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Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs

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Abstract: The phylogenetic relationships of anastomosis groups (AG) of *Rhizoctonia* associated with *Ceratobasidium* and *Thanatephorus* teleomorphs were determined by cladistic analyses of internal transcribed spacer (ITS) and 28S large subunit (LSU) regions of nuclear-encoded ribosomal DNA (rDNA). Combined analyses of ITS and LSU rDNA sequences from 41 isolates representing 28 AG of *Ceratobasidium* and *Thanatephorus* supported at least 12 monophyletic groupings within *Ceratobasidium* and *Thanatephorus.* There was strong support for separation of *Ceratobasidium* and *Thanatephorus,* however, six sequences representing different AG of *Ceratobasidium* grouped with certain sequences within the *Thanatephorus* clade. Phylogenetic analysis of ITS sequence data from 122 isolates revealed 31 genetically distinct groups from *Thanatephorus* (21 groups) and *Ceratobasidium* (10 groups) that corresponded well with previously recognized AG or AG subgroups. Although phylogenetic analysis of ITS sequences provided evidence that several AG of *Ceratobasidium* may be more closely related with some AG from *Thanatephorus,* these relationships were not as strongly supported by bootstrap analysis.

Key Words: basidiomycetes, binucleate *Rhizoctonia*, phylogeny, *Rhizoctonia solani*, taxonomy

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INTRODUCTION

The *Rhizoctonia* species complex includes a wide array of genetically diverse basidiomycetes that are frequently associated with plants and soil. Many species of *Rhizoctonia* are economically important plant pathogens, as well as being saprophytes on decaying organic matter, whereas others are mycorrhizal symbionts of orchids and mosses (Warcup and Talbot 1966, Currah et al 1987, Carling et al 1999, Cubeta and Vilgalys 2000). Because of the high level of genetic diversity in morphology, pathology and physiology, taxonomic uncertainty still surrounds fungi classified as *Rhizoctonia.* Also, many isolates of *Rhizoctonia* do not reproduce sexually and are known only from their asexual stage (anamorph). However, when the sexual stage (teleomorph) has been observed, the potential taxonomic characters obtained from this stage are similar to each other and/or lacking (Anderson 1982, Parmeter and Whitney 1970). A number of teleomorph genera are connected with *Rhizoctonia* anamorphs, including *Botryobasidium* Donk, *Ceratobasidium* Rogers, *Thanatephorus* Donk, *Tulasnella* Schröt., *Uthatobasidium* Donk and *Waitea* Warcup & Talbot (Andersen 1996). Because a close phylogenetic relationship between *Ceratobasidium* and *Thanatephorus* has been previously suggested by other researchers based on an examination of septal pore and teleomorph characters (Andersen 1996, Moore 1996, Müller et al 1998, Talbot 1970, Tu and Kimbrough 1978), this study focused primarily on examining the molecular systematics of these two genera.

The most widely studied species of *Rhizoctonia, Rhizoctonia solani* Kühn, is associated with a teleomorph *Thanatephorus cucumeris* (Frank) Donk (Talbot 1970, Tu and Kimbrough 1978). However, most taxonomists agree that *R. solani* is not a single species but rather a species complex. Since isolates in the *R. solani* species complex are highly variable, confusion exists about how to classify isolates into groups and whether these groups might represent species or some other taxonomic rank (Talbot 1965, Parmeter and Whitney 1970, Tu and Kimbrough 1978, Ogoshi 1987).

The most useful system for classification of fungi within the *R. solani* complex is based largely on anastomosis grouping (AG) (Ogoshi 1987, Carling

1996). However, the determination of relationships based on anastomosis behavior of individual isolates has also been uncertain since this fungus can exhibit different types of hyphal fusion within the same AG (Carling 1996). Currently 14 AG of *R. solani* are recognized (AG-1 to AG-13 and AG-BI). Seven of 14 AG (AG-1, AG-2, AG-3, AG-4, AG-6, AG-8, and AG-9) have been further divided into subgroups to reflect differences observed in frequency of anastomosis, fatty acid and isozyme patterns, pathogenicity, thiamine requirement, and cultural appearance among isolates (Ogoshi 1987, Stevens-Jonks and Jones 2001). A similar classification system has been developed for *Ceratobasidium,* where seven and 19 anastomosis groups have been described from the US (CAG-1 to CAG-7) and Japan (AG-A to AG-S), respectively (Burpee et al 1980, Ogoshi 1987). One anastomosis group of *Ceratobasidium* (AG-B) has been further divided into subgroups based on cultural characteristics and frequency of anastomosis (AG-Ba, AG-Bb, and AG-Bo). Although the AG system has provided a useful criterion for characterizing *Ceratobasidium* and *Thanatephorus,* the relationship of AG and AG subgroups to species or other taxonomic units has not been formally established. At least 14 of the 26 recognized AG associated with a *Ceratobasidium* teleomorph lack a species epithet for their respective *Rhizoctonia* anamorph. The challenge of determining species or other taxonomic units is also exacerbated because 17 AG are not associated with a well-defined species of *Ceratobasidium* based on examination of morphological characters.

With the advent of DNA-based molecular techniques, there has been a resurgence of interest in *Rhizoctonia* taxonomy. Several researchers (González 1992, Boysen et al 1996, Kuninaga et al 1997, Boidin et al 1998, Johanson et al 1998, Salazar et al 1999, 2000) have examined the phylogenetic and taxonomic relationships of AG and AG subgroups of *R. solani* using sequence analysis of the internal transcribed spacer (ITS 1 and ITS 2) and 5.8S regions of nuclear-encoded ribosomal DNA (rDNA). Results from these studies have shown that there is no sequence variation in the 5.8S region, while the ITS regions display a high level of sequence variation among isolates of *Ceratobasidium* and *Thanatephorus.* Most of these studies have concluded that molecular relationships are largely congruent with relationships inferred from hyphal anastomosis reactions. However, no taxonomic decisions about the phylogenetic groupings were made in the previous studies.

In this paper molecular systematics methods were used to test the hypotheses that 1) *Ceratobasidium* and *Thanatephorus* represent distinct evolutionary lineages of fungi with *Rhizoctonia* anamorphs and 2) anastomosis groups represent the most fundamental evolutionary units within *R. solani.* The application of phylogenetic data for unambiguous identification of *Rhizoctonia* anamorphs of *Ceratobasidium* and *Thanatephorus* is also presented.

