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Genetic variation and host specificity of *Phytophthora citrophthora* isolates causing branch cankers in Clementine trees in Spain

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Abstract Considerable tree losses have been observed during the past few years in Spain due to Phytophthora branch canker of clementines caused by *Phytophthora citrophthora*. The emergence of this disease led to the speculation that either the pathogen has evolved increasing its aggressiveness or specificity to clementines. A total of 134 isolates of *P. citrophthora* collected from 2003 to 2005 in 135 citrus orchards in Spain and 22 reference isolates were analyzed genotypically and phenotypically to determine the structure of the population. Genotypic diversity was evaluated by means of Inter-Simple Sequence Repeat (ISSR) markers. Among the phenotypic characteristics examined, sporangial characters, sexual behavior, growth rates and colony morphology of the isolates at different temperatures were studied. The aggressiveness and host-specificity of selected isolates were evaluated by pathogenicity tests on sweet oranges and clementines under field conditions. Phytophthora branch canker of clementines was associated mainly with one genotype (P-1), which included 88% of the isolates obtained from branches. Strains isolated years before the first disease outbreak clustered also with this major genotype, thus it may be considered as a predominant population. Thirteen other minor genotypes were determined, but most contained only one isolate. Although there was wide variation in the morphological and physiological characters, all Phytophthora isolates obtained from branch cankers were sexually sterile and showed a characteristic petalloid colony pattern. As in previous greenhouse studies, pathogenicity tests under field conditions demonstrated that clementines and their hybrids were more susceptible to *P. citrophthora* than sweet oranges. However, no evidence was found to support the hypothesis that the emergence of the disease was associated with more aggressive or host-specific forms of *P. citrophthora*.

Keywords ISSR · Microsatellites · *Phytophthora parasitica* · Sexual mating

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Introduction

Citrus diseases such as root rot, foot rot, gummosis, and brown rot of fruit caused by *Phytophthora*

citrophthora (Smith and Smith) Leonian, and *P. nicotianae* Breda de Haan (Syn. *P. parasitica* Dastur) have been present for many years in Spain (Fawcett 1936; Urquijo et al. 1961; Tuset 1977, 1983; Erwin and Ribeiro 1996). To date, these diseases have been managed effectively through cultural practices and fungicide applications (Tuset 1977, 1983; Alvarez et al. 2008b). However, a new syndrome caused by *P. citrophthora* was first recorded in Spain in 2002 and, since then, considerable tree losses have been observed (Alvarez et al. 2008a, b, 2009). In Spain, the disease affects almost exclusively clementine (*Citrus clementina* Hort. *ex* Tan.) cultivars and their hybrids (Alvarez et al. 2008a). Diseased trees show cankers and gum exudations on the above-ground parts on the scion, especially on the major limbs, whereas rootstocks generally remain healthy. The trunk and secondary branches are affected by the expansion of these lesions and, eventually, the entire tree may collapse and die (Alvarez et al. 2008a). Phytophthora branch canker has also been described in the winter rainfall citrus-growing areas of South Africa affecting mainly clementine cultivars (Schutte 2007). In contrast to other Phytophthora diseases, fungicide schedules currently applied in Spain showed a limited degree of control of branch cankers (Alvarez et al. 2008b).

The reasons behind the appearance of this new syndrome in branches are still unknown. Emergence and re-emergence of plant diseases can be a consequence of changes in cultural practices or an increase in the susceptibility of the host population. However, they are frequently associated with the introduction of new pathogen genotypes (Madden 2001; Brasier 2008) as well as with changes in the predominant pathogen populations as a result of several processes of variation, such as sexual recombination, interspecific hybridization, mutation, or mitotic and parasexual recombination (Goodwin 1997; Stukenbrock and McDonald 2008).

In Corsica (France), a new *P. citrophthora* population of the A2 mating type was detected affecting citrus trees. These new strains were more aggressive to scion cultivars than the predominant population, which was conversely more aggressive to rootstocks (Cohen et al. 2003; Vernière et al. 2004). Evidence of genetic differentiation and host specialization has been described for other *Phytophthora* species such as *P. cactorum* (Hantula et al. 1997; Eikemo et al. 2004; Bhat et al. 2006), *P. capsici* (Lamour and Hausbeck 2000; Lamour and Hausbeck 2001), *P. cinnamomi* (Linde et al. 1997), *P. infestans* (Abu-El Samen et al. 2003; Flier et al. 2007) and *P. megasperma* (Hamm and Hansen 1981).

The analysis of the variability in genetics, phenotypes and aggressiveness of *P. citrophthora* isolates from affected citrus-growing areas in Spain could explain whether this epidemic resulted from the introduction of new genotypes or whether the predominant *P. citrophthora* populations may have increased in their aggressiveness or evolved to some degree of host specificity.

In the last few years, molecular techniques have been widely applied to study genetic variability in plant pathogen populations. Among them, Inter-Simple Sequence Repeat (ISSR), also known as microsatellites, has been used for identifying intraspecific patterns within Oomycetes (Zietkiewicz et al. 1994; Hantula et al. 1996, 1997; Knapova and Gisi 2002; Cohen et al. 2003). Studies on the population structure of some *Phytophthora* species using molecular tools have also found phenotypic variability and host specificity associated to intraspecific genetic differentiation (Day et al. 2004; Flier et al. 2007). However, in other cases, this intraspecific genetic differentiation has not been correlated with differences in phenotypic characters and aggressiveness to the host. Hüberli et al. (2001) studied a set of morphological and physiological characters within a clonal lineage of *P. cinnamomi* in Australia and found great variation in some phenotypes, identifying two main clusters of isolates in the population. In addition, Knapova and Gisi (2002) found variability in some phenotypic traits within a *P. infestans* population in France and Switzerland. Therefore, combining studies of genetic and phenotypic intraspecific differentiation with pathogenicity assays allow testing of the hypothesis that Phytophthora branch canker of clementines is caused by a phylogenetically distinct and host-specific lineage of *P. citrophthora*.

