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18S rDNA AND β-TUBULIN DIVERSITY IN *RHIZOCTONIA ZEAE* VOORHEES

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Abstract

Waitea circinata Warcup & Talbot, which is related to the anamorphic form-genus *Rhizoctonia* D.C., is a world wide distributed soil-borne fungal pathogen. Several molecular based studies have been published based on rDNA-ITS sequences to determine the phylogenetic relations between and within the different *Rhizoctonia* groups. It is generally accepted that phylogenies depending on a single gene may be misleading. In this study, we phylogenetically analysed three varieties of *Waitea circinata* (var. *circinata*, var. *zeae* and var. *oryzae*) using the nucleotide sequences of two commonly used markers 18S rDNA and β-tubulin for identification. Our phylogenetic analysis clearly supported the distinction of the three varieties. In *W. circinata* var. *zeae*, we determined a total of three lineages, two of which are distributed worldwide and one is unique for Americas. We observed both of the global lineages in Turkey and due to their dispersion, the invasion of these two lineages to Turkey probably occurred in different periods of times. Additionally, our results in this study once again suggested the Americas as the origin for *W. circinata* var. *zeae*.

Key words: *Rhizoctonia zeae*, 18S rDNA, Beta-Tubulin.

Introduction

When Voorhees (1934) isolated some phytopathogenic fungi as causal agents of sclerotial rot of corn (*Zea mays* L.) in Florida, USA, he identified them as a new fungal species according to some anamorphic criteria including sclerotia and hyphae morphology and named it as *Rhizoctonia zeae* Voorhees. A couple of years later, Ryker & Gooch (1938) isolated another *Rhizoctonia*-like pathogen from rice in Luisiana, USA, and they classified this pathogen as a new species using similar criteria and named it as *Rhizoctonia oryzae*. *Waitea circinata*, the teleomorphic stage of this two anamorphic form species were identified from Australian soil by Warcup & Talbot (1962). Oniki *et al.* (1985) assigned the anamorphic form species *R. zeae* and *R. oryzae* to the teleomorphic species *W. circinata* and identified two groups, WAG-Z and WAG-O, on the basis of hyphal incompatibility. Gunnell (1986) studied a collection of isolates from different localities including Australia, New Zealand and USA and identified three varieties in *W. circinata* (var. *circinata*, var. *zeae* and var. *oryzae*) depending on morphological features of their anamorphic stages. Leiner and Carling (1994) verified this separation by observing both vegetative and perfect stages of these groups and suggested to combine *R. zeae*, *R. oryzae* and *R. circinata* as varieties of a single anamorphic form species *R. circinata* (var. *zeae*, var. *oryzae*, var. *circinata*) instead of naming them as seperate form species. Although this nomenclature seemed to be accepted, in most publications these taxa are still named as seperate form species (e.g. *R. zeae*). Recently two new *W. circinata* related fungi, *W. circinata* var. *agrostis* and var. *prodigus* were identified from creeping bentgrass and Kentucky bluegrass in Japan (Toda *et al.,* 2007) and from *Palspatum vaginatum* Swartz (Seashore Palspatum) in USA (Kammerer *et al.,* 2011), respectively.

For characterization and evaluation of genetic diversity among different species and populations molecular markers are valuable tools (Bakht *et al.,* 2013). As molecular techniques, protein analyses such as isozymes and allozymes have widely use for systematic purposes, however more useful data are now obtained from DNA analyses (Siripipatthana *et al.,* 2014). In the last two decades molecular methods, including phylogenetic analysis based on rDNA-ITS nucleotide sequences, rDNA-ITS RFLP (Restriction Fragments Length Polymorphism) analysis, RAPD (Random Amplified Polymorphic DNA) analysis and FAME (Fatty Acid Methyl Esters) compositions have also been commonly used in *W. circinata* taxonomy. In one of the earliest studies, Mushika *et al.* (1995) reported three groups in *W. circinata* which corresponded to var. *zeae*, var. *oryzae* and var. *circinata*, due to the RAPD profile and rDNA-ITS RFLP patterns. Subsequently, Priyatmojo *et al.* (2002) clearly discriminated the three varieties of *W. circinata* mentioned above using cellular fatty acid compositions. Although there are many molecular techniques available for taxonomic studies as mentioned above, phylogenetic analysis based on rDNA-ITS nucleotide sequences is the most common one and the discrimination of the five varieties of *W. circinata* have been verified with this method in several recently published studies (Sharon *et al.,* 2006; Toda *et al.,* 2007; de la Cerda *et al.,* 2007; Kammerer *et al.,* 2011; Aydin *et al.,* 2013).

