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A PCR-based assay for the detection and identification of *Pyrenochaeta lycopersici*

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Abstract

The isolation of *Pyrenochaeta lycopersici*, causal agent of corky root of tomato, is difficult because of its slow growth and poor sporulation. Identification is complicated due the existence of two morphologically similar forms, Types 1 and 2, that differ in several physiological and molecular features. For the rapid and unambiguous identification of isolates, two oligonucleotide primer pairs were designed using ITS region sequences. Specific PCR products of 147 and 209 bp were obtained for isolates of Type 1 and Type 2, respectively. Specificity of both primer pairs was verified using several fungal and bacterial species. As little as 0.7 pg of target DNA could be detected with the protocol. A nested PCR procedure was necessary for the detection of the fungus in plant tissue. This technique will be of use in epidemiological studies and in the implementation of control strategies.

Introduction

Corky-root, caused by *Pyrenochaeta lycopersici*, is an important soil-borne disease of tomato (*Lycopersicon esculentum*) and other Solanaceous crops worldwide. On the roots and rootlets of infected plants the fungus causes necrotic brown lesions that soon become corky with swollen, cracked and furrowed bark surfaces (Punithalingam and Holliday, 1973; Pohronezny and Volin, 1991). Corky roots are often invaded by other pathogens. The fungus produces solitary dark pycnidia, containing unicellular conidia on simple, septate branched conidiophores (Schneider and Gerlach, 1966). Microsclerotia of different shape and size are formed on infected roots and on greyish aerial mycelium of the fungus when grown in artificial media. When present, these features are usually sufficient for the identification of the fungus. However, diagnosis of the disease is sometimes hampered by several factors. Pycnidia of the fungus have never been observed on naturally infected roots and, once isolated, the fungus rarely sporulates in pure culture (Hockey and Jeves, 1984). Furthermore, obvious symptoms of the disease on young tomato plants and on other hosts, such as melon, are not always present (Infantino et al., 2000). The fungus grows slowly and is frequently associated with other pathogenic and saprophytic fungi present in the soil, making isolation procedures laborious and tedious. Several protocols for the isolation of *P. lycopersici* from infected roots and from soil have been published. The use of semi-selective media (Grove and Campbell, 1987; Tamietti and Valentino, 1990; Cascone et al., 2000) partially reduces the problem of competition with other microorganisms, but the results are sometimes unsatisfactory.

Data concerning the variability of *P. lycopersici* are scarce and sometimes conflicting. Infantino et al. (2003) found a low degree of molecular variability among an Italian collection of isolates of the fungus. In contrast Sugiura et al. (2003), on the basis of cultural and molecular characteristics

(mycelial growth rate, pathogenicity sequence of rDNA-ITS region, RAPD analysis), demonstrated the existence of two forms of *P. lycopersici*, naming them Type 1 and Type 2. The availability of a rapid and reliable detection method is thus needed to improve disease diagnosis. Among the most recent diagnostic techniques available for fungi, those based on the ribosomal DNA clusters 5.8S, 18S, 28S, and the spacers between them (ITS1 and ITS2) are among the most widely used for species definition, diagnosis and detection of fungi (Milgroom, 1997; Bridge and Arora, 1998; Martin et al., 2000). PCR-based techniques have proven to be very reliable in detection of soil-borne fungi causing diseases of important crops, such as *Monosporascus* vine decline of melon (Lovic et al., 1995) and *Plasmodiophora brassicae* of crucifers (Faggian et al., 1999). The aim of the present work is to develop a rapid PCR-based diagnostic test to identify and detect *P. lycopersici* directly from infected tomato tissues and to distinguish between the two types of *P. lycopersici*.

Materials and methods

Fungal isolates and culture conditions

The isolates of *P. lycopersici* used in this study are listed in Table 1. They originated from a number of European countries, Japan and the USA. A sub-set of 15 isolates was used for sequence analysis. In Table 2 a list of other species used to confirm specificity of the primer is provided. We used fungal and bacterial species commonly found during the isolation procedure for *P. lycopersici* or pathogens of other crops used in rotation with tomato (i.e. muskmelon) in Italy. Among the taxonomically related *Pyrenochaeta* species, we have considered *Herpotrichia parasitica* and *Phoma terrestris* because they could be found in agricultural soils. The isolates were either present in a collection at the Istituto Sperimentale per la Patologia Vegetale, Rome, Italy (I.S.Pa.Ve.) or obtained from international collections. Actively growing cultures of single hyphal tip isolates were grown on potato dextrose agar (PDA) (Oxoid, UK) at 22 ± 2 °C, exposed to 12 h alternate cycles of darkness and NUV (Philips TLD 18W/08 Blacklight Blue Fluorescent Lamp, peak 360 nm). Working cultures of the isolates were maintained on PDA and sub-cultured only twice to prevent genetic variation during the experiments. For long-term storage, the isolates were kept under liquid nitrogen at I.S.Pa.Ve.

