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 TAPESIA YALLUNDAE ET TAPESIA ACUFORMIS
 (54) Title: PCR-BASED DETECTION AND QUANTIFICATION OF TAPESIA YALLUNDAE AND TAPESIA ACUFORMIS

(57) **Abrégé/Abstract:**

The present invention provides primers and probes for use in TaqMan™ quantitative PCR assays for the detection of *Tapesia yallundae* (syn. *Pseudocercospora herpotrichoides* W-type) and *Tapesia acuformis* (syn. *Pseudocercospora herpotrichoides* R-type). The present invention also provides primers and probes for use in TaqMan™ quantitative PCR control assays for the detection of wheat DNA.

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(54) Title: PCR-BASED DETECTION AND QUANTIFICATION OF *TAPESIA YALLUNDAE* AND *TAPESIA ACUFORMIS*

(57) Abstract: The present invention provides primers and probes for use in TaqManTM quantitative PCR assays for the detection of *Tapesia yallundae* (syn. *Pseudocercospora herpotrichoides* W-type) and *Tapesia acuformis* (syn. *Pseudocercospora herpotrichoides* R-type). The present invention also provides primers and probes for use in TaqManTM quantitative PCR control assays for the detection of wheat DNA.

PCR-Based Detection and Quantification of *Tapesia yallundae* and *Tapesia acuformis*

The present invention relates to the use of primers and probes in TaqMan™ quantitative PCR assays for the detection of *Tapesia yallundae* (syn. *Pseudocercospora herpotrichoides* W-type) and *Tapesia acuformis* (syn. *Pseudocercospora herpotrichoides* R-type). The use of these assays enables the detection of specific fungal pathogens and their quantification in plant populations. The invention also relates to the use of primers and probes in TaqMan™ quantitative PCR assays for the detection of host wheat DNA for use as an endogenous reaction control.

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), *Pseudocercospora* (to MBC-type fungicides) and *Mycosphaerella fijiensis* to triazoles to mention just a few (Jones and Clifford; Cereal Diseases, John Wiley, 1983).

Cereal species are grown world-wide and represent a major fraction of world food production. Although yield loss is caused by many pathogens, the necrotizing pathogens *Septoria* and *Pseudocercospora* are particularly important in the major cereal growing areas of Europe and North America (Jones and Clifford; Cereal Diseases, John Wiley, 1983). In particular, the differential symptomology caused by different isolates and species of these fungi make the accurate predictive determination of potential disease loss difficult. Consequently, the availability of improved diagnostic techniques for the rapid and accurate identification of specific pathogens will be of considerable use to field pathologists.

Eyespot of wheat is caused by the pathogens *Tapesia acuformis* and *Tapesia yallundae*. These have previously been considered varieties of the same species *Pseudocercospora herpotrichoides* (Fron) Deighton. Wheat, rye, oats and other grasses are susceptible to the eyespot disease, which occurs in cool, moist climates and is prevalent in Europe, North and South America, Africa and Australia. Wheat is the most susceptible cereal species, but isolates have been identified that are also virulent on other cereals. The R-strain of the fungus (*Tapesia acuformis*), for example, has also been isolated from rye and grows more slowly on wheat than the W-strain (*Tapesia yallundae*) which has been isolated from wheat. Eyespot is restricted to the basal culm of the plant and can kill tillers or plants outright; however, it more usually causes lodging and/or results in a reduction in kernel size and number. Yield losses associated with eyespot are of even greater magnitude than those associated with *Septoria tritici* and *Septoria nodorum*. Typical control measures for eyespot include treatment with growth regulators to strengthen internodes, as well as fungicide treatment. However, the differing susceptibility of cultivars to different strains of the fungus render the predictive efficacy of fungicide treatments difficult.

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.

TaqMan™ chemistry and the ABI7700 (Perkin Elmer, Applied Biosystems Division, Foster City, CA) provide a means of creating precise, reproducible quantitative assays of DNA and RNA. The foundation of TaqMan™ chemistry is the polymerase chain reaction (PCR). In conventional PCR assays, oligonucleotide primers are designed complementary to the 5' and 3' ends of a DNA sequence of interest. During thermal cycling, DNA is first heat denatured. The sample is then brought to annealing and extension temperatures in which the primers bind their specific complements and are extended by the addition of nucleotide tri-phosphates by *Taq* polymerase. With repeated thermal cycling, the amount of template DNA is amplified.

In TaqMan™ chemistry, an oligonucleotide probe is designed that is complementary to the sequence region between the primers within the PCR amplicon. The probe contains a fluorescent reporter dye at its 5' end and a quencher dye at its 3' end. When the probe is intact, its fluorescent emissions are quenched by the phenomena of fluorescent resonance energy transfer (FRET). During thermal cycling, the probe hybridizes to the target DNA downstream of one of the primers. TaqMan™ chemistry relies on the 5' exonuclease activity of *Taq* polymerase to cleave the fluorescent dye from the probe. As PCR product accumulates, fluorescent signal is increased. By measuring this signal, the amplified product can be quantified. This method allows the quantitation of disease pressure by targeting pathogen DNA. In combination with the PCR primers, the probe provides another level of specificity in assays to differentiate pathogens.

The present invention thus provides:

an oligonucleotide primer selected from the group consisting of SEQ ID NOs:3-6, 8-23, 25-26, 28, 30, 42, and 43, in particular, wherein said primer is selected from the group consisting of SEQ ID NOs:3-6, 8-23, 25-26, 28, and 30

- a pair of oligonucleotide primers, wherein at least one of said primers is the oligonucleotide primer as mentioned hereinbefore
- a pair of oligonucleotide primers mentioned hereinbefore, wherein said pair consists of SEQ ID NO:14 and SEQ ID NO:18 or SEQ ID NO:3 and SEQ ID NO:8
- an oligonucleotide primer mentioned hereinbefore, wherein said primer is selected from the group consisting of SEQ ID NOs:42 and 43
- a pair of oligonucleotide primers, wherein at least one of said primers is the oligonucleotide primer consisting of SEQ ID NOs:42 and 43

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- a pair of oligonucleotide primers, wherein said pair consists of SEQ ID NO:42 and SEQ ID NO:43.

The invention further provides

- methods for the detection of a fungal pathogen, in particular of *Tapesia yallundae* and *Tapesia acuformis*, comprising:
 - (a) isolating DNA from a plant leaf infected with a 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.
- methods for the detection of a fungal pathogen, in particular of *Tapesia yallundae* and *Tapesia acuformis*, comprising:
 - (a) isolating DNA from plant tissue infected with said fungal pathogen;
 - (b) amplifying a part of the Internal Transcribed Spacer sequence of said fungal pathogen using said DNA as a template in a polymerase chain reaction with a pair of primers according to claim 3; and
 - (c) detecting said fungal pathogen by visualizing the amplified part of the Internal Transcribed Spacer sequence.

The invention further provides diagnostic kit used in detecting a fungal pathogen, comprising the primer of as mentioned hereinbefore.

The invention further provides

methods for the detection of wheat DNA, comprising:

- (a) isolating DNA from wheat tissue infected with a pathogen;
- (b) subjecting said DNA to polymerase chain reaction amplification using a pair of primers according to the invention; and
- (c) detecting said wheat DNA by visualizing the product or products of said polymerase chain reaction amplification.

