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Molecular identification of *Pythium aphanidermatum* isolates by using semi-specific primers

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Abstract

Identification of *Pythium aphanidermatum* is difficult and time-consuming due to its morphological and biological characteristics. in the current study semi-specific polymerase chain reaction (PCR) was developed for identification of *P. aphanidermatum*. Fourteen isolates collected from different areas of Iran were studied. On the basis of the fragment obtained in the random amplified polymorphic DNA (RAPD), after purification, cloning and sequencing, the primers were designed. The designed primers could not distinguish *P. aphanidermatum* isolates from *P. deliense* isolate. Then, new primers were designed based on differences of the sequence of fragments obtained from the semi-specific PCR. In order to generate the highest specificity and efficiency, annealing temperature and extension time were optimized for the primer pairs. Six other *Pythium* species as well as isolates of *Phytophthora drechsleri, Rhizoctonia solani* and *Fusarium solani* were used to assess the designed primers. Results showed that the primers designed amplified the 656bp and 618pb fragments in all isolates of *P. aphanidermatum* and *P. deliense* whereas no amplification was observed in other isolates. The optimal annealing temperature and the best extension time were considered to be 66 °C and 60 sec., respectively. So, the primers designed are able to identify the isolates of *Pythium aphanidermatum* and to separate them from other fungi causing root rot of sugar beet and other species of *Pythium* except *P. deliense*.

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Introduction

The genus *Pythium* belongs to oomycets which are a group of mycelium-containing (fungus-like) organisms and known as water molds. Most pathogenic oomycets of plant roots such as *Pythium* have been classified in order of *Pythiales* and family of *Pythiaceae* (West *et al*., 2003). So far, over 120 *Pythium* species have been identified (Martin, 1991). *Pythium* is among the important soil-borne pathogens which causes a great loss to sugar beet yield in most beet production areas of Iran annually. This genus has a vast global distribution and a wide host range and is a cause of destructive diseases on field crops and ornamental plants (Drenth and Guest, 2004). All of *Pythium* species are not pathogenic, pathogenicity of various species can be different and symptoms of seedlings are often similar to those of the pathogens of *Pythium* spp., *Rhizoctonia solani, Phytophthora drechsleri* and *Aphanomyces cochlioides*. So, identification of *Pythium* species is mainly conducted based on morphological and biological characteristics which could be highly variable and so requires extensive microscopic studies. These make the identification of the species very difficult. Therefore, it is highly important to rapidly and precisely identify the pathogen which results in adoption of proper methods for disease control. In recent years, several deoxyribonucleic acid (DNA)-based molecular approaches have been developed for detection and identification of different *Pythium* species which have shown high precision in differentiation of species (Olive and Bean, 1999). Use of DNA probes is a useful and effective method for identification of *Pythium* species. By using this method, *Pythium oligandrum and Pythium sylvaticum* were identified (Martin, 1991). Molecular characteristics of important and pathogenic species of *Pythium* were studied based on ribosomal DNA-random fragment length polymorphism (rDNA-RFLP); In this study, seven restriction enzymes were used and isolates with finger sporangium were separated from those with round sporangium and were classified in two distinct groups. Data did not show any differences between the isolates of *Pythium aphanidermatum* and *Pythium butleri*. They showed that intraspecific differences in the isolates of the species *P. aphanidematum, P. butleri* and *P.deliense* were very low (Wang and White, 1997). The isolates of *Pythium aphanidermatum* were separated from the other *Pythium* species by using the OPB-08, OPA-15 and OPA-03 primers. In this research, it was shown that *P. aphanidematum* could be separated from *P. deliense* by random amplified polymorphic DNA (RAPD) analysis (Herreo and Klemsdal, 1998). In Japan, 48 isolates of *Pythium irregulare*, isolated from different hosts and geographical regions were compared based on molecular, morphological and physiological characteristics. On the basis of RAPD analysis and internal transcribed spacer (ITS) sequencing, the isolates were divided into four groups among which the groups I and II, and the groups III and VI had close relationship with each other. The groups I and II consisted of isolates which had been isolated from different hosts and geographical regions. The groups III and VI consisted of seven isolates all of which had been isolated from sugar beet but from different geographical regions at different times (Matsumoto *et al*, 2000). The isolates of *Pythium spinosum* were divided into the varieties *Pythium spinosum* var. *spinosum* and *Pythium spinosum* var. *sporangiferum* (Gherbaway and Abdelzaher, 2002). In 2003, the species *Pythium aphanidermatum and Pythium dimorphum* were distinguished by using the specific primers designed on the basis of the ITS-rDNA region (Wang and White, 2003). Eighty four *Pythium* isolates collected from different hosts were studied by using amplified fragment length polymorphism (AFLP) fingerprinting (Grazon *et al*., 2005). By using AFLP fingerprinting, pathogenicity, genetic diversity and susceptibility to Metalaxyl were studied on 73 *Pythium aphanidermatum* isolates collected from different areas of Oman (Al-Sadi *et al*., 2008). By using multiplex-polymerase chain reaction (PCR), five *Pythium* species including *Pythium aphanidermatum, P. arrhenomanes, P. graminicola, P. torulosum* and *P. vanterpoolii* were identified and separated (Asano *et al*., 2010). According to past research, the *Pythium aphanidermatum* species is one of the most important causes of damping-off and