Isolation procedure

Symptomatic tomato roots were cut into 2–3 cm fragments and thoroughly washed with tap water with the addition of some drops of detergent, rinsed with tap water, dipped for 5 sec in ethyl alcohol, then submerged for 1 min in mercuric chloride ($HgCl_2$ 0.1%). The fragments were then rinsed five times with sterile distilled water (SDW) and dried on sterile blotter paper. Three to five 2–5 mm long symptomatic root fragments were aseptically excised with a sterile scalpel and placed on 9 cm diam Petri dishes containing PDA amended with 100 μg ml^{-1} each of streptomycin sulphate and ampicillin (Sigma®, St. Louis, MO, USA). Plates were incubated as described and checked daily for possible contamination.

Artificial inoculation

Isolate I.S.Pa.Ve. ER 1278 for Type 1 and isolate I.S.Pa.Ve. ER 1211 for Type 2 were used for artificial inoculations. Inoculum was prepared by growing the fungal isolates in flasks containing 200 g autoclaved millet kernels moisted with 100 ml water. After incubating for one month at 23 °C one volume of the inoculum was carefully mixed with 9 volumes of sterile soil:peat:sand (2:1:1) mixture. For each isolate, 20 seedlings of the susceptible tomato cv. Corbarino were transplanted at the second true leaf stage into plastic pots containing the infested soil, then maintained in a greenhouse under natural illumination at a temperature of 18–22 °C for 40 days. An equal number of seedlings were grown on uninfested soil as the control.

DNA preparation, amplification, sequencing and phylogenetic analysis

Total genomic DNA of *P. lycopersici* isolates was extracted using a modification of the procedure described by Cenis (1992): 8-day-old mycelium was gently scraped off a PDA Petri dish surface, transferred directly into 1.5 ml Eppendorf tubes containing 300 μl of lysis buffer (200 mM

Table 1. List of *Pyrenochaeta lycopersici* isolates under study: code, number in other collections, host, GenBank accession number, origin and PCR amplification with primer pair Plyc1-F/R specific for Type 1, and Plyc2-F/R specific for Type 2

Code I.S.Pa.Ve.	No. in other collections	Host	GenBank accession	Origin	Type 1	Type 2
E.R 854		tomato	AY649583	Italy		•
E.R 876		tomato	AY649584	Italy		•
E.R 925		tomato	AY649585	Italy		•
E.R 955	CBS 306.65	tomato	AY649587	Germany		•
E.R 958	CBS 267.59	tomato	AY649589	Holland	•	
E.R 1149		tomato	AY649590	Italy	•	
E.R 1203		tomato	AY649591	Italy	•	
E.R 1214		tomato	AY649592	Italy	•	
E.R 1241		tomato	AY649596	USA	•	
E.R 1250	MAFF 712039	tomato	AY649597	Japan	•	
E.R 1251	MAFF 712040	tomato	AY649588	Japan		•
E.R 1252	CBS 282.72	soil	AY649593	Holland	•	
E.R 1257	ATCC 48567	tomato	AY649594	USA	•	
E.R 1278		tomato	AY649595	Italy	•	
E.R 1135		melon	AY649586	Italy		•
E.R 823		tomato		Italy		•
E.R 824		tomato		Italy		•
E.R 825		tomato		Italy		•
E.R 826		tomato		Italy		•
E.R 853		tomato		Italy		•
E.R 857		tomato		Italy		•
E.R 859		tomato		Italy		•
E.R 921		tomato		Italy		•
E.R 922		tomato		Italy		•
E.R 926		tomato		Italy		•
E.R 929		tomato		Italy		•
E.R 930		tomato		Italy		•
E.R 931		tomato		Italy		•
E.R 936		tomato		Italy		•
E.R 941		tomato		Italy		•
E.R 944		tomato		Italy		•
E.R 945		tomato		Italy		•
E.R 953		tomato		Italy		•
E.R 954		tomato		Italy		•
E.R 969		tomato		Italy		•
E.R 972		tomato		Italy		•
E.R 980		tomato		Italy		•
E.R 981		tomato		Italy		•
E.R 1136		tomato		Italy		•
E.R 1141		tomato		Italy		•
E.R 1142		tomato		Italy		•
E.R 1143		tomato		Italy		•
E.R 1145		tomato		Italy		•
E.R 1151		tomato		Italy		•
E.R 1156		tomato		Italy		•
E.R 1157		tomato		Italy	•	
E.R 1158		tomato		Italy		•
E.R 1160		tomato		Italy		•
E.R 1202		tomato		Italy		•
E.R 1205		tomato		Italy		•
E.R 1207		tomato		Italy		•
E.R 1208		tomato		Italy		•
E.R 1209		tomato		Italy		•
E.R 1210		tomato		Italy		•
E.R 1211		tomato		Italy		•
E.R 1212		tomato		Italy		•

Table 1. (Continued.)

