(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199926243 B2
(10) Patent No. 744803

(54) Title
Detection of wheat and barley fungal pathogens using the polymerase chain reaction

(51)[6] International Patent Classification(s)
C12Q 001/68

(21) Application No: 199926243

(22) Application Date: 1999 .02 .18

(87) WIPO No: WO99/42609

(30) Priority Data

(31) Number	(32) Date	(33) Country
09/026601	1998 .02 .20	US

(43) Publication Date : 1999 .09 .06
(43) Publication Journal Date : 1999 .11 .04
(44) Accepted Journal Date : 2002 .03 .07

(71) Applicant(s)
Syngenta Participations AG

(72) Inventor(s)
James Joseph Beck

(74) Agent/Attorney
DAVIES COLLISON CAVE,1 Little Collins Street,MELBOURNE VIC 3000

(56) Related Art
EMBL AC:Y08750
EMBL AC:Y10923
2ND INT. SEED TESTING ASSOC. (ISTA)-SIMPOSIUM AUG 96,CAB INT

L02443/99

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 — A1

(11) International Publication Number: **WO 99/42609**

(43) International Publication Date: 26 August 1999 (26.08.99)

(21) International Application Number: PCT/EP99/01058

(22) International Filing Date: 18 February 1999 (18.02.99)

(30) Priority Data:
09/026,601 20 February 1998 (20.02.98) US

~~NOVARTIS PHARMA AG~~

(71) Applicant *(for all designated States except AT US)*: ~~NOVARTIS AG [CH/CH], Schwarzwaldallee 215, CH-4058 Basel (CH).~~

(71) Applicant *(for AT only)*: NOVARTIS–ERFINDUNGEN [AT/AT]; Verwaltungsgesellschaft m.b.H., Brunner Strasse 59, A–1235 Vienna (AT).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: BECK, James, Joseph [US/US]; 114 Ripley Court, Cary, NC 27513 (US).

(74) Agent: BECKER, Konrad; Novartis AG, Corporate Intellectual Property, Patent & Trademark Dept., CH–4002 Basel (CH).

(71) Syngenta Participations AG, Schwarzwaldallee 215, CH-4058, Basel (CH).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETECTION OF WHEAT AND BARLEY FUNGAL PATHOGENS USING THE POLYMERASE CHAIN REACTION

(57) Abstract

Internal Transcribed Spacer (ITS) DNA sequences from the ribosomal RNA gene region are described for species and strains of wheat fungal pathogens, including *Pyrenophora tritici–repentis* and *Pyrenophora teres*. Specific primers from within these sequences are identified as being useful for the identification of the fungal isolates using PCR–based techniques.

DETECTION OF WHEAT AND BARLEY FUNGAL PATHOGENS USING THE POLYMERASE CHAIN REACTION

The present invention relates to the use of primers in polymerase chain reaction assays for the detection of fungal pathogens in wheat and barley. The use of these primers enables the detection of specific isolates of fungal pathogens and the monitoring of disease development in plant populations.

Diseases in plants cause considerable crop loss from year to year resulting both in economic deprivation to farmers and, in many parts of the world, to shortfalls in the nutritional provision for local populations. The widespread use of fungicides has provided considerable security against plant pathogen attack. However, despite $1 billion worth of expenditure on fungicides, worldwide crop losses amounted to approximately 10% of crop value in 1981 (James, 1981; *Seed Sci. & Technol.* 9: 679-685).

The severity of the destructive process of disease depends on the aggressiveness of the pathogen and the response of the host. One aim of most plant breeding programs is to increase the resistance of host plants to disease. Typically, different races of pathogens interact with different varieties of the same crop species differentially, and many sources of host resistance only protect against specific pathogen races. Furthermore, some pathogen races show early signs of disease symptoms, but cause little damage to the crop. Jones and Clifford (1983; Cereal Diseases, John Wiley) report that virulent forms of the pathogen are expected to emerge in the pathogen population in response to the introduction of resistance into host cultivars and that it is therefore necessary to monitor pathogen populations. In addition, there are several documented cases of the evolution of fungal strains that are resistant to particular fungicides. As early as 1981, Fletcher and Wolfe (1981; *Proc. 1981 Brit. Crop Prot. Conf.*) contended that 24% of the powdery mildew populations from spring barley and 53% from winter barley showed considerable variation in response to the fungicide triadimenol and that the distribution of these populations varied between varieties, with the most susceptible variety also giving the highest incidence of less susceptible types. Similar variation in the sensitivity of fungi to fungicides has been documented for wheat mildew (also to triadimenol), *Botrytis* (to benomyl), *Pyrenophora* (to organomercury), *Pseudocercosporella* (to MBC-type fungicides) and *Mycosphaerella fijiensis* to triazoles to mention just a few (Jones and Clifford; Cereal Diseases, John Wiley, 1983).

Wheat is currently the most important agricultural commodity in international markets and occupies about 20% of the world's farmed land (1977; Compendium of Wheat Diseases, Amer. Phytopath. Soc. page 1). Eightly percent of the world's supply of wheat is grown in North America, Europe, China, and the Soviet Union. Approximately 20% of the worldwide production of wheat is lost to disease annually.

Pyrenophora tritici-repentis (Died.) Drechs. (syn. *P. trichostoma* (Fr.) Fckl.), anamorph *Drechslera tritici-repentis* (Died.) Shoem. (syn. *Helminthosporium tritici-repentis* Died.), causes tan spot also known as yellow spot of wheat worldwide (1977; Compendium of Wheat Diseases, Amer. Phytopath. Soc. page 42). It has resulted in wheat yield losses from 3 to 50 % in Australia, South America, and North America and has been recently considered the most important foliar wheat disease in North Dakota (Zhang *et al.*, 1997; Phytopathology. Vol.87:154-160). It can also contribute to leaf-spotting complexes with other foliar pathogens. Current disease control measures include fungicide application and cultural practices including destroying wheat stubble, using pathogen-free seed and wide row plant spacing to reduce foliage density and relative humidity in the wheat canopy.

Pyrenophora teres Drechs., anamorph *Drechslera teres* (Sacc.) Shoem. (syn. *Helminthosporium teres* Sacc.) causes net blotch primarily in barley; however, sporadic infections also occur in wheat (Jones and Clifford; Cereal Diseases, John Wiley, 1983). Typical yield losses due to net blotch are between 10 to 40%. Yield losses can approach 100% in fields containing susceptible cultivars (1982; Compendium of Barley Diseases, Amer. Phytopath. Soc. page 22). In addition to affecting overall grain yield and weight, the disease also reduces the carbohydrate content. This reduces malt extract yield and therefore lowers the brewing quality of the grain.

In view of the above, there is a real need for the development of technology that will allow the identification of specific races of pathogen fungi early in the infection process. By identifying the specific race of a pathogen before disease symptoms become evident in the crop stand, the agriculturist can assess the likely effects of further development of the pathogen in the crop variety in which it has been identified and can choose an appropriate fungicide if such application is deemed necessary.

In view of the above, an advantage of at least one embodiment of the invention is that a method for the identification of specific races of pathogen fungi early in the infection process is provided. The invention therefore provides Internal Transcribed Spacer (ITS) DNA sequences that show variability between different fungal pathotypes. Such DNA sequences are useful in the

method of the invention as they can be used to derive primers for use in polymerase chain reaction (PCR)-based diagnostic assays. These primers generate unique fragments in PCR reactions in which the DNA template is provided by specific fungal pathotypes and can thus be used to identify the presence or absence of specific pathotypes in host plant material before the onset of disease symptoms.

In a preferred embodiment, the invention provides novel ITS1 and ITS2 DNA sequences for the fungal pathogen *Pyrenophora tritici-repentis*. In another preferred embodiment, the invention provides ITS-derived diagnostic primers for the detection of *Pyrenophora tritici-repentis*. In an additional preferred embodiment, the invention provides novel ITS-derived diagnostic primers that are useful for the detection of not only *Pyrenophora tritici-repentis*, but also, surprisingly, *Pyrenophora teres* and *Drechslera sorokiniana*. The present invention therefore addresses a long-felt but unfulfilled need to identify different pathotypes of plant pathogenic fungi, especially those that cause tan spot in wheat.

This invention provides the possibility of assessing potential damage in a specific crop variety-pathogen strain relationship and of utilizing judiciously the diverse armory of fungicides that is available. Furthermore, the invention can be used to provide detailed information on the development and spread of specific pathogen races over extended geographical areas. The invention provides a method of detection that is especially suitable for diseases with a long latent phase.