Furthermore, the invention provides oligonucleotide probes for use in amplification-based detection of a fungal Internal Transcribed Spacer sequence, wherein said probe comprises:

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- (a) a nucleotide sequence complementary to at least 10 consecutive nucleotides of a sequence selected from the group consisting of: ITS1 of *Tapesia yallundae*, ITS2 of *Tapesia yallundae*, ITS1 of *Tapesia acuformis* and ITS2 of *Tapesia acuformis*;
- (b) a fluorescent reporter dye at a 5' end of said nucleotide sequence; and
- (c) a quencher dye at a 3' end of said nucleotide sequence.

The invention further provides oligonucleotide probes according as mentioned hereinbefore, wherein said nucleotide sequence is complementary to at least 10 consecutive nucleotides of a sequence selected from the group consisting of: nucleotides 31-263 of SEQ ID NO:37, nucleotides 420-570 of SEQ ID NO:37, nucleotides 31-262 of SEQ ID NO:38, and nucleotides 419-568 of SEQ ID NO:38, but in particular wherein said nucleotide sequence is selected from the group consisting of: SEQ ID NO:7, SEQ ID NO:24, SEQ ID NO:27, and SEQ ID NO:29.

The invention further provides oligonucleotide probes for use in amplification-based detection of wheat DNA, wherein said probe comprises:

- (a) a nucleotide sequence complementary to at least 10 consecutive nucleotides of SEQ ID NO:41 or SEQ ID NO:44;
- (b) a fluorescent reporter dye at a 5' end of said nucleotide sequence; and
- (c) a quencher dye at a 3' end of said nucleotide sequence.

The invention further provides an oligonucleotide primer pair/probe set for quantifying fungal DNA, wherein said primer pair consists of the pair of primers according to the invention and the probe is SEQ ID NO:24, SEQ ID NO:7 or SEQ ID NO:44.

In order to ensure a clear and consistent understanding of the specification and the claims, the following definitions are provided:

Gene: refers to a coding sequence and associated regulatory sequences wherein the coding sequence is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Examples of regulatory sequences are promoter sequences, 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns.

Identity: The percentage of sequence identity is determined using computer programs that are based on dynamic programming algorithms. Computer programs that are preferred

within the scope of the present invention include the BLAST (Basic Local Alignment Search Tool) search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. Version BLAST 2.0 (Gapped BLAST) of this search tool has been made publicly available on the Internet (currently <http://www.ncbi.nlm.nih.gov/BLAST/>). It uses a heuristic algorithm which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences which share only isolated regions. The scores assigned in a BLAST search have a well-defined statistical interpretation. Said programs are preferably run with optional parameters set to the default values.

Plant: refers to any plant, particularly to seed plants.

Plant material: refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, pollen tubes, ovules, embryo sacs, egg cells, zygotes, embryos, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant

The present invention is drawn to methods of identification and quantification of different species of plant pathogenic fungi. The invention provides primer and probe DNA sequences useful in TaqManTM quantitative PCR assays. Such DNA sequences are useful in the method of the invention as they are used in polymerase chain reaction (PCR) and TaqManTM-based diagnostic assays. These primers generate unique fragments in PCR reactions in which the DNA template is provided by specific fungal pathogens. In combination with the hybridization of the TaqManTM probe, they can be used to detect and quantify the specific pathogens in host plant material before the onset of disease symptoms.

In a preferred embodiment, the invention provides ITS-derived diagnostic primers and TaqManTM probes for the detection of *Tapesia yallundae* (syn. *Pseudocercospora herpotrichoides* W-type) and *Tapesia acuformis* (syn. *Pseudocercospora herpotrichoides* R-type).

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 quantification of disease pressure on a given crop.

Kits useful in the practice of the invention are also provided. The kits find particular use in the identification and quantification of the fungal pathogens *Tapesia yallundae* and *Tapesia acuformis*.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NOs:1-34 are the following oligonucleotide probes and primers useful for PCR-based detection of the fungal pathogens *Tapesia yallundae* and *Tapesia acuformis*:

Sequence Identifier	Oligo Name	Target	Oligo Sequence (5'->3')
SEQ ID NO:1	ITS1	Fungal 18S rDNA	tccgtaggtgaacctgcgg
SEQ ID NO:2	ITS4	Fungal 25S rDNA	tcctccgcttattgatatgc
SEQ ID NO:3	J103W	<i>Tapesia yallundae</i> (W)	ggctaccctacttggtag
SEQ ID NO:4	J104W	<i>Tapesia yallundae</i> (W)	cctgggggctaccctacttg
SEQ ID NO:5	J105W	<i>Tapesia yallundae</i> (W)	gggggctaccctacttggtag
SEQ ID NO:6	J106W	<i>Tapesia yallundae</i> (W)	tgggggctaccctacttggtag
SEQ ID NO:7	J107W	<i>Tapesia yallundae</i> (W)	(FAM)-ttagagtcgtcaggcctctcggagaagc- (TAMRA)
SEQ ID NO:8	J108W	<i>Tapesia yallundae</i> (W)	attattcaagggtggaggtcctga
SEQ ID NO:9	J109W	<i>Tapesia yallundae</i> (W)	aagggtggaggtctgaaccag
SEQ ID NO:10	J110W	<i>Tapesia yallundae</i> (W)	aagggtggaggtctgaacca
SEQ ID NO:11	J111W	<i>Tapesia yallundae</i> (W)	caagggtggaggtctgaacc

SEQ ID NO:12	J112R	<i>Tapesia acuformis</i> (R)	tcaagggtggagggtctgaacc
SEQ ID NO:13	J100R	<i>Tapesia acuformis</i> (R)	gggccaccctacttcggtaa
SEQ ID NO:14	J101R	<i>Tapesia acuformis</i> (R)	gaaatcctgggggccaccctctc
SEQ ID NO:15	J102R	<i>Tapesia acuformis</i> (R)	cctgggggccaccctact
SEQ ID NO:16	J113R	<i>Tapesia acuformis</i> (R)	gccaccctacttcggtaagggt
SEQ ID NO:17	J114R	<i>Tapesia acuformis</i> (R)	caccctacttcggtaagggttagagtc
SEQ ID NO:18	J115R	<i>Tapesia acuformis</i> (R)	aggtaattattcaagggtggaggt
SEQ ID NO:19	J116R	<i>Tapesia acuformis</i> (R)	aggtaattattcaagggtggaggtc
SEQ ID NO:20	J117R	<i>Tapesia acuformis</i> (R)	aaggtaattattcaagggtggaggt
SEQ ID NO:21	J118R	<i>Tapesia acuformis</i> (R)	ttattcaagggtggaggtctgg
SEQ ID NO:22	J119R	<i>Tapesia acuformis</i> (R)	tattcaagggtggaggtctgga
SEQ ID NO:23	J120R	<i>Tapesia acuformis</i> (R)	cctgccaagcaacaaaggta
SEQ ID NO:24	J121R	<i>Tapesia acuformis</i> (R)	(FAM)-cgggcctctcggagaagcctgg-(TAMRA)
SEQ ID NO:25	J122R	<i>Tapesia acuformis</i> (R)	cctacttcggtaagggttagagtcgt
SEQ ID NO:26	J123R	<i>Tapesia acuformis</i> (R)	tctccgagaggcccgac
SEQ ID NO:27	J124R	<i>Tapesia acuformis</i> (R)	(FAM)-aagcctggccagacctccaccc-(TAMRA)

