EXECUTIVE SUMMARY FOR 1998

Project Title: The Biology and Management of Spring Dead Spot in Bermudagrass

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Project Summary

Spring dead spot (SDS) is a serious disease of bermudagrass along the northern range of its adaptation in the USA. Three distinct root-rotting fungi called *Ophiosphaerella herpotricha*, *O. korrae*, and *O narmari* (formerly *Leptosphaeria korrae* and *L. narmari*) cause this disease. The purpose of our research is to learn more about the distribution and biology of these SDS pathogens, and based on this understanding, to develop more effective strategies for managing this disease.

Diseased bermudagrass stolons and roots were sampled from golf courses in Kansas Oklahoma, and Kentucky. *O. herpotricha* and *O. korrae* were recovered from samples in all states, with *O. herpotricha* being slightly more abundant. *O. narmari*, previously reported only in Australia, was detected for the first time in North America from samples collected in Oklahoma and Kansas.

Little is known about the population structure of SDS pathogens on a local and regional scale. Similarities among isolates of *O. herpotricha* are being analyzed by amplified fragment length polymorphism (AFLP) analysis. Preliminary analysis suggests that on individual fairways, there is a mosaic of clones of *O. herpotricha*. However, on a regional scale there appears to significant genetic heterogeneity among isolates. Less diversity has been detected among isolates of *O. korrae* and *O. narmari*.

Field and greenhouse studies are being conducted to evaluate the resistance of seed- and vegetatively propagated bermudagrass selections to spring dead spot. Field trials in Oklahoma indicated that several bermudagrass entries including Guymon, Sundevil, Midlawn, Midfield, Ft. Reno, and Mirage were more resistant to spring dead spot. We are currently developing greenhouse and laboratory methods to more rapidly screen bermudagrass selections for disease resistance. Furthermore, we are determining whether there are differences in pathogenicity to bermudagrass selections among the three SDS pathogens.

Various cultural and chemical control strategies have been proposed to control spring dead spot. We established a trial 1998 to evaluate the effects of some of these control recommendations, alone and in combination, for suppression of SDS. Treatments include summer aerification and verticutting, soil acidification with ammonium sulfate, and fungicide treatments.

Results for 1998 and Plans for 1999

Distribution of SDS pathogens. Our objective is to determine the distribution of the three pathogens associated with SDS. Because these fungi are extremely difficult to identify by traditional diagnostic techniques, we initially had to develop techniques for rapidly identifying these pathogenic fungi. We previously had developed species-specific DNA oligonucleotide primers to identify *O. herpotricha* and *O. korrae* but more recently Wetzel et. al. (in press) developed primers for identification of *O. narmari.* These primers have been successfully tested and are capable of differentiating the three SDS pathogens relatively quickly.

Surveys in Kansas and Oklahoma in 1996 indicated that *O. herpotricha* was the only pathogen associated with spring dead spots on two intensively sampled golf courses (>200 samples/course). On a third course, *O. herpotricha* was the most abundant pathogen, but *O. korrae* and *L. narmari* were also present, sometimes within a few meters of one another. This was the first time *O. korrae* was isolated from bermudagrass in the southern Great Plains and it was also the first report of *O. narmari* in North America (see manuscript by Wetzel et al., in press).

In 1998, approximately 20 SDS samples were collected from bermudagrass fairways on each of four golf courses in Kentucky. *O. herpotricha* was the only fungus recovered from diseased bermudagrass on two of the courses whereas *O. korrae* was the only fungus recovered from a third course. Isolates collected from the fourth course were *O. korrae* except for one *O. herpotricha* isolate. No *O. narmari* isolates were recovered from any of the Kentucky samples. These results are similar to surveys conducted in Kansas and Oklahoma. They suggest that SDS pathogens may not be uniformly distributed within a region or even on a single fairway. The reason for this uneven distribution isn't clear, although pathogen frequency may be associated with its original colonization of and movement on vegetatively propagated bermudagrass selections. If only a single pathogen was introduced on bermudagrass stolons or roots, then it would tend to be dominant in a given location. The presence of multiple SDS pathogens may indicate repeated introductions of SDS fungi on infected sod or stolons.

In 1999 we plan to continue sampling for spring dead spot in other states. Because of the time involved in culturing from samples, the number of courses sampled in each state will be reduced from four to three. We plan to sample from at least one state (New Mexico or California) from the western region of the United States because the composition of SDS pathogens in this area is not presently known.

We are also interested in understanding the amount of genotypic diversity among and within the SDS pathogens. Analysis of the DNA sequence of ribosomal introns and the internal transcribed spacer regions (ITS) indicated that the three SDS pathogens are closely related (see manuscript by Wetzel et al. for details). As a result we have proposed placing all three pathogens in the genus *Ophiosphaerella*. This reflects the common morphological and pathogenic characteristics of these fungi.

We are currently testing the genotypic diversity within a fungal species on on a local and regional scale. SDS isolates were collected on golf courses in Kansas and Oklahoma and from locations throughout North America and Australia. Genotypic similarity among these isolates is being analyzed by amplified fragment length polymorphism (AFLP) analysis. Preliminary analysis suggests that on individual fairways, there is a mosaic of clones of *O. herpotricha*. This indicates that vegetative propagation and movement of this fungus is occurring on the golf course. However, there does not appear to be clonality among isolates from different regions. Studies on the diversity among *O. korrae* and *O. narmari* are also underway.

Aggressiveness of SDS pathogens. We plan to test the aggressiveness of each of the three SDS pathogens in field studies at Manhattan and Wichita KS, and Stillwater OK. The bermudagrass cultivars 'Jackpot' and 'Midlawn" were inoculated in Manhattan and Wichita respectively, in September 1997 with oats infested with one of the three SDS pathogens. Bermudagrass cultivar trials at Oklahoma State University were also inoculated in September 1997 (in cooperation with D. Martin, OSU) with the three SDS pathogens. None of the field plots exhibited symptoms of SDS in the spring of 1998. This was not unexpected, since field symptoms take two years to develop. We will continue to monitor the development of SDS in these plots in the spring of 1999.

Screening bermudagrass selections for resistance to SDS. Field and greenhouse studies were conducted to evaluate the resistance of seed and vegetatively propagated bermudagrass entries to spring dead spot disease caused by O. herpotricha. In Kansas greenhouse studies, O. herpotricha caused root discoloration and root weight reductions in all entries tested. However, In Kansas field plots, root weight reductions were not different among entries and were not correlated with disease severity ratings. In Oklahoma trials that were inoculated with O. herpotricha, Guymon, Sundevil, Midlawn, Midfield, Ft. Reno, Mirage, and several experimental seed-propagated entries were most resistant to spring dead spot. Severity of spring dead spot among bermudagrass entries was correlated with freeze injury that occurred during the first winter after planting.