MATERIALS AND METHODS

Isolates and DNA extraction.—Sixty isolates representing 28 AG of *Ceratobasidium* and *Thanatephorus* were included in this study (TABLE I). Isolates were grown in 20 mL of potato dextrose broth (Difco) for 3–5 d at 25 C, harvested by filtration, lyophilized, ground to a fine powder in liquid nitrogen and stored at −20 C. Genomic DNA was extracted according the miniprep method of Raeder and Broda (1985). Mycelium was suspended in 500 mL of extraction buffer (15 mM NaCl, 50 mM Tris [pH 8.0], 10 mM Na_2EDTA, 1% [w/v] SDS) for 15 min. The solution was extracted with an equal volume of chloroform-isoamyl alcohol 24:1 (v/v) and centrifuged at 13 200 rpm for 15 min. The upper aqueous layer was incubated with 50 mL of RNase A (5 mg/mL) at 37 C for 30 min. The solution was re-extracted with an equal volume of chloroform-isoamyl alcohol 24:1 (v/v), and centrifuged at 13 200 rpm for 15 min. The upper aqueous layer was mixed with 0.1 volumes of 3 M sodium acetate and mixed, and 1.8 volumes of cold absolute ethanol was then added to precipitate DNA. The pellet was collected, rinsed with 70% ethanol, and dried under a vacuum.

DNA amplification and sequencing.—Prior to amplification, genomic DNA was purified on a 0.6% low-melting-point agarose gel, cut out of the gel and dissolved up to 0.001 μg per μL in distilled water. Approximately 0.01 μg of purified DNA was amplified by the polymerase chain reaction (PCR). Reactions for PCR amplification were performed in a 50 μL mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 μL of each of the four deoxynucleoside triphosphates, 5 pmol of each primer, 10 μL of template and 2.5 units of *Taq* polymerase. The amplifications were performed with a thermal cycler 480 from Perkin-Elmer (Norwalk, Connecticutt). The cycle parameters were an initial denaturation at 96 C for 5 min, followed by 25 cycles consisting of denaturation at 96 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min, and a final extension for 7 min at 72 C. The oligonucleotide primers ITS1 (or ITS5) and ITS4 (White et al 1990) were used for amplification and sequencing of the internal transcribed spacer (ITS) region of the nuclear-encoded rDNA region, while primers LROR, LR22R, LR3, and LR5 were used to amplify and sequence the 5′-portion of the large subunit (=28S RNA) rDNA region (Hopple and Vilgalys 1999).

Amplified DNA from each isolate was purified by adding 0.3 volumes of 7.5 M ammonium acetate and followed by 2.5 volumes of cold 95% ethanol. The pellet was collected, rinsed with 70% ethanol, and dried under a vacuum. Amplified DNA was sequenced using dye terminator chemistry (Applied Biosystems, Perkin Elmer) or a thermo sequenase

TABLE I. Anastomosis (AG/CAG) and subgroup designation, origin and source of isolates of *Rhizoctonia* species with *Ceratobasidum* and *Thanatephorus* teleomorphs and their GenBank accession numbers

Anastomosis group/ subgroup	Isolate	Origin (Source)[1]	Region sequenced		GenBank accession number[2]
			ITS1 and ITS2	28S	
Thanatephorus (R. solani)					
AG-1-IA	1Rs (ATCC 66159)	Soybean, US (14)	*	*	AF354060
	2Rs (ATCC 66158)	Rice, US (14)	*		AF354097
	A-10	Rice, Japan (10)	*		AB000010
	Cs-Gi	Rice, Japan (10)	*		AB000016
	Cs-Ka	Rice, Japan (10)	*		AB000017
	T68 (IMI 358761)	Rice, Philippines (8)	*		AJ000197
	T5 (IMI 360366)	Rice, Vietnam (8)	*		AJ000199
	T58 (IMI 360021)	Dayflower, Ivory Coast (8)	*		AJ000200
AG-1-IB	36Rs (ATCC 66150)	Turfgrass, US (4)	*	*	AF354059
	SFBV-1	Sugar beet, Japan (10)	*		AB000038
	001-7	Soil, Japan (10)	*		AB000025
	SHIBA-1	Turfgrass, Japan (10)	*		AB000039
AG-1-IC	3Rs (ATCC 44661)	Pine, Canada (1)	*	*	AF354058
	PS-1	Sugar beet, Japan (10)	*		AB000029
	RH-28	Sugar beet, Japan (10)	*		AB000035
AG-2-1	8Rs (ATCC 44658)	Soil, Australia (1)	*	*	AF354063
	56Rs (ATCC 62805)	Potato, US (6)	*		AF354105
	P-2	Potato, US (6)	*		AB000026
	P-5	Potato, US (6)	*		AB000027
	R123	Cabbage, Japan (10)	*		AB000030
AG-2-2 IIIB	15Rs	Mat rush, Japan (AO)	*		AF354116
AG-2-2 IV	BC-10 16Rs	Sugar beet, Japan (10)	*		AB000014
		Sugar beet, Japan (13)	*		AF354117
AG-3	4Rs (ATCC 14006)	Potato, US (12)	*	*	AF354064
	5Rs (ATCC 44660)	Potato, US (1)	*		AF354107
	42Rs (ATCC 14701)	Potato, US (2)	*		AF354106
	1	Tobacco, US (18)	*		AB000001
	30	Tobacco, US (18)	*		AB000002
	1600	Tobacco, US (18)	*		AB000004
	1614	Tobacco, US (18)	*		AB000005
	OKA-6	Tomato, Japan (10)	*		AB000023
	OKA-9	Tomato, Japan (10)	*		AB000024
	ST3-1	Potato, Japan (10)	*		AB000041
	ST4-1	Potato, Japan (10)	*		AB000042
	ST6-3	Potato, Japan (10)	*		AB000043
AG-4 HGI	AH-1 (ATCC 76126)	Peanut, Japan (10)	*	*	AB000012, AF354118
	78-23R-3	Spinach, Japan (13)	*		AB000007
	Chr-3	Chrysanthemum, Japan (13)	*		AB000015
	GM-3	Soybean, Japan (13)	*		AB000018
	Pf-10	Sugar beet, Japan (13)	*		AB000028
	R97	Sugar beet, Japan (13)	*		AB000031
	Me 8-2A	Cantaloupe, Spain (3)	*		RSU19952
	Me 8-4A (Me84)	Cantaloupe, Spain (3)	*		RSU19954
	Me 8-7A (Me87)	Cantaloupe, Spain (3)	*		RSU19956
	Rh13	Soil, Spain (3)	*		RSU19960
AG-4 HGII	Rh-165 (ATCC 76127)	Sugar beet, Japan (10)	*		AB000033
	77-26R-1	Sugar beet, Japan (13)	*		AB000006
	78-3R-9	Sugar beet, Japan (13)	*		AB000008
	78-4R-26	Sugar beet, Japan (13)	*		AB000009
	HI521-21	Soil, Japan (13)	*		AB000020