SEQ ID NO:28	J125R	<i>Tapesia acuformis</i> (R)	aaggatcattaatagagcaatggatagac
SEQ ID NO:29	J126R	<i>Tapesia acuformis</i> (R)	(FAM)-cgccccgggagaaatcctgg-(TAMRA)
SEQ ID NO:30	J127R	<i>Tapesia acuformis</i> (R)	tgggggccaccctacttc
SEQ ID NO:31	JB537	<i>Tapesia yallundae</i> (W)	gggggctaccctacttggtag
SEQ ID NO:32	JB541	<i>Tapesia yallundae</i> (W)	ccactgatttagaggccgag
SEQ ID NO:33	JB540	<i>Tapesia acuformis</i> (R)	gggggccaccctacttcggtaa
SEQ ID NO:34	JB542	<i>Tapesia acuformis</i> (R)	ccactgatttagaggccgcaa

SEQ ID NO:35 is a forward sequencing primer.

SEQ ID NO:36 is a reverse sequencing primer.

SEQ ID NO:37 is a DNA sequence for the Internal Transcribed Spacer of *Tapesia acuformis* (syn. *P. herpotrichoides* R-type), NRRL accession no. B-21234, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nucleotides 1-30), Internal Transcribed Spacer 1 (nucleotides 31-263), 5.8 S rRNA gene (nucleotides 264-419), Internal Transcribed Spacer 2 (nucleotides 420-570), and 5' end of the large subunit rRNA gene (nucleotides 571-627).

SEQ ID NO:38 is a DNA sequence for the Internal Transcribed Spacer of *Tapesia yallundae* (syn. *P. herpotrichoides* W-type), NRRL accession no. B-21231, comprising in the 5' to 3' direction: 3' end of the small subunit rRNA gene (nucleotides 1-30), Internal Transcribed Spacer 1 (nucleotides 31-262), 5.8 S rRNA gene (nucleotides 263-418), Internal Transcribed Spacer 2 (nucleotides 419-569), and 5' end of the large subunit rRNA gene (nucleotides 570-626).

SEQ ID NO:39 is a consensus DNA sequence of the partial ITS region PCR-amplified from wheat extracts from three different locations (Barton, Elmdon, Teversham) infected with *Tapesia acuformis*, comprising in the 5' to 3' direction: partial Internal Transcribed Spacer 1 sequence, 5.8 S rRNA gene, and partial Internal Transcribed Spacer 2 sequence.

SEQ ID NO:40 is a consensus DNA sequence of the partial ITS region PCR-amplified from wheat extracts from three different locations (Barton, Elmdon, Teversham) infected with *Tapesia yallundae*, comprising in the 5' to 3' direction: partial Internal Transcribed Spacer 1 sequence, 5.8 S rRNA gene, and partial Internal Transcribed Spacer 2 sequence.

SEQ ID NO:41 is the nucleotide sequence of the gene for cytochrome b-559 in wheat chloroplast DNA (Hird, *et al.*, *Mol. Gen. Genet.* 203: 95-100 (1986)).

SEQ ID NOS:42-44 are the following oligonucleotide primers and probe useful for PCR-based detection of wheat chloroplast DNA:

Sequence Identifier	Oligo Name	Primer Name	Oligo Sequence (5'->3')
SEQ ID NO:42	Forward Primer	WCP2	cagtgcgatggctggctatt
SEQ ID NO:43	Reverse Primer	WCP3	cgttgatgaactgcattgct
SEQ ID NO:44	TaqMan TM Probe	WCP1	(VIC)-acggactagctgtacctactgtttttcttgggatc-(TAMRA)

The present invention provides unique DNA sequences that are useful in identifying and quantifying different pathotypes of plant pathogenic fungi. Particularly, the DNA sequences can be used as primers in TaqManTM PCR-based analysis for the identification of fungal pathotypes. The DNA sequences of the invention include primers and probes derived from Internal Transcribed Spacer (ITS) sequences of the ribosomal RNA gene regions of particular fungal pathogens, which are capable of identifying the particular pathogen. The 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*, *Pseudocercospora*, 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 particular 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. Once having determined the ITS sequences of a pathogen, these sequences can be aligned with other ITS sequences. In this manner, primers can be derived from the ITS sequences. That is, primers can be designed based on regions within the ITS sequences that contain the greatest differences in sequence among the fungal pathotypes. These sequences and primers based on these sequences can be used to identify specific pathogens.

Sequences of representative oligonucleotide primers derived from ITS sequences are disclosed in SEQ ID NOs:1-34. The sequences find use in TaqManTM quantitative PCR-based identification of the pathogens of interest.

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 Schlessner *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 *Mycosphaerella musicola*.

The TaqMan™ methodology has recently been used in medical research for the quantitative detection of herpes simplex virus (HSV) DNA in clinical samples (*J. Clin. Microbiol.* 37(6): 1941-7 (June, 1999)) in veterinary medicine for the detection of parasitic microbes in host animals (*J. Clin. Microbiol.* 37(5): 1329-31 (May, 1999)), and has been shown to be useful in the screening of ground beef for bacterial pathogens (*Appl. Envir. Micro.* 62(4): 1347-1353 (Apr., 1996)). Only recently has the TaqMan™ method been used for the identification and/or quantification of fungal pathogens in crop plants (*Phytopathology.* 89 (9): 796-804 (1999)).

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.

The ITS sequences are compared within each pathogen group to locate divergences that might be useful to test in TaqMan™ PCR assays to distinguish the different species and/or strains. From the identification of divergences, numerous primers are synthesized for each probe and tested in TaqMan™ assays. Templates used for TaqMan™ assays are firstly purified pathogen DNA, and subsequently DNA isolated from infected host plant tissue. Thus, it is possible to identify probe-primer combinations that are diagnostic, *i.e.* that identify one particular pathogen species or strain but not another species or strain of the same pathogen.

Preferred primer-probe 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-probe combinations that fulfill this criterion for *Tapesia yallundae* and *Tapesia acuformis*. The

primers and probes 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 or probe. Primer-probe combinations designed to a specific fungal pathogen's ITS region can be used in combination with a primer or probe made to a conserved sequence region within the ribosomal gene's coding region to detect amplification of species-specific PCR fragments. In general, primers should have a theoretical melting temperature (T_M) near 59°C to achieve good sensitivity and should be void of significant secondary structure and 3' overlaps between primer combinations. Primer pairs' T_M s are typically within 2°C of one another. 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 approximately 5-30 nucleotide bases long. Probes are generally designed to have a T_M 10°C higher than that of the primers. All wheat extractions contain the host wheat DNA as well as any fungal pathogen DNA present. Thus, an endogenous control assay targeting the wheat DNA can be run on extracts to account for any differences among sample extractions. The present invention describes a control assay targeting the cytochrome b-559 gene. The cytochrome b-559 gene is a conserved gene among wheat varieties, necessary for the life of the host plant. These control assays provide a control against false negatives. That is, a negative result for fungal DNA that could be attributed to inhibition of the PCR reaction is verified by an endogenous control assay. These control assays also provide a target against which the fungal DNA quantity is normalized for sample to sample comparison. The present invention describes the use of these control assays in reactions separate from the fungal pathogen assays and in multiplexed reactions. 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 container, such as tubes or vials. One of the containers may contain unlabeled or detectably labeled DNA primers. The labeled DNA primers 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 TaqManTM 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, and other consumables of the like.