Kits useful in the practice of the invention are also provided. The kits find particular use in the identification of the fungal pathogen *Pyrenophora tritici-repentis*.

In particular, the present invention provides an isolated, substantially pure DNA molecule from the ribosomal RNA gene region of a fungal pathogen, wherein said DNA molecule comprises at least one of ITS1 and ITS2 of *Pyrenophora tritici-repentis*. The present invention further provides a method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) determining the nucleotide sequence of either ITS1 or ITS2 of *Pyrenophora tritici-repentis*;

(b) designing at least one PCR primer having sequence identity with at least 10 nucleotides of the nucleotide sequence determined in (a);

(c) isolating DNA from plant tissue suspected to be infected with *Pyrenophora tritici-repentis*;

(d) subjecting the DNA of (c) to polymerase chain reaction amplification using the primer or primers of (b); and

(e) detecting *Pyrenophora tritici-repentis* by visualizing the product or products of the polymerase chain reaction amplification of (d).

In a preferred embodiment, the present invention provides an Internal Transcribed Spacer sequence isolated from the ribosomal RNA gene region of *Pyrenophora tritici-repentis*, wherein said Internal Transcribed Spacer sequence is selected from the group consisting of: nucleotides 31-208 of SEQ ID NO:29; nucleotides 366-526 of SEQ ID NO:29; nucleotides 31-208 of SEQ ID NO:30; nucleotides 366-526 of SEQ ID NO:30; nucleotides 31-208 of SEQ ID NO:31; nucleotides 366-526 of SEQ ID NO:31; nucleotides 31-208 of SEQ ID NO:32; nucleotides 366-526 of SEQ ID NO:32; nucleotides 31-208 of SEQ ID NO:33; nucleotides 366-527 of SEQ ID NO:33; nucleotides 31-208 of SEQ ID NO:34; nucleotides 366-526 of SEQ ID NO:34; nucleotides 31-208 of SEQ ID NO:35; nucleotides 366-527 of SEQ ID NO:35; nucleotides 31-208 of SEQ ID NO:36; nucleotides 366-526 of SEQ ID NO:36; nucleotides 31-209 of SEQ ID NO:37; nucleotides 367-527 of SEQ ID NO:37; nucleotides 31-208 of SEQ ID NO:38; nucleotides 366-526 of SEQ ID NO:38; nucleotides 31-208 of SEQ ID NO:39; nucleotides 368-534 of SEQ ID NO:39; nucleotides 31-208 of SEQ ID NO:40; and nucleotides 366-527 of SEQ ID NO:40; and nucleotides 31-209 of SEQ ID NO:41; and nucleotides 367-536 of SEQ ID NO:41.

The present invention further provides an oligonucleotide primer for use in amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence, wherein said primer has sequence identity with at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of the invention. The present invention also provides a pair of oligonucleotide primers for use in the amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence, wherein at least one of said primers is the oligonucleotide primer having sequence identity with at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of the invention. The present invention still further provides a diagnostic kit used in detecting a fungal pathogen, comprising at least one primer having sequence identity with at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of the invention.

According to another embodiment, the present invention provides a method for the detection of a fungal pathogen, comprising:

(a) isolating DNA from a plant leaf suspected to be infected with a fungal pathogen;

(b) subjecting said DNA to polymerase chain reaction amplification using at least one primer according to the invention; and

(c) detecting said fungal pathogen by visualizing the product or products of said polymerase chain reaction amplification.

In one embodiment of this method of the invention, the fungal pathogen is *Pyrenophora tritici-repentis*. In another embodiment of this method of the invention, the fungal pathogen is *Pyrenophora teres*. In yet another embodiment of this method of the invention, the fungal pathogen is *Drechslera sorokiniana*.

The present invention also provides a method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) designing at least one PCR primer having sequence identity with at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of the invention;

(b) isolating DNA from plant tissue suspected to be infected with *Pyrenophora tritici-repentis*

(c) subjecting the DNA of (b) to polymerase chain reaction amplification using the primer or primers of (a); and

(d) detecting *Pyrenophora tritici-repentis* by visualizing the product or products of the polymerase chain reaction amplification of (c).

The present invention further provides a method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) designing at least one polynucleotide label probe comprising:

(i) a nucleic acid region having a sequence that is complementary to at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of the invention, and

(ii) a label portion comprising either a label that provides a signal when said probe hybridizes with another nucleic acid molecule, or a binding site for said label;

(b) isolating DNA from plant tissue suspected to be infected with *Pyrenophora tritici-repentis*

(c) hybridizing the DNA of (b) with the probe or probes of (a); and

(d) detecting *Pyrenophora tritici-repentis* by detecting the label, wherein detection of the label indicates that the hybridization of (c) has occurred.

In another embodiment, the present invention is directed to an oligonucleotide primer for use in the amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence, wherein said primer is selected from the group consisting of SEQ ID Nos:7-28. Preferably, the invention provides a pair of oligonucleotide primers for use in the amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence, wherein at least one of said primers is selected from the group consisting of SEQ ID Nos:7-28. In an especially preferred embodiment, the pair of primers is selected from the following primer pairs: SEQ ID NO:7 and SEQ ID NO:9; SEQ ID NO:11 and SEQ ID NO:15; SEQ ID NO:7 and SEQ ID NO:14; SEQ ID NO:1 and SEQ ID NO:9; SEQ ID NO:8 and SEQ ID

NO:4; SEQ ID NO:17 and SEQ ID NO:20; SEQ ID NO:18 and SEQ ID NO:19; SEQ ID NO:1 and SEQ ID NO:19; SEQ ID NO:7 and SEQ ID NO:19; SEQ ID NO:8 and SEQ ID NO:19; SEQ ID NO:10 and SEQ ID NO:19; SEQ ID NO:13 and SEQ ID NO:19; SEQ ID NO:23 and SEQ ID NO:26; SEQ ID NO:23 and SEQ ID NO:19; SEQ ID NO:24 and SEQ ID NO:19; and SEQ ID NO:27 and SEQ ID NO:28. In a preferred embodiment, the pair of oligonucleotide primers is either SEQ ID NO:1 and SEQ ID NO:9 or SEQ ID NO:1 and SEQ ID NO:19. In another preferred embodiment, the pair of oligonucleotide primers is SEQ ID NO:8 and SEQ ID NO:4.

The present invention also provides method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) isolating DNA from a plant leaf suspected to be infected with *Pyrenophora tritici-repentis*;

(b) amplifying a part of the Internal Transcribed Spacer sequence of *Pyrenophora tritici-repentis* using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers selected from the following primer pairs: SEQ ID NO:7 and SEQ ID NO:9; SEQ ID NO:11 and SEQ ID NO:15; SEQ ID NO:7 and SEQ ID NO:14; SEQ ID NO:1 and SEQ ID NO:9; SEQ ID NO:8 and SEQ ID NO:4; SEQ ID NO:17 and SEQ ID NO:20; SEQ ID NO:18 and SEQ ID NO:19; SEQ ID NO:1 and SEQ ID NO:19; SEQ ID NO:7 and SEQ ID NO:19; SEQ ID NO:8 and SEQ ID NO:19; SEQ ID NO:10 and SEQ ID NO:19; SEQ ID NO:13 and SEQ ID NO:19; SEQ ID NO:23 and SEQ ID NO:26; SEQ ID NO:23 and SEQ ID NO:19; SEQ ID NO:24 and SEQ ID NO:19; and SEQ ID NO:27 and SEQ ID NO:28; and

(c) detecting *Pyrenophora tritici-repentis* by visualizing the amplified part of the Internal Transcribed Spacer sequence.

In yet another embodiment, the present invention provides a method for detecting *Pyrenophora teres*, comprising:

(a) isolating DNA from a plant leaf suspected to be infected with *Pyrenophora teres*;

(b) amplifying a part of the Internal Transcribed Spacer sequence of *Pyrenophora teres* using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers that is either SEQ ID NO:1 and SEQ ID NO:9 or SEQ ID NO:1 and SEQ ID NO:19; and

(c) detecting *Pyrenophora teres* by visualizing the amplified part of the Internal Transcribed Spacer sequence.