A more rapid, uniform inoculation technique must be developed to screen large numbers of bermudagrass genotypes in the greenhouse. Past attempts to correlate greenhouse and field inoculation studies have been unsuccessful (Baird et al., 1998). A new graduate student, Fanny Iriarte will start in January 1999 to work on the development of a rapid screening technique for SDS.

Integrated turfgrass management for control of SDS. A field plot for studying the effects of various cultural and chemical practices was established in the summer of 1998. The experimental design was a split plot with intensive soil aerifications (aerifications in June and July) as main plots and fertilizer, fungicide and growth regulator combinations as subplots. Plots will be rated in the spring for severity of spring dead spot.

Graduate Students Associated with USGA Project

Henry Wetzel III : Ph.D. candidate, expected completion date December 1998
Fanny Iriarte: Ph.D. candidate, expected starting date January 1999

Molecular Evidence for the Presence of *Ophiosphaerella narmari* n. comb., a Cause of Spring Dead Spot of Bermudagrass, in North America

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The phylogenetic relationships among fungi that cause spring dead spot disease of bermudagrass in Australia and the United States were studied from nucleotide sequence data of the internal transcribed spacer (ITS) region of the rDNA. High levels of sequence similarity were observed among *Ophiosphaerella herpotricha*, *O. korrae* and *Leptosphaeria narmari*. These species clustered into a distinct clade that was distant from other *Leptosphaeria* and *Phaeosphaeria* species. Based on sequence data and previous descriptions of the ascoma and ascospore characteristics of these species, we propose a taxonomic transfer of *L. narmari* to *O. narmari*. Oligonucleotide primers specific for *O. narmari* were developed from ITS1 and ITS2 regions to help identify non-sporulating isolates. These primers amplified all isolates of *O. narmari*, including those from the United States, but not those of *O. herpotricha*, *O. korrae* or other ectotrophic root-inhabiting fungi. This is the first report of *O. narmari* in North America. Two Group I introns were found in the small subunit rDNA of some isolates of *O. korrae* and *O. narmari*, but not *O. herpotricha*. A 425-nucleotide intron in *O. narmari* and *O. korrae* was inserted two nucleotides downstream from the ITS5 conserved primer sequence. These introns exhibited an 80% sequence identity. In addition, a second 431-nucleotide intron in *O. narmari* was similar in size and location to one in *O. korrae*.

Key Words: Internal Transcribed Spacers (ITS) 1 & 2, Group I Intron, *Ophiosphaerella herpotricha*, *Ophiosphaerella korrae*, and ectotrophic root-infecting fungi.

Spring dead spot (SDS) is regarded as the most destructive disease of bermudagrass (*Cynodon dactylon* (L.) Pers.), also known as couch grass, and of bermudagrass hybrids (*C. dactylon* X *C. transvaalensis* Burtt-Davy) in Australia, New Zealand and throughout the transition climatic zone of the United States (Jackson, 1993). The disease results in the formation of circular or arc-shaped patches of dead turf as bermudagrass breaks winter dormancy in early spring. Dead patches are slowly recolonized by bermudagrass during the summer, but symptoms may reappear in the same location in successive years (Jackson, 1993).

SDS is caused by three ectotrophic, root-rotting fungal species. Walker and Smith (1972) reported *Leptosphaeria narmari* J.C. Walker & A.M. Sm. as the primary cause of SDS in Australia. Jackson (1987) also later identified *L. narmari* to be the cause of SDS in New Zealand. Walker and Smith (1972) also found a less common species, *L. korrae* J. C. Walker & A. M. Sm., that was associated with SDS in Australia. *L. korrae* was subsequently found to be the cause of patch symptoms of bermudagrass in California (Endo, Orh & Krausman, 1985) and Maryland (Crahay, Dernoeden & O'Neill, 1988). Tisserat, Pair & Nus (1989) later demonstrated that a third fungus, *Ophiosphaerella herpotricha* (Fr.:Fr.) J. C. Walker, was a cause of SDS in Kansas and Oklahoma.

The taxonomic status of the fungi associated with SDS is in a state of flux. Smith (1965) first described *Ophiobolus herpotrichus* (Fr.) Sacc. as a cause of SDS in Australia. However, this isolate was later redescribed as *L. korrae* by Walker & Smith (1972). More recently, Shoemaker & Babcock (1989) reclassified *L. korrae* as *Ophiosphaerella korrae* (J.C. Walker & A.M. Sm.) Shoem. & Babc. They concluded that ascoma cell walls of this species lacked the scleroplectenchyma characteristic to many *Leptosphaeria* species, and that the presence of fusiform ascospores without enlarged cells, appendages, or a sheath more closely matched the description of *Ophiosphaerella* Speg. (Walker, 1980). Shoemaker and Babcock (1989) also reclassified *L. narmari* as *Phaeosphaeria narmari* (J.C. Walker & A.M. Sm.) Shoem. & Babc.. Although they noted that ascocarps and ascospores of *P. narmari* are morphologically similar to *O. korrae*, they argued that the ascospore shape and differences in the ascoma wall layers more closely aligned this species with *Phaeosphaeria* Miyake.

Much like their taxonomy, the geographic distribution of SDS fungi is still unclear. *O. korrae* is widespread on bermudagrass in North America (Jackson, 1993) and also has been found in the Sydney metropolitan area of Australia (Walker & Smith, 1972). *O. korrae* also causes a patch disease called necrotic ring spot of several cool-season turfgrass species, including Kentucky bluegrass (*Poa pratensis* L.), annual bluegrass (*P. annua* L.), and creeping red fescue (*Festuca rubra* L. *rubra* Smith) in North America (Worf & Stewart, 1986; Landschoot, 1996; Dernoeden, Zhang & Wetzel, 1995). *L. narmari* has been reported on bermudagrass in New Zealand (Jackson, 1987) and Australia (Smith, 1971; Walker & Smith, 1972; Hawkes, 1987), but has not been reported from the United States. *O. herpotricha* is a common cause of SDS in the central United States (Tisserat *et al.*, 1989), but has not been reported on bermudagrass in Australia.

Determining the distribution of SDS pathogens has been hampered by the inability to rapidly identify isolates recovered from bermudagrass. Although these fungi can be differentiated on the basis of ascospore morphology, ascocarps rarely occur in nature (Jackson, 1993) and cannot easily be induced from certain isolates (Chastagner & Hammer, 1997). Mycelial characteristics and growth rates of these fungi in culture overlap and are not diagnostic (Walker & Smith, 1972). Species-specific DNA probes and PCR primers for *O. korrae* and *O. herpotricha* have been developed (Tisserat, Hulbert & Nus, 1991; Sauer, Hulbert & Tisserat, 1993; Tisserat, Hulbert & Sauer, 1994; O'Gorman *et al.*, 1994) but have not been developed for *L. narmari*.