The examples below show typical experimental protocols that can be used in the selection of suitable primer and probe sequences, the testing of primers and probes for selective and diagnostic efficacy, and the use of such primers and probes for disease and fungal isolate detection and quantification. Such examples are provided by way of illustration and not by way of limitation.

EXAMPLES

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) and 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 Fungal genomic DNA Extraction

Table 1 provides a listing of the fungal test 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 are 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
358	Tapesia acuformis	Novartis ¹	---
308	Tapesia acuformis	Novartis ¹	---
44643	Tapesia yallundae	ATCC ²	Germany
44614	Tapesia yallundae	ATCC ²	Ireland
60973	Tapesia acuformis	ATCC ²	Germany

42040	<i>Pseudocercospora herpotrichoides</i> var. <i>herpotrichoides</i>	ATCC ²	---
62012	<i>Pseudocercospora aestiva</i>	ATCC ²	Germany
24425	<i>Septoria nodorum</i>	ATCC ²	Montana
26517	<i>Septoria tritici</i>	ATCC ²	Minnesota
38699	<i>Septoria glycines</i>	ATCC ²	Illinois
22585	<i>Septoria passerini</i>	ATCC ²	Minnesota
26380	<i>Septoria avenae f.sp. triticea</i>	Bergstrom/ Ueng ³	Minnesota
52182	<i>Ceratobasidium cereale</i>	ATCC ²	Ohio
11404	<i>Drechslera sorokiniana</i>	ATCC ²	Minnesota
R-5391	<i>Fusarium culmorum</i>	Nelson ⁴	Germany
4551	<i>Fusarium moniliforme</i>	Novartis ¹	Indiana
R-8637	<i>Fusarium graminearum</i>	Nelson ⁴	Morocco
T-534	<i>Fusarium poae</i>	Nelson ⁴	Pennsylvania
18222	<i>Gerlachia nivalis</i>	ATCC ²	United Kingdom
093	<i>Microdochium nivale var. majus</i>	Novartis ¹	---

¹Novartis Agribusiness Biotechnology Research, Inc., Research Triangle Park, NC, USA

²American Type Culture Collection, Rockville, Maryland, USA

³Dr. Gary Bergstrom, Cornell University, and Dr. Peter Ueng, USDA-ARS, Beltsville, Maryland.

⁴Dr. Paul Nelson, Penn State University, State College, Pennsylvania

EXAMPLE 2: DNA Extraction from Wheat Stem Tissue

DNA is extracted from wheat stem tissues (identified in Table 2) as follows:

(1) Up to 25 wheat samples are placed on a clean surface. A sterile scalpel is used to cut the stem just above the first tiller or root. Another cut is made 4 cm above this cut. This 4 cm section constitutes the stem tissue sample which is pooled with the additional wheat samples for bulk maceration.

(2) The stem sample is placed in a Bioreba (Reinach, Switzerland) heavy duty plastic bag (cat#490100). The plant tissue is weighed, plastic bag with sample minus the tare (weight of the plastic bag).

(3) An equal volume (mL) of Muller Extraction Buffer (0.1% w/v Tween-80; 0.040 M Tris base; 0.15 M Sodium chloride; 0.1% w/v Bovine serum albumin (Pentex Fraction V); 0.01% w/v Sodium azide; 0.20 M EDTA; pH to 7.7, Store at 4°C) is added per weight (g) of wheat tissue. Tissue is macerated using a Bioreba Homex 6 homogenizer set at 70. The tissue is ground until fibrous.

(4) Extraction juice is aliquoted into Eppendorf tubes on ice.

(a) Extracts are boiled for 5 minutes.

(b) Boiled extracts are kept on ice. The boiled extract is microfuged for 5 minutes at 12,000 x G.

(c) 1:20 dilutions of the supernatant are made from the microfuged extract in dH₂O.

(d) The diluted extracts are stored on ice until ready to use.

Table 2: Origin of Wheat Samples Used in Primer and Probe Development

Sample	Description	Origin
W(Barton)	<i>Eyespot infected wheat</i>	United Kingdom
W(Elmdon)	<i>Eyespot infected wheat</i>	United Kingdom
W(Teversham)	<i>Eyespot infected wheat</i>	United Kingdom
R(Barton)	<i>Eyespot infected wheat</i>	United Kingdom
R(Elmdon)	Eyespot infected wheat	United Kingdom
R(Teversham)	Eyespot infected wheat	United Kingdom

Table 3: Origin of Wheat Samples Used for Assay Development

Sample	Description	Origin
1999 H	Uninfected wheat	Greenhouse
1999 #5	Eyespot infected wheat	Fairfield, WA
1999 #6	Eyespot infected wheat	Genesee, ID
1999 #8	<i>Eyespot infected wheat</i>	Walla Walla, WA
1999 #10	<i>Eyespot infected wheat</i>	Connell, WA

1999 #16	<i>Eyespot infected wheat</i>	Connell, WA
1999 #21	<i>Eyespot infected wheat</i>	Colfax, WA
1999 #23	<i>Eyespot infected wheat</i>	Colfax, WA
1999# 33	Eyespot infected wheat	Athena, OR
1999 #38	Eyespot infected wheat	Leland, ID
1999 #41	Eyespot infected wheat	Coulee City, WA
1999 #43	Eyespot infected wheat	Genesee, ID
1999 #46	Eyespot infected wheat	Leland, ID
1999 #47	Eyespot infected wheat	Leland, ID
1999 #54	Eyespot infected wheat	Wilur, WA
1999 #56	Eyespot infected wheat	Ritzville, WA
1999 #57	Eyespot infected wheat	Sprague, WA
1999 #72	Eyespot infected wheat	Grangeville, IO
1999 #73	Eyespot infected wheat	Grangeville, IO
1999 #74	Eyespot infected wheat	Grangeville, IO
1999 #80	Eyespot infected wheat	Ritzville, WA
1999 #82	Eyespot infected wheat	Edwall, WA
1999 #84	Eyespot infected wheat	Genesee, ID
1999 #93	Eyespot infected wheat	Davenport, WA
1999 #88	Eyespot infected wheat	Wilbur, WA
1999 #89	Eyespot infected wheat	Coulee City, WA
1999 #94	Eyespot infected wheat	Plummee, ID
1999 #95	Eyespot infected wheat	Pendleton, OR
1999 #96	Eyespot infected wheat	Harrington, WA
1999 #100	Eyespot infected wheat	Creston, WA
1999 #108	Eyespot infected wheat	Wilbur, WA
1999 #111	Eyespot infected wheat	Ferdinand, ID

EXAMPLE 3: Isolation and Sequencing of the Internal Transcribed Spacer (ITS)

Region DNA from *Tapesia yallundae* and *Tapesia acuformis* Infected Wheat Samples

Approximately 420-bp truncated ITS region fragments are PCR-amplified from wheat extracts identified in Table 2 infected with *Tapesia yallundae* using the *Tapesia yallundae*-specific primers JB537 (SEQ ID NO:31) and JB541 (SEQ ID NO:32). Similarly, the *Tapesia acuformis* truncated ITS fragments are amplified from *Tapesia acuformis*-infected wheat extracts using *Tapesia acuformis*-specific primers JB540 (SEQ ID NO:33) and JB542 (SEQ ID NO:34). Polymerase chain reactions are performed with the GeneAmp Kit from Perkin-Elmer (Foster City, CA; part no. N808-0009) using 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, containing 200 μM of each dTTP, dATP, dCTP, and dGTP, 50 pmol each primer, 2.5 units of *Taq* polymerase and 1 μl 1:10 diluted wheat extract in a final volume of 50 μl. Reactions are run at 94°C for 15 s and 1 min. at 75°C for 35 cycles in a Perkin-Elmer Model 9700 thermal cycler.

