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شناسایی و ردیابی اختصاصی *Phytopthora drechsleri*، *P. cryptogea* و *P. erythroseptica* با واکنش زنجیره‌ای پلیمراز

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چکیده

گونه‌های *Phytophthora drechsleri*، *P. cryptogea* و *P. erythroseptica* بیمارگرهای گیاهی اُامیستی خویشاوندند و از نظر ریخت‌شناختی به یک‌دیگر شباهت دارند. برای تمایز این آرایه‌ها از یک‌دیگر و از سایر گونه‌هایی که صفات ریخت‌شناختی همگرا دارند، شیوه‌ای بر اساس واکنش زنجیره‌ای پلیمراز ساده و تودرتو ابداع شد. بدین منظور مجموعه‌ای از جدایه‌ها مربوط به میزبان‌های مختلف، که نماینده‌ی تنوع موجود در ژن‌های هسته‌ای و میتوکندریایی این گونه‌ها بودند، بررسی شد. بر اساس توالی فواصل ترانویسی شده‌ی داخلی (آی‌تی‌اِس) و زیرواحد ۱ سیتوکروم اکسیداز سی، شش عدد آغازگرِ واکنش زنجیره‌ای پلیمراز اختصاصی برای *P. drechsleri* و همچنین بر اساس زیرواحد ۱ سیتوکروم اکسیداز سی سه عدد آغازگر اختصاصی برای *P. cryptogea* و *P. erythroseptica* طراحی و واسنجی شد. واکاوی‌ها نشان داد که بهترین نامزد برای شناسایی جدایه‌های *P. drechsleri* مجموعه‌ی ITS-DF2 و ITS-DR2 بود که محصولی ٥٦٧ جفت بازی را فزون‌سازی می‌کرد. استفاده از آغازگرهای COX-CF1 و COX-CR2 بهترین مجموعه برای تمایز *P. cryptogea/P. erythroseptica* از سایر گونه‌ها بود و موجب فزون‌سازی قطعه‌ای ٤١٥ جفت‌بازی شد. واکاوی نقشه‌ی آنزیم‌های برشی این دو گونه نشان داد که جایگاه آنزیم برشی غیرپالیندرومی *MnI* I در فزونه‌ی جدایه‌های *P. erythroseptica* منحصر به فرد است و از آن می‌توان برای تمایز این گونه از *P. cryptogea* استفاده کرد. نتایج این مطالعه نشان داد که استفاده از واکنش زنجیره‌ای تودرتو برای ردیابی این گونه‌ها حداقل ۱۰۰ برابر حساس‌تر از روش سنتی است.

کلیدواژه: *Phytophthora drechsleri*، *Phytophthora cryptogea*، *Phytophthora erythroseptica*، اُامیکوتا، فاصله‌ی ترانویسی شده‌ی داخلی، زیرواحد ۱ سیتوکروم اکسیداز سی، شناسایی، ردیابی

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Species-specific PCR identification and detection of *Phytophthora drechsleri, P. cryptogea* and *P. erythroseptica*

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Abstract

Phytophthora drechsleri, P. cryptogea and *P. erythroseptica* are phylogenetically closely related Oomyceteous plant pathogens which are morphologically similar. In order to discriminate these taxa from each other and from species with convergent morphological characteristics a simple as well as a nested-PCR based method was developed. A collection of isolates of each species from different hosts representing world-wide diversity of species were examined for unique regions of nuclear as well as mitochondrial genes. Six candidate PCR primers were designed and calibrated for species-specific amplification of *P. drechsleri* based on the DNA sequences of rDNA internal transcribed spacer regions and the cytochrome c oxidase subunit I, and also three candidate PCR primers specific for *P. cryptogea* and *P. erythroseptica* were designed and calibrated based on cytochrome c oxidase subunit I. Studies showed that the best primer set for identification of *P. drechsleri* was the combination of ITS-DF2 and ITS-DR2, which amplified a 567 bp band. The combination of COX-CF1 and COX-CR2 was the best set for discrimination of *P. cryptogea/P. erythroseptica* from other species which amplified a 415 bp product from both species. A restriction map analysis of *P. cryptogea/P. erythroseptica* indicated that the non-palindromic *Mnl* I enzyme restriction site was unique to amplicons of *P. erythroseptica* isolates and could be employed to distinguish this species from *P. cryptogea*. Based on this study, nested-PCR was at least 100 times more sensitive than conventional PCR for detection of these species.

Keywords: *Phytophthora drechsleri*, *Phytophthora cryptogea*, *Phytophthora erythroseptica*, *Oomycota*, Internal transcribed spacer, cytochrome c oxidase subunit I, identification, detection

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Introduction

The identification and discrimination of *Phytophthora cryptogea* Pethybridge & Lafferty (1919) and *Phytophthora drechsleri* Tucker (1931) has been a matter of controversy for more than 75 years. These two soil-borne plant pathogenic oomycetes are morphologically similar and phylogenetically related species which were considered as a species complex with ambiguous species boundaries (Erwin *et al.* 1983, Erwin & Ribeiro 1996, Mills *et al.* 1991). Recent findings however showed that these species are distinct taxa (Cooke *et al.* 2000, Kroon *et al.* 2004, Blair *et al.* 2008, Mostowfizadeh-Ghalamfarsa *et al.* 2010) with three lineages distinguished among *P. cryptogea* isolates (Mostowfizadeh-Ghalamfarsa *et al.* 2010). Studies based on multiple gene genealogy analysis also showed that the homothallic species, *Phytophthora erythroseptica* Pethybridge (1913), appears to have evolved from within one group of *P. cryptogea* (Mostowfizadeh-Ghalamfarsa *et al.* 2010).