Code I.S.Pa.Ve.	No. in other collections	Host	GenBank accession	Origin	Type 1	Type 2
E.R 1213		tomato		Italy		•
E.R 1236		tomato		Italy		•
E.R 1237		tomato		Italy		•
E.R 1238		tomato		Italy		•
E.R 1242		tomato		USA		•
E.R 1243		tomato		Italy		•
E.R 1244		tomato		Italy		•
E.R 1262		tomato		Italy		•
E.R 1263		tomato		Italy		•
E.R 1264		tomato		Holland		•
E.R 1277		tomato		Italy		•
E.R 1315		tomato		Italy		•
E.R 1316		tomato		Italy		•
E.R 1317		tomato		Italy		•
E.R 1318		tomato		Spain	•	
E.R 1319		tomato		Spain	•	
E.R 1320		tomato		Spain	•	
E.R 1321		tomato		Spain	•	
E.R 1147		melon		Italy		•
E.R 1159		melon		Italy		•
E.R 1216		melon		Italy		•
E.R 1217		melon		Italy		•
E.R 1219		melon		Italy		•
E.R 1312		melon		Italy		•
E.R 1313		melon		Italy		•
E.R 1314		melon		Italy		•
E.R 1310		watermelon		Italy		•
E.R 1311		watermelon		Italy		•

Underscored are the isolates used for sequence analysis. In bold are isolates also present in the paper of Sugiura et al. (2003).

Tris–HCl pH 8.5, 250 mM NaCl, 25 mM EDTA) and homogenised for 3 min using Eppendorf micro pestles (Eppendorf, Hamburg, Germany); 25 µl of 0.5% SDS were then added and tubes were placed at 65 °C for 10 min. After addition of 150 µl of 3 M sodium acetate, pH 5.2, the tubes were placed at −20 °C for 10 min, centrifuged at 10,000× *g* for 30 min and the supernatant transferred to a new tube. The DNA was precipitated by adding an equal volume of isopropanol and left for at least 5 min at room temperature. After 10 min centrifugation at 10,000× *g*, the pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µl TE-buffer. The ITS of the nuclear rDNA including the 5.8S rDNA region was amplified by PCR using the universal primers ITS4 (5′-TCCTCCGCTTATTGATATG C-3′) and ITS5 (5′-GCAAGTAAAAGTCGTA

Table 2. List of microrganisms used to evaluate the specificity of the primers developed for identification and detection of *Pyrenochaeta lycopersici*

Alternaria sp. I.S.Pa.Ve. ER 1171
Ascochyta rabiei I.S.Pa.Ve. ER 33
Cladosporium sp. I.S.Pa.Ve. ER 1169
Colletotrichum coccodes I.S.Pa.Ve. ER 1168
Diaporthe sojae I.S.Pa.Ve. ER 1342
Fusarium solani I.S.Pa.Ve. ER 1167
Fusarium oxysporum I.S.Pa.Ve. ER 1240
Herpotrichia parasitica CBS 218.77
Macrophomina phaseolina I.S.Pa.Ve. ER 783
Monosporascus cannonballus I.S.Pa.Ve. ER 1200
Myrothecium sp. I.S.Pa.Ve. ER 1172
Phoma terrestris CBS 335.87
Pseudomonas syringae pv. *tomato* I.S.Pa.Ve. MCB 412
Rhizoctonia solani I.S.Pa.Ve. ER 1343
Rhizopycnis vagum I.S.Pa.Ve. ER 940
Sclerotium rolfsii I.S.Pa.Ve. ER 1153
Verticillium dahliae I.S.Pa.Ve. ER 1180

ACAAGG-3′) (White et al., 1990). PCR amplifications were carried out in a total volume of 25 µl containing 0.6 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), 2.5 µl of 10 × PCR buffer (10 Mm Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.25 µM of each primer, 0.2 mM each of dNTPs and 10 ng of genomic DNA. For each amplification, the reaction mix without DNA template was used as the negative control. Each amplification was repeated three times. The temperature cycling parameters were as follows: 2 min denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 sec, 53 °C for 30 sec, 72 °C for 1 min, and a final elongation step of 7 min at 72 °C. The reactions were run using a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, USA). The amplified products were analysed by electrophoresis through 1.5% agarose gel in 0.5 × TAE buffer, stained with ethidium bromide and visualised under ultraviolet (UV) light (100 bp DNA Ladder Plus). The gels were visualised and analysed by the GEL DOC 2000® documentation system (BioRad Laboratories, Inc. USA). PCR products from the amplification of each of the isolates were purified with the Microcon PCR purification kit (Millipore Corporation, Bedford, MA, USA) and sequenced in both directions using primers ITS5 and ITS4 (Gene Lab – ENEA, Rome, Italy). Phylogenetic analyses were conducted using MEGA version 3.0 (Kumar et al., 2004).

Primer design

For each isolate, the amplified sequences of both strands of the ITS fragment were aligned by CLUSTAL W (Higgins et al., 1994) and manually adjusted by using Chromas (version 1.45) to obtain a consensus sequence. All sequences were submitted to BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) in order to find the most similar sequences in GenBank. The Primer 3 analysis software (Rozen and Skaletsky, 2000) was used to design specific primer pairs for *P. lycopersici* identification. Parameters were set in order to obtain amplified products of different sizes. Oligonucleotide primers were synthesised by GeneLab – ENEA, Rome, Italy. Sequences were deposited in GenBank under accession numbers AY649583–AY649597 inclusive.