The PCR products are cloned into the pCR®2.1-TOPO TA-cloning vector using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA; part no. K4550-40) according to manufacturer's directions. Clones containing the ITS fragment inserts are sequenced using the TA cloning vector's FORWARD (5'-gtaaaacgacggccagt-3'; SEQ ID NO:35) and REVERSE (5'-caggaaacagctatgac-3'; SEQ ID NO:36) primers. Sequencing is performed on an ABI PRISM 377™ DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, California).

EXAMPLE 4: Synthesis and Purification of Oligonucleotides

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

EXAMPLE 5: Selection of Species-Specific Primers and Probes

A multiple sequence alignment is made of ITS region consensus sequences of *Tapesia yallundae* (SEQ ID NO:40) and *Tapesia acuformis* (SEQ ID NO:39) obtained from infected wheat tissue as described in Example 3. Also included in the alignment are ITS region sequences from *Tapesia yallundae* and *Tapesia acuformis* fungal DNAs referenced in U.S. Patent No. 5,585,238 (SEQ ID NO:37 and SEQ ID NO:38, respectively). PCR primers and TaqMan™ probes are designed to the regions that contain the greatest differences in sequence between the fungal species. This produces primers and probes designed to be specific to either *Tapesia acuformis* or *Tapesia yallundae*. The oligonucleotide primers and probes shown below in Tables 3 and 4 are synthesized according to Example 4. The

previously described (U.S. Patent No. 5,585,238) *Tapesia yallundae*-specific primers JB537 (SEQ ID NO:31) and JB541 (SEQ ID NO:32), and *Tapesia acuformis*-specific primers JB540 (SEQ ID NO:33) and JB542 (SEQ ID NO:34) are also synthesized. In addition, the ribosomal gene-specific primers ITS1 (SEQ ID NO:1) and ITS4 (SEQ ID NO:2) published by 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 4: Primers and Probes for TaqMan™ Amplification of *Tapesia acuformis* DNA

Sequence Identifier	Oligo Name	Target	Oligo Sequence (5'->3')
SEQ ID NO:1	ITS1	Fungal 18S rDNA	tccgtaggtgaacctgcgg
SEQ ID NO:2	ITS4	Fungal 25S rDNA	tctccgcttattgatatgc
SEQ ID NO:12	J112R	<i>Tapesia acuformis</i> (R)	tcaagggtggaggtctgaacc
SEQ ID NO:13	J100R	<i>Tapesia acuformis</i> (R)	gggccaccctacttcggtaa
SEQ ID NO:14	J101R	<i>Tapesia acuformis</i> (R)	gaaatcctgggggccaccctacttc
SEQ ID NO:15	J102R	<i>Tapesia acuformis</i> (R)	cctgggggccaccctact
SEQ ID NO:16	J113R	<i>Tapesia acuformis</i> (R)	gccaccctacttcggttaagggt
SEQ ID NO:17	J114R	<i>Tapesia acuformis</i> (R)	caccctacttcggttaagggttagagtc
SEQ ID NO:18	J115R	<i>Tapesia acuformis</i> (R)	aggtaatttattcaagggtggaggt
SEQ ID NO:19	J116R	<i>Tapesia acuformis</i> (R)	aggtaatttattcaagggtggaggtc
SEQ ID NO:20	J117R	<i>Tapesia acuformis</i> (R)	aaggtaatttattcaagggtggaggt

SEQ ID NO:21	J118R	<i>Tapesia acuformis</i> (R)	ttattcaaggggtggagggtctgg
SEQ ID NO:22	J119R	<i>Tapesia acuformis</i> (R)	tattcaaggggtggagggtctgga
SEQ ID NO:23	J120R	<i>Tapesia acuformis</i> (R)	cctgccaagcaacaaggta
SEQ ID NO:24	J121R	<i>Tapesia acuformis</i> (R)	(FAM)-cgggcctctcggagaagcctgg-(TAMRA)
SEQ ID NO:25	J122R	<i>Tapesia acuformis</i> (R)	cctacttcggtaaggtttagagtcgt
SEQ ID NO:26	J123R	<i>Tapesia acuformis</i> (R)	tctccgagaggcccgac
SEQ ID NO:27	J124R	<i>Tapesia acuformis</i> (R)	(FAM)-aagcctggccagacctccacc-(TAMRA)
SEQ ID NO:28	J125R	<i>Tapesia acuformis</i> (R)	aaggatcattaatagagcaatggatagac
SEQ ID NO:29	J126R	<i>Tapesia acuformis</i> (R)	(FAM)-cgccccgggagaaatcctgg-(TAMRA)
SEQ ID NO:30	J127R	<i>Tapesia acuformis</i> (R)	tgggggccaccctacttc
SEQ ID NO:33	JB540	<i>Tapesia acuformis</i> (R)	gggggccaccctacttcggtaa
SEQ ID NO:34	JB542	<i>Tapesia acuformis</i> (R)	ccactgatttagaggccgcaa

Table 5: Primers and Probes for TaqMan™ Amplification of *Tapesia yallundae* DNA

Sequence Identifier	Primer Name	Target	Oligo Sequence (5'→3')
SEQ ID NO:1	ITS1	Fungal 18S rDNA	tccgtaggtgaacctgagg
SEQ ID NO:2	ITS4	Fungal 25S rDNA	tcctccgcttattgatatgc

SEQ ID NO:3	J103W <i>Tapesia yallundae</i> (W)	ggctaccctacttggtag
SEQ ID NO:4	J104W <i>Tapesia yallundae</i> (W)	cctgggggctaccctacttg
SEQ ID NO:5	J105W <i>Tapesia yallundae</i> (W)	gggggctaccctacttggtag
SEQ ID NO:6	J106W <i>Tapesia yallundae</i> (W)	tgggggctaccctacttggtag
SEQ ID NO:7	J107W <i>Tapesia yallundae</i> (W)	(FAM)-ttagagtcgtcaggcctctcggagaagc- (TAMRA)
SEQ ID NO:8	J108W <i>Tapesia yallundae</i> (W)	atttattcaagggtggaggctctga
SEQ ID NO:9	J109W <i>Tapesia yallundae</i> (W)	aagggtggagggtctgaaccag
SEQ ID NO:10	J110W <i>Tapesia yallundae</i> (W)	aagggtggagggtctgaacca
SEQ ID NO:11	J111W <i>Tapesia yallundae</i> (W)	caagggtggagggtctgaacc
SEQ ID NO:31	JB537 <i>Tapesia yallundae</i> (W)	gggggctaccctacttggtag
SEQ ID NO:32	JB541 <i>Tapesia yallundae</i> (W)	ccactgatttagaggccgcgag

EXAMPLE 6: Initial Screening of the Primer-Probe Library

The species-specific primer libraries designed in Example 5 are tested in initial TaqMan™ screens. Primer and probe combinations are tested for their ability to amplify from the target pathogen's DNA. All other reaction conditions are held constant (1X TaqMan™ Universal Master Mix (Perkin Elmer, Norwalk, CT; part no. N430-4447), 200 nM each primer, 100 nM probe, 0.04 ng/μL fungal target genomic DNA, thermal cycling: 50°C for 2 min., 95°C for 10 min., 40 cycles of 95°C for 15 s, 60°C for 60 s). Pathogen-specific primers and probes are determined by identifying those that best amplify the targeted DNA.

