Geographic Distribution and rDNA-ITS Region Sequence Diversity of *Waitea circinata* var. *circinata* Isolated from Annual Bluegrass in the United States

Chi-Min Chen and **Karla A. de la Cerda,** Department of Plant Pathology, University of California, Riverside 92521; **John E. Kaminski,** Department of Plant Science, University of Connecticut, Storrs 06269; and **Greg W. Douhan** and **Francis P. Wong,** Department of Plant Pathology, University of California, Riverside 92521

ABSTRACT

Chen, C.-M., de la Cerda, K. A., Kaminski, J. E., Douhan, G. W., and Wong, F. P. 2009. Geographic distribution and rDNA-ITS region sequence diversity of *Waitea circinata* var. *circinata* isolated from annual bluegrass in the United States. Plant Dis. 93:906-911.

Waitea circinata var. *circinata* is the causal agent of brown ring patch, an emergent disease of turfgrass in the United States. Forty-two isolates from annual bluegrass were obtained from California, Connecticut, Idaho, Illinois, Massachusetts, New York, Ohio, Oregon, and Rhode Island. Almost all isolates produced white to orange sclerotia (bulbils), 2 to 5 mm in size, that turned dark brown after 21 days on ¼-strength potato dextrose agar. The ribosomal DNA internal transcribed spacer regions and 5.8S region (ITS) were analyzed by restriction fragment length polymorphism (RFLP) analysis using *Msp*I and sequencing to attempt identification of the isolates. Some isolates were heterozygous at the *Msp*I restriction site, results not found in previous reports using the RFLP technique for identification. Four additional nucleotide positions were found to be variable within ITS based on sequence analysis, including two indels and two additional heterozygous positions. A total of 17 ITS haplotypes were found, and there was no obvious relationship between ITS haplotype and the geographic distribution of the isolates. Results of this work indicate that *W. circinata* var. *circinata* is present in multiple states and provide an initial understanding of the diversity of the pathogen in the United States.

Waitea circinata var. *circinata* has recently been described as the causal agent of brown ring patch in Japan on creeping bentgrass (*Agrostis stolonifera* L.) (21), and in the United States on annual bluegrass (*Poa annua* L.) and rough bluegrass (*Poa trivialis* L.) (3,5). *W. circinata* var. *circinata* has a *Rhizoctonia* anamorph, and is related to *R. oryzae* Ryker & Gooch (*Waitea circinata* var. *oryzae*) and *R. zeae* Voorhees (*Waitea circinata* var. *zeae*) (8,10).

In the United States, brown ring patch was first confirmed as a disease in western Washington state in 2003 and recently characterized from annual bluegrass putting greens in the western United States (California, Nevada, and Washington) (5). It was recognized as a new disease and commonly referred to as "Waitea patch" until it was realized that the same pathogen was responsible for the disease on annual bluegrass in the United States and creeping bentgrass in Japan (5,21).

Symptoms on annual bluegrass as described by de la Cerda et al. (5) include thin yellow rings or semicircles, several centimeters to one meter in diameter, that can turn brown or necrotic and often have a sunken appearance. Symptoms are typically observed at a range of 15 to 35°C maximum daytime air temperature. *W. circinata* var. *circinata* appears to frequently colonize the foliage, crowns, upper roots, and thatch in affected areas. This appears to make control difficult in some cases as multiple fungicide applications are often needed to eradicate the pathogen from the crowns, upper roots, and thatch for complete control. To complicate matters, the disease is superficially similar to other *Rhizoctonia* diseases such as leaf and sheath spot (*R. zeae* and *R. oryzae*) or yellow patch (*R. cerealis* van der Hoeven) that are reported to occur most frequently at high (>28°C) and low temperatures (<24°C), respectively (4,17).

In the spring and summer of 2006, yellow rings on annual bluegrass putting greens were reported from more than 50 California golf courses and multiple locations in the Midwest and northeastern United States (K. Dannenberger, M. Boehm, J. Rimmelspach, D. Settle, and J. Kaminski, *personal communication*). These areas include golf course putting greens from Ohio, Illinois, Indiana, Pennsylvania, Connecticut, New York, Massachusetts, Maine, and Rhode Island. In these cases, a *Rhizoctonia*-like fungus was recovered from symptomatic turf, but it was not clear which species was present.

The objectives of this study were to (i) confirm the presence of *W. circinata* var. *circinata* as a pathogen of annual bluegrass in these diverse locations in the United States using morphological methods, restriction fragment length polymorphism (RFLP), and sequence analysis of the intergenic spacer (ITS) region, and (ii) assess the diversity of the pathogen ITS region. With regard to the latter objective, identification of a species by ITS region sequence is a useful tool, but there are limited numbers of *W. circinata* var. *circinata* isolates that have been characterized (5,21). Assessing the ITS region diversity from a larger number of isolates has implications for improving ITS-based identification methods and would also provide a preliminary indication of intraspecies diversity and the pathogen population biology. For example, no ITS diversity may indicate a recent introduction and spread of this pathogen, whereas variability within this region may indicate that this species was previously present and that other factors such as management practices or environmental changes were responsible for the apparent increase of this "new" disease.

