A PCR-Based Assay by Sequence-Characterized DNA Markers for the Identification and Detection of *Aphanomyces euteiches*

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ABSTRACT

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Polymerase chain reaction (PCR) products were identified and amplified from isolates of *Aphanomyces euteiches* and *A. cochlioides*. The products were cloned and sequenced, and the data were used to design pairs of extended PCR primers to amplify sequence-characterized DNA markers. The primer pair OPC7-FS-30 and OPC7-RS-25 amplified a single 1,332-bp product from all isolates of *A. euteiches* that were not amplified from any other isolates tested. A single 718-bp product was selectively amplified only from isolates of *A. cochlioides*

with the primer pair OPB10-FS-25 and OPB10-RS-25. *A. euteiches* was detected in roots of several varieties of field-grown peas collected from a root rot trial site. PCR also detected *A. euteiches* in the organic fraction of field soil samples. Both pairs of extended primers were used in a multiplex reaction to unambiguously discriminate between *A. euteiches* and *A. cochlioides*. Both pairs of primers were used in two-step PCR reactions in which annealing and extension was done in a single step at 72°C. This reduced the time required for amplification of the diagnostic PCR product and its resolution by electrophoresis to less than 3 h.

Additional keywords: *Aphanomyces* root rot, sequence-characterized amplified region.

Root rot of peas caused by the oomycete *Aphanomyces euteiches* Dreschs., is the most globally destructive soilborne disease of peas (*Pisum sativum* L.) (22). The disease is prevalent throughout North America, Europe, Australia, New Zealand, and Japan (7,8,22). In the United States, annual losses in pea production in Wisconsin and Minnesota attributed to *A. euteiches* have been estimated at 10%, although individual fields can suffer complete loss (7). The pathogen causes root rot on other legume species including alfalfa (*Medicago sativum* L.) (5) and snapbean (*Phaseolus vulgaris* L.) (25). Previous reports have demonstrated that *A. euteiches* is a pathogen that exhibits considerable variation between isolates in virulence (6), pathogenicity (5,10,17–20), and DNA polymorphisms (17–19).

Often it is difficult to accurately diagnose *Aphanomyces* root rot due to the confounding presence of other soilborne organisms such as *Pythium* and *Rhizoctonia*. An artificial medium has been developed that uses metalaxyl, benomyl, and vancomycin (MBV) to selectively enhance the recovery of *A. euteiches* from infected plant tissue (24). The use of MBV medium requires several days and a confirmation based on microscopic analysis of the pathogen culture, and the selectivity of this medium is reduced when metalaxyl-tolerant isolates of *Pythium* are present (24). Alternatively, Kraft and Boge (11) have developed a polyclonal antiserum for the diagnosis of *Aphanomyces* root rot based on

enzyme-linked immunosorbent assay. The antiserum is highly specific for *A. euteiches*, but the fungus cannot be detected in roots until 3 days after infection (12).

A practice employed to limit losses from *Aphanomyces* root rot is the avoidance of heavily infested fields (3,7,22,28). Previous studies have demonstrated that the severity of root rot in peas is closely related to population densities of the pathogen in soil (3,26,27). The technique for assaying soil for the presence of the pathogen involves testing bulked field soil samples for the ability to cause root rot in pea seedlings under greenhouse conditions (28). A wet-sieving/baiting technique is used for more closely approximating inoculum potential of *A. euteiches* in specific sites under field locations (13,21). This method can be used to detect statistically significant differences in inoculum potential between areas within a field, fields, and locations (13). Oospores can be directly quantified microscopically from soil samples by staining with lactophenol cotton blue (3), however, this procedure is extremely time consuming and laborious. Additionally, oospores are typically found in the organic fraction of soil (21,22), making it very difficult to accurately quantify their numbers in soil by microscopic techniques.

A specific assay by polymerase chain reaction (PCR) could facilitate the detection of *A. euteiches* in both plant tissue and soil. Many plant pathogens, including several species of oomycetes, have been detected with PCR primers that are specific for the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (2,4,14–16,33). Lee et al. (14) designed four PCR primers based on ITS sequences that could be used to unambiguously discriminate between *Phytophthora capsici* (Leonian), *P. cinnamomi* (Rands), *P. megakarya* (Brasier and Griffin), and *P. palmivora* (E. J. Butler). Similarly, a PCR probe specific for the ITS region was designed that selectively amplified target DNA from all tested isolates of *Pythium ultimum* (Trow) but not from any other *Pythium* spp. (16). Oligonucleotides that are specific for several different *Pythium* and *Phytophthora* spp. have been bound

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to nylon membranes and probed with labeled PCR products corresponding to ITS sequences (15). This approach, using multiple DNA samples arrayed on a membrane in a reverse dot blot format, has been successfully used to identify oomycetes at both the species and genus level (15).

Recently, primers corresponding to conserved domains in either the ITS region or the actin gene coding region were used to distinguish *A. cochlioides* (Dreschs), a pathogen that infects sugar beet (*Beta vulgaris* L.) (22), from other sugar beet pathogens or closely related organisms. *A. cochlioides* was distinguished from other sugar beet pathogens by restriction digestion of PCR products generated with ITS primers (32). Both *A. cochlioides* and *A. euteiches* amplified a single 0.9-kb product using primers specific to the actin gene (32). However, the two species could be distinguished when the 0.9-kb product was digested with a mixture of three different restriction enzymes.

