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Assessing genetic diversity in the web blight pathogen *Thanatephorus cucumeris* (anamorph = *Rhizoctonia solani*) subgroups AG-1-IE and AG-1-IF with molecular markers

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Abstract

Web blight, an important foliar disease of dry beans in the Americas, is a challenge to manage. We studied genetic variation of 92 isolates of *Rhizoctonia solani* subgroups AG-1-IE and AG-1-IF using DNA fingerprinting methods and mycelial compatibility grouping. The isolates were collected over 13 years from bean fields in the Dominican Republic, Honduras and Puerto Rico. Cluster and AMOVA analysis of combined data from two universal rice primers and two internal sequence repeats revealed significant genetic variation among and within populations of both subgroups. Variation was influenced by geographic origin and sampling year for AG-1-IE isolates and geographic origin for AG-1-IF isolates. Mycelial compatibility of paired isolates was mostly scored as incompatible in both subgroups and supported many unique phenotypes. Only two isolates of AG-1-IE displayed mycelial compatibility and DNA fingerprints, suggesting clonal origin. Genetic variation in these AG-1-IE and AG-1-IF isolate populations may explain the lack of durable resistance to web blight reported in dry beans.

Keywords: *Phaseolus vulgaris*, DNA markers, DNA polymorphism, anastomosis groups, population genetics

Introduction

Web blight (WB), caused by aerial isolates of *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris*

Frank Donk), is an economically important disease of dry edible beans (*Phaseolus vulgaris* L.) grown in the humid tropical highlands and lowlands of Latin America and the Caribbean (LAC) (Gálvez et al. 1989). The disease causes millions of dollars in yield losses due to destruction of leaves or seed blemishes that reduce value (Godoy-Lutz et al. 1996). Over the past decades, the disease has spread within the LAC region and recently to countries in Africa and South America, especially Ecuador (Masangano and Miles 2004; Wortman 2006; JS Beaver, Univ. Puerto Rico, personal communication). The disease is spread by mycelial bridges between plants, rain-splashed sclerotia, infested soil debris (Gálvez et al. 1989) and airborne basidiospores (Cardenas-Alonso 1989; Echandi 1965). Deployment of resistant cultivars is the most economical management option; however, there are no available commercial varieties with WB resistance. Other management options include cultural practices such as minimum tillage and the use of costly fungicides, neither of which are highly effective, and chemical use can have negative environmental concerns (Beaver and Osorio 2009; Godoy-Lutz et al. 2008).

As a species, *R. solani* is composed of a large complex assemblage of anastomosis groups (AGs) that are genetically distinct nonmating populations showing a wide diversity in morphology, pathogenicity and physiology

(Kuninaga et al. 1997; Ogoshi 1987; Sneh et al. 1991). Isolates of *R. solani* can exist simultaneously as bi- or multinucleate homokaryons or heterokaryons possessing either homothallic (asexual), bipolar or heterothallic mating systems resulting in a variety of mechanisms for generating genetic diversity within AGs (Adams 1996; Cubeta and Vilgalys 1997). Web blight is mostly caused by five genetically different subgroups: AG-1-IA, AG-1-IB, AG-1-IE, AG-1-IF and AG-2-2WB (Godoy-Lutz et al. 2008). To a lesser extent, other isolates of AG-4 and AG-2-2 IV have been associated with WB symptoms (Carling et al. 2002; Gálvez et al. 1989). Isolates of AG-1-IE and AG-1-IF are the most widespread and collectively more virulent than other subgroups causing WB in the LAC region and have overcome cultivars with partial resistance (Beaver et al. 2008; Godoy-Lutz et al. 1996, 2000). Isolates of AG-1-IE and -IF produce abundant mycelia and sclerotia under field conditions that could contribute to the spread of clonal isolates through soil, water and contaminated symptomless seed to distant locations (Cardenas-Alonso 1989; Gálvez et al. 1989; Godoy-Lutz et al. 1996, 2003).

Identifying the genetic structure of pathogen populations will provide a basis to determine characteristics and the evolutionary processes that shaped those populations in agroecosystems (McDonald and Linde 2002).

Molecular biology has provided techniques that exploit the naturally occurring variation in DNA for identifying strains and clonal lineages within a species and for discriminating and classifying populations at different taxonomic levels (Xu 2006). Molecular approaches have been used to establish that AGs and other subgroups are genetically isolated and represent highly divergent evolutionary units (Cubeta and Vilgalys 1997; Rosewich et al. 1999).

Despite the economic importance of the WB pathogen, there is no published information regarding bean-infecting populations of AG-1-IE and AG-1-IF, including the distribution of genetic diversity within and among their populations and how their reproduction mode may have influenced their population fitness. Isolates within each of these subgroups are morphologically and culturally indistinguishable regardless of the country of origin. When the ITS 5.8S rDNA region was used to characterize 68 WB isolates, little or no polymorphism was detected among isolates from geographically distant locations; thus, field populations of these subgroups appear to be genetically homogenous (Godoy-Lutz et al. 2008). However, no attempts have been made to determine the extent of intra-population variability in *R. solani* AG-1-IE and AG-1-IF. DNA fingerprints generated by dominant neutral molecular markers have been employed for initial assessment of genetic diversity and population structure for AG-2, AG-3, AG-4, AG-8, AG-9 and AG-1-IB (Ceresini et al. 2002; Duncan et al. 1993; Grosch et al. 2011; Justesen

