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Detection and identification of *Phytophthora* species in southern Italy by RFLP and sequence analysis of PCR-amplified nuclear ribosomal DNA

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Abstract

In four neighbouring regions of southern Italy, Basilicata, Campania, Apulia and Calabria, pepper and zucchini plants showing Phytophthora blight symptoms, tomato plants with either late blight or buckeye rot symptoms, plants of strawberry showing crown rot symptoms and declining clementine trees with root and fruit rot were examined for *Phytophthora* infections by means of polymerase chain reaction (PCR) assays, using primers directed to nuclear ribosomal DNA (rDNA) repeat sequences. All diseased plants and trees examined tested positive. The detected fungal-like organisms were differentiated and characterized on the basis of primer specificity as well as through extensive restriction fragment length polymorphism (RFLP) and sequence analysis of PCR-amplified rDNA. *Phytophthora capsici* was identified in diseased pepper and zucchini plants, *P. infestans* was identified in tomato with late blight symptoms whereas buckeye rot-affected tomatoes and diseased strawberry plants proved to be infected by *P. nicotianae* and *P. cactorum*, respectively. Declining clementine trees were infected with *P. citrophthora* and *P. nicotianae* in about the same proportion. Also, thirty-one pure culture-maintained isolates of *Phytophthora* which had previously been identified in southern Italy by traditional methods but were never examined molecularly, were examined by RFLP and sequence analysis of PCR-amplified nuclear rDNA. Among these, an isolate from gerbera which had previously been identified by traditional methods only at genus level, was assigned to *P. tentaculata*. For the remaining pure culture-maintained isolates examined, the molecular identification data obtained corresponded with those delineated by traditional methods. Most of the diseases examined were already known to occur in southern Italy but the pathogens were molecularly detected and fully characterized at nuclear rDNA repeat level only from other geographic areas, very often outside Italy. A new disease to southern Italy was the Phytophthora blight of zucchini. This is also the first report on the presence and molecular identification of *P. tentaculata* from Italy.

Abbreviations: DNTPs – deoxyribonucleoside triphosphates; ITS – internal transcribed spacer; PCR – polymerase chain reaction; DNA RDNA – ribosomal DNA; RFLP – restriction fragment length polymorphism.

Introduction

Species of *Phytophthora* (meaning 'plant-destroyer' in Greek) are an extremely broad host-range group of plant pathogenic organisms affecting several hundred plant species worldwide, including vegetable and fruit crops, ornamental plants and forest trees (Erwin and Ribeiro, 1996; Agrios,

1997). Many diseases they cause are of great economic importance due to their devastating effects. Detection and identification of some *Phytophthora* species have traditionally been based upon microscopic examination of morphological characters, isolation of the pathogen on selective media, and occasionally on physiological traits. However, lack of reliable morphological markers and a considerable morphological plasticity occurring in some taxa may lead to inaccurate identification. Isolation of *Phytophthora* species on culture media is difficult because most of these plant pathogenic organisms tend to be slow-growing on agar and the optimal method varies from species to species. In addition, once in culture, morphological characters typical of a given taxon are not consistently and stably expressed (Duncan and Cooke, 2002). Host specificity and symptomatology are also not the most appropriate tools in detecting and identifying *Phytophthora* species. Some species attack only one or two species of host plants, but others may cause similar symptoms on many different kinds of host plants whereas more than one species may be involved in a given disease. Infections caused by *Phytophthora* species on roots and stems, especially in trees and shrubs, very often escape observations because the affected plants at first show only non-specific symptoms similar to those of drought and starvation, and then plants quickly become weakened and susceptible to other pathogens or other abiotic factors that are mistakenly considered as the causes of the death of the plants (Agrios, 1997).

During the last years, considerable progress has been made in the use of DNA-based methods for detection, identification, and classification of *Phytophthora* species (Ristaino et al., 1998; Wangsomboondee and Ristaino, 2002; Kong et al., 2003; Martin and Tooley, 2004). In particular, specific and sensitive detection methods have been developed, mainly based on polymerase chain reaction (PCR) assays (Bonants et al., 1997; Trout et al., 1997; Ristaino et al., 1998, 2001; Schubert et al., 1999; Grote et al., 2002; Ippolito et al., 2002). It has also become possible to distinguish, characterize and classify *Phytophthora* species on a phylogenetic basis, using restriction fragment length polymorphism (RFLP) and sequence analysis of PCR-amplified nuclear ribosomal DNA (rDNA) (Crawford et al., 1996; Ristaino et al., 1998; Cooke and Duncan, 1997; Cooke et al., 2000a, b; Cohen et al., 2003; Appiah et al., 2004). The phylogenetic and taxonomic relationships of individual species of *Phytophthora* and their relationships to each other and to other fungal-like organisms can be established using DNA techniques. In contrast to undefined genomic DNA fragments, the nuclear rDNA is a very attractive marker which provides valuable molecular information on fungi and stramenopiles (Chromista). In eukaryotes, in general it consists of highly repeated units, arranged in tandem, with each unit containing genes encoding the 18S, 5.8S and 28S rRNA respectively, interspaced with regions (internal transcribed spacer [ITS] regions, ITS1 and ITS2) which do not encode any functional part of the ribosome. The rRNA genes are highly conserved and are suitable for determining relationships among distantly related organisms whereas ITS region sequences are much more variable, even among quite closely related organisms. Sequence differences in the ITS regions are useful for distinguishing *Phytophthora* species (Cooke et al., 2000a, b; Duncan and Cooke, 2002). Additionally, the high copy number of rDNA sequences present in the nuclear genome makes them ideal targets for detection compared to single-copy genes.

In this work, *Phytophthora* infections were directly detected in tissues of diseased vegetable and fruit crop plants in southern Italy by means of PCR amplification using primers directed to nuclear rDNA sequences. The detected Phytophthoras along with several other *Phytophthora* isolates which had previously only been identified in southern Italy by traditional methods were characterized using RFLP and sequence analysis of PCR-amplified nuclear rDNA.