The objectives of our study were: (1) to assess the level of genotypic and phenotypic intraspecific diversity in the *P. citrophthora* population; (2) to determine the sexuality of this population; and (3) to determine if Phytophthora branch canker of clementines in Spain was associated with more aggressive or host-specific forms *P. citrophthora*.

Materials and methods

Sampling

Sampling was conducted from 2003 to 2005 in 135 citrus commercial orchards mainly affected by Phytophthora branch canker across the provinces of Tarragona (3 orchards), Castellón (16 orchards), Valencia (55 orchards), Alicante (6 orchards), and Murcia (7 orchards) in Eastern Spain, and Huelva (48 orchards) in southwestern Spain. At least five symptomatic trees were examined carefully in each orchard, collecting affected tissues from below ground and above ground parts of the tree and eventually fruits affected by brown rot. The province, citrus cultivar, rootstock, and the age of the trees were recorded for each affected orchard. Isolations from diseased tissues (bark, roots or fruits) were made on PARBPH selective medium (Jeffers and Martin 1986). After 48 h of incubation, hyphal tips from each developed colony were transferred to PDA Petri dishes. A total of 305 isolates of *Phytophthora* spp. were obtained from these samples. The main isolated species was *P. citrophthora* and a few isolates of *P. nicotianae* were recovered (Alvarez et al. 2008a). Plugs of PDA medium colonized by the mycelium of each isolate were preserved in 15 ml vials containing sterile distilled water at 14°C until used.

A total of 134 *P. citrophthora* isolates were selected arbitrarily from the total number of isolates in the collection. Geographical origin, citrus species and affected organs on the tree are summarized in Table 1. In addition, reference isolates of *Phytophthora* spp. obtained from culture collections were also included in the study (Table 2).

Morphological analysis

A total of 134 Spanish isolates of *P. citrophthora* from citrus and all reference *P. citrophthora* (22) and *P. nicotianae* (14) isolates (Table 2) were grown individually on V8 juice agar (2 g $CaCO_3$, 200 ml V8 juice and 15 g agar in 800 ml distilled water) medium for 4 days in darkness at 24°C. Fifteen 5-mm-diameter agar plugs extracted from the advancing margin of the colony of each isolate were placed in plastic Petri dishes containing 15 ml of 1.5% sterile soil extract. Dishes were incubated under fluorescent light for 3 to 5 days at 24°C.

Table 1 *Phytophthora citrophthora* isolates grouped according to geographic origin, citrus host, tree organ and distribution of genotypes according to the CGA marker

Host	Location						ISSR CGA genotypes													Total
	Tarragona	Castellón	Valencia	Alicante	Murcia	Huelva	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	P-10	P-11	P-12	P-13	P-14	
Citrus species																					
Clementines[a]	1	21	23	2	0	65	100	1	1	1	1	1	1	1	3	1	1	0	0	1	113
Sweet oranges	2	0	12	0	0	3	14	0	0	0	0	0	0	0	0	0	0	1	1	0	16
Lemons	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Tree organs																					
roots	2	1	9	1	0	5	14	1	0	0	0	0	0	0	0	1	0	1	0	0	17
trunk	1	0	5	0	5	12	12	0	0	0	0	0	0	0	1	0	0	0	0	0	13
branches	0	20	19	1	0	51	91	0	1	1	1	1	1	1	2	0	1	0	0	1	101
fruit	0	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	3
Total	3	21	35	2	5	68	119	1	1	1	1	1	1	1	3	1	1	1	1	1	134

[a] Including clementine hydrids

Table 2 Characteristics of reference *Phytophthora* isolates included in the study

Isolate details				Mating type[a]	Colony pattern[b]	ISSR		Source (reference)[e]
Code	Specie	Host	Year of isolation			CGA[c]	GT[d]	
CECT 2352	*P. citrophthora*	citrus	1990	S	pe	P-1	C-1	CECT-Spain
CECT 2353	*P. citrophthora*	citrus	1984	S	pe	P-1	C-1	CECT-Spain
Ph 119	*P. citrophthora*	citrus	–	S	pe	P-1	C-1	INRA-France
CTO 144	*P. citrophthora*	citrus	–	A2	ste	–[f]	C-1	INRA-France
PA 55	*P. citrophthora*	citrus	1998	S	pe	P-1	C-1	INRA-France
PA 34	*P. citrophthora*	citrus	1997	A1	ste	P-9	C-1	INRA-France
PA 67	*P. citrophthora*	citrus	1998	S	pe	P-1	C-1	INRA-France
PA 36	*P. citrophthora*	citrus	1998	A2	ste	P-12	C-1	INRA-France
Pc1	*P. citrophthora*	citrus	1998	S	pe	P-1	C-1	IVIA-Spain
Pc2	*P. citrophthora*	citrus	1994	S	pe	P-1	C-1	IVIA-Spain
C-31	*P. citrophthora*	citrus	2004	A1	ste	P-12	C-1	UC-Chile
PO118	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
PO252	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
PO098	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
PO157	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
PO328	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
PO323	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
PO316	*P. citrophthora*	ornamentals	2003	S	ste	P-11	C-1	Poland
Ps-74	*P. citrophthora*	*Pinus*	2006	S	ste	P-11	C-1	GIHF-Spain
Ps-121	*P. citrophthora*	*Euonymus*	2007	S	ste	P-11	C-1	GIHF-Spain
Ps-127	*P. citrophthora*	loquat	2007	S	ste	P-11	C-1	GIHF-Spain
Ps-134	*P. citrophthora*	fig	2007	S	ste	P-11	C-1	GIHF-Spain
Ph 186S	*P. nicotianae*	citrus	–	A1	–	–	C-2	UNIBA
Ph 115	*P. nicotianae*	citrus	2000	A1	–	–	C-2	INRA-France
PAR 113	*P. nicotianae*	citrus	–	A1	–	–	C-2	INRA-France
UNP-12	*P. nicotianae*	citrus	2005	A2	–	–	C-2	UNP-Peru
UNP-19	*P. nicotianae*	citrus	2005	A1	–	–	C-2	UNP-Peru
Phy 019	*P. nicotianae*	citrus	2003	A1	–	–	C-2	GIHF-Spain
Phy 021	*P. nicotianae*	citrus	2003	A1	–	–	C-2	GIHF-Spain
Phy 025	*P. nicotianae*	citrus	2003	A2	–	–	C-2	GIHF-Spain

