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REVIEW PAPER

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The advancing identification and classification of *Rhizoctonia* spp. using molecular and biotechnological methods compared with the classical anastomosis grouping

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Abstract Anamorphic classification of *Rhizoctonia* spp. has been based on young cell nuclear numbers and hyphal fusion to anastomosis groups (AGs), in addition to the teleomorph classification. The widespread development of molecular biology techniques has added modern tools to support classification of organisms according to their genetics and evolutionary processes. These various methods have also been used in recent years for classification of *Rhizoctonia.* Data are continuously accumulating in the literature and the sequences in databases, which are readily available for researchers in the network systems. In the present review, attempts were made to describe and compare the advantages and disadvantages of the various methods for the classification of *Rhizoctonia* spp. Currently, the rDNA-internal transcribed spacer (ITS) sequence analysis seems to be the most appropriate method for classification of *Rhizoctonia* spp. Data of all the appropriate multinucleate *Rhizoctonia* (MNR) accumulated in GenBank were analyzed together in neighbor-joining (NJ) and maximum-parsimony (MP) trees supplemented with percent sequence similarity within and among AGs and subgroups. Generally, the clusters of the isolate sequences were supportive of the AGs and subgroups based on hyphal fusion anastomosis. The review also indicates inaccuracies in designation of sequences of some isolates deposited in GenBank. The review includes detailed analyses of the MNR groups and subgroups, whereas complementary descriptions of the binucleate *Rhizoctonia* (BNR), uninucleate *Rhizoctonia* (UNR), and comprehensive interrelationships among all the currently available MNR, BNR, and UNR groups and subgroups in GenBank are to be discussed in a subsequent review article.

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Introduction

Morphotaxonomic criteria, which continue to be valid, are not always sufficiently accurate or convenient to taxonomically define isolates of the form-genus *Rhizoctonia*. Classification of *Rhizoctonia* spp. was first based on characterization of the cell nuclear condition [multinucleate (MNR), binucleate (BNR), and uninucleate (UNR)] and the ability of hyphae to anastomose with tester isolates of designated anastomosis groups (AGs) (Sneh et al. 1991). Although the anastomosis method is accurate, valid, and currently used, it is sometimes impossible to determine to which AG an isolate belongs by anastomosis, because certain isolates do not anastomose with representatives of any known AG while some isolates have lost their capability to self-anastomose (Hyakumachi and Ui 1987). On the other hand, isolates of certain AGs anastomose also with isolates of more than one AG [e.g., AGs 2 (including BI), 3, 6, and 8] (Sneh et al. 1991; Carling 1996). In addition, determination of AGs by hyphal anastomosis requires meticulous microscopic experience, and it is a time-consuming procedure. Several molecular techniques have been found to simplify and/or more accurately classify isolates of this form-genus.

The present review describes the advances in various molecular techniques for classification of *Rhizoctonia* spp. compared with the classical anastomosis grouping method. DNA sequences encoding ribosomal RNA genes, especially the internal transcribed spacer regions (ITS1 and ITS2) flanking the 5.8S subunit, have been very useful for evaluating phylogenetic and taxonomic relationships and determining genetic diversity in fungal species (Bruns et al. 1991). Among the various molecular classification methods used for classification of *Rhizoctonia* spp., the rDNA-ITS sequence analysis seems to be the currently most appropriate one. A comprehensive approach for the identification and classification of *Rhizoctonia* spp. isolates is attempted

Table 1. Relative efficacies of the various molecular methods used for classification of *Rhizoctonia* spp.

Method	Different AG	Same AG	Subgroups within AG	Individuals
Nucleic acids				
DNA–DNA hybridization		+++	+++	
RFLP				
18S, 28S rDNA	+++	+	+	
ITS rDNA		+++	+	
AT-rich DNA		+	+	+++
Single-copy nuclear DNA		+	+	+++
DNA fingerprinting				
RAPD				+++
AFLP				+++
DNA sequencing				
18S, 28S rDNA	+++	+++	+	
ITS rDNA	+++	+++	+++	
Electrophoretic karyotyping		+	+	+
Proteins				
Isozymes		+	+	+++
Zymograms		+	+	+++
Cellular fatty acids	+++	+	+	

AG, anastomosis groups; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; ITS, internal transcribed spacer
+++, range that can be expected to yield better results; +, range that might be expected to yield less suitable results

in the present review. It is based on rDNA-ITS sequence alignment analysis (by which the genetic relatedness of the isolates is exhibited by clustering of isolate sequences in a tree), complemented with detailed percent sequence similarity within and among AGs and subgroups; these are compared with the anastomosis grouping method.

The review includes detailed analyses of the MNR groups and subgroups, while complementary descriptions of the BNR, UNR, and comprehensive interrelationships among all currently available in GenBank MNR, BNR, and UNR groups and subgroups will be discussed in a subsequent review article.

Comparison of molecular and biochemical methods for classification of *Rhizoctonia* spp.

The classic taxonomy of the anamorph "form-genus" *Rhizoctonia*, which includes three major groups: MNR (teleomorphs *Thanatephorus* and *Waitea*), BNR (teleomorphs *Ceratobasidium* and *Tulasnella*), and UNR (teleomorph *Ceratobasidium*) has been essentially based on hyphal fusion that divided *Rhizoctonia* spp. into the well-established anastomosis groups (AGs) and further into subgroups. The introduction of polymerase chain reaction (PCR) and various molecular and biochemical tools in recent decades have confirmed the genetic relatedness validity of the AGs and greatly advanced the accuracy of its classification. The variety of these methods have also been used to develop rapid PCR-based diagnostic tools for accurate identification of the isolates to AGs and their subgroups. Furthermore, studies on the genetic structure of natural *Rhizoctonia* spp. field populations using various molecular markers have been emerging in recent years.

The various molecular methods used for classification of *Rhizoctonia* spp. include isozyme analysis, total cellular fatty acids analysis, electrophoretic karyotyping, DNA–DNA hybridization, DNA fingerprinting based on random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), repetitive probe, AT-rich DNA restriction fragment length polymorphism (RFLP), single-copy nuclear RFLP, rDNA RFLP, and rDNA sequence analysis. The various molecular methods differ in their ease of use and their efficacy to determine genetic relationships and diversity among the isolates. The genetic studies of *Rhizoctonia* spp. using these various techniques are reviewed with attempts to emphasize the advantages and drawbacks of each of these methods (Table 1).

Biochemical methods

Isozyme analysis

Isozymes are defined as multiple molecular forms of a certain enzyme, which are actually encoded by different alleles or numerous loci, scattered over the genome. Electrophoretic separation of isozymes relies on the existence of genetic polymorphisms, resulting in amino acid substitutions that are responsible for the differences in relative mobilities among allelic forms on an electrophoretic gel. Isozyme analysis has potential use as (1) an indicator for genetic diversity, as isozymes indicate high levels of variation in closely related isolates within a fungal group or subgroup, and as (2) markers for population biology of *Rhizoctonia* spp., because it enables simultaneous examination of a large number of isolates. Isozyme analysis has been used to determine relationships and diversity among isolates of different AGs and subgroups of MNR (Liu et al. 1990; Liu and Sinclair 1992, 1993; Laroche et al. 1992) and of BNR (Damaj et al. 1993). Generally, isozyme electrophoretic patterns provided good indications for genetic di-

versities among AGs, within the subgroups, and reconfirmed the genetic basis of the AG concept. Based on 10 enzyme systems, at least 6 subgroups within AG 1 and 5 subgroups within AG 2 were distinguished (Liu and Sinclair 1992, 1993). However, the use of isozyme analysis alone was not always sufficient for the identification of the previously known AG 1 subgroups. Therefore, this method may provide information about individual isolates with limited variations within a fungal population, although a large population is required for determining the frequency of distribution of the particular alleles and locus.

Isozyme profiles were also used to differentiate between isolates of AG 3 and AG 9 from potato fields. In addition, cluster analysis of isozyme banding patterns could differentiate AG 3 isolates to subgroups (IIA, IIB, and IIC). The Canadian and American isolates (subgroup IIC) were grouped together, although they were genetically distant from those originating from Asia and Europe (subgroup IIB) (Laroche et al. 1992). These results were supported by Ceresini et al. (2002), who suggested that AG 3 genotypes from the northern United States and Canada migrated into North Carolina by introduction of infested seed potato tubers. Isozyme phenotypes supported the separation of BNR isolates into four genetically distinct groups (Damaj et al. 1993). These groups are congruent with five of seven rDNA groups identified by 28S rDNA RFLP patterns (Cubeta et al. 1991).

Rhizoctonia solani isolates are known to produce pectin esterases and polygalacturonases defined as a pectic zymogram. Analysis of the pectic zymogram is one of the approaches for the detection of isozymes. This method successfully differentiated among AG 8 subgroups (Sweetingham et al. 1986; Neate et al. 1988; Cruikshank 1990; MacNish and Sweetingham 1993; MacNish et al. 1993, 1994), AG 2 field isolates pathogenic to flower bulbs (Schneider et al. 1997) or to tobacco (Nicoletti et al. 1999), and AG 1-IA isolates from rice paddies (Banniza and Rutherford 2001).

At least five pectic zymogram-pattern groups were determined among AG 8 isolates (ZG1-1–ZG1-5). Isolates of the same ZG may originate from the same clone (C3 hyphal anastomosis reaction) or different clones (C2 reaction), whereas isolates between different ZGs produce a C2 reaction type (MacNish et al. 1994).

Using DNA fingerprinting, Matthew et al. (1995) indicated that many different clones may exist within each zymogram group of AG 8. The ZG method was successfully used in studying AG 8 field isolate distribution patterns. ZG groups could not be differentiated by hyphal fusion frequency (MacNish et al. 1994) or by rDNA-ITS sequence analysis (Kuninaga, unpublished data). The different ZGs were related to disease symptoms, and thus this method could also be useful for disease management caused by AG 8 isolates (Sweetingham et al. 1986; Neate et al. 1988; MacNish and Sweetingham 1993). This method was also good for easy identification of AG 2-1 2t isolates (pathogenic to tulips at relatively cool temperatures) (Schneider et al. 1997).