MATERIALS AND METHODS

Isolate collection and maintenance. Most isolates were collected from diseased *P. annua* in 2006, but two isolates collected in 2005 and 2007 were included in this study. A list of isolates used in this study and their geographic origin is shown in Table 1. For isolates collected in the western United States, a single symptomatic leaf blade was placed on ¼-strength potato dextrose agar in 100-mm petri plates (¼-PDA; 4.95 g PDA, 5.63 g granulated agar [Fisher Scientific, Pittsburgh, PA] per 500 ml of deionized water [dH_2O]) and incubated at room temperature. After 24 to 48 h, developing colonies on the plates were examined; isolates were identified as *Rhizoctonia* spp. by the diagnostic 90-degree branching of developing mycelia and colony appearance (18). Each isolate was obtained by transferring a 1- to 2-

Corresponding author: F. P. Wong
E-mail: frank.wong@ucr.edu

The first two authors are considered co–first authors who contributed equally to this work.

Current address of J. E. Kaminski: Department of Crop and Soil Sciences, The Pennsylvania State University, State College, PA 16802.

*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 1 appears in color in the online edition.

Accepted for publication 29 April 2009.

doi:10.1094/PDIS-93-9-0906

mm hyphal tip to a new ¼-PDA plate with the aid of a dissecting needle and stereomicroscope. For all other isolates, infected plants were placed onto PDA and emergent hyphae subcultured onto new PDA 24 to 48 h later. These isolates were subsequently shipped to the Wong lab and hyphal tipped as described above. Unless otherwise noted, isolates were maintained in an incubator at 28°C without supplemental light and transferred to new ¼-PDA every 21 days for the duration of the study.

Morphological characterization. A 5-mm plug of each isolate was transferred to ¼-PDA and full-strength PDA (19.5 g PDA per 500 ml dH_2O) and maintained in the dark at 28°C. Identification of isolates was based on the width of several vegetative hyphae from 1-week-old cultures, as measured with an optical micrometer (Leica 12.5MZ, Leica Microsystems Inc., Bannockburn, IL), and diagnostic sclerotial (bulbil) and colony morphology from 21-day-old cultures as described by previous studies (1,5,18,21). Morphological characterization was performed three times for each isolate.

DNA extraction and PCR amplification of ITS sequences. DNA extraction and ITS sequence amplification were performed based on methodologies developed by de la Cerda et al. (5). Briefly, for each isolate, DNA was obtained from approximately 10 mg of mycelia scraped from cultures grown on PDA at 28°C for 7 days. The PureGene DNA extraction kit (Gentra Systems, Minneapolis, MN) was used for DNA extraction following the manufacturer's instructions. Five microliters of each extraction was separated on a 1.5% agarose-TBE gel by electrophoresis and stained with SYBR Green I nucleic acid stain (Molecular Probes, Eugene, OR) to estimate quantity and quality of DNA prior to PCR.

Amplification of an approximate 650-bp portion of the rDNA-ITS region was performed using ITS1F (5′ CTTGGTCAT TTAGAGGAAGTAA 3′) (6) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (23). For each isolate, approximately 100 ng of DNA was used for PCR in a 20-µl reaction volume with a final concentration of 0.2 mM each dNTP (Invitrogen, Carlsbad, CA), 0.375 µM of each oligonucleotide primer, 1× PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, and 0.5 units of *Taq* polymerase (Invitrogen). Amplification was performed in a PTC-200 thermocycler (MJ Research, Waltham, MA) using one cycle of 94°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. PCR products were examined by electrophoresis with 5 µl of each PCR products in 1.5% agarose-TBE gel to determine the success of amplification for each reaction. Products were stored at –20°C for further restriction enzyme digestion and sequencing.

Restriction enzyme digestion analysis of amplified ITS-sequences. As reported by Toda et al. (21), RFLP analysis of the ITS region with *Hap*II could differentiate *W. circinata* var. *circinata* from *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*. This procedure was used with the intent of rapidly screening and identifying the isolates as different *W. circinata* varieties. One microgram of the amplified rDNA-ITS region was digested with 10 units of *Msp*I (New England Biolabs, Ipswich, MA), an isoschizomer of *Hap*II. Reactions were incubated for at least 1 h at 37°C, and the products were separated and visualized by gel electrophoresis in 2% agarose-TBE gels.

ITS sequencing and cloning. ITS regions from the isolates were amplified as described above, and sequencing in both directions was performed at the Core Instrumentation Facility (CIF) of the University of California's (UC) Institute of Integrative Genome Biology at UC Riverside. The sequencing reactions failed for some isolates (discussed in the results section), and it was suspected that this was due to indels. Therefore, these ITS PCR products were cloned using the TOPO TA sequencing cloning system (Invitrogen) as described by the manufacturer. Positive clones were transferred to 100 µl of Luria-Bertani (LB) broth amended with 100 µg/ml ampicillin (Sigma Chemical Co., St. Louis, MO) in sterile 96-well microtiter plates and incubated overnight at 37°C. Five clones from each isolate were reamplified using ITS1F and ITS4, and sequencing was performed as described above. The sequences were edited using Sequencher (version 4.6, Gene Codes Corporation, Ann Arbor, MI), Clustal X (version 1.81) (20) was used for alignment, and MacClade version 4 (11) was used for visual editing.