Materials and methods

Plant material, pathogen isolates and culture conditions

Sampling of diseased and non-symptomatic plants was carried out from 2000 through 2003, mainly during growing seasons, in four neighbouring regions of southern Italy. A total of 42 pepper (*Capsicum annuum*) plants showing typical symptoms of Phytophthora blight disease was sampled in several commercial fields of pepper located in Agri valley (20 plants), Metapontino (10 plants)

and Vulture (12 plants) plains. All samples were taken in the vegetable-growing areas in the Basilicata. Diseased peppers were sampled in Apulia (12 plants) and Calabria (5 plants). Samples from late blight-infected tomato were collected in Vulture plain (8 plants), in Apulia near Foggia (10 plants) and Campania region near Battipaglia (5 plants). Five tomato plants showing mainly buckeye rot symptoms were sampled in the Metapontino plain. Ten plants of zucchini (*Cucurbita pepo*) with symptoms of damping-off, foliar blight, stem lesions and crown, root and fruit rot were collected in the Agri valley. Crown rot-affected cultivated strawberry (*Fragaria* × *ananassa*, 14 plants), as well as wild strawberry (*F. vesca*) showing crown rot and/or declining symptoms were collected in the Metapontino plain (5 plants), Agri valley (5 plants) and Campania region (15 plants), respectively. Clementine (*Citrus clementina*) (9 trees) (Ci.cl.1 through Ci.cl.9) that were 15 years-old, grown in a high-intensity orchard and showing stem gummosis, root and fruit rot symptoms, were collected in the Metapontino plain. Several non-symptomatic field-collected and healthy greenhouse-grown plants of the species examined were used as healthy controls.

Fifteen isolates of *P. capsici* from pepper, one isolate of *P. capsici* each from eggplant, tomato and musky gourd, two and one isolates of *P. infestans* from tomato and potato respectively, two isolates of *P. cactorum* from strawberry, two isolates of *P. syringae* and one of *P. citricola* (CITR-LE) all from lemon, one isolate of *P. nicotianae* each from tomato and tobacco, and one isolate each of *Phytophthora* sp. (TEN-GE), *P. cinnamomi* and *P. citrophthora* from gerbera, chestnut and *Citrus* sp. respectively, were also included in this study. These isolates hereafter referred to as 'pure culture-maintained isolates' had previously been identified in southern Italy by traditional methods but were never examined by molecular technologies (Cristinzio and Noviello, 1980; G. Cristinzio and I. Camele, unpublished data). Reference isolates of *P. capsici* (provided by J.B. Ristaino, North Carolina State University, Raleigh, USA) and *P. cactorum* (provided by E. Seemüller, Biologische Bundesanstalt, Dossenheim, Germany), whose molecular identification had already been determined, were included for comparison. Further information on these reference isolates is given elsewhere (Stammler and Seemüller, 1993; Stammler et al., 1993; Ristaino et al., 1998). All pure culture-maintained isolates including the reference isolates were maintained at 5 °C on oatmeal agar and were grown routinely at 20 °C on French bean or V8-juice agar.

DNA isolation

DNA from all pure culture-maintained isolates and reference isolates was isolated from fresh mycelium which was scraped from agar plates by a scalpel and transferred to a 1.5 ml reaction tube. DNA was isolated from approximately 0.1 g of mycelium employing a method previously described (Bonants et al., 1997). For DNA isolation from diseased and healthy plants, rootlet samples were collected from strawberries and clementines whereas stem, leaf and fruit tissues were taken from pepper, tomato and zucchini plants. Rootlets were carefully washed, blotted dry, cut into small pieces with scissors and ground (1 g) in liquid nitrogen with mortar and pestle to a fine powder. Stem, leaf and fruit lesions were excised as aseptically as possible and processed similarly. Tissue powder was extracted according to the DNA isolation protocol of Doyle and Doyle (1990). Some mycelium and plant samples were also extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

Primers and PCR amplification

Several primer pairs were employed for PCR amplification (Table 1). All primers were directed to nuclear rDNA sequences. The universal primer pair ITS6/ITS4 amplifies a ribosomal fragment that extends from the 3′-enol 3′-end of the 18S rRNA gene to the 5′-enol 5′-end of the 28 rDNA, thus including the ITS1 and ITS2 regions and the 5.8 rDNA, from many eukaryotes (White et al., 1990; Cooke et al., 2000a). The primer DC6 which primes in the 3′-region of the 18S rRNA gene, in combination with ITS4, specifically amplifies a DNA fragment from *Phytophthora*, *Pythium* and downy mildews (Bonants et al., 1997). Primers P1 and P2 were derived from the terminal regions of the DNA fragment PS3003 of *P. fragariae* var. *rubi* (Stammler and Seemüller, 1993). However, these primers also amplified the target DNA from other *Phytophthora* species (Bonants et al., 1997).

Table 1. Details of oligonucleotide primers used in this study

Primer	Primer sequence (5′ – 3′)	Sense	Target	References
ITS6	GAA GGT GAA GTC GTA ACA AGG	Forward	18S rDNA	Cooke et al. (2000a)
ITS4	TCC TCC GCT TAT TGA TAT GC	Reverse	28S rDNA	White et al. (1990)
DC6	GAG GGA CTT TTG GGT AAT CA	Forward	18S rDNA	Bonants et al. (1997)
P1	CCG TTA CTA GGG GAA TCC TT	Forward	PS3003	Stammler and Seemüller (1993)
P2	TTC ATT TTC GGA TAG AAC CG	Reverse	PS3003	Stammler and Seemüller (1993)
DC1	ACT TAG TTG GGG GCC TGT CT	Forward	ITS1 rDNA	Bonants et al. (1997)
DC5	CGC CGA CTG GCC ACA CAG	Reverse	ITS2 rDNA	Bonants et al. (1997)
B5	TGA GAT GCC ACC CGC AGC A	Reverse	ITS2 rDNA	Bonants et al. (1997)
ADF1	TAC TGT GGG GAC GAA AGT CCT	Forward	ITS1 rDNA	Boersma et al. (2000)
ADR1	CCG ATT CAA AAG CCA AGC AAC T	Reverse	ITS2 rDNA	Boersma et al. (2000)
DC3	CCA ATA GTT GGG GGT CTT ATT	Forward	ITS1 rDNA	Boersma et al. (2000)
DC8	AAT TCA AAA GCC AAG CCA CC	Reverse	ITS2 rDNA	Boersma et al. (2000)
Pc2B	GTT TGT GCT TCG GGC CGA GG	Forward	ITS2 rDNA	Ippolito et al. (2002)
Pc7	GCA GAA AAG CAT ACA ATA AGC GCC TGT	Reverse	ITS2 rDNA	Ippolito et al. (2002)

