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Intraspecific diversity within avocado field isolates of *Rosellinia necatrix* from south-east Spain

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Abstract Fifty-five isolates of *Rosellinia necatrix*, the cause of common avocado white root rot disease, were collected from south-east Spain and characterised according to their virulence behaviour and their molecular patterns to assess broader levels of genetic diversity. Virulence properties were revealed by in vitro inoculation on avocado plants. Differences in reaction types showed variability among these isolates. No sequence differences were observed when the internal transcribed spacer 1 (ITS1) and ITS2 regions and DNA fragments of the β-tubulin, adenosine triphosphatase and translation elongation factor 1 genes were explored in representive isolates from five virulence groups. Random amplified polymorphic DNA (RAPD) amplifications were also performed for each isolate using 19 random primers. Four of these primers revealed polymorphism among isolates and repetitive and discriminative bands were used to build an unweighted pair group with arithmetic mean tree. However, RAPD clustering showed low stability, and no correlation between RAPD and virulence groups was observed, possibly indicating high levels of sexual recombination.

Keywords *Persea americana*RAPD-PCR White root rot

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Rosellinia necatrix (teleomorph: *Dematophora necatrix*) is a mitotic soilborne ascomycetous fungus that causes white root rot on a wide range of plant species (Khan 1959). Avocado plantations were established in the coastal area of south-east Spain in the late 1960s; white root rot of the crop is considered an epidemic in this zone at the present time (Pérez-Jiménez et al. 2002). Infection by the pathogen causes wilting, chlorosis, and die-back which can occur within a few weeks (López-Herrera et al. 1998).

Effective control of this disease is primarily based on using disease-free plants in a pathogen-free soil (Schena et al. 2002). Soil solarization has been employed to control the disease in Spanish orchards (López-Herrera et al. 1999). Recently, successful chemical control has been achieved in avocado greenhouses with the contact fungicide fluazinam (López-Herrera and Zea-Bonilla 2007).

In the present study we used different markers to examine molecular polymorphisms among Spanish isolates of *R. necatrix* from different avocado growing areas and to assess broader levels of genetic diversity in order to design strategies for disease management. Firstly, 55 isolates were collected from avocado orchards within 250 km of the Mediterranean coastal avocado-growing area of south-east Spain over a

period of 13 years, from 1988 to 2001. Isolates were taken from affected trees arbitrarily sampled in every disease patch found in an orchard during the surveys. Small pieces of roots with white root rot symptoms were disinfested in 2% NaOCl for 3 min, dried between sterile filter papers and plated onto potato dextrose agar medium (potato dextrose agar (PDA), Difco, Detroit, MI, USA). Cultures were maintained on PDA at 25°C in darkness.

Initially, the pathogenicity of the 55 isolates was tested on avocado plants obtained by in vitro embryo culture from seeds of cv. Topa-Topa (Pliego-Alfaro 1988), according to the method described by Sztejnberg and Madar (1980). Five replicated pots were prepared per treatment. During the development of the experiment, daily readings of aerial symptoms of the plants were carried out according to a scale of 1=healthy plant to 5=dead plant. Data were analysed by analysis of variance. Means of severity symptoms at 15 days after inoculation were compared according to least significant difference tests. Five different virulence groups were established among the 55 *R. necatrix* isolates based on the average avocado disease severity 15 days after inoculation (Table 1).

In this work, we attempted to correlate disease virulence and genetic diversity. Firstly, we explored the genetic variability in the ITS1 and ITS2 regions and DNA fragments of the β-tubulin, adenosine triphosphatase (ATPase) and translation elongation factor 1 (*tef1*) genes from 14 (ITS1), 9 (ITS2), 12 (β-tubulin and ATPase) and 8 (*tef1*) isolates representing the virulence groups based on pathogenicity tests. The primers used for amplification and sequencing had been previously described for analysing genetic variability in fungi (Glass and Donaldson 1995; Kullnig-Gradinger et al. 2002; White et al. 1990). Polymorphism was not observed among isolates after sequencing analysis of 197 bp of the ITS1 region (isolates 12, 16, 17, 31, 51, 68, 96, 100, 119, 201, 268, 289, 290 and 320), 165 bp of the ITS2 region (isolates 12, 17, 51, 68, 72, 100, 109, 246, 247 and 269), 355 pb of a β-tubulin fragment (isolates 12, 16, 17, 31, 51, 68, 72, 119, 197, 201, 247 and 320) and 150 bp of a *tef1* fragment (isolates 12, 17, 51, 68, 106, 107, 109 and 117). The 1-kb fragment of the ATPase gene showed, on agarose gels, identical restriction patterns for the ten selected isolates analysed (isolates 12, 16, 17, 31, 51, 68, 72, 119, 197, 201, 247 and 320), after digestion with *HaeIII* or *TaqI* enzymes.