Primer specificity and sensitivity test

Specificity of the primers was tested by amplifying genomic DNA extracted from all the *P. lycopersici* isolates present in the I.S.Pa.Ve. collection, from several soil-borne fungal species isolated from tomato and melon, and from DNA of *Lycopersicon esculentum* roots (Tables 1 and 2). Cultural growth, DNA extraction and PCR conditions were as described above, except that the annealing temperature was increased to 62 °C to improve stringency and reduce mismatching (Innis and Gelfand, 1990). The amplified products were run in 2% agarose gel and visualised as described. For all isolates, PCR amplification with the universal primers ITS4 and ITS5 and amplification without DNA template were done to avoid false negatives and detect possible contamination. Each amplification was done at least three times. Sensitivity of the PCR was assayed using 10-fold DNA dilution series ranging from 70 ng to 0.7 pg using *P. lycopersici* isolate I.S.Pa.Ve. ER 1278 for Type 1 and isolate I.S.Pa.Ve. ER 963 for Type 2.

DNA extraction and PCR amplification from plant tissue

Roots of the tomato plants artificially inoculated with isolate of each type and of several naturally infected plants collected from tomato fields of central and southern Italy, were washed thoroughly with tap water, rinsed with SDW, blotted dry, cut into 1-cm segments and stored at −80 °C until use. Total DNA was extracted from 2 g of symptomatic root tissues by adopting the protocol described for DNA extraction from mycelium. DNA extracted from uninoculated and/or from symptomless tomato roots was used as the negative control. A nested-PCR procedure was utilised: a first amplification with the universal primer ITS4 and ITS5 was done; the amplified product was then diluted 1:50 with Milli-Q® ultrapure sterile water (Millipore, Billerica, MA, USA) and used as a template for amplification with the specific primer. Parameters of the first amplification were the same as those used for amplification with the *P. lycopersici* specific primers described above. Presence of the fungus in symptomatic roots was confirmed by isolation on PDA. In order to confirm the specificity of the primer used, specific amplicons for both types of *P. lycopersici* were

directly sequenced at GeneLab, Enea, Rome. The sequences obtained were aligned with those representative of each type.

Results

Isolation of the fungus

Pyrenochaeta lycopersici was re-isolated from symptomatic roots of all 20 plants artificially inoculated with Type 1 and Type 2 isolates. It was constantly isolated from naturally infested plants, even if sub-culturing of putative *P. lycopersici* mycelium was sometimes necessary to avoid contamination with other faster-growing species. *Pyrenochaeta lycopersici* isolates never sporulated under the cultural conditions used. *Fusarium* spp., *Colletotrichum coccodes* and *Rhizopycnis vagum* were among the other pathogenic species most frequently isolated. The latter, recently reported on tomato in Italy (Porta-Puglia et al., 2001), has morphological traits (colony colour, production of microsclerotia) very similar to *P. lycopersici* and its identification was impossible until pycnidia were produced, usually after two weeks of incubation.

ITS amplification and sequence analysis

The amplification with the universal primer ITS4 and ITS5 gave amplification products of approximately 600 bp for all *P. lycopersici* isolates. The sequence analysis of the rDNA-ITS sequences of *P. lycopersici* isolates under study clearly distinguished two subgroups, one formed by six isolates belonging to Type 2 (I.S.Pa.Ve. ER 876, I.S.Pa.Ve. ER 854, I.S.Pa.Ve. ER 925, CBS 306.65, MAFF 712040, and I.S.Pa.Ve. ER 1135) and another formed by nine isolates belonging to Type 1 (CBS 267.59, I.S.Pa.Ve. ER 1149, I.S.Pa.Ve. ER 1203, I.S.Pa.Ve. ER 1214, I.S.Pa.Ve. ER 1241, MAFF 712039, CBS 282.72, ATCC 48567 and I.S.Pa.Ve. ER 1278). DNA sequence homology between the two groups was 89–90%, while 99–100% similarity was observed