Phy 039	*P. nicotianae*	citrus	2003	A1	–	–	C-2	GIHF-Spain
Phy 047	*P. nicotianae*	citrus	2003	A2	–	–	C-2	GIHF-Spain
Phy 048	*P. nicotianae*	citrus	2003	A1	–	–	C-2	GIHF-Spain
Phy 060	*P. nicotianae*	citrus	2003	A1	–	–	C-2	GIHF-Spain
Phy 076	*P. nicotianae*	citrus	2004	A2	–	–	C-2	GIHF-Spain
Phy 084	*P. nicotianae*	citrus	2004	A2	–	–	C-2	GIHF-Spain
Ph 111	*P. citricola*	*Chamaecyparis*	–	–	–	–	C-3	INRA-France
CIT 61	*P. citricola*	Citrus	–	–	–	–	C-3	INRA-France

[a] Mating type. S. sterile; A1; A2

[b] Colony pattern observed on PDA medium. pe. petalloid; spe. slightly petalloid; ros. rosette; ste. stellate; sts. stellate striated and; wp. no pattern

[c] CGA. genotypes generated by ISSR analysis. P-1 to P-14

[d] GT. genotypes generated by ISSR analysis. C-1. *P. citrophthora*; C-2. *P. nicotianae*. C-3. *P. citricola*

[e] *CECT* Colección Española de Cultivos Tipo, Spain; *INRA* Institut National de la Recherche Agronomique, France; *IVIA* Instituto Valenciano de Investigaciones Agrarias, Spain; *UNIBA* Università di Bari, Italy; *UNP* Universidad Nacional de Piura, Peru; *PUC* Pontificia Universidad Católica, Chile; *GIHF* Grupo de Investigación en Hongos Fitopatógenos, Spain

[f] Not determined

Sporangial production was assessed by direct observation using a stereoscopic microscope.

Mycelial strands of each *Phytophthora* isolate containing mature sporangia and eventually chlamydospores in sterile water were examined microscopically at 400× magnification. The length and width of 25 intact primary sporangia were measured and the presence or absence of chlamydospores was also recorded. The length: width (L:W) ratio of the sporangia was calculated and other sporangial characteristics such as shape and papillation were recorded.

Sexual behavior

All the Spanish isolates of *P. citrophthora* and the reference strains of *P. citrophthora* and *P. nicotianae* were analyzed by sexual crossing on V8 juice agar by pairing with A1 (PA 34 and Ph 186S) and A2 (PA 36 and Phy 047) tester strains of *P. citrophthora* and *P. nicotianae* (Table 2). Mycelial plugs (5 mm in diameter) extracted from actively growing colonies in PDA medium of the unknown isolate were placed 2 cm from a plug of the known A1 or A2 isolate in the plate (90 mm in diameter) (Ribeiro 1978). There were two replicate plates per isolate. The plates were transferred to an incubator at 24°C. After incubation for 2 to 5 weeks in the dark, plates were examined microscopically for the presence of oospores where mycelia of the known and unknown isolates intermingled. Isolates that produced oospores when paired with the A1 tester strain, but did not produce oospores with the A2 tester strain were designated A2. Isolates that formed oospores when paired with the A2 tester and did not form when paired with the A1 isolate were designated A1. The characteristics of the sexual structures were recorded and the diameter of 25 oogonia per isolate and their oospores were measured.

Growth at different temperatures and colony morphology

All the 134 isolates of *P. citrophthora* in this study and all the reference isolates of *P. citrophthora* and *P. nicotianae* were assayed in this test. Inoculum discs of each isolate, 5 mm in diameter, were cut with a sterile cork borer from the colony margins of 5-day-old cultures grown on potato dextrose agar (PDA) (Biokar Diagnostics, France) and transferred,

mycelial side down, to the centre of individual Petri-plates containing 10 ml of PDA. Plates were sealed with Parafilm® and incubated at 16, 20, 24, 28, 32 and 35°C in the dark for 5 days. There were three replicate plates for each isolate-temperature combination. A preliminary study with eight isolates showed that at temperatures less than 16°C, growth was slow with no significant differences among isolates (*data not shown*).

Colony growth of each combination was measured along two lines intersecting at right angles at the centre of the inoculum disc. The radial growth rate (mm day^{-1}) was calculated by taking the average of all radial measurements, subtracting the inoculum disc radius and dividing by five. Growth rates per day were calculated for the different temperatures and maximum average data were adjusted to a regression curve. The best polynomial model was chosen from several combinations of terms, based on the significance of the estimated parameters ($P<0.05$) and coefficients of determination (R^2). The optimum growth temperature was derived from the growth curve equation obtained for each isolate tested. The colony pattern and nature of margin were registered for each isolate-temperature combination 5 days after incubation. Replications were performed for the isolates that showed anomalous data in at least one plate in the test of growth at different temperatures and in the isolates that had differences in phenotypical traits in the colony aspect test.

Multivariate analyses

The relationship among the morphological and physiological characters evaluated were analyzed using a multivariate factorial analysis (Peña 2002), using the software Statgraphics Plus 5.1 (Manugistics Inc., Rockville, MD, USA). The aim was to detect an intraspecific group of *P. citrophthora* isolates associated with Phytophthora branch canker of clementines. Among the quantitative variables, the length, width, and L:W ratio of sporangia as well as the optimum growth temperature for each isolate were studied. The qualitative variables evaluated were assigned the values of 0 or 1 and the following were analyzed: host [clementines and clementine hybrids vs. other citrus species (sweet orange and lemon cultivars)]; tree organs [branch infections vs. other infection sites (trunk, roots and fruit)]; mating system (heterothallic vs. sterile); shape of sporangia [obpyriform vs. other shapes (ovoid, pyriform, obturbinate, elongate, distorted)]; papillation of sporangia [papillate vs. others (semi-papillate or non-papillate)]; chlamydospores (presence or absence); growth at 24, 32 and 35°C (growth vs. no growth), colony pattern at 24°C [petalloid vs. others (slightly petalloid, rosette, stellate, stellate striated and no pattern)] and margin of the colony (regular vs. irregular).