RESULTS

Morphological identification of isolates. All isolates examined in this study were morphologically similar to *W. circinata* var. *circinata* isolates as previously described by Toda et al. (21) and de la Cerda et al. (5) based on hyphal width and mycelial and sclerotial morphology. The production of a thick buff colored mycelial mat on PDA often obscured the production

Table 1. Isolates used in this study

Isolate name	Location	Date collected	Source	GenBank accession no.
AGCCSJ-2.1	San Jose, CA	2006/7/7	F. P. Wong	FJ755849
AGTC-4	Hayden Lake, ID	2007/7/5	H. C. Wetzel	FJ755890
AVCCPL-0.1	Placentia, CA	2006/4/27	F. P. Wong	FJ755850
CCTRTR-1.1	Troy, NY	2006/7/17	J. E. Kaminski	FJ755851
CCTRTR-1.2	Troy, NY	2006/7/17	F. P. Wong	FJ755852
CDCGTC-9.2	Trabuco Canyon, CA	2006/7/7	F. P. Wong	FJ755853
CECCPO-0.1	Portland, OR	2006/7/7	F. P. Wong	FJ755854
CHGCSJ-0.1	San Jose, CA	2006/7/7	F. P. Wong	FJ755855
CWCCPL-5.1	Pleasanton, CA	2006/7/7	F. P. Wong	FJ755856
DCGCSL-0.1	San Luis Obispo, CA	2006/6/22	F. P. Wong	FJ755857
ECGCES-0.1	Escondido, CA	2006/5/29	F. P. Wong	FJ755858
FVGCAU-0.1	Aurora, IL	2006/7/19	D. M. Settle	FJ755859
GMGCGR-13.1	Graegle, CA	2006/7/7	F. P. Wong	FJ755860
HVGCCO-0.1	Corona, CA	2006/5/29	F. P. Wong	FJ755861
LACCLA-1.1	Los Angeles, CA	2006/3/1	F. P. Wong	FJ755862
LEGCLE-0.1	Lexington, MA	2006/7/17	J. E. Kaminski	FJ755863
LRCCLR-18.2	Thousand Oaks, CA	2005/12/1	F. P. Wong	FJ755864
MECCWS-11.1	Woodside, CA	2006/4/27	F. P. Wong	FJ755865
MLGCOB-0.1	Oak Brook, IL	2006/7/19	D. M. Settle	FJ755866
MPCCPB-12S.1	Pebble Beach, CA	2006/5/22	F. P. Wong	FJ755867
NLCCNL-0.1	New London, CT	2006/7/17	J. E. Kaminski	FJ755889
NSCCGL-0.1	Glenview, IL	2006/7/19	D. M. Settle	FJ755868
NWCCNO-0.1	Norwich, CT	2006/7/17	J. E. Kaminski	FJ755869
ONCCST-0.1	Stratford, CT	2006/7/18	J. E. Kaminski	FJ755870
PPGCAU-0.1	Aurora, IL	2006/7/19	D. M. Settle	FJ755871
PTCCMA-0.1	Marysville, CA	2006/7/7	F. P. Wong	FJ755872
RHCCTE-3.1	Temecula, CA	2006/3/1	F. P. Wong	FJ755873
RHCCTE-15.2	Temecula, CA	2006/3/1	F. P. Wong	FJ755874
RMCCHO-0.1	Hollister, CA	2006/7/7	F. P. Wong	FJ755875
SCCCHB-18.1	Huntington Beach, CA	2006/7/7	F. P. Wong	FJ755876
SDGCSD-0.1	San Dimas, CA	2006/5/22	F. P. Wong	FJ755877
SICCNA-0.1	Napa, CA	2006/6/1	F. P. Wong	FJ755878
TECCMA-0.1	Marblehead, MA	2006/7/19	J. E. Kaminski	FJ755879
TPCGLJ-18S.1	La Jolla, CA	2006/6/5	F. P. Wong	FJ755880
TPCGLJ-18S.2	La Jolla, CA	2006/6/5	F. P. Wong	FJ755881
TVCCSJ-0.1	San Jose, CA	2006/5/29	F. P. Wong	FJ755882
WACCRU-0.1	Rumford, RI	2006/7/20	J. E. Kaminski	FJ755883
WRCCAL-0.1	Albany, NY	2006/7/17	J. E. Kaminski	FJ755884
WSCCLA-5.1	Los Angeles, CA	2006/6/15	F. P. Wong	FJ755885
WTCCWT-0.1	Watertown, CT	2006/7/17	J. E. Kaminski	FJ755886
YOGTYO-0.1	York, ME	2006/7/21	J. E. Kaminski	FJ755887
06-487	Columbus, OH	2006/6/1	J. Rimelspach	FJ755888