TABLE I. Continued

Anastomosis group/ subgroup	Isolate	Origin (Source)[1]	Region sequenced: ITS1 and ITS2	Region sequenced: 28S	GenBank accession number[2]
	Rh-131	Sugar beet, Japan (13)	*		AB000032
	Rh-264	Sugar beet, Japan (13)	*		AB000034
	RR5-2	Sugar beet, Japan (13)	*		AB000036
	UHBC	Sugar beet, Japan (13)	*		AB000045
	Pin JRS1	Pine, Spain (3)	*		RSU19958
	Pin JRS3	Pine, Spain (3)	*		RSU19959
	RSA	Snapbean, Spain (3)	*		RSU19964
	7Rs (ATCC 44662)	Alfalfa, US (1)	*	*	AF354074
	18Rs	Sugar beet, Japan (13)	*	*	AF354072
	30Rs (ATCC 48803)	Unknown, Canada (5)	*	*	AF354073
AG-4 HGIII	6Rs (ATCC 42127)	Conifer, US (5)	*	*	AF354077
	44Rs (ATCC 14007)	Sugar beet, US (12)	*	*	AF354075
	45Rs (ATCC 10177)	Sugar beet, US (9)	*	*	AF354076
AG-5	10Rs	Soybean, Japan (13)	*	*	AF354078
	19Rs	Soybean, Japan (13)	*		AF354112
	31RS	Sugar beet, Japan (15)	*		AF354113
	K31	Pine, Japan (13)	*		AB000021
AG-6 HG-I	72Rs	Soil, Japan (13)	*	*	AF354061
	70Rs	Soil, Japan (13)	*		AF354102
	UBU-1-A	Soil, Japan (13)	*		AF354103
	HAM1-1	Soil, Japan (13)	*		AB000019
AG-6 GV	74Rs	Soil, Japan (13)	*	*	AF354062
	75Rs	Soil, Japan (13)	*		AF354104
	HN1-1	Soil, Japan (13)	*		AF354101
	NKN2-1	Soil, Japan (10)	*		AB000022
AG-7	76Rs	Soil, Japan (6)	*	*	AB000003, AF354096
	63Rs	Soil, Japan (6)	*		AF354099
	21RS	Soil, Japan (6)	*		AF354098
	91ST8057-2A-RSA	Soil, US (17)	*		AF354100
AG-8	33Rs	Barley, Scotland (4)	*	*	AF354066
	(ZG1-2)SA50	Oats, Australia (7)	*	*	AF354067
	(ZG1-3)SA1512	Barley, Australia (16)	*	*	AF354068
	(ZG1-4)88351	Barley, Australia (11)	*	*	AF354069
	A68	Wheat, Australia (11)	*	*	AB000011, AF354119
AG-9	65Rs (ATCC 62804)	Potato, US (6)	*		AF354109
	111Rs	Potato, US (6)	*		AF354108
	116Rs	Potato, US (6)	*	*	AF354065
	S4R1-TX	Potato, US (6)	*		AB000037
	V12M-TP	Potato, US (6)	*		AB000046
AG-10	W45b3	Wheat, US (13)	*		AF354111
	(ZG9)91614	Barley, Australia (11)	*	*	AF354071
AG-11	(ZG-3)R1013	Lupine, Australia (20)	*	*	AF354079
	Roth16	Soybean, US (17)	*		AF354114
	Roth24	Soybean, US (17)	*		AF354115
AG-BI	22Rs	Soil, Japan (13)	*	*	AF354070
	AII-4	Soil, Japan (13)	*		AF354110
	TE2-4	Soil, Japan (13)	*		AB000044
Unknown	T62 (IMI 360038)	Rice, Ivory Coast (8)	*		AJ000201
	T6 (IMI 369673)	Soil, Benin (8)	*		AJ000202
Ceratobasidium (binucleate *Rhizoctonia* spp.)					
AG-A	C-662	Soil, Japan (13)	*	*	AF354092
	SN-2	Soil, Japan (13)	*		AB000040
AG-Ba	C-460	Rice, Japan (13)	*	*	AF354088

TABLE I. Continued

Anastomosis group/ subgroup	Isolate	Origin (Source)[1]	Region sequenced		GenBank accession number[2]
			ITS1 and ITS2	28S	
AG-Bb	C-455	Rice, Japan (13)	*	*	AF354087
	C2 (IMI 375129)	Rice, Ivory Coast (8)	*		AJ000191
	C1 (IMI 062599)	Rice, West Malaysia (8)	*		AJ000192
	C3 (IMI 375130)	Rice, Japan (8)	*		AJ000193
	C6 (IMI 375133)	Rice, Japan (8)	*		AJ000194
AG-Bo	SIR-2	Sweet potato, Japan (13)	*	*	AF354091
AG-D	C-610	Unknown, Japan (13)	*	*	AF354090
AG-F	SIR-1	Sweet potato, Japan (13)	*	*	AF354085
AG-H	STC-9	Soil, Japan (13)	*	*	AF354089
AG-L	FK02-1	Soil, Japan (13)	*	*	AF354093
AG-O	FK06-2	Soil, Japan (13)	*	*	AF354094
AG-Q	C-620	Soil, Japan (13)	*	*	AF354095
CAG-1	BN1	Turfgrass, US (4)	*	*	AF354086
CAG-3	BN31	Peanut, US (4)	*	*	AF354080
CAG-4	BN38	Soybean, US (4)	*	*	AF354081
CAG-5	BN37	Cucumber, US (4)	*	*	AF354082
CAG-6	BN74 (ATCC 13247)	*Erigeron*, US (4)	*	*	AF354083
CAG-7	BN22 (FL FTCC585)	*Pittosporum*, US (4)	*	*	AF354084
Unknown	Rh2815	Broadbean, Spain (19)	*		RSU19962
	521 (RH2815L)	Soil, Israel (19)	*		RSU19963

[1] Isolates provided by; 1 = N. Anderson; 2 = K. Barker; 3 = M. Boysen; 4 = L. Burpee; 5 = E. Butler; 6 = D. Carling; 7 = A. Dube; 8 = A. Johannson; 9 = J. Kotila; 10 = S. Kuninaga; 11 = G. MacNish; 12 = G. Papavizas; 13 = A. Ogoshi; 14 = N. O'Neill; 15 = S. Naito; 16 = S. Neate; 17 = C. Rothrock; 18 = D. Shew; 19 = B. Sneh; and 20 = M. Sweetingham.