Similarly, AG 1-IA isolates from one field were of the same zymogram pattern, but had a variety of genomic fingerprinting patterns generated by the SSR (simple sequence repeats)-PCR method (Banniza and Rutherford 2001), indicating that isolates of the same ZG are either clonal or very closely related. However, in the absence of other genetic markers, pectic enzyme analysis may not be sufficiently sensitive to discern a clone. The ZG method may serve as a good system to identify MNR AGs and subgroups. Isolates of different AGs had different ZG patterns, and there were unique ZG patterns within each AG (Cruikshank 1990; MacNish et al. 1994; Banniza and Rutherford 2001). However, still more isolates representing all the AGs should be tested to confirm that ZGs are limited to the same AG and subgroup.

Fatty acids analysis

Fatty acid methyl ester (FAME) analysis had been routinely used to characterize and differentiate closely related strains in bacteria and yeast. This method is based on the analysis and identification of FAME derivatives using gas-liquid chromatography coupled with a computer software package [Microbial Identification System; Microbial ID (MIDI), Newark, DE, USA]. A slightly modified MIDI method was successfully used to characterize MNR AGs (Johnk and Jones 1992). Subgroups of the following MNR AGs were differentiated with this method: AG 2 (Johnk and Jones 1993; Matsumoto and Matsuyama 1999), AG 3 (Johnk et al. 1993), and AG 4 (Johnk and Jones 2001). However, AG 1-IA could not be differentiated from AG 1-IB, although AG 1-IC isolates were distinct (Johnk and Jones 1994; Priyatmojo et al. 2001). FAME profiles coupled with another molecular method could differentiate AG 1 (the coffee necrotic leaf spot pathogen) as a new AG 1 subgroup named AG 1-ID (Priyatmojo et al. 2001).

Whole-cell fatty acid analysis was used to characterize and distinguish isolates of different *R. solani* AGs. However, this method was not sufficiently accurate to distinguish between isolates of AG 4 and AG 7 (Baird et al. 2000). Clustal analysis of all fatty acids of isolates of three varieties of *Waitea* (*Rhizoctonia*) *circinata* clearly separated them into three distinct groups (Priyatmojo et al. 2002a). This method was also used for the identification of additional MNR and BNR AGs pathogenic to rice (Matsumoto et al. 1996, 1997; Lanoiselet et al. 2005) and turfgrass (Priyatmojo et al. 2002b).

Several technical issues may be problematic when the use of this method is considered. Cultural conditions such as culture age and temperature, which are critical parameters, are known to influence fatty acid composition. Uniformity of extraction protocol such as the saponification step, which is initiated to break the fungal cell wall to expose the phospholipids bilayer membrane, is also critical. Currently, results of fatty acid composition from different laboratories are inconsistent because of differences in cultural conditions and extraction procedures. Standardization of the method may be required to obtain better comparative results (Lanoiselet et al. 2005).

Electrophoretic karyotyping

Pulsed field gel electrophoresis (PFGE) is a method that enables size separation of very large DNA fragments (up to 12 Mb). Application of PFGE to separate chromosomal DNA in agarose matrices is a powerful method for fungal genomic research. It was initially used for separating *Saccharomyces cerevisiae* chromosomes (Carle and Olson 1984; Schwartz and Cantor 1984) but was subsequently widely used in many other fungi, including *Rhizoctonia* spp. (Wako et al. 1991; Keijer et al. 1996). The first estimation of chromosome number and genome size of an AG 4 isolate was reported by Wako et al. (1991). It was considered to have 6 chromosomes, of 0.8–3.8 Mb size range, and a total genome size of 11 Mb. In a subsequent study (Keijer et al. 1996), a considerable variation in karyotype among MNR isolates (of AGs 2-1, -3, and -4) was evident, and the chromosome number was at least 11. The chromosome size ranged from 0.6 to 6 Mb with an estimated genome size of 37–46 Mb. Karyotypes were smaller within an AG than among the AGs, and there was no strict correlation between karyotype and AGs. The consequent conclusion was that variation in karyotype is reflected in the phenotypic variation of the fungi, with generally less diversity within an AG than between AGs. Gross chromosomal rearrangements such as insertion (e.g., by transposable elements), deletions, duplication, and translocations, and the presence of B chromosomes could be suggested as sources for karyotype variation in *R. solani*.

Conventional cytological studies indicated that six chromosomes are present in *R. solani*. The conventional cytology by light microscopy may be misleading because of the inaccuracy of fungal chromosome staining and their small size, resulting in lower estimated chromosome numbers than those of PFGE for the same fungus. However, PFGE has limitations in the resolution of large chromosomes (about <6 Mb) as well as comigrating chromosomes of equal size, which might lead to the underestimation of chromosome number. Combination of PFGE with the Southern hybridization method is highly suitable for determining the localization of genetic markers in the chromosomes. Using this approach for chromosomal location of the rRNA gene cluster in AG 3 isolates, the ribosomal repeats were located in a single chromosome, whereas in AG 2-1 and AG 4 isolates the gene repeats were located in two different chromosomes (Keijer et al. 1996).

Analysis of genomic fingerprinting

RAPD (random amplified polymorphic DNA)

The RAPD analysis method is based on the use of single short oligonucleotide primers of arbitrary sequences for amplification of DNA segments distributed randomly throughout the genome. It is a fast and simple technique that does not require any prior knowledge of the analyzed genomes. The pattern of the amplified bands could be used for genomic fingerprinting, and the polymorphisms in the pattern of bands amplified from genetically distinct individuals behaved as Mendelian genetic markers (Welsh and McClelland 1990; Williams et al. 1990). RAPD markers have been successfully used for the following applications: (1) construction of genetic maps, (2) analysis of population genetic structure, (3) fingerprinting of individuals, and (4) targeting markers to specific regions of the genome.

The RAPD method successfully provided new genetic markers for genomic fingerprinting of several *R. solani* AGs: AG 1 (Toda et al. 1998; Pascual et al. 2000), AG 2 (Toda et al. 2004), AG 3 (Bounou et al. 1999), AG 8 (Tommerup et al. 1995; Yang et al. 1995), and AG 9 (Yang et al. 1996). The technique has been also used to understand the genetic relatedness of BNR AG-D (Nicolson and Parry 1996), UNR (Lilja et al. 1996), and *Rhizoctonia* endophytes (Shan et al. 2002).

Using the RAPD-PCR method, with isolates of MNR AGs 1 and 8 from Australia and Japan, Duncan et al. (1993) demonstrated considerable polymorphism among *R. solani* populations even within the same geographical region and showed that isolates from different geographical regions could be differentiated. RAPD was also a very useful method for rapid typing of MNR isolates (Toda et al. 1999a). The genetic relatedness among 41 isolates belonging to 11 MNR AGs was assessed by the fragment pattern analysis of amplified genomic DNA by RAPD-, ERIC- (enterobacterial repetitive intergenic consensus), and REP- (repetitive extragenic palindromic) PCR. Most of the isolates were grouped according to their AGs or subgroups by the RAPD, ERIC, and REP fingerprints. However, there was a significant high level of heterogeneity among the isolates of AGs 1-IC, 2, 3, and 4.

Despite its relatively easy use, the RAPD assay has several drawbacks that must be considered. Despite the reliability and reproducibility of RAPD patterns that were examined in standard reaction conditions (Tommerup et al. 1995), the results from different laboratories may not always be compatible. In addition, some analytical problems are also associated with the RAPD method. RAPD has only two alleles (amplification or nonamplification) for each amplicon locus. Individual bands are dominant and therefore may not effectively distinguish homozygotes from heterozygotes by the interpretation of DNA fingerprints from RAPD patterns.

However, some of these limitations could be overcome via conversion of RAPD amplicons into sequence-characterized amplified regions (SCARs). Several researchers have explored the use of informative RAPD markers to generate SCAR primers. Specific primer sets have been reported to develop a reliable and sensitive assay for the detection of AG 4 and AG 8 isolates infecting wheat (Brisbane et al. 1995; Bounou et al. 1999), AG 2-2 LP isolates (Toda et al. 2004), and BNR AG-G isolates in soil and plant samples (Leclerc-Potvin et al. 1999).

AFLP (amplified fragment length polymorphism)

AFLP provides a novel and very powerful fingerprinting method for DNA from any origin or complexity. It is based on selective PCR amplification of restriction fragments from a digest of the total genomic DNA. Typically, 50–100 restriction fragments are amplified and detected on denaturing polyacrylamide gels. AFLP is likely to be useful for DNA fingerprinting because a large number of loci can be screened in one reaction (Vos et al. 1995).

Currently, AFLP fingerprinting was used only by Ceresini et al. (2002) to evaluate the genetic diversity of field populations of AG 3 subgroups (PT and TB) in North Carolina, based on two independent criteria of AFLP markers and somatic compatibility. Each of the PT isolates (32) had distinct AFLP phenotypes, whereas 28 AFLP phenotypes were found among the TB isolates (36). Clones (somatically compatible of the same AFLP phenotype) were identified only in the TB population, whereas no clones could be detected among isolates of the PT population. In this study, based on a priori knowledge about the biology, ecology, and epidemiology of each pathogen, the hypothesis that the TB populations are more genetically diverse than the PT populations was evaluated. However, the results rejected the hypothesis because the TB isolates were represented by fewer AFLP phenotypes and somatic compatible groups than the PT isolates.