DNA sequence comparisons of the conserved and of the internal transcribed spacer (ITS) regions of ribosomal genes have been used to infer phylogenetic and taxonomic relationships among a variety of fungi (Saenz, Taylor & Gargas, 1994; Lee & Taylor, 1992; Bunting *et al.*, 1996) and these sequences have been used to study relationships among SDS pathogens. Tisserat *et al.* (1994) noted a high degree of similarity in the ITS1 and ITS2 DNA sequences between *O. herpotricha* and *O. korrae*. This molecular evidence supports the morphologically based taxonomic revisions forwarded by Shoemaker and Babcock (1989). Wetzel, Hulbert & Tisserat (1996) have also described preliminary DNA sequence similarity between *L. narmari*, *O. herpotricha* and *O. korrae*.

The objective of our study was to examine the phylogenetic relationships among the three fungi that cause SDS by comparing ribosomal ITS and intron DNA sequences. Based on the ITS sequence similarity among the three species, we propose a nomenclature change from *L. narmari* to *Ophiosphaerella narmari* (J.C. Walker & A.M. Sm.) n. comb. [= *Leptosphaeria narmari* (J.C. Walker & A.M. Sm.) (Walker & Smith, 1972) = *Phaeosphaeria narmari* (J.C. Walker & A.M. Sm.) Shoem. & Babc. (Shoemaker and Babcock, 1989)]. We also developed species-specific oligonucleotide primers to detect *O. narmari*, and have demonstrated the presence of *O. narmari* for the first time in North America.

MATERIALS AND METHODS

Fungal isolates

Two-hundred and twenty-five *O. herpotricha* isolates from the United States, 159 *O. korrae* isolates from Australia, Canada, and the United States, and 49 *O. narmari* isolates from Australia and the United States were evaluated for the presence or absence of introns in the nuclear small (NS) ribosomal DNA genes. This was accomplished with two sets of polymerase chain reaction (PCR) primers, ITS4 and ITS5 or NS5 and NS6, which amplify the entire ITS1-5.8S-ITS2 rDNA array or a portion of the conserved NS rDNA regions, respectively. Fungal isolates that were used in rDNA sequencing are listed in Table 1.

DNA Extractions and Isolate Identification

Fungal isolates were grown in 100 ml of modified yeast extract broth (5 g l^{-1} sucrose, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$-$7H_2O$, 0.5 g l^{-1} KCl, 10 mg l^{-1} $FeSO_4$-$7H_2O$, 1 g l^{-1} yeast extract, and 0.2 g l^{-1} asparagine) at room temperature (22 to 26 °C) on an orbital shaker for six days. The fungal mycelium was lyophilized prior to DNA extraction. DNA was extracted and resuspended for amplification in polymerase chain reaction (PCR) assays as described by Tisserat *et al.* (1991), except that DNA was precipitated in 2 vol. of 95% ethanol instead of 0.8 vol. isopropanol.

O. herpotricha and *O. korrae* isolates were identified by with species-specific PCR primers as described by Tisserat *et al.* (1994). U.S.A. isolates thought to be *O. narmari* were initially identified by comparing the ITS1 and ITS2 regions with those of known isolates from Australia (Table 1).

Oligonucleotide primers specific to *O. narmari* were then designed from ITS1 and ITS2 regions to discriminate against *O. herpotricha* and *O. korrae* DNA's. The DNA sequences of the *O. narmari*-specific primers in the 5' to 3' direction are as follows: OnITS1, CCAAGYGTAGAACAAACTAT and OnITS2, GGTCAAACGTGATAAAAGGG. The 'Y' designates a degenerate 50% C and 50% T base composition at that position in the OnITS1 primer sequence due to nucleotide variability among *O. narmari* isolates at that position.

PCR Conditions and DNA Cloning and Sequencing

We used 10 µl PCR reactions, except in DNA cloning procedures where we used 50 or 100 µl volumes. The PCR reactions contained 10 pmol of each primer; 0.8 unit *Taq* DNA polymerase; 200 µ M each of dATP, dCTP, dGTP and dTTP (Amersham Life Science Inc., Arlington Heights, IL) in 10X PCR buffer (4.0 M KCl, 0.5 M Tris-HCl pH 9.0, and 0.01% vol. Triton X-100); 2.0 mM $MgCl_2$; and 10 to 100 ng of fungal DNA.

We conducted PCR amplifications of *O. herpotricha* and *O. korrae* DNA in a programmable thermal cycler (MJ Research, PTC-100, Watertown, MA). Tubes were placed directly onto a preheated 94°C block and incubated for 5 min, then amplified for 26 cycles (94 °C for 30 sec, 65 °C for 45 sec, and 76 °C for 1.5 min). Specific *O. narmari* amplifications with primers OnITS1 and OnITS2 were performed by incubating tubes at 94 °C for 2.0 min, then amplifying for 16 cycles at 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 30 sec; followed by 15 cycles at 94 °C for 30 sec, 60 °C for 25 sec, and 72 ° C for 1.0 min; and a final extension at 72 °C for 7 min. DNA amplification with ITS4 and ITS5 or NS5 and NS6 primer pairs were similar to the *O. narmari* primers except that tubes were placed directly onto a preheated 94 °C block.

Following amplification, we confirmed amplification by electrophoresis at 100 V for 30 min in a 1% agarose gel in 0.5X TBE buffer with ethidium bromide (EtBr) at 0.08 µg ml^{-1} of agarose solution. The DNA products were viewed with transmitted ultraviolet light.

The ITS regions and part of the nuclear small subunit (SSU) rDNA were amplified using the conserved primers ITS4/ITS5 and NS5/NS6, respectively (White *et al.*, 1990). The primers were modified to include *Eco*R1 sites on the 5' ends. Amplified products were purified using the Wizard™

PCR Preps (Promega, Madison, WI), and then digested with the *Eco*R1 enzyme (10 unit/μl) at 37 °C for 2.5 hr. The products were ligated overnight at 15 °C into *Eco*R1-cut pUC19 plasmid in the presence of T4 DNA ligase (Life Technologies™, Gaithersburg, MD). The ligated plasmid was then mixed with 50 μl of electrocompetent *Escherichia coli* DH5α MCR cells and electroporated at 550 volts for 5 milliseconds using a Electronic Genetics® T-100™ electroporation system (Biotechnologies and Experimental Research Inc., San Diego, CA).

All clones were sequenced at the Nucleic Acid Research Facility (Iowa State University, Ames, IA). SeqPup (v 0.6f, Don Gilbert, 1996), a biosequence editor and analysis application for the Macintosh™, was used for DNA sequence alignment and comparisons. CLUSTAL W (Thompson, Higgins & Gibson, 1994) was used through the Baylor College of Medicine Search Launcher, http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html, (Smith *et al.*, 1996) for multiple sequence alignments. Nucleotide distances were estimated by the Jukes-Cantor model (Jukes & Cantor, 1969) in MEGA (Kumar, Tamura, & Nei, 1993). We used the complete deletion of sequence gaps for comparisons among sequences of other *Leptosphaeria* and *Phaeosphaeria* species to the fungal species that cause SDS but coded the gaps within the data set as a single insertional event regardless of the number of nucleotides per event. Pairwise distances between DNA sequences compared were constructed with PAUP version 3.1 (Swofford, 1993) and a branch and bound search was used to find the most parsimonious tree. Each analysis was subjected to a bootstrap test with 1000 replications (Felenstein, 1985) to determine the reproducibility of the relationships described within the respective tree.