We applied the random amplified polymorphic DNA (RAPD) fingerprinting technique in 58 *R. necatrix* isolates from avocado. The 55 Spanish isolates tested for pathogenicity and three additional strains also isolated from avocado were included: the Spanish strain *R. necatrix* 123 from [Spanish Type Culture Collection (Valencia, Spain), Coleccion Española de Cultivos Tipo 2817], and strains *R. necatrix* 122 and *R. necatrix* 199 from Israel and Mexico, respectively. Nineteen arbitrary decamers (OPF1–OPF9, OPF12, POF14-OPF20, OPC10, OPC13 and OPC16; Operon Technologies) were tested for reproducible RAPD patterns. Polymerase chain reaction (PCR) reactions were carried out in a total volume of 50 µl containing 25 ng of genomic DNA, 0.36 µM concentration of primer, 100 µM of each deoxyribonucleotide triphosphate and 2 U of *Taq* polymerase (Biotools) in 1X standard buffer with 2 mM $MgCl_2$. The amplification programme used was 35 cycles of 5 s at 93°C, 36 s at 36°C and 1 min at 72°C. PCR amplification products were visualized on 1.2% agarose gels.

Four primers, OPC10, OPC13, OPF3 and OPF12, gave polymorphic fragments among the 58 *R. necatrix* isolates. Comparison of the profiles obtained with the selected primers was done on the basis of the presence versus absence (1/0) of all, weak and strong,

Table 1 Pathogenicity of 55 avocado *Rosellinia necatrix* isolates tested in avocado plants

Severity index (1–5)	Isolates
5.0 a	320
4–4.6 a, b	11,13, 31, 70, 100, 107, 110, 117, 119, 200, 201, 202, 203, 204, 205, 246, 247, 268, 269, 284, 289, 290
3–3.8 b, c	10, 15, 16, 18, 19, 32, 48, 51, 52, 67, 68, 69, 71, 96, 98, 99, 106, 108, 109, 111, 116, 118, 244, 245, 285
2–2.8 c, d	17, 30, 33, 50, 97
1–1.6 d	12, 49

Intervals followed by the same letter do not differ significantly (Fisher's test, 95%)

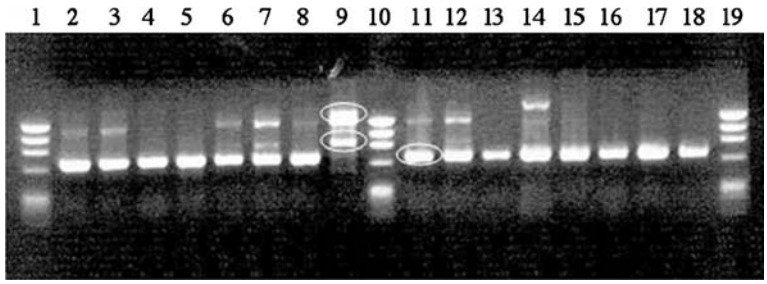

Fig. 1 Random amplified polymorphic DNA profiles of 16 *Rosellinia necatrix* isolates (15 isolates representing five virulence groups and a Mexican isolate, *R. necatrix* 199) obtained by using primer OPF3. Lanes 1, 10 and 19 show a DNA marker (ϕX174*HaeIII*, Promega). Lanes numbered 2 to 9 and 11 to 18 represent isolates 10, 31, 19, 48, 71, 117, 269, 199, 12, 17, 33, 49, 96, 109, 200 and 320, respectively. The scored bands are indicated by a circle

RAPD products of the same size. A total of 43 DNA fragments, showing polymorphism among isolates, were amplified by the four primers indicated above. As an example, amplification products generated with primer OPF3 in 16 isolates (15 Spanish isolates, representing five virulence groups, and the Mexican isolate, *R. necatrix* 199) are shown in Fig. 1. To confirm the repeatability of the 43 RAPD bands, 19 isolates were retested. New biomass production and DNA extraction and amplification were carried out. Significant strong RAPD bands were always repetitive and showed low discrimination between the 19 isolates retested. As an example, the 700-bp band (Fig. 2) was repetitive with primer OPF3. As a result, 15 RAPD bands were dropped from the analysis and the number of scored DNA fragments generated by individual primers was: five (OPF3), five (OPC13), eight (OPF12) and ten (OPC10). Genetic similarity and grouping divergence among 58 isolates of *R. necatrix* across the 28 scored loci were examined using the phylogenetic software package Tool for Population Genetic Analyses version 1.3. The algorithm for genetic distance described by Nei (1987) was used and a dendrogram based on the unweighted pair group method with arithmetic mean (UPGMA) was generated from 1,000 bootstrap replicates.

The UPGMA dendrogram is shown in Fig. 3 and no relationship was observed between the genetic distance of each isolate and its location in a pathogenicity group. Neither was a correlation found among the genetic distance of each isolate and locations from where they were isolated. Although RAPD clusters observed after UPGMA analysis showed low stability, three groups were identified at a genetic distance of 0.40. The major group contained most of the 58 isolates, including 51 Spanish isolates and the isolate *R. necatrix* 122 from Israel. A second group contained five isolates. Finally, the Mexican isolate, *R. necatrix* 199, was the furthest located, supported by a 100% bootstrap value. However, the major and second groups displayed bootstrap values <50%, and so they may not be biologically significant. Two additional *R. necatrix* apple isolates from Israel (data not shown) were also located in the major group with most of our avocado isolates, but the RAPD groups did not correlate with the host tree where isolates were obtained. These results agree with Kiyoshi et al. (1998) who observed no relationship between the

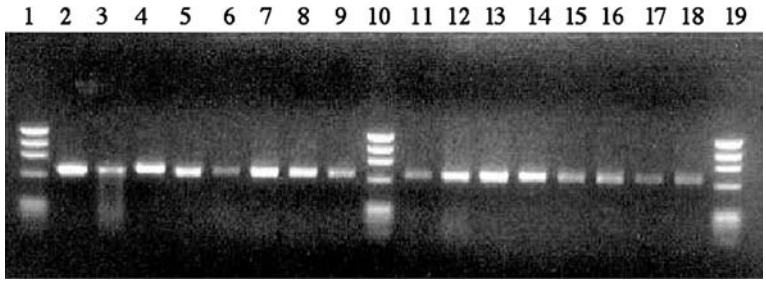

Fig. 2 RAPD repeated test. Amplification patterns obtained from newly produced biomass of 16 *Rosellinia necatrix* isolates using primer OPF3. Lanes 1, 10 and 19 show a DNA marker (ϕX174*HaeIII*, Promega). Lanes numbered 2 to 9 and 11 to 18 represent isolates 320, 70, 107, 110, 117, 118, 68, 69, 96, 109, 118, 17, 33, 97, 12 and 49, respectively

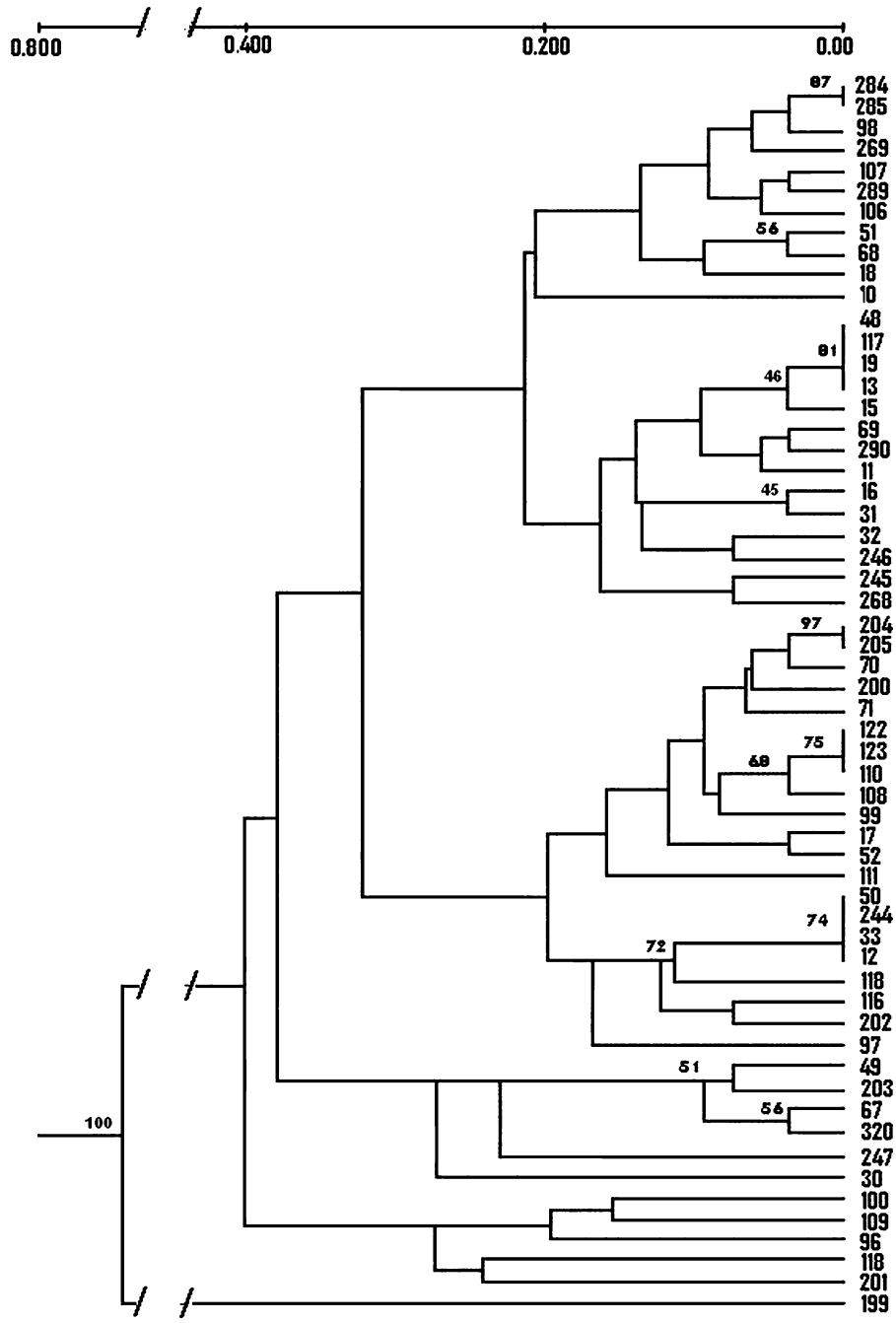

Fig. 3 Dendrogram of genetic distance among 58 avocado *Rosellinia necatrix* isolates generated using UPGMA with bootstrap of 1,000 replicates of 28 RAPD markers obtained with four arbitrary decamer primers. The 58 *R. necatrix* isolates are as follows: the 55 Spanish isolates tested for pathogenicity (Table 1), the Spanish strain *R. necatrix* 123 (CECT 2817), and strains *R. necatrix* 122 and *R. necatrix* 199 from Israel and Mexico, respectively. Bootstrap values above >50% are indicated

genetical distance of each *R. necatrix* strain and the plants or places of isolation. The existence of significant but minor variability in the Spanish collection of *R. necatrix* isolates detected in this RAPD study may be due to the meiotic recombination detected in southeast Spain avocado root systems where *Dematophora*-state ascospores have been isolated.

It can be concluded that using DNA sequencing of the typical genes commonly applied in fungal taxonomy, all *R. necatrix* isolates collected from Spanish avocados did not show molecular polymorphism. After the RAPD study, the genetic diversity found in the same collection of *R. necatrix* was moderately low and no correlation with pathogenicity was detected.

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