The main disadvantage of the AFLP markers is that alleles cannot be easily recognized. Allelic fragments will be scored as independent although actually they are not, which could lead to an overestimation of variation. Nevertheless, AFLP analysis has significant potential as a tool for studying the population genetics of *Rhizoctonia* spp.

RFLP analysis

DNA fingerprints based on RFLPs have been widely used in fungi. Multilocus minisatellite fingerprints based on hybridization of one probe to repetitive DNA sequences have greater resolution for clone differentiation. Repetitive probes were developed for specific fingerprint patterns of AG 8 (Matthew et al. 1995; Whisson et al. 1995) and AG 3 isolates (Balali et al. 1996). This method is more reproducible than RAPD but is technically more laborious. In addition, RFLP analysis requires cloning, Southern blotting, and labeling the probes, and relatively large amounts of DNA are required from each isolate.

Similar to the RAPD and AFLP markers, multilocus minisatellite fingerprints are also dominant markers that limit their potential use in population genetic analysis. Rosewich et al. (1999) developed this new approach using a single-copy RFLP marker that could unambiguously distinguish homozygotes from heterozygotes; it is more appropriate for elucidating the evolutionary processes. Using these seven codominant markers, they studied the population genetics of AG 1-IA RAPD. Among 182 isolates of the AG collected from six commercial fields in Texas, 36 multilocus RFLP genotypes were identified. As there was no evidence for population subdivision, all the isolates from the rice-growing regions in Texas were considered to be a part of a single AG 1-IA population. However, four of the seven loci were in Hardy–Weinberg equilibrium (HWE). In three loci, a significant departure from HWE was observed. They suggested that the loci not in HWE could be explained by a recent bottleneck in population size. The possible explanation was that the rice pathogen was evidently introduced from Asia in the recent past.

Codominant PCR-RFLP markers were used to detect individual genotypes of AG 3-PT from five fields of different counties in North Carolina. Multilocus PCR-RFLP genotypes (MRG) were determined by combining a specific PCR product and restriction enzymes for each of seven polymorphic loci. The analyzed allelic information from the seven single-locus PCR-RFLP markers yielded 32 MGR groups from 104 isolates, indicating high levels of gene flow among populations (Ceresini et al. 2002, 2003). Subsequently, this method was used to study the migration of the AG 3-PT populations from potato seed production sources (northern United States and eastern Canada) to recipient fields (North Carolina). Analysis of the molecular variation indicated that only little variation in genotypes existed between the seed source and the recipient populations or between subpopulations within each region, suggesting that the AG 3-PT populations in the recipient country fields originated from the source country fields.

These multilocus RFLP methods enable unambiguous scoring of isolates genotypes by enabling the distinction of homozygotes from heterozygotes. This method is highly efficient as only small amounts of DNA are initially required. A single-locus RFLP analysis is not likely to play an important role in population genetics studies of *Rhizoctonia* spp.

AT-rich RFLP

AT-DNA RFLPs are generated by digestion of total DNA with *Hae*III. This enzyme digests nuclear DNA to fragments <1 kb whereas AT-rich DNA is cleaved less frequently, resulting in discrete large MW bands. DNA fragments obtained by this method were presumed to be mitochondrial in a number of fungi (Banniza et al. 1999).

Distinct AT-DNA RFLP patterns were obtained for isolates belonging to different MNR AGs, whereas a lower variability was evident for AG 1-IA isolates from various countries. Isolates obtained from one field experiment consisted of only one identical RFLP pattern, but varied in their simple sequence repeat (SSR)-PCR, indicating that they are definitely not of one clone (Banniza and Rutherford 2001). These results suggest that the AT-rich DNA RFLP method may be suitable for studying different MNR AGs but may not be sufficiently sensitive for distinguishing among isolates or groups of isolates from different geographical regions.

DNA–DNA hybridization

The genome size of *R. solani* based DNA–DNA reassociation kinetics and electrophoretic karyotyping analysis is estimated to be about 28–46 Mb, and the repetitive sequences are considerably smaller (8%–17%) (Kuninaga 1996). Fungal genomes are considerably smaller than those of higher plants and therefore, may be highly appropriate for DNA hybridization studies. The DNA–DNA hybridization method using spectrophotometric or isotopic assays was performed in the mid-1980s on *R. solani* (Kuninaga and Yokosawa 1985; Vilgalys 1988). The first clear demonstration that anastomosis grouping may be the best indicator of the whole genomic DNA similarities in *R. solani* was confirmed by this method. DNA similarities among isolates within the same AG were confirmed to be >80% whereas those of isolates among AGs were only <30% (Carling and Kuninaga 1990; Kuninaga 1996). DNA hybridization measures the average DNA similarity of cross-hybridizing sequences, which undoubtedly includes paralogous and orthologous genes. In *R. solani*, however, this method proved appropriate for determining that AGs may clearly represent distinct biological species (Sneh et al. 1991; Kuninaga 1996, 2002). However, the hierarchy of AGs could not be resolved by comparing DNA hybridization similarity values because of low hybridization levels among different AGs. More detailed hierarchical phylogenetic groupings of AGs and their subgroups were successfully obtained by the following more-appropriate methods, which have been introduced since the early 1990s.

Analysis of ribosomal RNA genes

The readily available PCR primer sets (White et al. 1990) and fungal gene sequences in GenBank facilitated and enhanced comparable fungal studies, including *Rhizoctonia* spp., focused on rDNA analysis.

rDNA RFLP

In most fungi, rDNA includes a tandemly repeated array of the three rRNA genes separated by transcribed and nontranscribed spacers with different evolutionary rates. In a haploid genome of the MNR AG 4 isolates the rRNA genes are arrayed in about 59 repeated units (Cubeta et al. 1996). The initial studies using RFLP analysis after hybridization with an rDNA probe indicated that isolates within *R. solani* AGs were characterized by one or more unique RFLP patterns (Vilgalys and Gonzalez 1990; Jabaji-Hare et al. 1990). In addition, considerable rDNA variations were found even among isolates of the same subgroup in AGs 1 and 2. On the other hand, the use of RFLP based on Southern blotting had limited success. The length of mutations, comigration of nonhomologous fragments, and multiple restriction sites render the use of RFLP patterns only with probes less appropriate for estimation of genetic relatedness.

The method based on rDNA-RFLP without hybridization with a probe provides more-detailed restriction analyses. This method includes digestion of PCR-amplified rDNA with a restriction enzyme, separation of the resulting DNA fragments according to their size by gel electrophoresis, and a comparison of the restriction patterns.

PCR-generated rDNA-RFLP was first used for 28S rDNA of BNR (Cubeta et al. 1991). It was first performed in the rDNA-ITS region for MNR (Liu and Sinclair 1992, 1993; Liu et al. 1993). The following RFLP studies were considerable for MNR (Liu et al. 1995; Kanematsu and Naito 1995; Keijer et al. 1996; Julian et al. 1996; Schneider et al. 1997; Hyakumachi et al. 1998; Meyer et al. 1998; Nicoletti et al. 1999; Priyatmojo et al. 2001; Godoy-Lutz et al. 2003) and for BNR, using 28S rDNA (Mazzola 1997; Martin 2000) or rDNA-ITS (Sen et al. 1999; Toda et al. 1999b; Kasiamdari et al. 2002; Hyakumachi et al. 2005). PCR-generated rDNA RFLP analyses supported a genetic basis for the classic anastomosis groups concept. Nevertheless, isolates within AGs are more genetically variable than presumed. These polymorphism variations enabled the establishments of new subgroups within the existing AGs such as in MNR: AG 2–3 (bean foliar blight pathogen), and AG 1-1D (coffee necrotic leaf spot pathogen) (Kanematsu and Naito 1995; Priyatmojo et al. 2001). Godoy-Lutz et al. (2003) provided additional genetically different types within AG 1 and AG 2-2. Similarly, Toda et al. (1999b) differentiated BNR isolates of AG-D to AG-D-(I) (*Rhizoctonia*-patch and bentgrass winter-patch pathogens) and AG D-(II) (elephant-footprint pathogen) by RFLP analysis of the rDNA-ITS regions. However, as in RFLP analysis, a number of fragment differences are used to estimate the degree of nucleotide divergence, and the presence of insertions and deletions ("indels") are potential sources for errors because a single length mutation may result in changes in more than one fragment.

RFLP banding pattern analysis of ITS regions indicated at least 6 subgroups (1A–1F) in MNR AG 1 and 5 subgroups in AG 2 (2A–2E; it was subsequently found by anastomosis that 2E actually belongs to AG 3) (Liu and Sinclair 1992; Liu et al. 1993). Further construction of the ITS rDNA restriction maps for these 11 subgroups indicated that DNA length mutations, "indel" events of the short piece of DNA, were common among these isolates. It is therefore suggested that heterogeneous RFLP phenotypes detected in these studies would be attributed to ITS length variation, as many restriction sites are common in these regions. The complexity of ITS restriction phenotypes were demonstrated within the BNR AG-I (Sen et al. 1999). Determination of relatedness of AG-I based solely on banding patterns might yield erroneous estimation and consequently lead to inaccurate conclusions.

RFLP analysis of the 28S rDNA was effective for characterizing of BNR isolate to AGs (-Ba, -C, -D, -F, -H, -I, -J, and -K). However, AG-J isolates have been excluded from *Rhizoctonia* spp. because they have clamp connections (Cubeta et al. 1991). Variations in restriction patterns of

28S rDNA were observed, however, among isolates within each of AGs -I, -J, and -Q (Mazzola 1997), as well as also among isolates within each of AGs -A and -G (Martin 2000). Although the 28S rDNA RFLP marker system is considered to be useful for identification of BNR AGs, caution should still be used when RFLP patterns generated from a few restriction enzymes are interpreted.