Given its morphological similarity, *P. drechsleri* is traditionally discriminated from *P. cryptogea* by its ability to grow well at and above 35 °C (Tucker 1931). Other studies however show that the high-temperature criterion does not always correlate with the other identifying features (Klisiewicz & Beard 1976, Banihashemi & Ghaisi 1993) and as a result, some isolates were described as intermediate between both species (Flowers *et al.* 1973, Shepherd & Pratt, 1973, Klisiewicz, 1977, Stanghellini & Kronland 1982). Despite its convergent morphology, *P. erythroseptica* is a homothallic species. However, *P. drechsleri* has occasional homothallic behavior (Waterhouse 1963) and it seems that there are some intermediate isolates of *P. cryptogea* which are also homothallic (David E. L. Cooke, unpublished data). These could be a source of error in the identification of these species. On the other hand, there are some superficially similar taxa of *Phytophthora* that grow at or above 35ºC such as *P. cajani* (Amin *et al.* 1978), *P. melonis* (Ho *et al.* 2007) and *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.* 2008) that have been mistaken for either *P. drechsleri* or high-temperature tolerant *P. cryptogea* isolates. In the absence of clear morphological or physiological criteria for accurate identification of these species the objective of this study was therefore to develop diagnostic molecular tools for identification of *P. drechsleri*, *P. cryptogea* and *P. erythroseptica* isolates using species-specific PCR primers.

Material and Methods

Origin and Maintenance of Isolates

Details of the isolates examined in this study are listed in Table 1. The isolates were sourced from the culture collections of the authors. Isolates were stored on cornmeal agar (CMA; ground corn extract 40 g l^{-1}, agar 15 g l^{-1}) slopes at 15 °C. Routine stock cultures for research studies were also grown on CMA at 20 °C.

DNA Extraction

Isolates were grown in 50 ml still culture of pea broth (boiled extract of 125 g frozen green peas in 1000 ml distilled water at pH 6.2) at 20 ºC. After vacuum filtration, the mycelia was washed with sterilized distilled water, freeze-dried and stored at -20 °C. Freeze-dried mycelia were homogenized using sea sand (Fluka, Germany) and a plastic disposable pestle. DNA was extracted from homogenized preparation using a Puregene DNA extraction kit, Flowgen (Lichfield, England) according to the manufacturer's instructions. The amount of DNA obtained was estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

Primer Design

Sequenced regions of β-tubulin gene, translation elongation factor 1 α gene, elicitin gene, internal transcribed spacers 1, 2 and 5.8S gene of rDNA (ITS), and cytochrome c oxidase gene subunit I (*COX*) from *ca* 72 *Phytophthora* species along with a collection of *P. cryptogea*, *P. drechsleri*, and *P. erythroseptica*e isolates from different hosts and matrices from previous studies (Mostowfizadeh-Ghalamfarsa *et al.* 2010) were recovered from GenBank using the Nucleotide Sequence Search Program provided by the National Center for Biotechnology Information (NCBI, http://www3.ncbi.nlm.nih.Gov/Entrez) (Bethesda, MD, USA). Multiple sequence alignment of each gene was made using ClustalX

Table 1. Species-specific amplification of DNA sequences from different *Phytophthora* species by designed primer sets for *Phytophthora drechsleri, P. cryptogea* and *P. erythroseptica*.