et al. 2003; Meinhardt et al. 2002; Yang et al. 1995). A comprehensive review of molecular methods used for characterization and classification of *Rhizoctonia* spp. has been published by Sharon et al. (2006). Other dominant molecular markers that have been used in recent fingerprinting studies for *R. solani* are universal rice primers (URP) (Kang et al. 2002) and inter simple sequence repeats (ISSR) (Gupta et al. 1994). Despite some limitations inherent with their dominant nature, the aforementioned markers have been extensively used for eukaryotic organisms because they are cost effective, highly reproducible, have multiple polymorphic loci, are appropriate for closely related individuals and no prior knowledge of the genome is needed for their use (Gupta et al. 1994; Nybom 2004; Sharma et al. 2005; Xu 2006). DNA fingerprinting complemented with another independent criteria such as mycelial compatibility pattern has been suggested for providing preliminary information on mating system and genetic structure of *R. solani* (Cubeta and Vilgalys 1997; Vilgalys and Cubeta 1994). The clonal composition, defined as where two or more isolates are somatically compatible and share the same DNA marker phenotype, has been examined in field populations of AG-3 and AG-1-IA (Campos and Ceresini 2006; Ceresini et al. 2002).

The objective of this study was to determine genetic structure of populations of *R. solani* isolates within subgroups AG-1-IE and AG-1-IF, collected from Honduras, Puerto Rico and the Dominican Republic over 13 years based on DNA fingerprinting and mycelial compatibility.

Materials and methods

Isolate collection and DNA extraction

The 92 multinucleate isolates examined in this study originated from web blight symptomatic leaves of individual *P. vulgaris* plants collected over 13 years and stored at the University of Nebraska-Lincoln. Isolates were collected in Honduras (37 isolates), Dominican Republic (15 isolates) and Puerto Rico (40 isolates) (Table 1) from locations where breeders screen lines for WB resistance and the disease is endemic. Isolates were stored in sugar beet seeds at 4°C and were activated by plating on water agar (WA) and transferring to Difco potato dextrose agar (PDA) after 48 h. Three to four days later (before isolates began producing sclerotia), five agar plugs from the edge of the growing mycelia were introduced into 50 ml of sterile V-8 liquid medium (1.5 g CaCO₃ + 100 ml V-8 juice + 400 ml of de-mineralized sterile water) in a deep Petri dish and were grown at 25°C without shaking under constant light until a mat of mycelia reached the edges of the plate and before sclerotia developed. Mycelial mats were harvested by

Table 1. Isolates of *Rhizoctonia solani* from *Phaseolus vulgaris* examined in this study

AG-subgroup	Isolate code	Origin	Collector	Year	GenBank accession
AG-1-IA	TC751	Zamorano, HON	J.R. Steadman	2007	JF946720
AG-1-IE	H1	Jamastran, HON	F. Saladin	1994	NS
AG-1-IE	H2	Las Acacias, HON	F. Saladin	1994	NS
AG-1-IE	H14	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H17	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H18	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H20	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H22	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H23	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H24	Jamastran, HON	N. Escoto	1995	NS
AG-1-IE	H25	Jamastran, HON	N. Escoto	1995	JF946725
AG-1-IE	Jamastran	Jamastran, HON	J. Venegas	2005	DQ447860
AG-1-IE	J2	Jamastran, HON	J. Venegas	2005	JF946722
AG-1-IE	LosLimones	Los Limones, HON	J. Venegas	2005	DQ447861
AG-1-IE	L2	Los Limones, HON	J. Venegas	2005	JF946719
AG-1-IE	PR5	La Isabela, PR	J. Beaver	1995	AF308629
AG-1-IE	PR11	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR19	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR20	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR21	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR25	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR27	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR28	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR33	La Isabela, PR	J. Beaver	1995	JF946724
AG-1-IE	PR34	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR36	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR39	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR42	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR43	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR45	La Isabela, PR	J. Beaver	1995	DQ447877
AG-1-IE	PR48	La Isabela, PR	J. Beaver	1995	JF946725
AG-1-IE	PR49	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR50	La Isabela, PR	J. Beaver	1995	NS
AG-1-IE	PR0632	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0633	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0634	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0635	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0636	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0637	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0638	La Isabela, PR	G. Godoy-Lutz	2006	JF946733
AG-1-IE	PR0639	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0640	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0641	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0642	La Isabela, PR	G. Godoy-Lutz	2006	JF946735
AG-1-IE	PR0643	La Isabela, PR	G. Godoy-Lutz	2006	JF946728
AG-1-IE	PR0644	La Isabela, PR	G. Godoy-Lutz	2006	NS

(continued)

Table 1. Isolates of *Rhizoctonia solani* from *Phaseolus vulgaris* examined in this study (continued)