Our objective in this study was to develop a PCR assay that could discriminate isolates of *A. euteiches* from other closely related species. We chose to develop a system based on sequence-characterized amplified regions (SCARs) (23), and investigated the use of PCR for detecting the presence of the fungus in plant roots and soil samples. SCARs are produced with primers designed based on sequence information obtained from cloned random amplified polymorphic DNA (RAPD) (23). We report here, the design of PCR primers that amplify a single SCAR from all tested isolates of *A. euteiches*. The primers were used to detect the presence of the pathogen in both the roots of peas grown in natural field soil and in naturally infested soil samples with a rapid two-step thermocycling profile. We have also designed a pair of SCAR primers that amplify a single PCR product from all tested isolates of *A. cochlioides*. The complete nucleotide sequences of both SCAR products have been determined.

MATERIALS AND METHODS

Isolates and DNA extraction. Isolates in Table 1 were cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit). To obtain mycelia for DNA extractions, a 5-mm hyphal plug from the edge of an actively growing culture was used to seed 100 ml of potato dextrose broth (PDB; Difco). Cultures were grown for 7 days at 22°C in a rotary shaker. The culture filtrate was removed and the mycelial mats were washed three times in 250 ml of wash buffer (100 mM Tris (pH 8.0), 150 mM NaCl, and 5 mM EDTA). Mycelial mats were lyophilized and stored at -80°C prior to extraction of nucleic acids.

Two-hundred nanograms of lyophilized mycelia were ground in N₂ (l). Powdered mycelia were vortexed for 2 min in 3 ml of extraction buffer (200 mM Tris [pH 8.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate). The aqueous phase was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and an extraction in an equal volume of chloroform/isoamyl alcohol (24:1). Total nucleic acid was precipitated with 0.5 vol of 7.5 M ammonium acetate and 3 vol of 95% ethanol. After collecting the precipitate by centrifugation, the pellet was washed in 70% ethanol and resuspended in Tris-EDTA ([TE] 10 mM Tris and 1 mM EDTA [pH 8.0]). RNA was removed from the preparations by the addition of RNAase (RNAase +; 5'→3' Inc., Boulder, CO). The sample was extracted with organic solvents and DNA was precipitated with ethanol and salt as mentioned above. DNA concentrations were determined by ultraviolet absorbance at 260 nm, and DNA was diluted to 10 ng/μl for use in PCR reactions. DNA was prepared as described above for all isolates listed in Table 1 except *P. infestans*-180 and *P. infestans*-543, for which purified DNA was provided by H. Judelson (University of California, Riverside).

RAPD PCR. Approximately 50-ng samples of DNA were used in 25-μl (vol) amplification reactions containing 15 mM Tris-HCl

(pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 μM each of dATP, dCTP, dTTP, and dGTP, 15 ng of a single RAPD 10-mer primer (Operon Inc., Alameda, CA), and 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR reaction was done in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with the following amplification conditions: an initial 9 min denaturation at 95°C followed by 40 cycles of 1 min at 94°C, 1 min at 37°C, 1 min at 72°C, and a final extension step for 7 min at 72°C. Each DNA sample × primer combination was repeated at least twice. Entire PCR reactions (25 μl) were resolved by electrophoresis in 1.4% agarose gels (Ultrapure Agarose, Life Technologies Inc., Gaithersburg, MD) in 1× Tris-borate-EDTA buffer (90 mM Tris-borate and 2 mM EDTA). Gels were stained with ethidium bromide, and PCR products were visualized under ultraviolet light.

Cloning and sequencing PCR products. PCR products from RAPD reactions were visualized and products that were present only in every isolate of *A. euteiches* or every isolate of *A. cochlioides* were identified and excised from agarose gels. DNA was gel purified using the GeneClean kit (Bio 101 Inc., Vista, CA). The purified PCR products were cloned into the vector pCR2.1 TOPO (TOPO TA; Invitrogen Corp., Carlsbad, CA), according to manufacturer's instructions. Plasmid DNA was isolated from transformed *Escherichia coli* by the alkaline lysis method (29), digested with *Eco*R1, and the restriction digestion products were visualized by gel electrophoresis to identify clones containing the PCR products. Inserts were commercially sequenced (Genomed Synthesis Inc., San Francisco) by the dideoxy-chain termination method (30) prior to designing extended primers to detect SCARs. Results are presented based on the use of two different pairs of extended primers, the sequences of which are listed below. The underlined sequences correspond to the sequences of the original random 10-mer primer used in the RAPD reactions that produced the SCAR pairs

OPC7-FS-30 5' GTCCCCGACGACAACACCAAGAAAGACAACG 3'
OPC7-RS-25 5' GTCCCCGACGAGGTTGGTGGAAGTG 3'

OPB10-FS-25 5' CTGCTGGGACATCCAGGAATGAGAC 3'
OPB10-RS-25 5' CTGCTGGGACCAAATCGCGTGCAGG 3'

SCAR reactions. Approximately 50-ng samples of DNA were used in 25-μl (vol) amplification reactions containing 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 μM each of dATP, dCTP, dTTP, and dGTP, 200 nM each SCAR primer, and 2.5 units of AmpliTaq Gold DNA polymerase. Annealing temperatures were optimized for each pair of SCAR primers as previously described (23). The optimal thermocycling profiles for both pairs of SCAR primers considered in this paper were as follows: 9 min denaturation at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 70°C, 1 min at 72°C, and a final extension step for 7 min at 72°C.

A more rapid two-step PCR for detecting the presence of SCAR markers was developed with the same PCR reaction conditions as described above and with the following thermocycling profile: an initial 9 min denaturation at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 72°C, and a final extension step for 7 min at 72°C. Each DNA sample × primer × pair combination was repeated at least two times. Entire PCR reactions were resolved by electrophoresis as described above.