In still another embodiment, the present invention provides a method for detecting *Drechslera sorokiniana*, comprising:

(a) isolating DNA from a plant leaf suspected to be infected with *Drechslera sorokiniana*;

(b) amplifying a part of the Internal Transcribed Spacer sequence of *Drechslera sorokiniana* using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers that is SEQ ID NO:8 and SEQ ID NO:4; and

(c) detecting *Drechslera sorokiniana* by visualizing the amplified part of the Internal Transcribed Spacer sequence.

The following is a brief description of the sequences in the sequence listing ("nt's" = nucleotide numbers in the sequence):

SEQ ID NO:1 - Oligonucleotide Primer ITS1.
SEQ ID NO:2 - Oligonucleotide Primer ITS2.
SEQ ID NO:3 - Oligonucleotide Primer ITS3.
SEQ ID NO:4 - Oligonucleotide Primer ITS4.
SEQ ID NO:5 - M13 Universal-20 Primer.
SEQ ID NO:6 - Reverse Primer used in Example 2.
SEQ ID NO:7 - Oligonucleotide Primer JB629.
SEQ ID NO:8 - Oligonucleotide Primer JB630.
SEQ ID NO:9 - Oligonucleotide Primer JB631.
SEQ ID NO:10 - Oligonucleotide Primer JB632.
SEQ ID NO:11 - Oligonucleotide Primer JB633.
SEQ ID NO:12 - Oligonucleotide Primer JB634.
SEQ ID NO:13 - Oligonucleotide Primer JB635.
SEQ ID NO:14 - Oligonucleotide Primer JB636.
SEQ ID NO:15 - Oligonucleotide Primer JB637.
SEQ ID NO:16 - Oligonucleotide Primer JB638.
SEQ ID NO:17 - Oligonucleotide Primer JB651.
SEQ ID NO:18 - Oligonucleotide Primer JB652.
SEQ ID NO:19 - Oligonucleotide Primer JB653.
SEQ ID NO:20 - Oligonucleotide Primer JB654.
SEQ ID NO:21 - Oligonucleotide Primer JB655.
SEQ ID NO:22 - Oligonucleotide Primer JB656.
SEQ ID NO:23 - Oligonucleotide Primer JB657.
SEQ ID NO:24 - Oligonucleotide Primer JB658.

SEQ ID NO:25 - Oligonucleotide Primer JB659.

SEQ ID NO:26 - Oligonucleotide Primer JB660.

SEQ ID NO:27 - Oligonucleotide Primer JB675.

SEQ ID NO:28 - Oligonucleotide Primer JB676.

SEQ ID NO:29 - Consensus DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolates 6715, 119-2-3, DL22, PTR4A, 44184, 205, 403, 109, 407, 1316, and 223, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:30 - Consensus DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 6715 clones 2 and 4, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:31 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 119-2-3 clone 2-2, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:32 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate DL22 clone 1-1, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:33 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate PTR4A clone 2-3, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-527), and 5' end of the large subunit rRNA gene (nt's 528-580).

SEQ ID NO:34 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 44184 clone 3-1, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365),

Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:35 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 205 clone 4-2, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-527), and 5' end of the large subunit rRNA gene (nt's 528-580).

SEQ ID NO:36 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 403 clone 5-2, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:37 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 109 clone 6-2, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-209), 5.8S rRNA gene (nt's 210-366), Internal Transcribed Spacer 2 (nt's 367-527), and 5' end of the large subunit rRNA gene (nt's 528-580).

SEQ ID NO:38 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 407 clone 7-3-2, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-526), and 5' end of the large subunit rRNA gene (nt's 527-579).

SEQ ID NO:39 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 1316 clone 8-1, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-367), Internal Transcribed Spacer 2 (nt's 368-534), and 5' end of the large subunit rRNA gene (nt's 535-587).

SEQ ID NO:40 - DNA sequence of the ITS region from *Pyrenophora tritici-repentis* isolate 223 clone 9-2, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's 1-30), Internal Transcribed Spacer 1 (nt's 31-208), 5.8S rRNA gene (nt's 209-365), Internal Transcribed Spacer 2 (nt's 366-527), and 5' end of the large subunit rRNA gene (nt's 528-580).

SEQ ID NO:41 - DNA sequence of the ITS region from *Pyrenophora teres* isolate 36570 clone 10-1, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nt's

1-30), Internal Transcribed Spacer 1 (nt's 31-209), 5.8S rRNA gene (nt's 210-366), Internal Transcribed Spacer 2 (nt's 367-536), and 5' end of the large subunit rRNA gene (nt's 537-590).

The present invention provides unique DNA sequences that are useful in identifying different pathotypes of plant pathogenic fungi. Particularly, the DNA sequences can be used as primers in PCR-based analysis for the identification of fungal pathotypes. The DNA sequences of the invention include the Internal Transcribed Spacer (ITS) sequences of the ribosomal RNA gene regions of particular fungal pathogens as well as primers derived from these regions that are capable of identifying the particular pathogen. These ITS DNA sequences from different pathotypes within a pathogen species or genus, which vary between the different members of the species or genus, can be used to identify those specific members.

Biomedical researchers have used PCR-based techniques for some time and with moderate success to detect pathogens in infected animal tissues. Only recently, however, has this technique been applied to detect plant pathogens. The presence of *Gaumannomyces graminis* in infected wheat has been detected using PCR of sequences specific to the pathogen mitochondrial genome (Schlesser *et al.*, 1991; *Applied and Environ. Microbiol.* 57: 553-556), and random amplified polymorphic DNA (*i.e.* RAPD) markers were able to distinguish numerous races of *Gremmeniella abietina*, the causal agent of scleroderris canker in conifers. U.S. Patent No. 5,585,238 (herein incorporated by reference in its entirety) describes primers derived from the ITS sequences of the ribosomal RNA gene region of strains of *Septoria, Pseudocercosporella,* and *Mycosphaerella* and their use in the identification of these fungal isolates using PCR-based techniques. In addition, WO 95/29260 (herein incorporated by reference in its entirety) describes primers derived from the ITS sequences of the ribosomal RNA gene region of strains of *Fusarium* and their use in the identification of these fungal isolates using PCR-based techniques. Furthermore, U.S. Patent No. 5,800,997 (herein incorporated by reference in its entirety) describes primers derived from the ITS sequences of the ribosomal RNA gene region of strains of *Cercospora, Helminthosporium, Kabatiella, and Puccinia* and their use in the identification of these fungal isolates using PCR-based techniques.

Ribosomal genes are suitable for use as molecular probe targets because of their high copy number. Despite the high conservation between mature rRNA sequences, the non-transcribed and transcribed spacer sequences are usually poorly conserved and are

thus suitable as target sequences for the detection of recent evolutionary divergence. Fungal rRNA genes are organized in units, each of which encodes three mature subunits of 18S (small subunit), 5.8S, and 28S (large subunit). These subunits are separated by two Internal Transcribed Spacers, ITS1 and ITS2, of around 300 bp (White *et al.*, 1990; In: PCR Protocols; Eds.: Innes *et al.*; pages 315-322). In addition, the transcriptional units are separated by non-transcribed spacer sequences (NTSs). The ITS and NTS sequences are particularly suitable for the detection of specific pathotypes of different fungal pathogens.

The DNA sequences of the invention are from the Internal Transcribed Spacer sequences of the ribosomal RNA gene region of different plant pathogens. The ITS DNA sequences from different pathotypes within a pathogen species or genus vary among the different members of the species or genus. After determining the ITS sequences of a pathogen, these sequences can be aligned with other ITS sequences. Primers can then be derived from the ITS sequences. That is, primers can be designed based on regions within the ITS sequences having the greatest differences in sequence among the fungal pathotypes. The sequences and primers based thereon can be used to identify specific pathogens.

Particular DNA sequences of interest include ITS DNA sequences from *Pyrenophora tritici-repentis* and *Pyrenophora teres*. Such ITS DNA sequences are disclosed in SEQ ID NOs: 29-41. Sequences of representative oligonucleotide primers derived from these ITS sequences are disclosed in SEQ ID NOs: 7-28. The sequences find use in the PCR-based identification of the pathogens of interest. Therefore, based on Applicants' disclosure of the present invention, the fungal pathogen responsible for tan spot in wheat can, for the first time, be detected by a PCR-based diagnostic assay.