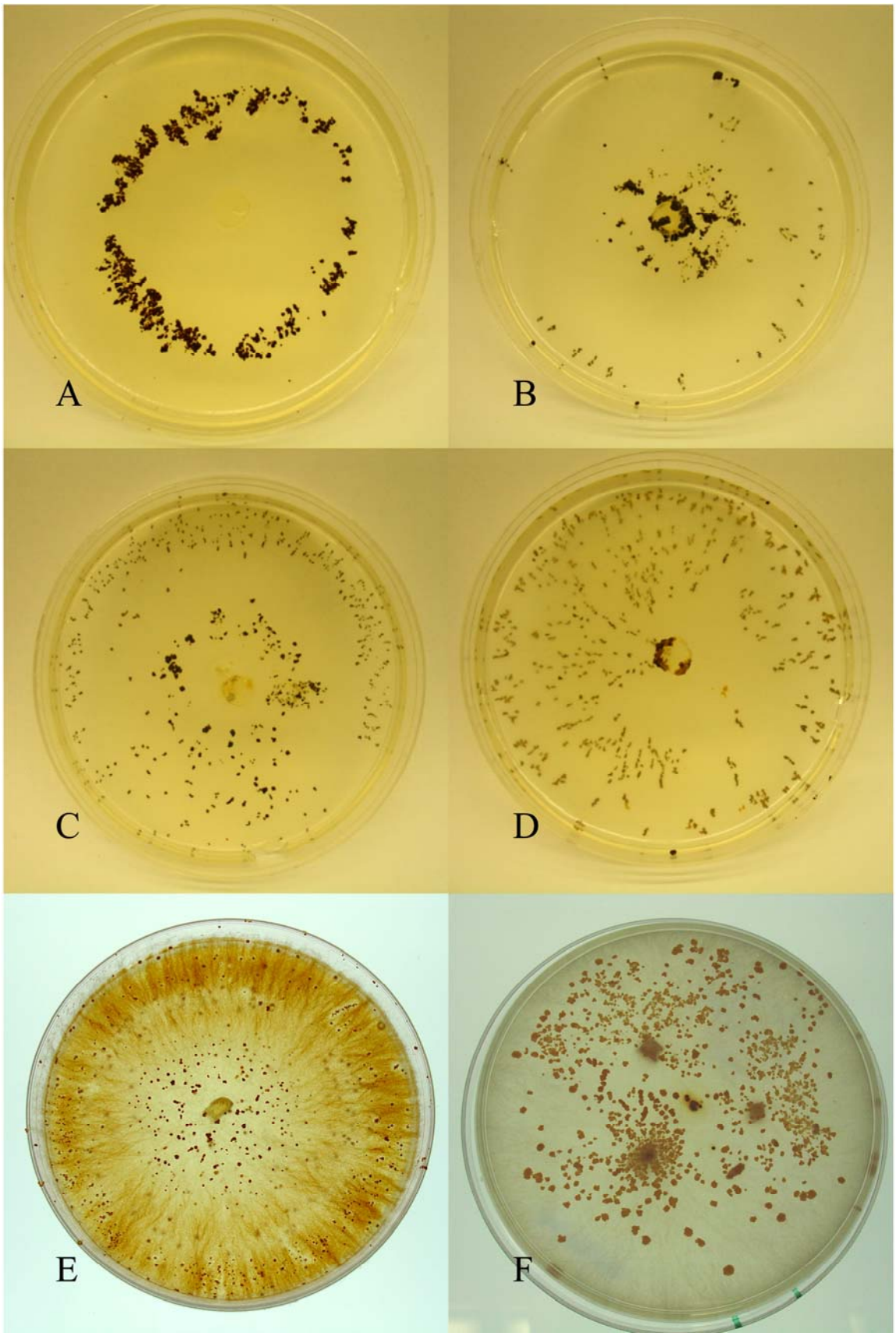

Fig. 1. Morphology of *Waitea circinata* var. *circinata* isolates on ¼-strength potato dextrose agar after 21 days incubation. Variations in morphology include **A,** dark brown sclerotia on the agar surface, **B and C,** mixture of large and small dark brown sclerotia on top of and embedded in the agar, and **D,** mixture of orange, whitish, and dark brown sclerotia. Morphology of isolates can vary, but these are clearly distinguishable from other *W. circinata* varieties such as **E,** *Rhizoctonia zeae* (*W. circinata* var. *zeae*), which produces reddish sclerotia, and **F,** *Rhizoctonia oryzae* (*W. circinata* var. *oryzae*), which produces pink to salmon sclerotia after 21 days incubation on ¼-strength potato dextrose agar.

and morphology of the diagnostic sclerotia of the isolates, thus the morphological characteristics on ¼-PDA were more uniform for identification purposes. All isolates produced thin buff colored mycelia on ¼-PDA, but the best diagnostic feature was the production of irregular 2- to 5-mm white to orange sclerotia within 7 to 14 days that turned dark brown by 21 days of incubation. Two isolates (PTCCMA-0.1, NWCCNO-0.1) did not turn dark brown after 21 days, retaining a white to orange color. Some others had a mixture of dark brown, orange, and white sclerotia on the media. For individual isolates, sclerotia were produced on the medium surface, embedded in the medium, or a combination of both. Some isolates often had variation in morphology and the location of sclerotial production (on or in the media) during the three replicated tests, while others were morphologically consistent in all three tests. Although these variations in colony morphology were present on ¼-PDA, the presence of dark brown or orange sclerotia was always associated with *W. circinata* var. *circinata* isolates, and these features were clearly distinct from the two other *W. circinata* varieties, *R. oryzae* and *R. zeae* (Fig. 1).

