 1985, Jian et al 1997) and potential for biological control of *Rhizoctonia* disease of plants (Castanho and Butler 1978, Zanzinger et al 1984). A specific dsRNA, named M2, first was identified in *R. solani* AG-3 from potato and has been associated with the reduced ability of the fungus to cause disease on potato, a phenomenon referred to as hypovirulence (Jian et al 1997). It also has been hypothesized that this dsRNA is involved in the regulation and use of the carbon source quinic acid by the fungus for saprotrophic growth (Liu C et al 2003a, b). Because of the potential importance of the M2 dsRNA in the ecology and pathology of *R. solani* AG-3 (Liu C et al 2003a, b) there has been an increased interest in determining whether this dsRNA occurs in other related fungi in the *Rhizoctonia* species complex.

Although dsRNA have been identified in most AG belonging to *R. solani* no information is available regarding the occurrence of specific dsRNA in different fungi in the *Rhizoctonia* species complex. This complex is composed of genetically diverse fungi associated with plants and soil and includes plant pathogens, saprotrophs and mutualists (Cubeta and Vilgalys 2000). Fungi in the *Rhizoctonia* species complex have been placed in at least 34 AG, which are defined based on their somatic hyphal interactions (Ogoshi 1987, Carling 1996). In general isolates that recognize and undergo hyphal fusion (anastomosis) with each other are related genetically and belong to the same AG. The placement of fungi in the *Rhizoctonia* species complex into different AG is supported by recent phylogenetic analyses of genes that encode for ribosomal RNA (rDNA) and β -tubulin suggesting that they represent genetically isolated

TABLE I. Origin of isolates of *Rhizoctonia solani* (teleomorph = *Thanatephorus*) and binucleate *Rhizoctonia* (teleomorph = *Ceratobasidium*), anastomosis group (AG) and occurrence of the M2 dsRNA using reverse transcription polymerase chain reaction (RT-PCR)

Isolate	AG	Host	Origin ^a (Source) ^b	M2 ^c
<i>Thanatephorus cucumeris</i>				
Cs-Ka	1-IA ^d	<i>Oryza sativa</i>	JP (SK)	–
Cs-Gi	1-IA	<i>O. sativa</i>	JP (SK)	–
TC05USAS KAT D13	1-IA	<i>Glycine max</i>	LA (PC)	–
TC05USAS KAT D18	1-IA	<i>G. max</i>	LA (PC)	+
Rh509	2-2IV	<i>Beta vulgaris</i>	JP (SN)	–
SA-1	2-2IV	<i>Glycine max</i>	JP (SN)	–
Rhs 1A1 ^e	3	<i>Solanum tuberosum</i>	ME (ST)	+
T2	3	<i>Nicotiana tabacum</i>	NC (DS)	+
T3	3	<i>N. tabacum</i>	NC (DS)	+
T4	3	<i>N. tabacum</i>	NC (DS)	–
T5	3	<i>N. tabacum</i>	NC (DS)	+
Rs58	3	<i>S. tuberosum</i>	NC (PC)	+
Bs69	3	<i>S. tuberosum</i>	AK (DC)	+
Tom7b	3	<i>Solanum lycopersicum</i>	NC (KI)	–
Tom19a	3	<i>S. lycopersicum</i>	NC (KI)	–
Tom21f	3	<i>S. lycopersicum</i>	NC (KI)	–
Tom22b	3	<i>S. lycopersicum</i>	NC (KI)	–
OHT1-1	6	Soil	JP (AO)	+
<i>Thanatephorus practicola</i>				
T1	4 HGI	<i>N. tabacum</i>	NC (DS)	+
Chr-3	4 HGI	<i>Chrysanthemum</i>	JP (AO)	+
AH-1	4 HGI	<i>Arachis hypogaea</i>	JP (SK)	+
<i>Ceratobasidium cornigerum</i>				
FA52909	K	Unknown	JP (AO)	–
BN4	A	Soil	GA (ATCC34969)	+
HOKK	A	<i>Rosa</i>	JP (MH)	–
4dt800	A	<i>Rosa</i>	JP (MH)	–
RH3	E	Unknown	Unknown	–
BN31	E	<i>A. hypogaea</i>	GA (LB)	–
BN38	F	<i>G. max</i>	GA (LB)	+
Rh359c	S	<i>Rhododendron</i>	MS (TR, WC)	–
<i>Ceratobasidium cereale</i>				
STWP2	D I	<i>Agrostis stolonifera</i>	JP (AT)	–
TG-SDS-1	D I	<i>Zoysia</i>	JP (AT)	–
BRGWP2	D I	<i>A. stolonifera</i>	JP (AT)	–
KT-1-1	D II	<i>Zoysia</i>	JP (AT)	–
OK-EF-1	D II	<i>Zoysia</i>	JP (AT)	–
KOU-04-1FW	D III	<i>Zoysia</i>	JP (TH)	–
KOU-04-18FW2	D III	<i>Zoysia</i>	JP (TH)	–
<i>Ceratobasidium oryzae-sativa</i>				
C-455	Bb	<i>O. sativa</i>	JP (AO)	–
<i>Ceratobasidium spp.</i>				
AOO1C	C	Soil	JP (AO)	–
70B	C	Soil	JP (AO)	–
AH-9	G	<i>A. hypogaea</i>	JP (AO)	–
P9023	G	<i>Musa sp.</i>	NC (EE)	–
C-653	G	Unknown	JP (AO)	–
Rh328f	G	<i>Rhododendron</i>	MS (TR, WC)	–
Cre-V3a	I	Unknown	Unknown	–
184	J	Unknown	ND (Nelson)	–
SIR-1	F	<i>Ipomoea batatas</i>	JP (AO)	+

TABLE I. Continued

Isolate	AG	Host	Origin ^a (Source) ^b	M2 ^c
BN37	R	<i>Cucumis sativus</i>	GA (LB)	+
MWR-26	U	<i>Rosa</i>	JP (MH)	+

^a AK, Alaska; GA, Georgia; JP, Japan; LA, Louisiana; ME, Maine; MS, Mississippi; NC, North Carolina; ND, North Dakota.

^b AO, A. Ogoshi; AT, A. Tanaka; DC, Don Carling; DS, D. Shew; EE, E. Echandi; KI, Kelly Ivors; LB, L. Burpee; MH, M. Hyakumachi; PC, Paulo Ceresini; SK, S. Kuninaga; SN, S. Naito; ST, S. Tavantzis; TH, T. Hayakawa; TR, T. Rinehart; WC, W. Copes.

^c +, M2 dsRNA was detected based on RT-PCR using primer pair P35 and P36; –, M2 dsRNA was not detected based on RT-PCR using primer pair P35 and P36.

^d Isolates of *R. solani* AG1-IA represent *Thanatephorus cucumeris* (syn. *Corticium*) *sasakii* Shirai (Matsumoto).

^e Rhs 1A1 originated as a sector from the virulent field isolate Rhs 1AP; M2 was identified and sequenced from Rhs 1A1 (Lakshman et al 1998).

groups or species (Kuninaga et al 1997, González et al 2001, González et al 2006).

In this study we hypothesized that the M2 dsRNA will be present in closely related isolates of AG-3 and other members of the *Rhizoctonia* species complex. Our second hypothesis was that the M2 dsRNA will be genetically diverse and unique evolutionary lineages (haplotypes) of the M2 dsRNA will be associated with specific groups of *Rhizoctonia* fungi. The objectives of this study were (i) to determine the occurrence and genetic diversity of the M2 dsRNA in *Rhizoctonia* fungi and (ii) to infer haplotypes and the ancestral relationships of the M2 dsRNA with phylogenetic and coalescent analyses.

MATERIALS AND METHODS

Isolates.—Eight isolates of *R. solani* AG-3 from tobacco and tomato, 10 of *R. solani* (teleomorph = *Thanatephorus*) and 27 of binucleate *Rhizoctonia* (teleomorph = *Ceratobasidium*) (TABLE I), representing respectively 4 and 13 AG were examined.

Preparation of fungal tissue.—Isolates were grown in 25 mL potato dextrose broth in 250 mL Erlenmeyer flasks by inoculating each flask with a 5 mm diam agar plug. After incubation 5–7 d at 24 C mycelial mats were washed three times with sterile deionized water, freeze dried (lyophilized) and stored at –80 C. Lyophilized tissue was ground into a fine powder in liquid nitrogen with a mortar and pestle and used for RNA extraction.

RNA extraction.—Total RNA was extracted from approximately 50 mg of ground mycelium with TRIZOL[®] Reagent (Invitrogen, Carlsbad, California) according to manufacturer's instructions. Lithium chloride was used to improve RNA isolation by removing excessive amounts of carbohydrates (Salzman et al 1999, Liu C et al 2003b). RNA pellets were suspended in 40 µL RNase-free water and stored at –20 C and used in RT-PCR.

Reverse transcription polymerase chain reaction.—Total RNA (4 µL) was denatured at 100 C for 1 min in the presence of

3 µL RNase-free water and 1 µL 10 µM gene-specific primer P36 (5'-GGGGCTTCTGGCGGAAAGAA-3') (Liu C et al 2003b). Integrated DNA Technologies Inc., (Coralville, Iowa) synthesized and provided all oligonucleotide primers. P36 was used in reverse-transcription of the total RNA for the 3'- end (bases 2437–3570) of the M2 dsRNA for synthesis of complementary DNA (cDNA) based on the conservation of this region. After denaturation the reaction was chilled immediately on an ethanol-ice bath. RNA was reverse-transcribed in a total volume of 20 µL with 200 U Superscript II[™] Reverse Transcriptase (Invitrogen, Carlsbad, California), 500 µM of each dNTP and 20 U RNase OUT (Invitrogen, Carlsbad, California) at 42 C for 90 min. The reaction was heat-inactivated at 70 C for 10 min. cDNA were stored at –20 C and used as a template for amplification with PCR.

PCR was performed in a total volume of 50 µL containing 2 µL aliquot of undiluted cDNA template, 1.25 U *Taq* polymerase (Qiagen Inc., Valencia, California), 10× reaction buffer containing 15 mM MgCl₂ (Qiagen Inc., Valencia, California), 200 µM of each dNTP (Promega, Madison, Wisconsin) and 1 µM target-specific primers P35 (5'-GTCATTACATGCAGCTTACC-3') (Liu C et al 2003b) and P36. The cycling parameters were 1 min at 94 C, 35 cycles of 1 min at 94 C, 45 s at 55 C, 1 min at 72 C, followed by a final synthesis step at 72 C for 7 min. Amplicons were analyzed by gel electrophoresis with a 1% agarose gel in 0.5× Tris-Boric-EDTA (TBE) buffer. RT-PCR products were viewed after staining with ethidium bromide by UV transillumination. The primers used to detect the M2 dsRNA were based on previous studies that found that primer specificity allowed the detection of the M2 dsRNA with PCR even in low titer as compared to detection using northern blot analysis or gel electrophoresis (Lakshman and Tavantzis 1994, Lakshman et al 1998).

Sequencing.—RT-PCR products were recovered from a 1% SeaPlaque[®] GTG[®] agarose gel (Cambrex Bio Science Rockland Inc., Rockland, Maine) and purified with the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California) according to manufacturer's instructions. Purified products were sequenced with Big Dye Terminator Chemistry (version 3.1, Applied Biosystems, Foster City,

California) and submitted to the Duke IGSP Sequencing and Genetic Analysis Facility in Durham, North Carolina. Forward and reverse chromatograms were aligned and adjusted by visual examination with Sequencher™ (Gene Codes Corp., Ann Arbor, Michigan). Sequences have been submitted to GenBank (accession Nos. EF423919–EF423928).

Phylogenetic analysis of M2 dsRNA.—Forward and reverse chromatograms were aligned and cDNA sequence ambiguities were resolved by visual examination with Sequencher. cDNA sequences were analyzed with SNAP Workbench (suite of nucleotide analysis programs) (Price and Carbone 2005). The sequences were aligned with Clustal W version 1.7 (Thompson et al 1994). The combined sequence alignment was collapsed into unique haplotypes excluding insertions or deletions (indels) and excluding infinite sites violations with SNAP Map (Aylor et al 2006). A phylogenetic analysis was performed with unweighted parsimony (PAUP* 4.0) (Swofford 1998) with bootstrap analysis using 500 resamples of parsimony-informative sites to provide statistical confidence on the reliability of clades in the inferred parsimony tree.

Site compatibility analysis was performed to identify homoplasious variable sites arising from recombination or recurrent mutation. The overall compatibility matrix for parsimony informative and uninformative sites in the multiple sequence alignment was generated and viewed respectively with SNAP Clade and Matrix (Bowden et al 2008). This also allowed for viewing of nonrecombining partitions or blocks of compatible sites in the multiple sequence alignment. We used SNAP CladeEx (Bowden et al 2008) to find the largest nonrecombining block of segregating sites so that all sites included in a block were compatible. This was important because subsequent coalescent analyses assumed that all variation is compatible and consistent with an infinite sites mutation model.

Coalescent analysis of M2 dsRNA.—We applied a coalescent-based model that assumes no recombination and provides estimates of mutation ages, divergence times and the time to the most recent common ancestor (TMRCA) of the sample sequences. Coalescent theory is based on the idea that all genes or alleles in a given population essentially are inherited from a single ancestor shared by all members of the population, known as the most recent common ancestor (MRCA). Eventually all lineages coalesce into a single lineage, the MRCA of the sample. A gene genealogy describes the inheritance relationships, and the gene of interest undergoes coalescence to the common ancestor. Coalescent analyses were done with Genetree version 9.0 (Griffiths and Tavaré 1994). We performed multiple independent simulations of the coalescent to estimate the ages of mutation and the TMRCA of the M2 dsRNA genealogy with the highest root probability (Carbone et al 2004). Genetree requires a starting value of θ , or $2N_e\mu$, where N_e is the effective population size and μ is the neutral mutation rate for simulating probability distributions in the coalescent. Coalescent-based genealogies were inferred assuming Watterson's θ , θ_w , (Watterson 1975) and a constant population size model. Independent simulations

with five different starting random number seeds and 1 000 000 simulations of the coalescent for each run were performed to assess convergence. Four additional genealogies were inferred with 10 000 000 simulations for each run. If the independent coalescent runs converged to different genealogies we examined the two genealogies with the highest root probabilities to determine if there were any significant topological differences. If the two genealogies were concordant we selected one for subsequent analyses. To estimate TMRCA we first calculated a maximum likelihood estimate of θ based on the genealogy with the highest root probability and then performed coalescent simulations using the estimated θ to determine the ages of mutations on the genealogy.

A minimal ancestral recombination graph of M2 dsRNA haplotypes.—We reconstructed the ancestral history of haplotypes with a parsimony approach that assumes both mutation and recombination backward in time. This was done with kwarg, a heuristic implementation of the branch and bound method implemented in beagle (Lyngsø et al 2005, Carbone et al 2007). Whereas beagle computes minimum recombination histories with an exhaustive approach, kwarg implements a heuristic search for plausible histories and therefore does not guarantee to find a minimal recombination history. With increasing complexity of recombination in the data searching for shared histories using kwarg is more feasible than estimating the exact number of recombination events with beagle. We performed 10 independent kwarg searches to verify that similar ancestries were found among all sampled ancestral recombination graphs (ARG).

RESULTS

Occurrence of the M2 dsRNA.—M2 dsRNA was detected in isolates of AG-3 from tobacco but was not detected in the isolates from tomato (TABLE I). The M2 dsRNA was detected in *R. solani* AG-1-IA, AG-4 and AG-6; and binucleate *Rhizoctonia* AG-A, AG-F, AG-R, and AG-U (TABLE I). Additional isolates were examined and the M2 dsRNA was not detected in representative isolates of *Waitea circinata* Warcup & Talbot (87NEP, 87NE9, S7-6-1Ash, M008, and C-504), or isolates within the cantharelloid clade: *Cantharellus cibarius* Fr. (JWR-740), *Clavulina cristata* (Holmsk.) J. Schröt (MY-6), *Craterellus cornucopioides* (L.) Pers. (ECS-807-T), *Tulasnella pruinosa* Bourd. & Galzin (HHB-1208-Sp), *Tulasnella sp.* (PB01), *Uthatabasidium fusisporum* (J. Schröt.) Donk (HHB-10215-Sp), *Botryobasidium sp.* (546-SS-1), *R. endophytica* Saksena & Vaartaja, or *R. globularis* Saksena & Vaartaja (data not shown).

Phylogenetic Analysis of M2 dsRNA.—The 3' region, consisting of a total of 1000 bp of the M2 dsRNA, was sequenced to examine the genetic diversity among isolates of different AGs. This region has been found

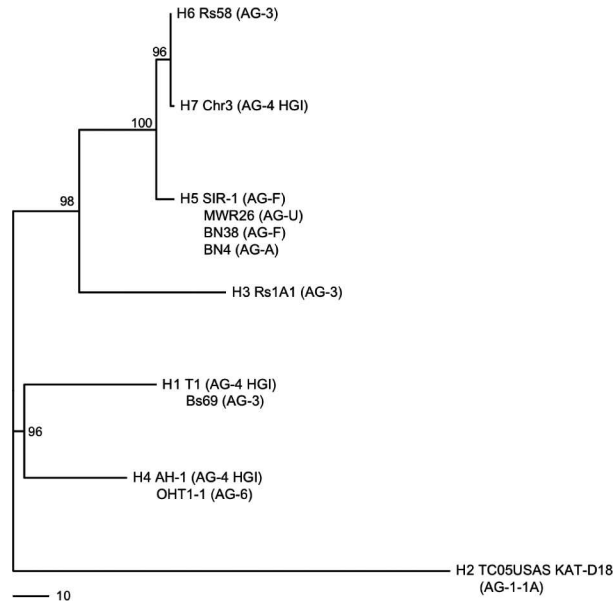


FIG. 1. The single most parsimonious tree based on the 3' region of the M2 double-stranded RNA from *Rhizoctonia solani* (teleomorph = *Thanatephorus*) AG-1-IA, AG-3, AG-4, AG-6, and binucleate *Rhizoctonia* (teleomorph = *Ceratobasidium*) AG-A, AG-F and AG-U. Numbers above interior branches are bootstrap percent support values for 500 resamplings. Tree length = 255 steps, consistency index = 0.8980, homoplasy index = 0.1020, retention index = 0.7400 and rescaled consistency index = 0.6645.

in previous studies to represent a conserved region of the genome that is useful for detecting the M2 dsRNA (Lakshman and Tavantzis 1994, Lakshman et al 1998, Charlton 2007). Isolates included in the phylogenetic analysis were *R. solani* AG-1-IA (TC05USAS KAT-D18), AG-3 (Bs69 from potato), AG-4 (Chr3, AH-1, and T1), AG-6 (OHT1-1), AG-A (BN4), AG-F (BN38 and SIR-1), AG-U (MWR26) and two reference

isolates of *R. solani* AG-3, Rhs 1A1 and Rs58 (accession nos. EF423929 and EF423940). The latter two isolates represent divergent evolutionary lineages (haplotypes) from potato (Lakshman et al 1998, Charlton 2007). Rhs 1A1 is the original isolate from which the M2 dsRNA was identified and sequenced (Lakshman et al 1998). There were a total of 229 variable sites of which 63 were parsimony-informative within the 3' region. The distribution of isolates within each M2 dsRNA haplotype was not uniquely associated with a specific AG except for isolates of *Ceratobasidium*. A parsimony analysis resulted in one most parsimonious tree with a consistency index of 0.8980 and length of 255 steps (FIG. 1). All interior branches were supported in >95% of 500 bootstrap replicates.

Coalescent Analysis of M2 dsRNA Haplotypes.—The distribution of isolates associated with the coalescent analysis are shown in TABLE II. The coalescent simulations using Genetree resulted in two genealogies with the highest root probability. These two genealogies had similar topologies with only a few differences in the position of two mutations in the two deepest branches of the genealogy. The two trees were in the top five of all simulations performed. One of the two gene genealogies with the highest root probability is shown in FIG. 2. The rooted M2 dsRNA genealogy shows the ancestral history among isolates of different AGs as well as the number of mutations separating isolates. The isolate of *R. solani* AG-1-IA (haplotype H2) has a long history of separation from the other isolates and is one of two distinct evolutionary lineages that diverged from the common ancestor of the entire sample. The M2 dsRNA also appears to have been recently acquired by isolates of *Ceratobasidium* based on the recent divergence of haplotype H5 from H6 in the genealogy.

TABLE II. Frequency of haplotypes of M2 dsRNA found in anastomosis groups (AG) of *Rhizoctonia solani* (teleomorph = *Thanatephorus*) and binucleate *Rhizoctonia* (teleomorph = *Ceratobasidium*) based on coalescent analysis of the 3' region of the M2 double-stranded RNA

Haplotype (frequency)	Isolate	AG	Teleomorph
H1 (2)	T1	AG-4 HGI	<i>Thanatephorus</i>
	Bs69	AG-3	<i>Thanatephorus</i>
H2 (1)	TC05USAS KAT-D18	AG-1-IA	<i>Thanatephorus</i>
H3 (1)	Rhs1A1	AG-3	<i>Thanatephorus</i>
H4 (2)	AH-1	AG-4 HGI	<i>Thanatephorus</i>
	OHT1-1	AG-6	<i>Thanatephorus</i>
H5 (4)	SIR-1	AG-F	<i>Ceratobasidium</i>
	MWR26	AG-U	<i>Ceratobasidium</i>
	BN4	AG-A	<i>Ceratobasidium</i>
	BN38	AG-F	<i>Ceratobasidium</i>
H6 (2)	Rs58	AG-3	<i>Thanatephorus</i>
	Chr3	AG-4 HGI	<i>Thanatephorus</i>

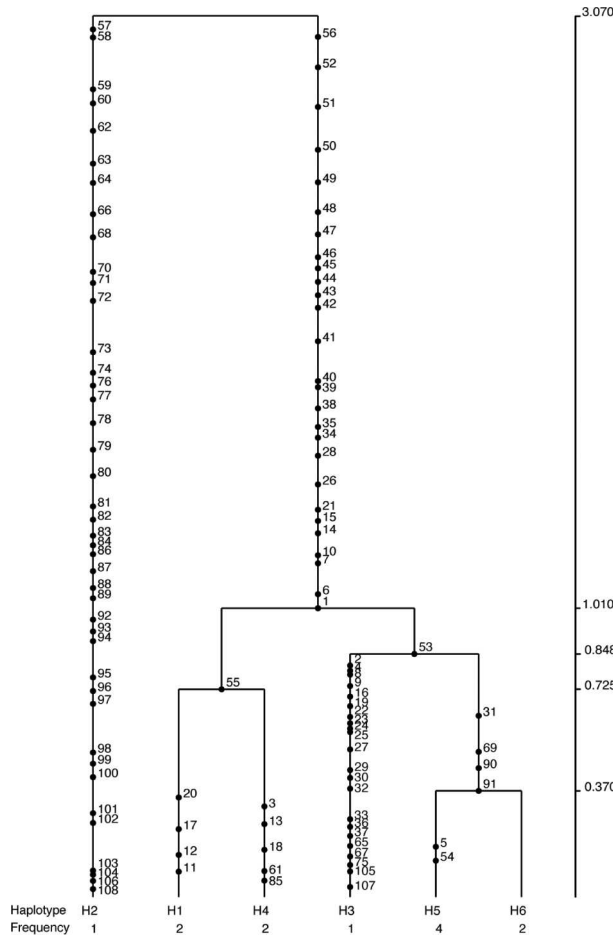


FIG. 2. One of two coalescent-based gene genealogies with the highest root probability showing the distribution of mutations of the M2 dsRNA (likelihood is 6.1377×10^{-22} , $SD = 1.5586 \times 10^{-1}$). The inferred genealogy is based on five starting random number seeds, using 1 000 000 simulations of the coalescent for each run and an additional four runs with 10 000 000 simulations with a likelihood estimate of $\theta = 27.5$ (Watterson's estimate of $\theta = 35.76$) assuming a constant population size model. The direction of divergence is from the top of the genealogy (past) to the bottom (present). The numbers below the tree designate distinct haplotypes and their observed frequencies (See TABLE II). The numbers along the lines in the coalescent tree represent the number of mutations. The time scale on the right shows the estimated TMRCA, in coalescent time units of N_e generations, where N_e is the effective population size.

A Minimal Ancestral Recombination Graph of M2 dsRNA Haplotypes.—In the ARG, M2 dsRNA haplotypes H1, H3, H4 and H7 have undergone recombination before coalescing to their most recent common ancestor back in time, whereas haplotypes H2, H6 and H5 coalesce without recombination in the recent past (FIG. 3). Haplotype H6 (AG-3) shares a recent common ancestor with H3 (AG-3) and a more distant ancestor with H7 (AG-4) in the ARG.

Haplotype H3 (AG-3) had undergone at least four recombination events before coalescing with H2 (AG-1-IA). Haplotype H5, which includes four isolates of *Ceratobasidium* belonging to three different anastomosis groups, also showed a complex pattern of reticulation with the sampled M2 dsRNA haplotypes. Similar patterns of descent were also observed in nine additional ARGs sampled by kwarg. This analysis suggests that either H2 (AG-1-IA) or H5 (AG-A, AG-F, AG-U) were appropriate outgroups for phylogenetic analysis of the M2 dsRNA.

DISCUSSION

In this study three different analyses were used to infer the genetic relatedness and ancestry of the M2 dsRNA from fungi belonging to the *Rhizoctonia* species complex: (i) parsimony analysis of all data, assuming no recombination; (ii) coalescent analysis with a nonrecombining partition of the data; and (iii) parsimony analysis of all data, assuming recombination (i.e. ARG). The parsimony and coalescent analyses assume no recombination and suggested that the M2 dsRNA found in *Ceratobasidium* was more closely related to those found in *Thanatephorus*. The coalescent analysis also suggested that *Ceratobasidium* diverged more recently and might have acquired the M2 dsRNA. The ancestral recombination graph provided evidence for the relationship of four of the M2 dsRNA haplotypes found in isolates of *Thanatephorus* and that isolates of *Ceratobasidium* represented a unique and divergent evolutionary lineage. This relationship was not captured by parsimony and coalescent analyses without recombination and is more congruent with what is known about the phylogenetic relationships and hyphal anastomosis of the fungi. In this study population-based analyses such as the coalescent and ARG were informative for addressing questions related to the evolutionary relationships of the M2 dsRNA in *Rhizoctonia* fungi that belong to a diverse species complex, where the taxonomic boundaries are not well delineated.

All three methods indicated that the M2 dsRNA found in *Ceratobasidium* represented a unique haplotype (H5) that did not include any isolates of *Thanatephorus* and provided different inferences about the genetic relatedness of the M2 dsRNA. However only the ARG showed that the M2 dsRNA in *Ceratobasidium* are distinct from *Thanatephorus*. Some caution should be exhibited when applying phylogenetic methods that assume no recombination, particularly when analyzing dsRNA data. Although we have observed signatures of recombination in the M2 dsRNA from *R. solani* AG-3 (Charlton 2007) the nature of this recombination is not currently known.

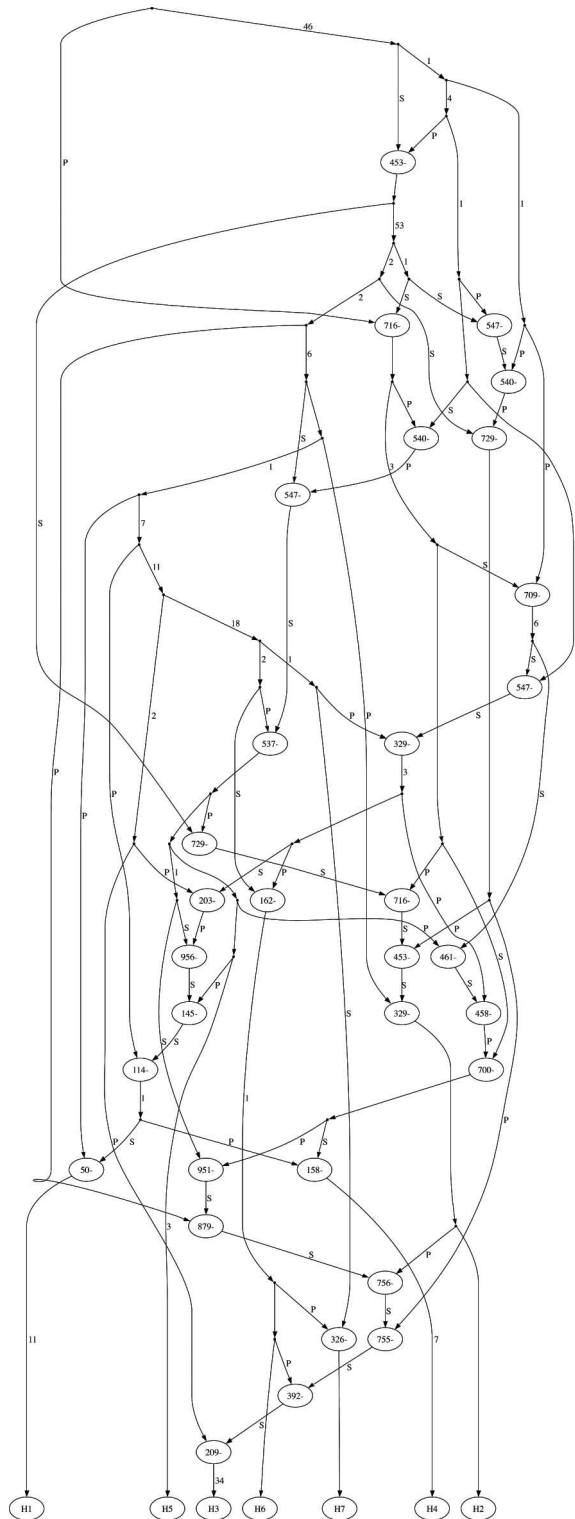


FIG. 3. One possible ancestral recombination graph (ARG) for M2 dsRNA haplotypes generated with kwarg showing a minimum of 32 recombination events backward in time. The graph was rooted by specifying haplotype H2 as outgroup. Haplotypes are indicated with H followed by a number (e.g. H1) enclosed in ovals at the base of the ARG. Recombination events are illustrated in the interior of the

Many studies have investigated the occurrence of dsRNA in fungi (Ghabrial 1998) and similar studies have been conducted in *R. solani* (Bharathan 2005, Kim et al 1996, Kousik et al 1994, Robinson and Deacon 2002). However most of these studies have not focused on determining the occurrence of a specific dsRNA. A study by Voth et al (2006) focused on the detection of a specific dsRNA, Umv-H1, from the corn smut fungus *Ustilago maydis*. They found Umv-H1 in 34.2% (66 of 232) of isolates of *U. maydis* in the USA population and 100% of the 44 isolates examined in the population from Mexico. Peever et al (1997) also examined *Cryphonectria parasitica* for the occurrence of specific hybridization groups of the CHV-1, CHV-2 and CHV-3 hypoviruses and found that they were present in 28% of the isolates sampled. Because of the potential importance of M2 on the parasitic and saprobic behavior of the fungal host we were interested in determining whether this dsRNA was present in other *Rhizoctonia* fungi.

In this study we hypothesized that the M2 dsRNA would be present in closely related isolates of AG-3 and other members of the *Rhizoctonia* species complex. The M2 dsRNA was found in isolates of *R. solani* AG-1-IA, AG-3 from potato and tobacco, AG-4, and AG-6, and in binucleate *Rhizoctonia* AG-A, AG-F, AG-R and AG-U. Using the primers described previously we were unable to detect the M2 dsRNA in the small subset of isolates of *R. solani* AG-2-2IV, AG-3 from tomato, binucleate *Rhizoctonia* AG-Bb, AG-C, AG-D, AG-E, AG-G, AG-I, AG-J, AG-K and AG-S (TABLE I) or fungi related to *Ceratobasidium* and *Thanatephorus* in the cantharelloid clade (data not shown). In our study only a small sample of representative isolates of *Ceratobasidium*, *Thanatephorus* and fungi in the cantherelloid clade (Binder et al 2005) were examined and a more extensive sampling from diverse regions is needed to determine whether the M2 dsRNA occurs in these fungi.

The occurrence of the M2 dsRNA in *Ceratobasidium* and *Thanatephorus* is interesting because they are thought to represent noninterbreeding populations within the *Rhizoctonia* species complex. In general dsRNA viruses have been shown to occur in the same species of fungi in nature (Ghabrial 1998). However

ARG as ovals with a number inside that refer to the variable position where the event occurred. Two lineages converge at each recombination event (interior oval in the ARG); one will contribute with the suffix (S) and the other with the prefix (P) of the new recombinant. Numbers along the branches indicate the number of mutations for a lineage to coalesce with its most recent common ancestor in the ARG.

the occurrence of the dsRNA, CHV-1, from *Cryphonectria parasitica* was found in two isolates of an unidentified species of *Cryphonectria* (Liu YC et al 2003). Also there has been a report of a specific dsRNA occurring in two distinct genera, the large dsRNA of an isolate of *Sclerotinia homoeocarpa* that has been shown to be conspecific with the OnuMV3a dsRNA of *Ophiostoma novo-ulmi*, with a 92.4% nucleotide sequence identity (Deng et al 2003). These studies suggest that the host range of dsRNA might not be limited to a fungal species.

The subsample of isolates where the M2 dsRNA was detected provided us with a unique opportunity to test our second hypothesis that the M2 dsRNA would be genetically diverse and that unique evolutionary lineages (haplotypes) of the M2 dsRNA would be associated with specific groups of *Rhizoctonia* fungi. Four haplotypes contained at least two isolates often from different AG. Two of six haplotypes were represented by only a single isolate, Rhs 1A1 (AG-3) and TC05USAS KAT-D18 (AG-1-IA). The M2 dsRNA was present in three isolates of AG-4 HGI, but these isolates were represented by three different haplotypes. Each of these haplotypes had a unique history of mutation based on coalescent analysis, demonstrating the diversity of this dsRNA within an AG. Reconstruction of the ancestral history with ARG is important because it provided ancillary information about the relative contribution of mutation and recombination to how these haplotypes were related through descent. This information was not captured by parsimony analysis that assumes no recombination. Based on the coalescent analysis and ARG, the AG-3 isolate Rhs 1A1 has respectively 22 and 34 mutations that have occurred recently. Rhs 1A1 originated as a sector from a field isolate Rhs 1AP that might have given rise recently to these mutations in the laboratory. Although Rhs 1A1 represents a unique haplotype with a different mutational history, a similar haplotype has been found in field populations of *R. solani* AG-3 (Charlton 2007). Of interest, the dsRNA found in Rhs 1A1 and other isolates used in this study are stable and have been maintained for more than 12 y under laboratory conditions with minimal changes in nucleotide sequence (Lakshman and Tavantzis 1994, Charlton 2007).

The coalescent analysis coupled with the ARG suggested that *Thanatephorus* (AG-1-IA) might represent an ancestral lineage of *Rhizoctonia* fungi. This is consistent with experimental evidence that this group of fungi represents a distinct species based on morphological and DNA-based characteristics (Parameter 1970, Ogoshi 1987, Vilgalys 1988, Vilgalys and González 1990, Stalpers and Andersen 1996, Kuniyama et al 1997, González et al 2001, González et al

2006). Although the ARG provided a similar ancestral inference about AG-1-IA, the analysis also suggested the possibility that *Ceratobasidium* might represent a unique and distinct ancestral lineage with a complex pattern of descent that is deeply rooted in the ARG.

Haplotypes of the M2 dsRNA were not associated uniquely with geographic origin or AG, and there was no compelling evidence for co-evolution of this dsRNA with anastomosis group affinity of *Ceratobasidium* or *Thanatephorus*. Therefore our second hypothesis was rejected because we were unable to associate unique haplotypes of the M2 dsRNA with specific groups of *Rhizoctonia* fungi. However a large sample of isolates that incorporates analysis of other regions of the dsRNA genome is required to more accurately reflect the occurrence and genetic diversity of the M2 dsRNA. A more complete understanding of the evolutionary relationships and ancestry of the M2 dsRNA might provide insight into its effect(s) on the fungal host.

ACKNOWLEDGMENTS

We thank Lisa Bukovnik (Duke University, IGSP Sequencing and Genetic Analysis Facility) for help with the sequencing, the Center for Forest Mycology Research (Madison, Wisconsin) for providing cultures for this project and Pam Purvear for assistance in literature searches. The authors also thank Dilip Lakshman and Narayanaswamy Bharathan for helpful comments regarding this research. This research was supported by the USDA CSREES grants (2004-35400-14429, 2005-34500-15893 and 2006-35604-16666).

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