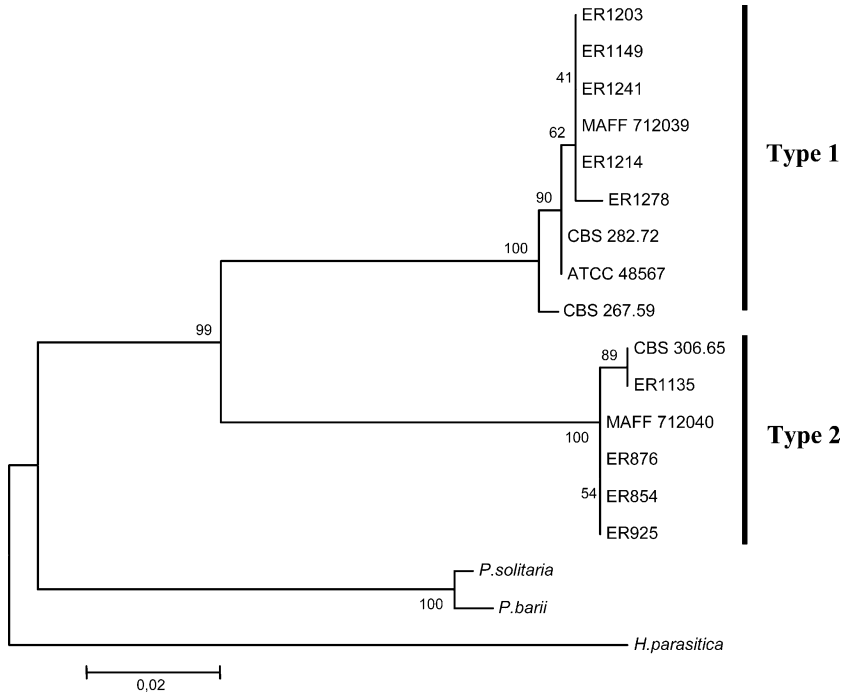

Figure 1. Neighbour-Joining tree based on phylogenetic analysis of the ITS1-5.8S gene-ITS2 sequences using a Kimura 2-parameter model. The numbers at branch points are the percentage of 1000 bootstrapped data set that supported the specific internal branches. The phylogenetic tree showed the clear subdivision of the isolates into two well defined groups. Accession AF466301.1 (*Paraphaeospaheria solitaria*), AF466303.1 (*Paraphaeospaheria barii*) and AF525676.1 (*Herpotrichia parasitica*) were used as outgroups. The scale bar is in the units of the number of substitutions per site.

within each group. BLAST searches were performed separately for the two groups. *Paraphaeosphaeria solitaria* AF466301.1 (e-150) for Type 1 and *Paraphaeosphaeria barii* AF466303.1 (e-143) for Type 2 isolates were the species with the maximum sequence homologies. These two sequences were used, along with *Herpotrichia parasitica*, AF525676.1, as out-groups for the construction of a phylogenetic tree (Figure 1).

Primer design and PCR amplification

Based on the sequence analysis of the isolates, two primer pairs were selected: Plyc1-F (5′-GTAGG