Multiplex PCR. All DNA samples were tested in multiplex PCR reactions containing all four extended primers (OPC7-FS-30, OPC7-RS-25, OPB10-FS-25, and OPB10-RS-25). Approximately 50-ng samples of DNA were used in 25-μl (vol) amplification reactions containing 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 μM each of dATP, dCTP, dTTP, and dGTP, 200 nM each SCAR primer, and 2.5 units of AmpliTaq Gold DNA polymerase. PCR reactions were performed with the following thermocycling profile: 9 min denaturation at 95°C,

followed by 40 cycles of 1 min at 94°C, 1 min at 70°C, 1 min at 72°C, and a final extension step for 7 min at 72°C. Each DNA sample × primer × pair combination was repeated at least two times. Entire PCR reactions were resolved by electrophoresis as described previously.

Detection of *A. euteiches* in roots of field-grown peas. In May 2000, a field in Milton-Freewater, Oregon, used for over 10 consecutive years as a pea cultivar testing site for resistance to root rot was surveyed for plants infected with *A. euteiches*. Plants were selected that exhibited stunting and chlorosis. Plants that did not exhibit symptoms were selected as healthy controls. Plants were harvested at early bloom stage. After the selected plants were removed from the field, the roots were vigorously washed in tap water, rinsed in ddH₂O, and blotted dry on sterile filter paper. DNA was extracted from ≈200 mg of root tissue using the *FastDNA* kit (Bio 101). The procedure involved placing the root tissue in a microcentrifuge tube with a DNA extraction buffer and homogenizing the tissue for two 15-s periods at an intensity setting of 5.0 (FastPrep FP 120, Bio 101). The DNA was bound to a matrix by a binding buffer and eluted with 100 µl of sterile-distilled water. DNA concentrations were determined by ultraviolet absorbance at 260 nm, and DNA was diluted to 10 ng/µl for use in PCR reactions. Approximately 50-ng samples of DNA were used in 25-µl (vol) amplification reactions with primers OPC7-FS-30 and OPC7-RS-25. Reaction conditions, thermocycling profiles, and electrophoresis were done as described above. Each DNA sample × primer × pair combination was repeated at least two times.

DNA isolation from soil organic debris. Samples of organic debris from field soils previously determined by the wet-sieve/baiting technique (21) to be infested with *A. euteiches* were used for DNA isolations. Soil samples used for this analysis were Warden silt loam (coarse-silty, mixed mesic, Xerollic Camborthic) (31). Organic debris was separated from the soil mineral fraction by the wet-sieving technique as previously described (13). DNA was extracted from 500 mg of organic debris using the *FastDNA* spin kit (Bio 101) (1,9). The procedure was essentially identical to that described above for the isolation of DNA from root tissue, except that in the case of isolation of DNA from soil organic debris, the sample was homogenized for 30 s at an intensity setting of 5.5. DNA was also isolated from 500-mg samples of total field soil according to the same procedure. DNA concentrations were determined by ultraviolet absorbance at 260 nm, and DNA was diluted to 10 ng/µl for use in PCR reactions. Approximately 50-ng samples of DNA were used in 25-µl (vol) amplification reactions with primers OPC7-FS-30 and OPC7-RS-25. Reaction conditions, thermocycling profiles, and electrophoresis were done as described above. Each DNA sample × primer × pair combination was repeated at least two times. DNA extracted from organic debris that tested negative for the fungus based on the wet-sieve/baiting technique (21) was used as a control in PCR reactions.

RESULTS

SCAR primers specific for *A. euteiches*. Twenty random 10-mer primers were tested in RAPD reactions with the isolates listed in Table 1. Three 10-mer primers, OPC7, OPC11, and OPC14 produced RAPD markers that were amplified by all the tested isolates of *A. euteiches*. These three PCR products were cloned and sequenced, and the sequences were used to design pairs of SCAR primers. The primer pair of OPC11-5-2FS (5'-AAA-GCTGCGGAGCACGGACATCTT-3') and OPC11-5-2RS (5'-AAAGCTGCGGCAAGAGTGGTGGCC-3') amplified a single band of ≈2.5 kbp in all tested isolates of *A. euteiches* and *A. cochlioides*, suggesting that the SCAR was highly specific to the genus *Aphanomyces* (data not shown). Similarly, the primer pair of OPC14-5-3FS (5'-TGCGTGCTTGCCACAGGTTCCGACC-3') and OPC14-5-3RS (5'-TGCGTGCTTGTAGGATGTATTGAC-3')

amplified a single band of ≈3.0 kbp in all tested isolates of *A. euteiches* and *A. cochlioides*. However, this primer pair also amplified a very faint band of the same size from *M. pinodes*, *R. solani*, and *P. ultimum* (data not shown). Because our objective was to produce a SCAR that was specific for *A. euteiches*, these two primer pairs were not considered further.

TABLE 1. Fungal isolates evaluated by polymerase chain reaction (PCR) with sequence-characterized amplified region (SCAR) primers^a