root rot of sugar beet which has been reported from different areas of the country (Mahmoudi *et al.*, 2005).

In the current research, semi-specific primers were designed and used for the first time for rapid identification of the pathogen *Pythium aphanidermatum* in sugar beet based on the sequence of the fragment obtained from RAPD test.

Materials and methods

Details of the isolates studied are presented in Table 1. Some of the isolates were obtained from the collection of the Plant Pathology Department of Sugar Beet Seed Institute, Karaj, Iran. These isolates had been collected from the beet fields of the main beet production areas of the country during 2005-2009. Also, some isolates were obtained from the collection of the Plant Protection Department of Shiraz and Hamadan Universities.

Fungal Cultures and DNA extraction

In order to produce mycelial mass, each isolate was cultured on 100ml of liquid culture medium (10 g sucrose, 1 g yeast extract in 1 liter distilled water) and transferred onto a shaker with 120 rpm speed (Wang and White, 1997). After 7-10 days, content of each flask were passed through No.1 whatman filter paper by using vacuum pump and Büchner funnel and washed with sterile distilled water. The mycelial mass was placed into aluminum foil cover and kept at -20 °C. By using the modified method of Dellaporta *et al.* (1983), DNA was extracted from the all isolates of *Pythium* spp. and also from some isolates of *Rhizoctonia solani*, *Phytophthora drechsleri* and *Fusarium solani*. The quantity and quality of extracted DNA was determined by using 0.8% agarose gel and spectrophotometer.

RAPD-PCR

In order to obtain the band of interest with the approximate size of 2kb for *P. aphanidermatum*, RAPD test was conducted by using the primer OPA-15 with the sequence 5'- TTCCGAACCC -3'. Also, in order to make sure that DNA of the isolates used is able to amplify, RAPD test was carried out prior to PCR by using the RAPD standard 10 mer primer according to the following conditions: 1 µl of DNA per reaction at the concentration of 40 ng/µl, 2 µl of 10X buffer, 1.2 µl of 2.5 mM dNTP, 1.8 µl of 20 mM $MgCl_2$, 2 µl of primer at the concentration of 30 ng/µl and 0.2 µl (one unit) of SmarTaq polymerase. The PCR for RAPD test was done at the final volume of 20 µl for each reaction. The PCR reaction for RAPD test was conducted in thermocycler (BioRad) in the following steps: 5' initial denaturation at 95 °C, 40 cycles including denaturation for 45" at 95 °C, annealing for 45" at 34 °C, primer extension for 100" at 72 °C and one step of 10' for final extension at 72 °C for completing the length of the amplified fragments. Then, electrophoresis of RAPD reaction products was conducted on 1.2% agarose gel at the voltage of 100, and the gel was stained by ethidium bromide and photographed by UV Tech. Finally, the banding pattern of the isolates was determined on the gel.