DNA extraction

Mycelium of all Spanish and reference *Phytophthora* isolates grown on PDA for 7 days at 24°C in the dark were scraped and mechanically disrupted by grinding to a fine powder with liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N. A. Plant Miniprep Kit (Omega Bio-tek, USA) following the manufacturer's instructions. DNA was stored at −20°C.

Analysis of ITS sequences of the isolates

This analysis was conducted to support the morphological and physiological identification of *P. citrophthora* isolates. Amplification of the ITS region was performed in a volume of 25 μl using a reaction mixture of 1x PCR buffer, 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.4 μM of ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) and ITS6 (5′- GAAGGTGAAGTCGTAACAAGG-3′) (Cooke and Duncan 1997), 1 U of DNA *Taq* polymerase (Dominion MBL, Córdoba, Spain) and 1 μl of template DNA. Amplification was carried out on a Peltier Thermal Cycler-200 (MJ Research). The cycling parameters were an initial step of 4 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and an elongation step at 72°C for 60 s. A final extension was performed at 72°C for 10 min. The amplification products were separated in a 1.5% agarose gel (agarose D-1 Low EEO, Conda, Madrid, Spain) in a 0.5× TBE buffer, and stained with 0.1% ethidium bromide. The molecular size of the amplification products was estimated using Gene Ruler™ 100 bp Ladder Plus (Fermentas, Germany). PCR products were purified with MBL-PCR QuickClean (Dominion MBL, Córdoba, Spain) and sequenced in both directions. The sequences

obtained were compared with *Phytophthora* sequences available in the EMBL/GenBank database.

In another study searching for variability among *P. citrophthora* isolates in this region, the sequences were also edited using the program DNAman (version 4.03, Lynnon BioSoft, Quebec, Canada) and phylogenetic analysis were carried out using MEGA version 3.1 (Kumar et al. 2004). The genetic distances were calculated using the Kimura 2-parameter model. For phylogenetic inference, the neighbour-joining (NJ) method was used, and the topology of the tree was tested with 1000-bootstrap trials.

ISSR analysis of the isolates

ISSR amplifications were carried out with the same PCR kit as described above, but using the concentrations proposed by Cohen et al. (2003). The ISSR primers used were GT (5′-YHY$(GT)_7$ G-3′) (Hantula et al. 1997) and CGA (5′- DHB$(CGA)_5$ -3′) (Hantula et al. 1996). The samples were denatured at 95°C for 5 min, after which 35 cycles of amplification were carried out (denaturation at 95°C for 30 s, annealing for 45 s at 50°C (GT) or 57°C (CGA) and elongation at 72°C for 2 min). After the last cycle, a final extension at 72°C was carried out for 3 min. At least three independent PCR amplifications were performed for each isolate. Amplification products were analyzed by electrophoresis in gels as mentioned above.

A comparison of CGA profiles was carried out on the basis of presence/absence (1/0) of the amplification products of the same length. Only clear and reproducible bands were included in the analysis. A binary matrix combined the complete data records for all the isolates. Genetic similarity was calculated based on Dice´s coefficient (Dice 1945) with the SimQual software. A dendrogram was constructed after cluster analysis of the similarity coefficients by means of the unweighted pair-group method (UPGMA) using the SAHN and TREE software. The Mantel test (Mantel 1967) estimated the degree of fit the cophenetic matrix and the matrix upon which the clustering was based. NTSYSpc version 2.02 (Exeter Software, Setauket, NY) was used to perform these analyses.

Pathogenicity

The aggressiveness of six randomly selected isolates from the P-1 genotype (Phy 016, Phy 035, and Phy 058) and the P-9 genotype (Phy 011, Phy 028, and Phy 051) was evaluated. The study was carried out in six commercial clementine orchards, two of cv. Clemenules, two of cv. Hernandina and two of cv. Fortune, and six sweet orange [*C. sinensis* (L.) Osbeck] orchards, one of cv. Lane Late, one of cv. Navelate, one of cv. Navelina and three of cv. Valencia Late). Orchards were located in Valencia province and trees were 9- to 14-years old. In each orchard, six citrus trees were selected randomly for inoculations. In each tree, six lignified branches (~25–30 mm-diameter) located at the same height in the inner part of the canopy were randomly selected. Selected branches were surface-disinfested around the inoculation points with 70 % ethanol before inoculation. A 5-mm-diameter disk from the bark was removed with a cork borer and a PDA agar plug colonized with mycelium was placed in contact with the cambium. The inoculation point was covered with moist cotton wool, sealed with a strip of Parafilm® and wrapped with foil paper to prevent drying (Alvarez et al. 2008a, b, 2009). Each branch was inoculated with one *P. citrophthora* isolate. Thus, the scion of each tree was inoculated with all six isolates at the same time.

Evaluations were performed 4 weeks after inoculation. The surface of the branch or rootstock bark was scraped and canker area traced on transparent plastic sheet and quantified by means of the software Assess (American Phytopathological Society. St. Paul MN). The size of the inoculation wound was subtracted to provide the actual lesion area. Re-isolations onto PARBPH were made to confirm that cankers resulted from infections by *P. citrophthora*.

Linear regression analyses were performed using Statgraphics Plus 5.1 in order to relate canker area values to the genotype of the isolate and the citrus species and cultivar inoculated. The interaction between isolate genotype and citrus species was also investigated by multiple regression analysis.

