Diversity of *Phytophthora capsici* in Northwest Spain: Analysis of Virulence, Metalaxyl Response, and Molecular Characterization

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ABSTRACT

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Phytophthora crown rot, caused by *Phytophthora capsici*, is potentially the most destructive disease of pepper in Spain. Phenotypic and genetic diversity of 16 *P. capsici* isolates collected from 11 farms in northwest Spain was characterized based on virulence, mating type, sensitivity to metalaxyl, and genetic analysis using random amplified polymorphic DNA (RAPD) methods. Low variability was observed among the isolates in their metalaxyl response, with 87.5% being highly sensitive. No isolates of the A2 mating type were detected. More variability was found in the virulence assay, and isolates were classified into two groups according to their pathogenicity on a set of four pepper cultivar differentials. Genetic variation examined with eight RAPD primers generated 92 polymorphic bands and revealed the existence of different patterns among isolates. Cluster analysis using the unweighted pair-group method with arithmetic averages (UP-GMA) separated the Spanish isolates into three RAPD groups and established a relationship between the Spanish population and a representative worldwide group of isolates. No correlation was found between groups obtained by RAPD analysis and groups defined by virulence or metalaxyl response.

Phytophthora capsici Leon. is a soil-borne oomycete pathogen of pepper (*Cap-sicum annuum* L.) that was first identified as the causative agent of chile wilt in New Mexico (42). Since then, *P. capsici* has been identified worldwide and can infect numerous plant species (22). *P. capsici* can infect different parts of the pepper plant and, depending on the point of infection, may cause several disease syndromes on roots, crown, branches, leaves, and fruit. In Galicia (NW Spain), *Phytophthora* crown rot is the most destructive disease of pepper. The aerial phase of the disease is much less common (50,56). In *Phytophthora* crown rot, the pathogen penetrates the plant through the base of the stem, causing first necrosis in that area. At the same time, the plant becomes withered while the fungus advances through both the stem and the root, causing obstruction in the vascular system. Infected tissues become dry, sunken, and turn dark in color, with death quickly following.

There are chemical treatments, especially phenylamide fungicides, which are used to manage *P. capsici*. However, due to the multiple disease syndromes caused by

this fungus, chemical control of *P. capsici* is sometimes ineffective; additionally, it often fails after repeated use due to the appearance of new genotypes insensitive to fungicides. Biological control has also been unsuccessful (48). Another effective management method is the use of resistant pepper cultivars. Different sources of resistance to *P. capsici* have been reported in pepper. Among the genotypes that exhibit resistance to *Phytophthora* crown rot are USDA PI201234, USDA PI201232, Serrano Criollo de Morelos-331 (SCM 331), and SCM 334. SCM 331 and SCM 334 have shown a higher degree of resistance to *P. capsici* than USDA PI201234 and USDA PI201232 (7,24,25,53). However, as with fungicides, resistance can be overcome by the development of new pathogenic genotypes. Therefore, despite best management efforts, *P. capsici* cannot always be managed effectively. For these reasons, it is necessary to search for a sustainable method for disease management, which necessitates a better understanding of the genetic variability in populations, spatially and temporally. By understanding population genetics, adequate decisions can be made about disease management with fungicides and breeding programs.

Research on genotypic diversity has been carried out with different *Phytophthora* spp. (43,51,55,66). *P. capsici* is a heterothallic species with two mating types, A1 and A2, which interact to produce sexual spores (oospores). The presence of both mating types in the same area

provides a means for creation of potentially more virulent phenotypes through sexual recombination (29,43,45). Other possible sources of genetic variation are mutations or outcrossings between different races (64). In *P. capsici*, the presence of pathotypes and physiological races have been reported (24,52). Furthermore, it has been suggested that the phenomenon of outcrossing could not only occur between individuals of the same species, but also between different species. Genetic variants were created in the laboratory by fusion of *P. capsici* and *P. nicotianae* zoospores. These new hybrid genotypes varied in their host ranges, pathogenicity, and virulence (20,21).

Although virulence testing using a set of host differentials has been traditionally used to identify markers for genetic variation studies, it has several disadvantages. Differential screening is labor-intensive, time-consuming, and often generates variable results that are influenced by the environment and inoculation techniques. The recent development of genetic markers based on polymerase chain reaction (PCR), such as random amplified polymorphic DNA (RAPD) (65), offers a virtually unlimited number of selectively neutral markers from which to randomly select a sufficient number to conduct population genetic analyses. DNA polymorphism in these markers arises from nucleotide pair substitutions, deletions, inversions, and translocations (63). RAPD analyses have been widely used to study the genetic variation in oomycete populations (13,47,57).