Isolate	Host	Location	OPC7 ₁₃₃₂	OPB10 ₇₁₈
<i>Aphanomyces</i> spp.				
<i>A. euteiches</i> -2	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -5	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -8	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -A9	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -12	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -14	<i>Pisum sativum</i>	WI	+	—
<i>A. euteiches</i> -15	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -16	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -20	<i>Pisum sativum</i>	OR	+	—
<i>A. euteiches</i> -29	<i>Phaseolus vulgaris</i>	WI	+	—
<i>A. euteiches</i> -122	<i>Medicago sativa</i>	MN	+	—
<i>A. euteiches</i> -467	<i>Pisum sativum</i>	WI	+	—
<i>A. euteiches</i> -483	<i>Pisum sativum</i>	WI	+	—
<i>A. euteiches</i> -617	<i>Medicago sativa</i>	WI	+	—
<i>A. euteiches</i> -Ae5	<i>Pisum sativum</i>	FR	+	—
<i>A. euteiches</i> -Ae7	<i>Pisum sativum</i>	FR	+	—
<i>A. euteiches</i> -Lanc42	<i>Medicago sativa</i>	WI	+	—
<i>A. euteiches</i> -NC1	<i>Medicago sativa</i>	NC	+	—
<i>A. euteiches</i> -PA3	<i>Medicago sativa</i>	PA	+	—
<i>A. euteiches</i> -SP7	<i>Pisum sativum</i>	WA	+	—
<i>A. euteiches</i> -WI98	<i>Medicago sativa</i>	WI	+	+
<i>A. cochlioides</i> -Bern4C	<i>Beta vulgaris</i>	MI	—	+
<i>A. cochlioides</i> -Russ4B	<i>Beta vulgaris</i>	MI	—	+
<i>A. cochlioides</i> -B24	<i>Beta vulgaris</i>	MN	—	+
<i>A. cochlioides</i> -C2	<i>Beta vulgaris</i>	ND	—	+
<i>A. cochlioides</i> -C22	<i>Beta vulgaris</i>	ND	—	+
<i>A. cochlioides</i> -R64	<i>Beta vulgaris</i>	MN	—	+
<i>A. cochlioides</i> -Tx8	<i>Beta vulgaris</i>	TX	—	+
<i>A. cochlioides</i> -Tx51	<i>Beta vulgaris</i>	TX	—	—
<i>Achlya</i> spp.				
<i>A. conspicua</i>	<i>Oryza sativa</i>	LA	—	—
<i>A. klebsiana</i>	<i>Oryza sativa</i>	LA	—	—
<i>A. americana</i>	Soil	NC	—	—
<i>A. recurva</i>	Soil	MD	—	—
<i>A. diffusa</i>	Soil	MD	—	—
<i>Fusarium</i> spp.				
<i>F. oxysporum</i> -F15	<i>Pisum sativum</i>	OR	—	—
<i>F. oxysporum</i> -F22	<i>Pisum sativum</i>	OR	—	—
<i>F. oxysporum</i> -F33	<i>Pisum sativum</i>	OR	—	—
<i>F. oxysporum</i> -F34	<i>Pisum sativum</i>	MN	—	—
<i>F. solani</i> -F7	<i>Pisum sativum</i>	ID	—	—
<i>F. solani</i> -F12	<i>Pisum sativum</i>	WA	—	—
<i>Thelaviopsis</i> spp.				
<i>T. basicola</i> -T10	<i>Pisum sativum</i>	WA	—	—
<i>T. basicola</i> -T11	<i>Pisum sativum</i>	WA	—	—
<i>Rhizoctonia</i> spp.				
<i>R. solani</i> -R1	<i>Phaseolus vulgaris</i>	MD	—	—
<i>R. solani</i> -R2	<i>Pisum sativum</i>	WA	—	—
<i>Pythium</i> spp.				
<i>P. ultimum</i> -P3	<i>Phaseolus vulgaris</i>	WI	—	—
<i>P. ultimum</i> -P8	<i>Pisum sativum</i>	WA	—	—
<i>P. ultimum</i> -P9	<i>Pisum sativum</i>	ID	—	—
<i>P. aphanidermatum</i>	<i>Cucumis melo</i>	AZ	—	—
<i>P. dissotomicum</i>	<i>Lactuca sativa</i>	AZ	—	—
<i>Mycosphaerella</i> sp.				
<i>M. pinodes</i> -4B	<i>Pisum sativum</i>	NZ	—	—
<i>Phytophthora</i> spp.				
<i>P. infestans</i> -180	<i>Solanum tuberosum</i>	NY	—	—
<i>P. infestans</i> -543	<i>Solanum tuberosum</i>	MX	—	—

^a All locations are in the United States except FR = France; NZ = New Zealand; and MX = Mexico. + = SCAR product amplified; — = SCAR product not amplified. *Achlya conspicua* (ATCC 52604), *A. klebsiana* (ATCC 52605), *A. americana* (ATCC 14565), *A. recurva* (ATCC 16088), and *A. diffusa* (ATCC 16111).

Primer OPC7 amplified a product of ≈ 1.3 kbp from all isolates of *A. euteiches*, but not from any of the other organisms tested (Fig. 1A). This PCR product was isolated from two isolates of *A. euteiches* (2 and PA3), and the products from each isolate were cloned and sequenced. Both sequences were identical (data not shown) and represented a fragment of 1,332 bp submitted to GenBank (Accession no. AF228037). The sequence has a G + C content of 51.9%. No significant homology could be detected with any sequences present in GenBank. These sequences were used to design the SCAR primer pair OPC7-FS-30 and OPC7-RS-25. The thermocycling profile was optimized so only a single PCR product

(OPC7₁₃₃₂) was amplified from all isolates tested of *A. euteiches*, whereas no PCR products were amplified from other fungal isolates (Table 1; Fig. 1B). All reactions were repeated at least two times, and results were very consistent.