In this study we phylogenetically analysed a wide collection of *W. circinata* var. *zeae* and additionally some var. *oryzae* and var. *circinata* isolates from different localities and hosts using nucleotide sequences of 18S rDNA (SSU) and β-tubulin genes. With these analyses, we aimed to better understand the systematic positions and the genetic borders of these taxa. As explained above, although there are molecular studies which verifies the separation of these five varieties of *W. circinata*, there are not many studies which examine the comprehensive genetic diversity of each variety. In the current study, we especially aimed to examine the genetic diversity of *W. circinata* var. *zeae*. Such studies may be important to better understand the diversity and the distribution of this group of fungi around the world and also to determine the potential host-pathogen specificities which is the result of co-evolution between isolates and the related hosts. Such information may help us to better manage this globally distributed plant pathogen.

Materials and Methods

Isolates: The names, sources and hosts of *W. circinata* isolates used in this study are listed in Table 1. All isolates were stored with the dried oat method of Sneh *et al.* (1991).

Molecular methods: The mycelium of *W. circinata* isolates (Table 1) were prepared as explained in Gurkanli *et al.* (2009) and genomic DNA isolations from the mycelium were made according to the method of Carling *et al.* (1987). Extracted genomic DNA were stored at -20ºC prior to use.

For full-length amplification and sequencing of 18S rDNA, primer sets NS1/NS4 and NS3/NS8 (White *et al.,* 1990) and for partial amplification and sequencing of the gene, primer set NS1 (White *et al.,* 1990)/Wc-18S_879 (TTT CAC CTC TAG CAA CGC, This study) were used. Amplification of β-tubulin gene was performed using primer set B36F/B12R (Thon & Royse, 1999). PCR programs used for the amplifications are given in Table 2. For all amplifications, a 50 µl PCR reaction was prepared using, genomic DNA<1 µg, 1.5 mM $MgCl_2$, 1.25 Unit Taq polymerase (Promega, Go-Taq Flexi DNA Polymerase), 2.5 mM dNTP mix (Amresco), 10 µl of 5X PCR buffer (Promega, Go-Taq Green Buffer), 0.6 pmol (final con.) of each primer and ddH_2O. Amplifications were made with a MWG Primus thermal cycler (Germany) and products were electrophoresed on 1% agarose gel (Amresco, Solon, Ohio) prepared in 1X TBE (Tris-Borate-EDTA) buffer. After staining with ethidium bromide, gels were visualized with the GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK).

Nucleotide sequencings were made commercially by Macrogen, Korea using the sequencer ABI3730XL. All new nucleotide sequences obtained in this study were deposited in GenBank under accession numbers KT347100-KT347133 (Table 1).

Phylogenetic analysis: 18S rDNA and β-tubulin nucleotide sequencings were performed from both strands with the same primers used for the PCR amplifications (Table 2). Nucleotide strands were assembled with SeqMan II module of the LASERGENE 99 system (Applied Biosystem). Multiple nucleotide sequence alignments of our new sequences together with the related sequences obtained from NCBI databank (see Figure legends) were generated using ClustalX (Thompson *et al.,* 1997) and then optimized by hand with BioEdit (Hall, 1999). To determine the best fitting substitution model for our data sets, the Akaike information criterion (AIC; Akaike, 1974) and Bayesian information criterion (BIC) tests were applied with jModelTest v. 0.1 package program (Posada, 2008; Guindon & Gascuel, 2003). Neighbor-joining (NJ; Saitou & Nei, 1987), Maximum-Likelihood (ML; Felsenstein, 1981) and Maximum-Parsimony (MP) algorithms were used to determine the phylogenetic relations among isolates. NJ and MP analysis were applied using PAUP* v. 4.0b10 (Swofford, 2003) where ML analysis were applied using PYHML v. 3.0 (Guindon & Gascuel, 2003). MP analysis were performed with heuristic search approach using TBR swapping algorithm with 10 random repetitions. In the case of more than one most parsimonious tree, a consensus tree was generated. Bootstrap analysis for ML and MP trees were conducted with 1000 and for NJ tree was conducted with 10 000 pseudo-replications.