[2] GenBank numbers with an "AF" prefix represent isolates sequenced in this study.

dye terminator cycle sequencing pre-mix kit (Amersham Life Science) as described by the manufacturer. The sequencing products were separated in a 6% polyacrylamide gel using an ABI-373A automated sequencer (Perkin-Elmer, Foster City, California).

Data analyses.—Two aligned sequence data sets were developed for phylogenetic analyses. The first data set included 41 rDNA sequences spanning both ITS and adjacent 5′-end LSU regions. The second data set included 122 ITS sequences (40 sequences from the first data set, 19 new sequences from this study and 63 previously characterized sequences from GenBank) (Boysen et al 1996, Johanson et al 1998, Kuninaga et al 1997). The complete list of isolates, sequences, and GenBank deposition numbers is presented in TABLE I.

For each data set, sequences were aligned using the Clustal V program (Higgins et al 1992) within the Megalign computer software package (Lasergene, DNASTAR Inc.) and later adjusted by visual examination. Regions of ambiguous alignment were excluded from further phylogenetic analysis. Other regions containing single-nucleotide insertions or deletions were included in the phylogenetic analyses, with gaps treated as missing data. The final data sets used for phylogenetic analysis are available from the authors and have also been deposited with TreeBASE (http://herbaria.harvard.edu/treebase/, number SN931).

Phylogenetic analysis was performed using the maximum parsimony criterion in PAUP* (Swofford 2000). Because both data sets were too large to perform complete tree-searches, alternative search strategies which maximized the exploration of larger "tree-space" (Maddison et al 1992, Olmstead et al 1993, Moncalvo et al 2000, Soltis et al 1998) were employed. This approach included full heuristic searches with simple taxon addition sequences when possible (with TBR swapping and MAXTREES unlimited), as well as multiple searches using random addition sequences, NNI swapping and MAXTREES set to between 2–10 trees at each step. Branch support was assessed by bootstrap analysis (Felsenstein 1985) based on 500 replicate heuristic searches using the "fast bootstrap" option in PAUP*. Because appropriate outgroups were not available, all phylogenies were midpoint rooted. An initial attempt included *Tulasnella arinosa* and *Botryobasidium intertexum* as potential outgroups, but sequence alignment was problematic due to low similarity and ambiguous correspondence of nucleotides. Additional tree topologies were also evaluated by constraining phylogenetic searches to seek trees which were consistent with alternative taxonomic hypotheses and then testing these against the most-parsimonious trees using Templeton's nonparametric test in PAUP*. Genetic divergence between ITS sequences were calculated in PAUP* (Swofford 2000) using Kimura's 2-parameter distance measure with base frequencies estimated from the data and gamma parameter = 0.5.

RESULTS

Combined analysis of ITS and LSU rDNA sequence data.—Alignment of the 5′ end of the LSU region was easily accomplished by visual examination and associated with the highly conserved nature of this region with most length mutations involving insertions or deletions of a single nucleotide (Hopple and Vilgalys 1999). In contrast to the LSU region, the ITS regions (particularly ITS1) contained many small deletions and insertions of one to several nucleotides, or were difficult to align. These variable regions did not align among isolates but were specific for certain AG subgroups of *Thanatephorus.* The combined ITS and LSU data set consisted of at total of 1643 aligned nucleotide positions, which was reduced to 1517 positions after removal of regions with ambiguous alignment.

Heuristic searches revealed three most-parsimonious trees with a length of 2130, CI = 0.483, and RI = 0.71; one of the most parsimonious trees is shown in FIG. 1. A strict consensus of all three trees differed only in the placement of several unsupported branches. Phylogenetic analysis of the combined ITS and LSU data set supported several monophyletic groupings as follows: within *Ceratobasidium* (FIG. 1), AG-Ba with AG-Bb (clade 1); AGL with AG-O (clade 2); AG-A with AG-Bo (clade 3); AG-D with CAG-1 (clade 4); and AG-F with CAG-5 (clade 7); and within *Thanatephorus,* AG-4 HGI and AG-4 HGII (clade 5); AG-4 HG III (clade-6); AG-6 HGI and AG-6 GV (clade 8); AG-1-IA and AG-1-IC (clade 9); AG-2–1 and AG-9 (clade 10); AG-8 (clade 11); and AG-5 with AG-11 (clade 12).

Within the combined ITS and LSU tree, there was strong support (93% bootstrap value) for separation of two major groups of sequences corresponding largely but not completely with taxonomic division of isolates according to their associated teleomorph (*Ceratobasidium* italicized, and Thanatephorus not italicized). However, six sequences representing *Ceratobasidium* anastomosis groups CAG-3, CAG-4, CAG-5, CAG-6, CAG-7, and AG-F were found to group with other sequences belonging to the larger *Thanatephorus* clade. Phylogenetic searches in which *Ceratobasidium* and *Thanatephorus* were constrained to be monophyletic revealed trees that were significantly longer (22 additional steps) than the most parsimonious trees when tested using the Templeton test ($p < 0.02$). Based on combined ITS and LSU sequences, we can reject the hypothesis that *Ceratobasidium* and *Thanatephorus* as morphologically circumscribed represent mutually monophyletic groups.

Analysis of ITS rDNA sequence data.—The ITS data matrix included 853 aligned positions, of which 270 positions had to be excluded from analysis because they could not be unambiguously aligned. Of the remaining 583 positions, 162 were phylogenetically informative. Parsimony analysis yielded over 5000 equally parsimonious trees with a length of 535 (CI = 0.551, RI = 0.872). One of the most parsimonious trees is shown as a phylogram in FIG. 2 together with fast bootstrap support values for branches with support.