All tested isolates of *A. euteiches* produced the characteristic SCAR (OPC7₁₃₃₂) when a two-step thermocycling profile was used. Several isolates were tested with the two-step thermocycling profile to determine the minimum number of cycles needed to produce the SCAR. The SCAR was clearly produced in as few as 25 cycles from all isolates (data not shown). These results indicate that the primer pair OPC7-FS-30 and OPC7-RS-25 could be used

Fig. 1. Agarose gels showing **A**, Amplification products from polymerase chain reaction (PCR) of genomic DNA by the random 10-mer primer OPC7 (5'-GTCCCGACGA-3'). The arrow points to the PCR product amplified by all isolates of *Aphanomyces euteiches* that was cloned and sequenced. **B**, Amplification products from PCR of genomic DNA by the primer pair OPC7-FS-30 (5'-GTCCCGACGACACAACACCAAGAAAGACAACG-3') and OPC7-RS-25 (5'-GTCCCGACGAGTTGGTGGCAAGTG-3') with a two-step PCR thermocycling profile (40 cycles of 1 min at 95°C followed by 1 min at 72°C). Entire PCR reactions (25 μ l) were electrophoresed. Lane 1: 123-bp ladder (Gibco-BRL, Gaithersburg, MD); Lanes 2 through 9: isolates 2, A9, 12, 15, NC1, AC7, 122, and PA3 of *A. euteiches*, respectively; Lane 10: *Mycosphaerella pinoides*-4B; Lane 11: *Fusarium solani*-F7; Lane 12: *F. oxysporum*-F22; Lane 13: *Pythium ultimum*-PIII; Lane 14: *Thelaviopsis basicola*-T10; Lane 15: *Rhizoctonia solani*-R2; Lane 16: *Phytophthora infestans*-180; Lanes 17 to 19: isolates B24, Russ4B, and Tx8 of *A. cochlioides*, respectively; **A**, Lane 20: H₂O; and **B**, Lane 20: 1-kb ladder (Gibco-BRL).

to discriminate *A. euteiches* from all other tested organisms by a thermocycling profile that could be completed in ≈ 90 min.

SCAR primers specific for *A. cochlioides*. Primer OPB10 amplified a PCR product ≈ 700 bp from all isolates of *A. cochlioides* (Fig. 2A). This PCR product was isolated from two isolates of *A. cochlioides* (C2 and Tx8), and both bands were cloned and sequenced. Both sequences were identical (data not shown) and these sequences represented a 718-bp fragment (GenBank Accession no. AF228038) with a G+C content of 47.1%. The amino acid sequence coded by OPB10₇₁₈ had significant homology ($\leq 52\%$) with many Ca²⁺-transporting ATPase sequences present in

GenBank. However, OPB10₇₁₈ does not code for a complete open reading frame for any gene product. These sequences were used to design the SCAR primer pair OPB10-FS-25 and OPB10-RS-25. The thermocycling profile was optimized so only a single PCR product (OPB10₇₁₈) was amplified from all isolates tested of *A. cochlioides*, and no PCR products were amplified from other organisms (Table 1 and Fig. 2B). All reactions were repeated at least two times, and results were very consistent.

Interestingly, when the sequence of the primer OPB10-FS-25 was extended to include four more nucleotides (5'-TTTCG-3'; primer OPB10-FS-29), the specificity of the SCAR primer pair

Fig. 2. Agarose gels showing **A**, amplification products from polymerase chain reaction (PCR) of genomic DNA by the random 10-mer primer OPB10 (5'-CTGCTGGGAC-3'). The arrow points to the PCR product amplified by all isolates of *Aphanomyces cochlioides* that was cloned and sequenced. **B**, Amplification products from PCR of genomic DNA by the primer pair OPB10-FS-25 (5'-CTGCTGGGACATCCAGGAATGAGAC-3') and OPB10-RS-25 (5'-CTGCTGGGACCAAATCGCGTGCAGG-3') with a two-step PCR thermocycling profile (40 cycles of 1 min at 95°C followed by 1 min at 72°C). Entire PCR reactions (25 μ l) were electrophoresed. Lane 1: 123-bp ladder (Gibco-BRL, Gaithersburg, MD); Lanes 2 through 6: isolates 2, 5, 8, A9, and 12 of *A. euteiches*; Lanes 7 through 14: isolates Tx8, R64, Russ4B, C22, C2, B24, Tx51, and Bern4C of *A. cochlioides*; Lane 15: *Mycosphaerella pinoides*-4B; Lane 16: *Rhizoctonia solani*-R1; Lane 17: *Fusarium solani*-F7; Lane 18: *F. oxysporum*-F33; Lane 19: *Pythium ultimum*-PIII; and Lane 20: *Phytophthora infestans*-180.

was reduced from the species to the genus level. The primer pair of OPB10-RS-25 and OPB10-FS-29 (5'-CTGCTGGGACATC-CAGGAATGAGACTTCG-3') amplified a single PCR product that comigrated with OPB10₇₁₈, but the resulting SCAR product was selectively amplified from all tested isolates of *A. euteiches* and *A. cochlioides*. No SCAR products were amplified from any of the other fungal species tested (data not shown).

Multiplex PCR by SCAR primers. Multiplex PCR reactions were performed by both pairs of SCAR primers (OPC7-FS-30, OPC7-RS-25, OPB10-FS-25, and OPB10-RS-25). Results presented in Figure 3 show that the specificity of the primers was not affected by a multiplex reaction. Single PCR products corresponding to OPC7₁₃₃₂ and OPB10₇₁₈ were amplified from *A. euteiches* (Lane 7) and *A. cochlioides* (lane 8), respectively, when all four primers were used together in a multiplex reaction. Both SCAR products were produced in a multiplex reaction when a mixture of DNA from both species was amplified (lane 9). All isolates of *A. euteiches* amplified a single band corresponding to OPC7₁₃₃₂, and all isolates of *A. cochlioides* amplified a single band corresponding to OPB10₇₁₈, whereas no PCR products were produced in any of the other fungal isolates under multiplex PCR conditions (Table 1). All reactions were repeated at least two times, and results were very consistent.