Sequencing of RAPD products and designing of semi-specific primers

After making sure that the fragment of interest has been amplified in the PCR reaction, purification of the amplified product was conducted by using the Vivantis-GF-1 kit, Malaysia. The fragment of interest was cloned in the plasmid pTG19 – T and after transformation of *Escherichia coli* cells (by using the modified method of Sambrook and Russell, 2001), the clones containing the fragment of interest were selected by Colony-PCR using M13 primers. Extraction of plasmid was conducted on the basis of alkaline bacterial lysis with minor modifications (Sambrook and Russell, 2001). For sequencing, the cloned fragment was sent to Bioneer Company (Bioneer Korea). The cloned fragment was sequenced by using universal primers. The sequence of the RAPD product was used to design the semi-specific primers with the Oligo 5 software.

Study of the specificity of the primers designed based on the RAPD-derived fragment

In order to study the specificity of the primers, first the primers designed based on the RAPD-derived

fragment were assessed. For doing this, all isolates of *Pythium* spp. and the three isolates *Rhizoctonia solani*, *Phytophthora drechsleri* and *Fusarium solani* were investigated. The PCR was performed at the final volume of 20 µl per reaction. The mixture of PCR reaction included 1 µl of DNA at the concentration of 40 ng/µl, 2 µl of 10X buffer, 1 µl of 2.5 mM dNTP, 1.4 µl of 20 mM $MgCl_2$, 1 µl of each primer at the concentration of 30 ng/µl and 0.2 µl (one unit) of SmarTaq polymerase. After distribution of the PCR reaction mixture to the tubes and addition of the template DNA, microtubes were transferred into thermocylcer (Biometra). In order to gain the highest specificity and efficiency, the annealing temperature and the time of primer pair extension were optimized. So, the annealing temperature was investigated in the range of 56-66 °C for the all isolates.

Sequencing of the semi-specific PCR products

The semi-specific PCR products were purified by Bioneer Korea and sequenced directly. The sequences obtained were converted to FASTA by CLUSTALW software and aligned and compared by using the BLAST (NCBI) software in the Genebank. This way, the differences among the sequence of the fragments of the *P. aphanidermatum* and *P. deliense* isolates were determined. On the basis of the sequence of the fragments obtained, the new primer pairs were designed from the nucleotides different in the two sequences by using the Oligo 5 software.

Study of the specificity of the primers designed based on the semi-specific PCR

In order to study the specificity of the primers designed based on differences of the fragment sequence of the isolates of *P. aphanidermatum* and *P. deliense* obtained in the semi-specific PCR reaction and distinguish these two pathogens, the gradient PCR was conducted for these isolates at the range of 56-70 °C. The PCR was performed at the final volume of 20 µl per reaction. The mixture of PCR reaction included 1 µl of DNA at the concentration of 40 ng/µl, 2 µl of 10X buffer, 1.2 µl of 2.5 mM dNTP, 1.3 µl of 20 mM $MgCl_2$, 1 µl of each primer at the concentration of 30 ng/µl and 0.2 µl (one unit) of SmarTaq polymerase. Assessment of PCR reaction products was conducted on 1.2% agarose gel at the voltage of 100. After electrophoresis, the gel was stained for 10-15 min in the solution containing ethidium bromide and then washed-out for 7 min in distilled water. Finally, the bands produced were photographed by UV Tech. Finally, the banding pattern of the isolates was determined on the gel. The specificity of the designed primers was investigated based on the presence and absence of the expected band.

Results

RAPD-PCR

OPA-15 ten mer primer produced a fragment of approx. 2000bp in the isolates of *P. aphanidermatum*. This fragment was obtained from the all isolates of *P. aphanidermatum*. So, on the basis of the sequence of the fragment obtained in the RAPD test, the primer pairs were designed for rapid identification of the isolates of *P. aphanidermatum*. In the RAPD test, the amplification ability of the all isolates was evaluated by using the standard primer (internal check for PCR reactions). In this test, all isolates displayed amplification ability by the standard primer (Table 2). Thus, it can be said that all isolates had high amplification ability.

Sequencing of RAPD products and designing of primers

In order to design primer, the 2000bp fragment obtained in the RAPD test was sequenced by Bioneer Korea. The sequenced obtained was converted to FASTA by CLUSTALW software and aligned by using the BLAST (NCBI) software in the Genebank. So, the fragment obtained showed 64% homology with putative messenger ribonucleic acid (mRNA) of pathogen *Phytophthora infestans*. In the primer pairs designed based on the fragment obtained in the RAPD test (PA-F1and PA-R1), the sequence of the forward primer is based on the sequence of the RAPD primer sequence and located at the position of its 6^{th} base in the sequenced fragment of the PCR product, whereas the sequence of the reverse primer was

located outside the sequence of the RAPD primer (Table 3).