AG-subgroup	Isolate code	Origin	Collector	Year	GenBank accession
AG-1-IE	PR0645	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0646	La Isabela, PR	G. Godoy-Lutz	2006	JF946723
AG-1-IE	PR0647	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0648	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0649	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PR0650	La Isabela, PR	G. Godoy-Lutz	2006	NS
AG-1-IE	PRRS12	Aguado, PR	R. Echavez-Badel	1996	DQ447862
AG-1-IE	Sabaneta	Sabaneta, HON	J. Venegas	2005	JF946734
AG-1-IE	TC752	Zamorano, HON	J.R. Steadman	2007	NS
AG-1-IE	TC753	Zamorano, HON	J.R. Steadman	2007	JF946721
AG-1-IE	TC756	Zamorano, HON	J.R. Steadman	2007	NS
AG-1-IE	TC757	Zamorano, HON	J.R. Steadman	2007	NS
AG-1-IE	TC758	Zamorano, HON	J.R. Steadman	2007	NS
AG-1-IE	TC759	Zamorano, HON	J.R. Steadman	2007	NS
AG-1-IF	DRBV1	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV3	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV5	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV6	San Juan Valley, DR	G. Godoy-Lutz	1994	JF946729
AG-1-IF	DRBV7	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV8	San Juan Valley, DR	G. Godoy-Lutz	1994	DQ447882
AG-1-IF	DRBV22	San Juan Valley, DR	G. Godoy-Lutz	1994	AF308626
AG-1-IF	DRBV24	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV32	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV35	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV36	San Juan Valley, DR	G. Godoy-Lutz	1994	JF946730
AG-1-IF	DRBV40	San Juan Valley, DR	G. Godoy-Lutz	1994	DQ447878
AG-1-IF	DRBV42	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV49	San Juan Valley, DR	G. Godoy-Lutz	1994	NS
AG-1-IF	DRBV98	San Juan Valley, DR	G. Godoy-Lutz	1994	DQ447883
AG-1-IF	H26	Jamastran, HON	N. Escoto	1995	NS
AG-1-IF	H27	Jamastran, HON	N. Escoto	1995	JF946731
AG-1-IF	H28	El Barro, HON	N. Escoto	1995	NS
AG-1-IF	H29	El Barro, HON	N. Escoto	1995	JF946732
AG-1-IF	H31	El Barro, HON	N. Escoto	1995	NS
AG-1-IF	H32	El Barro, HON	N. Escoto	1995	AF308627
AG-1-IF	H33	El Barro, HON	N. Escoto	1995	DQ447880
AG-1-IF	H34	El Barro, HON	N. Escoto	1995	DQ447881
AG-1-IF	H36	El Barro, HON	N. Escoto	1995	NS
AG-1-IF	H37	El Barro, HON	N. Escoto	1995	NS
AG-1-IF	H38	El Barro, HON	N. Escoto	1995	DQ447879
AG-1-IF	H40	El Barro, HON	N. Escoto	1995	NS
AG-1-IF	PR064	La Isabela, PR	G. Godoy-Lutz	2006	JF946726
AG-1-IF	PR0671	La Isabela, PR	G. Godoy-Lutz	2006	JF946727
AG-4	TC754A	Zamorano, HON	J.R. Steadman	2007	NS
AG-4	TC754B	Zamorano, HON	J.R. Steadman	2007	NS
AG-4	TC755	Zamorano, HON	J.R. Steadman	2007	NS

HON Honduras, PR Puerto Rico, DR Dominican Republic, NS isolate not sequenced

filtration and stored at -20°C , and 50 mg portions were used for genomic DNA extractions. A commercial DNA extraction kit, UltraClean Plant DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used to extract DNA and eliminate culture pigments absorbed by mycelia during growth.

Determination of subgrouping by specific primers

Assuming that most isolates belonged to AG-1 based on previous classification by Godoy-Lutz et al. (2008), initial PCR amplification was performed with primers WB-A (for determination of AG-1-1E) and WB-B (for AG-1-1F). A few isolates that did not amplify were tested with other primers suggested by Carling et al. (2002) and Godoy-Lutz et al. (2008). PCR amplifications were performed by adding 1.0 μl DNA (80–100 ng), 5 μl of $10\times$ *Taq* DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM MgCl_2 and 0.1% (v/v) Triton X-100 when diluted 1:10), 4 μl of 1.25 mM of each dNTP, 0.8 μl of each 20 μM primers (forward and reverse) (Invitrogen, Carlsbad, CA, USA) and 0.4 μl of *Taq* DNA polymerase (5 U/ μl) (Promega, Madison, WI, USA) to a 50 μl reaction mixture prepared with sterile nuclease free water. Amplifications were performed in a PTC-100 Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: 1 cycle of 94°C for 2 min; followed by 30 cycles of 94°C for 40 s, $53\text{--}62^{\circ}\text{C}$ (depending upon the primer) for 1 min and 72°C for 1 min; completed by a final extension at 72°C for 5 min.