Species	Isolate		Year of isolation	Host (Matrix)	Location	Amplification using				
	Local	International				ITS-D1[a]	ITS-D2[b]	Cox-D1[c]	Cox-C1[d]	Cox-C2[e]
P. cactorum	SCRP27	IMI296524	1985	*Rubus idaeus*	Wales	+	-	-	-	-
P. cajani	SCRP66	IMI320064	1987	*Cajanus* sp.	India	-	-	-	-	-
P. cambivora	SCRP67	IMI296831	1985	*Rubus idaeus*	Scotland	-	-	-	+	-
P. capsici	SCRP103	IMI352321	1989	*Piper nigrum*	India	+	-	-	-	-
P. cinnamomi	SCRP115	CBS270.55	1993	*Chamaecyparis lawsoniana*	Netherlands	-	-	-	-	-
P. citricola	SCRP130		1986	*Rubus idaeus*	Scotland	-	-	-	-	-
P. citrophthora	SCRP179	IMI332632		*Actinidia* sp.	Chile	+	-	-	-	-
P. cryptogea G I*	SCRP214		1973	*Gerbera jamesonii*	France	-	-	-	+	+
	SCRP205	IMI34684	?	*Solanum tuberosum*	Northern Ireland	-	-	-	+	+
	SCRP207	IMI045168	1951	*Solanum lycopersicum*	New Zealand	-	-	-	+	+
	SCRP206		?	?	England	-	-	-	+	+
	SCRP212		1987	*Solanum lycopersicum*	France	-	-	-	+	+
	SCRP219		1983	*Solanum lycopersicum*	France	-	-	-	+	+
	SCRP225		1995	*Ozothamnus* sp.	England	-	-	-	+	+
	SCRP226	IMI382781	1999	*Solanum lycopersicum*	South America	-	-	-	+	+
	SCRP229		1987	*Rubus idaeus*	England	-	-	-	+	+
	SCRP230	IMI323058	1988	*Rubus idaeus*	England	-	-	-	+	+
	SUC4		1992	?	USA	-	-	-	+	+
P. cryptogea G II	SCRP204	IMI379121 (3134)	?	*Abies nobilis*	Ireland	-	-	-	+	+
	SCRP210		?	*Abies nobilis*	USA	-	-	-	+	+
	SCRP221		?	*Rubus idaeus*	Australia	-	-	-	+	+
	SCRP223		1995	*Choisya* sp.	England	-	-	-	+	+
	SCRP235	IMI129907	?	Soil	Australia	-	-	-	+	+
	SUC2		1992	*Solanum melongena*	Iran	-	-	-	+	+
	SUKv15		2002	*Beta vulgaris*	Iran	-	-	-	+	+
	SUSt1		2002	*Beta vulgaris*	Iran	-	-	-	+	+
	SUSt3		2002	*Beta vulgaris*	Iran	-	-	-	+	+
	SCRP201	IMI260685, CBS468.81	1981	*Begonia eliator*	Germany	-	-	-	+	+
	SCRP213		1972	*Gerbera jamesonii*	France	-	-	-	+	+
	SCRP217		?	*Solanum melongena*	Spain	-	-	-	+	+

Table1. Continued.

Species	Isolate		Year of isolation	Host (Matrix)	Location	Amplification using				
	Local	International				ITS-D1[a]	ITS-D2[b]	Cox-D1[c]	Cox-C1[d]	Cox-C2[e]
	SCRP228	IMI303922	1985	*Rubus idaeus*	Ireland	-	-	-	+	+
						-	-	-		
P. cryptogea G III	SCRP209		?	*Juglans hindsii*	USA	-	-	-	+	+
	SCRP220		1989	*Rosmarinus officinalis*	France	-	-	-	+	+
	SCRP731		2003	*Rosmarinus officinalis*	Italy	-	-	-	+	+
	SCRP732		2003	*Rosmarinus officinalis*	Italy	-	-	-	+	+
P. erythroseptica	SCRP238	ATCC36302	1997	*Solanum tuberosum*	USA	-	-	-	+	+
	SCRP240		?	*Solanum tuberosum*	Netherlands	-	-	-	+	+
	SCRP241		?	*Solanum tuberosum*	Netherlands	-	-	-	+	+
	SCRP242		?	*Solanum tuberosum*	Australia	-	-	-	+	+
P. drechsleri	SCRP222		?	*Solanum tuberosum*	Wales	+	+	+	-	-
	SCRP232	ATCC46724, CBS292.35	1935	*Beta vulgaris* var. *altissima*	USA	+	+	+	-	-
	SCRP236	IMI040500	1949	*Solanum tuberosum*	Argentina	+	+	+	-	-
P. drechsleri	SCRP239	IMI340632	1990	*Oryza sativa*	USA	+	+	+	-	-
	SUAh4		2002	*Beta vulgaris*	Iran	+	+	+	-	-
	SUAk2		2002	*Beta vulgaris*	Iran	+	+	+	-	-
	SUC5		1992	?	USA	+	+	+	-	-
	SUC18		1992	*Beta vulgaris*	Iran	+	+	+	-	-
	SUC20		1993	*Helianthus annus*	Iran	+	+	+	-	-
	SUKv3		2002	*Beta vulgaris*	Iran	+	+	+	-	-
	SUSa1		2002	*Beta vulgaris*	Iran	+	+	+	-	-
	SUSa2		2002	*Beta vulgaris*	Iran	+	+	+	-	-
	SUSd3		2002	*Beta vulgaris*	Iran	+	+	+	-	-
	SUSr1		2002	*Beta vulgaris*	Iran	+	+	+	-	-
P. infestans	sc 03.26.3.3		2003	*Solanum tuberosum*	Scotland	+	-	-	-	-
P. inundata	SCRP644	IMI389751	1972	*Salix* sp.	UK	-	-	-	-	-
P. insolita	SCRP385	IMI288805	1979	Soil	Taiwan	-	-	-	-	-
P. katsurae	SCRP389	IMI 382396	?	*Theobroma cacao*	N. Sulawasi	+	-	-	-	-
P. lateralis	SCRP390	IMI040503	1942	*Chamaecyparis lawsoniana*	USA	-	-	-	-	-
P. medicaginis	SCRP407		1999	*Medicago sativa*	Iran	-	-	-	-	-

Table1. Continued.