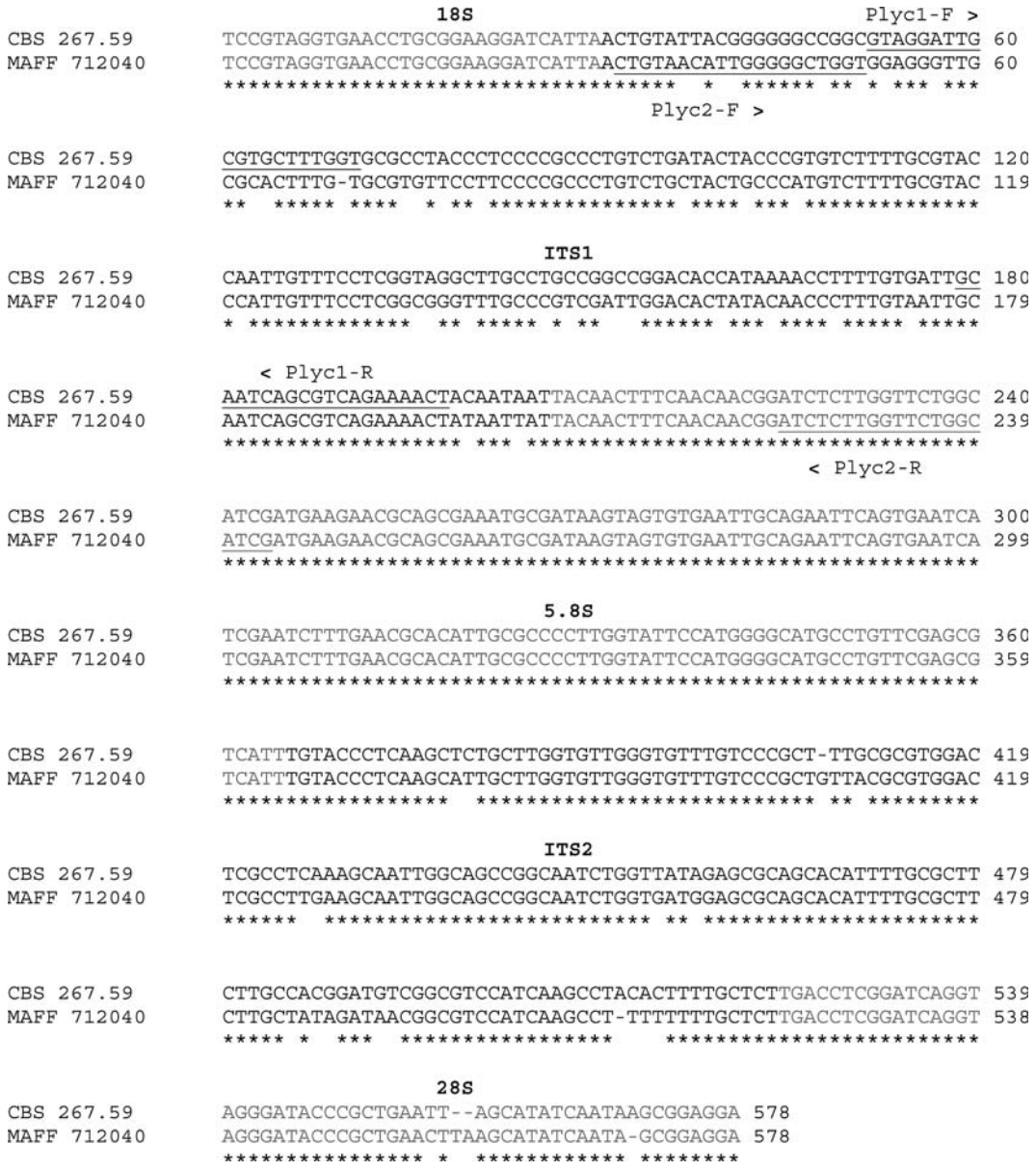

Figure 2. 5′–3′ sequence of the ITS region of *Pyrenochaeta lycopersici* CBS 267.59 and *P. lycopersici* MAFF 712040. The position of primer Plyc1-F and Plyc1-R (specific for Type 1 isolates) and of Plyc2-F and Plyc2-R (specific for Type 2 isolates) is underscored. The complete sequence of 5.8S gene and the partial sequence of 18S and 28S genes are in shade.

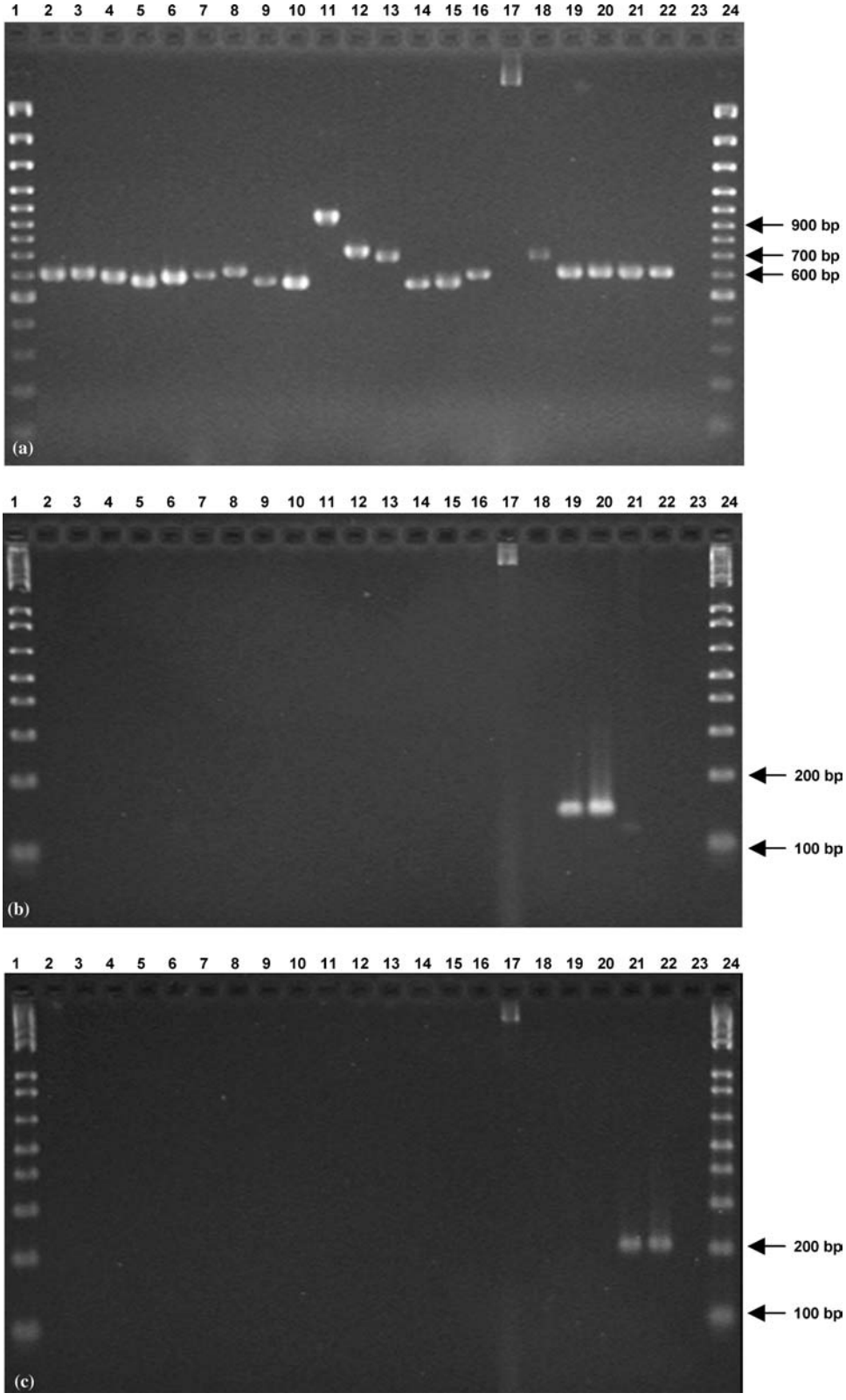
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
900 bp
700 bp
600 bp
(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
200 bp
100 bp
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
200 bp
100 bp
(c)