DNA polymorphism analysis

Isolates were tested with 10 markers that have been reported to be polymorphic and reliable in characterizing genetic diversity in *R. solani* (Sharma et al. 2005; Zala et al. 2008). Only four molecular markers, URP: URP2R (5'-CCCAGCAACTGATCGCACAC-3') and URP6R (5'-GCAAGCTGGTGGGAGGTAC-3') (Sharma et al. 2005), and ISSR: ISSR10 (5'-CACCACCACCAC-CAC-3') (Sharma et al. 2005) and (GACA) $_4$ (Ureña-Padilla et al. 2002), were informative for assessing diversity. Other markers that were tested but either did not amplify or were not polymorphic for most isolates included (TGTC) $_4$ (Ureña-Padilla et al. 2002), (GTG) $_5$ (Longato and Bonfante 1997; Meinhardt et al. 2002), URP13R (5'-TACATCGCAAGTGACACAGG-3'), URP17R (5'-AATGTGGGCAAGCTGGTGGT-3'), ISSR2 (5'-ACT-GACTGACTGACTG-3') (Sharma et al. 2005) and TC06 (CAG) $_{11}$ (5'-CAGAGATACGTCCAGCAACG-3') (Zala et al. 2008). Attempts to modify amplification conditions such as adjusting DNA quantity and concentrations of *Taq* DNA polymerase, MgCl_2 or dNTPs were unsuccessful, and these markers were not used in characterizing isolates. PCR amplifications for URP2R,

URP6R, ISSR10, and (GACA) $_4$ were performed by adding 3.0 μl of DNA (<250 ng) to 25 μl of PCR Master Mix 2X (50 $\mu\text{l}/\text{ml}$ *Taq* DNA polymerase in reaction buffer pH 8.5, 400 μM each dATP, dGTP, dCTP and dTTP, 3 mM MgCl_2) (Promega), 1.5 μl of 20 μM primers (forward and reverse) (Invitrogen) and sterile nuclease free water up to 50 μl . Amplification conditions for each marker, performed in a PTC-100 Thermal cycler (Bio-Rad), were as follows: (GACA) $_4$ —1 cycle of 95°C for 5 min; followed by 34 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 1.5 min; completed by a final extension at 72°C for 10 min; URP2R and URP6R—1 cycle of 94°C for 4 min; followed by 35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min; completed by a final extension at 72°C for 10 min; ISSR10—1 cycle of 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 2 min; completed by a final extension at 72°C for 10 min.

All PCR amplification products were size-fractionated by gel electrophoresis in 1.5% (w/v) Ultra Pure Agarose (Invitrogen) in $0.5\times$ TBE buffer (0.045 mol/l Tris, 0.045 mol/l Boric acid, and 0.001 mol/l EDTA) at 100 V. A 100-bp DNA Ladder (Invitrogen) was included in each gel for standardization. Ethidium-bromide-stained amplified fragments were recorded digitally using the ChemiDoc EQ System with the Quantity One 1-D Analysis software version 4.6.2 (Bio-Rad). All amplifications were repeated twice and included a positive control (known subgroup isolate) and a negative control (no DNA).

ITS 5.8S rDNA sequence analysis

The 5.8 rDNA-ITS region of 18 isolates, in addition to 13 previously described isolates (Godoy-Lutz et al. 2008), representing AG-1 subgroups was amplified with primers ITS4 and ITS5 (White et al. 1990). The PCR amplification protocol described earlier for specific primers, except for 20 $\mu\text{mol}/\text{l}$ ITS4 and ITS5 primers (Invitrogen) were used. The amplifications were performed in a PTC-100 Thermal cycler under conditions similar to those used for subgroup specific primers, except that the annealing temperature was set at 55°C . PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and sequenced using both primer strands by an Applied Biosystems (Foster City, CA, USA) Model 3100 Genetic Analyzer at the Genomics Core Research Facility at the University of Nebraska-Lincoln. Sequence data for the complete ITS 5.8S rDNA region of the isolates was manually checked and edited to remove portions of the 18S and 28S rDNA using the Multiple Sequence Alignment of Clustal W software (Thompson et al. 1994). Sequence data was analyzed by a parsimony method with the program PAUP version 4.02 beta (Swofford 2003). Nucleotide substitutions were

equally weighted and unordered and alignment gaps were considered as missing data. Distance matrix analysis was conducted with the maximum parsimony algorithm, omitting all sites with gaps. The phylogenetic tree was rooted with isolate R9 (AG-2-2 IV, GenBank AB054863). Confidence intervals in tree topologies were estimated by bootstrap analysis with 1000 replicates. Only nodes with bootstrap values of more than 50% were considered to be significant. The final tree was visualized with the program TreeDyn (Chevenet et al. 2006). Gene sequences of other *R. solani* WB isolates were added to the group being examined. The sequences correspond to AG-1-1A [GenBank accession AF308631 (isolate Cuba 2) and DQ447859 (isolate Cuba1)], AG-1-1E [GenBank accession DQ447877 (isolate PR45), DQ447873 (isolate N1), AF308629 (isolate PR5), DQ447862 (isolate PRRs12), DQ447861 (isolate Los Limones), DQ447860 (isolate Jamastran)] and AG-1-1F [GenBank accession DQ447878 (isolate DRBV40), AF308626 (isolate DRBV22), AF308627 (isolate H32), DQ447879 (isolate H38), DQ447882 (isolate DRBV8)].

Mycelial compatibility test

The mycelial compatibility of 21 isolates of AG-1-1E and 13 isolates of AG-1-1F, chosen randomly from UPGMA tree clusters, was conducted on culture media. Each isolate was grown on PDA for 2–3 days and paired with one another in all possible combinations by placing 5 mm agar plugs taken from the edge of the growing mycelia onto PDA in a 90 mm Petri dish, spaced 5 cm apart. Plates were incubated at $21 \pm 1^\circ\text{C}$ until the mycelia from each isolate met or formed sclerotia (ca. 2 weeks). Two plates of each isolate combination were prepared. A modified rating based on Ceresini et al. (2002) was used. Mycelial interaction was scored as compatible when mycelia merged, or incompatible when a visible clear zone or a sclerotial barrier was formed in the contact zone between paired colonies.