Species	Isolate		Year of isolation	Host (Matrix)	Location	Amplification using				
	Local	International				ITS-D1[a]	ITS-D2[b]	Cox-D1[c]	Cox-C1[d]	Cox-C2[e]
P. megasperma	SCRP435	IMI133317	1968	*Malus sylvestris*	Australia	-	-	-	-	-
P. melonis	SUD26		1983	*Cucumis melo*	Iran	-	-	-	-	-
P. nicotianae	SCRP468	IMI268688	?	*Citrus* sp.	Trinidad	+	-	-	-	-
P. palmivora	SCRP526		?	*Hevea brasiliensis*	Thailand	-	-	-	-	-
P. pistaciae	SUD44		1993	*Pistacia vera*	Iran	-	-	-	-	-
P. quercina	SCRP541		1995	*Quercus robur*	Germany	-	-	-	-	-
P. ramorum	Alex1		2003	*Rhododendron* sp.	Scotland	-	-	-	-	-
P. syringae	SCRP654		1996	*Fagus sylvatica*	Germany	-	-	-	-	-
P. sojae	SCRP555		?	*Glycine max*	USA	-	-	-	+	-

+=Positive PCR product. -=Negative PCR product. [a] *Combination of ITS-DF1 & ITS-DR1 primers.* [b] *Combination of ITS-DF2 & ITS-DR2 primers.* [c] *Combination of COX-DF1 & COX-DR1 primers.* [d] *Combination of COX-CF1 & COX-CR1 primers.* [e] *Combination of COX-CF1 & COX-CR2 primers.*
* *P. cryptogea phylogenetic groups.*

(Thompson *et al.* 1997) with subsequent visual adjustment. Sequences were examined for conserved regions unique to *P. cryptogea*, *P. drechsleri,* and *P. erythroseptica*. The selected primers were further analyzed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Primers then evaluated for criteria such as melting temperature (T_m), self-dimerization, self-annealing, potential hairpin formation and G-C content using Oligo Calculator (http://www.basic.northwestern.edu/ biotools/oligocalc.html) (Kibbe 2007).

Having compared the specificity and sensitivity of primer sets, ITS-based and cytochrome c oxidase-based primers were selected for nested-PCR. Universal forward ITS6 (Cooke & Duncan 1997) and reverse ITS4 (White *et al.* 1990) primers were applied as external primers. Primer ITS6 is similar to ITS5 (White *et al.* 1990), but modified according to the *P. megasperma* 18S rDNA sequence (Förster *et al.* 1990) to allow more efficient amplification in *Phytophthora* spp. (Cooke & Duncan 1997). In case of cytochrome c oxidase-based primers COXF4N and COXR4N primer set (Kroon *et al.* 2004) are applied as external primers.

PCR Protocol

Amplifications were performed in a CG1-96 thermocycler (Corbett Research, Australia). PCR was carried out in 25 µl reactions containing 2.5 µl of 10× PCR buffer Promega, Southampton, England), 100 mM of BSA, 100 mM dNTPs, 1.5 mM of $MgCl_2$ (for ITS based primers) or 2.5 mM of $MgCl_2$ (for others), 1 mM of each primer, 0.4 U *Taq* DNA polymerase (Promega, Southampton, England) and 100 ng target DNA.

In case of ITS-based primers PCR was originally carried out with a program of 95 °C for 2 min (initial denaturation) followed by 30 cycles of 95 °C for 20 s, a gradient of annealing temperature from 55-68 °C for 25 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min for both simple and nested-PCR. In case of other primers PCR was originally carried out with a program of 95 °C for 2 min (initial denaturation) followed by 35 cycles of 95 °C for 30 sec, a

gradient of annealing temperature from 55-68 °C for 30 sec, 72 °C for 50 sec, and a final extension of 72 °C for 10 min for both simple and nested-PCR. Annealing temperatures were 55 and 52 for ITS and *COX* universal primers, respectively. Successful amplification was confirmed by gel electrophoresis (1 h at 80 V) on 1.0% agarose gels in 1× TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light.

PCR conditions, including annealing temperature and the time of annealing were optimized to maximize the yield of the desired amplification product while minimizing levels of non-specific products.

Specific Identification and Detection of Species

Primer specificity. To determine specificity of the primers, PCR was conducted on the high quality genomic DNA of various morphologically and molecularly characterized *Phytophthora* species (Table 1) using the specific primer sets.

Primer sensitivity. To resolve the sensitivity of the primers, spectrophotometrically quantified DNA was serially diluted with HPLC water over 10 orders (100 ng-10 fg) of magnitude (Table 4). Sensitivity of detection was then determined using PCR Beads (puReTaq™, Reasy-To-Go™ PCR Beads, Amersham Biosciences, UK) and each specific primer sets for both conventional and nested-PCR.

Detection of isolates in host tissues

In order to detect isolates in naturally infected tissues, DNA from diseased pistachio (*Pistacia vera*) roots naturally infected by *P. drechsleri* (from Kerman, Iran), as well as diseased sugar beet (*Beta vulgaris*) roots infected by *P. cryptogea* (from Shiraz, Iran), were tested by putative species specific primers (ITS-D2 and COX-C2 sets) through direct and nested PCR. DNA extraction performed according to Mostowfizadeh-Ghalamfarsa & Mirsoleimani (2013) and amplified products sequenced.

Sequencing of amplified products

The amplification products were purified through GenJET PCR purification kit (Fermentas, Ontario, Canada) to remove excess primers and nucleotides. PCR products were sequenced (Tech Dragon, Hong Kong, China) in forward and reverse orientation using the primers used for amplification and a dye terminator cycle sequencing kit (BigDye® Terminator V 3.1, Applied Biosystems, CA, USA) on a 3730 ×l DNA Analyzer (Applied Biosystems) according to the manufacturer's instruction.