Isolates of several MNR and BNR AGs are known to include indels of 400 bp in their 28S rRNA subunit (Cubeta et al. 1991; Mazzola 1997; Meyer et al. 1998; Martin 2000). Based on their sequence analysis, it was confirmed that *R. solani* isolates have about 480-bp indels and that the presence or absence of indels may not be obviously correlated with the corresponding AGs (Kuninaga, unpublished data). The presence of indels interferes with the accuracy of the conclusions drawn from the 28S rDNA RFLP analysis method and its inferences.

rDNA sequence analysis

Most of the aforementioned drawbacks associated with the rDNA RFLP method are avoided by the use of the rDNA sequence analysis method, which has been widely used for *Rhizoctonia* spp. since the mid-1990s (Boysen et al. 1996; Kuninaga et al. 1997; Johanson et al. 1998; Boidin et al. 1998; Salazar et al. 1999; 2000b; Kuninaga et al. 2000a,b; Pope and Carter 2001; Carling et al. 2002; Toda et al. 2004). Currently, the DNA sequence information seems to offer the most accurate way for establishing the taxonomic and phylogenetic relationships for *Rhizoctonia* spp.

Generally the 5.8S region was found to be conserved in *R. solani* (Kuninaga et al. 1997), while some sequence variations were found in this region among BNR AGs isolates (Kuninaga, unpublished data), On the other hand, higher nucleotide sequence variations in both ITS1 and ITS2 were found among isolates of different AGs and subgroups than within AGs, as well as host range and other biological criteria (Kuninaga et al. 1997; Kuninaga 2002).

The genetic relatedness of *R. solani* AGs was comprehensively studied by Kuninaga et al. (1997). A percent similarity higher than 96% of the ITS sequence was evident for isolates within an AG subgroup, 66%–100% for isolates of different subgroups within an AG, and 55%–96% for isolates among different AGs. AG 2 is highly heterogenic and includes a considerable number of subgroups, which are subdivided according to their morphology, virulence, nutritional requirements, and hyphal anastomosis frequency. Currently, subgroups 2-1, 2-2 IIIB, 2-2 IV, 2-2 LP, 2-3, 2-4, and 2-BI are included in AG 2 (Carling et al. 2002). Subgrouping of AG 2 using ITS sequence analysis was consistent with the above-listed subgroups (Salazar et al. 1999, 2000a; Carling et al. 2002).

Similarly, the rDNA-ITS sequence analysis most accurately divided subgroups within AG 1 (Kuninaga et al. 1997; Toda et al. 2004), AG 3 (Kuninaga et al. 2000a), AG 4 (Boysen et al. 1996; Kuninaga et al. 1997), and AGs 6 (Pope and Carter 2001). The mycorrhizal isolates belonging to AGs 6 and 12 were placed on separate clusters in the tree, despite their functional similarities; these AGs are likely to have a separate evolutionary background. In addition, AG 6 isolates from the Southern Hemisphere (Australia and Africa) were distinctly divided from the isolates of the Northern Hemisphere (Japan and the United States), indicating that isolates from these geographically separate regions have evolved independently.

Combined analyses of the phylogenetic relationships of BNR (*Ceratobasidium*) and MNR (*Thanatephorus*) by cladistic analyses of ITS and 28S rDNA sequences revealed a total of 31 genetically distinct groups (21 MNR and 10 BNR groups), which corresponded well with previously recognized AGs and subgroups (Gonzalez et al. 2001). The phylogenetic analysis further suggested that certain AGs are not monophyletic and that there is a greater taxonomic support for AG subgroups than for AGs.

Fewer studies were reported on rDNA-ITS sequence analyses of BNR isolates than on MNR isolates (González et al. 2002; Otero et al. 2002; Ma et al. 2003; Hyakumachi et al. 2005; Sharon et al., in preparation). Hyakumachi et al. (2005) reported on two new BNR AGs according to their phylogenetic tree based on the rDNA-ITS sequence analysis: AG-T (the cut flower root and stem rot pathogen, AG-CUT) and AG-U (the miniature roses pathogen, AG-Min). The isolates of AG-T were subsequently found to actually belong to AG-A, indicating that AG-T had been inaccurately designated (Sharon, Sneh, Kuninaga, and Hyakumachi, unpublished data). Pathogenic BNR isolates of strawberries were identified by rDNA-ITS sequence analysis in consistent clades to AGs -A, -F, -G, -I, and -K (Sharon et al., in preparation).

Otero et al. (2002) studied endophytic BNR isolates using ITS sequence analysis. Of these isolate sequences, two main clades appeared on the phylogenetic tree. One (22 of 26 isolates) was located close to AG-Q, while the other (2 isolates) was close to AG-H. One of the other two isolates was located in the AG-A clade. All the isolates from this study except for one (jto 109) had a low percent (%) of ITS sequence similarity with that of previously recognized BNR AG (Kuninaga, unpublished data). Ma et al. (2003) compared ITS sequences of *Epulorhiza* (synonym, *R. repens*) type BNR isolates from orchids with database isolate sequences. The ITS sequence identity ranged from 88% to 100% among isolates of *E. repens* and from 98% to 100% among isolates of *E. calendulina*, whereas between the species it was only 18%–44%. No matching sequences have been found in the GenBank for *E. repens* and *E. calendulina*.

The above-described information indicates that additional work with many more BNR isolates may identify additional new AGs and subgroups and provide significant support for the accuracy of phylogenetic analysis of rDNA-ITS sequences for BNR isolates.

A significant advantage in the use of rDNA sequence analysis is apparent from its applicability for constructing PCR primers for diagnostic purposes. Use of the ITS regions for the production of specific primers has proved to be a successful strategy for developing diagnostic assays for subgroups within *R. solani* AGs (Kuninaga 2003).

Specific primers designed from unique regions within the ITS regions have been developed for detection and identification of *Rhizoctonia* spp. isolates: for the rice sheath disease complex [(caused by *R. solani*, *R. oryzae*, and *R. oryzae-sativae* (Johanson et al. 1998)], as well as for differentiation of *R. oryzae* and AG 8 (wheat pathogens) (Mazzola et al. 1996). PCR primers developed for identification of MNR subgroups of AG 2 (2-1, 2-2 IIIB, 2-2 IV, 2-2 LP, 2-3, 2-4, and 2-BI) have led to the production of diagnostic primers for rapid and reliable identification of these seven subgroups (Salazar et al. 2000b; Carling et al. 2002). Specific primer sets were also developed to differentiate between the two AG 3 subgroups, 3-PT and 3-TB (Kuninaga et al. 2000a; Matthew et al. 1995). Real-time PCR assays using TaqMan probes have been described for AG 3-PT from soil and on potato tubers (Lee et al. 2002). Specific PCR primers designed from the 28S rDNA region were successfully used for the identification of MNR subgroups of AG 1 (-IA, -IB, and -IC) and AG 2 (2-1 and 2-2) (Matsumoto 2002).

The ITS-based specific PCR primers may be used in a simple assay for detection of the pathogen in plant material in the absence of visual black scurf disease symptoms and in soil at a level of 0.5 mg sclerotia/g soil (Lee et al. 2002).

Detailed classification of MNR isolates to AGs and subgroups by rDNA-ITS sequence analysis

Cluster analyses based on rDNA-ITS sequence of representatives of anastomosis groups and subgroups of *Rhizoctonia* spp. isolates have been reported (Liu and Sinclair 1992; Kasimadri et al. 2002; Kuninaga et al. 1997; Salazar et al. 2000a; Gonzalez et al. 2001; Carling et al. 2002; Toda et al. 2004). Applications of rDNA-ITS sequence analysis are expected to greatly advance our knowledge regarding *Rhizoctonia* spp. taxonomy because it enables a direct comparison of sequence data from different research groups. The publication of rDNA-ITS sequences and their deposition in databases facilitate the identification and confirmation of AG affinities of new or existing *Rhizoctonia* spp. isolates without the need to actually use the various AG-representative isolates or the hyphal fusion procedure. However, with the accumulation of additional sequence data, there are concerns regarding the existence of inaccurate data in the databases available in GenBank, such as mislabeled isolates and other errors introduced during sequence analysis (as are subsequently described). Such errors have already been included in scientific publications and copied by researchers in their subsequent publications. When sequence data that are very difficult to align are used, specific caution should be taken to avoid inaccurate and misleading conclusions.

In several publications, rDNA-ITS sequences of only one isolate per AG were used for cluster analysis (Gonzalez et al. 2001, 2002; Salazar et al. 2000). When the sequence was from an incorrectly designated isolate as discussed by Sharon et al. (in preparation), misinterpretation of the results and wrong conclusions were drawn. In these publications, there were no considerations in the discussion to isolates or clusters that are located in unclear locations in the trees. In addition, as a result of significant variations in sequences of some isolates within AGs or subgroups, which is defined in percent similarity, the use of several isolates per group or subgroup and not only one isolate per group is expected to increase the reliability of the analysis and to verify the location of the isolates in their correct separate clusters. Also, isolates of a certain subgroups are located in different subclusters in between other subgroups as in the case of AGs 2 2-IIIB, 2 2-IV, and 2 2-LP (Carling et al. 2002; Fig. 1). Such diversity could not be observed using only one or two isolates.

Currently, the rDNA-ITS sequences deposited in GenBank vary significantly; from longer rDNA-ITS sequences of AG 2-2IV (isolate BC10) of about 678 bp to shorter sequences of *W.* (*R.*) *circinata* of about 570 bp. Multiple alignment containing ITS sequences lacking a substantial portion of the start or the end resulted in an inaccurate analysis and therefore were omitted from the summarized isolate sequences. To obtain a better standardization of the deposited rDNA-ITS sequences in GenBank, it is recommended to deposit only complete sequences; starting with the end of the conserved area of 18S (primers ITS1-TCCGTAGGTGAACCTGCGG or ITS5-GGAAG TAAAAGTCGTAACAAGG) and ending at the beginning of the conserved area of the 28S region (primer ITS4-TCCTCCGCTTATTGATATGC).