Methods for the use of the primer sequences of the invention in PCR analysis are well known in the art. For example, see U.S. Patent Nos. 4,683,195 and 4,683,202, as well as Schlesser *et al.* (1991) *Applied and Environ. Microbiol. 57*:553-556. See also, Nazar *et al.* (1991; *Physiol. and Molec. Plant Pathol.* 39: 1-11), which used PCR amplification to exploit differences in the ITS regions of *Verticillium albo-atrum* and *Verticillium dahliae* and therefore distinguish between the two species; and Johanson and Jeger (1993; *Mycol. Res.* 97: 670-674), who used similar techniques to distinguish the banana pathogens *Mycosphaerella fijiensis* and *Mycospharella musicola.*

The ITS DNA sequences of the invention can be cloned from fungal pathogens by methods known in the art. In general, the methods for the isolation of DNA from fungal isolates are known. See, Raeder & Broda (1985) *Letters in Applied Microbiology 2*:17-20;

Lee *et al.* (1990) *Fungal Genetics Newsletter 35*:23-24; and Lee and Taylor (1990) In: *PCR Protocols: A Guide to Methods and Applications*, Innes *et al.* (Eds.); pages 282-287.

Alternatively, the ITS sequences of interest can be determined by PCR amplification. In an exemplified embodiment, primers to amplify the entire ITS region are designed according to White *et al.* (1990; In: PCR Protocols; Eds.: Innes *et al.* pages 315-322), and the amplified ITS sequence is subcloned into the pCRII cloning vector. The subcloned sequence included the left hand ITS (ITS1), the right hand ITS (ITS2), as well as the centrally located 5.8S rRNA gene. This is undertaken for several isolates of *Pyrenophora tritici-repentis* and from an isolate of *Pyrenophora teres*.

The determined ITS sequences are compared within each pathogen group to locate divergences that are useful to test in PCR to distinguish the different species and/or strains. The ITS DNA sequences that are determined are shown in SEQ ID NOs: 29-41. From the identification of divergences, numerous primers are synthesized and tested in PCR-amplification. Templates used for PCR-amplification testing are firstly purified pathogen DNA, and subsequently DNA isolated from infected host plant tissue. Thus, it is possible to identify pairs of primers that are diagnostic, *i.e.* that identify one particular pathogen species or strain but not another species or strain of the same pathogen. Primers are also designed to regions highly conserved among the species to develop genus-specific primers as well as primers that identify any of several fungal pathogens that cause a certain disease. For example, primers are developed to detect both *P. teres* and *P. tritici-repentis*.

Preferred primer combinations are able to distinguish between the different species or strains in infected host tissue, *i.e.* host tissue that has previously been infected with a specific pathogen species or strain. This invention provides numerous primer combinations that fulfill this criterion for *P. teres* and *P. tritici-repentis*. The primers of the invention are designed based on sequence differences among the fungal ITS regions. A minimum of one base pair difference between sequences can permit design of a discriminatory primer. Primers designed to a specific fungal DNA's ITS region can be used in combination with a primer made to a conserved sequence region within the ribosomal DNA's coding region to amplify species-specific PCR fragments. In general, primers should have a theoretical melting temperature between about 60 to about 70 °C to achieve good sensitivity and should be void of significant secondary structure and 3' overlaps between primer combinations. Primers generally have sequence identity with at least about 5-10 contiguous nucleotide bases of ITS1 or ITS2. In preferred embodiments, primers are

anywhere from about 5-30 nucleotide bases long and are preferably at least 10 nucleotide bases long.

As an alternative to the above-described PCR diagnostic techniques, a specific fungal DNA's ITS region can be used to design polynucleotide label probes, each comprising a label portion and a nucleic acid region having a sequence that is complementary to at least about 5-10 contiguous nucleotides of ITS1 or ITS2. In preferred embodiments, the complementary nucleic acid regions of such probes are anywhere from about 5-30 nucleotide bases long and are preferably at least 10 nucleotide bases long. The label portion of the probes may comprise either a label that provides a signal or a binding site for such a label. Polynucleotide label probes such as these may be used in fungal detection methods that involve the following: (a) designing at least one polynucleotide label probe comprising a nucleic acid region having a sequence that is complementary to at least about 5-10 contiguous nucleotide bases of ITS1 or ITS2 of a fungal pathogen, such as *Pyrenophora tritici-repentis*, and a label portion comprising either a label that provides a signal when said probe hybridizes with another nucleic acid molecule or a binding site for said label; (b) isolating DNA from plant tissue infected with the fungal pathogen; (c) hybridizing the DNA of (b) with the probe or probes of (a); and (d) detecting the fungal pathogen by detecting the label, wherein detection of the label indicates that the hybridization of (c) has occurred and the fungal pathogen is present. Labels may be designed and detected according to any means known in the art, such as through the use of radioisotopes, fluorescence, or a planar optical waveguide. *See*, for example, U.S. Pat. No. 4,868,105, WO 95/33197, and WO 95/33198, all of which are incorporated herein by reference.

The present invention lends itself readily to the preparation of "kits" containing the elements necessary to carry out the process. Such a kit may comprise a carrier being compartmentalized to receive in close confinement therein one or more containers, such as tubes or vials. One of the containers may contain unlabeled or detectably labeled DNA primers or probes, which may be present in lyophilized form or in an appropriate buffer as necessary. One or more containers may contain one or more enzymes or reagents to be utilized in PCR reactions. These enzymes may be present by themselves or in admixtures, in lyophilized form or in appropriate buffers. Finally, the kit may contain all of the additional elements necessary to carry out the technique of the invention, such as buffers, extraction reagents, enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, transfer materials, autoradiography supplies, and the like.

EXAMPLES

The examples below show typical experimental protocols that can be used for isolating ITS sequences, selecting suitable primer sequences, testing primers for selective and diagnostic efficacy, and using such primers for disease and fungal isolate detection. The examples are provided by way of illustration and not by way of limitation. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory, Cold Spring Harbor, NY (1989); by T.J. Silhavy, M.L. Berman, and L.W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984); and by Ausubel, F.M. *et al., Current Protocols in Molecular Biology*, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Example 1: Fungal Isolates and Genomic Fungal DNA Extraction

See Table 1 for a listing of the fungal isolates used and their source. Fungi are grown in 150 ml potato dextrose broth inoculated with mycelial fragments from PDA (Potato Dextrose Agar) cultures. Cultures are incubated on an orbital shaker at 28°C for 7-11 days. Alternatively, mycelia may be isolated directly from a PDA plate. Mycelia are pelleted by centrifugation and then ground in liquid nitrogen, and total genomic DNA is extracted using the protocol of Lee and Taylor (1990; In: *PCR Protocols: A Guide to Methods and Applications*; Eds.: Innes *et al.*; pages 282-287).

Table 1: Source of Test Isolates

Isolate	Organism	Source	Origin
6715	*Pyrenophora tritici-repentis*	ATCC[1]	---
44184	*Pyrenophora tritici-repentis*	ATCC[1]	Australia
205	*Pyrenophora tritici-repentis*	Novartis[2]	Västerås, Sweden
109	*Pyrenophora tritici-repentis*	Novartis[2]	Vallskog, Sweden
403	*Pyrenophora tritici-repentis*	Novartis[2]	Vattholma, Sweden
223	*Pyrenophora tritici-repentis*	Novartis[2]	Västerås, Sweden
407	*Pyrenophora tritici-repentis*	Novartis[2]	Vattholma, Sweden
1316	*Pyrenophora tritici-repentis*	Novartis[2]	Vallskog, Sweden
PTR 3B	*Pyrenophora tritici-repentis*	L. Francl[3]	North Dakota
PTR 4A	*Pyrenophora tritici-repentis*	L. Francl[3]	North Dakota
PTR 1	*Pyrenophora tritici-repentis*	L. Francl[3]	North Dakota
148-1-1	*Pyrenophora tritici-repentis*	L. Francl[3]	North Dakota
119-2-3	*Pyrenophora tritici-repentis*	L. Francl[3]	North Dakota
83-A-3-1	*Pyrenophora tritici-repentis*	L. Francl[3]	North Dakota
DL22	*Pyrenophora tritici-repentis*	L. Francl[3]	Minnesota
36570	*Pyrenophora teres f. teres*	ATCC[1]	Denmark
11404	*Drechslera sorokiniana*	ATCC[1]	Minnesota
44234	*Rhizoctonia cerealis*	ATCC[1]	Netherlands
R-9420	*Fusarium graminearum*	P. Nelson[4]	Washington
62215	*Fusarium culmorum*	ATCC[1]	Switzerland
18222	*Microdochium nivale*	ATCC[1]	Scotland
44643	*Pseudocercosporella herpotrichoides*-W type	ATCC[1]	Germany
308	*Pseudocercosporella herpotrichoides*-R type	Novartis[5]	---
24425	*Septoria nodorum*	ATCC[1]	Montana
26517	*Septoria tritici*	ATCC[1]	Minnesota
60531	*Cladosporium herbarum*	ATCC[1]	New Zealand
52476	*Cercospora arachidicola*	ATCC[1]	Oklahoma
HS-1	*Helminthosporium sativum*	Novartis[2]	Sweden
DT-1	*Pyrenophora teres*	Novartis[2]	Sweden
DR-1	*Pyrenophora tritici-repentis*	Novartis[2]	Sweden