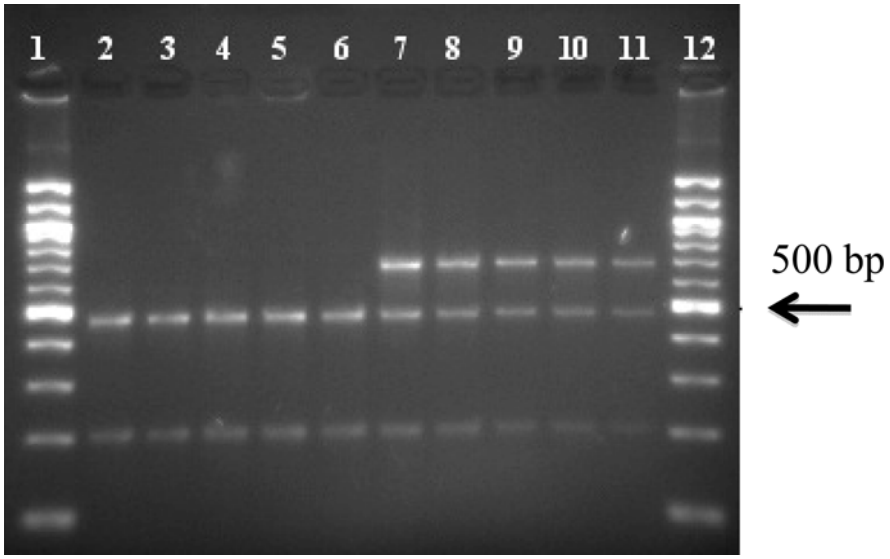

Fig. 2. *Msp*I digestion of amplified ribosomal DNA intergenic spacer (rDNA-ITS) regions from representative isolates (1 unit per 100 ng DNA) followed by gel electrophoresis in a 2% agarose-TBE gel. Lanes 2 to 6 represent isolates that are homozygous at the *Msp*I/*Hap*II restriction site yielding two fragments of sizes ~170 and 470 bp. Lanes 7 to 11 represent isolates that were heterozygous at the *Msp*I restriction site leading to the production of fragments of sizes ~170 and 470 bp and uncut polymerase chain reaction product (~650 bp). Lanes 1 and 12 are a 100-bp ladder.

RFLP and sequence analysis. Initial RFLP analyses showed that digestion of the amplified ITS-regions with *Msp*I produced the diagnostic 470- and 170-bp bands when visualized by gel electrophoresis (Fig. 2). However, six isolates analyzed in this manner produced bands of 650 (uncut), 470, and 170 bp (Fig. 2). Sequencing analysis indicated that these isolates were heterozygous at the *Msp*I/*Hap*II restriction site (nucleotide position 183), and therefore presumably only part of the DNA was being digested. In total, five nucleotide positions were found to be variable within the ITS; two indels and three heterozygous positions (including position 183 mentioned above). The two single-base-pair indels within the poly A/T stretches were first putatively identified from failed sequencing reads. In these cases, sequence reads of the amplified ITS regions appeared normal until nucleotide positions 235 and or 438; sequencing reads past these points were unreadable due to the frame shift in the sequencing read caused by the indels. The presence of variable poly A/T regions at these locations was confirmed through cloning and sequencing multiple clones (Table 2). Seventeen unique ITS haplotypes were found, and most of the isolates belonged to haplotype 3 (Table 2). No obvious relationships between isolate geographical origin and haplotype were observed.

Table 2. Heterozygous and indel positions present in the ITS1F/ITS4 amplified sequence of the ribosomal DNA intergenic transcribed spacer (rDNA-ITS) region for selected isolates of *Waitea circinata* var. *circinata*

	Nucleotide position (bp)[y]							
ITS haplotype	**149**	**183[z]**	**235**	**438**	**439**	**Isolate**	**Occurrence**	**Origin**
1	C	C	T/-	T/-	A	SICCNA-0.1	1	CA
2	C	C	T	T/-	A/G	CWCCPL-5.1	1	CA
3	C	C	T	-	G	AGCCSJ-2.1, AGTC-4, AVCCPL-0.1, CCTRTR-1.2, CDCGTC-9.2, CHGCSJ-0.1, DCGCSL-0.1, ECGCES-0.1, HVGCCO-0.1, MLGCOB-0.1, MPCCPB-12S.1, RHCCTE-15.2, SDGCSD-0.1, TECCMA-0.1, TPGCLJ-18S.2, WSCCLA-5.1, WTCCWT-0.1, WRCCAL-0.1, 06-487	19	CA, CT, ID, IL, NY, OH, MA
4	C	C	T/-	-	G	GMGCGR-13.1	1	CA
5	C	C	T/-	-	A/G	LACCLA-1.1, TPGCLJ-18S.1	2	CA
6	C	T/C	T	T/-	A/G	PTCCMA-0.1, RHCCTE-3.1	2	CA
7	C	T/C	T/-	-	G	ONCCST-0.1	1	CT
8	C	T/C	T	-	G	YOGTYO-0.1, CECCPO-0.1, CCTRTR-1.1	3	ME, OR, NY
9	G	C	T/-	-	A	LEGCLE-0.1, NWCCNO-0.1	2	MA, CT
10	G	C	-	-	A/G	FVGCAU-0.1	1	IL
11	G	C	-	-	A	SCCCHB-18.1	1	CA
12	C/G	C	T/-	-	G	NLCCNL-0.1	1	CT
13	C/G	C	T/-	-	A/G	PPGCAU-0.1, RMCCHO-0.1	2	IL, CA
14	C/G	C	T/-	-	A	WACCRU-0.1	1	RI
15	T/G	C	-	-	A/G	LRCCLR-18.2	1	CA
16	T	C	T/-	-	A/G	MECCWS-11.1	1	CA
17	T	C	T/-	-	G	NSCCGL-0.1, TVCCSJ-0.1	2	IL, CA