Results

To determine the total genetic divergence of 18S rDNA within *W. circinata*, we obtained nearly complete sequence (approximately 1700 bp) of the gene for the selected isolates (BK1, GH700+1, Mm-4-3, Brazil, M003, CrT2, Yakintas) representing different biological varieties (*W. circinata* var. *circinata*, var. *oryzae* and var. *zeae*). As a result, each of *W. circinata* var. *oryzae* (GH700+1 and Mm-4-3) and var. *circinata* (BK1) varieties revealed a single and unique 18S rDNA haplotype which showed 99.4% nucleotide similarity (9 substitutions within 1667 aligned nucleotides) with each other (Table 3). On the other hand, three different haplotypes appeared within fully sequenced *W. circinata* var. *zeae* isolates (Brazil; Yakakent; M003 and CrT2) with a nucleotide sequence similarity higher than 99.8% (2 substitutions within 1667 alligned nucleotides). Additionally, *W. circinata* var. *zeae* isolates showed 99.5-99.6% (6-8 substitutions) and 99.7-99.8% (3-5 substitutions) nucleotide sequence similarities with *W. circinata* var. *oryzae* and var. *circinata* isolates, respectively (Table 3). Our results showed that all of the variations within *W. circinata* var. *zeae* haplotypes occurred in the first half of the gene. That is why we designed a new reverse primer, Wc-18S_879 which amplifies approximately the first 880 bases of 18S rDNA with NS1 as forward primer. In addition to the full length sequenced isolates, we partially sequenced (approximately 840 bp) the 18S rDNA from *W. circinata* isolates, AS7S-11A, C-504, Rcl246, Rss318, Rss319, Agillar, Sariyurt, Yakintas, Yakakent, CHTS-1 ABC, Hungary with using the primer set mentioned above. Phylogenetic analysis were carried out with 782 aligned nucleotides with 32

segregating sites. AIC and BIC tests suggested TIM3 and K80 substitution models, respectively. MP analyses resulted in four most parsimonious trees (length: 34 step; CI: 0.941; RI: 0.667; HI: 0.059). In Fig. 1, NJ tree drawn with TIM3 model which gave the highest bootstrap values was given and the bootstrap values derived from MP and ML analysis have also been presented in parenthesis on the related nodes. Three lineages appeared within *W. circinata* isolates in all trees that were produced with NJ, MP and ML methods (Fig. 1). All *W. circinata* var. *zeae* isolates except for isolate Brazil grouped within the Lineage-I that was comprised of two haplotypes (Haplotype-A and -B) and the node combining these haplotypes was supported with 73% bootstrap values both in the NJ and ML trees. Isolates from Americas (USA06), Far East (Rcl246, Rss318, Rss319, As7S-11A, M003) with one exception (C-504 from Japan) and also some Turkish originated isolates (Agillar, JR-8 and CrT2) showed the Haplotype-A within the Lineage-I. Most of the Turkish originated isolates (CrT21, Yakintas, CHTS-1 ABC, Sariyurt and Yakakent) and also isolates C-504 (from Japan) and Hungary shared the Haplotype-B within the Lineage-I. Interestingly, isolate Brazil which showed the third *W. circinata* var. *zeae* haplotype (Haplotype-C) grouped with *W. circinata* var. *circinata* isolates (BK1) in the Lineage-II that appeared as sister to the first one with 74%, 74% and 72% bootstrap values in NJ, MP and ML trees, respectively. *W. circinata* var. *oryzae* isolates (Mm4-3 and GH700+1) constituted the Lineage-III that appeared as sister to the first two with 100% bootstrap values in NJ, MP and ML trees (Fig. 1).

Table 1. Sources, origins and accession numbers for 18S rDNA and β-tubulin nucleotide sequences of the *Waitea circinata* isolates used in this study.