Phylogenetic analysis of the ITS sequence data revealed numerous well supported terminal groupings (bootstrap support = 72–100%) that correspond with previously recognized AG or AG subgroups within *Thanatephorus* (AG-1-IA and AG-1-IC, AG-1-IB; AG-6 HGI and AG-6 GV; AG-2–1 and AG-9; AG-4 HGI and AG-4 HGII, AG-4 HGIII; and AG-3; AG-5; AG-7; AG-8; AG-10; AG-11; AG-BI) and *Ceratobasidium* (AG-F and CAG-5; AG-L and AG-O; CAG-1 and AG-D; and AG-Ba and AG-Bb) (FIG. 2). At least 31 genetically distinct groupings could be identified based on differences in their ITS sequences from *Thanatephorus* (21 groups) and *Ceratobasidium* (10 groups). Within any single genetic group, ITS sequences showed little or no genetic divergence. Within an AG, sequence divergence among strains from the same AG varied as follows: AG-1 (from 2–4%), AG-2 (1.4%–3.5%), AG-4 (1–3%) and AG-6 (0–0.5%). Genetic divergence between unrelated groups of AG within *Thanatephorus* varied more widely, from 0.9% (between AG-2–1 and AG-9) to 8.1% (between AG-1-IA and AG-BI). Although sampling within *Ceratobasidium* was more limited, sequence divergence among genetic groups of *Ceratobasidium* appears to be greater than within *Thanatephorus,* ranging from 1.1% (between AG-Bo and AG-A) up to 16.1% (CAG-1 and CAG-3). The highest levels of sequence divergence were observed between isolates of *Thanatephorus* and *Ceratobasidium,* ranging from 1.9% between CAG-6 and AG-9, up to 16.5% between AG-1-IB and CAG-1.

Phylogenetic analysis of ITS sequences also suggested that several *Ceratobasidium* anastomosis groups might be more closely related with other groups from *Thanatephorus* than with other groups from *Ceratobasidium.* For example, phylogenetic analysis placed both CAG-4 and CAG-6 basal to the AG-4 clade (FIG. 2). However, these relationships are not strongly supported by bootstrap analysis and by other equally parsimonious trees. Phylogenetically constrained trees (with *Thanatephorus* and *Ceratobasidium* each constrained to be monophyletic) were not significantly longer (four extra steps) than the most parsimonious ITS trees (Templeton test, $p = 0.21–0.25$). Based on ITS evidence alone, therefore, monophyly of each teleomorph genus cannot be rejected.

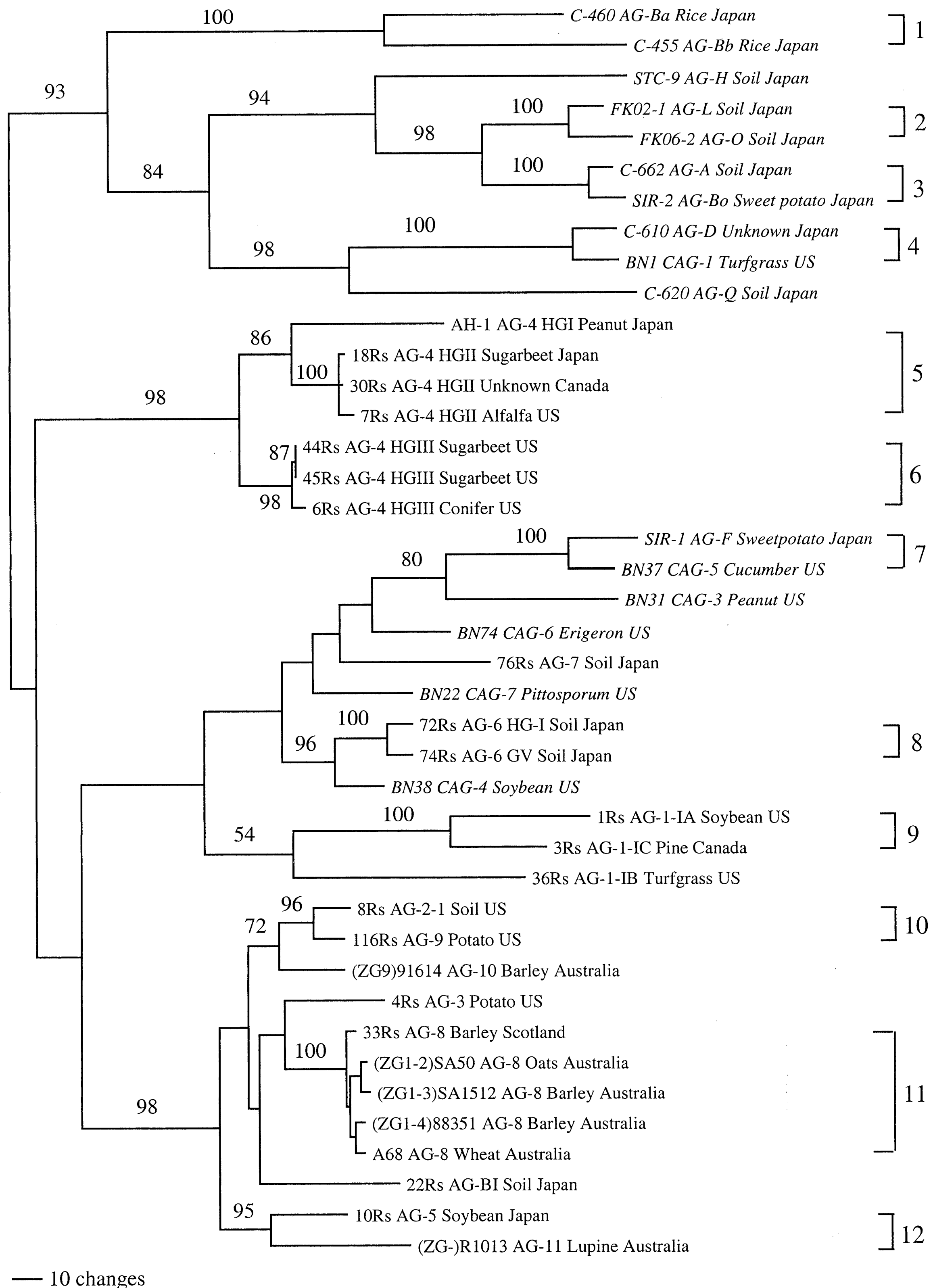

FIG. 1. One of three most parsimonious trees based on phylogenetic analyses with PAUP* Swofford 2000) of internal transcribed spacer (ITS) and adjacent 28S large subunit (LSU) regions of nuclear-encoded ribosomal DNA (rDNA). This analysis includes 41 isolates representing 28 anastomosis groups of *Ceratobasidium* and *Thanatephorus*. Tree length is 2130 steps with 462 phylogenetically informative characters. Consistency index (CI) = 0.483, and retention index (RI) = 0.71. The relative support for each clade is indicated by bootstrap values on branches.