Also, the following species-specific primer pairs were used: DC1/DC5 or B5 that specifically amplifies DNA of *P. fragariae* (Bonants et al., 1997), ADF1/ADR1 that is specific for *P. cactorum* (Boersma et al., 2000), DC3/DC8 that is specific for *P. nicotianae* (Boersma et al., 2000), and Pc2B/Pc7 that specifically amplifies the target DNA from *P. citrophthora* (Ippolito et al., 2002). Amplifications were performed with an automated thermal cycler (Hybaid) in a 50 µl reaction volume containing 125 µM of the four dNTPs, 0.5 µM of each primer, 1 U of DyNAzyme EXT DNA polymerase, 1× polymerase buffer (both Finnzymes), 1–3 µl of template DNA (20–50 ng), and water. The reaction mixture was subjected to 35 cycles at the following incubations: 30 s denaturation at 95 °C (120 s for the first cycle), 30 s annealing at 55 °C (65 °C with primers DC1/DC5 or B5, and DC3/DC8) and 60 s (150 s with primers P1/P2) extension at 72 °C (10 min for the final cycle). In nested PCR assays, initial amplification ('first round') was carried out with either primers DC6/ITS4 or P1/P2. The products obtained were re-amplified with primers ITS6/ITS4 and the species-specific primers mentioned above, respectively. For amplification with nested primers, either 1 µl of undiluted PCR products or 3 µl of 1:50 dilution obtained in the initial amplification were used as template in the same reaction mixture as in the first round. Five microliters of PCR products were analyzed by gel electrophoresis in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) in the presence of 0.5 µg ml^{-1} ethidium bromide. DNA bands were visualized using a UV transilluminator.

RFLP analysis

Ten microliters of PCR products obtained with the primer pair ITS6/ITS4 were separately digested with *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II and *Hae*III restriction endonucleases following the manufacturer's instructions (MBI Fermentas). Seven to twelve microliters of the digests were used to resolve the restriction fragments on either vertical 5% polyacrylamide gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8) or 3% horizontal agarose gel. After electrophoresis, the DNA was visualized with ethidium bromide as described above. Molecular weights were determined using the 1 kb DNA ladder (BRL Life Technologies).

Sequence analysis

The ITS6/ITS4 PCR products (approximately 5 µg) were separeted by electrophoresis in 1.5% agarose gel. Fragments with sizes corresponding to the expected amplified sequences were excised from the gel and eluted using the QIAquick gel extraction kit (Qiagen). DNA fragments were either sequenced directly or cloned prior to sequencing. For cloning, DNA fragments were ligated into plasmid vector pGEM-T (Promega) and recombinant plasmid used to transform *Escherichia coli* strain DH5α. Selected recombinant clones

were screened for rDNA inserts of the specific *Phytophthora* species by PCR followed by RFLP analysis as described above. All cloned fragments showed the same RFLP profiles as the PCR-amplified DNA used for cloning. Plasmid DNA was purified using the NucleoSpin system kit (Macherey-Nagel). Sequencing of both strands was performed by a commercial service (Bio Molecular Research and Biotechnology Centre, University of Padua, Padua, Italy). Primers for sequencing PCR products were the same as for PCR amplification whereas the standard primers SP6 and T7 were used for sequencing the cloned fragments. Sequences were then assembled and edited using DNASTAR's LaserGene software (DNASTAR) and consensus sequences were generated. These were then used as query sequences in a BLAST 2.0 search (Altschul et al., 1977) and were trimmed to remove all 18S and 28S rDNA sequences. Sequence alignments were performed by using CLUSTAL, version 5, using DNASTAR's LaserGene software (DNASTAR). The GenBank accession numbers of sequences determined in this study and others retrieved from the GenBank database are given in Table 2.

Results

Amplification of Phytophthora *rDNA by direct ('one-round') and nested PCR*

With primer pairs ITS6/ITS4 and DC6/ITS4, the target DNA was amplified from all samples collected from diseased pepper, tomato and zucchini plants (data not shown). The target DNA was also amplified from all pure culture-maintained isolates including the *P. capsici* and *P. cactorum* reference isolates. No visible PCR products were obtained on template DNA isolated from diseased strawberry plants and clementine trees. With the primer pair P1/P2 all pure culture-maintained isolates including the reference ones tested positive while DNA from diseased plants was not amplified with these primers (data not shown). When the amplification products obtained with primers DC6/ITS4 were re-amplified with primer pair ITS6/ITS4 in nested PCR assays, an amplification product whose size was in the range typical of *Phytophthora* (Cooke et al., 2000b) was obtained from all samples taken from diseased pepper, tomato, zucchini, strawberry plants and diseased clementine trees as well as from all pure culture-maintained isolates including the *P. capsici* and *P. cactorum* reference isolates (Figure 1). Nested PCR assays employing species-specific primer pairs in the second round were also used to detect mixed infections in diseased strawberry plants and clementine trees. When the amplification products obtained in the first round with primer pairs DC6/ITS4 or P1/P2 were re-amplified with primers ADF1/ADR1, all symptomatic strawberry plants examined tested positive (Figure 2). Similarly, the *P. cactorum* pure culture-maintained isolates as well as the *P. cactorum* reference isolate reacted positive. The primer pair DC1/DC5 or B5 did not initiate amplification in template DNA from strawberry plants in nested PCR assays (data not shown). Among the clementine trees that tested positive in nested PCR with primers DC6/ITS4 followed by ITS6/ITS4 five trees (Ci.cl.1, Ci.cl.2, Cicl.3, Ci.cl.6 and Cicl.9) gave positive results when DC6/ITS4 were followed by the primer pair Pc2B/Pc7 whereas the remaining trees (Ci.cl.4, Cicl.5, Ci.cl.7 and Cicl.8) tested positive when DC3/DC8 were used as nested primers (Figure 2, and data not shown). Species-specific primer pairs Pc2B/Pc7 and DC3/DC8 amplified the target DNAs in both direct and nested PCR also from *P. citrophthora* and *P. nicotianae* pure culture-maintained isolates, respectively. DNA was not amplified from healthy control plants by either direct or nested PCR assays.