Isolate *W. circinata*	Locality and host	Source	18S rDNA rDNA Acc. No	β-tubulin Acc. No
M003	Aichi-Japan	No Data	KT347100	KT347120
var. *zeae*	Soil			
AS-7S-11a	No Data	No Data	KT347101	
var. *zeae*	No Data			
C-504	Ishikawa-Japan	No Data	KT347102	KT347121
var. *zeae*	Soil			
Brazil	Brazil	(Poltronieri *et al.*, 2002)	KT347103	KT347122
var. *zeae*	*Z. mays*			
Rcl246	Taiwan	Dr. TF Hsieh	KT347104	KT347123
var. *zeae*	*Z. mays*			
Rss318	Taiwan	Dr. TF Hsieh	KT347105	
var. *zeae*	*Sorgum*			
Rss319	Taiwan	Dr. TF Hsieh	KT347106	
var. *zeae*	-			
Rz55-1	Samsun-Turkey	Dr. I. Erper	KT347107	KT347124
var. *zeae*	No Data			
Rz55-2	Samsun-Turkey	Dr. I. Erper	KT347108	
var. *zeae*	No Data			
Yakintas	Amasya-Turkey	(Eper *et al.*, 2006)	KT347109	KT347125
var. *zeae*	Onion			
CrT2	Samsun-Turkey	(Gurkanli, 2005)	KT347110	KT347126
var. *zeae*	Tobacco soil			
CrT21	Samsun-Turkey	(Gurkanli, 2005)	KT347111	KT347127
var. *zeae*	Tobacco soil			
Yakakent	Samsun-Turkey	(Gurkanli, 2005)	KT347112	
var. *zeae*	Tobacco soil			
JR-8	Artvin-Turkey	(Demirci & Eken, 1999)	KT347113	
var. *zeae*	Johnsongrass			
CHTS1-ABC	Isparta-Turkey	Prof. G. Karaca	KT347114	KT347128
var. *zeae*	No Data			
USA06	USA	Dr. T.C. Paulitz	KT347116	KT347129
var. *zeae*	No Data			
Hungary	Hungary	(Vajna & Oros, 2005)	KT347119	KT347130
var. *zeae*	*L. erene*, *Festuca* sp.			
BK1	Erzurum-Turkey	Demirci (1998)	KT347115	KT347131
var. *circinata*	*H vulgare*			
GH700+1	Gifu-Japan	No Data	KT347117	KT347132
var. *oryzae*	Zoysiagrass			
Mm-4-3	Japan	No Data	KT347118	KT347133
var. *oryzae*	Zoysiagrass			

Table 2. PCR protocols and primers used in this study.

Gene	Primer	ID	D	A	E	FE
18S rDNA	NS1/NS4[1]	95°C 3 min	94°C 1 min	51°C 1 min	72°C 1.5 min	72°C 5 min
18S rDNA	NS3/NS8[1]	95°C 2 min	94°C 1 min	60°C 1 min	72°C 1.5 min	72°C 10 min
18S rDNA	NS1[1] / Wc-18S_879[2]	95°C 5 min	95°C 45 s	55°C 45 s	72°C 2 min	72°C 4 min
β-Tubulin	B36F/B12R[3]	94°C 2 min	94°C 1 min	58°C 45 s	72°C 1.5 min	72°C 5 min

[1]White *et al.,* 1990; [2]This study; [3]Thon & Royse, 1999

ID: Initial denaturation, **D:** Denaturation, **A:** Annealing, **E:** Extention, **FE:** Final extention

Table 3. β-Tubulin (shaded with grey) and 18S rDNA (non-shaded) nucleotide sequence percent similarities among *Waitea circinata* isolates used for phylogenies.

	M003	C-504	Brazil	Rcl246	Rz55-1	Yakintas	CrT2	CrT21	Yakakent	CHTS1-ABC	USA06	Hungary	BK1	GH700+1	Mm-4-3
M003	-	99.7	98.1	100	100	97	100	97.5	-	97.5	100	99.7	82	93	93
C-504	-	-	98.3	99.7	99.7	97.3	99.7	97.8	-	97.8	99.7	100	82.3	93.3	93.3
Brazil	99.8	-	-	98.1	98.1	96.7	98.1	97.3	-	97.3	98.1	98.3	82.6	92.7	92.7
Rcl246	-	-	-	-	100	97	100	97.5	-	97.5	100	99.7	82	93	93
Rz55-1	-	-	-	-	-	97	100	97.5	-	97.5	100	99.7	82	93	93
Yakintas	-	-	-	-	-	-	97	99.4	-	99.4	97	97.3	81.8	92.7	93
CrT2	100	-	99.8	-	-	-	-	97.5	-	97.5	100	99.7	82	93	93
CrT21	-	-	-	-	-	-	-	-	-	100	97.5	97.8	82.3	93.3	93.5
Yakakent	99.9	-	99.9	-	-	-	99.9	-	-	-	-	-	-	-	-
CHTS1-ABC	-	-	-	-	-	-	-	-	-	-	97.5	97.8	82.3	93.3	93.5
USA06	-	-	-	-	-	-	-	-	-	-	-	99.7	82	93	93
Hungary	-	-	-	-	-	-	-	-	-	-	-	-	82.3	93.3	93.3
BK1	99.7	-	99.8	-	-	-	99.7	-	99.7	-	-	-	-	82.3	82.3
GH700+1	99.5	-	99.6	-	-	-	99.5	-	99.5	-	-	-	99.4	-	99.7
Mm-4-3	99.5	-	99.6	-	-	-	99.5	-	99.5	-	-	-	99.4	100	-