The specific objectives of this study were to: (i) study the phenotypic variability of isolates from the *P. capsici* population in northwest Spain by determining virulence, mating type, and response to metalaxyl; (ii) use RAPDs to determine whether phenotypic variation can be detected at the DNA level; and (iii) assess the genetic variation within a population of Spanish isolates and determine the relatedness between them and a representative worldwide group of *P. capsici* isolates.

MATERIALS AND METHODS

Isolate origin. Isolates of *P. capsici* were obtained from various sources (Table 1). Sixteen were from infected roots of pepper plants of cultivar Padrón collected during a survey in Galicia (NW Spain) carried out in 1998. These isolates were selected based on morphology and viru-

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lence from 35 isolates obtained from 11 farms (with only one field per farm) within a 7-km-wide area in Padrón municipality, where production of this cultivar is concentrated. This survey targeted farms where growers had observed disease symptoms. Samples were collected between 15 May and 24 June when plants were in the vegetative stage and between 28 July and 24 September when plants were producing fruit (56). Three isolates were kindly supplied by Centro de Investigaciones Agrarias de Mabegondo (CIAM, Spain), and 10 were supplied by the Scottish Crop Research Institute (SCRI, Scotland).

Virulence assays. Sixteen *P. capsici* isolates, collected in 1998 and representing a range of morphological types, were compared for their virulence on pepper. All isolates were cycled through pepper cultivar Padrón within 6 months of the virulence assay. Four cultivars or lines of pepper were used for the virulence assay: Yolo Wonder (susceptible), line SCM 331 (resistant), line PI201234 (partially resistant) (7,24), plus the susceptible local cultivar Padrón, which was included because of its economic value in NW Spain. Plants were grown separately in pots in a chamber at 25°C and a photoperiod of 16 h light and 8 h darkness until they were 3 weeks old. They were then inoculated using a soil drench with a 5-ml suspension containing

10⁴ zoospores ml⁻¹ of an isolate. The suspension of zoospores was prepared according to Larkin et al. (38). Each virulence assay was conducted two times. Disease severity was rated for 20 days after inoculation on a scale of 0 to 5, where 0 = no visible symptoms, 1 = ≤50% of stem with water-soaked lesions and/or ≤50% of leaves wilted, 2 = >50% of stem with water-soaked lesions or >50% of leaves wilted, 3 = ≤50% of stem with water-soaked lesions and >50% of leaves wilted or >50% of stem with water-soaked lesions and ≤50% of leaves wilted, 4 = >50% of stem with water-soaked lesions and >50% of leaves wilted, 5 = dead plant. Disease severity ratings were used to calculate area under disease progress curves (AUDPC) according to the formula described by Campbell and Madden (12). AUDPC data were analyzed with a nonparametric one-way Kruskal-Wallis test followed by a multiple comparison procedure according to Conover (15). Additionally, percentage of diseased plants (incidence) was recorded during the time-course of study. Incidence data were analyzed using a logistic regression.

Mating type. Isolates were tested for mating type by placing transfer plugs 5 cm apart from known mating types (A1 and A2) on V8 agar medium. The presence of oospores was scored 7 to 10 days after

incubation at 25°C. Both *P. capsici* A1 (CBS 521.77) and A2 (CBS 370.72) isolates were obtained from Centraalbureau voor schimmelcultures (CBS).

Metalaxyl sensitivity. Responses to the fungicide metalaxyl were determined by growing the isolates on V8 juice agar media amended with Ridomil (250 mg metalaxyl ml⁻¹) suspended in sterile distilled water and added to V8 juice agar cooled to 49°C at 0, 0.1, 1, 5, 10, and 100 µg ml⁻¹ (54). Agar plugs (3 mm diameter) were placed on plates of different concentrations and incubated in the dark at 24°C. At 3, 4, 5, 6, and 7 days after inoculation, the radius of each colony was recorded using two measurements taken at a 90° angle. Three replicate petri dishes per fungicide level were tested for each isolate and each assay was repeated twice. Percent growth of an isolate on amended media was calculated by subtracting the inoculation plug diameter (3 mm) from the diameter of each colony and dividing the average diameter of the colony on amended plates by the average diameter of the colony on nonamended control plates. An isolate was scored as sensitive (S) if colony growth on media amended with 5 µg ml⁻¹ metalaxyl was less than 40% of the isolate growth on nonamended media. Intermediate isolates (I) exhibited growth on media amended with 5 µg ml⁻¹ greater than 40% of that on nonamended media, but growth on media amended with 100 µg ml⁻¹ metalaxyl less than 40% of that on nonamended media. Resistant isolates (R) exhibited growth on media amended with 100 µg ml⁻¹ greater than 40% of that on nonamended media (54). Furthermore, the effective concentration (EC₅₀) for each isolate was calculated by plotting the percentage of growth of each isolate against log₁₀ of the metalaxyl concentration. The point on the regression line at which 50% of the isolate growth was inhibited is the EC₅₀ value.

DNA extraction. To extract DNA, isolates were grown in still culture in pea broth for 1 week at 24°C (16). Mycelia were collected and freeze-dried for extended storage at -20°C. Total genomic DNA was extracted according to Raeder and Broda (58) with some modification. Freeze-dried mycelia were ground in a mortar with sodium dodecyl sulfate (SDS) extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, and 5% SDS). Extraction with phenol/chloroform/isomyl alcohol (25:24:1, vol/vol) was then carried out. Precipitation was carried out with cold isopropanol, and the pellet was washed with 70% ethanol and resuspended in sterile double-distilled water. The concentration of DNA was measured spectrophotometrically at 260 nm.

RAPD analysis. To determine genetic similarity among *P. capsici* isolates, RAPD fragments were generated for all isolates. RAPD primers were selected by screening genomic DNA from six isolates with dif-

Table 1. *Phytophthora capsici* isolates used in this study

Isolate	Year of isolation	Country of origin	Source ^w	Host or location
UDC141Pc ^x	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC142Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC175Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC188Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC192Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC196Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC199Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC201Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC242Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC245Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC248Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC265Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC275Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC288Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC309Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
UDC3810Pc	1998	Spain	UDC (Spain)	<i>Capsicum annuum</i>
BE-3 ^y	2001	Spain	CIAM (Spain)	<i>Capsicum annuum</i>
BE-4 ^y	2001	Spain	CIAM (Spain)	<i>Capsicum annuum</i>
RO-4 ^y	2001	Spain	CIAM (Spain)	<i>Capsicum annuum</i>
IMI 040502	NA ^z	Type	SCRI (Scotland)	<i>Capsicum annuum</i>
IMI 223314	NA	Iran	SCRI (Scotland)	Soil
IMI 325900	NA	Brazil	SCRI (Scotland)	<i>Theobroma cacao</i>
IMI 379381	NA	Pakistan	SCRI (Scotland)	<i>Capsicum annuum</i>
IMI 379384	NA	Pakistan	SCRI (Scotland)	<i>Capsicum annuum</i>
IMI 149560	NA	Mexico	SCRI (Scotland)	<i>Capsicum annuum</i>
IMI 149561	NA	Mexico	SCRI (Scotland)	<i>Capsicum</i> sp.
IMI 352321	NA	India	SCRI (Scotland)	<i>Piper nigrum</i>
IMI 329706	NA	India	SCRI (Scotland)	<i>Piper nigrum</i>
IMI 230564	NA	Nigeria	SCRI (Scotland)	<i>Capsicum</i> sp.

^w UDC = Universidade da Coruña, CIAM = Centro de Investigaciones Agrarias de Mabegondo, SCRI = Scottish Crop Research Institute.

^x *P. capsici* with UDC were from Padrón (NW Spain). Equal first two numbers indicates the same farm and field.

^y *P. capsici* isolates from different locations in NW Spain (BE = Betanzos, RO = O Rosal).

^z NA = not available.

ferent morphology and virulence. Twenty primers from Advanced Biotechnologies (Germany) were screened and eight (ABA17, ABA3, ABA20, ABA10, ABA9, ABA7, ABA13, and ABA18) were selected to generate RAPD fragments. Primers were selected for their ability to generate reproducible and well-separated DNA bands after two separate experiments for each primer. Amplification reactions were carried out in a 20- μ l reaction mixture containing 100 ng of DNA, 0.2 μ M of primer, 100 μ M of each dNTP (Bioline), and 0.4 Units of *Taq* DNA polymerase (Roche) in 1 $\times$ PCR reaction buffer (Roche). Amplification was performed in a programmable thermal cycler (Primus 96 PCR-System) with the following conditions: 30 s denaturation at 94°C, 40 cycles at 92°C for 30 s, annealing at 35°C for 1 min, extension at 72°C for 2 min. Amplified products were resolved in 1.5% agarose gels in TBE buffer, stained with ethidium bromide, and photographed under UV light. RAPDs were performed twice to confirm reproducibility of amplification products, and only those bands that appeared consistently were considered for further analysis. Fragments were then scored as present (1) or absent (0), and a binary data matrix was obtained for each data set. Bands of the same mobility were scored as identical. There was no correction for band intensity, and all bands ranging from 100 to 1,500 bp were scored. Cluster analysis of the data matrix was performed by the unweighted paired-group method of arithmetic averages (UPGMA), and a dendrogram depicting the relatedness among the 29 *P. capsici* isolates was constructed. Genetic similarities were computed between all pairs of isolates using the formula given by Nei and Li (49): $S = 2b_{ij}/(b_i + b_j)$, where b_{ij} is the number of amplicons shared by two isolates i and j , and b_i and b_j are the total number of amplicons found in isolates i

and j , respectively. Analyses were performed using the Multivariate Statistical Package (MVSP) version 3.1 (Kovach Computing Services).

RESULTS

Virulence assay. A set of 16 *P. capsici* isolates was tested for virulence on four pepper genotypes that differed in their degree of susceptibility to the pathogen. Noninoculated control plants did not show any symptoms. Based on disease incidence and AUDPC calculations of disease severity ratings, significant differences among cultivars and isolates were observed (Tables 2 and 3). Initial symptoms appeared 2 to 8 days postinoculation, and maximal disease ratings were achieved 5 to 15 days after inoculation, depending on the cultivar and isolate interaction. In some cases, no symptoms were observed during the entire study period.

Susceptible cultivar Yolo Wonder was more susceptible to all the *P. capsici* isolates tested than accessions PI201234 and SCM 331. Differences between PI201234 and SCM 331 were observed. On average, symptoms developed on 63% of plants of susceptible cultivar Yolo Wonder, but only on 15.3% of PI201234 plants and 3.5% of SCM 331 plants. Yolo Wonder showed the highest AUDPC global mean (43.2, mean of all the data of all the isolates), while that of PI201234 was substantially lower (11.2) and that of SCM 331 the lowest (3.07). The local cultivar, Padrón, displayed an infection pattern similar to that of Yolo Wonder but had a higher percentage of infected plants (68%) and higher AUDPC (50.2).

P. capsici isolates showed varying levels of virulence. All were able to infect susceptible pepper cultivars Yolo Wonder and Padrón, but only 37.5% of isolates caused more than 95% of these plants to become diseased; 25% of *P. capsici* isolates were able to infect both PI201234 and SCM

331; 25% of isolates infected PI201234 but not SCM 331; 12.5% infected SCM 331 but not PI201234; and 37.5% infected neither PI201234 nor SCM 331.

Incidence data were analyzed for each isolate by comparing the proportion of diseased plants for each pepper genotype with that in SCM 331, using a logistic regression ($P < 0.05$) (Table 2). SCM 331 was used as reference since it was most resistant to *P. capsici*, and it was assumed that the low incidences ($\leq 20\%$) seen in SCM 331 were due to the fact that it is not a 100% pure line for the character resistance, so there is a small percentage of susceptible individuals (7). Incidence data revealed that all the isolates were pathogenic to Yolo Wonder. Only isolate UDC248Pc lacked virulence to Padrón, the plant disease incidence being just 5%, not significantly different from the incidence in SCM 331 ($P = 0.05$). In the case of PI201234, several isolates were pathogenic and their incidences were statistically different from those in SCM 331. This is also the case for isolates UDC175Pc, UDC192Pc, UDC199Pc, UDC242Pc, and UDC275Pc.

Analysis of AUDPC values was carried out only for data from the susceptible genotypes. Many plants of the resistant cultivars did not develop symptoms of disease, thus the AUDPC was zero. An initial trial was performed analyzing the AUDPC data by a two-way ANOVA, but both the absence of normality and the evidence of interactions led to the use of nonparametric one-way Kruskal-Wallis test on the medians ($P < 0.05$) followed by a multiple comparison procedure according to Conover (15) (Table 3). This analysis revealed significant variability among the isolates in virulence based on AUDPC. Isolates UDC142Pc, UDC192Pc,

Table 2. Percentage of plants of each pepper cultivar exhibiting symptoms 15 days after inoculation with *Phytophthora capsici* isolates^z

Isolate	Padrón	Yolo Wonder	PI201234	SCM 331
UDC141Pc	25*	15*	0	0
UDC142Pc	100*	100*	25	15
UDC175Pc	30*	35*	55*	5
UDC188Pc	35*	30*	0	0
UDC192Pc	100*	95*	30*	0
UDC196Pc	100*	95*	35	20
UDC199Pc	40*	50*	20*	0
UDC201Pc	100*	95*	0	5
UDC242Pc	100*	75*	30*	0
UDC245Pc	100*	70*	0	5
UDC248Pc	5	30*	0	0
UDC265Pc	55*	40*	0	0
UDC275Pc	100*	100*	50*	15
UDC288Pc	70*	95*	25	10
UDC309Pc	100*	70*	0	0
UDC3810Pc	90*	75*	5	0

^z * Indicates significant differences with respect to incidence of isolate causing symptoms on SCM 331 based on comparing the proportions by logistic regression ($P < 0.05$). For every combination of isolate and cultivar, 10 plants were analyzed in each of two experiments.

Table 3. AUDPC^z values for *Phytophthora capsici* isolates on susceptible cultivars Padrón and Yolo Wonder

Isolate	Padrón	Yolo Wonder
UDC248Pc	3.6 a ^z	11.0 a
UDC141Pc	15.2 ab	4.5 a
UDC175Pc	20.7 ab	23.0 ab
UDC188Pc	25.7 ab	20.3 ab
UDC199Pc	27.7 ab	33.7 abc
UDC265Pc	30.5 ab	17.9 a
UDC245Pc	42.9 ab	36.0 abc
UDC288Pc	54.1 cd	70.9 ef
UDC3810Pc	55.1 bc	39.3 abc
UDC201Pc	69.9 cd	53.4 bcd
UDC242Pc	72.0 de	42.4 bcd
UDC309Pc	73.3 de	50.7 cde
UDC142Pc	75.4 de	75.3 f
UDC192Pc	77.6 de	72.6 f
UDC275Pc	78.8 e	75.5 f
UDC196Pc	79.9 e	64.8 def

^z Data are means of 20 plants. Different letters within the same column indicate significant differences among medians based on a Kruskal-Wallis test ($P < 0.05$) followed by a multiple comparison procedure according to Conover (15).

UDC275Pc, and UDC196Pc were highly virulent on both cultivars Padrón and Yolo Wonder. Isolates UDC248Pc and UDC141Pc were hypovirulent (of low virulence) on cultivars Padrón and Yolo Wonder. Some isolates behaved with different degrees of virulence depending on the cultivar. UDC288Pc was of intermediate virulence on Padrón but was one of the most virulent on Yolo Wonder. UDC242Pc was intermediately virulent on Yolo Wonder and highly virulent (hypervirulent) on Padrón. Therefore, some specificity for the cultivars was observed for some isolates. Origin of the isolates was not related to virulence; isolates from the same farm had different AUDPC values. For example, there were significant differences between UDC141Pc and UDC142Pc, these being hypovirulent and hypervirulent, respectively. Additionally, UDC199Pc showed low to intermediate virulence, whereas isolates UDC192Pc and UDC196Pc were highly virulent.

Mating type. All Spanish *P. capsici* isolates were of the A1 mating type. Oospores were produced only when plates of each isolate included the standard isolate of the A2 mating type.

Metalaxyl sensitivity assays. The addition of metalaxyl to the culture medium significantly reduced growth rates in most of the isolates tested (Table 4). Of the 16 Spanish isolates tested, the majority (87.5%) was classified as sensitive, because the percent growth rates on media with 5 µg ml⁻¹ metalaxyl relative to the control ranged from 0 to 33.5% but never surpassed 40%. Only one isolate was considered intermediate, the percentage of growth on media with 100 µg ml⁻¹ metalaxyl being less than 40%. Just one isolate was resistant; the percentage of growth on media with 100 µg ml⁻¹ being greater than 40%. Effective concentration (EC₅₀) values ranged from 0.53 to 4.65 µg ml⁻¹ (Table 4). Among the isolates, 37.5% had EC₅₀ values between 0.1 and 1 µg ml⁻¹,

and 62.5% between 1 and 5 µg ml⁻¹. In the first group (EC₅₀ values between 0.1 and 1 µg ml⁻¹), all isolates were classified as sensitive, whereas in the second group (EC₅₀ values between 1 and 5 µg ml⁻¹), 80% were considered sensitive, one intermediate, and one resistant. No relationship was observed between metalaxyl response and geographical origin of the isolates.

RAPD analysis. Of the 20 RAPD primers tested on 29 *P. capsici* isolates, only 8 were suitable for further investigation. The remaining 12 primers produced no bands, poor patterns, or nonreproducible results. The eight primers selected generated simple banding patterns that revealed DNA polymorphisms among *P. capsici* isolates. Examples of the comparative banding profiles of DNA of isolates using primer ABA17 are shown in Figure 1. Using these primers, a total of 153 bands were scored, of which 92 (60.2%) were polymorphic. The number of amplified products generated by each primer varied between 14 and 26 bands, and the size of the products was within the range 100 to 1,500 bp. When fingerprints of these isolates were compared, some bands common to all isolates were observed, while others were unique to just one or a few isolates. In most cases, the polymorphism was characterized by the presence of certain amplification products from some isolates.

The results obtained with all eight RAPD primers were combined, and a tree was constructed using UPGMA cluster analysis based on the coefficient by Nei and Li (Fig. 2) (49). The use of alternative coefficients (e.g., Jaccard's coefficient) did not significantly alter the resulting dendrogram. Values of the similarity coefficient of RAPD data ranged from 0.31 to 0.99. There was little variation in RAPD patterns among Spanish isolates and a high degree of variation in patterns among isolates from different geographical areas. Spanish isolates clustered together with 75% similarity in RAPD profiles, whereas the other *P. capsici* isolates clustered with 50% similarity, except for isolate IMI 325900, which had a highly distinctive pattern, showing a similarity of only 31%. These results indicate less variation within the Spanish population than among the isolates from different origins. The percentage of similarity in RAPD fragment patterns between Spanish isolates and the nearest neighbor belonging to another country was 73%, while the farthest isolate was 31% similar.

At a similarity level of 85%, the dendrogram showed that hierarchical clustering separated the isolates into three major RAPD groups and 10 minor groups (G1-G13). G1 included nine isolates, G2 seven isolates, and G3 only three isolates. G1 and G2 clustered together with a similarity of 82%, whereas G3 clustered with the other groups at 75% similarity. The RAPD groups G4-G13 each contained one isolate

Table 4. Metalaxyl sensitivity and effective concentration value (EC₅₀) of Spanish *Phytophthora capsici* isolates

Isolate	Metalaxyl sensitivity ^z	EC ₅₀ (µg ml ⁻¹)	
		Mean	Range
UDC141Pc	S	0.87	0.78-0.97
UDC142Pc	S	2.01	1.81-2.22
UDC175Pc	I	4.51	2.99-6.02
UDC188Pc	S	1.90	1.79-2.01
UDC192Pc	S	0.91	0.72-1.11
UDC196Pc	S	1.67	1.52-1.82
UDC199Pc	S	1.53	1.31-1.74
UDC201Pc	S	2.08	1.82-2.34
UDC242Pc	S	1.47	1.42-1.53
UDC245Pc	S	2.11	2.07-2.15
UDC248Pc	S	0.85	0.61-1.10
UDC265Pc	S	0.82	0.50-1.13
UDC275Pc	S	1.35	1.23-1.47
UDC288Pc	S	0.59	0.47-0.71
UDC309Pc	R	4.65	4.62-4.67
UDC3810Pc	S	0.89	0.88-0.92

^z S = sensitive, I = intermediate, R = resistant.

Fig. 1. Random amplified polymorphic DNA (RAPD) profile generated by primer ABA17 in *Phytophthora capsici* isolates.

and corresponded with isolates from different countries. These isolates did not cluster with any other isolate at this similarity level (85%) and were considered minor groups. In G1, two subgroups were differentiated at 90% and five minor groups at 95% similarity. In the same way, in G2, from 90 to 95% similarity, four to six subgroups were separated. The highest level of similarity (99%) was found between genotypes UDC141Pc and UDC142Pc, which were obtained from the same field. With regard to geographic origin, the Spanish genotypes fell into three different clusters (G1, G2, and G3), most of them being included in G1 and G2. Among them, isolates from the same locations and even from the same field belonged to different clusters. Thus, isolates BE-3 and BE-4, both from the same region, were located in clusters G3 and G1 of the dendrogram, respectively. Isolates UDC192Pc, UDC196Pc, and UDC199Pc, from the same field, were included in three different groups. Each of the representative worldwide genotypes constituted only one group, but genotypes from the same geographical region clustered together. Mexican isolates IMI 149560 and IMI 149561 clustered together with a similarity coefficient of 0.69, isolates from Pakistan (IMI 379384 and IMI 379381) presented a similarity percentage of 81%, while IMI 352321 and IMI 329706 (both from India) showed 64%.

The RAPD markers differentiated the Spanish isolates into three different RAPD genotypes. The differentiation of these

genotypes was not related to the phenotypic characters, such as virulence, mating type, and metalaxyl sensitivity. Only isolates UDC309Pc and UDC175Pc, which had the highest EC_{50} values, were closely related, with a Nei and Li coefficient of 0.94.

DISCUSSION

P. capsici is one of the most destructive pathogens affecting pepper. In NW Spain, it is responsible for one of the most common diseases of this crop, Phytophthora crown rot, and is one of the major limiting factors in pepper production (56).

Genetic resistance in pepper to *P. capsici* was first reported by Kimble and Grogan (33). Since then, several different screening methods have been developed to distinguish resistant from susceptible individuals (4,8,9,24). Cultivars exhibiting resistance include line 29, PI201232, PI201234, as well as 19 lines grouped together as Criollo de Morelos (6,7,8, 24,30). Results in this work regarding resistance of SCM 331 and PI202324 confirmed those from previous studies (5,7,8,32). Serrano Criollo de Morelos-331 showed the highest degree of resistance to multiple isolates of *P. capsici*. PI201234 showed moderate resistance and Yolo Wonder was the most susceptible. The local cultivar, Padrón, responded much like Yolo Wonder and was considered to have a similar susceptibility level.

Most of the Spanish isolates were considered moderately virulent, and only a few were either highly virulent or hy-

povirulent on both Padrón and Yolo Wonder. Similar results have been reported by Hwang et al., who suggested the existence of variation in virulence of naturally occurring *P. capsici* isolates (32). Oelke and Bosland reported the existence of physiological races within *P. capsici* based on distinct reactions to pepper differentials (52). Gil Ortega et al., however, proposed the presence of two vertical pathotypes, P0 and P1, within *P. capsici* (24). P0 includes all *P. capsici* isolates that are able to overcome the resistance of susceptible cultivars but not that of PI201232 and SCM 334, while P1 refers to isolates that overcome susceptible cultivars and PI20232 resistance but not that of SCM 334. According to Gil Ortega et al., isolates UDC175Pc, UDC192Pc, UDC199Pc, UDC242Pc, and UDC275Pc can be considered P1 because they are pathogenic to both Yolo Wonder and PI201234 (24). The other isolates would be P0, because they are only pathogenic to Yolo Wonder. However, in some cases, even after statistical testing, a reasonable doubt remains because of disease incidence on PI201234 and SCM331. This was the case for isolates UDC142Pc, UDC288Pc, and especially UDC196Pc, which caused disease in 25 to 35% of plants of PI201234. Such values were not significantly different from those obtained for SCM 331, in which the same isolates are responsible for some diseased plants. This is probably due to the fact that SCM 331 and PI201234 are not completely pure lines and a small but relevant number of susceptible individuals appear at random

Fig. 2. Unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis on random amplified polymorphic DNA (RAPD) markers of *Phytophthora capsici* genotypes from Spain and representative worldwide genotypes based on Nei and Li similarity coefficient.

in the assays (7). For the same reason, isolate UDC199Pc actually could be pathotype P0, because its disease incidence in PI201234 is really low (20%), even though it is significantly different from that obtained for SCM 331.

In general, isolates of *P. capsici* collected in different locations in Galicia (NW Spain) from pepper plants were highly sensitive to metalaxyl. Only one isolate was considered resistant and another was classified as moderately resistant. These isolates also displayed great variability in EC₅₀ values. Isolates from the same farm did not show the same sensitivity to metalaxyl. Therefore, a clear correlation between the previous fungicide applications in a given farm and the metalaxyl effective concentrations for isolates from that farm could not be established. Furthermore, no relationship was found between the virulence of isolates and their response to metalaxyl. Some studies in *P. infestans* have demonstrated that metalaxyl-resistant isolates have a better fitness than sensitive isolates (14,17). However, other studies in both *P. infestans* and *P. capsici* have suggested that no correlation exists between fitness and metalaxyl resistance and that metalaxyl insensitivity does not have significant costs outside of metalaxyl selection pressure (26,37,41). The high variability observed in EC₅₀ values was unexpected considering that no isolates of A2 mating type were detected in the fields and sexual recombination appears to play an important role in generating fully insensitive isolates (31). Possibly the variability is due to asexual variation as shown in *P. infestans* (1), or the A2 mating type was much less common than the A1 mating type and too few isolates were tested to detect it.

In the RAPD analysis, Spanish isolates clustered into three different but close groups. These three groups were far from isolates from other countries. Among worldwide isolates, IMI 230564 was the most similar to the Spanish isolates (similarity coefficient = 0.73) and also the closest geographically. The farthest isolates within the dendrogram were those from Brazil and India, which were also the farthest geographically. However, in the case of India, divergence between these isolates and those from Spain could be attributed to different hosts rather than to their origin. In fact, isolates from Mexico, which is close to Brazil geographically, showed more similarity with isolates from Spain, which also were obtained from pepper. These results suggest a possible correlation between genetic similarity and the host or the origin of the isolates. However, a more thorough study with a larger number of isolates would be required to confirm this hypothesis.

Despite previous results that RAPD analysis is useful for detection of genetic variation among *P. capsici* isolates, no

virulence specific RAPD fragments were identified in this study. Thus, isolates with different degrees of virulence may have the same RAPD patterns. Virulence is usually considered the most important phenotypic marker in studying populations of plant pathogens. However, virulence/avirulence loci represent only a small portion of the total genetic variation that may exist among different isolates within a pathogen population. Molecular markers such as RAPD are neutral markers, independent of host selection, and are usually used to assess total genetic variation that is not directly affected by hosts, or environmental factors that influence the expression of phenotypes.

In the same way, variation observed in isolate response to the fungicide metalaxyl was not related to *P. capsici* groups defined by RAPD markers. This result is not surprising, since, as explained above, RAPD markers detect variation that is distributed throughout the genome, whereas response to metalaxyl represents defined loci on the genome (65). This lack of correspondence was found in other *Phytophthora* spp. (2,40,45), where correlation between groups defined by RAPD markers, virulence, mating type, and response to metalaxyl was not observed.

Despite the absence of correlations between RAPD markers and virulence or metalaxyl sensitivity, RAPD analysis results provide useful information about the genome. In this work, the information provided by RAPD analysis suggests that the isolates from Spain have considerable levels of genetic variability.

All Spanish *P. capsici* isolates examined in this study were found to be of the A1 mating type, as were the six Spanish isolates previously studied by Andrés et al. (3). For this reason, variation in population may be a product of mutations rather than sexual recombination (35–37). Mutations that cause slight changes in DNA sequences throughout the genome can account for the levels of polymorphism observed. Mutation is the primary source of new genetic variation in oomycetes (27). These mutations, in most cases, can be neutral and cause no observable changes in phenotypes, but it is not impossible that at least part of the genotypic variation observed with these markers could be attributed to spontaneous mutations. Fast rates of genetic change due to mutation at virulence loci have been discovered within *P. infestans* and *P. sojae* (18,19,23,29,61). Isolates of *P. sojae* from the United States that shared a single restriction fragment length polymorphism (RFLP) genotype were differentiated into seven races, six of which probably arose from race 1 by mutation (23). Mutations may also have occurred for fungicide resistance (27). Extreme sensitivity to metalaxyl seems to be the ancestral phenotype for the US-1 clonal lineage of *P. infestans*. However,

metalaxyl-resistant US-1 isolates were obtained in the Philippines (34), where US-1 is the only known lineage. Mutation rates do not need to be excessively high to explain the observed changes in fungicide sensitivity and virulence. Other possible mechanisms of genetic variability during asexual reproduction in *Phytophthora* spp. include mitotic crossing over, gene conversion, and extra-chromosomal elements (2,27). Mitotic recombination probably could play an important role in generating genotypic variability. However, due to the dominant nature of RAPD markers (65), it was not possible to obtain concrete evidence that mitotic recombination was contributing to the genotypic variation observed. Another potential explanation for variation is parasexual recombination. There is some evidence of parasexual recombination in *Phytophthora* spp. in the laboratory, although it has not been confirmed with molecular markers (39,44,60). Another potential source of genetic variation could arise from the hybridization between individuals of different species. Interspecific hybridization has been suggested by several authors as a means by which genetic variability can be generated in populations of *Phytophthora* spp. (11,21,27,62). However, this phenomenon has been scarcely described in nature (46,59). Recently in Galicia, the presence of *P. nicotianae* isolates, which cause similar symptoms to those caused by *P. capsici*, has been reported (3). These two pathogens are heterothallic species that inhabit a wide range of soil habitats. In many agrosystems, however, it is rare that both compatibility types needed for sexual reproduction are present, and consequently, genetic variability generated via sexual processes is rare (20). The two organisms could potentially colonize the same plant and interact producing a new pathogenic hybrid. Indeed, genetic variants were created in the laboratory by fusion of *P. capsici* and *P. nicotianae* zoospores, and it was observed that these new hybrid genotypes varied in their host ranges, pathogenicity, and virulence (20,21). Goodwin and Fry (28) were able to cross closely related *P. infestans* and *P. mirabilis* to create viable species hybrids. The challenge is to detect the occurrence of these events that generate new pathogen genotypes in nature and differentiate these new genotypes from the parent isolates (10,46). Therefore, further study will be required to test the hypothesis of possible outcrossing between *P. capsici* and *P. nicotianae* in Spanish fields.

Although more research would be needed to determine the origin and nature of the extensive genetic variation revealed by RAPD analysis, both phenotypical and molecular characterization of Spanish *P. capsici* isolates carried out in this work provide a better understanding of the population dynamics as well as its relationship

with other isolates from different continents and hosts. Increased understanding of the Spanish *P. capsici* population will be useful for the development of improved management strategies.

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