In the present review, the rDNA-ITS sequences of all the isolates designated to MNR AGs available in GenBank (during 2005) were initially retrieved and analyzed with the Clustal W program from DDBJ (DNA Data Bank of Japan, Research Organization of Information and Systems, National Institute of Genetics, Shizuoka, Japan: http://www.ddbj.nig.ac.jp) for multiple alignment, and additionally manual multialignments were performed using the Genedoc program, version 2.5.000 (Nicholas et al. 1997). Manual alignment has to be meticulously performed, even though there may be more than one correct alignment. The anastomosis grouping provides the researcher the framework for choosing the most appropriate manual alignment. The MP analysis frequently provides more than one accurate tree, and the researcher can either choose the most appropriate tree according to past knowledge or create a consensus tree based on all the most parsimonious trees. As it was technically impossible to include all the available MNR isolate sequences in one tree, three neighbor-joining (NJ) trees were initially prepared, using the Clustal W program at DDBJ. One tree included the AGs 1, 2, and 3 and their subgroups (see Fig. 1). The second tree included AGs 4, 5, and 6 (Fig. 2), and the third tree included AGs 7, 8, 9, 10, 11, 12, and *R. circinata* (Fig. 3). Representative isolates (distantly located in each clusters) from each AG and each subgroup were selected for the subsequent analysis of all the MNR AGs together in one consensus maximum-parsimony (MP) tree (Fig. 4), using the programs "dnapars," followed by the consense program that generates a consensus tree from all the best possible trees. Bootstrap values were calculated with the seqboot program using 1000 replicates. "dnapars," "consense," and "seqboot" were all

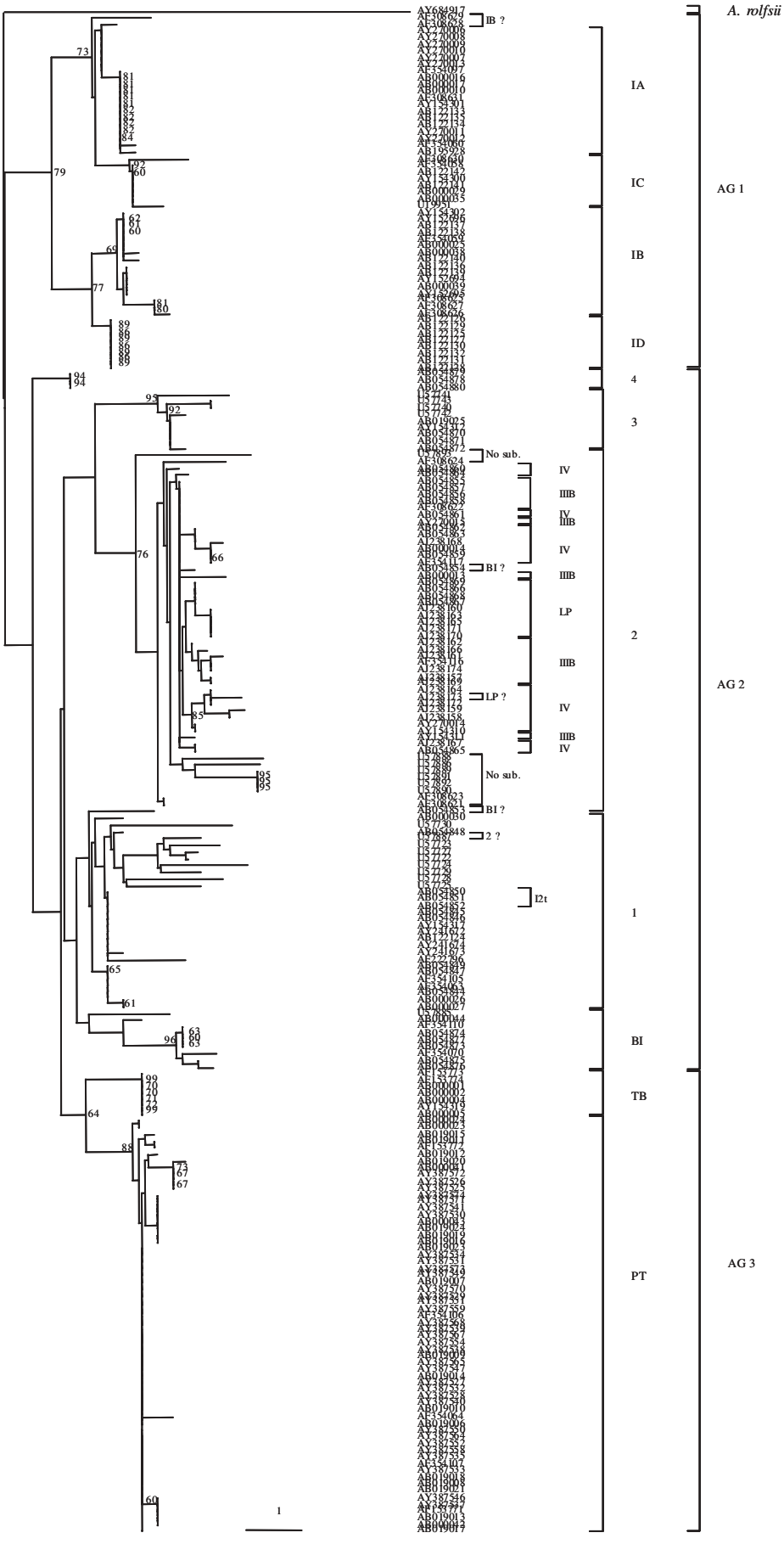

Fig. 1. A neighbor-joining tree of anastomosis groups AGs 1, 2, and 3 of multinucleate *Rhizoctonia* spp. isolates (accession #s) available in GenBank, clustered according to multiple alignment of rDNA-ITS sequence analysis. The distances were determined according to Kimura's two-parameter model. The *bar* indicates one base change per 10 nucleotide positions. Bootstrap values over 60% are positioned alongside the branches with 111 trials. Isolate AY684917 [*Athelia* (*Sclerotium*) *rolfsii*] was used as an outgroup (its *horizontal line* in the figure represents only 25% of its actual distance). The AGs and sub-groups for the clusters are indicated. To reduce the denseness of the Figure 29 isolate sequences, highly similar to the other AG 3 PT were deleted. The tree number in the TreeBase is SN2960

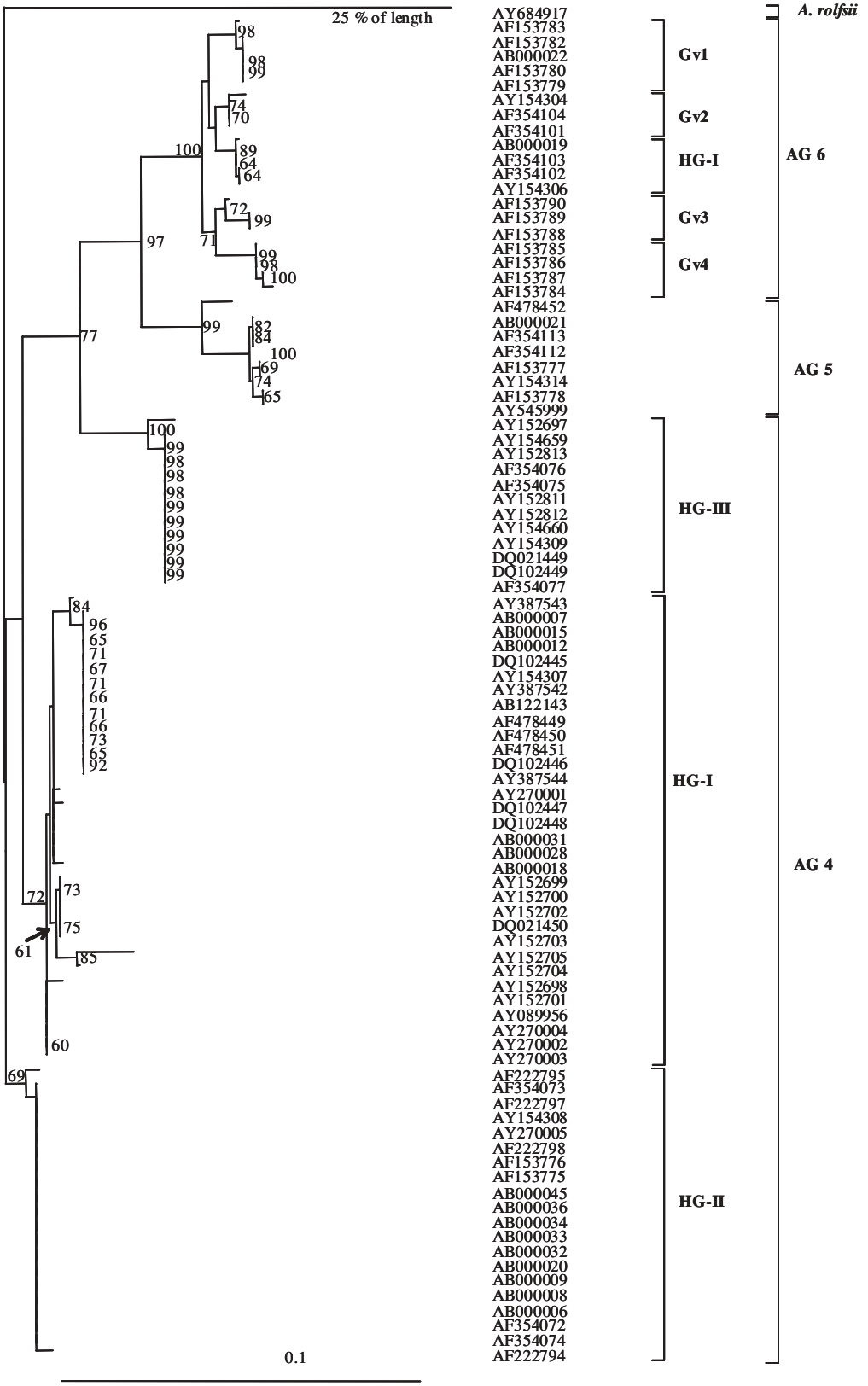

Fig. 2. A neighbor-joining tree of AGs 4, 5 and 6 of multinucleate *Rhizoctonia* spp. isolates (accession #s) available in GenBank, clustered according to multiple alignment of rDNA-ITS sequence analysis (see legend of Fig. 1). The tree number in the TreeBase is SN2960