EXAMPLE 7: TaqMan™ Primer Optimization

Once a primer pair specific for the targeted pathogen's DNA has been identified, the primer concentrations are optimized in a single TaqMan™ run. A matrix of different concentrations of the forward primer are run against those of the reverse primer with all other reaction conditions held constant (1X TaqMan™ Universal Master Mix (Perkin Elmer), 100 nM probe, 0.4 ng/μL fungal target genomic DNA, thermal cycling: 50°C for 2 min., 95°C for 10 min., 40 cycles of 95°C for 15 s, 60°C for 60 s).

EXAMPLE 8: TaqMan™ Probe Optimization

Once optimal primer concentrations are determined as in Example 7, the probe concentration is optimized. With primers at their optimal concentrations, different concentrations of probe are run in a typical TaqMan™ run. The probe concentration that gives the best signal in reporting the PCR amplification is chosen. The optimal primers and probe for quantification of *Tapesia acuformis* and *Tapesia yallundae* are recorded along with their optimal reaction concentrations (Tables 5 and 6, respectively). The *Tapesia acuformis* and *Tapesia yallundae* assays are established with an annealing temperature of 60°C over 35 cycles.

Table 6: Primer and Probe Combinations Specific for *Tapesia acuformis*

Target	Oligo	Sequence Identifier	Primer Name	Optimized Concentration (nM)
<i>Tapesia acuformis</i> (R)	Forward Primer	SEQ ID NO:14	J101R	50
	Reverse Primer	SEQ ID NO:18	J115R	900
	TaqMan™ Probe	SEQ ID NO:24	J121R	700

Table 7: Primer and Probe Combinations Specific for *Tapesia yallundae*

Target	Oligo	Sequence Identifier	Primer Name	Optimized Concentration (nM)
<i>Tapesia yallundae</i> (W)	Forward Primer	SEQ ID NO:3	J103W	300
	Reverse Primer	SEQ ID NO:8	J108W	300
	TaqMan™ Probe	SEQ ID NO:7	J107W	200

EXAMPLE 9: Determination of TaqMan™ Assay Specificity to Fungal Genomic DNA

The TaqMan™ assay is validated against a panel of DNA from other cereal pathogens for cross-reactivity (Table 1). TaqMan™ reactions are prepared using the optimal primer and probe concentrations as determined in Examples 7 and 8 and tested against 0.2 ng/μL of the genomic DNA from the cereal pathogens as prepared in Example 1. Depending on the results, changes are made to the thermal cycling parameters to make the assay more stringent. These include changing the annealing/extension temperature or the number of cycles in the run. A successful TaqMan™ assay is sensitive to sub-picogram amounts of target DNA without any cross-reactivity to the panel of cereal pathogens or the plant DNA. In Table 8 results of the *Tapesia acuformis* (R-type) and *Tapesia yallundae* (W-type) assays documented under Example 8 are shown. C_T values are used to show amplification among isolates screened. Those isolates with a C_T value of 35 give no amplification with the assays.

Table 8: Results of *Tapesia acuformis* TaqMan™ Assay on Fungal Genomic DNA Samples

Isolate	Organism	C _T Value	
		R-type assay	W-type assay
358	<i>Tapesia acuformis</i>	18.52	35
308	<i>Tapesia acuformis</i>	18.65	35
44643	<i>Tapesia yallundae</i>	35	
44614	<i>Tapesia yallundae</i>	35	17.18
60973	<i>Tapesia acuformis</i>	31.36	35
42040	<i>Pseudocercospora herpotrichoides</i> var. <i>herpotrichoides</i>	35	18.7
62012	<i>Pseudocercospora aestiva</i>	35	35
24425	<i>Septoria nodorum</i>	35	35
26517	<i>Septoria tritici</i>	35	35
38699	<i>Septoria glycines</i>	35	35
22585	<i>Septoria passerini</i>	35	35
26380	<i>Septoria avenae f.sp. triticea</i>	35	35
52182	<i>Ceratobasidium cereale</i>	35	35

11404	Drechslera sorokiniana	35	35
R-5391	Fusarium culmorum	35	35
4551	Fusarium moniliforme	35	35
R-8637	Fusarium graminearum	35	35
T-534	Fusarium poae	35	35
18222	Gerlachia nivalis	35	35
093	Microdochium nivale var. majus	35	35

Note: C_T value or threshold cycle, represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. The Sequence Detection software generates a Standard Curve of C_T vs. (LogN) Starting Copy Number for all standards and then determines the starting copy number of unknowns by interpolation.

EXAMPLE 10: Determination of TaqMan™ Assay Specificity to Pathogen in Infected Wheat

Wheat samples are identified as *Tapesia acuformis* and/or *Tapesia yallundae* infected based on analysis using the assays described in Example 3. Wheat samples are also tested using the primer combinations listed in Table 6 and the PCR conditions in Example 8. Using Sequence Detection Systems software (Perkin Elmer-Applied Biosciences), the amplification of pathogen DNA from the wheat samples is quantified against a standard curve of the fungal target's genomic DNA (Table 9). Results for the *Tapesia acuformis* specific assay are presented in Table 10. DNA from *Tapesia acuformis* is detected and quantified in all infected samples. Results for the *Tapesia yallundae* specific assay are presented in Table 11. DNA from *Tapesia yallundae* is detected and quantified in all infected samples. No cross-reactivity is observed in uninfected wheat tissue for either assay.