We sequenced approximately 430 bp of the β-tubulin gene from 15 *W. circinata* isolates representing different 18S rDNA haplotypes obtained in this study. Phylogenetic analyses were carried out with 374 aligned nucleotides with 116 segregating sites. AIC and BIC tests suggested TrN+G (G: 0.257) and HKY+G (G: 0.256) substitution models, respectively. MP analyses gave three most parsimonious trees (length: 153 steps; CI: 0.948; RI: 0.896; HI: 0.052). In Fig. 2, NJ tree drawn with HKY+G model was given and the bootstrap values derived from MP and ML analysis have also been presented in parenthesis on the tree. In the phylogram, three biological varieties of *W. circinata* (var. *zeae*, var. *oryzae*, var. *circinata*) were clearly seperated from each other and formed different monophyletic groups (Fig. 2). *W. circinata* var. *zeae* and var. *oryzae* were appeared as sister to each other and the node combining these varieties was supported with 100% bootstrap values in all three phylograms drawn with NJ, ML and MP algorithms. The β-tubulin nucleotide sequence similarities among these varieties were determined as between 92.7-93.5% (Table 3). In all three phylograms the third variety, *W. circinata* var. *circinata*, was appeared as sister to the var. *zeae* and var. *oryzae* monophyletic group with 100% bootstrap values. The β-tubulin nucleotide sequence similarity between *W. circinata* var. *circinata* and var. *zeae* was determined as 81.8-82.6%, and was determined as 82.3% between *W. circinata* var. *circinata* and var. *oryzae*. A total of five haplotypes were determined among *W. circinata* var. *zeae* isolates that grouped in two lineages with 96.7-99.4% nucleotide sequence similarities (Table 3). The node combining these lineages was supported with 100%, 96% and 55% bootstrap values in NJ, MP and ML trees, respectively (Fig. 2). The Lineage-I that had three haplotypes was comprised of isolates from diverse origins. Two isolates, C-504 (Japan) and Hungary showed the first haplotype in the Lineage-I. The second haplotype that was shared by isolates USA06, Rcl246 (Taiwan), M003 (Japan), CrT2 and Agillar (Turkey) appeared as sister to the first one with 97%, 74% and 95% bootstrap values in NJ, MP and ML trees, respectively. Isolate Brazil showed the third haplotype within the Lineage-I which seems more ancestoral (with 79% and 68% bootstrap values in NJ and ML trees, respectively) to the first two haplotypes. As an unexpected result, three isolates from Turkey (CrT21, CHTS1-ABC and Yakintas) showed quite different β-tubulin haplotypes from other *W. circinata* var. *zeae* isolates and formed a separate monophyletic group (Lineage-II). The node combining the two haplotypes of the Lineage-II was supported with 100%, 99% and 100% bootstrap values in NJ, MP and ML trees, respectively (Fig. 2).