RFLP analysis

Following separate digestion with *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II and *Hae*III restriction endonucleases, all ITS6/ITS4 amplicons from pepper, zucchini and isolates of *P. capsici* from pure culture showed the same restriction profiles with each of these enzymes. These profiles were identical to those of the American reference isolate of *P. capsici* (Figures 3, 4, and data not shown). Number and size of fragments obtained by digestion with the mentioned endonucleases are shown in Table 3. After digestion of the PCR products with each endonuclease, all rDNA samples from diseased wild and cultivated strawberry as well as the *P. cactorum* pure culture-maintained isolates had the same restriction profiles and were identical to those of

Table 2. *Phytophthora* species or isolates examined in this study by sequence analysis of nuclear ribosomal DNA repeat (ITS1, 5.8S RNA and ITS2)

Species	Isolate	Plant host	Geographic origin	GenBank accession No.	Reference/Collector(s)
P. capsici	IMI352321	*Piper nigrum*	India	AF266787	Cooke et al. (2000a)
P. capsici	KACC40177	*Lycopersicon esculentum*	Korea	AF228079	S.-B. Hong, H.-J. Jee, S.-H Kim and S.-J. Go
P. capsici		*Capsicum annuum*	Taiwan	AY251662	L.-C Huang and R.-F Liou
P. capsici	CAP-TO*	*Lycopersicon esculentum*	southern Italy	AJ854284	This paper
P. capsici	CAP-PE*	*Capsicum annuum*	southern Italy	AJ854285	This paper
P. capsici	Cu.pe.1**	*Cucurbita pepo*	southern Italy	AJ854286	This paper
P. capsici	Cu.pe.2**	*Cucurbita pepo*	southern Italy	AJ854287	This paper
P. cactorum		*Rubus idaeus*	Wales	AF266772	Cooke et al. (2000a)
P. cactorum	KACC40174	*Pyrus sinensis*	Korea	AF087480	S.-B. Hong, H.-J. Jee, S.-H Kim and S.-J. Go
P. cactorum	CAC-ST*	*Fragaria* × *ananassa*	southern Italy	AJ854288	This paper
P. cactorum	Fr.an.1**	*Fragaria* × *ananassa*	southern Italy	AJ854289	This paper
P. cactorum	Fr.ve.1**	*Fragaria vesca*	southern Italy	AJ854290	This paper
P. infestans	KACC40706	*Lycopersicon esculentum*	Korea	AF228084	S.-B. Hong, H.-J. Jee, S.-H Kim and S.-J. Go
P. infestans	IMI66006	*Solanum tuberosum*	The Netherlands	AF266779	Cooke et al. (2000a)
P. infestans	INF-TO*	*Lycopersicon esculentum*	southern Italy	AJ854291	This paper
P. infestans	INF-PO*	*Solanum tuberosum*	southern Italy	AJ854292	This paper
P. infestans	Ly.es.LB**	*Lycopersicon esculentum*	southern Italy	AJ854293	This paper
P. nicotianae	6134-A2	Unknown	China	AY208128	Z. Zhang, Y. Wang and X. Zheng
P. nicotianae	NIC-TO*	*Lycopersicon esculentum*	southern Italy	AJ854294	This paper
P. nicotianae	NIC-TB*	*Nicotiana tabacum*	southern Italy	AJ854295	This paper
P. nicotianae	Ci.cl.5**	*Citrus clementina*	southern Italy	AJ854296	This paper
P. syringae	CBS 132.23	Unknown	United Kingdom	AF380146	Man in't Veld et al. (2002)
P. syringae	MI296829	*Rubus idaeus*	Scotland	AF266803	Cooke et al. (2000a)
P. syringae	SYR-LE1*	*Citrus limon*	southern Italy	AJ854297	This paper
P. syringae	SYR-LE2*	*Citrus limon*	southern Italy	AJ854298	This paper
P. citrophthora	PA68	*Poncirus trifoliata*	Corsica	AY228575	Cohen et al. (2003)
P. citrophthora	IMI332632	*Actinidia chinensis*	Chile	AF266785	Cooke et al. (2000a)
P. citrophthora	Ci.cl.3**	*Citrus clementina*	southern Italy	AJ854299	This paper
P. cinnamomi	6-4-4c	Unknown	California	AY302182	M. Garbelotto, T.T. Sarvi and K. Ivors
P. cinnamomi	CIN-CH*	*Castanea sativa*	southern Italy	AJ854300	This paper
P. cryptogea	IMI045168	*Lycopersicon esculentum*	New Zealand	AF266796	Cooke et al. (2000a)
P. tentaculata	CBS552.96	*Chrysanthemum leucanthemum*	Germany	AF266775	Cooke et al. (2000a)
P. tentaculata	TEN-GE*	*Gerbera jamesonii*	southern Italy	AJ854302	This paper
P. citricola	CIT 7	*Quercus robur*	Germany	AJ007370	Schubert et al. (1999)
P. citricola	IMI031372	*Rubus idaeus*	*Ireland*	AF266788	Cooke et al. (2000a)
P. citricola	CITR-LE*	*Citrus limon*	southern Italy	AJ854301	This paper

* isolates maintained in pure culture which had previously been identified by traditional methods.
** isolates detected by PCR assays in tissues of naturally infected field-collected plants.

the German reference isolate *P. cactorum*. Samples from late blight-diseased tomato and from *P. infestans* pure culture-maintained isolates yielded the same restriction profiles with each enzyme whereas those from buckeye rot-affected tomato and the clementine trees Ci.cl.4, Cicl.5, Ci.cl.7 and Cicl.8 proved to be indistinguishable from *P. nicotianae* pure culture-maintained isolates (examples of restriction patterns are given in Figures 3 and 4). The PCR products obtained from the clementine trees Ci.cl.1, Ci.cl.2, Cicl.3, Ci.cl.6 and Cicl.9 all gave the same restriction patterns as isolate of *P. citrophthora* obtained from pure culture. The isolates of *P. syringae* obtained from pure culture had a unique RFLP pattern and differed from other species examined with all restriction enzymes tested. The isolate TEN-GE showed the same *Alu*I, *Rsa*I, *Sau*3AI, *Hha*I and *Hin*fI restriction