Figure 3. PCR amplification products using the universal primer pair ITS 4/5 (a) and the *Pyrenochaeta lycopersici* specific primer pair Plyc1-F/R (b) and Plyc2-F/R(c). Lane1 and lane 24, 100 bp DNA Ladder Plus (MBI Fermentas); lane 2, *Herpotrichia parasitica* I.S.Pa.Ve ER 960; lane 3, *Myrothecium* sp. I.S.Pa.Ve. ER 1172; lane 4, *Fusarium solani* I.S.Pa.Ve. ER 1167; lane 5, *F. oxysporum* I.S.Pa.Ve ER 1240; lane 6, *Alternaria* sp. I.S.Pa.Ve. ER 1171; lane 7, *Colletotrichum coccodes* I.S.Pa.Ve. ER 1168; lane 8, *Monosporascus cannonballus* I.S.Pa.Ve ER 1200; lane 9, *Cladosporium* sp. I.S.Pa.Ve. ER 1169; lane 10, *Phoma terrestris* CBS 335.87; lane 11, *Rhizopycnis vagum* I.S.Pa.Ve. ER 940; lane 12, *Rhizoctonia solani* I.S.Pa.Ve. ER 1343; lane 13, *Sclerotium rolfsii* I.S.Pa.Ve. ER 1153; lane 14, *Verticillium dahliae* I.S.Pa.Ve. ER 1180; lane 15, *Ascochyta rabiei* I.S.Pa.Ve. ER 33; lane 16, *Diaporthe sojae* I.S.Pa.Ve. ER 1342; lane 17, *Pseudomonas syringae* pv. *tomato* I.S.Pa.Ve. MCB 412; lane 18, *Lycopersicon esculentum*; lane 19, *Pyrenochaeta lycopersici* I.S.Pa.Ve. ER 1278; lane 20, *P. lycopersici* I.S.Pa.Ve. ER 1319; lane 21, *P. lycopersici* I.S.Pa.Ve. ER1316; lane 22, *P. lycopersici* I.S.Pa.Ve. ER 1211; lane 23, water.

ATTGCGTGCTTTGGT-3′) and- Plyc1-R (5′-AGTTTTCTGACGCTGATTGC-3′) that produced a specific band of 147 bp only with isolates of Type 1 and the Plyc2-F (5′-CTGTAACATTGGG GGCTGGT-3′) and Plyc2-R (5′-CGATGCCAG AACCAAGAGAT-3′) that produced a band of 209 bp with isolates of Type 2. The locations of the specific primer for both types in 5.8 gene-ITS region are shown in Figure 2. Plyc1-F and Plyc1-R were placed within the ITS1; Plyc2-F was inside the ITS1 while Plyc2-R was placed within the conserved 5.8S region. *In silico* BLAST search for primer pair specificity showed no homologies with other microorganisms for either Plyc1-F or Plyc2-F. Specificity of the primer was confirmed by *in vivo* amplification. Primer pair Plyc1- F and Plyc1-R successfully amplified a 147-bp PCR product only from DNA of the Type 1 isolates, while a 209-bp PCR product was obtained only from Type 2 isolates when Plyc2- F and Plyc2-R were used. No amplification was observed when DNA of tomato and DNA of the species listed in Table 2 was used as a template for both primer pairs (Figure 3). Specificity was further confirmed by the analysis of the sequence of the PCR products obtained by amplification with each specific primer. As little as 0.7 pg of target DNA could be amplified by both primer pairs (Figure 4).

Detection of Pyrenochaeta lycopersici *in infected roots*

PCR amplification of DNA extracted from all the artificially infested plants gave an amplification