Results

Morphological analysis

Spanish isolates of *P. citrophthora* showed variable sporangial shape with obpyriform and ovoid as the most common. High variation in the sporangial

dimensions were also observed with the following lengths and widths (ranges in brackets): (24.9–) 43.2 (–61.1) × (18.9–) 30.7 (–43.4) μm and with a L:W ratio of (1.2–) 1.4 (–1.6). Fifty-three percent of the isolates had papillate sporangia and 47% showed semi-papillate sporangia. A total of 52% of the isolates formed chlamydospores. With the exception of one isolate (Phy 037), all isolates were sexually sterile. Isolate Phy 037 produced globose oogonia and amphyginous antheridia when paired with the tester strains PA 34 (*P. citrophthora*) and Ph 186S (*P. nicotianae)* (A1), 3 or 5 weeks after pairing. The oospores were spherical and plerotic, measuring (26.3–) 27.7 (28.4) μm in diameter.

Growth at different temperatures and colony morphology

The optimum growth temperature of the isolates was variable, ranging from 22.9 to 28.9°C. There was also considerable variation in colony pattern within this species. Petalloid growth was the most common colony pattern in all *P. citrophthora* isolates at 16 (89.3%), 20 (91.1%), 24 (88.4%) and 28°C (81.2%). Other colony patterns recorded were: slightly petalloid, rosette, stellate, stellate striated and no pattern (Table 4). *P. citrophthora* isolates grew at 5 and 32°C, but showed no growth at 35°C.

Multivariate analyses

The two factors that had the highest eigenvalues or proper values (4.54 and 2.55, respectively) were extracted. They accounted for approximately 51% of the total variance (32.5 and 18.3% for Factors 1 and 2, respectively). The Factor 1 divided the isolates on an interspecific base and the Factor 2 was defined mainly by intraspecific traits. The main contributing variables for Factor 1 were optimum growth temperature, tree organs, mating system, growth temperature at 24°C and 35°C, and colony pattern at 24°C. The main contributing variables for Factor 2 were sporangial length and width, L:W ratio, papillation and the presence of chlamydospores (Table 3). The projection of all *Phytophthora* isolates on the plane for Factor 1 clearly separated them into three groups (Fig. 1). One group clustered 133 Spanish isolates of *P. citrophthora* and reference isolates of *P. citrophthora*. The second group was composed by 13 Spanish and reference isolates of *P. nicotianae.* One heterothallic isolate of *P. citrophthora* (Phy 037) from Spain and three reference heterothallic isolates (C-31, CTO 144, and PA 36), identified as *P. citrophthora,* clustered separately between the two main groups. Factor 2 did not separate any specific group; however, the analysis showed that isolates that had larger and wider sporangia frequently produced more chlamydospores than isolates with smaller and narrower sporangia.

Table 3 Parameters for factors 1 and 2 extracted from the multivariate factorial analysis

Parameter[a]	Factor 1	Factor 2
Length (L) of sporangia	0.0541	0.8798[b]
Width (W) of sporangia	0.1318	0.7216[b]
L:W ratio	−0.0923	0.7494[b]
Optimum growth temperature	−0.7845[b]	−0.2746
Host	−0.1576	0.1246
Tree organ	−0.7185[b]	0.2346
Mating system	0.8922[b]	−0.0726
Shape of sporangia	0.3232	−0.1232
Papillation of sporangia	−0.2958	−0.5648[b]
Presence of chlamydospores	0.3608	0.5260[b]
Growth at 24°C	−0.8968[b]	0.0242
Growth at 32°C	0.0281	−0.0378
Growth at 35°C	−0.9354[b]	−0.0597
Colony pattern at 24°C	−0.7641[b]	0.1275
Margin of colony at 24°C	−0.2929	−0.0690

[a] Qualitative variables studied: host [clementines and hybrids vs. other citrus species (sweet oranges and lemons cultivars)]; tree organs [branch infections vs. other infection sites (trunk, roots and fruit)]; mating system of isolates (heterothallic vs. sterile); shape of sporangia [obpyriform vs. other shapes (ovoid, pyriform, obturbinate, elongate, distorted)]; papillation of sporangia [papillate vs. others (semi-papillate or non-papillate)]; chlamydospores (presence or, absence); growth at 24, 32 and 35°C (growth vs. no growth), colony pattern at 24°C [petalloid vs. others (slightly petalloid, rosette, stellate, stellate striated and no pattern)] and margin of colony (regular vs. irregular)

[b] Main contributing variables for factors 1 and 2

Analysis of ITS sequences of the isolates

The comparison of the sequences of the Spanish isolates with the sequences of this region available in the GenBank database confirmed the identity of the species *P. citrophthora* previously identified by mean

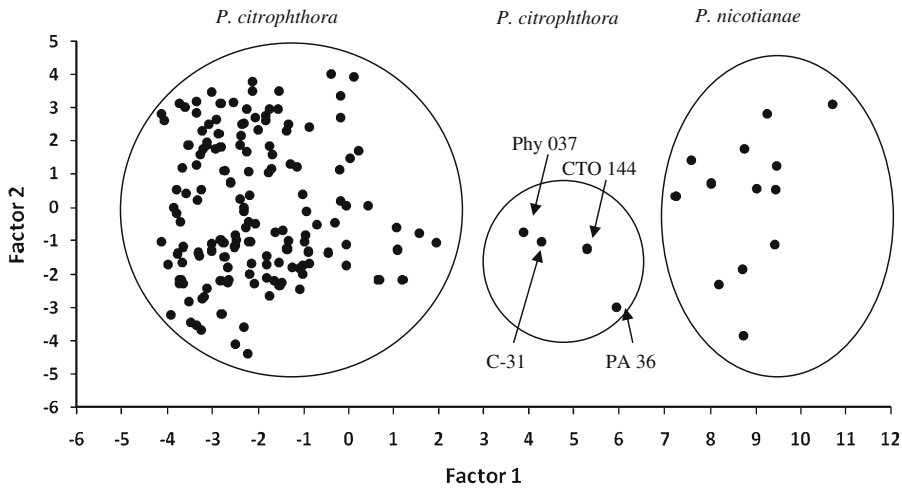

Fig. 1 Projection of *Phytophthora* isolates on the plane of factors 1 and 2 from multivariate factorial analysis

morphological and physiological traits. In addition, the alignment of the isolates showed only one single base pair change in the ITS region, showing that this region is quite homogeneous and did not reveal any variability.