Restriction Fragment Identification

In order to discriminate *P. cryptogea* from *P. erythroseptica*, restriction maps were provided using sequences of COX-CF1 and COX-CR2 primer sets PCR product (NEBcutter ver.2, New England Biolabs, UK) (http://tools.neb.com/NEBcutter2/index .php). Maps were compared for differential unique restriction sites. An appropriate enzyme was selected according to the differences in length of the fragments. PCR products were digested in accordance with the manufacturers' instructions. Digested bands were visualized by electrophoresis in 1% (w/v) agarose gels in 1× TBE buffer.

Results

Primer Design

Six PCR primers specific for *P. drechsleri* were designed based on ITS and *COX* genes (Table 2). For *P. cryptogea* and *P. erythroseptica* three specific primers were designed against the *COX* gene sequences (Table 2). No eligible candidate specific for each of three species was found in any of the other genes examined. Optimized PCR conditions for each putative species-specific primer pairs are summarized in Table 3.

Specificity and Sensitivity of the Designed Primers

The expected size of the amplification product for each set of species-specific primer is shown in Table 3. When each designed primer set was used, an amplicon of the expected size was obtained with DNA from all morphologically and molecularly well-characterized target species tested (Table 1). The ITS-D2 (combination of ITS-DF2 and ITS-DR2), COX-D1 (combination of COX-DF1 and COX-DR1), and COX-C2 (combination of COX-CF1 and COX-DR2) set did not amplify purified

Table 2. The putative specific primers designed for detection of *Phytophthora drechsleri*, *P. cryptogea* and *P. erythroseptica*.

Target Species	Primer	Primer sequence	orientation	Target DNA	Accession number[a]	Primer location[b]	Length (bp)
P. drechsleri	ITS-DF1	5′ GCT TTT TCT GCT GCG GCG 3′	Forward	ITS[c]	AY659442	633-650	18
	ITS-DF2	5′ CTC TAT CAT GGC GAC CGC C 3′	Forward	ITS	AY659442	95-113	19
	ITS-DR1	5′ TCA GGT CCA ATT GAG ATG CA 3′	Reverse	ITS	AY659442	789-808	20
	ITS-DR2	5′ CAC CAG TCC ATC CCG CCG 3′	Reverse	ITS	AY659442	649-663	18
	COX-DF1	5′ TAG TCA AGT TTC TGC GGC A 3′	Forward	*COX*[d]	AY659582	263-281	18
	COX-DR1	5′ TCA TGT AAA GCG ATA TCT AGG 3′	Reverse	*COX*	AY659582	584-604	21
P. cryptogea/ *P. erythroseptica*	COX-CF1	5′ TAG TCA AGT TTC RGC AGC A 3′	Forward	*COX*	AY659565	266-283	19
	COX-CR1	5′ WGT ATC ATG TAA AGC AAT ATC TAA T 3′	Reverse	*COX*	AY659565	587-611	25
	COX-CR2	5′ AAA TCC RGT AAA AAT ACC G 3′	Reverse	*COX*	AY659565	662-680	19

[a] Reference to the GenBank accession containing the DNA sequence, on which the primer is based. [b] Reference to the location of the primer within the original DNA sequence. [c] Internal transcribed spacers 1, 2 and 5.8S gene of rDNA. [d] Cytochrome c oxidase subunit I. The best primers are in bold letters.

Table 3. Optimized PCR conditions for *Phytophthora drechsleri*, *P. cryptogea* and *P. erythroseptica* putative species-specific primer pairs.

Primer	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final Extension	Length (bp)*
ITS-D1[a]	95(120)**	30	95(20)	66(25)	72(60)	72(600)	174
ITS-D2[b]	95(120)	30	95(20)	65(25)	72(60)	72(600)	567
COX-D1[c]	94(120)	35	94(20)	66(25)	72(50)	72(600)	347
COX-C1[d]	94(120)	35	94(20)	56(25)	72(50)	72(600)	354
COX-C2[e]	94(120)	35	94(20)	63(25)	72(50)	72(600)	415

* Average amplicon length. ** Temperature 'ºC' (time 's').
[a] Combination of ITS-DF1 & ITS-DR1 primers. [b] Combination of ITS-DF2 & ITS-DR2 primers. [c] Combination of COX-DF1. & COX-DR1 primers. [d] Combination of COX-CF1 & COX-CR1 primers. [e] Combination of COX-CF1 & COX-CR2 primers.

DNA from other *Phytophthora* species tested (Table 1, Fig. 1 & 2). In contrast, the ITS-D1 (combination of ITS-DF1 and ITS-DR1) and COX-C1 (combination of COX-CF1 and COX-DR1) sets amplified 6 and 2 species, respectively, other than the target species (Table 1). Comparison of the primer sequences using Primer-Blast revealed that none of the putative species-specific primers matched with sequences from any other *Phytophthora* species.