[y] Nucleotide position is relative to the end of the ITS1F priming site. A single nucleotide represents a homozygous position, two nucleotides represent a heterozygous position, and - represents no T at this position relative to the other sequences since these are poly T stretches of sequence data. Position 235 consists of 5 or 6 poly T's, and position 438 consists of 8 or 9 poly T's.

[z] Nucleotide position 183 corresponds to the variable position within the *Msp*I/*Hap*II cutting site (5′ CCGG 3′).

DISCUSSION

An issue that emerged while performing this work was the taxonomy and name of the brown ring patch pathogen. Currently, only the species *Waitea circinata* Warcup & Talbot (22) is recognized, and the use of *W. circinata* varieties to describe the pathogen causing brown ring patch and the teleomorphs of *R. oryzae* and *R. zeae* (8) may be taxonomically invalid at this time. The use of *Rhizoctonia circinata* varieties *circinata*, *oryzae,* and *zeae* to describe the anamorphs of *W. circinata,* as proposed by Leiner and Carling (10), is lacking a formal taxonomic description according to the International Code of Botanical Nomenclature, and the use of *Chrysorhiza zeae* as an anamorphic name for *W. circinata*, as proposed by Stalpers and Andersen (19), does not appear to be adequate for distinguishing theses three pathogens from each other. However, it is clear that brown ring patch is caused by a *Rhizoctonia* species distinct from *R. oryzae* and *R. zeae* (5,8,10,21), and for the time being, the use of *W. circinata* and the variety designation is very practical in the context of describing the pathogen causing brown ring patch and its relationship to other *Rhizoctonia* species. However, additional work must be performed to resolve the taxonomy of this group, which is outside of the scope of the studies presented here.

In this work, we present information on the recent development and emergence of *W. circinata* var. *circinata* as a common pathogen of annual bluegrass in the United States. We have shown that *W. circinata* var. *circinata* is present as a pathogen of annual bluegrass across a wide geographic distribution in the United States and completed a preliminary assessment of genetic diversity within the pathogen based on ITS sequences. This analysis also revealed unexpected ITS variability compared to previous studies (5,21), which could potentially impact diagnostic methods used to identify this pathogen.

Previously, *W. circinata* var. *circinata* was shown to be a pathogen of annual bluegrass from nine locations in California, Nevada, and Washington in the United States (5) and of rough bluegrass in two locations in California (3). In this work, we show that the pathogen is also present across a wide range of locations including Connecticut, Idaho, Illinois, Massachusetts, New York, Ohio, Oregon, and Rhode Island. A larger sample of California isolates also shows that the pathogen is widely distributed within the state. Additional reports of the disease from locations in the Midwest and eastern United States require further confirmation of the presence of the pathogen, but our data presented here do indicate that *W. circinata* var. *circinata* is widespread in the United States (Fig. 3).

Data obtained from our disease diagnostic clinic at UC Riverside indicate that *W. circinata* var. *circinata* is the dominant *Rhizoctonia* disease of annual bluegrass in California. From 2006 to 2008, more than 90 *Rhizoctonia* isolates were obtained from California annual bluegrass putting green samples showing symptomatic yellow rings. Only two of these were identified as *R. zeae* (*W. circinata* var. *zeae*), none as *R. oryzae* (*W. circinata* var. *oryzae*), and two as *R. cerealis* (F. Wong, *unpublished data*); the rest appeared to be *W. circinata* var. *circinata* based solely on morphological characteristics and timing of the disease (15 to 35°C maximum daytime temperatures with the majority of samples arriving during periods of 25 to 30°C maximum daytime temperature). Of some >500 annual bluegrass putting green samples we examined in the UC Riverside turf disease diagnostic clinic during this period, none were found to be affected by *R. solani*, suggesting that *W. circinata* var. *circinata* is favored in this semi-arid environment on annual bluegrass putting greens. As discussed by de la Cerda et al. (5), and reinforced by the data presented in this study, it does appear that *W. circinata* var. *circinata* is a common and widespread pathogen in this geographic area. Other areas where *W. circinata* var. *circinata* isolates were obtained (the midwestern and northeastern United States) tend to have more diseases caused by other *Rhizoctonia* species, and it is unclear at this time how frequently brown ring patch occurs in relation to these other *Rhizoctonia* diseases.