RESULTS

Fungal isolates collected from SDS-affected bermudagrass across the United States have, for the most part, been identified by us as either *O. herpotricha* or *O. korrae* (Tisserat *et al.*, 1991; Sauer *et al.*, 1993). However, 37 non-sporulating isolates were collected from the United States (Oklahoma, Kansas and California) that exhibited cultural characteristics similar to Australian isolates of *O. narmari*. We also suspected that these isolates were *O. narmari* because they failed to amplify with species-specific primers developed to detect *O. herpotricha* and *O. korrae* and failed to hybridize to specific repetitive

DNA sequence probes pOH29 or pLK88 from *O. herpotricha* and *O. korrae*, respectively (data not shown).

The ITS1 and ITS2 regions of seven North American isolates thought to be *O. narmari* were sequenced and compared to those of *O. herpotricha*, *O. korrae*, and Australian isolates of *O. narmari* (Figs 1-2). High levels of sequence similarity were observed among all *Ophiosphaerella* species and they were tightly grouped in a clade distinct from *Leptosphaeria* and *Phaeosphaeria* species (Figs 3-4). The ITS sequences of the seven North American isolates thought to be *O. narmari* were nearly identical to Australian isolates of *O. narmari*. Sequence variation among the North American and Australian *O. narmari* isolates was ≤ 2.2% in the ITS1 and ITS2 regions. Two Californian *O. namari* isolates were identical to two of the Australian isolates in the ITS2 and different at only three nucleotides (1.8%) in the ITS1 sequence (Fig. 1-2). The *O. namari* isolates differed from the *O. herpotricha* isolate at 3.0-3.8 % of the ITS1 and ITS2 nucleotides and they differed from the *O. korrae* isolates at 4.9-7.3 % of the nucleotides.

The UPGMA and cladistic analyses consistently separated the ITS sequences of the fungal isolates into three distinct groups (Figs 3-4). These groups correspond to the three species groupings that have been designated based on morphological characters. They also correspond to the presence or absence of repetitive DNA sequences detected by the *O. herpotricha* and *O. korrae* specific probes. Taken together, these results clearly demonstrate that the three SDS pathogens are distinct species.

We developed species-specific oligonucleotide primers to more rapidly identify isolates of *O. narmari*. We designed PCR primers OnITS1 and OnITS2 to match regions in the ITS1 and ITS2 of *O. narmari* that were divergent from *O. herpotricha* or *O. korrae* (Figs 1-2). The primers amplified a 468-base pair (bp) fragment from all North American and Australian isolates of *O. narmari*. The primers did not amplify DNA from *O. herpotricha*, *O. korrae*, or other ectotrophic, root-inhabiting fungal species (Fig. 5).

We detected a 425-nucleotide insertion two bp downstream of the ITS5 conserved primer in the small subunit (SSU) rDNA of nine *O. namari* isolates and three *O. korrae* isolates (Fig. 6). Analysis of PCR products amplified with the ITS4 and ITS5 primers indicated that the insertion occurred in 43 of 49

O. namari isolates and 51 of 159 *O. korrae* isolates. The insertions in both species had regions that were homologous to the P, Q, R, and S consensus sequences, respectively, of a group 1 intron summarized by Cech (1988) (Fig. 6). There was ≤ 1.4% variation in the intron nucleotide sequences among the two Australian and seven North American *O. narmari* isolates. Five polymorphisms resulted from nucleotide transitions and were distributed throughout the sequence. Intron sequences among the three *O. korrae* isolates were identical. The introns in the two species were not only positioned at identical sites in the SSU rDNA, but also exhibited some sequence homology. The *O. narmari* DAR 20806 and *O. korrae* 2BB-85 sequences differed from each other by 20%. We did not detect this intron in any of the 225 *O. herpotricha* isolates.

The majority of the PCR amplifications employing the ITS4/ITS5 primers resulted in either the expected 590-bp or 1019-bp amplicons, corresponding to the fragments with or without the intron, respectively. However, two *O. korrae* isolates from the U.S.A. simultaneously produced both fragment sizes with the ITS4/ITS5 primers. This may indicate that the intron was not fixed within the tandem rDNA array.

We found a second 431-bp insertion in the SSU rDNA of 33/49 *O. narmari* isolates following amplification with NS5/NS6 primers. Analysis of the insertion in eight different *O. narmari* isolates showed that these insertions also had regions corresponding to the P, Q, R, and S conserved sequences of group I introns (Fig. 7). There was ≤1.2% variation in intron nucleotide sequence among the one Australian and seven North American *O. narmari* isolates (Fig. 7). Three of the nucleotide changes were transitions and two were transversions. These were distributed throughout the sequence. We did not detect this intron in any of 199 *O. herpotricha* or 120 *O. korrae* isolates. There were also 26 *O. herpotricha* and 39 *O. korrae* isolates that failed to produce any PCR product(s), indicating that the NS5 or NS6 primer sites in these isolates may have deletions or insertions that did not allow for proper annealing and amplification as discussed by Gargas, DePriest & Taylor (1995). In addition, we detected a unique insertion within the NS5/NS6 region in 3 of 119 North American *O. korrae* isolates. The insertion appears to be approximately 110 bp larger than the 431-bp intron (data not shown). We did not sequence this insertion, but did characterize *Rsa*I and *Hinc*II restriction site polymorphisms. The

insertion shared the *Rsa*I site spanning nucleotides 49-52 and a *Hinc*II site spanning nucleotides 282-287 (Fig. 7) with the unique *O. korrae* and *O. narmari* PCR amplicons from the NS5/NS6 primer pair (data not shown). The insertion did differ from the 431-bp *O. narmari* intron by lacking two additional *Rsa*I sites at nucleotides 102-105 and 109-112 (Fig. 7).

The majority of the PCR amplifications of *O. narmari* DNAs with the NS5/NS6 primers resulted in either the expected 307-bp or 738-bp amplicons, corresponding to the fragments with or without the intron, respectively. However, one *O. narmari* isolate from Australia and one of the three *O. korrae* strains with this insertion simultaneously produced both fragment sizes following amplification with the NS5/NS6 primers indicating that the intron was not fixed within the tandem rDNA array.

DISCUSSION

We report, for the first time, the presence of *O. narmari* in North America. Although we were unable to produce fertile ascocarps in any of our isolates, the ITS sequences of seven North American isolates examined were nearly identical to known isolates of *O. narmari* from Australia. Furthermore, *O. narmari*-specific primers amplified all North American isolates thought to be *O. narmari* and these isolates failed to hybridize to *O. herpotricha* or *O. korrae* specific repetitive DNA probes.