Sequencing of the semi-specific PCR product and designing of new primers

The fragments obtained from the semi-specific PCR were aligned and compared by using the BLAST (NCBI) software in the Genebank and so the difference between the sequence of the fragments of *P. aphanidermatum* and *P. deliense* was determined. Thus, each fragment showed 73% homology with the complementary DNA (cDNA) fragment of the pathogen *Aphanomyces euteiches*. There was 99% homology between the two fragments obtained from the isolates of *P. aphanidermatum* and *P. deliense*. The sequence alignment of the fragments obtained from these two pathogens showed that these two fungi differ in five nucleotides. So, the difference between the two species locates in the amplified region of the genome in the positions of the bases 5, 7, 8, 566 and 575. The primer pairs PA-F2 and PA-R2 were designed based on the sequence of the semi-specific PCR products and with regard to the difference between the sequences of the fragments obtained for the isolates of *P. aphanidermatum* and *P. deliense*, so that the forward primer was designed on the basis of the sequence of the RAPD primer and at the position of its 2nd base, whereas the reverse primer was designed on the basis of the sequence difference between the two isolates. So, the primer PA-R2 starts from the nucleotide 564 and ends at the nucleotide 583 (Table 3).

Table 1. Characteristics of the isolates used in the current study.

No	Code	Name	Area (province)	Sampling date	Host
1	PA-8	*P. aphanidermatum*	West Azerbaijan	2007	Sugar beet
2	PA-22	*P. aphanidermatum*	Kermanshah	2007	Sugar beet
3	PA-24	*P. aphanidermatum*	West Azerbaijan	2007	Sugar beet
4	PH-16	*P. helicoides*	Hamadan	_____	_____
5	POe-17	*P. oedochilum*	Hamadan	_____	_____
6	PI-18	*P. intermedium*	Hamadan	_____	_____
7	PD-19	*P. deliense*	Shiraz	_____	Sugar beet
8	POi-25	*P. oligandrum*	Hamadan	2007	Sugar beet
9	PU-27	*P. ultimum*	Kermanshah	2007	Sugar beet
10	PU-67	*P. ultimum*	Kermanshah	2007	Sugar beet
11	Ph-38	*Phytophthora drechsleri*	Kermanshah	2007	Sugar beet
12	Ph-68	*Phytophthora drechsleri*	Kermanshah	2008	Sugar beet
13	FS	*Fusarium solani*	Uromieh	_____	Sugar beet
14	RH	*Rhizoctonia solani*	Kermanshah	1999	Sugar beet

Table 2. Ability to amplify the different species of *Pythium* and other fungi of root rot by using the RAPD and designed primers for *P. aphanidermatum*.

Isolate No.	Isolate	Amplification		
		RAPD	Primer sets	
		Standard primer	PA-F1,PA-R1	PA-F2, PA-R2
PA-8	*P. aphanidermatum*	+	+	+
PA-22	*P. aphanidermatum*	+	+	+
PA-24	*P. aphanidermatum*	+	+	+
PH-16	*P. helicoides*	+	-	-
POe-17	*P. oedochilum*	+	-	-
PI-18	*P. intermedium*	+	-	-
PD-19	*P. deliense*	+	+	+
POi-25	*P. oligandrum*	+	-	-
PU-27	*P. ultimum*	+	-	-
PU-67	*P. ultimum*	+	-	-
Ph-38	*Phytophthora drechsleri*	+	-	-
Ph-68	*Phytophthora drechsleri*	+	-	-
FS	*Fusarium solani*	+	-	-
RH	*Rhizoctonia solani*	+	-	-

*This primer is used to confirm the ability to amplify the extracted DNA.