In this study, we found that a majority of *W. circinata* var. *circinata* isolates shared common morphological features when grown on ¼-PDA that can help in the diagnosis of the pathogen from field samples. Variation in sclerotial size, location on and in the media, timing of production in culture, and color were present, but generally the sclerotia of these isolates could be produced submerged in the medium, on the medium surface, or in both in combination, were 2 to 5 mm in size, and were initially white to orange with most of the sclerotia turning dark brown over time. No correlation was found between morphological variation and ITS haplotype or geographic origin. The development of the sclerotia of *W. circinata* var. *circinata* is distinct from *W. circinata* var. *zeae* isolates, which produce smaller sclerotia submerged and embedded in the agar that develop a reddish color over time. However, *W. circinata* var. *circinata* may be confused with *W. circinata* var. *oryzae*, since the latter is often described as having large sclerotia with a salmon color (15), which can be potentially confused with the orangish color of developing (and some older) *W. circinata* var. *circinata* sclerotia. A larger study comparing the sclerotial morphology of a range of *W. circinata* isolates would allow us to determine the usefulness of this feature for the clear differentiation of *W. circinata* varieties. For the time being, a combination of morphological characteristics in culture and molecular testing would be recommended for correct identification of *W. circinata* var. *circinata* from diagnostic samples.

Molecular identification of *W. circinata* var. *circinata* by sequence analysis of the ITS region appears to be reliable, as the sequences were quite similar to each other and divergent from other *Rhizoctonia* species as shown in previous studies (5,21). ITS sequence has been shown to be very useful for separating *Rhizoctonia* species and anastomosis groups and also supports the separation of *Waitea* spp. from *Thanatephorus* spp. and *Ceratobasidium* spp., additional teleomorphic genera with *Rhizoctonia* anamorphs (9,16). However,

Fig. 3. Geographic distribution of *Waitea circinata* var. *circinata* isolates identified in this study. Circles represent the approximate geographic origin of isolates used in this study, while squares represent the origin of previously characterized isolates (5). Outline map generated using Smart Draw (San Diego, CA).

the presence of indels in the ITS region interfered with direct sequencing of PCR products from some isolates in this study, which has also been determined for other basidiomycetes, including *Rhizoctonia* species (7). Even when no indels are present, we found a heterozygous position in the diagnostic RFLP site that was not previously reported by de la Cerda et al. (5) and Toda et al. (21) which was most likely due to the limited number of isolates examined in either study. Finding heterozygosity is also not unexpected in a dikaryotic fungus and has also been found in other *Rhizoctonia* species (2,14). Obviously, any diagnostic tool used to identify a target fungal species is only as robust as the number and diversity of isolates used to screen for such markers. Therefore, the utility of the RFLP analysis based on digestion of ITS with *Msp*I or *Hap*II to differentiate varieties of *W. circinata* merits further examination. Possibly, there are *W. circinata* var. *circinata* isolates that are completely lacking the restriction enzyme recognition site and isolates of *W. circinata* var. *zeae* and *W. circinata* var. *oryzae* that have it. Only additional studies examining a larger number of *W. circinata* isolates, of all varieties, would elucidate this.

One objective of this study was to use ITS sequences in an initial attempt to assess the overall diversity of the *W. circinata* var. *circinata* isolates in order to begin to understand the population biology of this apparent "newly emerging" disease. ITS sequence diversity was limited to only five nucleotide positions, but the presence of indels and heterozygosity resulted in a total of 17 unique ITS variants with no obvious relationship between isolate origin and ITS haplotype. This would support the hypothesis that the emergence of the pathogen as a problem is due to changes in turfgrass cultural practices or environmental conditions. This is also supported by preliminary data from amplified fragment length polymorphism (AFLP) analyses showing that genome-wide polymorphisms are common (C. Chen, *unpublished data*). Moreover, isolates described as *W. circinata* var. *circinata* were isolated from turf in California in the mid-1980s (8), isolated from soil in Alaska in the mid-1990s (10), and reported as a pathogen of rice in California (8), suggesting the wide distribution of this species. Additional comparison of the amplified ITS regions used in this study aided by Blastn comparisons of ITS sequences previously deposited in GenBank also revealed nearly identical sequences associated with isolates identified as *R. oryzae* from wheat in the Pacific Northwest (12,13). Although it is unknown if these are truly the same as our *W. circinata* var. *circinata* populations affecting annual bluegrass in the United States, it does suggest that this is a more widespread pathogen of grasses in the United States than previously recognized. Additional studies using AFLP analysis and multilocus sequencing approaches are currently being pursued in order to more robustly test the hypothesis that *W. circinata* var. *circinata* is not a recently introduced species and to help better characterize the population structure of this pathogen.

Identification of the factors contributing to the development of the disease on turfgrass in response to either changes in management programs for turfgrass or potentially larger issues such as global climate change are of the highest importance. The current challenges with this pathogen are to understand how to best manage this disease on golf course putting greens, with respect to the impact of fungicide applications and cultural controls, and to determine what factors have caused this disease to emerge as a major and widespread problem.