Data analysis

Banding patterns for each marker were observed and band sizes noted. Each amplicon representing one allele was scored as either “0” for absence or “1” for presence on each gel. DNA fingerprints were converted to binary data matrices, which were used directly for comparison by two band-based similarity coefficients: Jaccard’s coefficient (1901) and Dice’s coefficient (1945), which is equivalent to the Nei–Li coefficient (1979). Cluster analysis using genetic distance was performed by the SAS/STAT Program (Anonymous 2004, Cary, NC, USA) using the UPGMA (unweighted pair grouping

by mathematical averages) clustering algorithm to generate dendrograms to connect isolates. The Mantel test for matrix correspondence was used to correlate genetic distances calculated from the URP and ISSR marker data to determine whether they could be combined further using 5000 permutations. The binomial matrix was also analyzed with the software PAST (Paleontological STatistics Version 2.01) (Hammer et al. 2001) for comparison with other similarity indexes for UPGMA and neighbor-joining cluster algorithms and visualization of dendrograms.

A nonparametric analysis of molecular variance (AMOVA) was used to determine genetic variability among populations as described by Excoffier et al. (1992). A Euclidean distance matrix generated from the binary matrix was calculated for AMOVA, and both were performed with the GenAlEx 6.3 (Peakall and Smouse 2006) software. AMOVA allows the hierarchical partitioning of genetic variation among and within populations and the estimation of variance components and Wright’s F_{ST} statistic analogs [designated as Phi (Φ) statistic] (Excoffier et al. 1992). Φ can range from 0, indicating no differentiation within the overall population and its subpopulation, to a theoretical maximum of 1, indicating complete differentiation (Peakall and Smouse 2009). Data sets for AG-1-1E and AG-1-1F were analyzed separately. For AG-1-1E, hierarchical AMV was partitioned among geographical locations, among sampling year and within isolate populations. For AG-1-1F, the variance was partitioned among geographical locations and within isolate populations. All data sets were tested using 9999 permutations.

Other statistics for measuring genetic variation with dominant markers, such as number of total and specific or unique alleles, percentage of polymorphic loci and Shannon’s index of diversity were also calculated with GenAlEx. We estimated Shannon’s index of diversity to quantify phenotypic diversity within populations as revealed by each primer. This index does not require the assumption of Hardy–Weinberg equilibrium.

Results

Determination of subgrouping based on specific primers and ITS 5.8S rDNA region

Isolates from Puerto Rico, Honduras and the Dominican Republic that were not previously assigned to an anastomosis subgroup were classified as AG-1-1E based on the size of the band at 540 bp, and AG-1-1F based on the size of band at 510 bp after being amplified with WB-A and WB-B. The anastomosis subgroup results and isolate collection data are presented in Table 1. Isolate

TC751 was assigned to subgroup AG-1-IA, and isolates TC754A, TC754B and TC755 were assigned to subgroup AG-4 on the basis of specific primer amplifications (Godoy-Lutz et al. 2008; Kuninaga et al. 1997).

Alignment of the sequences and phylogenetic tree analysis of the full ITS 5.8S rDNA region of 18 selected *R. solani* WB isolates in this study from Puerto Rico, Honduras and Dominican Republic and sequences of 12 known *R. solani* WB isolates in the GenBank (Godoy-Lutz et al. 2008) agree with the AG subgroup assignment based on subgroup-specific primer amplifications.

The sequence alignment showed 577 sites conserved and 61 parsimonious informative sites. The strict

consensus tree out of 1000 equally parsimonious trees was 186 steps long, CI 90.892 and RI 0.108 (Figure 1). Sequences of the 18 WB isolates have been deposited in the NCBI GenBank database, and the accession numbers are listed in Table 1.

Genetic variation and population structures using URP and ISSR primers

Of the 10 primers tested, only four produced unambiguous, high-intensity amplicons yielding polymorphic patterns that showed 100% repeatability. The rest of the primers either failed to generate amplicons or generated weak nonrepeatable patterns for most isolates

Figure 1. Strict consensus of 1000 equally parsimonious trees based on ITS rDNA sequence data from 30 web blight isolates of AG-1. The tree length is 186 steps, CI 0.892 and RI 0.108. The tree is rooted to isolate R9 (AG-2-2 IV). Numbers above branches indicate bootstrap percentiles from 1000 repeats using PAUP version 4 (Swofford 2003).

within both subgroups. The data obtained for each of the primers is summarized in Table 2. Unique or specific amplicons for each population within subgroups were detected. Internal simple sequence repeat primers (ISSR10 and [GACA]₄) resulted in the smallest number of bands and yielded 4–46% polymorphism, whereas universal rice primers (URP2R and URP6R) yielded 36–80% polymorphism in isolates of both subgroups. Shannon's index of diversity varied according to the primer with URP2R showing the highest index with an average of 0.30 for AG-1-IE and 0.25 for AG-1-IF. These results were reflected in the primer data dendrograms when they were generated for each primer (Supplementary Figure S1–S10).

When Mantel correlation analysis was used between matrices for both primer systems, high positive correlations ($r = 0.7509$ and 0.7534 $P < 0.001$, respectively) were obtained. This allowed data from 101 loci, from all four primers, to be combined for cluster analysis and other population estimates. UPGMA based on Jacard's distances generated the highest cophenetic correlation value of 0.9725 with a 1000 replicate bootstrap to provide the best representation of similarity (Figure 2). With a few exceptions, most isolates clustered together as either AG-1-IE or AG-1-IF supporting a population structure defined by geographic location and year of sampling or by geographic location, respectively.

AMOVA revealed significant genetic variation among and within grouping factors for each subgroup. The AG-1-IE subgroup showed significant variation due to geographic location (28%), sampling year (35%) and within isolate population (37%) (Table 3). AG-1-IF subgroups showed that 27% of the total variation was due to geographic location and 73% to within isolate population. Values of Φ above 0.25 were significant for all grouping factors in both subgroups (Table 3).

Mycelial compatibility test

When paired in culture, isolates of AG-1-IE and AG-1-IF formed either a sclerotial barrier or a clear demarcation or line in the contact zone between the paired isolate colonies. Few paired isolates scored as compatible (Figure 3). For AG-1-IE, only 5 of 210 pair combinations (excluding self pairings) from the same sampled location or sampling year displayed a compatible reaction (Supplementary Figure S11). These five isolate pairs were PR20/PR28, PR0635/PR0637, PR0635/PR0638 from Puerto Rico; Jamastran/J2 and TC756/TC757 from Honduras. Of the remaining pair combinations, 26 were incompatible and formed a clear zone or demarcation in the contact zone of the paired isolates, and 179 were incompatible and formed a sclerotial barrier in the contact zone.

Table 2. Genetic diversity estimates of populations of *R. solani* AG-1-IE and AG-1-IF in this study based on selected primers

Isolate origin, population	Primers											
	URP2R				URP6R				ISSR10			
	N	NA	NS	%P	I	N	NA	NS	%P	I	N	NA
Puerto Rico, AG-1-I	37	20	4	80	0.279 (0.05)	12	3	44	0.136 (0.04)	4	3	20
Honduras, AG-1-IE	24	18	2	68	0.323 (0.05)	10	1	36	0.162 (0.04)	5	3	16
Dominican Republic, AG-1-IF	17	13	3	44	0.247 (0.06)	12	4	44	0.158 (0.04)	8	4	32
Honduras, AG-1-IF	12	12	2	48	0.272 (0.06)	11	3	44	0.195 (0.05)	4	0	4
											NS	NA
											%P	I
											(GACA) ₄	
											0	23
											4	42
											2	34
											5	46
											0.029 (0.01)	0.145 (0.04)
											0.093 (0.02)	0.183 (0.04)

Values in parentheses represent standard deviation of the means

N = isolates/population

NA = alleles or bands/population

NS = bands unique or specific to a single population

%P = percentage of polymorphic loci, \

I = Shannon's diversity index

Figure 2. Dendrogram depicting relationships among *Rhizoctonia solani* WB isolates, generated from Jaccard's genetic distance matrix by using UPGMA cluster algorithm of PAST program (Hammer et al. 2001). Cophenetic $r = 0.9755$, bootstrap 1000 replicates.

Figure 3. Macroscopic mycelial compatibility tests between isolates of *Rhizoctonia solani* AG-1-IE (top row) and AG-1-IF (bottom row). Interactions were scored as incompatible (a, d), showing a distinct barrier of sclerotia between paired isolates; incompatible (b, e), showing clear demarcation between paired isolates; or compatible (c, f), mycelia merged with no distinct demarcation or sclerotial barrier between paired isolates.

Table 3 Analysis of molecular variance (AMOVA) of *R. solani* WB isolates of AG-1-IE and AG-1-F based on combined universal rice primers and inter simple sequence repeat primer data

Subgroups	Source of variation	df	SS	% Variation	Φ	P value
AG-1-IE	Among locations	1	114.43	28	0.281	0.000
	Among sampling years	2	94.45	35	0.490	0.000
	Within isolate populations	57	173.86	37	0.633	0.000
	Total	60	382.75			
AG-1-IF	Among locations	1	32.91	27	0.265	0.000
	Within isolate populations	27	146.13	73		
	Total	28	179.04			

P value for randomization test for Φ is based on 9999 permutations across the full data set

Similarly for AG-1-IF, 4 of 78 pair combinations from the same sampled location displayed a compatible reaction: H29/H32, H29/H33, H31/H38 and H32/H33 from Honduras (Supplementary Figure S12). Of the remaining pair combinations, 30 were incompatible and formed a clear zone or demarcation in the contact zone of the paired isolates, and 44 were incompatible and formed a sclerotial barrier in the contact zone.

Discussion

Isolates of AG-1-IE and AG-1-IF that are morphologically and culturally indistinguishable and cause web blight of dry beans, produced unique phenotypic fingerprint patterns with URP and ISSR primers. In addition, these isolates produced predominantly incompatible mycelial interactions in culture and, when

considered with the DNA fingerprint data, exhibit significant genetic variation among and within isolate populations.