Study of the specificity of the primers designed

In order to identify the isolates of *P.aphanidermatum*, semi-specific primers were designed based on the 2000bp fragment obtained in the RAPD reaction. The specificity of the primers designed was determined by studying the results of the PCR for the all isolates used in the current research (Table 1). Results obtained based on the primers designed showed that the isolates of *P.aphanidermatum* are similar to each other. So, it can be said that these two primers are able to identify the isolates of *P.aphanidermatum*. These primers were able to distinguish the isolates of *P.aphanidermatum* from other species of *Pythium* such as *P .ultimum, P. oligandrum, P. helicoides, P. oedochilum* and *P. intermedium* except *P. deliense* (Table 2). Thus, a fragment of 656bp was obtained for the all isolates of *P. aphanidermatum* and the isolate *P. deliense*. Also, these primers were able to distinguish the isolates of *P. aphanidermatum* from the other root rot agents of sugar beet such as *Phytophthora drechsleri, Fusarium solani* and *Rhizoctonia solani* (Fig. 1). Results of the gradient PCR at the temperature range of 56-70 °C for the designed primer pairs PA-F2 and PA-R2 showed no differentiation between the fungi *P. aphanidermatum* and *P. deliense* (Fig. 2). The degree of specificity of the primer pairs PA-F2 and PA-R2 was studied by using *Pythium* isolates and also other root rot fungi such as *Phytophthora drechsleri, Fusarium solani* and *Rhizoctonia solani*. Results showed that these primer pair is able to identify the isolates of *P .aphanidermatum* and distinguish them from the other root rot agents and also other *Pythium* species except *P.deliense* (Table 3). The temperature regime of PCR reaction for the both primer pairs designed as 5' at 95 ˚C, and 40 cycles with 40" at 94 ˚C, 60" at 56-70 ˚C, 60" at 72 ˚C and final extension with 10' at 72˚C was optimized and performed. The optimum annealing temperature in the specific PCR was determined 66 ˚C.

Table 3. Sequence of the semi-specific primers designed based on the sequence of the fragments obtained from the RAPD test and semi-specific PCR.

Name of primer	Sequence of primer
PA-F1	5'-AACCCCGACTTCAGACAATG-3'
PA-R1	5'-GCCCTCGAACCACCACCACAC-3'
PA-F2	5'- TCCGAACCCCGACTTCAGA-3'
PA-R2	5'- GCTCTCTGTGAACGTTCCTA-3'

Discussion

The pathogen *Pythium aphanidermatum* is the most important root rot agent of sugar beet in central warm and southern parts of Iran. This pathogen can result in root rot and putrefaction at higher soil temperature and moisture and annually causes great yield losses in most beet production areas of Iran. Precise identification of the causal agent opens the way for applying correct strategies for disease management. Symptoms of seedling diseases of sugar beet due to different fungal agents are similar to each other and it is time-consuming to identify the *Pythium* species on the basis of morphological characteristics. So, applying molecular methods, especially use of different primers, can be rapid and precise for identification of the soil-borne pathogens such as *Pythium* species. Herreo and Klemsdal (1998) by using the OPA-15 and OPB-8 primers could distinguish the isolates of *P. aphanidermatum* from the other species of *Pythium*. RAPD test is simple and quick compared with other molecular methods and does not need extensive genetic information on the isolates under study. However, due to the low repeatability of this method, semi-specific primers were designed in the current research based on the information gained from the RAPD test for the first time to identify the isolates of *P. aphanidermatum*. The primers designed could identify the isolates of *P. aphanidermatum*. Also, these primers distinguished the pathogens of *P. aphanidermatum* from the other