ACKNOWLEDGMENTS

We thank the California State Golf Course Superintendents of America and its Northern California, Southern California, Hi-Lo Desert, San Diego, and Sierra Nevada Chapters for research funding used to conduct this work. The University of California Agricultural Experiment Station provided additional funding. We thank D. Settle of the Chicago District Golf Association, J. Rimelspach and M. Boehm, and H. Wetzel for their isolates collected from Illinois, Ohio, and Idaho, respectively.

LITERATURE CITED

1. Burpee, L., and Martin, B. 1992. Biology of *Rhizoctonia* species associated with turfgrasses. Plant Dis. 76:112-117.
2. Ceresini, P. C., Shew, H. D., Vilgalys, R. J., Gale, L. R., and Cubeta, M. A. 2003. Detecting migrants in populations of *Rhizoctonia solani* anastomosis group 3 from potato in North Carolina using multilocus genotype probabilities. Phytopathology 93:610-615.
3. Chen, C. M., Douhan, G. W., and Wong, F. P. 2007. First report of *Waitea circinata* var. *circinata* causing brown ring patch on *Poa trivialis* in California. Plant Dis. 91:1687.
4. Couch, H. B. 1995. Diseases of Turfgrass, 3rd ed. Krieger Publishing, Malabar, FL.
5. de la Cerda, K. A., Douhan, G. W., and Wong, F. P. 2007. Discovery and characterization of *Waitea circinata* var. *circinata* affecting annual bluegrass from the western United States. Plant Dis. 91:791-797.
6. Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for Basidiomycetes - Application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.
7. Gonzalez, D., Cubeta, M. A., and Vilgalys, R. 2006. Phylogenetic utility of indels within ribosomal DNA and beta-tubulin sequences from fungi in the *Rhizoctonia solani* species complex. Mol. Phylogenet. Evol. 40:459-470.
8. Gunnell, P. S. 1986. Characterization of the telemorphs of *Rhizoctonia oryzae-sativae*, *Rhizoctonia oryzae*, and *Rhizoctonia zeae*, and the effect of cultural practices on aggregate sheath spot of rice, caused by *Rhizoctnia oryzae-sativae*. Ph.D. thesis. University of California, Davis.
9. Hsiang, T., and Dean, J. D. 2001. DNA sequencing for anastomosis grouping of *Rhizoctonia solani* isolates from *Poa annua*. Int. Turfgrass Soc. Res. J. 9:674-678.
10. Leiner, R. H., and Carling, D. E. 1994. Characterization of *Waitea circinata* (*Rhizoctonia*) isolated from agricultural soils in Alaska. Plant Dis. 78:385-388.
11. Maddison, D. R., and Maddison, W. P. 2001. MacClade: Analysis of Phylogeny and Character Evolution. Sinauer Associates, Sunderland, MA.
12. Mazzola, M., Wong, O. T., and Cook, R. J. 1996. Virulence of *Rhizoctonia oryzae* and *R. solani* AG-8 on wheat and detection of *R. oryzae* in plant tissue by PCR. Phytopathology 86:354-360.
13. Okubara, P. A., Schroeder, K. L., and Paulitz, T. C. 2008. Identification and quantification of *Rhizoctonia solani* and *R. oryzae* using real-time polymerase chain reaction. Phytopathology 98:837-847.
14. Rosewich, U. L., Pettway, R. E., McDonald, B. A., and Kistler, H. C. 1999. High levels of gene flow and heterozygote excess characterize *Rhizoctonia solani* AG-1 IA (*Thanatephorus cucumeris*) from Texas. Fungal Genet. Biol. 28:148-159.
15. Ryker, T. C., and Gooch, F. S. 1938. *Rhizoctonia* sheath spot of rice. Phytopathology 28:233-246.
16. Sharon, M., Kuninaga, S., Hyakumachi, M., and Sneh, B. 2006. The advancing identification and classification of *Rhizoctonia* spp. using molecular and biotechnological methods compared with the classical anastomosis grouping. Mycoscience 47:299-316.
17. Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 2005. Compendium of Turfgrass Diseases, 3rd ed. American Phytopathological Society, St. Paul, MN.
18. Sneh, B., Burpee, L., and Ogoshi, A. 1991. Identification of *Rhizoctonia* Species. American Phytopathological Society, St. Paul, MN.
19. Stalpers, J. A., and Andersen, T. F. 1996. A synopsis of the taxonomy of teleomorphs connected with *Rhizoctonia* S. L. Pages 49-63 in: Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. B. Sneh, S. Jabaji-Hare, S. Neate, and G. Dijst, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.
20. Thompson, J. D., Plewniak, F., and Poch, O. 1999. A comprehensive comparison of multiple sequence alignment programs. Nucleic Acids Res. 27:2682-2690.
21. Toda, T., Mushika, T., Hayakawa, T., Tanaka, A., Tani, T., and Hyakumachi, M. 2005. Brown ring patch: A new disease on bentgrass caused by *Waitea circinata* var. *circinata*. Plant Dis. 89:536-542.
22. Warcup, J. H., and Talbot, P. H. B. 1962. Ecology and identity of mycelia isolated from soil. Trans. Br. Mycol. Soc. 45:495-518.
23. White T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White eds. Academic Press, San Diego, CA.