Comparison of the Sensitivities of Simple and Nested-PCRs

Comparison of direct and nested-PCR with species-specific primers as internal sets and universal primers showed that nested-PCR is more sensitive than the direct method in most cases. Nested-PCR was found to be at least 5000, 100, 1000, and 1000 times more sensitive for ITS-D1, ITS-D2, COX-D1, and COX-C2, respectively (Table 4). However, the strength of bands was

Table 4. The effect of DNA quantity (per µl sample) on PCR product band density of the putative species-specific primer sets for *Phytophthora drechsleri*, *P. cryptogea* and *P. erythroseptica* in direct and nested PCR.

Primer sets	DNA Quantity									
	100 ng	10 ng	1 ng	500 pg	100 pg	50 pg	10 pg	1 pg	100 fg	10 fg
ITS-D1[a]	+++	++	+	+	-	-	-	-	-	-
ITS-D2[b]	+++	+++	++	+	+	-	-	-	-	-
COX-D1[c]	++	+	-	-	-	-	-	-	-	-
COX-C1[d]	+++	+++	+++	+++	+++	++	-	-	-	-
COX-C2[e]	++	+	-	-	-	-	-	-	-	-
ITS6 & ITS4*	+++	++	+	+	-	-	-	-	-	-
Nested- PCR with ITS-D1	+++	+++	+++	+++	+++	+++	+++	+++	++	-
Nested- PCR with ITS-D2	+++	+++	+++	+++	+++	+++	++	+	-	-
COXF4N & COXR4N*	+++	+++	++	-	-	-	-	-	-	-
Nested- PCR with COX-D1	+++	+++	+++	+++	+++	++	+	-	-	-
Nested- PCR with COX-C1	+++	+++	+++	+++	++	+++	++	-	-	-
Nested- PCR with COX-C2	+++	+++	+++	+++	++	+	+	-	-	-

[a] Combination of ITS-DF1 & ITS-DR1 primers. [b] Combination of ITS-DF2 & ITS-DR2 primers. [c] Combination of COX-DF1. & COX-DR1 primers. [d] Combination of COX-CF1 & COX-CR1 primers. [e] Combination of COX-CF1 & COX-CR2 primers.

* External primer sets for nested PCR.

+++ = Very good. ++ = Good. + = Reasonable. - = No band.

ng = nano (10^{-9}) gram. pg = pico (10^{-12}) gram. fg = femto (10^{-15}) gram.

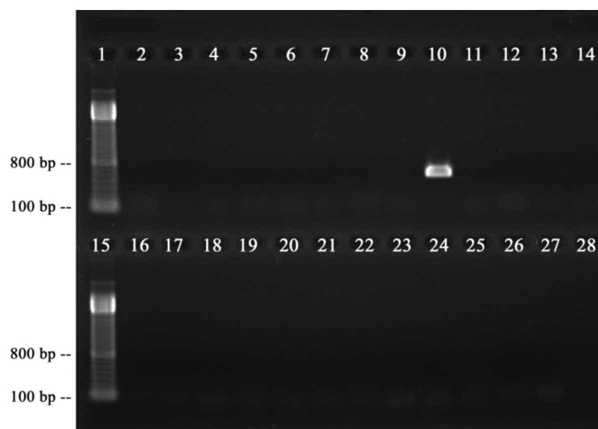

Fig. 1. Gel electrophoresis of DNA products of various *Phytophthora* species after PCR with primers ITS-DF2 and ITS-DR2. (1) 100 bp DNA ladder, (2) *P. cactorum*, (3) *P. cajani*, (4) *P. cambivora*, (5) *P. capsici*, (6) *P. cinnamomi*, (7) *P. citricola*, (8) *P. citrophthora*, (9) *P. cryptogea*, (10) *P. drechsleri*, (11) *P. erythroseptica*, (12) *P. infestans*, (13) *P. inundata*, (14) *P. insolita*, (15) 100 bp DNA ladder, (16) *P. katsurae*, (17) *P. lateralis*, (18) *P. medicaginis*, (19) *P. megasperma*, (20) *P. melonis*, (21) *P. nicotianae*, (22) *P. palmivora*, (23) *P. pistaciae*, (24) *P. quercina*, (25) *P. ramorum*, (26) *P. syringae*, (27) *P. sojae* and (28) negative control.

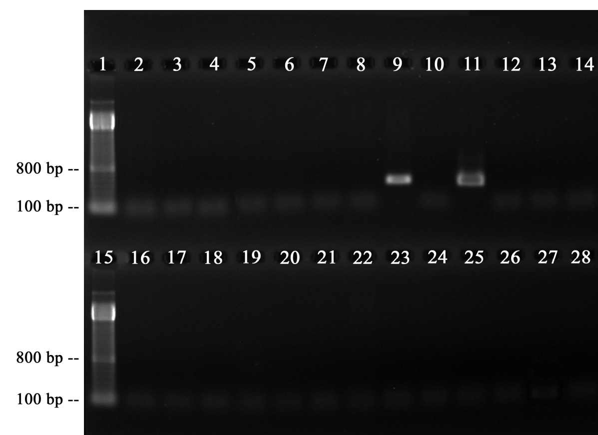

Fig. 2. Gel electrophoresis of DNA products of various *Phytophthora* species after PCR with primers COX-CF1 and COX-CR2. (1) 100 bp DNA ladder, (2) *P. cactorum*, (3) *P. cajani*, (4) *P. cambivora*, (5) *P. capsici*, (6) *P. cinnamomi*, (7) *P. citricola*, (8) *P. citrophthora*, (9) *P. cryptogea*, (10) *P. drechsleri*, (11) *P. erythroseptica*, (12) *P. infestans*, (13) *P. inundata*, (14) *P. insolita*, (15) 100 bp DNA ladder, (16) *P. katsurae*, (17) *P. lateralis*, (18) *P. medicaginis*, (19) *P. megasperma*, (20) *P. melonis*, (21) *P. nicotianae*, (22) *P. palmivora*, (23) *P. pistaciae*, (24) *P. quercina*, (25) *P. ramorum*, (26) *P. syringae*, (27) *P. sojae* and (28) negative control.