[1]American Type Culture Collection, Rockville, Maryland, USA
[2]Novartis Sweden AB, 261 23 Landskrona, Sweden
[3]Dr. Len Francl, North Dakota State University, Fargo, North Dakota, USA
[4]Dr. Paul Nelson, Penn State University, State College, Pennsylvania, USA
[5]Novartis Crop Protection Limited, Basel, Switzerland

Example 2: Isolation of the Internal Transcribed Spacer (ITS) Regions

Approximately 600-bp long internal transcribed spacer (ITS) region fragments are PCR-amplified from 10 ng of genomic DNA isolated from *P. tritici-repentis* isolates 6715, 119-2-3, DL22, PTR4A, 44184, 205, 403, 109, 407, 1316, 223 and *P. teres* isolate 36570 using 50 pmol of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'; SEQ ID NO:1) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; SEQ ID NO:4). PCRs are performed as described in Example 4. The ITS PCR products are cloned using the Invitrogen Corporation's (San Diego, CA) TA Cloning Kit (part no. K2000-01) using the PCR2.1 cloning vector. The DNA sequences of the ITS regions are determined by the dideoxy method using the Applied Biosystems (Foster City, CA) automated sequencer with the primers ITS1 (SEQ ID NO:1), ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'; SEQ ID NO:2), ITS4 (SEQ ID NO:4) and the M13 universal -20 (5'-GTAAAACGACGGCCAGT-3'; SEQ ID NO:5) and Reverse (5'-AACAGCTATGACCATG-3'; SEQ ID NO:6) primers. The ITS primers ITS1, ITS2, ITS3, and ITS4 are detailed in White *et al.* (1990; In: PCR Protocols; Eds.: Innes *et al.* pages 315-322).

Example 3: DNA Extraction from Wheat

DNA is extracted from wheat using a bulk maceration method. The bulk maceration method is used to isolate DNA from several naturally infected wheat leaves from the field to optimize the field sampling method for high throughput analysis.

The following is the protocol for the bulk maceration method:

(1) Place the appropriate number of wheat leaves in a Bioreba (Reinach, Switzerland) heavy duty plastic bag (cat#490100). Weigh the plant tissue, plastic bag with leaves minus the tare (weight of the plastic bag).

(2) Add an equal volume (ml) of Muller Extraction Buffer (0.1% w/v Tween-80; 0.04 M Tris-Cl, pH 7.7; 0.15 M NaCl; 0.1% w/v BSA-Pentex fraction V;

0.01% w/v sodium azide; 200 mM EDTA) per weight (g) of wheat tissue. Macerate the tissue using a Bioreba Homex 6 homogenizer set at 70. Grind the leaves until the tissue is fibrous.

(3) Pool the extracts from multiple bags, if used, and vortex well. Aliquot the extraction juice into eppendorf tubes on ice.

(a) Boil 100 µl of the concentrated extract for 5 minutes.

(b) Place the boiled extract on ice.

(c) Make a 1:10 dilution by adding 10 µl from the boiled, concentrated extract to 90 µl of sterile dH_2O.

(d) Store the diluted extracts on ice until ready to use.

Example 4: Polymerase Chain Reaction Amplification

Polymerase chain reactions are performed with the GeneAmp Kit from Perkin-Elmer/Cetus (Norwalk, CT; part no. N808-0009) using 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH8.3, containing 200 µM of each dTTP, dATP, dCTP, and dGTP, 50 pmol each primer, 2.5 units of *Taq* polymerase and 10 ng of genomic DNA or 1 µl of 1:10 diluted plant extract in a final volume of 50 µl. Reactions are run for 30 - 40 cycles of 15 s at 94°C; 15 s at 50°C - 70°C; and 45 s at 72°C in a Perkin-Elmer/Cetus Model 9600 thermal cycler. The products are analyzed by loading 10 µl of each PCR sample on a 1.0% agarose gel and electrophoresing.

Example 5: Synthesis and Purification of Oligonucleotides

Oligonucleotides (primers) are synthesized by, for example, either Integrated DNA Technologies (Coralville, IA) or Midland Certified Reagent Company (Midland, Texas).

Example 6: Selection of Species-Specific Primers

The ITS regions from the mulitiple isolates of *P. tritici-repentis* and the single isolate of *P. teres* are aligned. The oligonucleotide primers shown below in Table 2 are synthesized according to Example 5 based on analysis of the aligned sequences. Primers are designed to the regions that contain the greatest differences in sequence among the

fungal species. Primers are also designed to regions highly conserved among the species to develop genus-specific primers. In addition, the published ribosomal gene-specific primers ITS1, ITS2, ITS3 and ITS4 (White *et al.*, 1990; In: PCR Protocols; Eds.: Innes *et al.* pages 315-322) are synthesized for testing in combination with the primers specific for the ITS regions.

Table 2: Primers Designed for Fungal Detection

Primer Template	Primer	Primer Sequence
18S rDNA	ITS1	5'-TCCGTAGGTGAACCTGCGG-3' (SEQ ID NO:1)
5.8S rDNA	ITS2	5'-GCTGCGTTCTTCATCGATGC-3' (SEQ ID NO:2)
5.8S rDNA	ITS3	5'-GCATCGATGAAGAACGCAGC-3' (SEQ ID NO:3)
25S rDNA	ITS4	5'-TCCTCCGCTTATTGATATGC-3' (SEQ ID NO:4)
P. tritici-repentis	JB629	5'-GTACTACTTGTTTCCTTGGCG-3' (SEQ ID NO:7)
P. tritici-repentis	JB630	5'-TCAGTTGCAATCAGCGTCAG-3' (SEQ ID NO:8)
P. tritici-repentis	JB631	5'-TGGACAAGAGCGCAAATAATG-3' (SEQ ID NO:9)
P. tritici-repentis	JB632	5'-ATGAAGCCGGACTGGGATA-3' (SEQ ID NO:10)
P. tritici-repentis	JB633	5'-ATGAAGCCGGACTGGGATAGGG-3' (SEQ ID NO:11)
P. tritici-repentis	JB634	5'-CGCTGCCTTGCCCGTCTGGC-3' (SEQ ID NO:12)
P. tritici-repentis	JB635	5'-CGCTGCCTTGCCCGTCT-3' (SEQ ID NO:13)
P. tritici-repentis	JB636	5'-CATGGACGCGCGACCGC-3' (SEQ ID NO:14)
P. tritici-repentis	JB637	5'-CATGGACGCGCGACCGCGGC-3' (SEQ ID NO:15)
P. tritici-repentis	JB638	5'-CTCCGAAACCAGTAGGCC-3' (SEQ ID NO:16)
P. tritici-repentis	JB651	5'-GATAGGGCCTCGCTGCCTTGC-3' (SEQ ID NO:17)
P. tritici-repentis	JB652	5'-GATAGGGCCTCGCTGCCT-3' (SEQ ID NO:18)
P. tritici-repentis	JB653	5'-AAGGTTGAAAGAAGCTTCATGG-3' (SEQ ID NO:19)
P. tritici-repentis	JB654	5'-CAAAAGGTTGAAAGAAGCTTCATGG-3' (SEQ ID NO:20)
P. tritici-repentis	JB655	5'-AAGCCGGACTGGGATAGG-3' (SEQ ID NO:21)
P. tritici-repentis	JB656	5'-CAAAAGGTTGAAAGAAGC-3' (SEQ ID NO:22)
P. tritici-repentis	JB657	5'-GCCGGACTGGGATAGGGC-3' (SEQ ID NO:23)
P. tritici-repentis	JB658	5'-GGACTGGGATAGGGCCTC-3' (SEQ ID NO:24)
P. tritici-repentis	JB659	5'-GAAGCTTCATGGACGCGCG-3' (SEQ ID NO:25)
P. tritici-repentis	JB660	5'-GGCGAGTCTCGGGAGAGA-3' (SEQ ID NO:26)
P. tritici-repentis	JB675	5'-GCCGGACTGGGATAGGGCCTC-3' (SEQ ID NO:27)
P. tritici-repentis	JB676	5'-GGCGAGTCTCGGGAGAGAGAC-3' (SEQ ID NO:28)

Example 7: Determination of Primer Specificity to Purified Fungal Genomic DNA

PCRs are performed according to Example 4 using different primer combinations (Table 3) to amplify single specific fragments. Specific PCR amplification products are produced from primers designed from the ITS regions between the 18S and 25S ribosomal DNA subunits of each fungal strain of interest.