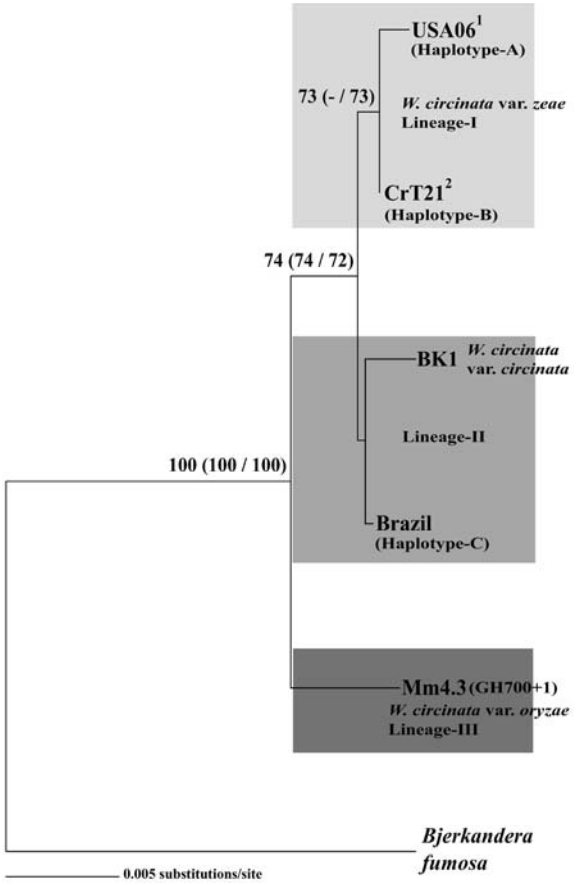

Fig. 1. NJ tree showing the phylogenetic relations among 18S rDNA haplotypes of three *Waitea circinata* biovars. (var. *zeae*, var. *oryzae* and var. *circinata*). The tree was drawn with TIM3 substitution model and rooted with *Bjerkandera fumosa* (DQ060086, Ze-ze *et al*., unpublished). On the tree, only the bootstrap values greater than 50% were shown and the bootstrap values of MP and ML trees were stated in parenthesis.
[1]Rcl246, Rss318, Rss319, As7S11A, Rz55-1, JR-8, M-003, CrT2
[2]C504, Hungary, Yakintas, CHTS1-ABC, Rz55-2, Yakakent

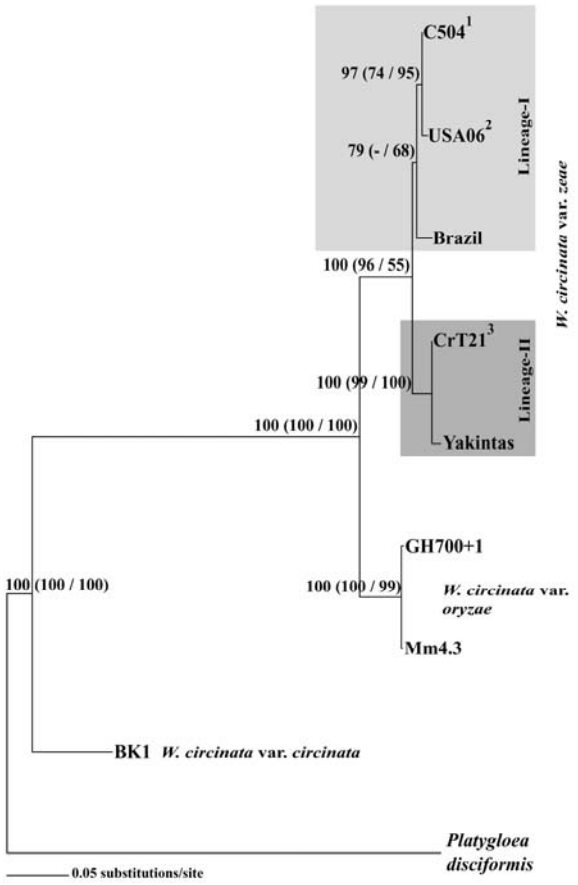

Fig. 2. NJ tree showing the phylogenetic relations among β-tubulin haplotypes of three *Waitea circinata* biovars. (var. *zeae*, var. *oryzae* and var. *circinata*). The tree was drawn with HKY+G (G: 256) substitution model and rooted with *Platygloea disciformis* (AY371531, Begerow *et al.,* 2004). On the tree, only the bootstrap values greater than 50% were shown and the bootstrap values of MP and ML trees were stated in parenthesis.
[1]Hungary, [2]CrT2, Rz55-1, Rcl246, M-003
[3]CHTS1-ABC