ISSR analysis of the isolates

The GT primer generated a distinctive and unambiguous pattern (C-1) identical in all *P. citrophthora* isolates, composed of two bands of 0.43 and 1.6 Kb. This primer also generated characteristic bands for reference isolates of *P. nicotianae* (C-2) and *P. citricola* (C-3) (Table 2). Isolates of each species showed unique ISSR bands that distinguished them from other species. On the other hand, the CGA primer generated 14 reproducible genome fingerprinting patterns equivalent to 14 genotypes in *P. citrophthora* (Tables 1 and 4; Fig. 2). A total of 22 reliable fragments, ranging from 0.5 to 2 Kb, were amplified. All of these were polymorphic and were distributed among two to eight bands per pattern. The PCR amplification generated across the 14 genotypes (from P-1 to P-14) using the primer CGA (Fig. 2). The most common banding pattern was composed of 119 isolates (P-1); whereas the rest of 13 banding patterns were composed of only one or three isolates (Table 1). Experiments were repeated

Table 4 Genotype groups of *Phytophthora citrophthora* isolated from citrus: sexuality and colony patterns

ISSR CGA marker	Representative isolates	Mating type[a]	Colony pattern[b]
P-1	Phy 016	S	petalloid
P-2	Phy 015	S	slightly petalloid
P-3	Phy 068	S	stellate
P-4	Phy 145	S	slightly petalloid
P-5	Phy 190	S	rosette
P-6	Phy 134	S	stellate
P-7	Phy 098	S	stellate
P-8	Phy 050	S	rosaceous
P-9	Phy 011	S	stellate-striate
P-10	Phy 065	S	stellate-striate
P-11	Phy 193	S	stellate
P-12	Phy 037	H	rosette
P-13	Phy 192	S	slightly petalloid
P-14	Phy 022	S	without pattern

[a] Mating type. S: sterile; H: heterothallic, A1 or A2. All *P. citrophthora* isolates belonging at the same genotype presented equal sexual behaviour

[b] Colony pattern observed 5 days after incubation on PDA medium. All *P. citrophthora* isolates belonging to the same genotype showed the identical aspect of the colony

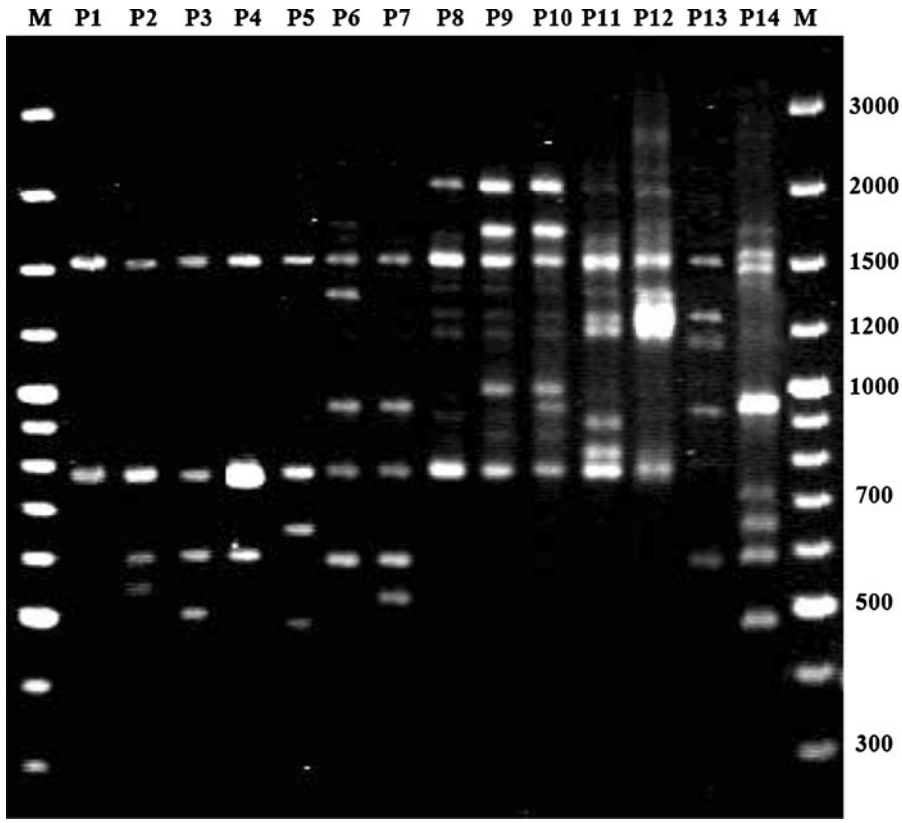

Fig. 2 Polymerase chain reaction profiles obtained for isolates of *Phytophthora citrophthora* by Inter-Simple Sequence Repeat primer CGA. P-1 (Phy 002), P-2 (Phy 015), P-3 (Phy 068), P-4 (Phy 145), P-5 (Phy 190), P-6 (Phy 134), P-7 (Phy 098), P-8 (Phy 050), P-9 (Phy 011), P-10 (Phy 065), P-11 (Phy 193), P-12 (Phy 037), P-13 (Phy 192), P-14 (Phy 022); M (molecular weight marker)

at least three times with identical results. Table 2 shows the distribution of groups according to the ISSR analyses on which the reference isolates of *Phytophthora* were grouped.