Table 9: Standard Curve of *Tapesia acuformis* and *T. yallundae* Genomic DNAs Run in Duplicate Against the R-type and W-type Assays, Respectively

R-type Assay	W-type Assay
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<i>Tapesia acuformis</i> #308 DNA	C _T Value	<i>Tapesia yallundae</i> #42040 DNA	C _T Value
5 ng	18.57 18.38	5 ng	18.13 17.92
500 pg	21.3 21.35	500 pg	21.83 22.02
50 pg	23.57 24.27	50 pg	25.26 25.37
5 pg	27.82 27.89	5 pg	29.53 29.88
500 fg	31.47 31.17	500 fg	33.32 35
50 fg	34.13 34.01	No Template Control	35 35
No Template Control	35 35		

Table 10: Results of the *Tapesia acuformis* TaqMan™ Assay on Wheat Extractions. Samples Are Run in Duplicate and are Documented with Results of Conventional PCR Assays

Sample Number	TaqMan™ Results for <i>Tapesia acuformis</i> assay				PCR Testing Results (0 to +5 scale)	
	CT Value	Template (pg)	Standard Deviation	Mean (pg)	<i>T. acuformis</i>	<i>T. yallundae</i>
H	35	0	0	0	-	-
	35	0	0	0		
6	35	2.50E-02	0	0.02	-	-
	35	2.50E-02	0	0.02		
57	31.07	4.60E-01	0.03	0.44	+	-
	31.20	4.20E-01	0.03	0.44		

47	31.13	4.40E-01	0.14	0.54	+	-
	30.62	6.40E-01	0.14	0.54		
84	33.68	7.00E-02	0.01	0.06	+	-
	33.96	5.70E-02	0.01	0.06		
23	29.42	1.50E+00	0.28	1.71	++	-
	29.10	1.90E+00	0.28	1.71		
46	28.67	2.60E+00	0.44	2.90	++	-
	28.37	3.20E+00	0.44	2.90		
73	30.54	6.70E-01	0.06	0.72	++	-
	30.37	7.60E-01	0.06	0.72		
21	27.34	6.80E+00	2.28	5.15	+++	-
	28.24	3.50E+00	2.28	5.15		
38	30.04	9.70E-01	0.71	1.47	+++	-
	29.05	2.00E+00	0.71	1.47		
43	26.12	1.60E+01	0.97	16.94	+++	-
	26.01	1.80E+01	0.97	16.94		
41	24.07	7.20E+01	19.75	57.57	++++	-
	24.75	4.40E+01	19.75	57.57		
72	28.01	4.20E+00	0.29	3.96	++++	-
	28.16	3.80E+00	0.29	3.96		
74	26.01	1.80E+01	3.03	19.75	++++	-
	25.71	2.20E+01	3.03	19.75		
5	26.72	1.10E+01	1.50	9.51	+++++	-
	27.03	8.50E+00	1.50	9.51		
82	26.74	1.00E+01	1.29	9.51	+++++	-
	27.01	8.60E+00	1.29	9.51		
93	26.05	1.70E+01	2.12	18.68	+++++	+
	25.82	2.00E+01	2.12	18.68		
96	24.07	7.10E+01	3.75	68.50	+++++	++
	24.18	6.60E+01	3.75	68.50		

Table 11: Results of the *Tapesia yallundae* TaqMan™ Assay on Wheat Extractions. Samples Are Run in Duplicate and are Documented with Results of Conventional PCR Assays

Sample Number	TaqMan™ Results for <i>Tapesia acuformis</i> assay				PCR Testing Results (0 to +5 scale)	
	CT Value	Template (pg)	Standard Deviation	Mean (pg)	<i>T. acuformis</i>	<i>T. yallundae</i>
H	35	0	0	0	-	-
	35	0	0	0		
6	35	0	0	0	-	-
	35	0	0	0		
82	33.41	4.5E-01	0.07	0.40	+++++	-
	33.78	3.6E-01				
94	33.29	5.2E-01	0.21	0.37	+	+
	34.68	2.2E-01				
108	34.41	2.6E-01	0	0.26	+++	+
	34.40	2.7E-01				
111	33.21	5.4E-01	0.02	0.53	++	+
	33.28	5.2E-01				
33	24.67	9.1E+01	37.45	64.30	++	++
	26.13	3.8 E+01				
54	28.09	1.2E+01	6.31	16.10	+++	++
	27.14	2.1E+01				
80	26.43	3.1E+01	3.62	34.03	++++	++
	26.18	3.7E+01				
95	29.98	3.8E+00	0.08	3.7	-	++
	30.03	3.6E+00				
100	27.16	2.0E+01	1.40	21.32	+++	+++
	27.01	2.2E+01				
8	25.63	5.1E+01	9.96	57.91	+	+++
	25.22	6.5E+01				

10	22.36	3.6E+02	79.1	418.46	++	+++
	21.91	4.7E+02				
16	23.77	1.6E+02	6.18	150.78	++	++++
	23.87	1.5E+02				
56	25.14	6.8E+01	2.26	66.56	++++	++++
	25.22	6.5E+01				
88	24.48	1.0E+02	21.89	85.90	++	++++
	25.09	7.0E+01				
89	23.87	1.5E+02	16.48	157.85	++++	+++++
	23.63	1.7E+02				

EXAMPLE 11: An Endogenous Control To Be Used With The Fungal Pathogen TaqMan™ Assays

All wheat extractions contain the host wheat DNA as well as any fungal pathogen DNA present. Thus, an endogenous control assay targeting the wheat DNA is run on extracts to account for any differences among sample extractions. These assays provide a control against false negatives. That is, a negative result for fungal DNA that could be attributed to inhibition of the PCR reaction is verified by this endogenous control assay. These assays also provide a target against which the fungal DNA quantity is normalized for sample to sample comparison.

EXAMPLE 12: Selection Of Endogenous Control Primers And Probes

Primers and probes for the amplification and detection of wheat chloroplast DNA are drawn to the coding sequence of the cytochrome b-599 gene (SEQ ID NO:41). Selection of primer and probe sequences is performed using the ABI Primer Express program (PE Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. This program selects TaqMan™ primer and probe sets optimized by melting temperature, secondary structure, base composition, and amplicon length. From the sets chosen by the software, a best set is selected by manually finding primers with the fewest number of thermodynamically stable bases at the 3' end. The primer/probe set chosen for the amplification of wheat DNA as an endogenous control is documented in Table 12. These are synthesized as in Example 4.

Table 12: Primer And Probe Combinations For An Endogenous Control Reaction Targeting Wheat (*Triticum aestivum*) Chloroplast DNA

Oligo	Sequence Identifier	Primer Name	Oligo Sequence (5'->3')
Forward Primer	SEQ ID NO:42	WCP2	cagtgcgatggctggctatt
Reverse Primer	SEQ ID NO:43	WCP3	cgttggatgaactgcattgct
TaqMan™ Probe	SEQ ID NO:44	WCP1	(VIC)-acggactagctgtacctactgttttttctgggatc-(TAMRA)

EXAMPLE 13: Use Of A TaqMan™ Assay To Quantify Wheat DNA In Wheat Extractions

Extractions of wheat tissue are made as in Example 2. The assay presented in Example 11 is run against these tissues as follows: Reactions are prepared in thin-walled optical grade PCR tubes (PE Applied Biosystems, Foster City, CA, USA). Reaction mixtures are made by bringing forward and reverse primer concentrations to 900 nM and probe concentration to 250 nM in a 1X solution of TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA). One microliter of 1:20 diluted wheat extract is added. Additionally, cross-reactivity with fungal DNA is tested by adding 1 µL of 5 ng/µL fungal DNA preparation. The reactions are carried out in a ABI 7700 instrument (PE Applied Biosystems, Foster City, CA, USA), thermal cycling: 50°C for 2 min., 95°C for 10 min., 40 cycles of 95°C for 15 s, 60°C for 60 s). The ABI 7700 software determines the CT value at which the fluorescence of each reaction reaches a threshold value of 0.4. This data is presented in Table 13. The CT values presented correspond inversely with the amount of wheat target DNA present in each sample. Samples in which a CT of 40 are reported show no amplification. Table 13 shows that the endogenous control assay detects the cytochrome b-559 gene in multiple varieties of wheat. The TaqMan™ assay for wheat chloroplast DNA also shows that different amounts of host DNA are present in each sample. By using dilutions of target DNA, a standard curve can be generated as described in Example 10 against which the wheat DNA can be quantified.