Table 3: ITS-Derived Diagnostic PCR Primers

Primer specificity	5' Primer	3' Primer	Approximate size of amplified fragment
P. tritici-repentis	JB629 (SEQ ID NO:7)	JB631 (SEQ ID NO:9)	390 bp
P. tritici-repentis	JB633 (SEQ ID NO:11)	JB637 (SEQ ID NO:15)	485 bp
P. tritici-repentis	JB629 (SEQ ID NO:7)	JB636 (SEQ ID NO:14)	411 bp
P. teres/ P. tritici-repentis	ITS1 (SEQ ID NO:1)	JB631 (SEQ ID NO:9)	500 bp
P. tritici-repentis/ D. sorokiniana	JB630 (SEQ ID NO:8)	ITS4 (SEQ ID NO:4)	433 bp
P. tritici-repentis	JB651 (SEQ ID NO:17)	JB654 (SEQ ID NO:20)	473 bp
P. tritici-repentis	JB652 (SEQ ID NO:18)	JB653 (SEQ ID NO:19)	498 bp
P. tritici-repentis/ P. teres	ITS1 (SEQ ID NO:1)	JB653 (SEQ ID NO:19)	448 bp
P. tritici-repentis	JB629 (SEQ ID NO:7)	JB653 (SEQ ID NO:19)	438 bp
P. tritici-repentis	JB630 (SEQ ID NO:8)	JB653 (SEQ ID NO:19)	357 bp
P. tritici-repentis	JB632 (SEQ ID NO:10)	JB653 (SEQ ID NO:19)	512 bp
P. tritici-repentis	JB635 (SEQ ID NO:13)	JB653 (SEQ ID NO:19)	482 bp
P. tritici-repentis	JB657 (SEQ ID NO:23)	JB660 (SEQ ID NO:26)	393 bp
P. tritici-repentis	JB657 (SEQ ID NO:23)	JB653 (SEQ ID NO:19)	508 bp
P. tritici-repentis	JB658 (SEQ ID NO:24)	JB653 (SEQ ID NO:19)	503 bp
P. tritici-repentis	JB675 (SEQ ID NO:27)	JB676 (SEQ ID NO:28)	392 bp

Example 8: Determination of Primer Specificity to Plant Tissue Infected with Fungi and Cross-Reactivity with Other Cereal Fungal Pathogens

Total genomic DNA is isolated as described in Example 3 from healthy wheat leaves and from wheat leaves infected with *P. tritici-repentis*. PCRs are performed as described in

Example 4 testing primer combinations listed in Table 3 against DNA from the wheat tissue. Purified fungal genomic DNAs are obtained as described in Example 1 and PCR assayed as described in Example 4 using the diagnostic primers. Other fungal DNA species and isolates are tested for the ability of the diagnostic primers to cross-react therewith. Results of such experiments are as follows:

P. tritici-repentis-specific primer combination JB675 (SEQ ID NO:27) and JB676 (SEQ ID NO:28) amplify a 392 bp fragment from DNA from all of the *P. tritici-repentis* isolates listed in Table 1 and from *P. tritici-repentis*-infected wheat tissue. This primer combination does not amplify a diagnostic fragment from healthy wheat tissue nor from purified genomic DNA from the following common cereal pathogens listed in Table 1: *P. teres, R. cerealis, D. sorokiniana, F. graminearum, F. culmorum, M. nivale, P. herpotrichoides* R- and W-pathotypes, *S. nodorum, C. herbarum, S. tritici, C. arachidicola* and *H. sativum*. Similar diagnostic results are obtained with the following *P. tritici-repentis*-specific primer combinations: JB658 (SEQ ID NO:24) and JB653 (SEQ ID NO:19); JB657 (SEQ ID NO:23) and JB653 (SEQ ID NO:19); JB657 (SEQ ID NO:23) and JB660 (SEQ ID NO:26); JB652 (SEQ ID NO:18) and JB653 (SEQ ID NO:19); and JB629 (SEQ ID NO:7) and JB631 (SEQ ID NO:9). *P. tritici-repentis*-specific primers JB633 (SEQ ID NO:11) and JB637 (SEQ ID NO:15) produce similar results.

The primers JB651 (SEQ ID NO:17) and JB654 (SEQ ID NO:20) amplify a 473 bp fragment from DNA from *P. tritici-repentis* isolates #6715 and #DL22. The primers amplify a smaller fragment, approximately 400 bp, from healthy wheat DNA. These primers do not amplify purified genomic DNA from the following common cereal pathogens listed in Table 1: *P. teres, R. cerealis, D. sorokiniana, F. graminearum, F. culmorum, M. nivale, P. herpotrichoides* R- and W-pathotypes, *S. nodorum, C. herbarum, S. tritici* and *C. arachidicola*.

Primer combination JB635 (SEQ ID NO:13) and JB653 (SEQ ID NO:19) amplify a 482 bp fragment; primer combination JB632 (SEQ ID NO:10) and JB653 (SEQ ID NO:19) amplify a 512 bp fragment; primer combination JB630 (SEQ ID NO:8) and JB653 (SEQ ID NO:19) amplify a 357 bp fragment; and primer combination JB629 (SEQ ID NO:7) and JB653 (SEQ ID NO:19) amplify a 438 bp fragment from DNA from *P. tritici-repentis* isolates #6715 and DL22. These primer combinations do not amplify any fragments from DNA from *D. sorokiniana* isolate #11404 and *P. teres* isolate #36570. Primer combination JB629 (SEQ ID NO:7) and JB636 (SEQ ID NO:14) amplify a 411 bp fragment from *P. tritici-*

repentis isolate #6715 DNA, but do not amplify from DNA from *D. sorokiniana* #11404 and *S. nodorum* #24425. The primers JB630 (SEQ ID NO:8) and ITS4 (SEQ ID NO:4) amplify a 433 bp fragment from *P. tritici-repentis* isolate #6715 DNA and also from DNA from *D. sorokiniana* #11404, but do not amplify from DNA isolated from *S. nodorum* #24425 and *S. tritici* #26517.

Primer combination ITS1 (SEQ ID NO:1) and JB653 (SEQ ID NO:19) amplify a 448 bp fragment from DNA isolated from *P. tritici-repentis* and also from *P. teres*. This primer combination does not amplify a diagnostic fragment from healthy wheat tissue nor from purified genomic DNA from the following common cereal pathogens listed in Table 1: *R. cerealis*, *D. sorokiniana*, *F. graminearum*, *F. culmorum*, *M. nivale*, *P. herpotrichoides* R- and W-pathotypes, *S. nodorum*, *C. herbarum*, *S. tritici*, *C. arachidicola* and *H. sativum*. Similar diagnostic results are obtained with the primer combination ITS1 (SEQ ID NO:1) and JB631 (SEQ ID NO:9).