O. narmari was recovered from bermudagrass samples collected in California, Oklahoma, and Kansas, but it is likely the fungus has a much wider distribution. Many bermudagrass hybrids are vegetatively propagated, and it would be relatively easy for an ectotrophic root pathogen such as *O. narmari* to be moved from one location to another on infected stolons (Pair, Crowe & Willis, 1986). In a preliminary study, we recovered *O. narmari* from only one of three three golf courses in Kansas and Oklahoma (approximately 200 SDS patches sampled per course) and at relatively low frequencies (<5%) compared to *O. herpotricha*. This suggests that *O. narmari* may not be as widespread or common as *O. herpotricha* in the central Great Plains region. In contrast, *O. narmari* is the most common cause of SDS in Australia (Jackson, 1993) while *O. herpotricha* has not been reported. Nevertheless, previous information on the distribution and frequency of all three SDS pathogens is suspect because of the difficulty in identifying non-sporulating cultures. The development of species-specific primers to *O. narmari*, coupled with those already available for *O. herpotricha* and *O. korrae* (Tisserat *et al.*, 1994;

O'Gorman *et al.*, 1994) should help in determining the abundance and geographical distribution of the three fungal species.

Sequence analysis of ITS regions has been used to estimate phylogenetic relationships among many fungi (Lee & Taylor, 1992; Carbone & Kohn, 1993; Bunting *et al.*, 1996). Our sequence data clearly demonstrate the close relationship among the three SDS pathogens. *O. narmari*, *O. korrae*, and *O. herpotricha* clustered into a monophyletic group distinct from other tested *Leptosphaeria* and *Phaeosphaeria* species. This grouping supports the transfer of *L. korrae* to *O. korrae* proposed by Shoemaker and Babcock (1989) but does not support their placement of *L. narmari* in *Phaeosphaeria*. They concluded that the texture of the ascocarp walls, the length of the ascospores, and a slight constriction of the first septum of the ascospore in *L. narmari* more closely matched the description of *Phaeosphaeria*. However, the ascospores of this species, while much shorter than either *O. korrae* or *O. herpotricha*, have the same general shape and color as other *Ophiosphaerella* species and lack the appendages, sheaths or swollen cells that typify *Phaeosphaeria*. Therefore, we believe that both the morphological characteristics of the ascocarp and ascospores and sequence data from the ITS regions support our transfer of *L. narmari* to *O. narmari.*

Group I introns have been detected in a number of fungi (Chen, Shearer & Crane,1996; Tan & Wong, 1996; Gargas *et al.*, 1995; Nishida, Blanz & Sugiyama, 1993; DePriest & Been, 1992). We report the presence of introns in two locations in the SSU rDNA of *O. narmari* and *O. korrae*. We initially had hoped that the presence, size and sequence variability of these introns might be useful as a diagnostic tool for identifying *O. korrae* and *O. narmari*. Tan and Wong (1996) suggested that introns present in *Gaeumannomyces graminis* (Sacc.) Arx & D. L. Olivier var. *avenae* (E. M. Turner) and *G. g.* var. *tritici* J. Walker might be used as indicators of host specificity. However, the introns in *O. korrae* and *O. narmari* were not consistently found in all isolates, and in the case of one *O. narmari* and three *O. korrae* isolates, did not appear to be fixed within the tandem rDNA array. The variable occurrence of introns in both species suggests that they are mobile, similar to the intron mobility reported in the lichen-forming ascomycete, *Cladonia chlorphaea* P. Browne (DePriest, 1993). We did not observe any association between the presence of an intron in an isolate and its geographic origin.

We found that the 425-bp intron present in some *O. narmari* isolates was inserted in the same position and had 80% sequence identity to the 425-bp intron in *O. korrae* 2BB-85. One explanation for this shared intron is that the insertion occurred before *O. korrae* and *O. narmari* diverged. If so, the intron sequences appear to be evolving faster than the ITS sequences since the introns of the two species varied at approximately 20% of the nucleotides while the ITS1 and ITS2 sequences only varied at about 6%. An alternative explanation is that the insertions in the two species occurred independently, but occurred in the same site due to an insertion site specificity of the intron. Analysis of insertion sites of group 1 introns in a variety of taxa has shown they favor specific sites in the rDNA (Gargas *et al.*, 1995). In fact, a distantly related ascomycete *Acanthostigmella brevispina* M.E. Barr & Rogerson gbL35291 (Untereiner, Straus, & Malloch, 1995) has a similar (>70% identity) 365 nucleotide intron in the exact same position as the 425-bp introns of *O. narmari* and *O. korrae* lending support to this alternative explanation.

We did not detect insertions in over 200 *O. herpotricha* isolates examined. Although we did not sequence the entire SSU rDNA, Dong, Chen & Crane (1998) did not report any insertions in an isolate of *O. herpotricha* following sequence analysis of the NS1/NS8 region. The absence of introns in *O. herpotricha* is somewhat surprising because this fungus exhibited a high ITS sequence similarity to both *O. narmari* and *O. korrae*. Whether introns similar to *O. narmari* or *O. korrae* were once present in *O. herpotricha* and subsequently deleted, or whether *O. herpotricha* was geographically isolated from the other species and never acquired the introns via horizontal transfer is not known.

We thank Percy Wong for supplying additional *O. narmari* isolates from Australia and Daniel Skinner for his advice and assistance with the phylogenetic analysis of the DNA sequence data. This research was sponsored in part by the GCSAA Foundation's Chapter Cooperative Research Program, Heart of America Golf Course Superintendents Association, Kansas Golf Course Superintendents Association, Kansas Turfgrass Foundation, and the United States Golf Association. Contribution number 98-456-J from the Kansas Agricultural Experiment Station.

Table 1. Isolate, type culture designation, host plant, location, and the collector of isolates in which internal transcribed spacer (ITS) ribosomal DNA was sequenced.

Isolate	Type culture designation	Host plant[b]	Location	Collector
Oh[a]KS27	ATCC 96411	Cd	Kansas, U.S.A.	Tisserat
OhKS66	ATCC 96412	Cd	Kansas, U.S.A.	Tisserat
OhA11-1-3	- -	Cd	Oklahoma, U.S.A.	Wetzel
Ok2BB-85	ATCC 96410	Pp	Wisconsin, U.S.A.	Worf
OkMich-5	- -	Pp	Michigan, U.S.A.	Vargas
OkKS59	ATCC 64686 or DAR 13726	Ac	N.S.W., Australia	Walker, J.C.
OkA11-84A	- -	Cd	Oklahoma, U.S.A.	Wetzel
OnDAR17497	DAR 17497a	Ss	N.S.W., Australia	Kuiper
OnDAR20806	DAR 20806	Pc	N.S.W., Australia	Campbell
OnDAR35070	DAR 35070	*Gramineae* Undetermined	Queensland, Australia	Dodman
OnCA1	- -	Cd	California, U.S.A.	Endo
OnCA3	- -	Cd	California, U.S.A.	Endo
OnA11-1-4	ATCC 201719	Cd	Oklahoma, U.S.A.	McCann
OnA11-4	- -	Cd	Oklahoma, U.S.A.	Wetzel
OnA11-96A	- -	Cd	Oklahoma, U.S.A.	Wetzel
OnA16-28A	- -	Cd	Oklahoma, U.S.A.	Wetzel
OnA16-57A	- -	Cd	Oklahoma, U.S.A.	Wetzel

[a] Isolate genus and species abbreviations are as follows: *Ophiosphaerella herpotricha* [Oh], *Ophiosphaerella korrae* [Ok], and *Ophiosphaerella narmari* [On].

[b] Host abbreviations are as follows: *Axonopus compressus* [Ac], *Cynodon dactylon* (L.) Pers. [Cd], *Pennisetum clandestinum* Hochst. Ex. Chinov. [Pc], *Poa pratensis* L. [Pp], and *Stenotaphrum secundatum* (Walt.) O. Kuntz [Ss].

```
              1                                                                    70
Ok2BB-85      CACGATAGTACAGGCCCCAAGTGCAGCACAAACTGTATGGGCGGGTTATGTCTATTACCCTTGTTTATTG
OkgbL18905    ..GA.............A.................C..................................
OkKS59        ...................................C..................................
OhKS27        .......................T..A.......ACGCA.A.............................
OnDAR17497    .......................T..A.......A.GC..A.............................
OnCA1&CA3     .......................T..A.......A.GC..A.............................
OnA11-1-4     .....................C.T..A.......A.GC..A....C........................

              71                                                                  140
Ok2BB-85      AGTACCTATGTTTCCTTGGTGGGCTTGCCTGCCAAAAGGACACCCCATTGAACCTATTTATTTTCAATCA
OkgbL18905    ................................................................T.....
OkKS59        ...............G................................................T.....
OhKS27        .............................C...............T...A.....T..............
OnDAR17497    .............................C...............T...A.....T..............
OnCA1&CA3     ....................A........................T...A.....T..............
OnA11-1-4     .............................C...............T...A.....T..............

              141                    164
Ok2BB-85      GCGTCTGAATAACAATAATAATTA
OkgbL18905    ........................
OkKS59        ........................
OhKS27        ............A...........
OnDAR17497    ............A..C........
OnCA1&CA3     ..A.........A..C........
OnA11-1-4     ............A..C........
```

Fig. 1. Comparisons of the ITS1 sequences (5' to 3') among *Ophiosphaerella korrae*, *O. herpotricha*, and *O. narmari* isolates. The *O. korrae* 2BB-85 sequence also represents *O. korrae* A11-84A; *O. herpotricha* KS27 also represents *O. herpotricha* KS66 and A11-1-3; *O. narmari* DAR 17497 also represents *O. narmari* DAR 20806 and DAR 35070; and *O. narmari* A11-1-4 represents *O. narmari* A11-4, A11-96A, A16-28A, and A16-57A sequences. The *O. korrae* gbL18905 sequence represents sequence from *O. korrae* isolate LK1 (O'Gorman *et al.*, 1994). In the alignment, a dot (.) indicates the base matches the Ok2BB-85 sequence. Underlined sequence represents the regions from which species-specific primers were designed. The species-specific ITS1 primer sequences for *O. korrae* and *O. herpotricha* were previously published by Tisserat *et al.* (1994). The underlined region in the OkgbL18905 sequence represents the Lk17s primer designed by O'Gorman *et al.* (1994). The species-specific ITS1 primer sequence for *O. narmari* is 5'CCAAGYGTAGAACAAACTAT3' where'Y' designates 50% 'T' and 50% 'C' base composition in the primer.

```
           1                                                                   72
Ok2BB-85   GTACCTTCAAGCTATGCTTGGTGTTGGGTGTTTGTCCACTCCACTGCGTCTGGACTCGCCTTAAAGCAATTG
OkKS59     ........................................................................
OhKS27     ............C.....................T...........T...............T.....
OnDAR17497 ..................................T.......C....T.A...................
OnDAR20806 ..................................T...........T.A...................
OnA11-1-4  ..................................T.......C....T.....................
OnA11-96A  ..................................T.......C....T.....................

           73                                                                 144
Ok2BB-85   GCAGCCTATATCTGGTTTTGAGCGCAGCACATTTTGCGTCTTACTGCTAGTTATGTGGGCACCCATTAAGCC
OkKS59     ................A.......................................................
OhKS27     ..........T.................................C......A.A.......A......
OnDAR17497 ..........T.........................................................
OnDAR20806 ..........T.....................................................
OnA11-1-4  ..........T.................................C.....................A..
OnA11-96A  ..........T.................................C.....................

           145          162
Ok2BB-85   TCTTTATCACGTTTGACC
OkKS59     ..................
OhKS27     .T................
OnDAR17497 CT................
OnDAR20806 CT................
OnA11-1-4  CT................
OnA11-96A  CT................
```

Fig. 2. Comparisons of the ITS2 sequences (5' to 3') among *Ophiosphaerella korrae*, *O. herpotricha*, and *O. narmari* isolates. Nucleotides 155 to 162 correspond to the conserved 5' end of *Saccharomyces cerevisiae* 28S rDNA sequence (gbJ01355). The *O. korrae* 2BB-85 sequence also represents *O. korrae* Mich-5 and A11-84A; *O. herpotricha* KS27 represents *O. herpotricha* KS66 and A11-1-3; *O. narmari* DAR 20806 represents *O. narmari* DAR 35070, CA1, and CA3; *O. narmari* A11-1-4 represents *O. narmari* A11-4; *O. narmari* A11-96A represents *O. narmari* A16-28A and A16-57A sequences. In the alignment, a dot (.) indicates the base matches the Ok2BB-85 sequence. Underlined sequence represents the regions from which species-specific primers were designed. The species-specific ITS2 primer sequences for *O. korrae* and *O. herpotricha* were previously published by Tisserat *et al.* (1994). The species-specific ITS2 primer sequence for *O. narmari* is 5'GGTCAAACGTGATAAAAGGG3' which represents the reverse complement sequence to the underlined portion of the *O. narmari* DAR 17497 sequence.

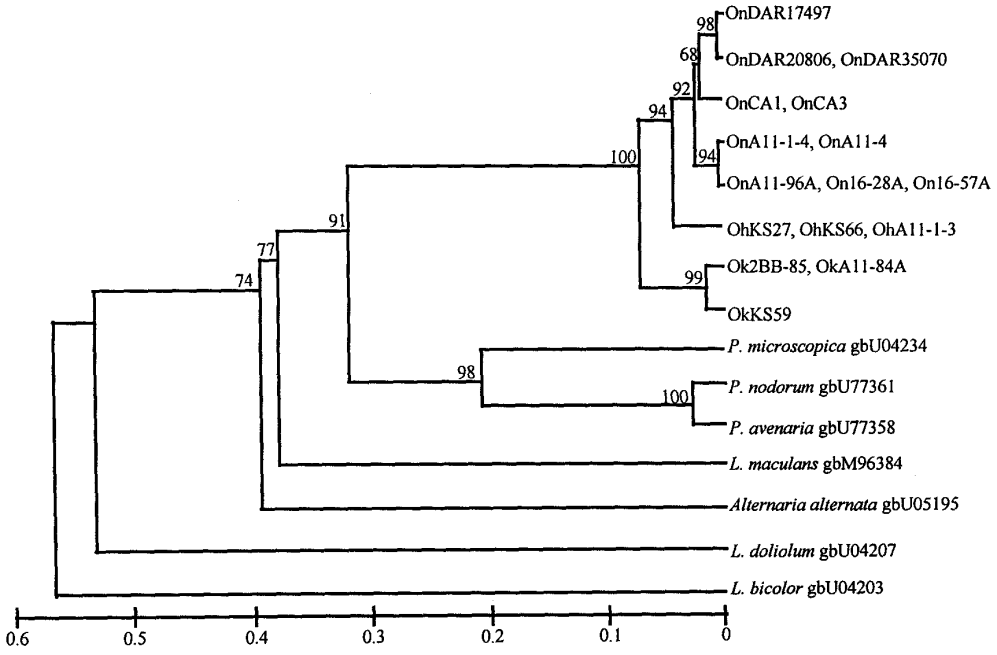

Fig. 3. Estimates of genetic distances, presented in this UPGMA tree, among *Leptosphaeria*, *Ophiosphaerella*, and *Phaeosphaeria* species based on ITS1 and ITS2 DNA sequence data. The data were subjected to 1000 bootstrap replicates, which corresponds to the values above the branches. Species followed by the designation (gb) represents the GenBank accession of the DNA sequence.

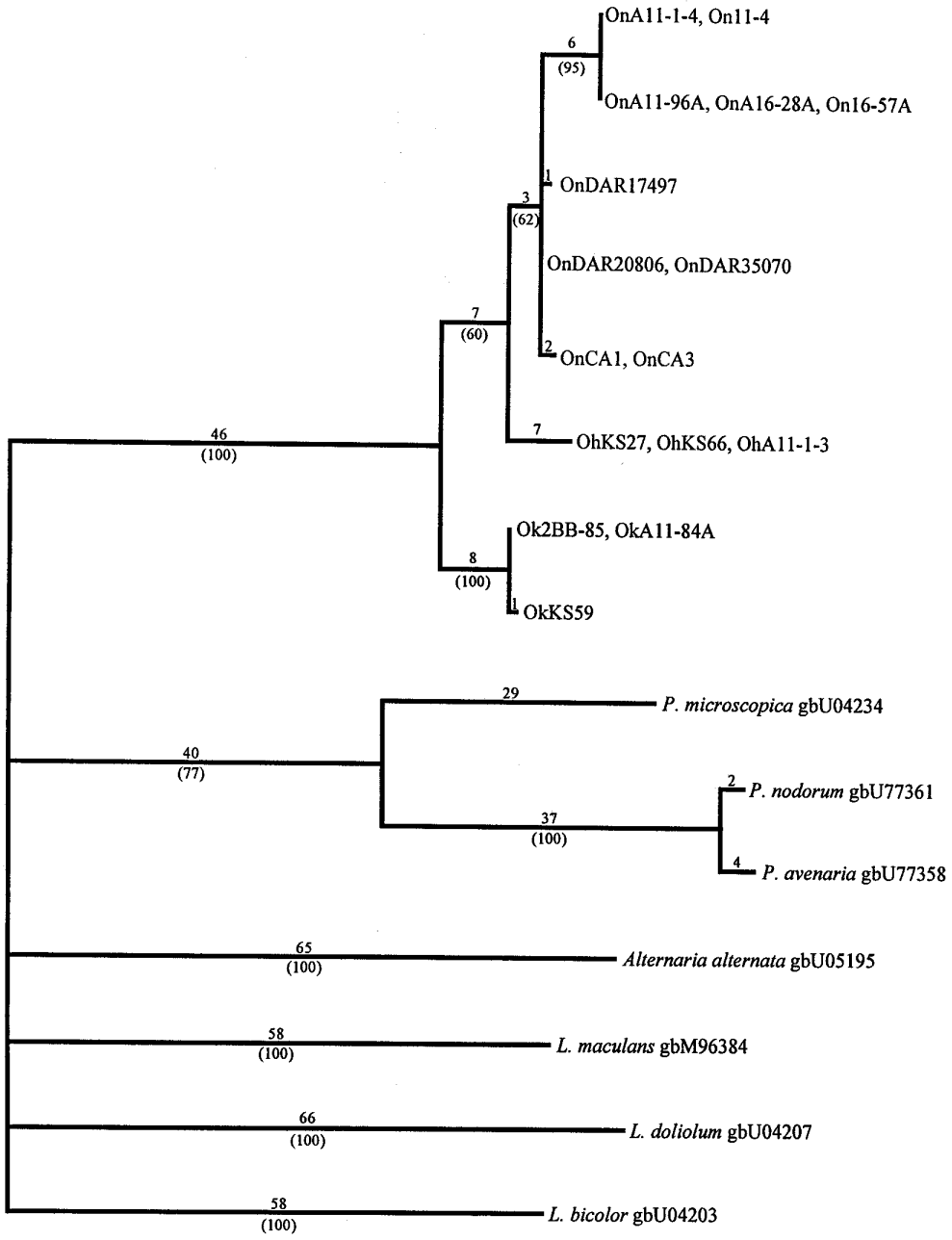

Fig. 4. Estimates of genetic relationships among *Leptosphaeria*, *Ophiosphaerella*, and *Phaeosphaeria* species based on ITS1 and ITS2 DNA sequence data. A branch and bound search found this tree the most parsimonious with 440 steps when *L. bicolor* gbU04203 was used as the outgroup. The consistency index (CI)=0.584, when uninformative characters are excluded. Branch lengths and bootstrap values from 1000 replicates are given above and below the branches, respectively. Species followed by the designation (gb) represents the GenBank accession of the DNA sequence.