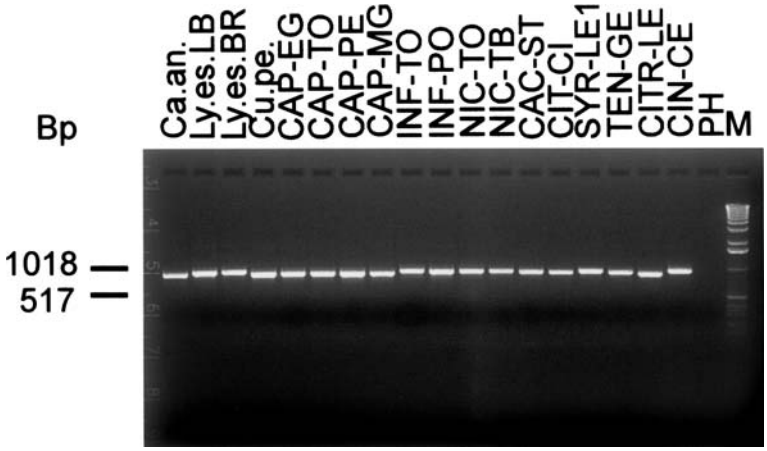

Figure 1. PCR amplification (examples) of *Phytophthora* ribosomal DNA fragments using primer pair DC6/ITS4 followed by nested primers ITS6/ITS4 from template DNA extracted from diseased plants and from *Phytophthora* isolates maintained in pure culture which had previously been identified by traditional methods. M, 1 kb DNA ladder (BRL Life Technologies); Ca.an., *Capsicum anuum* (pepper); Ly.es.LB, *Lycopersicon esculentum* (tomato) with late blight symptoms; Ly.es.BR, tomato showing mainly buckeye rot symptoms; Cu.pe., *Cucurbita pepo* (zucchini); CAP-PE, CAP-EG, CAP-TO and CAP-MG, isolates of *P. capsici* from pepper, eggplant, tomato and musky gourd respectively; INF-TO and INF-PO, *P. infestans* isolates from tomato and potato respectively; NIC-TO and NIC-TB, *P. nicotianae* from tomato and tobacco respectively; CAC-ST, *P. cactorum* from strawberry; CIT-CI, *P. citrophthora* from *Citrus* sp.; SYR-LE1, *P. syringae* from lemon; TEN-GE, *Phytophthora* sp. from gerbera; CITR-LE, *P. citricola* from lemon; CIN-CE, *P. cinnamomi* from chestnut; PH, healthy pepper.

profiles as *P. cactorum* isolates but had unique *Taq*I, *Mse*I and *Hae*III profiles. Following digestion with *Hpa*II, the restriction pattern of TEN-GE was identical to that of *P. infestans* and was due to the presence of two cleavage sites in the amplified sequences. The isolate CITR-LE showed the same *Rsa*I and *Mse*I restriction profiles as *P. citrophthora*. Each of these profiles resulted from the presence of three cleavage sites. However, after digestion with *Hha*I, *Hin*fI and *Hae*III, and *Sau*3AI and *Taq*I, the restriction patterns of CITR-LE were identical to those *P. capsici* and *P. cactorum*, respectively whereas the *Alu*I and *Rsa*I patterns of CITR-LE were unique. The iso-

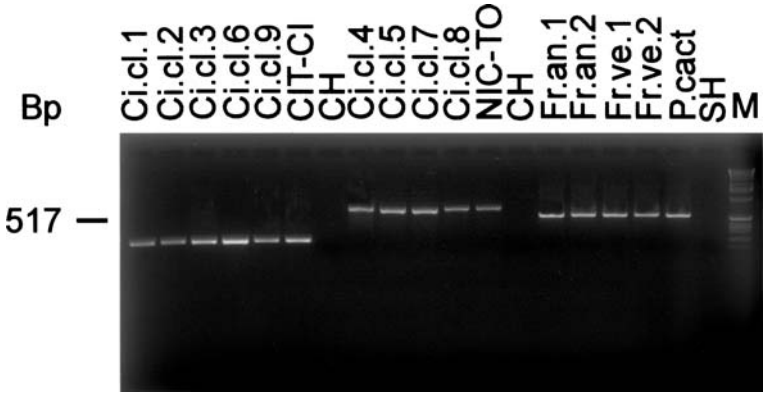

Figure 2. Detection of *Phytophthora* infections by nested PCR assays in declining *Citrus clementina* (clementine) trees (Ci.cl.1 to Ci.cl.9) and diseased *Fragaria* × *ananassa* (cultivated strawberry) plants (Fr.an.1 and Fr.an.2) and *F.vesca* (wild strawberry) plants (Fr.ve.1 and Fr.ve.2) using the following primer pair combinations: DC6/ITS4 followed by Pc2B/Pc7 (Ci.cl.1 through CIT-CI), DC6/ITS4 followed by DC3/DC8 (Ci.cl.4 through NIC-TO) and DC6/ITS4 followed by ADF1/ADR1 (Fr.an.1 through P.cact). CH, healthy clementine; SH, healthy cultivated strawberry; P. cact, German reference isolate of *P. cactorum*; CIT-CI and M, see Figure 1 for abbreviations.