While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and further embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the scope of the present invention.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

EDITORIAL NOTE FOR

26243/99

THE FOLLOWING SEQUENCE LISTING IS PART OF THE DESCRIPTION

THE CLAIMS FOLLOW ON PAGE 22

SEQUENCE LISTING

<110> Novartis AG

<120> DETECTION OF WHEAT AND BARLEY FUNGAL PATHOGENS USING THE POLYMERASE CHAIN REACTION

<130> PF/5-30426/A/CGC1984

<140>
<141>

<150> US 09/026,601
<151> 1998-02-20

<160> 41

<170> PatentIn Ver. 2.0

<210> 1
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer ITS1

<400> 1
tccgtaggtg aacctgcgg 19

<210> 2
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer ITS2

<400> 2
gctgcgttct tcatcgatgc 20

<210> 3
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer ITS3

<400> 3
gcatcgatga agaacgcagc 20

<210> 4
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer ITS4

<400> 4
tcctccgctt attgatatgc 20

<210> 5
<211> 17

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: M13
Universal-20 primer

<400> 5
gtaaaacgac ggccagt 17

<210> 6
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Reverse primer
used in Example 2

<400> 6
aacagctatg accatg 16

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB629

<400> 7
gtactacttg tttccttggc g 21

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB630

<400> 8
tcagttgcaa tcagcgtcag 20

<210> 9
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB631

<400> 9
tggacaagag cgcaaataat g 21

<210> 10
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB632

<400> 10
atgaagccgg actgggata 19

<210> 11
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB633

<400> 11
atgaagccgg actgggatag gg 22

<210> 12
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB634

<400> 12
cgctgccttg cccgtctggc 20

<210> 13
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer JB635

<400> 13
cgctgccttg cccgtct 17

<210> 14
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB636

<400> 14
catggacgcg cgaccgc 17

<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer JB637

<400> 15
catggacgcg cgaccgcggc 20

<210> 16
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB638

<400> 16

ctccgaaacc agtaggcc 18

<210> 17
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB651

<400> 17
gatagggcct cgctgccttg c 21

<210> 18
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB652

<400> 18
gatagggcct cgctgcct 18

<210> 19
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB653

<400> 19
aaggttgaaa gaagcttcat gg 22

<210> 20
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB654

<400> 20
caaaaggttg aaagaagctt catgg 25

<210> 21
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB655

<400> 21
aagccggact gggatagg 18

<210> 22
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB656

<400> 22
caaaaggttg aaagaagc 18

<210> 23
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB657

<400> 23
gccggactgg gatagggc 18

<210> 24
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB658

<400> 24
ggactgggat agggcctc 18

<210> 25
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB659

<400> 25
gaagcttcat ggacgcgcg 19

<210> 26
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB660

<400> 26
ggcgagtctc gggagaga 18

<210> 27
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB675

<400> 27
gccggactgg gatagggcct c 21

<210> 28
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer JB676

<400> 28
ggcgagtctc gggagagaga c 21

<210> 29
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 29
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc tttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgcccttta gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggagcgca gcacattatt tgcgctcttg 480
tccagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggatacccgc tgaacttaag catatcaata agcggagga 579

<210> 30
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 30
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc tttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgcccttta gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggagcgca gcacattatt tgcgctcttg 480
tccagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggatacccgc tgaacttaag catatcaata agcggagga 579

<210> 31
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 31
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc tttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgcccttta gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggarcgca rcacattatt tgcgctcttg 480
tccagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggatacccgc cgaacttaag catatcaata agcggagga 579

<210> 32
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 32
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc tttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240

tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc garactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggarcgca gcacattatt tgcgctcttg 480
tccagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggatacccgc tgaacttaag catatcaata agcggagga 579

<210> 33
<211> 580
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 33
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcaggattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcga 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc mraractcgc 420
cttaaaawcm ttggcmrccg gcctactggt ttcsgagmgc agcacattat ttgcgctctt 480
gtccagccgc ggtcgcgcgt ccatgaagct tctttcaacc ttttgacctc ggatcaggta 540
gggatacccg ctgaacttaa gcatatcaat aagcggagga 580

<210> 34
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 34
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggarcgca gcacattatt tgcgctcttg 480
tccagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggatacccgc tgaacttaag catatcaata agcggagga 579

<210> 35
<211> 580
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 35
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ytcgctgcct tgcccgtctg gcgccatatt cacccatgtc ttttkgcgta mtamttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tgggcagccg gcctactggt ttcggagmgc agcacattat ttgcgctctt 480
gtccagccgc ggtcgcgcgt ccatgaagct tctttcaacc ttttgacctc ggatcaggta 540
gggatacccg ctgaacttaa gcatatcaat aagcggagga 580

<210> 36
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 36
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60

ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggagcgca gcacattatt tgcgctcttg 480
tccagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggataccgc tgaacttaag catatcaata agcggagga 579

<210> 37
<211> 580
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 37
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttttnca gttgcaatca 180
gsgtcagcaa aacaaatgta atcaattaca actttcaaca acggatctct tggttctggc 240
atcgatgaag aacgcagcga aatgcgataa gtagtgtgaa ttgcagaatt cagtgaatca 300
tcgaatcttt gaacgcacat tgcgcccttt ggtattccaa agggcatgcc tgttcgagcg 360
tcatttgtac cctcaagctt tgcttggtgt tgggtgtctt gtctctctcc cgagactcgc 420
cttaaaatca ttggcagccg gcctactggt ttcggagcgc agcacattat ttgcgctctt 480
gtycarccgc ggtcgcgcgt ccatgaagct tctttcaacc ttttgacctc ggatcaggta 540
gggatacccg ctgaactwaa gcatatcaat aagcggarga 580

<210> 38
<211> 579
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 38
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgcgta ctacttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc gagactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggagcgca gcacattatt tgcgctcttg 480
tycagccgcg gtcgcgcgtc catgaagctt ctttcaacct tttgacctcg gatcaggtag 540
ggataccgc tgaacttaag catatcaata agcsgarga 579

<210> 39
<211> 587
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 39
tccgtaggtg aacctgcgga nggatcatwa cacaaatatg amccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgty tnttgcgta ctamttgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacc ttttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaattacaa ctttcaacaa cggatctctt ggttctggca 240
tcgatgaaga acscagcgaa atgcgataag tagtgtgaat tgcagaattc agtgcaatca 300
tcgaatcttt gaaacgcaca ttgcgccctt tggtatwcca aagggcatgc ctgttcgagc 360
gtcatttgta ccctccaagc tttgccttgg gtgttgggcg tcttgtctct ctcccsagac 420
tcgcyttaaa atcattgggc agcsggccta ctggtttccg gagcgcarca matttatttg 480
csctcttgtc mascogcggt cgcgcgtcca tgaarcttct tycaaccttt tgacctcgga 540
tcaggtaggg ataccogctg aacttaagca tatcaataag cggagga 587

<210> 40
<211> 580
<212> DNA
<213> Pyrenophora tritici-repentis

<400> 40
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aagccggact gggatagggc 60
ctcgctgcct tgcccgtctg gcgccatatt cacccatgtc tttttgsgta ctactkgttt 120
ccttggcggg tccgcccgcc aattggacct tattcaaacy ttttttttcag ttgcaatcag 180
cgtcagcaaa acaaatgtaa tcaatkacaa ctttcaacaa cggatctctc ggttctggca 240
tcgatgaaga acgcagcgaa atgcgataag tagtgtgaat tgcagaattc agtgaatcat 300
cgaatctttg aacgcacatt gcgccctttg gtattccaaa gggcatgcct gttcgagcgt 360
catttgtacc ctcaagcttt gcttggtgtt gggcgtcttg tctctctccc garactcgcc 420
ttaaaatcat tggcagccgg cctactggtt tcggagcgca gcacattatt tgcgctcttg 480
tccagccgcg gtccscscgt ccatgaagct tctttcaacc ttttgamctc ggatcaggta 540
gggatacccg ctgaacttaa gcatatcaat aagcggagga 580

<210> 41
<211> 590
<212> DNA
<213> Pyrenophora teres

<400> 41
tccgtaggtg aacctgcgga gggatcatta cacaaatatg aaggcagatt gggtagtccc 60
cgsttttggg ggtttgccca ttctggcgcc atattcaccc atgtcttttg cgtactactt 120
gtttccttgg cgggctcgcc cgccaattgg actttattca aacctttttt tattgcaatc 180
agcgtcagca aaacaatgta atcaattaca actttcaaca acggatctct tggttctggc 240
atcgatgaag aacgcagcga aatgcgataa gtagtgtgaa ttgcagaatt cagtgaatca 300
tcgaatcttt gaacgcacat tgcgcccttt ggtattccaa agggcatgcc tgttcgagcg 360
tcatttgtac cctcaagctt tgcttggtgt tgggcgtctt ttgtctctcc cccgagactc 420
gccttaaaaa cattggcagc cggcctactg gtttcggagc gcagcacatt atttgcgctc 480
ttgtycagcc gcggtcgcgc gtccatgaag ccttttttttt ttttcagcct tttgactctc 540
ggatcaggta gggatacccg ctgaacttaa gcatatcaat aagcggagga 590

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated, substantially pure DNA molecule from the ribosomal RNA gene region of a fungal pathogen, wherein said DNA molecule comprises at least one of ITS1 and ITS2 of *Pyrenophora tritici-repentis*.