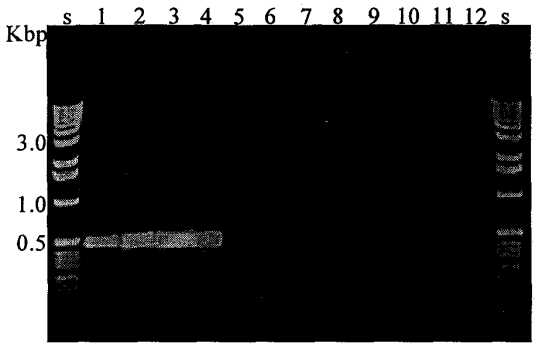

Fig. 5. Polymerase chain reaction (PCR) amplification products using primers OnITS1 and OnITS2: lanes 1 to 4 are *O. narmari* isolates DAR 35070, CA1, A11-1-4, and KS185; lanes 5 and 6 are *O. herpotricha* isolates KS66 and A11-1-3; lanes 7 and 8 are *O. korrae* isolates 2BB-85 and CA2; lane 9 is *Gaeumannomyces incrustans* isolate A#91; lane 10 is *G. graminis* var. *graminis* isolate FL-19; lane 11 is *Phaeosphaeria nodorum*; and lane 12 is primers without DNA template. PCR amplification products (10 µl of the 25µl reaction) were resolved in a 1.0% agarose gel at 100 V for 1.0 h.

```
           1                                                                                                  93
OnDAR20806 TGATTCTTCGCAATCCTTGCGGAAGCCTTTGCAGCTGGAAACAGTAGCTAGAGCGACAATAAATAATTCTGGCCTTATGCAAGTCAGCGCCTT
OnDAR35070 ..................................................................C.....................
OnCA1&CA3  ..................................................................C.....................
OnA11-1-4  ................................................................................................
Ok2BB-85  ..............................................C.........TA...C......A.................AT-..

           94                    P                                                                      186
OnDAR20806 GCTGGCAACACTTTCGAATTGCGGGAAACCCCTAAGAGCCTTTTACACCAAACCAGCTTGGGAAACCTTCTGGCGGCCCGTGGGAAACTACGG
OnDAR35070 ................................................................................................
OnCA1&CA3  ................................................................................................
OnA11-1-4  ................................................................................................
Ok2BB-85  .T....G.....................................................TC.........G.........T............A.

           187                                                                 Q                      277
OnDAR20806 GGTATGGTAACAGCTAAAAGGATGAGTTAGCGCT--TACAACGCTGATGAAATGGGCAATCCGCAGCGAAGCCCCTTTTGTTCATGGCAACTG
OnDAR35070 ...................................--.........................................................
OnCA1&CA3  ...................................--.........................................................
OnA11-1-4  ..........................G.....--C...G.....................................................
Ok2BB-85  ............T............C..A....CC.GT.GA.T.AG.............................GC....A...A..GT..

           278                                                                 R                      351
OnDAR20806 AGTAGC----------------TTGCTGCTGCAGTAACCAAACATGGGGAACGTTCACAGACTAAGTGGAAGTGGGTGGAGTTTAAG--CTC
OnDAR35070 ......----------------.....................................................................---...
OnCA1&CA3  ......----------------.....................................................................G.-...
OnA11-1-4  ......----------------.....................................................................G.-...
Ok2BB-85  G...AACAATAACGTAAAAATTA.GTT...A...AC..T.....................................G...AAA...

           352       S                                                                  425
OnDAR20806 TGCTTAAGATATAGTCGGGCCCCTTTGGAAACTCTGGGGATGTGGAATTGCCAGTAATTCTACAAATCAAGCTG
OnDAR35070 ...................................................................
OnCA1&CA3  ...................................................................
OnA11-1-4  ..............................................T....................
Ok2BB-85  ..............................A.......----------.G..T......A.
```

Fig. 6. Comparisons of the 425-nucleotide group 1 intron sequences (5' to 3') among *Ophiosphaerella narmari* and *O. korrae* isolates. These sequences were derived through the amplification of genomic DNA with the ITS4/ITS5 PCR primer pair. The *O. narmari* A11-1-4 sequence also represents *O. narmari* A11-4, A11-96A, A16-28A and A16-57A sequences; and the *O. korrae* 2BB-85 sequence also represents *O. korrae* Mich-5 and A11-84A sequences. In the alignment, a dot (.) indicates the base matches the OnDAR20806 sequence, and a dash (-) indicates a gap in reference to the OnDAR20806 sequence. This intron is positioned between bases 1767 and 1768 in reference to the 18S rDNA sequence (gbV01335) of *Saccharomyces cerevisiae*. The *O. narmari* DAR 20806 sequence is 80% identical to the *O. korrae* 2BB-85 sequence. The underlined areas of the sequence are those that have the highest identity to the P, Q, R, and S consensus sequences characteristic of a Group 1 intron.

```
                1                                                                                                  92
OnDAR35070      AAACACTAATGTTTTGCCGCAGTAGCTCTGCGCCAGGGGCCAGTAATGGTACGGTGGTGTTGTCGTTTAATCCCTAGTCTTTCTTTCCTATC
OnCA1&CA3       ................................................................................................
OnA11-1-4       ................................................................................................
OnA11-4         ................................................................................................
OnA11-96A       ................................................................................................

               93                                                                P                                184
OnDAR35070      TTGAAAGATGTACCTTGTACATCAGTTGTGATTTAGAAAAGGAAGGCGAGACCCTCAAATTGCGGGAAACTCGCGAAGCATTTAGCTACCAA
OnCA1&CA3       ................................................................................................
OnA11-1-4       .......G.....................................C..............................................
OnA11-4         .......G.....................................C..............................................
OnA11-96A       .......G.....................................................................................

               185                                                                                Q            276
OnDAR35070      GCTGTATTTCGAAAGATTGCAGTGGCCAGGTTAATCGCCTGGGTATGGTAACAAGGCTATCTGTATGTGACAATCCGCAGCCAAGCCCCGCT
OnCA1&CA3       ................................................................................................
OnA11-1-4       ................................................................................................
OnA11-4         ................................................................................................
OnA11-96A       ................................................................................................

               277                               R                                                          S     368
OnDAR35070      GTTTAGTTGACGTGGGGAAGGTTCAGAGACTAAATGGGGGTCGGTTTGATAATACTCAATCGCTAATATCAGGCTTAAGATATAGTCCGGGC
OnCA1&CA3       ..C.............................................................................................
OnA11-1-4       ................................................................................................
OnA11-4         ..C.............................................................................................
OnA11-96A       ..C.............................................................................................

               369                                                                                431
OnDAR35070      CCTGTCGAAAGGCAGTGGCATTTGCTAACTCGATTACCGGGTACCCAAAACTAACATTTTGCG
OnCA1&CA3       ...............................................................
OnA11-1-4       ........................C.........A.....................
OnA11-4         ........................C.........A.....................
OnA11-96A       ........................C.........A.....................
```

Fig. 7. Comparisons of the 431-nucleotide group 1 intron sequences (5' to 3'), positioned between bases 1165 and 1166, in reference to the 18S rDNA sequence (gbV01335) of *Saccharomyces cerevisiae*, among *Ophiosphaerella narmari* isolates. These sequences were derived through the amplification of genomic DNA with the NS5/NS6 PCR primer pair.The *O. narmari* A11-96A sequence also represents *O. narmari* A16-28A and A16-57A sequences. In the alignment, a dot (.) indicates the base matches the OnDAR35070 sequence. The underlined GTTGAC is the recognition sequence for the *Hinc*II restriction endonuclease. The three underlined GTAC sequences are the recognition sequence for the *Rsa*I restriction endonuclease. The additional underlined areas of the sequence are those that have the highest identity to the P, Q, R, and S consensus sequences characteristic of a Group 1 intron.