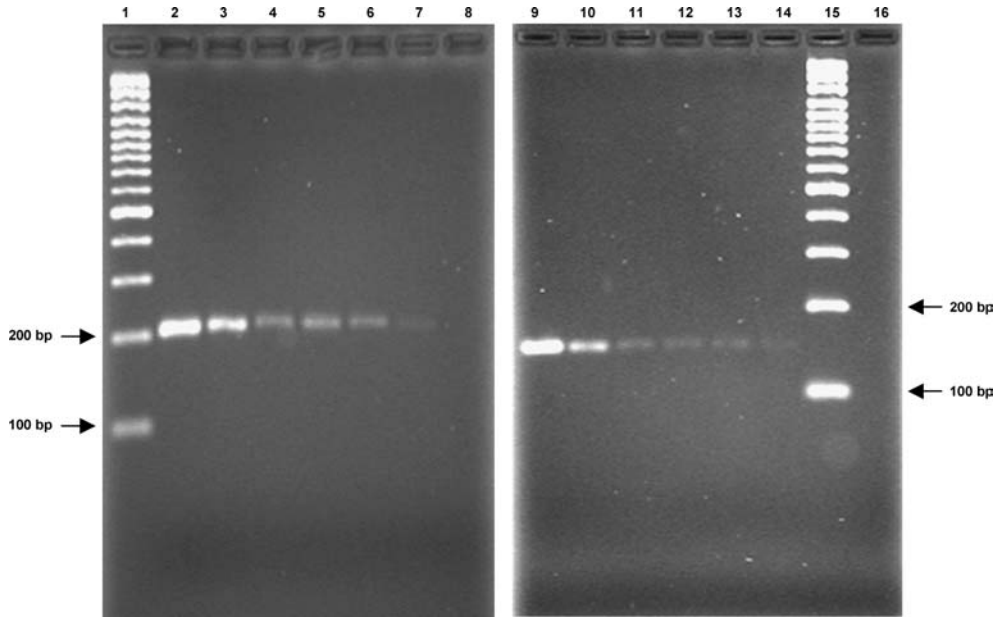

Figure 4. Sensitivity of the *Pyrenochaeta lycopersici* specific primers. Lane 1 and lane 15: 100 bp DNA Ladder Plus (MBI Fermentas); lanes 2–7: 10-fold dilution of genomic DNA of isolate I.S.Pa.Ve. ER 963 (Type 2) ranging from 70 ng to 0.7 pg; lane 8: water; lanes 9–14: 10-fold dilution of genomic DNA of I.S.Pa.Ve. ER 1278 (Type1) ranging from 70 ng to 0.7 pg; lane 16: water.

band of the expected size for each type. From field samples, only amplification products corresponding to Type 2 isolates were obtained. No amplification was observed when DNA of healthy and uninoculated plants was used as a template for the amplification. The PCR assay was constantly in agreement with the isolation of the pathogen on PDA.

Discussion

The unambiguous identification of *P. lycopersici* requires the presence and measurement of reproductive structures such as pycnidia, conidiophores and conidia, but their production on artificial media is not always successful. Only nine out of 19 Italian *P. lycopersici* isolates tested were able to sporulate when comparing several growth media (Infantino et al., 2003). The isolates used in this work were selected on the basis of morphological and cultural observations of the colonies on PDA or according to the results of Sugiura et al. (2003). Four isolates of Type 1 (ATCC 48567, I.S.Pa.Ve. ER 1278, I.S.Pa.Ve. ER 1149, and I.S.Pa.Ve. ER 1241) and several isolates of Type 2 sporulated on double strength V8 agar; measurements of both pycnidia and conidia fitted with those of the species (data not shown). Based on the sequence analysis of the ITS spacer region obtained in this work, the 15 isolates were subdivided in two distinct groups, corresponding to Type 1 and Type 2 described by Sugiura et al. (2003). The design of specific primer pairs for both types of *P. lycopersici* using the ITS region of rDNA allowed the rapid and unambiguous identification of the isolates belonging to the two different types. PCR products of 147 and 209 bp were obtained when the DNA of isolates belonging to Type 1 and Type 2, respectively, was used as a target for amplification with specific primer. The analysis of the entire collection of *P. lycopersici* isolates present at I.S.Pa.Ve. with both primer pairs showed that 14 out of 86 isolates tested were of Type 1. Of these, only four originated from Italy (Table 1). The use of the specific primers allowed also the detection of *P. lycopersici* in artificially and naturally infected roots, even if in close association with other fungi. The time needed for diagnosis was reduced from 15 days with the traditional protocol to only two using the nested-PCR procedure described.

PCR-based methods using rDNA have been used to distinguish species complex involved in soil-borne diseases, such as sheath-blight of rice (Johanson et al., 1998), brown stem of soybean (Chen et al., 1999), *Rhizoctonia solani* AG2 subgroups (Salazar et al., 2000) and *Alternaria* spp. of carrot (Konstantinova et al., 2002). The use of the specific primers for *P. lycopersici* developed here, in association with primers recently designed for *Colletotrichum coccodes* (Cullen et al., 2002) and for *Rhizopycnis vagum* (Ghignone et al., 2003), two species frequently associated with *P. lycopersici*, could improve our knowledge on the epidemiology and the role of each species in the development of the disease. The ITS spacer region sequences of both *P. lycopersici* Type 1 and Type 2 isolates obtained in this work, along with those of other species taxonomically closely related to *P. lycopersici*, will be used in future phylogenetic analyses of this species.

Studies are in progress in order to define the incidence, the geographic distribution and virulence of isolates of both types in Italy and abroad. The primers designed in this study offer a valid tool for studies on the epidemiology of the disease and for the implementation of control strategies, particularly important in view of the banning of methyl bromide in 2005.

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