The ITS 5.8S rDNA sequence of the 18 new isolates in this study (GenBank accessions JF946719 to JF946736) revealed polymorphisms that separated the AG-1 isolates into three clades representing subgroups of AG-1-IA, AG-1-IE and AG-1-IF in agreement with a previous report (Godoy-Lutz et al. 2008). Only a few isolates had a 100% match of their sequences. Most of the variability displayed within clades was associated with isolates collected from 2005 to 2007. The subgroup separation was congruent with the dendrograms generated by cluster analysis of combined marker data. The ITS rDNA region is considered appropriate for establishing phylogenetic relationships of *Rhizoctonia* AGs and subgroups within AGs (Cubeta and Vilgalys 1997; Kunita et al. 1997), and PCR primers based on highly conserved regions of the rDNA unit have been designed for diagnostic purposes for many fungi (Klich and Mullaney 2004). However, assessing population diversity of closely related species through polymorphism at the ITS 5.8S rDNA level has its limitations. It provides a relatively small amount of sequence data (ITS spacers <300 bp) with low discriminatory power, and the co-occurrence of multiple sequences present in a single individual makes its use unreliable and hinders comparative sequence analysis to answer questions at this hierarchical level (Muir and Schlötterer 1999). The occurrence of multiple sequences at the ITS1 level has been documented for AG-1 (Grosch et al. 2007), AG-3 (Justesen et al. 2003), and AG-4 (Boysen et al. 1996). For AG-1-IE and AG-1-IF, however, this phenomenon has not been observed; on the contrary, 80 and 66%, respectively, of isolate sequences have had a 100% match (Godoy-Lutz et al. 2008).

Not all of the primers used successfully with other *R. solani* subgroups amplified products or were informative for distinguishing polymorphism among the isolates used in this study. Based on the degree of polymorphism indicated by the percent of polymorphic loci generated and Shannon's index, URP2R was more suitable for studying genetic diversity in populations of both subgroups. In general, both URP and ISSR marker systems have been useful for evaluating variation in fungal populations (Aggarwal et al. 2010; Jana et al. 2005; Kiyosi et al. 2005; Sharma et al. 2005; Stodart et al. 2007). The UPGMA dendrogram showed two main clusters separating subgroups. The subclustering, with a few exceptions, was based on geographic location and year of sampling for isolates of AG-1-IE and on geographic location for isolates of AG-1-IF. Other fragmentation within subclusters, such as one isolate of each of AG-1-IA, AG-1-IE and of AG-1-IF, as well as three of AG-4 clustering within the

AG-1-IE group, cannot be explained although isolates of AG-1-IA are more closely related to AG-1-IE than to other subgroups of AG-1 (Godoy-Lutz et al. 2008; Grosch et al. 2007).

The AMOVA test rejected the null hypothesis of no genetic differentiation among the populations where $\Phi = 0$ ($F_{ST} = 0$, Wright's fixation index). The results for populations of both subgroups can be used to infer the existence of high genetic differentiation ($\Phi > 0.25$) that can result from multiple factors including reproduction strategies, mating system, gene flow, and geographic isolation (Cubeta and Vilgalys 1997). Within-isolate population variation for both subgroups was also statistically significant, which supports heterogeneous populations. A high percentage of within-isolate population variance was obtained for isolates of AG-1-IF congruent with the subclustering in the dendrogram. Isolates of this group were recently classified as a distinct population within Central and South America and the Caribbean, based on phylogenetic analysis (Grosch et al. 2007). Even though we were unable to induce sporulation under laboratory conditions, other authors have documented the development of the teleomorphic stage (basidia and basidiospores) in bean fields where AG-1 isolates are prevalent (Cardenas-Alonso 1989; Echandi 1965; Godoy-Lutz et al. 2000). Basidiospores, airborne microsclerotia and vegetative mycelia (rain-splashed locally and on seed for long distances) would contribute significantly to the temporal and spatial distribution of the pathogen (Gálvez et al. 1989). Also, the development of basidiospores by meiotic reproduction provides a mechanism for generating genetic diversity in a population (Cubeta and Vilgalys 1997). However, basidiospores are fragile and do not travel long distances (Echandi 1965).

Variation due to geographic location was significant. In a previous study, we reported that the majority of isolates within subgroups of AG-1-IE, AG-1-IF and AG-2-2 WB had identical sequences in the ITS 5.8S rDNA region even though they were collected from wild common beans and commercial dry beans representing different *Phaseolus* host gene pools in geographically separate locations (Godoy-Lutz et al. 2008). This result is very unusual considering the diploid and multinucleate nature of *R. solani*. One theory proposed is that gene flow due to seed movement of *R. solani* was involved in distributing similar genotypes among countries in the region regardless of the geographic distance. In the present study, however, the finding of significant genetic variation can be indicative of reduced gene flow that could result from geographical barriers between populations. This would be consistent with the fact that each of the three countries of isolate origin are geographically separated by water and would be unlikely to exchange bean seed due to different seed class preferences.