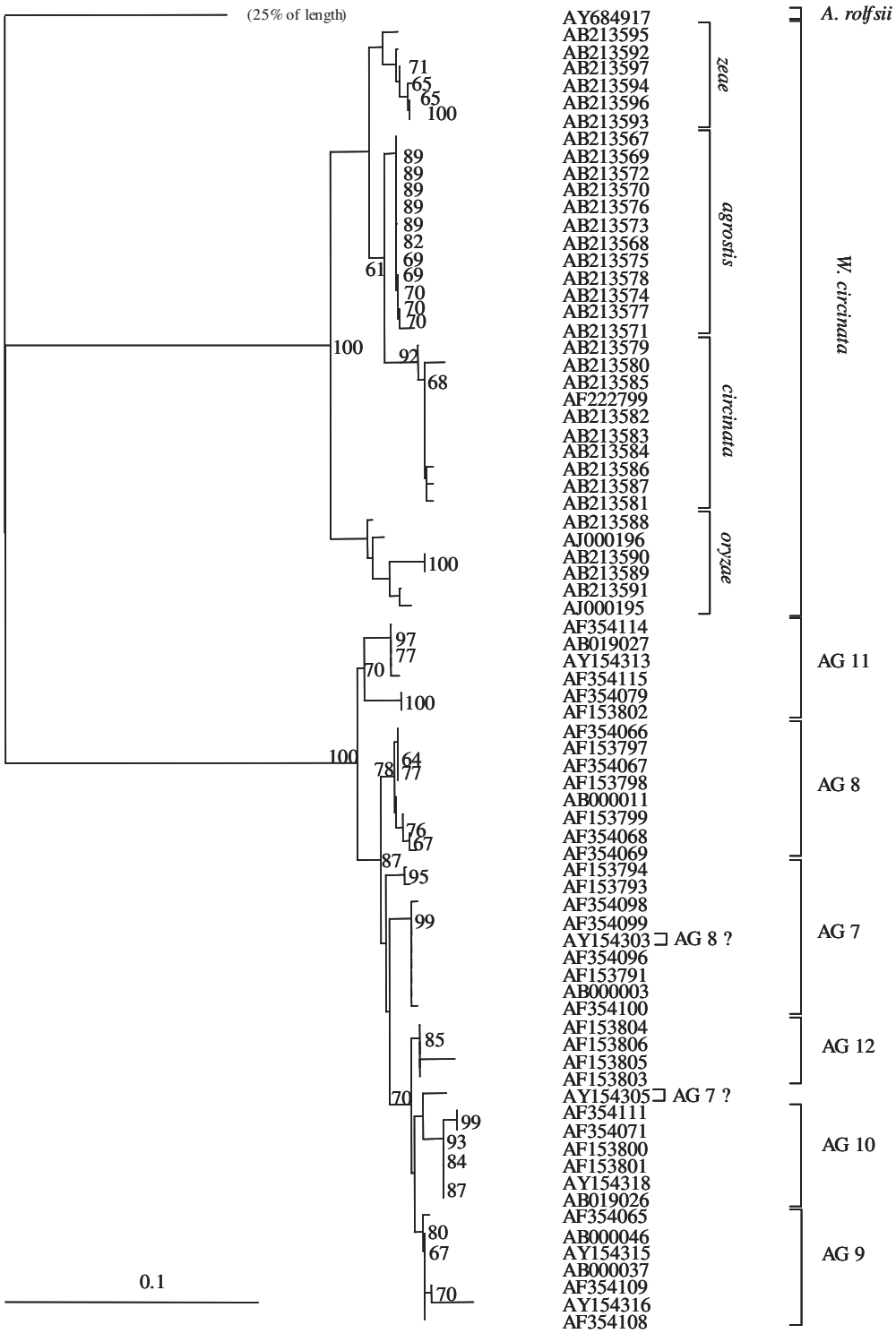

Fig. 3. A neighbor-joining tree of AGs 7, 8, 9, 10, 11, 12 and *Waitea* (*Rhizoctonia) circinata* of multinucleate *Rhizoctonia* spp. isolates (accession #s) available in GenBank, clustered according to multiple alignment of rDNA-ITS sequence analysis. (see legend of Fig. 1) Tree number in the TreeBase is SN2960

from the Phylip package 3.6 (J. Felsenstein 1989). All trees were viewed by TreeView version 1.6.6 (Page 1996). Percent sequence similarity of all the isolates within AGs and subgroups as well as among the AGs and subgroups [calculated with the MatGat program (Campanella et al. 2003)] are summarized in Table 2.

It was also important to include ITS sequences of all the isolates available in GenBank (summarized in Figs. 1–3) to obtain information on the rate extents of percent sequence similarities within the AGs (see Table 2). Statistically, the probability to obtain a wider range and, thus, a more-reliable representative result is higher when sequences of more isolates of the same AG or subgroup are analyzed. Attempting to determine a threshold of percent similarity of ITS sequences for differentiating isolates belonging to different AGs or subgroups could not be consistently obtained because in some instances there was an overlap between the percent sequence similarity ranges for isolates

Table 2. rDNA-ITS sequences percent similarity range of multinucleate *Rhizoctonia* AGs and subgroups

AG/subgroup	1	1 IA	1 IB	1 IC	1 ID	2	2 I	2 I2t	2 2	2 2 IIIB
1	91–100									
1 IA	85–92	96–100								
1 IB	85–100	85–90	91–100							
1 IC	83–92	87–92	83–88	96–100						
1 ID	83–100	86–88	91–93	83–86	100					
2	80–89	80–89	79–87	79–88	81–86	81–100				
2 I	81–89	82–89	82–87	81–88	83–86	81–100	92–100			
2 I2t	84–88	86–88	84–87	85–87	86	83–100	94–100	100		
2 2	79–86	80–85	79–86	79–84	81–85	81–100	81–88	83–87	92–100	
2 2 IIIB	78–86	81–85	81–86	78–84	82–85	82–100	82–87	84–87	91–100	94–100
2 2LP	80–85	83–85	82–85	80–83	84–85	83–99	83–87	85–86	93–99	94–99
2 2 IV	80–86	83–85	82–86	80–84	83–85	83–99	83–87	85–87	92–98	95–98
2 3	83–88	85–88	83–86	83–87	83–85	82–92	85–92	89–91	82–88	83–88
2 4	84–87	86–87	85–87	85–87	84–85	82–95	90–95	94	84–86	84–86
2 BI	81–85	82–85	81–84	80–83	83–84	83–87	84–88	86–87	83–87	83–87
3	83–88	86–88	84–88	84–88	83–88	82–94	84–83	84–84	84–85	84–86
3 TB	85–88	86–88	86–88	85–88	85	83–94	89–94	93	83–87	85–87
3 PT	83–88	86–88	83–87	84–87	83–85	82–94	89–94	92–93	82–87	83–87
4	80–88	83–87	84–88	80–86	83–87	81–88	82–88	84–87	81–87	83–87
4 HG I	80–88	83–87	84–88	80–86	86–87	81–88	82–88	84–87	81–87	83–87
4 HG II	82–88	84–86	86–88	82–85	83–87	82–87	83–87	84–85	82–86	84–86
4 HG III	80–87	84–86	85–87	80–83	85–86	81–86	83–86	84–85	81–85	83–86
5	83–87	84–87	84–86	83–87	84–85	82–93	86–91	90–91	82–87	84–87
6	80–90	82–90	80–87	83–90	81–85	78–91	80–90	84–90	78–85	79–86
6 HG I	83–88	86–88	83–87	86–88	84–85	81–90	85–90	89–90	81–85	84–86
6 GV1	80–88	82–88	80–86	83–88	81–85	78–90	80–90	84–89	78–84	79–84
6 GV2	83–89	86–89	83–86	86–89	84–85	80–90	85–90	89–90	80–85	83–86
6 GV3	84–90	87–89	84–87	88–90	84–85	80–90	84–90	89	80–84	82–84
6 GV4	83–90	87–90	83–87	88–90	84–85	81–91	85–90	89–90	81–85	83–86
7	84–90	87–90	84–89	87–91	85–87	80–89	84–89	87–89	80–85	82–85
8	83–89	87–89	83–87	86–89	84–85	81–91	85–91	89–90	81–86	83–86
9	84–89	86–89	84–87	85–89	84–86	82–97	89–97	93–95	82–88	84–88
10	61–88	61–88	83–87	84–88	83–84	81–95	88–95	92–93	81–86	83–86
11	82–87	84–87	83–87	82–86	83–85	82–95	86–91	90	82–88	83–88
12	84–91	87–91	85–89	84–88	85	82–90	85–90	89–90	82–88	85–87
W. circinata	63–68	64–67	63–67	64–68	63–66	60–68	62–67	64–67	60–65	61–64
zeae	64–67	64–66	64–65	65–67	64–65	62–68	63–67	65–67	62–65	62–64
agrostis	63–67	65–67	63–66	64–67	64–65	61–67	63–67	65–66	61–64	61–64
circinata	63–68	64–66	63–67	65–68	63–64	60–68	62–66	64–66	60–64	61–63
oryzae	64–68	65–66	64–66	65–68	64–66	60–68	62–67	64–67	60–65	61–64
A. rolfsii	59–63	59–61	60–63	62	62	61–65	61–64	63	61–63	61–63