Discussion

Although conventional methods such as anastomosis reactions and colony morphology are useful, in the last two decades molecular methods have become major tools in systematics of *Rhizoctonia*-related fungi. One of these methods, phylogenetic analysis based on nucleotide sequences of 18S-28S rDNA-ITS region has been commonly preferred by researchers because immense numbers of haplotypes from different localities have already accumulated in international data bases (GenBank and NCBI) that makes it possible to compare obtained sequences with other haplotypes from all over the world. Additionally, non-coding intergenic loci such as 18S-28S rDNA-ITS region accumulate high amounts of mutations (substitutions, insersions and deletions) which allows identification at the species level. Although this feature seems convenient for inferring phylogenetic relations within a certain anastomosis group (AG) or between closely related AGs, it may cause some errors for phylogenies concerning distantly related ones. The main reason of the possible error is the high amount of variations within ITS1 and ITS2 loci that makes the multiple allignment of the haplotypes more hypothetical (subjective) and as a result different phylogenetic relationships arise in different studies. For example, with analysing the nucleotide sequences of 18S-28S rDNA-ITS, Kuninaga *et al.* (1997) and Hsiang & Dean (2001) grouped *Rhizoctonia solani* AG-1 and AG-7 in the same lineage. On the other hand in Carling *et al.* (2002) and Sharon *et al.* (2006; 2008) these two AGs appeared in totally different lineages. Additionally, different phylogenetic relations among AGs of *Rhizoctonia* can also arise when using genetic markers other than 18S-28S rDNA-ITS. For instance, if we compare the topologies of phylogenetic trees derived from the combined analyses of 18S-28S rDNA-ITS, 28S rDNA (partial) and β-tubulin genes (González *et al.,* 2006) and the one solely derived from 18S-28S rDNA-ITS (Sharon

et al., 2008) significant differences in relationships among AGs can be seen. For all these reasons more than one locus should be used for inferring phylogenies among AGs of *Rhizoctonia* spp. From this perspective, we analysed a wide collection of *W. circinata* using 18S rDNA and β-tubulin genes to infer the true genealogy of the organism. 18S rDNA and β-tubulin phylogenies suggested unique lineages for each of *W. circinata* var. *circinata*, var. *oryzae* and var. *zeae* varieties. The only exception was isolate Brazil (var. *zeae*) which grouped with *W. circinata* var. *circinata* isolates in 18S rDNA tree. These results clearly supported the identification of these populations as seperate varieties of *W. circinata*. Among *W. circinata* var. *zeae* isolates as the main objective of the study, we determined three main groups due to 18S rDNA and β-tubulin haplotypes which are also supported with 18S-28S rDNA-ITS phylogeny in Aydın *et al*. (2013). On the other hand, there were no host accordance among the isolates sharing the same group which is possibly the result of polyphagous nature of the organism. The first group (isolates sharing the 18S rDNA Haplotype-A) comprised of isolates from northern part of Turkey (Black Sea region) including districts of Samsun and Artvin, in addition to the isolates from Far East and Americas. Although this group seems to have a wide distribution around the world, it is restricted to the North Anatolia in Turkey which suggests that the invasion of the group to Turkey occurred within a relatively recent period of time. On the other hand, most of the Turkish isolates from different parts of Turkey including Northern, Middle and West Anatolia appeared in the second group (isolates sharing the 18S rDNA Haplotype-B) together with a single Japanese isolate (C-504) and a Hungarian isolate. This distribution area suggests that this haplotype is dominant in Turkey and possibly in Eurasia and came to Turkey earlier than the first group. Interestingly, Turkish isolates in this group showed a unique β-tubulin haplotype that clearly indicates a common ancestor which suggest that the invasion of the group to Turkey occurred from a single source and dispersed to other localities of Turkey in the course of time. The third group was comprised of a single isolate, Brazil, which showed 18S rDNA haplotpe-C and interestingly this haplotype appeared as related with *W. circinata* var. *circinata* instead of other two var. *zeae* haplotypes (Fig. 1). This isolate also showed a unique β-tubulin haplotype (Fig. 2) and additionally formed a separate lineage (main monophyletic group-II) in 18S-28S rDNA-ITS phylogeny together with other Americas originated isolates in Aydın *et al.* (2013). Our results in this study together with Aydın *et al.* (2013) suggested Americas as the origin for *W. circinata* var. *zeae* because the genetic diversity of isolates from Americas is higher than those of other continents.

In conclusion, 18S rDNA, β-tubulin (this study) and 18S-28S rDNA-ITS phylogenies Aydın *et al.* (2013) suggested three lineages for *W. circinata* var. *zeae*. Two of the lineages have distributed worldwide where one of them is unique for Americas. We determined both of the global lineages in Turkey and due to their dispersion, the invasion of these two lineages to Turkey may have occurred in different period of times. Additionally, after Aydın *et al.* (2013) our results in this study suggested Americas as the origin for *W. circinata* var. *zeae* but this hypothesis must be confirmed with more isolates originating from the Americas.

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