2. A method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) determining the nucleotide sequence of the DNA molecule of claim 1;

(b) designing at least one PCR primer having sequence identity with at least 10 nucleotides of the nucleotide sequence determined in (a);

(c) isolating DNA from plant tissue suspected to be infected with *Pyrenophora tritici-repentis;*

(d) subjecting the DNA of (c) to polymerase chain reaction amplification using the primer or primers of (b); and

(e) detecting *Pyrenophora tritici-repentis* by visualizing the product or products of the polymerase chain reaction amplification of (d).

3. An Internal Transcribed Spacer sequence isolated from the ribosomal RNA gene region of *Pyrenophora tritici-repentis*, wherein said Internal Transcribed Spacer sequence is selected from the group consisting of: nucleotides 31-208 of SEQ ID NO:29; nucleotides 366-526 of SEQ ID NO:29; nucleotides 31-208 of SEQ ID NO:30; nucleotides 366-526 of SEQ ID NO:30; nucleotides 31-208 of SEQ ID NO:31; nucleotides 366-526 of SEQ ID NO:31; nucleotides 31-208 of SEQ ID NO:32; nucleotides 366-526 of SEQ ID NO:32; nucleotides 31-208 of SEQ ID NO:33; nucleotides 366-527 of SEQ ID NO:33; nucleotides 31-208 of SEQ ID NO:34; nucleotides 366-526 of SEQ ID NO:34; nucleotides 31-208 of SEQ ID NO:35; nucleotides 366-527 of SEQ ID NO:35; nucleotides 31-208 of SEQ ID NO:36; nucleotides 366-526 of SEQ ID NO:36; nucleotides 31-209 of SEQ ID NO:37; nucleotides 367-527 of SEQ ID NO:37; nucleotides 31-208 of SEQ ID NO:38; nucleotides 366-526 of SEQ ID NO:38; nucleotides 31-208 of SEQ ID NO:39; nucleotides 368-534 of SEQ ID NO:39; nucleotides 31-208 of SEQ ID NO:40; and nucleotides 366-527 of SEQ ID NO:40; and nucleotides 31-209 of SEQ ID NO:41; and nucleotides 367-536 of SEQ ID NO:41.

4. An oligonucleotide primer for use in the amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence, wherein said primer is selected from the group consisting of SEQ ID Nos:7-28.

5. A pair of oligonucleotide primers for use in the amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence, wherein at least one of said primers is the oligonucleotide primer of claim 4.

6. A pair of oligonucleotide primers according to claim 5, wherein said pair is selected from the following primer pairs:

SEQ ID NO:7 and SEQ ID NO:9;
SEQ ID NO:11 and SEQ ID NO:15;
SEQ ID NO:7 and SEQ ID NO:14;
SEQ ID NO:1 and SEQ ID NO:9;
SEQ ID NO:8 and SEQ ID NO:4;
SEQ ID NO:17 and SEQ ID NO:20;
SEQ ID NO:18 and SEQ ID NO:19;
SEQ ID NO:1 and SEQ ID NO:19;
SEQ ID NO:7 and SEQ ID NO:19;
SEQ ID NO:8 and SEQ ID NO:19;
SEQ ID NO:10 and SEQ ID NO:19;
SEQ ID NO:13 and SEQ ID NO:19;
SEQ ID NO:23 and SEQ ID NO:26;
SEQ ID NO:23 and SEQ ID NO:19;
SEQ ID NO:24 and SEQ ID NO:19; and
SEQ ID NO:27 and SEQ ID NO:28.

7. A diagnostic kit used in detecting a fungal pathogen, comprising at least one primer according to claim 4.

8. A method for the detection of a fungal pathogen, comprising:

(a) isolating DNA from a plant leaf suspected to be infected with a fungal pathogen;

(b) subjecting said DNA to polymerase chain reaction amplification using at least one primer according to claim 4, and

(c) detecting said fungal pathogen by visualizing the product or products of said polymerase chain reaction amplification.

9. The method of claim 8, wherein said fungal pathogen is *Pyrenophora tritici-*

repentis.

10. The method of claim 8, wherein said fungal pathogen is *Pyrenophora teres*.

11. The method of claim 8, wherein said fungal pathogen is *Drechslera sorokiniana*.

12. A method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) designing at least one PCR primer having sequence identity with at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of claim 3;

(b) isolating DNA from plant tissue suspected to be infected with *Pyrenophora tritici-repentis*;

(c) subjecting the DNA of (b) to polymerase chain reaction amplification using the primer or primers of (a); and

(d) detecting *Pyrenophora tritici-repentis* by visualizing the product or products of the polymerase chain reaction amplification of (c).

13. A method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) designing at least one polynucleotide label probe comprising:

(i) a nucleic acid region having a sequence that is complementary to at least 10 contiguous nucleotides of the Internal Transcribed Spacer sequence of claim 3; and

(ii) a label portion comprising either a label that provides a signal when said probe hybridizes with another nucleic acid molecule, or a binding site for said label;

(b) isolating DNA from plant tissue suspected to be infected with *Pyrenophora tritici-repentis*;

(c) hybridizing the DNA of (b) with the probe or probes of (a); and

(d) detecting *Pyrenophora tritici-repentis* by detecting the label, wherein detection of the label indicates that the hybridization of (c) has occurred.

14. A method for detecting *Pyrenophora tritici-repentis*, comprising:

(a) isolating DNA from a plant leaf suspected to be infected with *Pyrenophora tritici-repentis*;

(b) amplifying a part of the Internal Transcribed Spacer sequence of *Pyrenophora tritici-repentis* using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers according to claim 6; and

(c) detecting *Pyrenophora tritici-repentis* by visualizing the amplified part of the

Internal Transcribed Spacer sequence.

15. A pair of oligonucleotide primers according to claim 6, wherein said pair is either
SEQ ID NO:1 and SEQ ID NO:9; or
SEQ ID NO:1 and SEQ ID NO:19.

16. A method for detecting *Pyrenophora teres*, comprising:
(a) isolating DNA from a plant leaf suspected to be infected with *Pyrenophora teres*;
(b) amplifying a part of the Internal Transcribed Spacer sequence of *Pyrenophora teres* using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers according to claim 15; and
(c) detecting *Pyrenophora teres* by visualizing the amplified part of the Internal Transcribed Spacer sequence.

17. A pair of oligonucleotide primers according to claim 6, wherein said pair is SEQ ID NO:8 and SEQ ID NO:4.

18. A method for detecting *Drechslera sorokiniana*, comprising:
(a) isolating DNA from a plant leaf suspected to be infected with *Drechslera sorokiniana*;
(b) amplifying a part of the Internal Transcribed Spacer sequence of *Drechslera sorokiniana* using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers according to claim 17; and
(c) detecting *Drechslera sorokiniana* by visualizing the amplified part of the Internal Transcribed Spacer sequence.

19. An isolated, substantially pure DNA molecule from the ribosomal RNA gene region of a fungal pathogen according to claim 1, substantially as hereinbefore described with reference to the Examples.

20. A method of detecting *Pyrenophora tritici-repentis* according to any one of claims 2, 12, 13 and 14, substantially as hereinbefore described with reference to the Examples.

21. An Internal Transcribed Spacer sequence according to claim 3, substantially as hereinbefore described with reference to the Examples.

22. An oligonucleotide primer for use in the amplification-based detection of a fungal Internal Transcribed Spacer DNA sequence according to claim 4, substantially as hereinbefore described with reference to the Examples.

23. A diagnostic kit according to claim 7, substantially as hereinbefore described with

reference to the Examples.

24. A method for the detection of a fungal pathogen according to claim 8, substantially as hereinbefore described with reference to the Examples.

25. A method for detecting *Pyrenophora teres* according to claim 16, substantially as hereinbefore described with reference to the Examples.

26. A method for detecting *Drechslera sorokiniana* according to claim 18, substantially as hereinbefore described with reference to the Examples.

DATED this 5th day of December, 2001

Syngenta Participations AG

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants