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Reproductive Fitness and Random Amplified Polymorphic DNA Variation among Isolates of *Pratylenchus vulnus*[1]

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Abstract: The reproductive fitness of seven isolates of *Pratylenchus vulnus* from different geographical areas and hosts was assessed in monoxenic cultures (carrot), and greenhouse cultures (plum, sour orange, and quince). The genetic makeup of the different isolates was compared by Random Amplified Polymorphic DNA (RAPD-PCR). The apple (PvAP-S) and apricot (PvAT-F) isolates reproduced less in monoxenic cultures than the rose (PvRO-S) and walnut (PvWA-A and PvWA-U) isolates. On plum, the rose isolate (PvRO-S) reproduced better than the apple (PvAP-S) and walnut isolate from the United States (PvWA-U). On sour orange, the apple (PvAP-S), unknown origin (PvU-UK), and walnut isolate from Argentina (PvWA-A) multiplied well, whereas the walnut isolate from the United States (PvWA-U), apricot (PvAT-F), and rose (PvRO-S) did not. On quince, the apple (PvAP-S) and walnut (PvWA-U) isolates showed a higher reproduction than the one from unknown origin (PvU-UK). RAPD-PCR patterns among the seven *P. vulnus* isolates were similar, although high intraspecific varibility was detected. Very few bands of *P. neglectus* were shared by any population of *P. vulnus*. A high degree of similarity was found among the patterns corresponding to the rose (PvRO-S), apple (PvAP-S), walnut from the United States (PvWA-U), and unknown origin (PvUK-U) isolates. The apricot isolate (PvAT-F) was the most dissimilar among the seven isolates. No correlation could be established between the genetic variation of *P. vulnus* detected by RAPD-PCR and reproductive fitness. Results demonstrate high genetic varibility between geographically separated populations of *P. vulnus*.

Key words: genetic variability, *Pratylenchus vulnus*, nematode, RAPD-PCR, race, reproductive fitness, root-lesion nematode.

The root-lesion nematode *Pratylenchus vulnus* Allen & Jensen is an important pathogen attacking many pome, nut, and stone fruit crops in the Mediterranean area (7,22,24,28), California (5,14–16), and the southeastern United States (1).

Evaluation of commercial rootstocks in the last 20 years conducted in different geographical areas suggests that differences in both host range and pathogenicity exist, although no races have been described. In Italy, sour orange (*Citrus aurantium* L.) is considered a good host of *P. vulnus* (10). In contrast, a *P. vulnus* isolate used in a host range study in Spain did not attack six citrus species, including sour orange (23). Discrepancies in host suitability among American and European populations of *P. vulnus* on *Prunus* have also been reported (13,23,27). More recently, differences in relative pathogenicity have been found on M-26 apple (*Malus silvestis* L.) and GF-677 peach–almond hybrid (*Prunus persica* Stock. × *P. amygdalus* Batsch) rootstocks among six of the *P. vulnus* isolates used in this investigation (J. Pinochet, unpubl.).

Reproductive fitness together with virulence are major components of pathogenicity. Monoxenic cultures provide homogeneous conditions for inoculum, substrate, humidity, and temperature conditions, allowing a fairly accurate measure of the reproductive fitness. This permits comparison of the amount of reproduction of one isolate or strain with others on a given host (29).

Molecular techniques offer new possibilities to detect genetic differences with a level of resolution that could not be approached previously. Few such studies have been conducted on plant-parasitic nematodes, the majority dealing with root-knot (4,8,20,25) and cyst nematodes (3). One recent and promising technique is a variant of the Polymerase Chain Reaction

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(PCR) termed Random Amplified Polymorphic DNA (RAPD-PCR) (31). This technique is based on the use of short, 10-mer primers of arbitrary sequence. By lowering the annealing temperature in the amplification cycle, the primer anneals at loci distributed randomly in the genome, allowing the synthesis of highly polymorphic amplification products. The RAPD technique has shown its usefulness in the identification of species of *Meloidogyne* and *Heterodera,* as well as in detecting intraspecific variation among populations (3,4).

The variability among *P. vulnus* populations could be important in rootstock selection programs currently being conducted against this pest in the United States (6), Spain (22,23), and France (28). The purpose of this study was to evaluate the reproductive fitness of seven isolates of *P. vulnus* with excised root tissue and in greenhouse studies with three woody plant species, and to compare their genetic relationships using RAPD-PCR.

Materials and Methods

Seven populations of *P. vulnus* originating from different geographic locations and hosts were obtained from several sources (Table 1). The isolates were reared monoxenically on carrot (*Daucus carota* L.) disk cultures (17) and incubated at 23–24 C for several generations. Species identification was made by the Commonwealth Institute of Parasitology, St. Albans, United Kingdom, and verified by morphologic and morphometric studies conducted by the fourth author of this investigation.

Comparison of reproductive fitness in vitro: In an initial in vitro experiment, the rate of reproduction among seven isolates of *P. vulnus* was compared. Nematode inoculum was recovered from carrot disk cultures by adding sterile water to the cultures and collecting nematodes with a pipette. The nematodes were then surface sterilized with 2,000 ppm of streptomycin sulfate for 1 hour in 8-cm^3 glass vials followed by rinsing twice (centrifugation) in sterile water before inoculation of carrot sections of the cultivar Nantesa. Each carrot section (approximately 2.5 g) was placed in a 5 cm-d petri plates. To establish a new monoxenic culture, 10 gravid females from each isolate were individually collected with a micropipette and delivered to the surface of a carrot section in 0.1 ml water suspension. Ten cultures per isolate were prepared and incubated at 23–24 C. Forty-five days after inoculation, nematodes were extracted by collecting the washings from the petri plate with the carrot culture and macerating the corresponding carrot disk in a blender at 14,500 RPM for 30 seconds (three 10-second periods separated by 5-second intervals). The suspension was sieved through nested 0.150-mm and 0.025-mm sieves (100 and 500 mesh, respectively). Root tissue and debris collected on the 0.150-mm sieve were discarded. Nematodes were recovered from the 0.025-mm screen (two washings). Different stages of the nematode were counted under the compound microscope in 1-ml aliquots.

Table 1. Origin of seven isolates of *Pratylenchus vulnus* and one of *P. neglectus* used in this study.

Isolate	Geographic origin	Host	Source†
PvRO-S	Barcelona, Spain	Rose (*Rosa multiflora*)	IRTA
PvAP-S	Gerona, Spain	Apple (*Malus silvestris*)	IRTA
PvAT-F	Antibes, France	Apricot (*Prunus armeniaca*)	INRA
PvU-UK	Unknown	Unknown	CABI
PvWA-A	Córdoba, Argentina	Walnut (*Juglans nigra*)	UNC
PvWA-U	Idaho, USA	Walnut (*Juglans regia*)	UI
PvOL-I	Taranto, Italy	Olive (*Olea europea*)	INA
PnWA-U	Idaho, USA	Walnut (*Juglans regia*)	UI

† IRTA = Institut de Recerca i Tecnologia Agralimentàties; INRA = Institut National de la Recherche Agronomique; CABI = Commonwealth Agricultural Bureau International; UCN = Universidad Nacional de Córdoba; UI = University of Idaho; INA = Intituto di Nematologia Agraria.

Data were subjected to analysis by a one-way analysis of variance. Final nematode populations were $\log_{10}$ transformed (x + 1) for analysis. Means were compared by Tukey's multiple-range test ($P \leq 0.01$).

Comparison of reproductive fitness in greenhouse: Three separate greenhouse experiments were conducted to compare the reproductive fitness (on one host) and parasitic fitness (on different hosts) of the same nematode isolates (29) on GF 8-1 plum (*Prunus cerasifera* Ehrh. × *P. munsoniana*), sour orange, and BA-29 quince (*Cydonia oblonga* Miller) rootstocks. The three rootstocks were chosen based on existing information on the host suitability on *Prunus, Cydonia,* and *Citrus* to the nematode (12,13, 23,27). Sour orange was propagated from seeds. Plum and quince rootstocks were micropropagated axenically in nutrient agar (18), transferred to 40-ml pots containing peat soil and acclimatized in a high humidity chamber for 20 days. Plants were then transferred to 400-ml pots with a 10:1 (v:v) soil–peat mixture. The soil was a sandy loam (86% sand, 12% silt, 2% clay, pH 7.3, < 4% organic matter). Nematode suspensions from each isolated were recovered from monoxenic cultures and adjusted to deliver 100 nematodes per plant through four holes, 4 cm deep, and 2 cm from the base of the stem. Ambient temperature in the greenhouse fluctuated between 21 and 28 C. Plants were watered as needed and fertilized with full-strength Hoagland's nutrient solution (9) once a week. In each experiment, treatments were replicated eight times in a completely randomized design. Nematode reproduction was assessed 90 days after inoculation. Nematodes in soil were recovered by differential sieving using 0.150, 0.074, and 0.025-mm sieves (100, 200, and 500 mesh, respectively) and centrifugation-sugar-flotation (11). Nematodes in roots were extracted by cutting the whole root system into pieces ca. 1-cm long and macerating them in water with a commercial blender as described in the in vitro experiment.

Data were subjected to analysis by a one-way analysis of variance. Data on final nematode population densities were $\log_{10}$ transformed (x + 1) for analysis, and the means were compared by Tukey's multiple-range test ($P \leq 0.05$). In these three experiments, the Italian isolate (PvOL-I) was not tested.

Comparison by Random Amplified Polymorphic DNA: Nematode DNA was extracted according to a miniprep previously described (4). Two types of cultures were used for each *P. vulnus* isolate, one reared from a single gravid female and the other from several specimens hereon designated as multiple specimen isolate (all life stages). Another root-lesion nematode species, *Pratylenchus neglectus* (Rensch) Filipjev & Schuurmans Stekhoven, was included as an outgroup isolate for RAPD-PCR comparison. This isolate was designated as PnWA-U. DNA was extracted from a pellet of several thousand individuals, previously treated with 500 ppm streptomycin sulfate, and washed thoroughly with sterile water. All PCR reactions were conducted as described by Cenis (4). The primers used were purchased from Operon Technologies (Alameda, CA, USA). Twenty primes were chosen at random from sets A, B, and C. The reactions that gave adequate amplifications were replicated three times. For the analysis of RAPD data, an estimation of genetic similarity between the nematode populations was made by using the estimator $F = 2n_{xy}/n_x + n_y$, where n_{xy} are the bands shared by population x and y, and n_x and n_y are the total number of bands of each population. The Nei and Li similarity matrix was used to determine the coefficients of similarity (19).

Results

Reproductive fitness, in vitro: The PvAP-s (apple) and PvAT-F (apricot) isolates reproduced less ($P \leq 0.01$) in monoxenic carrot cultures than the PvRO-S (rose) and PvWA-A and PvWA-U (walnut) isolates at 45 days after inoculation with 10 gravid females per culture (Table 2). There were no differences in population increase among PvAP-S, PvAT-F, PvOL-I (olive), and PvU-UK (unknown origin) isolates. The female–male ratio was higher in the

TABLE 2. Comparative reproduction of seven isolates of *Pratylenchus vulnus* in monoxenic carrot cultures 45 days after inoculation with 10 gravid females at 23–24 C.

Nematode isolate	Nematode developmental stages (%)				Final nematode population	Female/male ratio
	Eggs	Larvae	Females	Males		
PvAP-S	94 (37)	114 (45)	35 (13)	12 (5)	255 a	2.6:1
PvAT-F	383 (49)	228 (29)	130 (17)	5 (38)	778 a	3.4:1
PvOL-I	66 (7)	582 (61)	145 (15)	168 (17)	954 ab	0.9:1
PvU-UK	382 (44)	280 (32)	108 (12)	103 (12)	872 ab	1:1
PvRO-S	2,084 (55)	1,058 (21)	662 (12)	589 (12)	5,079 b	1:1
PvWA-A	598 (14)	2,288 (52)	811 (19)	649 (15)	4,350 b	1.3:1
PvWA-U	333 (8)	2,532 (59)	700 (16)	715 (17)	4,280 b	0.9:1

Values in parentheses are percentages of the total population.

Data are means of 10 replications. Actual data are presented, but data were transformed to $\log_{10}(x + 1)$ for analysis. Means in column for each isolate followed by the same letter do not differ according to Tukey's multiple-range test ($P \leq 0.01$).

PvAP-S and PvAT-F isolates (2.6:1 and 3.4:1, respectively) in comparison to the rest of the isolates, which ranged between 0.9:1 and 1.3:1. In isolates PvAT-F, PvU-UK, and PvRO-S, eggs accounted for the largest percentage of the nematode population.

Reproductive fitness, in vivo: Isolate PvRO-S reproduced more ($P \leq 0.05$) than PvAP-S and PvWA-U on plum rootstocks (Table 3). No differences were found between the remaining isolates on this host. On sour orange, the isolates PvAP-S, PvU-UK, and PvWA-A multiplied well, whereas isolates PvWA-U, PvAT-F, and PvRO-S did not. On quince, PvAP-S and PvWA-U isolates showed a higher reproduction ($P \leq 0.05$) than PvU-UK. All isolates reproduced well on plum and quince. Only PvRO-S reached a higher ($P \leq 0.05$) final nematode population density on plum than on sour orange and quince. On sour orange, five isolates (PvAP-S, PvWA-U, PvU-UK, PvAT-F, and PvRO-S) reproduced less than on plum. Isolates PvAP-S, PvWA-U, PvAT-F, and PvRO-S also produced less on sour orange than on quince.

TABLE 3. Comparative reproduction of six isolates of *Pratylenchus vulnus* on plum, sour orange, and quince rootstocks at 90 days after inoculation with 100 nematodes per plant.

Nematode isolate	Final nematode density in roots and soil		
	Plum (GF 8-1)	Sour orange	Quince (BA-29)
PvAP-S	1,800 a B	620 b A	1,580 b B
PvWA-U	1,970 a B	0 a A	1,590 b B
PvU-UK	1,980 ab B	315 b A	530 a AB
PvAT-F	2,130 ab B	35 a A	470 ab B
PvWA-A	3,330 ab A	740 b A	1,270 ab A
PvRO-S	6,990 b C	0 a A	780 ab B

Data are means of eight replications. Actual data are presented, but data were transformed to $\log_{10}(x + 1)$ for analysis. For each *P. vulnus* isolate, means in each column followed by the same lowercase letter do not differ according to Tukey's multiple-range test ($P \leq 0.05$). For each rootstock, means in each row followed by the same uppercase letter do not differ according to Tukey's multiple-range test ($P \leq 0.05$).

Random Amplified Polymorphic DNA: Of 20 primers tested, only six (OP B-1, B-4, B-7, B-12, B-15, and B-17) were adequate for all the isolates evaluated. The patterns of amplified DNA bands obtained after reactions with primers OPB-07 and OPB-17 are depicted in Figure 1. No appreciable differences were noted in the bands obtained from single female cultures and multiple specimen cultures in any of the isolates. Similarities among the seven isolates of *P. vulnus* were detected, as well as some polymorphism. Very few bands of *P. neglectus* were shared by any population of *P. vulnus*. The reactions with the six primers used produced 59 scorable bands. Only the bands present in three replicated reactions were scored, and nonreproducible bands were not considered. The data of similarity among the isolates are presented in a similarity matrix in Table 4. *Pratylenchus neglectus* showed a very low (0.0 to 0.06) similarity with *P. vulnus* isolates. On the other hand, the group of *P. vulnus* is diverse enough to allow the separation of all of them with the six primers used. The similarity was higher than 0.86 among

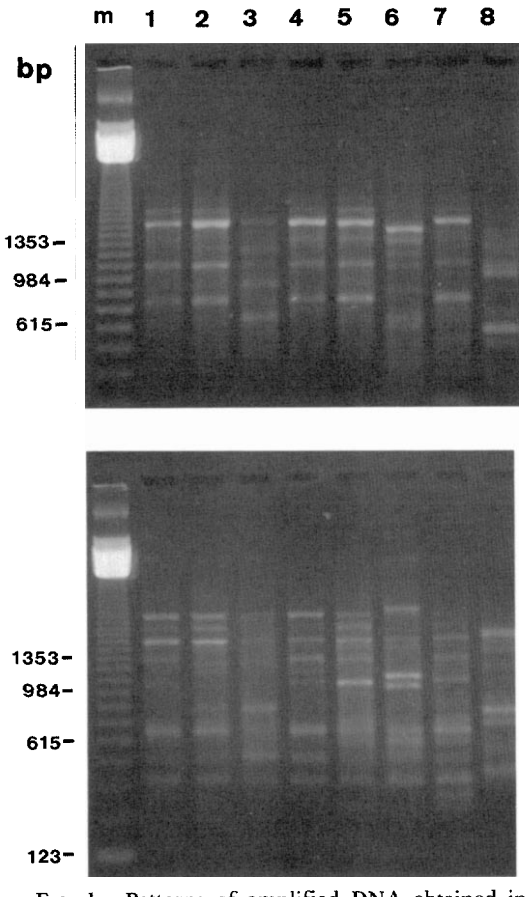

FIG. 1. Patterns of amplified DNA obtained in RAPD-PCR reactions with eight isolates of *Pratylenchus*. Lane m: Size marker, 123 bp ladder. Lanes 1–7, *P. vulnus*, isolates PvU-UK, PvAP-S, PvAT-F, PvRO-S, PvWA-U, PvWA-A, and PvOL-I, respectively. Lane 8, *P. neglectus*, isolate PnWA-U. Top panel, reactions made with primer OPB-7. Bottom panel, primer OPB-17.

four populations: PvU-UK, PvRO-S, PvAP-S, and PvWA-U. The most dissimilar isolate was PvAT-F, which had similarity values from 0.51 to 0.57 when compared with the rest of the *P. vulnus* isolates.

DISCUSSION

This study describes a range of host reactions in plum, sour orange, and quince among six isolates of *P. vulnus*. Some *P. vulnus* isolates readily attacked citrus (sour orange), whereas others were incapable of reproducing on this plant. This could explain discrepancies in the literature on the host-status of *P. vulnus* on several citrus species (12,10,23). Since races are mainly based on plant host response (2,30), two distinct races can be clearly distinguished among *P. vulnus* populations. The PvAP-S, PvU-UK, and PvWA-A isolates are citrus attacking forms, whereas PvWA-U, PvAT-F, and PvRO-S are nonattacking forms. Although a few specimens (mainly males) were detected in roots of two sour orange plants inoculated with PvAT-F, there is no evidence that it reproduced on sour orange. Final population (35 nematodes) was considerably lower than initial population, and these nematodes were probably survivors from the initial inoculum. Therefore, this isolate is considered a nonattacking form on citrus. When comparing in vitro with in vivo reproductive fitness, isolate PvRO-S showed the highest level of fitness on both carrot and plum of the isolates tested. The PvWA-A isolate showed a similar pattern on these hosts, and was also the fittest on sour orange.

Results obtained with RAPD-PCR analysis show its potential usefulness in the taxonomy of *Pratylenchus* spp. The patterns of amplified DNA bands of the *P. neglectus* isolate were clearly different from those of the *P. vulnus* isolates. This fact confirms the good resolution at the species level of the technique, which has also been reported with other nematode genera in previous studies (3,4). Comparison of populations of *Pratylenchus brachyurus* (Godfrey) Filipjev & Schuurmans Stekhoven has been attempted by isozyme analysis (21). Although *P. brachyurus* and *P. scribneri* Steiner were easily distinguished, only three enzymatic systems from the 18 studied provided useful polymorphisms. In contrast, the number of primers able to produce polymorphic markers in RAPD reactions is virtually unlimited. Another advantage of the method is its relative simplicity of operation and the small amount of material needed to perform the reaction. A single individual can be enough for a few reactions, although this was not performed in the present study. For the cited reasons, the RAPD techniques is a good alternative to the biochemical methods for nematode identification, as well as for detecting genetic variability within the spe-

TABLE 4. Nei and Li's similarity coefficients of seven isolates of *Pratylenchus vulnus* and one of *P. neglectus* from different hosts and geographic origin.

Isolate	PvU-UK	PvAP-S	PvAT-F	PvRO-S	PvWA-U	PvWA-A	PvOL-I	PnWA-U
PvU-UK	1.0							
PvAP-S	0.93	1.0						
PvAT-F	0.57	0.56	1.0					
PvRO-S	0.96	0.90	0.55	1.0				
PvWA-U	0.92	0.86	0.55	0.88	1.0			
PvWA-A	0.68	0.67	0.51	0.65	0.65	1.0		
PvOL-I	0.70	0.63	0.56	0.67	0.71	0.63	1.0	
PnWA-U	0.05	0.05	0.0	0.05	0.10	0.0	0.0	1.0

Coefficients were based on 59 scorable RAPD bands obtained with six random primers (OP B-1, B-4, B-7, B-12, B-16, and B-17).

cies. On the negative side, the technique lacks reproducibility in the weak stained bands, thus requiring three or four replicates for every reaction in order to determine which are the reproducible bands.

Abundant polymorphism allowed the characterization of each isolate used in the study. This indicates that considerable intraspecific variation exists in *P. vulnus,* and indeed similarities ranged from 0.51 to 0.96. However, the grouping of populations based on similarity of bands did not correlate with the grouping based on host preference. A similar situation has also been noted in *Meloidogyne* spp., in which four races of *M. incognita,* established on a host preference differential test, proved to be undistinguishible by RAPD markers (4). It could be that the genetics of host preference is based on one or very few genes; therefore, its detection would require the use of a wider set of primers. Although the RAPD similarity has been used in other studies as an aid to define geographic relatedness among nematode populations and the history of introductions (3), the present RAPD data grouping did not correlate well with the geographical origin of the *P. vulnus* isolates. This is not unusual since genetic diversity and geographic separation can be obscured by the intensive movement of infected plant material through different continents, which is likely to be the case with *P. vulnus.*

The identical patterns obtained between single female descendencies and multiple specimen descendencies from isolates of the same origin indicate that there was no contamination from other *Pratylenchus* species, confirming the genetic purity of each isolate. This control over each isolate was necessary because of frequent contamination with other members of the genus from soil and root samples, which require several culture transfers to achieve a pure culture.

Pratylenchus vulnus is an important pathogen of fruit tree crops in many parts of the world. The search for new sources of resistance, especially on *Prunus* (wild species) is a priority that will require a long-term effort to identify these sources, acquire the knowledge on inheritance of nematode resistance, and transmit this character into new commercial rootstocks. The existence of different races, as well as pathogenic forms, would make a breeding and selection process even more complex (26) and underlines the importance of knowing as much as possible about the existing variability of the nematode pathogen against which one is trying to breed. Although no correlation could be established between the genetic variability of *P. vulnus* using RAPD-PCR and reproductive fitness, our findings demonstrate the genetic diversity of this species, up to now unknown, and the possible practical implications that should be taken into account for successfully breeding rootstocks against *P. vulnus.*

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