Detection of *A. euteiches* in infected roots by SCAR primers. Pea plants of several varieties that exhibited symptoms of stunting and chlorosis typically observed in plants infected by *A. euteiches* were collected from a root rot field trial. SCAR primers OPC7-FS-30 and OPC7-RS-25 were used together to

successfully amplify a single PCR product from the roots of many of these plants that comigrated with the SCAR product amplified from purified *A. euteiches* DNA (Fig. 4). No PCR product was detected with amplification of DNA extracted from healthy pea roots (Fig. 4, lane 3), indicating that the amplification products are not the result of nonspecific amplification of host plant DNA. The SCAR primers successfully detected the presence of *A. euteiches* in 11 varieties (CMG-324F, CMG-330F, PLS-4743X, FR-665, PLS-526, DS-89043, Bolero, CMG-325-AF, PLS-224, Genie, and FR-720) and in 34 of 42 collected root samples. All reactions were repeated at least two times, and results were very consistent.

Detection of *A. euteiches* in soil organic debris by SCAR primers. *A. euteiches* could not be detected by SCAR primers OPC7-FS-30 and OPC7-RS-25 in DNA extracted from total soil samples that were previously determined by the wet-sieve/baiting technique to cause disease (data not shown). However, a single PCR product was amplified from DNA extracted from the organic fraction of these soil samples that comigrated with the SCAR product amplified from purified *A. euteiches* DNA (Fig. 5). No PCR product was detected with amplification of DNA extracted from an organic fraction that was determined previously by the wet-sieve/baiting technique to not cause disease (Fig. 5, lanes 9 and 10). The three soil samples presented in Figure 5 that amplified the SCAR were determined by the wet-sieve/baiting technique to be equally infectious (15/15 plants infected), but differences were observed between samples for the amount of PCR product amplified by the pair of SCAR primers. These differences may be due to the presence of inhibitors of polymerase in the DNA extracts.

DISCUSSION

This study demonstrated that SCARs can be used to unambiguously discriminate isolates of *A. euteiches* from other oomycetes, including several *Achlya* spp., *A. cochlioides* from diverse sources, and other soilborne fungi. The SCAR primers amplified a single diagnostic PCR product from all isolates, irrespective of the geographic origin or the host tissue from which they were isolated. Using a similar approach, primer pairs were designed that amplified a SCAR that was specific for isolates of *A. cochlioides*. Only 20 random 10-mer primers had to be screened for the successful development of diagnostic SCARs for both *A. euteiches* and *A. cochlioides*, suggesting that this approach was relatively efficient. Two other primer pairs were designed that amplified SCARs that could resolve isolates at the genus level.

The SCAR primers detected *A. euteiches* in infected roots of field-grown peas. Although several selected plants exhibited chlorosis and stunting that did not amplify the diagnostic SCAR, these symptoms are very general and could be the result of adverse environmental conditions or disease caused by other soilborne pathogens present at the site, such as *Fusarium oxysporum* or *R. solani*. The SCAR could be amplified from root tissue isolated from peas at the early bloom stage, suggesting that this may be an effective technique for diagnosing plants early in the growing season.

Currently, pea growers often have their fields assayed for the presence of the pathogen prior to planting. With this knowledge, the grower can make an informed decision as to whether it would be prudent to choose to cultivate a variety that has resistance to the fungus. This assay is typically done according to the soil indexing technique (28), which requires at least 2 week to complete and dedicated greenhouse space. The SCAR primers detected the presence of *A. euteiches* from DNA isolated from the organic debris fraction of field soil samples. The infectious nature of these samples has been confirmed previously by the wet-sieve/baiting technique. These results suggest that the primers may be useful for screening cultivated fields for the presence of the fungus prior

Fig. 3. Agarose gel showing products amplified from genomic DNA by a multiplex polymerase chain reaction (PCR) that included the primers OPC7-FS-30 (5'-GTCCCGACGACAACCAAGAAAGACAACG-3'), OPC7-RS-25 (5'-GTCCCGACGAGGTTGGTGGCAAGTG-3'), OPB10-FS-25 (5'-CTGCTGGGACATCCAGGAATGAGAC-3'), and OPB10-RS-25 (5'-CTGCTGGGACCAATCGC-GTGCAGG-3') with a two-step PCR thermocycling profile (40 cycles of 1 min at 95°C followed by 1 min at 72°C). Lane 1: 123-bp ladder (Gibco-BRL, Gaithersburg, MD); Lanes 2 through 4: *Aphanomyces euteiches*-2, H₂O, and *A. cochlioides*-C2, respectively, amplified with the primer pair of OPC7-FS-30 and OPC7-RS-25; Lanes 5 through 7: *A. euteiches*-2, H₂O, and *A. cochlioides*-C2, respectively, amplified with the primer pair OPB10-FS-25 and OPB10-RS-25; Lanes 8 through 11: *A. euteiches*-2, *A. cochlioides*-C2, a 1:1 mixture of DNA of *A. euteiches*-2 and *A. cochlioides*-C2, and H₂O, respectively, amplified with all four primers.

to planting. However, this technique might also amplify DNA from nonviable fungal structures, resulting in a false positive test. A false negative test might result from sampling soil from fields that have been out of pea production for several years. In this case, the organic fraction that is used for DNA extraction and typically contains oospores might be degraded, and the oospores could be present in the mineral fraction of the soil samples. Knowledge of the cropping history of the tested field would reduce the possibility of making this erroneous conclusion based on a negative PCR result.