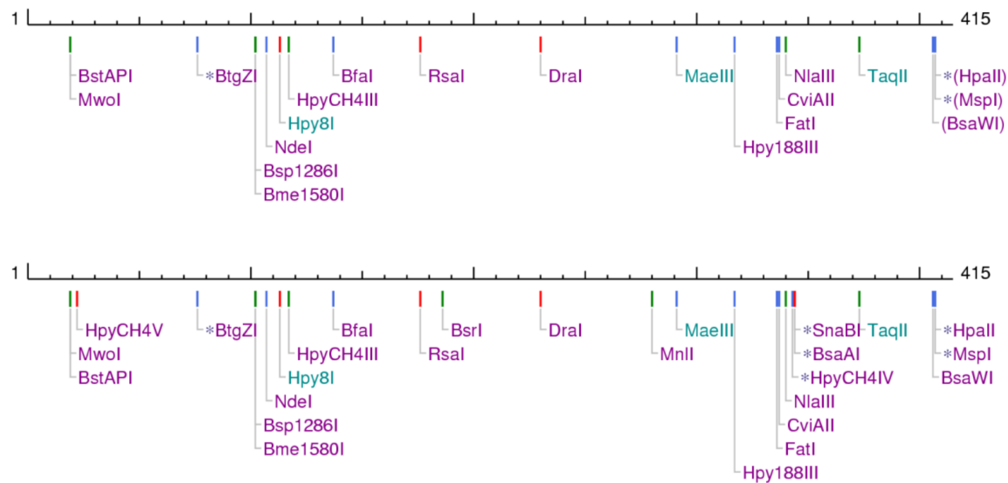

Fig. 3. One-site cutters' restriction map of the amplicon of COX-C2 primer set in *Phytophthora cryptogea* (upper panel) and *P. erythroseptica* (lower panel).

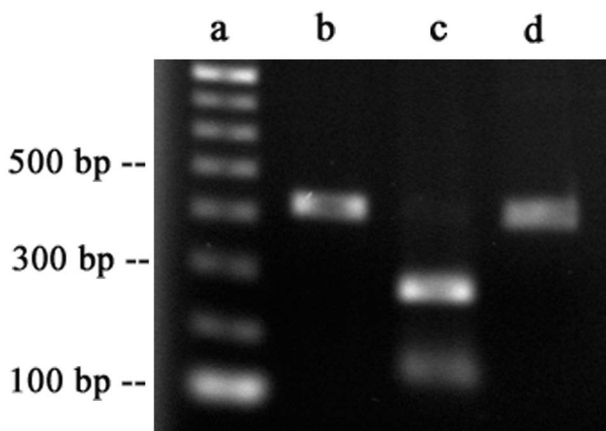

Fig. 4. Comparison of COX-C2 primer set PCR products: (a) 100 bp DNA ladder, (b) *Phytophthora erythroseptica* isolate SCRP242 intact fragment, (c) *P. erythroseptica* isolate SCRP242 fragment digested with *MnI* I and (d) *P. cryptogea* isolate SCRP207 fragment digested with *MnI* I.

much better in nested-PCR. The nested-PCR sensitivity was at least 5 times more sensitive for COX-C1 (Table 4).

Detection of Species in Infected Plant Samples

The ITS-D2 and COX-C2 primer sets detected their corresponding species in all naturally infected tissues examined. The resulting amplicons of both experiments were sequenced and their identity confirmed using a BLAST search (http://blast.ncbi.nlm.nih.gov). All bands were 100% identical to the expected fragment with a full coverage.

Restriction Fragment Identification

Comparison of restriction map analysis of *P. cryptogea* and *P. erythroseptica* indicated that there was only one restriction site which could discriminate amplicons of COX-C2 primer set (Fig. 3). The non-palindromic *MnI* I enzyme (New England Biolabs, UK) was unique for COX-C2 PCR product of the *P. erythroseptica* isolates. The recognition site was CCTC$(N)_6$N, which in this case was located on the reverse strand. *MnI* I was able to cut the 415 bp amplicon of *P. erythroseptica* into two fragments of 281 and 134 bp. Amplicons of the *P. cryptogea* isolates remained undigested (Fig. 4).