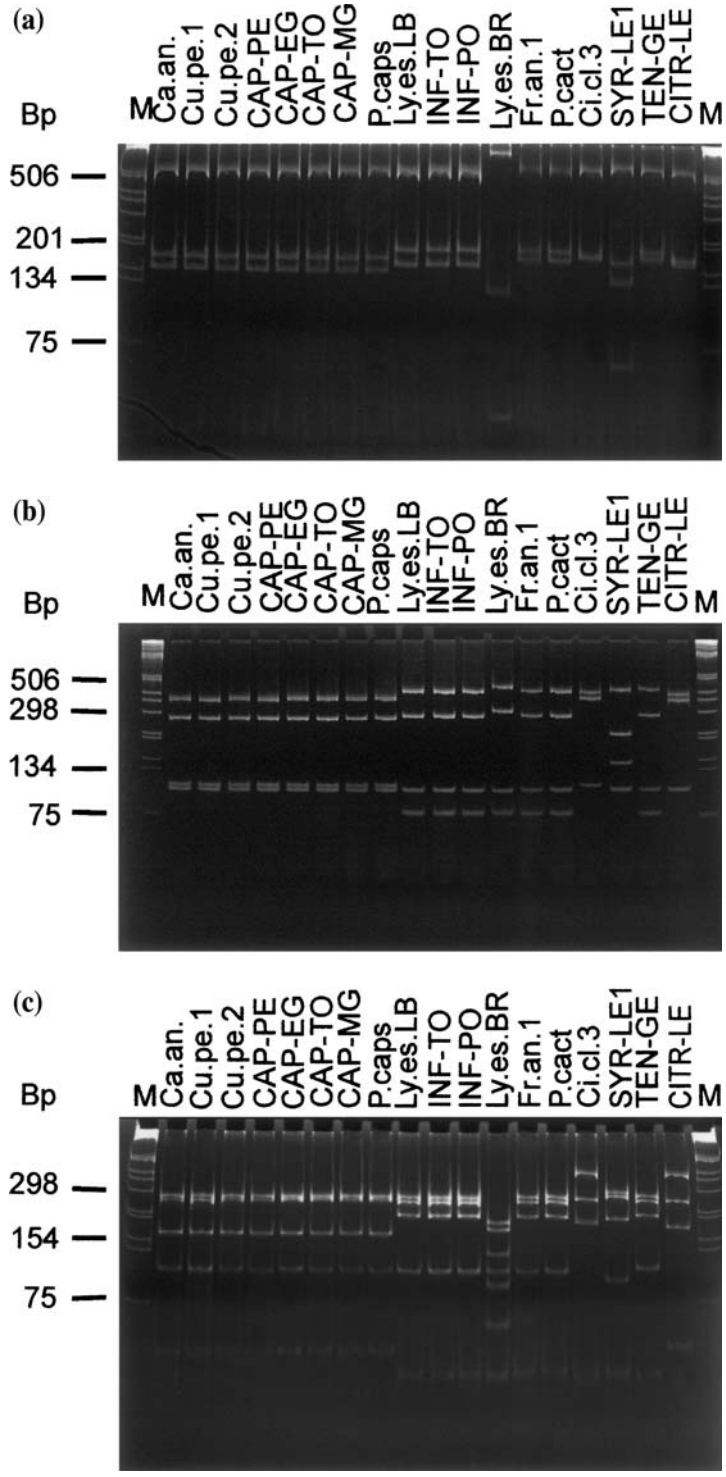

Figure 3. *Alu*I (a), *Rsa*I (b) and *Mse*I (c) restriction profiles of *Phytophthora* ribosomal DNA amplified by PCR assays using primer pair DC6/ITS4 followed by nested primers ITS6/ITS4 from template DNA extracted from diseased plants and from *Phytophthora* isolates maintained in pure culture including reference isolates. P. caps, American reference isolate of *P. capsici*; see Figures 1 and 2 for other abbreviations.

late of *P. cinnamomi* showed the same *Alu*I pattern as TEN-GE isolate but had *Rsa*I, *Hha*I, *Taq*I, *Hin*fI and *Hpa*II restriction profiles that differed from those of all other isolates examined (Figure 4(a), and data not shown).

Sequence analysis

Nucleotide sequence analysis of PCR-amplified rDNA revealed that CAP-TO, CAP-PE, Cu.pe.1 and Cu.pe.2 isolates each had a total ITS1, 5.8S and ITS2 region sequence length of 752 nucleotide residues like the Indian (GenBank accession No. AF266787) and Korean (GenBank accession No. AF228079) *P. capsici* isolates (Table 2). Isolates CAP-TO and CAP-PE proved to be identical as did Cu.pe.1 and Cu.pe.2. However, CAP-TO and CAP-PE isolates differed from the zucchini-infecting isolates at one position in ITS2 level resulting 99.9% ITS1, 5.8S and ITS2 region sequence similarity. CAP-TO (or CAP-PE) and Cu.pe.1 (or Cu.pe.2) isolates shared 99.7, 99.1 and 98%, and 99.6, 99.2 and 97.9% sequence similarity with the *P. capsici* isolates from India, Taiwan (GenBank accession no. AY251662) and Korea, respectively. Differences occurred mainly in the ITS2 region. The isolates infecting cultivated and wild strawberry from southern Italy (CAC-ST, Fr.an.1 and Fr.ve.1) had a total ITS1, 5.8S and ITS2 region sequence length of 792 residues and proved to be identical. They showed a sequence similarity of 99.9 and 99.2% with *P. cactorum* isolates from Wales and Korea, respectively (see Table 2). Isolates INF-TO, INF-PO and Ly.es.LB were identical. Each of these isolates shared a sequence similarity of 100 and 99.5% with *P. infestans* isolates from Korea and The Netherlands, respectively. The ITS1, 5.8S and ITS2 regions of NIC-TO, NIC-TB and Ci.cl.5 isolates had a total sequence length of 803 nucleotides. These isolates were nearly identical with similarity values ranging from 99.6 to 99.9 %. NIC-TO, NIC-TB and Ci.cl.5 isolates had sequence similarity of 99.8–99.9% with the Chinese *P. nicotianae* isolate. The isolate SYR-LE2 was identical to *P. syringae* CBS 132.23 isolate from the United Kingdom whereas isolate SYR-LE1 differed from them by one position in the ITS2 region. Isolate Ci.cl.3 showed a sequence similarity of 99.9% with the *P. citrophthora* PA68 isolate whereas isolate CIN-CH was identical to the Californian *P. cinnamomi* isolate. Isolates CITR-LE and TEN-GE proved most closely related to *P. citricola* CIT 7 isolate and *P. tentaculata* isolate sharing 98.8 and 98.5% sequence similarity, respectively. Southern Italian *Phytophthora* species from a variety of hosts differed in sequence similarity from 65% (NIC-TB or Ci.cl.5 *versus* SYR-LE2) to 95.1 % (CAP-TO or CAP-PE1 vs. CITR-LE).