Among the Spanish *P. citrophthora* strains, the similarity coefficient among representative isolates of the 14 genotypes ranged from 0.16 to 0.91 with an average of 0.48 (Fig. 3). The highest genetic

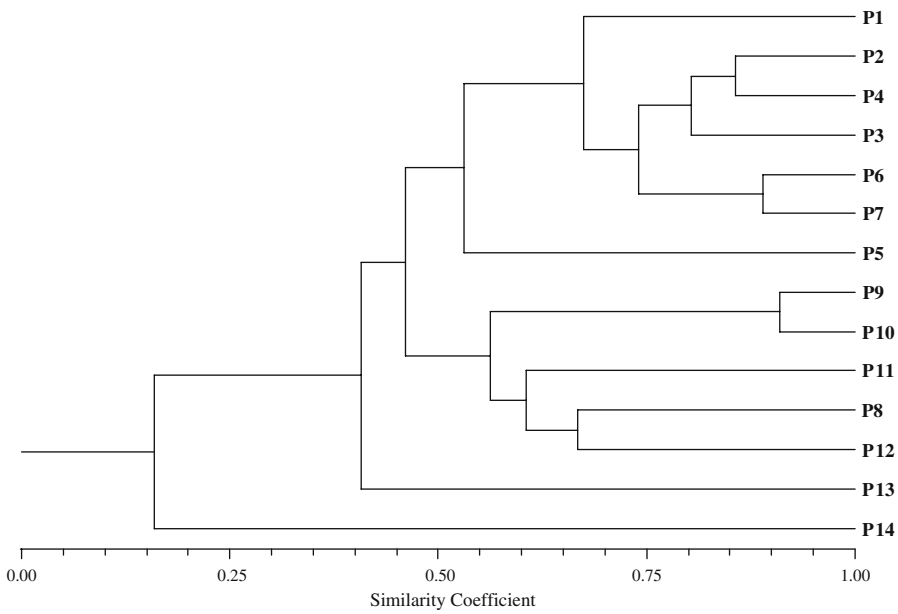

Fig. 3 Dendrogram showing similarities among genotypes of *Phytophthora citrophthora* based on Inter-Simple Sequence Repeat using simple matching coefficients. The tree was generated using the SAHN clustering program with the unweighted pair-group method with arithmetic average algorithm in NTSYS-pc

similarity coefficient (0.91) was found between the genotypes P-9 and P-10, whereas the lowest (0.16) was found between P-14 and the other groups. In the dendrogram that shows the genetic relationship among the 14 genotypes of *P. citrophthora* based on amplified CGA-ISSR fragments (Fig. 3), the variability within the population of *P. citrophthora* can be observed. However, this variation was only found in 15 isolates (equivalent to 13 genotypes) of the 134 analyzed.

Pathogenicity

Lesion areas were significantly ($P<0.05$) affected by the citrus species inoculated (Table 5). The analysis showed that clementines were more susceptible than sweet oranges. In contrast, isolate genotype and its interaction with the citrus species inoculated were not significant ($P>0.05$).

Discussion

As previously observed by Cohen et al. (2003), the use of the ISSR primer GT allowed clear discrimination among species of *Phytophthora*, whereas use of the CGA primer allowed separation among isolates of *P. citrophthora*. In contrast, the ITS region of rDNA did not reveal any variability, suggesting that this region may be homogeneous in this species. Strains of *P. citrophthora* isolated from branch cankers were mainly associated to one genotype (P-1), which included 88% of the isolates. Strains obtained from citrus roots, fruits, as well as 11 isolates from branch

Table 5 Regression analyses between host and pathogen variables and lesion sizes obtained in the pathogenicity tests

Variables	Regression analysis		
	Estimate	r^2	*P* value
Associated to the Host			
Citrus cultivars (vs. Hernandina)		10.27	0.0000
Constant	4.0836		0.0030
Clemenules	6.9171		0.0001
Fortune	2.0605		0.3587
Lane-Late	1.4523		0.5291
Navel-Late	0.2066		0.9132
Valencia-Late	−0.1807		0.9170
Citrus species (vs. clementines)		4.05	0.0010
Constant	7.96924		0.0000
Sweet oranges	−3.66511		0.0010
Associated with the Pathogen			
Isolate (vs. Phy 058)		0.31	0.9759
Constant	5.49936		0.0001
Phy 011	0.61681		0.7478
Phy 016	0.76538		0.6989
Phy 028	0.20063		0.9162
Phy 035	0.73254		0.7076
Phy 051	1.58837		0.4107
Isolate genotype (vs. P-1)		0.03	0.7790
Constant	5.97519		0.0000
P-9	0.31584		0.7790
Interaction	Multiple regression analysis		
		r^2	*P* value
Citrus species × isolate genotype		0.65	0.1709

cankers were included in 13 minor genotypes, most of them containing only one isolate. The number of genotypes in the present study was significantly higher than that reported by Cohen et al. (2003), which found four population groups within *P. citrophthora* from citrus using the same ISSR markers.

As in previous studies (Tuset 1977), the examination of a large sample of *P. citrophthora* isolates from citrus, mainly isolated from branch cankers, showed that sexual mating was rare. Only one of 134 isolates studied produced oospores. Furthermore, isolates clustered in the prevailing P-1 genotype were all sterile. Likewise, Cohen et al. (2003), found that about 70% of the isolates of *P. citrophthora* (76/109) recovered from the Corsican citrus growing-areas formed a large group of predominantly sterile isolates (G1). Three *P. citrophthora* isolates from Corsica pertaining to the G1 group included in the present study clustered with the predominant P-1 genotype. Therefore, this P-1 genotype may be considered equivalent to the G1 group found in Corsica. Other population groups identified by Cohen et al. (2003) were the G2 (heterothallic isolates) and G3 groups, which were represented in our study by the groups P-12 and P-9, respectively.

Based on comparisons with isolates from the previous survey conducted in Corsica, the G1 group of *P. citrophthora* identified by Cohen et al. (2003) was considered as the predominant population in this citrus area. Four *P. citrophthora* isolates from citrus obtained in Spain 4 to 18 years before the first disease outbreak clustered also with the predominant P-1 group. Thus, as in Corsica, P-1 might be considered as the main population of *P. citrophthora* in Spain. Although all the available accesions from culture collections were included in current study, more isolates of *P. citrophthora* from previous surveys probably would be necessary to validate this hypothesis. Phylogenetic analysis including isolates from the Mediterranean Basin and other affected areas such as South Africa would clarify the worldwide phylogeography of *P. citrophthora* causing branch cankers on clementines.