We could not detect *A. euteiches* in field soil samples without first separating the organic debris fraction from soil and isolating DNA from this fraction. Appropriate target sequences are likely to be limiting, given the small percentage of total DNA from field samples attributed to the pathogen. Separation of the organic fraction prior to DNA extraction was necessary due to very small yields of DNA from total soil. This is likely due to the low organic content of this soil, which averages less than 1% (31). We observed variation among samples for the level of amplification of the diagnostic SCAR which might have been the result of the co-extraction of compounds such as humic acid or tannins that inhibit the action of *Taq* polymerase (34).

The nucleotide sequence data obtained for the SCAR product OPC7₁₃₃₂ may provide valuable information for the development of a quantitative PCR assay for *A. euteiches* based on the use of a sequence detection system that quantifies PCR products with fluorescent-labeled oligonucleotides. The development of such an assay would facilitate the study of early events in the infection process and could be used to compare rates of pathogen multiplication and movement between resistant and susceptible genotypes. A quantitative PCR assay may provide a more precise alternative to counting oospores (3) for determining inoculum

levels present in soil. In the future we will design primers based on the nucleotide sequence of SCAR product OPC7₁₃₃₂ and attempt to develop quantitative PCR assays with these primers.

Our results show the successful development of a pair of SCAR primers, OPB10-FS-25 and OPB10-RS-25, that amplified a single PCR product (OPB10₇₁₈) that unambiguously discriminated all isolates tested of *A. cochlidioides* from *A. euteiches*, several *Achlya* spp., and other species of soilborne phytopathogenic fungi. Although only eight isolates of *A. cochlidioides* were included in the analysis, these isolates had diverse geographic origins. *A. euteiches* can be found in legumes in regions where sugar beet is cultivated (32), and this assay can readily discriminate these two species. We did not investigate if the SCAR primer pair could detect *A. cochlidioides* in infected plant materials or soil samples. Our research is limited to diseases of legume crops, but the sequences of these SCAR primers and the availability in GenBank of the sequence of the SCAR product OPB10₇₁₈ should make it possible for other pathologists or plant breeders to explore applications for SCARs in the study of *A. cochlidioides*.

Our results demonstrate several advantages inherent in the use of SCARs. The SCAR primer pairs developed in this study to detect *A. euteiches* and *A. cochlidioides* could efficiently anneal to template DNA at a temperature of 72°C. This allowed for their use with a two-step thermocycling profile, in which both the annealing of primers to template DNA and the subsequent extension of the newly synthesized strands of DNA were performed with a single step (72°C for 1 min), reducing the total time of the thermocycling reaction to ≈2 h. Because only a single PCR product was amplified under these thermocycling conditions, it could be unambiguously resolved on an agarose gel after a short period of electrophoresis. Both the thermocycling reaction and electrophoresis were easily completed in less than 3 h.

Fig. 4. Detection of *Aphanomyces euteiches* in DNA isolated from roots of pea cultivars collected at a root-rot field trial in Milton-Freewater, OR. Plants in the early bloom stage that exhibited chlorosis and stunting were selected. Agarose gel showing products amplified by the primer pair OPC7-FS-30 (5'-GTCCCCGACGA-CAACACCAAGAAAGACAACG-3') and OPC7-RS-25 (5'-GTCCCCGACGAGGTTGGTGCAAGTG-3') with a two-step polymerase chain reaction (PCR) thermocycling profile (40 cycles of 1 min at 95°C and 1 min at 72°C). Entire PCR reactions (25 µl) were electrophoresed. Lane 1: 123-bp ladder (Gibco-BRL, Gaithersburg, MD); Lane 2: purified *A. euteiches*-2 DNA; lane 3: healthy cv. Bolero (did not exhibit chlorosis or stunting); Lanes 4 through 6: three plants of cv. CMG-325-AF; Lanes 7 through 9: three plants of var. FR-720; Lanes 10 through 12: three plants of var. PLS-4743X; Lane 13: var. CMG-324F; Lanes 14 through 16: three plants of var. Genie; Lanes 17 through 19: three plants of var. FR-665; and Lane 20: var. Bolero.

Fig. 5. Detection of *Aphanomyces euteiches* in DNA isolated from the organic fraction of field soil samples. Agarose gel showing products amplified by the primer pair OPC7-FS-30 (5'-GTCCCGACGACAACACCAAGAAAGACAACG-3') and OPC7-RS-25 (5'-GTCCCGACGAGGTTGGTGGCAAGTG-3') with a two-step polymerase chain reaction (PCR) thermocycling profile (40 cycles of 1 min at 95°C and 1 min at 72°C). Entire PCR reactions (25 µl) were electrophoresed. Lane 1: 123-bp ladder (Gibco-BRL, Gaithersburg, MD); Lane 2: purified *A. euteiches*-2 DNA; Lanes 3 through 4: two repetitions of amplifications of DNA isolated from sample Spellman no. 8; Lanes 5 through 6: two repetitions of amplifications of DNA isolated from sample Athena Falls; Lanes 7 through 8: two repetitions of amplifications of DNA isolated from sample Priebe; and Lanes 9 through 10: two repetitions of amplifications of DNA isolated from sample Mattawa Small Balsom. Samples Spellman no. 8, Athena Falls, and Priebe had previously been determined by the wet-sieve/baiting technique (22) to contain *A. euteiches*.