AG/subgroup	5	6	6 HG I	6 GV1	6 GV2	6 GV3	6 GV4	7	8	9
5	96–100									
6	87–92	90–98								
6 HG I	91–92	92–98	99–100							
6 GV1	87–92	90–98	92–98	94–100						
6 GV2	91–92	92–98	97–98	92–97	99					
6 GV3	91–92	92–97	95–96	92–96	95–97	99–100				
6 GV4	90–91	90–97	95–96	90–95	95–96	96–97	99–100			
7	86–87	86–91	89–90	86–89	88–90	90–91	89–91	92–100		
8	88–89	88–91	90	88–89	90–91	90–91	90–91	91–92	99–100	
9	88–91	84–91	88–90	84–90	88–91	88–91	89–91	86–90	89–91	96–100
10	88–90	84–91	88–89	84–89	88–90	89–91	89–90	87–90	89–91	92–96
11	91–92	83–89	88–89	83–88	87–88	86–88	87–88	86–88	87–89	88–91
12	86–88	82–91	87–89	82–89	87–89	88–91	88–90	88–91	88–91	87–90
W. circinata	64–66	63–68	64–67	63–68	64–66	66–68	65–67	67–70	68–70	64–68
zeae	64–65	63–68	66–67	65–68	65–66	66–68	63–68	67–70	68–70	65–67
agrostis	65	63–67	64–65	63–67	64–66	66–67	63–67	68–70	68–70	65–67
circinata	65	63–67	64–65	63–67	64–66	66–67	63–67	67–69	69–70	65–68
oryzae	65–66	62–68	65–66	65–67	65–66	66–68	62–68	67–68	68–69	64–67
A. rolfsii	61–62	59–61	60–61	59–61	60	60	60–61	60–61	61–62	60

Table 2. *Continued*

AG/subgroup	2 2 LP	2 2 IV	2 3	2 4	2 BI	3	3 TB	3 PT	4	4 HG I	4 HG II	HG III
2 2LP	98–100											
2 2 IV	96–99	97–100										
2 3	85–86	85–87	95–100									
2 4	85	85–86	89–91	100								
2 BI	83–87	83–87	83–86	86–87	94–99							
3	84–87	84–87	88–91	93–94	86–89	95–100						
3 TB	85–87	85–87	89–91	94	86–89	95–96	100					
3 PT	84–86	84–87	88–91	93–94	86–88	95–96	95–96	98–100				
4	84–87	84–87	82–87	83–86	83–87	82–88	85–88	82–86	88–97			
4 HG I	84–87	84–87	83–87	83–86	83–87	83–88	86–88	83–86	88–97	94–100		
4 HG II	85–86	85–86	84–86	84–85	83–86	84–87	86–87	84–86	90–97	92–97	98–100	
4 HG III	84–85	84–86	82–85	85–86	84–85	82–86	85–86	82–83	88–92	88–92	90–91	99–100
5	85–86	85–86	91–93	86–87	84–87	88–91	91	88–90	87–90	87–90	87–89	87–88
6	80–83	80–86	84–91	80–85	79–86	83–90	85–90	83–89	82–90	84–90	83–89	82–88
6 HG I	84–85	84–86	87–90	84	84–85	88–90	89	88–89	86–90	87–90	88–89	86–87
6 GV1	80–84	80–85	84–89	80–84	79–85	83–90	85–90	83–89	82–90	84–90	83–89	82–87
6 GV2	83–85	83–86	87–90	85	84–85	88–90	89–90	88–89	87–90	87–90	88–89	87–88
6 GV3	82–83	82–84	87–90	85	83–85	87–90	89–90	87–89	85–90	86–90	87–89	85–87
6 GV4	84–85	83–86	87–91	85	84–86	88–90	90	88–89	86–90	86–90	88–89	86–87
7	83–84	83–85	86–89	84–85	82–84	86–89	88–89	86–89	83–87	83–87	84–87	84–85
8	84–85	84–86	88–90	85–86	85–86	89–91	90	89–91	84–87	85–87	86–87	84–85
9	85–88	85–88	89–92	88–90	84–89	90–94	91–94	90–93	83–87	83–87	84–86	83–85
10	84–85	84–86	89–91	92–93	84–87	92–94	93–94	92–94	83–87	84–87	84–86	83–85
11	85–87	84–88	91–95	85–86	84–88	88–90	89–90	88–89	82–87	83–87	83–85	82–84
12	85–86	85–88	85–89	85	84–86	86–89	88–89	86–89	84–88	85–88	85–86	84–86
W. circinata	61–64	61–64	65–68	62–66	61–64	64–68	64–66	64–68	61–65	61–65	61–64	61–63
zeae	63–64	62–64	66–68	63	62–64	65–67	65–66	65–67	61–65	61–65	61–63	62–63
agrostis	61–63	62–64	65–67	65–66	63–64	64–67	64–65	66–67	61–65	62–65	62–63	61–63
circinata	62–63	61–64	65–68	65–66	61–64	64–68	64–66	66–68	61–64	61–64	62–63	61–63
oryzae	61–64	61–64	65–68	62–63	61–64	64–66	64–65	64–66	61–64	61–64	62–63	61–63
A. rolfsii	61–62	62–63	62–64	63	64–65	62–65	62	64–65	60–61	60–61	60–61	60–61

AG/subgroup	10	11	12	*W. circinata*	*zeae*	*agrostis*	*circinata*	*oryzae*
10	99–100							
11	88–91	92–100						
12	87–90	87–89	98–100					
W. circinata	65–69	64–67	64–69	89–100				
zeae	65–66	65–67	65–67	91–95	98–100			
agrostis	66–67	65–67	65–67	89–95	94–95	98–100		
circinata	67–69	65–66	65–67	91–93	92–93	91–93	98–100	
oryzae	65–66	64–66	64–67	89–92	91–92	89–91	91–92	97–100
A. rolfsii	60	61–63	63–64	60–63	60–62	62–63	61–62	60–62

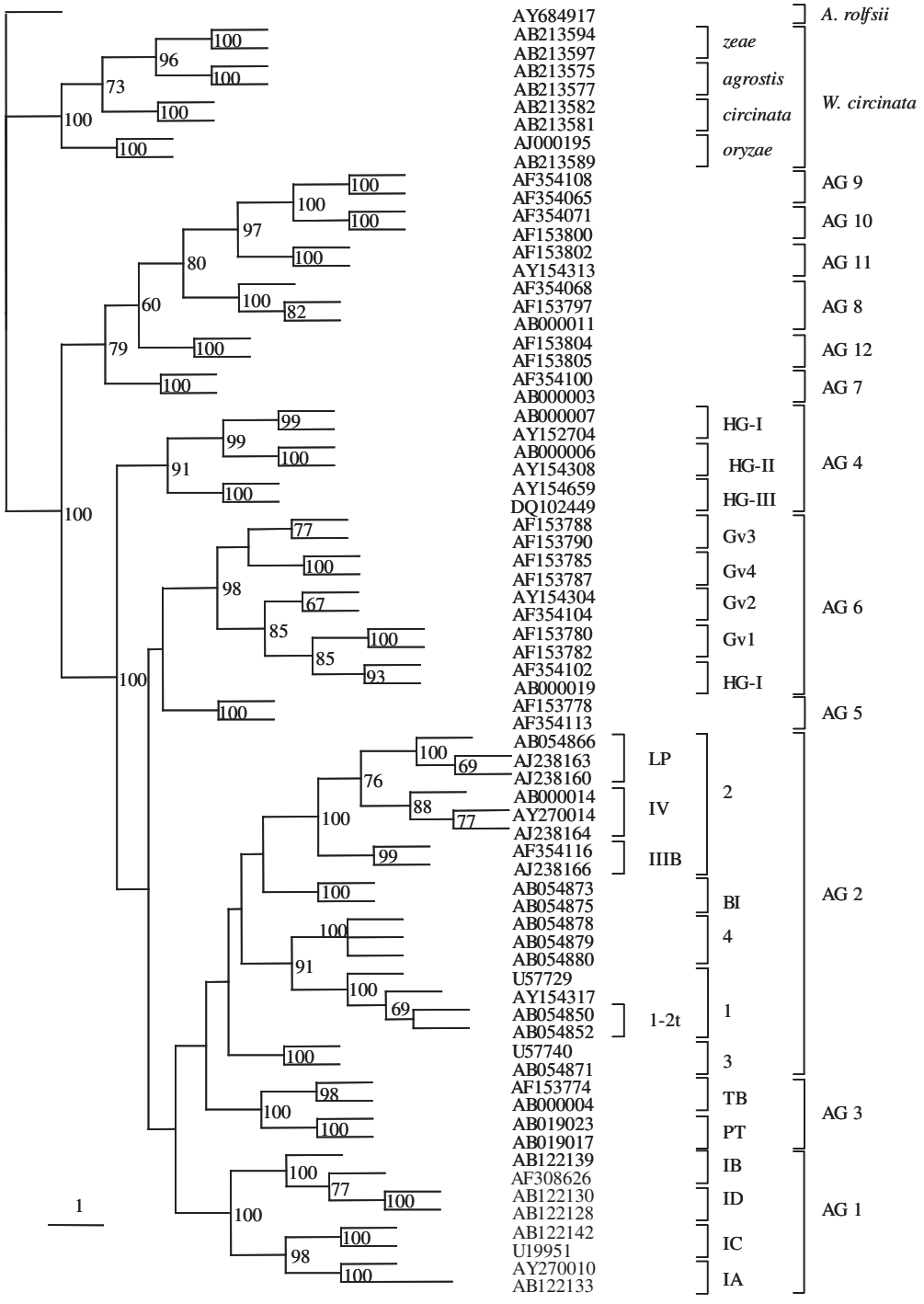

Fig. 4. A consensus tree assembling 587 most parsimonious trees composed of rDNA-ITS sequences of representative isolates (accession numbers) of all the MNR AGs and sub-groups from Figures 1, 2, and 3. The *bar* indicates one change. Bootstrap values of over 60 of 100 trials are positioned alongside the branches. Isolate AY684917 [*Athelia* (*Sclerotium*) *rolfsii*] was used as an outgroup. The AGs and sub-groups for the clusters are indicated. The tree number in the TreeBase is SN2960

within an AG with the percent similarity range among the different AGs. Cluster locations of different sequences (and consequently the AGs) in trees based on ITS sequence analysis are calculated by the relevant computer program used, which may result in different trees. Percent sequence similarity is calculated by analyzing the differences between two sequences, and therefore the results are not changed when different computer programs are used or by addition of new sequences. Therefore the combined use of cluster analysis and percent sequence similarity complement each other.