Discussion

With PCR assays using primers derived from nuclear rDNA sequences, *Phytophthora* infections were detected in tissues of naturally infected field-collected plants of pepper, zucchini, tomato, strawberry and clementine, sampled in several locations of four neighbouring regions of southern Italy: Basilicata, Campania, Apulia and Calabria. However, in samples from diseased strawberry plants and clementine trees, detectable amplification products were only obtained by nested PCR, in which amplification with universal fungal and/or fungal-like organism primers was followed by re-amplification with either universal primers or *Phytophthora* species-specific primer pairs. This indicates that the target DNA was present in very low concentration or was unevenly distributed in the tissue samples examined. Alternatively PCR inhibitory factors may occur in DNA extracts from plants. Most of the diseases examined were already known to occur in southern Italy but the pathogens were molecularly detected and fully characterized at nuclear rDNA level only from other geographic areas, very often outside Italy. However, detection of *Phytophthora* spp. from strawberry plants and *Citrus* trees by PCR technology using primers directed to ITS1, 5.8S and ITS2 region sequences has also been reported from southern Italy (Ippolito et al., 2002; Cacciola et al., 2003). The blight disease of zucchini was new to southern Italy. Prior to this work, a Phytophthora blight disease of zucchini was observed in northern Italy in the Piedmont region. In this case, the causal agent was characterized on physiological basis only (Tamietti and Valentino, 2001).

Differentiation and characterization of Phytophthoras detected in the previously mentioned vegetable and fruit crops in southern Italy along with thirty-one pure culture-maintained isolates of *Phytophthora* also from southern Italy, were primarily based on RFLP analysis of PCR-amplified

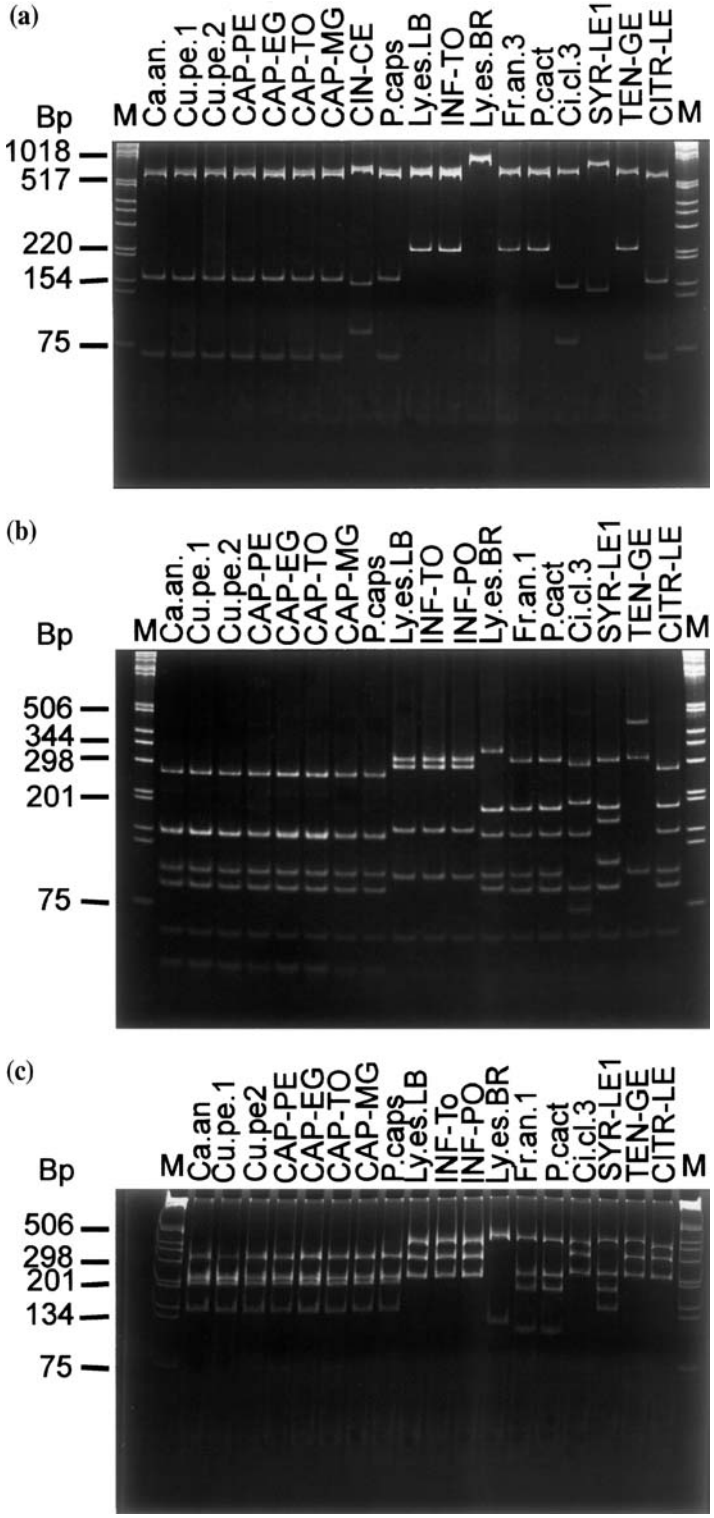

Figure 4. *Hha*I (a), *Taq*I (b) and *Hpa*II (c) restriction profiles of *Phytophthora* ribosomal DNA amplified by PCR assays using primer pair DC6/ITS4 followed by nested primers ITS6/ITS4. See Figures 1, 2 and 3 for abbreviations.

rDNA sequences, using nine frequently cutting enzymes. This straightforward method is widely used in several laboratories for studying *Phytophthora*. It also proved highly suitable in our work in which the several *Phytophthora* taxa could clearly be distinguished from each other and genetically characterized whereas unknown *Phytophthora* isolates could be assigned to a given taxon. *Phytophthora cactorum* was detected and identified in samples from all diseased strawberry plants tested. However, the quarantine-listed organism *P. fragariae* could not be detected in our work, neither alone nor in association with *P. cactorum*. Recently, Cacciola et al. (2003) reported for the first time detection of *P. fragariae* in strawberry plants grown in a plantation of the Calabria region, with no clear symptoms of the red core disease using nested PCR assays with nuclear rDNA primers. In this case, the planting material came from nursery stocks outside Italy. RFLP analysis and nested PCR assay by means of *Phytophthora* species-specific primers revealed that the declining clementine trees were infected with *P. citrophthora* and *P. nicotianae* in about the same proportion. In all the trees examined only one of the two species was detected but it can not be excluded that the two mentioned *Phytophthora* species or even others occurred together in one sample. It is possible that only the species with the highest titre was detected with the methods used.