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LITERATURE CITED

- Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., Jansen, J. L., Niehuis, J., and Triplett, E. W. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* 62:1935-1943.
- Bunting, T. E., Plumley, K. A., Clarke, B. B., and Hillman, B. I. 1996. Identification of *Magnaporthe poae* by PCR and examination of its relationship to other fungi by analysis of their nuclear rDNA ITS-1 regions. *Phytopathology* 86:398-404.
- Chan, M. K. Y., and Close, R. C. 1987. *Aphanomyces* root rot of peas. I. Evaluation of methods for assessing inoculum density of *Aphanomyces euteiches* in soil. *N. Z. J. Agric. Res.* 30:225-233.
- Chen, W., Gray, L. E., and Grau, C. R. 1996. Molecular differentiation of fungi associated with brown stem rot and detection of *Phialophora gregata* in resistant and susceptible soybean cultivars. *Phytopathology* 86:1140-1148.
- Delwiche, P. A., Grau, C. R., Holub, E. B., and Perry, J. B. 1987. Characterization of *Aphanomyces euteiches* isolates recovered from alfalfa in Wisconsin. *Plant Dis.* 71:155-161.
- Grau, C. R., Muehlchen, A. M., and Tofte, J. E. 1991. Variability in virulence of *Aphanomyces euteiches*. *Plant Dis.* 75:1153-1156.
- Hagedorn, D. J. (ed.). 1984. *Compendium of Pea Diseases*. The American Phytopathological Society, St. Paul, MN.
- Jones, F. R., and Drechsler, C. 1925. Root rot of peas in the United States caused by *Aphanomyces euteiches*. *J. Agric. Res.* 30:293-325.
- Kaur, R., Kumar, R., and Bachhawat, A. K. 1995. Selective recovery of DNA fragments from silica particles: Effect of A-T content and elution conditions. *Nucleic Acids Res.* 4932-4936.
- King, T. E., and Bissonette, H. 1954. Physiologic variability in *Aphanomyces euteiches*. (Abstr.) *Phytopathology* 44(suppl.):S495.
- Kraft, J. M., and Boge, W. L. 1994. Development of an antiserum to quantify *Aphanomyces euteiches* in resistant pea lines. *Plant Dis.* 78:179-183.
- Kraft, J. M., and Boge, W. L. 1996. Identification of characteristics associated with resistance to root rot caused by *Aphanomyces euteiches* in pea. *Plant Dis.* 80:1383-1386.
- Kraft, J. M., Marcinkowska, J., and Muehlbauer, F. J. 1990. Detection of *Aphanomyces euteiches* in field soils from Northern Idaho by a wet-sieving/baiting technique. *Plant Dis.* 74:716-718.
- Lee, S. B., White, T. J., and Taylor, J. W. 1993. Detection of *Phytophthora* species by oligonucleotide hybridization to amplified ribosomal DNA spacers. *Phytopathology* 83:177-181.
- Lévesque, C. A., Harlton, C. E., and de Cock, A. W. A. M. 1998. Identification of some oomycetes by reverse dot blot hybridization. *Phytopathology* 88:213-222.
- Lévesque, C. A., Vrain, T. C., and De Boer, S. H. 1994. Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA. *Phytopathology* 84:474-478.
- Malvick, D. K., Grau, C. R., and Percich, J. A. 1998. Characterization of *Aphanomyces euteiches* strains based on pathogenicity tests and random amplified polymorphic DNA analysis. *Mycol. Res.* 102:465-475.
- Malvick, D. K., and Percich, J. A. 1998. Variation in pathogenicity and genotype among single-zoospore strains of *Aphanomyces euteiches*. *Phytopathology* 88:52-57.
- Malvick, D. K., and Percich, J. A. 1998. Genotypic and pathogenic diversity among pea-infecting strains of *Aphanomyces euteiches* from the Central and Western United States. *Phytopathology* 88:915-921.
- Manning, M. A., and Menzies, S. A. 1984. Pathogenic variability in isolates of *Aphanomyces euteiches* from New Zealand soils. *N. Z. J. Agric. Res.* 27:569-574.
- Mitchell, J. E., Bhalla, H. S., and Yang, G. H. 1969. An approach to the study of the population dynamics of *Aphanomyces euteiches* in soil. *Phytopathology* 59:206-212.
- Papavizas, G. C., and Ayers, W. A. 1974. *Aphanomyces* species and their root diseases in pea and sugar beet. *U.S. Dep. Agric. Res. Tech. Bull.* 1485.
- Paran, I., and Michelmore, R. W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85:985-993.
- Pfender, W. F., Delwiche, P. A., Grau, C. R., and Hagedorn, D. J. 1984. A medium to enhance recovery of *Aphanomyces* from infected plant tissue. *Plant Dis.* 68:845-847.
- Pfender, W. F., and Hagedorn, D. J. 1982. *Aphanomyces euteiches* f. sp. *phaseoli*, a causal agent of bean root and hypocotyl rot. (Abstr.) *Phytopathology* 72(suppl.):S306.
- Pfender, W. F., and Hagedorn, D. J. 1983. Disease progress and yield loss in *Aphanomyces* root rot of peas. *Phytopathology* 73:1109-1113.
- Pfender, W. F., Rouse, D. I., and Hagedorn, D. J. 1981. A "most probable number" method for estimating inoculum density of *Aphanomyces euteiches* in naturally infested soil. *Phytopathology* 71:1169-1172.
- Reiling, T. P., King, T. H., and Fields, R. W. 1969. Soil indexing for pea root rot and the effect of root rot on yield. *Phytopathology* 50:287-290.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (eds.) 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. CSHL Press, Cold Spring Harbor, New York.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74:5463-5467.
- United States Department of Agriculture-SCS. 1985. Soil survey of Yakima county area Washington. U.S. Dep. Agric.
- Weiland, J. J., and Sundsbak, J. L. 2000. Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rRNA gene. *Plant Dis.* 84:475-482.
- Willits, D. A., and Sherwood, J. E. 1999. Polymerase chain reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barley varieties. *Phytopathology* 89:212-217.
- Zhou, J., Bruns, M., and Tiedje, J. M. 1996. DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* 62:316-322.