The locations of the isolates in the clusters consistently supported the anastomosis groups, confirming that anastomosis is solidly based on genetic relatedness. During performing the great number of analyses of the data and trees, it was evident that adding or subtracting a number of isolate sequences and/or changing the outgroup may change the position and order of the clusters and subclusters in the trees, and thus the various groups may change their relative locations along the tree. In some cases it also caused a splitting of a certain AG to two distant locations. The positions of the isolates and AGs in the clusters of the

consensus MP trees were kept more stable in response to such additions or deletions than in the NJ trees, although the NJ trees were frequently supported by corresponding MPs trees.

Several tree-constructing methods are used for rDNA-ITS sequence analyses: neighbor-joining (NJ), maximum-parsimony (MP), and most likelihood (ML) methods. The algorithms for preparing the NJ and the MP trees are basically different. Generally, when sequence variation of isolates includes wide differences in molecular evolutionary rate, the MP results generate less-appropriate trees than the NJ and ML methods. However, in MNR isolate sequences, the trees obtained by the MP program were more consistent than those by NJ trees. The ITS region of *R. solani* is generally very difficult to align because of excessive nucleotide insertions or deletions. These highly variable ITS regions may account for some inaccuracies in an NJ analysis and consequent trees.

Attempts to designate an order of numbers to clusters of several AGs along the tree (Gonzalez et al. 2001, 2002) are therefore inconsistent and may be misleading. Moreover, choosing one of the best MP trees might be less reliable then using a consensus tree combining all the best MP trees resulted in the analysis. Closer percent sequence similarities among certain AGs compared to more-distant similarities among others may indicate closer genetic relatedness between certain AGs or clusters of several AGs but may not be sufficient for all groups in the tree to determine a consistent order. The clusters in Fig. 4 clearly indicate that the MNR AGs are divided into the following groups of AGs: one group includes AGs 1, 2, and 3, and another group includes AGs 5 and 6. AG 4 (*R. praticola*) belonged to this group in a tree based on another alignment but seems to be separate from this group in Fig. 4. Another group includes AGs 7, 8, 9, 10, 11, and 12, while the four subgroups or subspecies (Toda et al. 2005, in preparation) of *R. circinata* are clearly of a separate group from all the rest.

It is important to emphasize that although *R. circinata* var. *zeae* (WAG-Z) and *R. circinata* var. *oryzae* were designated as distinctly separate AGs (Sneh et al. 1991), Ogoshi (1985) had already indicated that anastomosis was also observed between isolates of these two species or subspecies and that anastomosis frequencies of >30% were observed among isolates of each of all the four subspecies of *R. circinata* (Hyakumachi, unpublished data). This finding may indicate that according to the anastomosis reactions these four subspecies may be considered as subgroups of one AG. The percent sequence similarity ranges among these subspecies was 89%–100% whereas within the subspecies it was 97%–100%, and their positions in the trees (Figs. 3, 4) indicate that these are distinctly separated subgroups.

AG-2 is divided into the greatest number of subgroups, AGs 4, 6, and 3 are divided into fewer subgroups, while some AGs, i.e, 7, 8, 9, 10, 11, and 12, have not been divided at all. AG 9 has been divided into two subgroups, but there were not sufficient available sequences in GenBank to divide this group in the present analysis. AG7 may also include at least two subgroups (Kuninaga, unpublished data), corresponding with the Stuttgart group and the pine tree group based on anastomosis reaction and the whole-cell fatty acid composition (Baird et al. 2000). This result indicates differences in genetic diversity within an AG in different AGs. The current knowledge of more-defined subgroups in certain AGs than in others may also be influenced by the relatively greater research efforts invested in certain AGs that cause serious diseases to important crop plants, compared to other AGs, which were less investigated and consequently yielded considerably fewer isolate sequences.

The percent sequence similarity range within the subgroups and AGs that are not separated to subgroups is high (94%–100%), except for AGs 7, 11 (92%–100%), and 1-IB (91%–100%), which may indicate the existence of more subgroups in these AGs. This result will be supported by additionally accumulated information for new isolates. However, the percent similarity ranges within AGs that are separated to subgroups is lower: AG 1 (91%–100%), AG 2 (81%–100%), AG 4 (88%–97%), and *R. circinata* (89%–100%). On the other hand, the percent similarity range among the MNR AGs is around 78%–90%, except for some lower ranges for AG 10 with AG 1 (61%–88%), or for some AGs that are closer, such as AG 5 with AG 6 or 7 and 8 (91%–92%) or 9 and 10 (92%–96%), and subgroups of AGs which are closer to each other than among the AGs (90%–98%). *Rhizoctonia circinata* (teleomorph, *Waitea circinata*) is considerably more distant genetically from all of the other MNR groups (63%–70%). The genetic distance of *R. circinata* from the rest of the MNR is almost as remote as the distance of the outgroup *S. rolfsii* to the other MNR AGs (59%–65%) (see Table 2).

The data summarized in Table 2 indicate that a percent sequence similarity threshold (within subgroups or AGs) differentiating among anastomosis groups or subgroups could not be definitely determined, despite the fact that isolates of different AGs and subgroups are located in distinct clusters and subclusters (see Figs. 1–4), because there is some overlap in percent sequence similarity ranges within and among AGs. Therefore, a combination of rDNA-ITS sequence analysis and percent sequence similarity is mutually supportive. The knowledge of the anastomosis of isolates is also supportive to the results from all the molecular methods.

Including the ITS sequences of all the available isolates in the rDNA-ITS sequence analyses in the review enables also tracing inaccuracies and errors of certain deposited isolate sequences in GenBank. It is evident from the location of some isolates marked within or among the clusters in Figs. 1–3 that they have been inaccurately designated in the GenBank. For some of them, it is clear to which group their sequence belongs. For example, accession numbers AE308628 and -9 designated as AG 1-IB in GenBank are located in the AG 1-IA cluster (see Fig. 1), and it is likely that they actually belong to this subgroup. Some isolates are designated as AG 2 but not to their subgroups. Some may belong to certain known subgroups, while others may form another subgroup but need more research to support this conclusion. Accession number AY154303 designated as

AG 8 is located in the AG 7 cluster (indicating that it belongs to AG 7), and AY154305 designated as AG7 is located in a different cluster than the AG 7 isolates and is close to AG 10. It is not clear where this isolate belongs.

Parts of the AG 2-2IIIB and AG 2-2IV sequences are located together with AG 2-2LP in several locations in a subcluster of AG 2-2 (Carling et al. 2002; see Fig.1). They cannot be differentiated by the rDNA-ITS sequence analysis (see Table 2), and there is no significant difference between their percent sequence similarities. AG 2-2IIIB and AG 2-2IV differ probably by a mutation causing thiamine requirement (Sneh et al. 1991), which may not be manifested by a significant change in the rDNA-ITS sequence. The cold climate tulip isolates of AG 2-1-2t could not be distinguished from the rest of the AG 2-1 isolates, according to their location in the same cluster, and their sequence similarity with AG 2-1 is 94%–100% (see Fig. 1). The subgroup AG 2–4 is distant from the most of the other AG 2 subgroups (Fig. 1), AG 2-2 (84%–86% sequence similarity), AG 2–3 (89%–91%), and 2-BI (86%–87%), but is closer to AG 2-1 (90%–95%) and also to AG 3 (94%) (see Table 2).

AG 6 was reported to include the HG-I and GV subgroups. GV was already known to be a variable subgroup (Sneh et al. 1991), but it has not been further studied in this respect. The data gathered from GenBank and summarized in Figs. 3 and 4 and Table 2 indicate that the isolates gathered in GV can be actually divided into four subgroups, namely, GV1, GV2, GV3, and GV4.

The continuous accumulation of *Rhizoctonia* spp. isolate sequences in GenBank will undoubtedly generate, in the future, both some changes to the summarized information presented in this review and indications for the existence of new and currently unrecognized AGs and subgroups.

From the data summarized and analyzed in Fig. 4 and Table 2, it may be suggested that the alignment in the TreeBase of Fig. 4 (SN2960) for MNR isolates can be used to identify unknown MNR isolates into AGs and subgroups by manually adding the new sequences to the existing alignment in the TreeBase. Caution should be taken when new alignments are submitted to a computer program analysis in which the original manual alignment will be disregarded; they will be always observed to manually correct the mismatches appearing in the alignment. Even so, the new alignment might result in changes in the locations of isolates and subgroups or AGs along the tree, compared to the presented complete MNR tree (Fig. 4). Therefore, it should be also complemented with percent sequence similarity analysis. If the sequence of the unknown isolate is within 95%–100% similarity and its location is within a cluster of a certain AG or a subgroup, this indicates that the isolate belongs to that AG or subgroup. This procedure may facilitate the laboratory work required for precise identification of unknown MNR isolates or verify previous identification of isolates. In a subsequent review article, a similar tree and table of percent sequence similarity will be prepared for the identification of BNR isolates.

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