The host cultivar did not appear to influence the genetic variation among isolates of either subgroup (data not shown). Most of the studies on population genetics of *R. solani* or other plant pathogens using dominant markers such as RAPD, URP, AFLP or ISSR have reported that geographic location or host specificity or both had an effect on population structure (Aggarwal et al. 2010; Duncan et al. 1993; Jana et al. 2005; Sharma et al. 2005; Stodart et al. 2007). Because of the limitations of dominant markers, these studies are difficult to compare with other studies where co-dominant markers (microsatellites) were used. In dominant marker systems, homozygosity and heterozygosity are indistinguishable, meaning banding patterns of diploid individuals, such as *R. solani*, that are generated are representative of individual phenotypes rather than genotypes (Kosman and Leonard 2005). In our study, the use of cluster analysis and AMOVA enabled us to draw conclusions about within-population structure as an exploratory analysis (Bonin et al. 2007). The availability of statistical analysis methods and associated software for dominant marker data can help to circumvent the limitations associated with these markers; however, this approach needs to be carefully evaluated (Bonin et al. 2007).

In this study, most mycelial interactions of selected paired isolates were scored as incompatible. Only five pair combinations of AG-1-IE and four pair combinations of AG-1-IF were compatible and occurred between isolates collected in the same location and sampling year. A mycelial compatibility test is also termed as macroscopic vegetative interaction (MacNish et al. 1997) or somatic compatibility grouping (Ceresini et al. 2002) and can reflect anastomosis behavior at the microscopic level in some AGs (MacNish et al. 1997). Paired isolates grown on culture media are scored as compatible (equivalent to a C3 reaction in a microscopic anastomosis), when no reaction is observed in the contact zone between similar colonies such as in self pairings, or incompatible (equivalent to a C2 reaction in a microscopic anastomosis) when a visible demarcation line or raised hyphae is observed in the contact zone between colonies reflecting antagonistic responses (MacNish et al. 1997; Yang et al. 1993). The latter implies genetic and cytoplasmic isolation (Worrall 1994).

None of the isolate pair combinations scored as compatible can technically be considered clones since they do not share the same marker phenotype, with the exception of isolates PR0635 and PR0637. Therefore, the mycelial compatibility test indicates that selected field isolates of AG-1-IE and AG-1-IF examined in this study are unique phenotypes, which could support the occurrence of outcrossing events during the pathogen's life cycle. Most likely these isolates are heterokaryons because they were collected from naturally infected plants where cobweb-like mycelia that covered plants may have originated from different sources.

Our results show some similarities to studies conducted on populations of *R. solani* AG-3 from potato and tobacco hosts (Ceresini et al. 2002) and populations of AG-1-IA from soybeans (Campos and Ceresini 2006) where few or no clones (sharing both somatic compatibility and molecular marker phenotype) were identified. Also the range of incompatibility varied in intensity from "weak" clear demarcation to raised hyphae in the contact zone between paired isolates. The biological significance of the term "weak" macroscopic incompatible reaction is not known (Ceresini et al. 2002). The authors attributed the range of somatic interactions between pairings of isolates as consistent with recombination. On the other hand, Ceresini et al. (2002) attributed the lack of somatic compatibility of field isolates of AG-3 to multiple loci and/or multiple alleles controlling somatic compatibility within the subgroup. Somatic compatibility in *Rhizoctonia* species is still not well understood.

Future work on genetic variation of the WB pathogen will require more structured sampling of isolates of various subgroups from different fields and countries within a geographic region where seed exchange commonly occurs. The development of co-dominant microsatellite markers would facilitate studies attempting to understand population structure and dynamics. Recently, the population structure of *R. solani* subgroup AG-1-IA, causal agent of sheath blight of rice and other blights of maize and soybean from South America, United States, China and India has been studied by employing subgroup specific DNA co-dominant microsatellite simple sequence repeat (SSR) markers (Bernardes-de-Assis et al. 2009; Ciampi et al. 2008; González-Vera et al. 2010). Host specialization was shown to affect selection and the pattern of populations with different reproductive modes, leading the authors to propose a recombining reproductive mode for maize-infecting populations and a mixed reproductive mode (asexual and sexual) for the rice-infecting populations that includes recombination events followed by clonal expansion during the growing season (González-Vera et al. 2010). These results supported the hypothesis that characterizing populations of AG-1-IA can optimize the deployment of major resistance genes or single-pathway target fungicides as well as agricultural practices that lead to minimizing gene flow by reducing the spread of propagules via irrigation systems or contaminated machinery (González-Vera et al. 2010; McDonald and Linde 2002).

Similarly, knowledge of the population dynamics of AG-1-IE and AG-1-IF may explain the lack of durability of most bean germplasm accessions and recombinant inbred lines, which have exhibited some degree of field resistance in the past, but had to be reevaluated under contrasting field environments (Singh and Schwartz 2010). Little or no progress has been made in the identification of specific genes or QTL (quantitative trait loci)

for resistance to WB, inhibiting the deployment of varieties with high levels of resistance in the LAC region (Beaver and Osorio 2009). Bean breeders have attributed this hindrance to a lack of understanding of the pathogen variation and its significance for breeding for disease resistance (Singh and Schwartz 2010).

The economic importance and the widespread dissemination of the WB pathogen, which affects a crop that provides an important source of protein, fiber, antioxidants, and micronutrients to 500 million people in Latin America and Africa should merit more attention for sustainably managing this pathogen.

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Supplementary material follows the **References**.

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