Discussion

Two primer sets based on the ITS region of rDNA and another set based on *COX* gene were designed for *P. drechsleri*. No other suitable primer set was found in the other genes examined. The PCR product of ITS-D1 primer set contained only a part of ITS2 region of rDNA repeats. Although Primer-Blast search of the NCBI non-redundant nucleotide database did not match with sequence from any other *Phytophthora*, the ITS-D1 set amplified a clear band of approximately 260

bp from *P. cactorum*, *P. citrophthora*, *P. infestans*, *P. katsurae* and *P. nicotianae* and also a faint one for *P. capsici* (data not shown) which was different from the predicted 174 bp fragment of ITS-D1. This *ca* 260 bp band was also observed in *P. drechsleri* isolates in addition to the expected 174 bp band. Searching the alignments, we found an exact match of the 8 terminal nucleotides of the forward primer (ITS-DF1) in 551-568 nucleotide sites of the target rDNA. It could be the source of the nonspecific band according to the expected fragment size between these sites and the reverse primer. It is obvious that this set cannot be used as species-specific primer for *P. drechsleri*.

The PCR product of ITS-D2 primer set contained parts of ITS1, ITS2 and whole of the 5.8S subunit. ITS-D2 was highly specific for detection and discrimination of *P. drechsleri* and did not amplify any other species. The high specificity of this primer is due to variation in the ITS1 region of rDNA where ITS-DF2 was designed and also specificity of ITS-DR2 which did not match with any sequences from other *Phytophthora* species in Primer-Blast search. This primer set was also highly sensitive and able to detect as little as 100 pg μl^{-1} DNA in a sample using direct PCR. Nested PCR with ITS-DR2, and universal ITS4 and ITS6 was at least 100 times more sensitive than the conventional PCR.

The COX-D1 primer set was also highly specific for *P. drechsleri* and did not amplify any other *Phytophthora* species. It is due to the unique sequence of COX-DR1 which did not match with any sequence from other *Phytophthora* species in NCBI nucleotide database. Although the sensitivity of COX-D1 set was not very high (10 ng μl^{-1}), the specificity of this primer makes it a useful tool for identification of *P. drechsleri* as a backup to the ITS-D2 set. These two regions are suggested barcode sequences for *Phytophthora* species (Robideau *et al.* 2011). However, combination of COX-D1 set with external COXF4N and COXR4N universal primers in nested PCR made this set more than 1000 times more sensitive than the direct PCR.

In general, it appears that ITS-D2 primer set could be the best candidate for intraspecific detection and discrimination of *P. drechsleri*. The high sensitivity and specificity of this primer set make it practical for laboratory identification as well as, environmental monitoring and population studies. Although the application of universal ITS4 and ITS6 promotes the sensitivity of the detection, another alternative could be the DC6 forward and ITS4 reverse external primers which amplify the ITS regions of members of the orders *Peronosporales* and *Pythiales* (Bonants *et al.* 1997).

P. cryptogea has three major groups (namely G I, G II, and G III) (Mostowfizadeh-Ghalamfarsa *et al.* 2010) and it seems that *P. erythroseptica* has evolved as a separate homothallic line from *P. cryptogea* groups. The presence of these groups made the designing of "catch-all" species-specific primers a challenge. No other genes were found for designing primers for *P. cryptogea* due to a high level of genetic diversity between isolates and three different groups. Comparison of multiple alignment of *COX* single-copy gene of different *Phytophthora* spp. revealed differences between *P. cryptogea* and other *Phytophthora* spp. but not *P. erythroseptica*. One forward primer and two reverse primers were designed for *P. cryptogea* which tested as two separate combinations. COX-C1 set amplified *P. cambivora* and *P. sojae* in addition to *P. cryptogea* and *P. erythroseptica*. This could be because of mismatch primer amplification due to relatively low annealing temperature of this set (56 ºC). COX-C1 set was a highly sensitive primer pair that could detect as little as 50 pg μl^{-1} DNA in a sample.

COX-C2 primer set was highly specific for all *P. cryptogea* groups and did not amplify other *Phytophthora* species but *P. erythroseptica*. The amplification of *P. erythroseptica* supports the idea of *P. cryptogea* origin of *P. erythroseptica* isolates which behaves as a *P. cryptogea* group in the *Phytophthora* spp. multigene tree (Mostowfizadeh-Ghalamfarsa 2010). COX-C2 set was able to amplify as little as 10 ng μl^{-1} and 10 pg μl^{-1} DNA in a sample using direct and nested PCR, respectively. This primer is the best choice for molecular identification and discrimination of *P. cryptogea* groups and its sister taxon *P. erythroseptica* from other species.

The non-palindromic *Mnl* I enzyme restriction site was shown to be unique to the amplicon of COX-C2 primer set in *P. erythroseptica*. Although there is another one-site cutter (*Bsr* I) for *P. erythroseptica* (Fig. 3) this enzyme is also a two-site cutter in *P. cryptogea* and is thus not as suitable for restriction fragment identification. The

Mnl I digest fragments (134 and 281bp) provided more resolution in the gel electrophoresis (Fig. 4). The specificity, sensitivity, affordability and the easy method make this a practical and reliable molecular identification method for *P. erythroseptica*.

Although a collection of isolates from different hosts and geographical areas has been tested with these primer sets and Primer-Blast results were supportive of the findings being robust, in order to have more confidence for using the sets in detection studies, more environmental samples should be assessed by each species-specific set. Moreover, although not specifically tested here, these primers should be suitable for the development of a valuable detection method for these species in infested water and soil.

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