Strict asexual reproduction in *P. citrophthora*, together with the high proportion of isolates that clustered in a unique genotype were indicative of a clonal structure of the population of *P. citrophthora* causing branch cankers on clementines in Spain. Citrus has been cultivated for centuries in the Mediterranean Basin and some evidence indicates that *Phytophthora* has been interacting with citrus for much of this time (Bou 1879; Rullán 1896; Fawcett 1936; Erwin and Ribeiro 1996). In agricultural ecosystems, pathogen populations evolve adapting to host genotypes and prevailing environmental conditions, leading to the selection of predominant pathogen genotypes (McDonald and Linde 2002; Milgroom and Peever 2003; Stukenbrock and McDonald 2008). Results from the present study indicate that this may be the case of *P. citrophthora* on citrus in Spain.

Interestingly, the P-1 genotype was also prevalent in the new citrus-growing areas on the southwestern coast. In these areas, orchards were established in deforested areas and were planted with citrus produced in nurseries located on the eastern coast, where the P-1 genotype was prevalent even before the emergence of the disease. Spain has had a mandatory citrus certification program since 1961, which is currently regulated by Council Directive 92/34/EEC and the RD 929/1995. The program establishes that citrus planting stocks should be free of *Phytophthora* spp. based on visual examinations. In some cases, this type of inspection may not be sufficiently reliable, and the P-1 genotype might have been inadvertently spread from the eastern to the southwestern coast on nursery plants with latent infections or infested soil. However, although possible, this hypothesis cannot be validated since data from nurseries were not available for the study.

In general, *P. citrophthora* isolates included in this study showed similar morphological and physiological traits as those described by Tuset (1977), who first reported a detailed description of this species affecting citrus in Spain. However, the present study demonstrates a substantial variation in some phenotypes within the population of *P. citrophthora*. Surprisingly, isolates that showed a petalloid colony pattern matched with the isolates clustered in the P-1 genotype (Table 4). This result indicates that the colony pattern was a stable character and might be used as a phenotypic trait to infer intraspecific genotypic changes. Apphia et al. (2003) also found a relationship between colony patterns and the genotypic diversity in *P. capsici*. Waterhouse (1963) reported that certain *Phytophthora* species had distinctive colony patterns that persist

under a variety of cultural conditions. However, Hüberli et al. (2001) considered that this character is unstable and should be used with considerable caution, even at the interspecific level. Apart from colony pattern, no other morphological or physiological character was correlated with the intraspecific genetic differentiation obtained in the ISSR analysis.

The multivariate factorial analysis showed two groups, each of them containing isolates of *P. citrophthora* and *P. nicotianae*, respectively. Although sexual reproduction was the main contributing variable to separate these interspecific groups (Table 3, Fig. 1), *P. citrophthora* isolates were also separated from *P. nicotianae* by other traits such as growth rate, growth at 35°C and colony pattern (Tuset 1977; Erwin and Ribeiro 1996).

Heterothallic *P. citrophthora* isolates tested in this work shared the same genotype (P-12), forming an unique group. Irrespective of the geographical origin and mating type of these isolates, the CGA primer was also able to separate sterile and heterothallic isolates within the population. The heterothallic nature of *P. citrophthora* was not reported in the original description by Smith and Smith (Erwin and Ribeiro 1996). However, results of the current study and previous works (Cohen et al. 2003; Vernière et al. 2004; Vial et al. 2006) raise the question as to whether it is correct to define *P. citrophthora* as a sexually sterile species or whether sexually fertile and sterile isolates are in fact two different species. In addition to its role as a potential source of genetic variation, the presence of oospores in the life cycle of the pathogen has direct implication on its epidemiology as a means of dissemination and survival.

Sporangium size was heterogeneous in the species. However, the multivariate analysis showed that isolates with larger and wider sporangia produced chlamydospores more frequently. This aspect may have some biological implications and must be studied in depth. Sporangial shape and papillation did not have a significant effect on the separation of groups within *P. citrophthora*.

Lesion size from branch inoculations with *P. citrophthora* is in general highly variable (Alvarez et al. 2008b). This variability in lesion size accounts for the low r^2 values obtained in the pathogenicity tests. In these cases, one way to improve the statistical significance of the experiments is to increase the number of replications. However, due to the crop damage imposed by inoculations, this is rarely feasible in trials in commercial orchards. As reported in previous greenhouse studies (Alvarez et al. 2008a), pathogenicity tests with *P. citrophthora* under field conditions showed that clementines and their hybrids were more susceptible to *P. citrophthora* than sweet oranges. Nevertheless, susceptible clementine cultivars were widely grown in Spain decades before the first disease outbreak (Agustí 2000). Thus, it is unlikely that this new epidemic was induced by a change in host susceptibility.

No signifficant differences in lesion areas were detected between the two genotypes of *P. citrophthora* (P-1 and P-9) evaluated. Although, due to the limited number of isolates, it was not possible to include more genotypes, this result would indicate that Phytophthora branch canker of clementines in Spain was not associated with a more aggressive population of *P. citrophthora*. Furthermore, no evidence of host specialization was detected in the predominant P-1 genotype. These results may offer the advantage for citrus breeders to work with P-1 isolates to generate resistant clementine cultivars. Nevertheless, the high number of different genotypes among asexual progenies observed in this study may be indicative of potential evolution from the predominant population (P-1) which could determine future changes in its genetic structure.

In conclusion, Phytophthora branch canker of clementines in Spain was mainly associated with a major P-1 genotype of *P. citrophthora*, which may be considered as the predominant population. It was not possible to relate the emergence of the disease with either a genetic drift or the development of host-specific forms in *P. citrophthora*. The possible effect of agronomic and environmental factors in the emergence of this disease as well as the role of infected planting stock need to be further investigated.

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