Table 13: CT Values Reported For A TaqMan™ Assay Targeting Wheat Chloroplast DNA In Wheat And Fungal DNA Extractions

Sample Number	Wheat Variety	CT Value
6	Madsen	17.17
57	Madsen	19.48
73	Lambert	20.71
21	Brundage	18.9
41	Eltan	20.23
13	Mixed	19.99
5	Madsen	19.19
5 ng Tapesia acuformis DNA #308		40
NTC		40

Example 14: Multiplexing Of TaqMan™ Assays For Fungal Pathogens And Control Assay For Host DNA

The reaction presented in Example 13 is multiplexed with reactions for quantification of fungal DNA such that both tests take place in the same reaction tube. The probe and primers for *Tapesia acuformis* documented in Table 6 at their optimized concentrations are added to the reactions described in Example 13. These reactions are run as described on infected wheat tissue. The data presented here show that TaqMan™ fungal pathogen assays may be run in the same reaction tube as an endogenous control reaction for the wheat tissue.

Table 14: CT Values Reported For A TaqMan™ Assay Targeting Wheat Chloroplast DNA In Wheat DNA Extractions

Sample Number	Wheat assay CT Value	R-type Assay CT Value	Calculated	PCR Testing Results (0 to +5 scale)
---------------	-------------------------	--------------------------	------------	--

			Concentration (pg)	<i>T.</i> <i>acuformis</i>	<i>T.</i> <i>yallundae</i>
6	17.09	40	0	-	-
41	27.70	20.65	24.3	++++	-
13	30.9	19.99	3.69	+++++	+

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.

SEQUENCE LISTING

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YALLUNDAE AND TAPESIA ACUFORMIS

<130> PB/5-31084A

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<151> 1999-08-10

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<151> 1999-12-01

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- 4 -

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<210> 33
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<210> 40

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- 10 -

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 gactagctgt acctactgtt tttttcttgg gatcaatatc agcaatgcag ttcattccaac 480
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<220>
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<210> 43
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<400> 43
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<210> 44
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 <213> Artificial Sequence

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 <223> Description of Artificial Sequence: WCP1

<400> 44
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What is claimed is:

1. An oligonucleotide primer selected from the group consisting of SEQ ID NOs:3-6, 8-23, 25-26, 28, 30, 42, and 43.
2. An oligonucleotide primer according to claim 1, wherein said primer is selected from the group consisting of SEQ ID NOs:3-6, 8-23, 25-26, 28, and 30.
3. A pair of oligonucleotide primers, wherein at least one of said primers is the oligonucleotide primer of claim 2.
4. A pair of oligonucleotide primers according to claim 3, wherein said pair consists of SEQ ID NO:14 and SEQ ID NO:18.
5. A pair of oligonucleotide primers according to claim 3, wherein said pair consists of SEQ ID NO:3 and SEQ ID NO:8.
6. An oligonucleotide primer according to claim 1, wherein said primer is selected from the group consisting of SEQ ID NOs:42 and 43.
7. A pair of oligonucleotide primers, wherein at least one of said primers is the oligonucleotide primer of claim 6.
8. A pair of oligonucleotide primers according to claim 7, wherein said pair consists of SEQ ID NO:42 and SEQ ID NO:43.
9. A method for the detection of a fungal pathogen, comprising:
 - (a) isolating DNA from a plant leaf infected with a pathogen;
 - (b) subjecting said DNA to polymerase chain reaction amplification using at least one primer according to claim 2; and
 - (c) detecting said fungal pathogen by visualizing the product or products of said polymerase chain reaction amplification.

10. The method of claim 9, wherein said fungal pathogen is selected from *Tapesia yallundae* and *Tapesia acuformis*.
11. A method for the detection of a fungal pathogen, comprising:
 - (a) isolating DNA from plant tissue infected with said fungal pathogen;
 - (b) amplifying a part of the Internal Transcribed Spacer sequence of said fungal pathogen using said DNA as a template in a polymerase chain reaction with a pair of primers according to claim 3; and
 - (c) detecting said fungal pathogen by visualizing the amplified part of the Internal Transcribed Spacer sequence.
12. The method of claim 11, wherein said fungal pathogen is selected from *Tapesia yallundae* and *Tapesia acuformis*.
13. A diagnostic kit used in detecting a fungal pathogen, comprising the primer of claim 2.
14. A diagnostic kit used in detecting a fungal pathogen, comprising the pair of primers of claim 3.
15. A method for the detection of wheat DNA, comprising:
 - (a) isolating DNA from wheat tissue infected with a pathogen;
 - (b) subjecting said DNA to polymerase chain reaction amplification using a pair of primers according to claim 7; and
 - (c) detecting said wheat DNA by visualizing the product or products of said polymerase chain reaction amplification.
16. An oligonucleotide probe for use in amplification-based detection of a fungal Internal Transcribed Spacer sequence, wherein said probe comprises:
 - (a) a nucleotide sequence complementary to at least 10 consecutive nucleotides of a sequence selected from the group consisting of: ITS1 of *Tapesia yallundae*, ITS2 of *Tapesia yallundae*, ITS1 of *Tapesia acuformis* and ITS2 of *Tapesia acuformis*;
 - (b) a fluorescent reporter dye at a 5' end of said nucleotide sequence; and
 - (c) a quencher dye at a 3' end of said nucleotide sequence.

17. An oligonucleotide probe according to claim 16, wherein said nucleotide sequence is complementary to at least 10 consecutive nucleotides of a sequence selected from the group consisting of: nucleotides 31-263 of SEQ ID NO:37, nucleotides 420-570 of SEQ ID NO:37, nucleotides 31-262 of SEQ ID NO:38, and nucleotides 419-568 of SEQ ID NO:38.
18. An oligonucleotide probe according to claim 17, wherein said nucleotide sequence is selected from the group consisting of: SEQ ID NO:7, SEQ ID NO:24, SEQ ID NO:27, and SEQ ID NO:29.
19. An oligonucleotide probe for use in amplification-based detection of wheat DNA, wherein said probe comprises:
- (a) a nucleotide sequence complementary to at least 10 consecutive nucleotides of SEQ ID NO:41;
 - (b) a fluorescent reporter dye at a 5' end of said nucleotide sequence; and
 - (c) a quencher dye at a 3' end of said nucleotide sequence.
20. An oligonucleotide probe according to claim 19, wherein said nucleotide sequence is SEQ ID NO:44.
21. An oligonucleotide primer pair/probe set for quantifying fungal DNA, wherein said primer pair consists of the pair of primers according to claim 4 and the probe is SEQ ID NO:24.
22. An oligonucleotide primer pair/probe set for quantifying fungal DNA, wherein said primer pair consists of the pair of primers according to claim 5 and the probe is SEQ ID NO:7.
23. An oligonucleotide primer pair/probe set for quantifying wheat DNA, wherein said primer pair consists of the pair of primers according to claim 8 and the probe is SEQ ID NO:44.