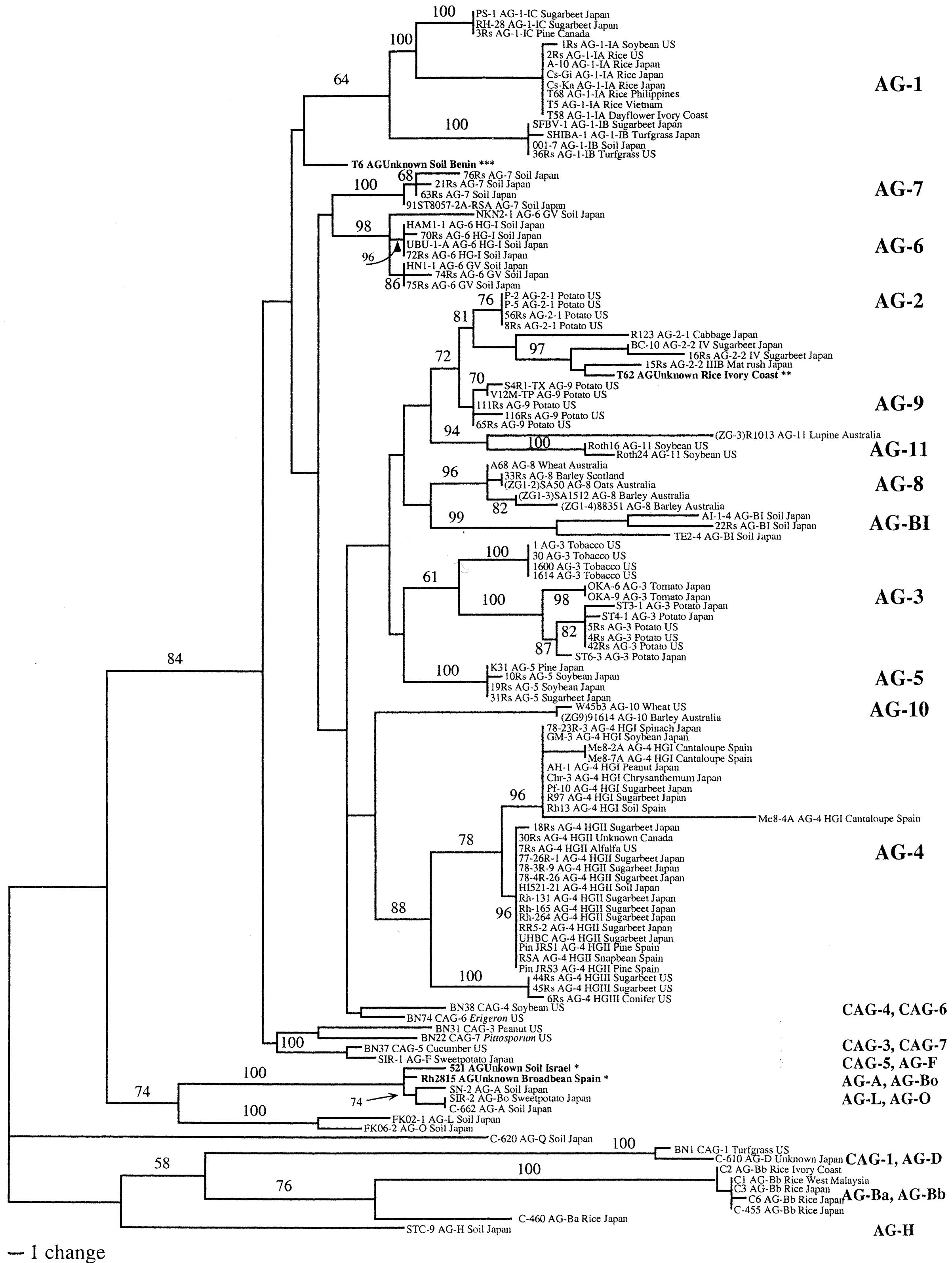

FIG. 2. Consensus tree based on phylogenetic analyses with PAUP* (Swofford 2000) of internal transcribed spacer (ITS) regions of nuclear-encoded ribosomal DNA (rDNA) from 122 isolates representing 28 anastomosis groups of *Ceratobasidium* and *Thanatephorus*. Tree length is 535 steps with 162 phylogenetically informative characters. Consistency index (CI) = 0.551 and retention index (RI) = 0.872. The relative support for each clade is indicated by bootstrap values on branches.

DISCUSSION

Phylogenetic distinction between Thanatephorus *and* Ceratobasidium.—Much controversy still surrounds the taxonomy of *Ceratobasidium* and *Thanatephorus,* and their phylogenetic placement within the Basidiomycota. Results from this study (FIGS. 1 AND 2) suggest that at least some isolates of *Thanatephorus* may be more closely related with other isolates from *Ceratobasidium.* However, these relationships were not as strongly supported by bootstrap analysis. Recent molecular evidence from small subunit rDNA sequence analysis suggests that *Thanatephorus, Ceratobasidium,* and *Waitea* (of the Ceratobasidiales) are closely related to each other and to other fungi belonging to the euagaric clade, which includes mushrooms and their allies (Hibbett and Thorn 2001).

When grown on nutrient medium, many isolates of *Thanatephorus* and *Ceratobasidium* often have a similar appearance (Kotila 1929, Parmeter et al 1967, Burpee et al 1980, Ogoshi 1987). In this study, all isolates were examined for their nuclear condition and *Rhizoctonia* species associated with *Ceratobasidium* had only two nuclei per hyphal cell, whereas those associated with *Thanatephorus* were multinucleate (3 or more nuclei per hyphal cell). In addition, each isolate of the six AG that clustered within the *Thanatephorus* clade was re-examined for its nuclear condition and found to be binucleate. Burpee et al (1980) reported that isolates of binucleate *Rhizoctonia* (representing CAG-3, CAG-4 and CAG-5) caused pre-and post emergence damping-off of bean, pea and tomato, and were often morphologically indistinguishable from *R. solani.* In absence of a teleomorph, Burpee et al (1980) assigned these isolates to *Ceratobasidium* based on their hyphal anastomosis reactions and binucleate nuclear condition, but also expressed some uncertainty about their taxonomic placement. Yokoyama and Ogoshi (1986) have observed hyphal fusion among isolates of *Ceratobasidum* (AG-F) and *Thanatephorus* (AG-6) which also suggested that certain isolates of *Ceratobasidium* and *Thanatephorus* may possibly be genetically related. However, since only a single isolate for each of the six AG of *Ceratobasidium* that clustered in the *Thanatephorus* clade was sequenced, more testing with additional isolates representing each of these AG is required to substantiate these relationships.

Few studies to date have examined taxonomic relationships among *Ceratobasidium* and *Thanatephorus* using DNA-based methods. Johanson et al (1998) studied ITS sequence data and found that isolates of *Ceratobasidium oryzae-sativae* (anamorph = *R. oryzae-sativae,* anastomosis group AG-Bb) were more closely related with *T. cucumeris* (=*R. solani* AG-1) than with *Waitea circinata* (anamorph = *R. oryzae,* WAG-O). Also, Boidin et al (1998) analyzed a rather large ITS data matrix to infer phylogenetic relationships among fungi with *Rhizoctonia* anamorphs, and concluded that *Ceratobasidium* was closely related to *Thanatephorus, Uthatobasidium* and *Waitea.* Our study extends previous research by analyzing a larger set of rDNA sequences from a greater variety of both multinucleate (*Thanatephorus*) and binucleate (*Ceratobasidium*) isolates. Statistical evidence from rDNA phylogenies also suggests that some isolates currently classified in *Ceratobasidium* based on nuclear condition and hyphal anastomosis reaction might be more correctly classified within *Thanatephorus.* To better resolve this question, additional phylogenetic analyses are still needed using isolates that represent outgroup taxa. Based on molecular as well as ultrastructural evidence, possible outgroup taxa to consider include *Waitea* or *Uthatobasidium,* as well as other members of the euagaric clade in the homobasidiomycetes (Hibbett and Thorn 2001). All of these species possess variably perforated parenthosomes.

Identification of anastomosis groups, AG subgroups and species based on rDNA sequences.—Numerous studies have demonstrated the tremendous genetic diversity that exists within both *Thanatephorus* and *Ceratobasidium,* manifested as an ever growing number of genetically distinct anastomosis groups and subgroups (Vilgalys and Cubeta 1994, Carling 1996, Kuninaga et al 1997, Salazar et al 2000). As more laboratories begin to collect sequence data for different isolates of *Rhizoctonia,* analysis of rDNA sequences can serve as an independent and convenient method for identifying genetically distinct groups within the *Rhizoctonia* species complex.

Because the ITS region is known to have a higher rate of molecular evolution than other ribosomal genes (Hibbett et al 1997), ITS sequences have been particularly useful for identifying previously undetected genetic groups at the species level. In a recent study, Kuninaga et al (1997) demonstrated the utility of rDNA ITS sequences for assessing genetic diversity and identification of AG and AG subgroups among 45 isolates of *Thanatephorus.* Isolates belonging within the same AG shared high sequence similarity (above 96%), whereas isolates from different AG showed significantly less similarity (55% or higher). In this study, we combined data primarily from the previous work of Kuninaga et al (1997) with new sequences from our lab and with sequences from other studies (Boysen et al 1996, Johanson et al 1998). For the 99 *Thanatephorus* isolates included in this study, at least 21 genetically distinct ITS groups could be identified. These 21 groups corresponded extremely

well with known groups based on hyphal anastomosis typing. In addition, at least 10 putative genetic groups were evident for 23 isolates of *Ceratobasidium* based on their ITS rDNA sequences. Interestingly, although fewer isolates of *Ceratobasidium* were investigated in our study, their genetic diversity was higher than for isolates of *Thanatephorus*. This observation suggests that *Ceratobasidium* may also harbor many additional as-yet-undescribed genetic groups.

In general, analysis of ITS and combined ITS and LSU sequence data provided similar conclusions about relationships of *Rhizoctonia* anamorphs of *Ceratobasidium,* as have been described in a previous studies based on RFLP analysis of LSU (Cubeta et al 1991). For example, AG-A and AG-Bo (*C. cornigerum*); AG-Ba (*C. setariae,* anamorph = *R. fumigata*) and AG-Bb (*C. oryzae-sativae*); AG-D and CAG-1 (*C. cereale* Murray and Burpee (anamorph = *R. cerealis* Van der Hoeven); AG-F and CAG-5 (*Ceratobasidium* sp.); and AG-L and AG-O (*Ceratobasidium* sp.) appear to represent independent evolutionary lineages that correspond to different species of *Ceratobasidium.* Additional sampling should reveal increased resolution of phylogenetic structure in these groups and phylogenetic accuracy will improve with the addition of replicates per taxon and with an increase in the number of characters (Smouse et al 1991, Graybeal 1998, Poe 1998).