soil-borne agents causing damping-off and root rot of sugar beet and other species of *Pythium* except *P. deliense*. In these primers, the start of sequence of the forward primer is at the position of the 6th base of the OPA-15 RAPD primer in the sequenced fragment of 2000bp, whereas the sequence of the reverse primer places in a position outside the sequence of RAPD primer which may be the same in the two species *P. aphanidermatum* and *P. deliense*. So, these two species were not distinguished by using these designed primers. In the studies of Herreo and Klemsdal (1998), the RAPD primers could distinguish the isolates of *P. aphanidermatum* from those of *P. deliense*. So, on the basis of differences in the sequence of the fragments obtained in the semi-specific PCR, the more specific primers for distinguishing these two species were designed. Results showed that these primers identified all isolates of *P. aphanidermatum* and distinguished them from the other species of *Pythium* and soil-borne agents of root rot and damping-off, whereas these primers also could not distinguish the two species *P.aphanidermatum* and *P. deliense*. Levesque *et al.* (1998) reported that the taxonomic status of *P. aphanidermatum* and *P. deliense* requires further investigation. Results obtained coincided with those of Wang and White (1997) who also were not able to distinguish these two species by designing the species-specific primers on the basis of the ITS1 region. Also, Moorman *et al.* (2002) reported that the sequence of the ITS1, 5.8S and ITS2 was similar for *P. aphanidermatum* and *P. deliense* and this region could not be used to distinguish these two species. Gherbaway *et al.* (2005) were not able to distinguish these two species of *Pythium* by using the V6, E15 and U19 RAPD primers. The primers designed in the current study are able to identify all species of *P. aphanidermatum* and distinguish them from the other root rot agents. In spite of specificity of the sequence of the primers designed for *P. aphanidermatum*, these primers were not able to distinguish *P. aphanidermatum* and *P. deliense* due to the high genetic similarity between the two species. According to the molecular studies conducted, the two species *P. aphanidermatum* and *P. deliense* have 99% similarity to each other which confirm the results of the morphological studies; however these two species have been located in two separate species (Lodhhi, 2007). The failure of the primers designed to distinguish the two species could be due to the molecular similarity between these two related species. However, the number of species studied in the current research is not sufficient to discuss on the change of the taxonomic position of the two species. Also, provided that more primers with ability to give more coverage of the genome of the two above-mentioned fungi are used, it is likely to achieve polymorphic and distinguishing primers in the future. Finally, sequencing of the whole genome of the two above-mentioned species can reveal the extent of their similarity or dissimilarity.

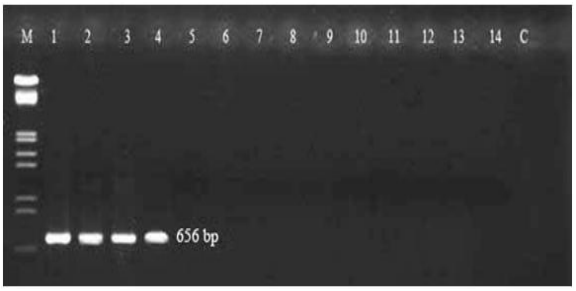

Fig. 1. Banding pattern of the 656 bp fragment amplified by the PA-R1 and PA-F1 primer pair designed in the isolates of *P. aphanidermatum* and *P. deliense*. The numbers 1-14 are the isolates of *P. deliense* (1), *P. aphanidermatum* (2-4), *P. ultimum* (5-6), *P. oligandrum, P. intermedium, P. helicoides, P. oedochilum*, *Phytophthora drechsleri* (11-12), *Fusarium solani, Rhizoctonia solani*. C: negative control (master mix without DNA). M: Marker for determining the size of DNA (Lambda DNA /*Eco*RI+*Hin*dIII Marker).

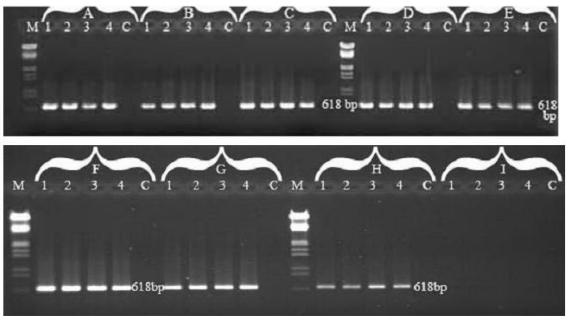

Fig. 2. Banding pattern of the 618 bp fragment amplified by the PA- F2 and PA-R2 primer pair at the

temperature range of 56-70 °C for the isolates of *P. aphanidermatum* and *P.deliense*. Numbers 1-2: isolates of *P. aphanidermatum* and the numbers 3-4: isolates of *P. deliense*. C: negative control (master mix without DNA). M: Marker for determining the size of DNA (Lambda DNA /*Eco*RI+*Hin*dIII Marker). A to I: PCR of semi-specific of the PA-F2 and PA-R2 primer pair with the annealing temperature of 56,57.5, 60.8, 62.4, 64, 65.5, 66.2, 67.6 and 70 °C respectively.

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