The use of additional restriction enzymes instead of only three as used by Cooke et al. (2000b) allows a more detailed characterization and further extends knowledge on rDNA RFLP profiles of the *Phytophthora* species examined. These profiles would be helpful in comparative studies aimed at identification of unknown *Phytophthora* isolates and would allow confirmation of the presence of putative restriction sites in the sequence data available from GenBank. It is well known that fragment sizes based on putative restriction site analysis do not always correspond to those deduced from enzymatic RFLP analysis due to some undetermined bases in the sequence data.

Comparisons of the restriction patterns obtained following digestion with each endonuclease employed did not reveal any polymorphism among all isolates of each *Phytophthora* species identified. This was not unexpected due to limits in resolving power either for some closely related species or at the intraspecific level (Schubert et al., 1999; Cooke et al., 2000a, b). In contrast, intraspecific polymorphism within five *Phytophthora* species has recently been observed by Martin and Tooley (2004) using RFLP analysis of PCR-amplified mitochondrially encoded cytochrome oxidase (*cox*) I and II genes. Thus, the *cox* gene marker should be taken into account for further work aimed at differentiation of isolates within the southern Italian *Phytophthora* species. Also, as revealed by RFLP and sequence analysis there was no evidence in the present study that sequence heterogeneity of nuclear rDNA repeats occurred for any isolate. Such sequence heterogeneity has previously been reported for *Phytophthora* isolates (see Martin and Tooley (2004), for references). The fragment sizes obtained by RFLP analysis of PCR-amplified rDNA sequences were in good agreement with the expected fragment sizes based on analysis of putative restriction sites for all sequences determined.

The pure culture-maintained isolate TEN-GE proved closely related at ITS1, 5.8S and ITS2 region sequence level to *P. tentaculata* and was tentatively assigned to this species. TEN-GE had been isolated into pure culture from gerbera plants grown in Campania region that showed crown and stem rot symptoms but its identity at species level had never been determined with either traditional or molecular methods. However, recent investigations revealed that TEN-GE isolate has the same morphological characters as *P. tentaculata* and it proved to be the causal agent of crown and stem rot of gerbera according to Koch's rule fulfilment (G. Cristinzio, unpublished data). To our knowledge, *P. tentaculata* had only been reported from Germany and Spain where it was isolated from ornamental plants such as *Chrysanthemum* spp., *Delphinium ajacis* and *Verbena* hybrid (Kröber and Marwitz, 1993; Moralejo et al., 2004). Thus, our findings represent the first evidence for the presence and molecular identification of *P. tentaculata* from Italy and show that this species has a more extended host range than previously described. Our data indicate that the the molecular identification of the species of *Phytophthora* in southern Italy corresponds well with identification by traditional taxonomic methods.

Table 3. Number and size of fragments obtained by digestion with several restriction endonucleases of ITS6/ITS4 amplicons from representative southern Italian *Phytophthora* isolates

Species (isolate)	Fragment size (bp) after digestion with:									Uncut amplicon
	*Alu*I	*Rsa*I	*Sau*3AI	*Mse*I	*Hha*I	*Taq*I	*Hin*fI	*Hpa*II	*Hae*III	
P. capsici (Cu.pe.1)	525	370	715	268	640	260	300	296	545	
	180	270	100	258	160	148	170	220	318	
	150	110	45	164	65	148	154	204		
		104		100		105	130	144		
		10*		50		90	100			
				23*		58	8*			
						48				
						7*				
Total size	855	864	860	863	865	864	862	864	863	864
P. infestans (INF-TO)	535	430	760	270	690	310	350	396	907	
	200	280	100	250	220	285	170	296		
	160	105	45	212		155	154	220		
	7*	78		100		100	130			
		10*		40		58	100			
				23*			8*			
				13*						
Total size	902	903	905	908	910	908	912	912	907	907
P. nicotianae (NIC-TO)	745	438	770	187	915	315	355	398	725	
	120	296	100	175		195	255	398	192	
	50	105	45	130		150	170	120		
		78		100		105	130			
				94		90	8*			
				94		58				
				60		7*				
				40						
				23*						
				13*						
Total size	915	917	915	916	915	920	918	916	917	915
P. cactorum (Fr.an.1)	535	430	760	270	690	305	350	390	720	
	200	280	100	250	220	195	170	220	190	
	170	105	45	212		150	150	185		
		78		100		105	130	108		
		10*		40		90	100			
				23*		58	8*			
				13*		7*				
Total size	905	903	905	908	910	910	908	903	910	903
P. citrophthora (Ci.cl.3)	540	410	745	380	660	290	330	370	492	
	177	370	105	255	160	200	172	296	320	
	177	110	45	187	78	150	155	230	90	
		10*		40		90	130			
				23*		74	100			
				10*		58	8*			
						23*				
						7*				
Total size	894	900	895	895	898	892	895	896	902	894
P. syringae (SYR-LE1)	540	420	768	295	775	298	340	380	745	
	168	215	110	270	150	195	280	220	175	
	130	150	45	200		165	165	180		
	68	105		96		108	130	136		
	13*	20*		40		90	8*	8*		
		10*		13*		58				
				12*		7*				
Total size	919	920	923	926	925	921	923	924	920	918

Table 3. Continued.

Species (isolate)	Fragment size (bp) after digestion with:									Uncut amplicon
	*Alu*I	*Rsa*I	*Sau*3AI	*Mse*I	*Hha*I	*Taq*I	*Hin*fI	*Hpa*II	*Hae*III	
P. tentaculata (TEN-GE)	542	425	762	268	685	440	350	390	598	
	190	290	100	255	220	300	165	296	165	
	178	105	45	205		105	158	222	150	
		78		106		58	130			
		10*		40		7*	100			
				23*			8*			
				13*						
Total size	910	908	907	910	905	910	911	908	913	908
P. citricola (CITR-LE)	535	395	730	375	650	270	320	360	566	
	175	365	100	255	160	195	162	296	310	
	165	105	45	168	65	150	152	220		
		10*		48		105	130			
				23*		90	104			
				10*		58	8*			
						7*				
Total size	875	875	875	879	875	875	876	876	876	872
P. cinnamomi (CIN-CE)	540	440	690	380	700	190	365	405	450	
	205	210	120	268	155	180	275	220	328	
	190	170	85	220	85	150	170	167	108	
		105	45	50		125	130	145	60	
		10*		23*		120				
						90				
						58				
						12*				
						7*				
Total size	935	935	940	941	940	932	940	937	946	939

* Expected fragments based on nucleotide sequence analysis but not identified in the gels.

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