Experimental results from this and previous studies that have examined rDNA ITS sequences of *R. solani* (González 1992, Boysen et al 1996, Kuninaga et al 1997, Boidin et al 1998, Johanson et al 1998, Salazar et al 1999, 2000) have several common themes: 1) most AG and AG subgroups represent genetically distinct groups which support previous separation based on hyphal anastomosis behavior, 2) certain AG are not monophyletic; and 3) there is greater taxonomic support for AG subgroups than AG. Given the genetic diversity that has been identified within an AG, only a few studies have explored the relationship of AG and subgroups to species or other taxonomic units. Boidin et al (1998) recognized four species: 1) *Thanatephorus microsclerotius* (Weber) Boidin, Mugmier & Canales including AG-1-IB; 2) *T. sasaki* (Shirai) Tu & Kimbrough including AG-1-IA and AG-1-IC; 3) *T. praticola* (Kotila) Flentje including AG-4; and 4) *T. cucumeris* including AG-2, 3, 5, 6, 8, 9 and BI. AG-7 was not included in any of the above species. A biochemical approach was used by Mordue et al (1989) to study the taxonomy of *Rhizoctonia* (12 isolates of *R. solani* representing eight AG and 13 species of *Rhizoctonia* from orchids) based on cultural characteristics, carbon and nitrogen utilization and enzyme production. Mordue et al (1989) recognized AG-4 as a distinct species, *Thanatephorus praticola,* while the remaining subgroups of *R. solani* were assigned to *T. cucumeris.* However, they did not recognize AG-1-IA (*T. sasakii* (Shirai) Tu & Kimbrough, the causal agent of sheath blight of rice), AG-1-IB (*Corticium microsclerotium* Weber (anamorph = *Rhizoctonia microsclerotia* Matz, the causal agent of web blight of bean), or AG-1-IC as taxa distinct from *T. cucumeris.* Kuninaga et al (1997) was able to separate subgroups within *R. solani* AG-1 (IA, IB and IC) and AG-4 (HGI and HGII) based on sequence analysis of rDNA ITS region and suggested that they represent independent evolutionary units. Several groups with higher support in a neighbor joining tree were evident (AG-4 = 97%; AG-2–2 IIIB and IV = 99%; AG-2–1 = 85%; AG-9 = 99%; AG-6 and AG-7 = 75%). Two clusters of AG-4 isolates corresponded to HGI and HGII; AG-6 to HG-I and GV, and AG-9 to TX and TP subgroups based on previous DNA/DNA hybridization studies. Subgroups of AG-1 represented a distinct cluster based on neighbor joining tree, but had moderate support with bootstrap analysis (63%). Results from ITS1 rDNA sequence analysis in this study are consistent with the results of Kuninaga et al (1997). However, the combined analysis of the ITS and LSU region grouped AG-1-IA and AG-1-IC, but not AG-1-IB together. Also, the combined analysis of the ITS and LSU region grouped AG-4 HGI and AG-4 HGII, but not AG-4 HGIII. Therefore, the hypothesis that AG represents the most fundamental evolutionary units within *Thanatephorus* (anamorph = *R. solani*) was rejected.

The availability of a large ITS database allowed an expanded phylogenetic analysis of *R. solani.* The ITS region was very difficult to align and exhibited more homoplasy than the LSU region. Although this alignment generated many indels in the ITS region, most were concentrated in six highly variable regions. In our analyses only phylogenetically informative characters were used. We chose to delete gapped (ambiguous) and variable regions based on rigorous examination of the effect of removing these regions of data (data not shown). Although the highly variable ITS1 rDNA region was useful for identifying individuals, it may be inappropriate for phylogenetic analysis due to excessive nucleotide deletions, insertions and substitutions (Kuninaga et al 1997). Therefore, we conducted an analysis with combined ITS and LSU sequence data. This illustrates the importance of striking a balance between alignment, homoplasy and phylogenetically informative characters to achieve the desired level of taxonomic resolution.

Several ITS sequences available in GenBank were problematic and not included in our analysis. These sequences were very difficult to align and appeared as long branches in our phylogenetic tree, suggesting

the presence of many autapomorphies associated with sequencing errors. As part of this study, three isolates (1556, AH-1, and A68) previously sequenced by Kuninaga et al (1997) were re-sequenced (76Rs, ATCC 76126, and A68, respectively, in this study) to provide a measure of sequence quality. The sequences differed by two nucleotides, were easy to align and no long branches were evident in the subsequent analysis. With the accumulation of additional sequence data in the future, it will be very important to maintain high sequence quality to minimize misinterpretation of data by including previously sequenced isolates as an internal control.

The potential utility for unambiguous identification of isolates of *Ceratobasidium* and *Thanatephorus* is of practical significance to many mycologists and plant pathologists. Isolates 521 and Rh2815, originally described by Boysen et al (1996) as *R. solani* AG-4, were later identified as anamorphs of *Ceratobasidium* based on analysis of the rDNA ITS region (Kuninaga et al 1997, Boidin et al 1998, Salazar et al 1999). These isolates were placed in our data matrix and results suggest that they may belong to AG-A of *Ceratobasidium* (indicated in bold with a single asterisk * in FIG. 2). Also when isolate T62 of Johanson et al (1998) was placed in the data matrix, it grouped with AG-2–2 IIIB (indicated in bold with two asterisks ** in FIG. 2). In this study, we were unable to associate isolate T6 of Johanson et al (1998) with a specific AG (indicated in bold wih three asterisks *** in FIG. 2), which provided an indication of the limitations for sole use of rDNA-based data for identification. The accumulation of a larger and high quality rDNA sequence database should establish a foundation for the development of species concepts in *Rhizoctonia* and testing hypotheses related to geographic subdivision, host, and ecological specialization. For example, do ecologically interacting isolates share a common gene pool, host and/or geographic preference? The analysis of large data sets will present some challenges in the future, but DNA data coupled with additional characters should facilitate unambiguous identification of *Ceratobasidium* and *Thanatephorus*.

The majority of taxonomic studies have employed phenetic and distance based methods to infer phylogenetic relationships. Numerical methods through distance matrices group taxa based on overall similarity (a combination of synapomorphies, symplesiomorphies, and homoplasy) and not for their phylogenetic relationships. In a phenogram it is not possible to evaluate the contribution of each character in the formation of the groups. Therefore, there was a recognized need for a robust analysis well rooted in hypothesis testing to examine the phylogenetic relationships within *Ceratobasidium* and *Thanatephorus*. Despite the limitations of the bootstrap, they provide some indication of the internal support for the *Ceratobasidium* and *Thanatephorus* clades (Sanderson, 1989). Results from combined analysis of ITS and LSU sequence data suggest that AG-1, AG-4, AG-6, and AG-8 represent well-defined and genetically isolated groups, while AG-2 and AG-3 are of multiple origin (polyphyletic).

Although the taxonomic ranking of AG has been debated for decades, AG-4 was considered a distinct species by Kotila in 1929, prior to the development of the AG concept, and by other researchers since (Talbot 1970, Ogoshi 1987, Saksena and Vaartaja 1961, Anderson 1982). Information that has been accumulating in the past few years based on molecular data has provided additional support for AG-4 as a distinct species. However, we are not certain whether subgroups within AG-4 should be considered as species. We have taken a conservative approach and suggest that AG-4, but not its associated subgroups, represents a species that should be given formal taxonomic status.

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