Genetic Diversity Among *Alternaria solani* Isolates from Potatoes in South Africa

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ABSTRACT

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Genetic diversity among isolates of *Alternaria solani*, the causal agent of early blight of potato, from various potato-growing regions in South Africa (SA), was determined using virulence assays, vegetative compatibility (VC) tests, and random amplified microsatellite (RAMS) primers. The virulence assays showed low virulence levels for the largest part of the population, but failed to otherwise characterize the population diversity. The VC tests revealed 19 VC groups (VCGs), indicating a relatively high level of diversity among the isolates. There was little correlation between geographic origin of isolates and VCGs. Analysis of RAMS profiles revealed 27% genetic diversity among 46 isolates. This value is relatively high for an asexually reproducing fungus, but is similar to values obtained previously by authors studying *A. solani*. Distance analysis of the RAMS profiles also provided no evidence for geographical clustering of isolates. VCG and RAMS profiles indicated that isolates are randomly spread across SA. This fact, together with the high diversity of *A. solani* in SA, indicates that the fungus has a high potential to adapt to resistant cultivars or fungicides. This information can aid in the breeding and deployment of *A. solani*-resistant potato varieties, and in early blight disease management in SA.

Additional keywords: epidemiology, *Solanum tuberosum*

The genus *Alternaria* is widespread and of great economic importance, as it causes destructive leaf spots, blights (foliar and blossom), blemishes, and damage to stored products or seed of numerous hosts (28). Mycologists around the world have studied this genus with respect to parasitism, host range, varietal resistance, physiology, taxonomy, and control (28). *Alternaria solani* Sorauer is one of the best-known and most economically important members of this genus and has also been the subject of various studies (28). This species is the causal agent of early blight of potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), and other members of the *Solanum* family. Early blight on potatoes causes major yield losses in most growing regions of the world. Disease symptoms are characteristic dark brown to black lesions with concentric rings, which produce a "target spot" effect.

Genetic analyses of plant pathogen populations are important in understanding epidemiology, host-pathogen coevolution, resistance management, and control meth-

ods (2,12,14,17,18). Methods commonly used to assess variation in fungal populations include virulence analyses, vegetative compatibility (VC), and biochemical and molecular analyses (11,12,17). Of these, molecular techniques such as isozyme analysis and the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) method have been used to study populations of *A. solani* in the United States (24,34). Various other authors have provided evidence for the existence of races of *A. solani* based on morphological, physiological, and virulence differences (3,8,20). However, little is known about the population diversity of *A. solani* in South Africa.

The amount and distribution of genetic variation within and between populations in phytopathogenic fungi is of interest in plant pathology, as it gives an indication of the potential for development of pathogenic specialization and fungicide resistance (1,13). The objective of this study was thus to determine the levels of genetic variation among *A. solani* isolates causing early blight on potatoes in South Africa. Information on the diversity and spatio-temporal distribution of this species on potato in South Africa can contribute to the development, assessment, and accurate deployment in time and space of resistant potato germ plasm.

In this study, three techniques were used to assess the intraspecific diversity of the

population of *A. solani* isolates, namely virulence assays, VC, and the random amplified microsatellites (RAMS) technique (6,37). The RAMS technique combines the advantages of RAPDs (no prior knowledge of the organism's sequence is required, and a single reaction can produce many polymorphic markers) with those of microsatellite analyses (efficient and accurate for intraspecific studies) (6,7). As the chance of finding polymorphisms using RAMS is greater than with other techniques, including RAPDs, it is a promising technique to use for studies of genetic variation (6) and was therefore chosen for this study. Correlation among virulence, VC, the RAMS profiles obtained, and geographic origin was also sought.

MATERIALS AND METHODS

Collection, identification, and maintenance of isolates. Potato production areas in South Africa are divided into 14 main regions, namely Limpopo (formerly Northern Province), North West, Gauteng, Mpumalanga, Northern Cape, Western Free State, Eastern Free State, KwaZulu-Natal, Sandveld, Ceres, South-Western Cape, Southern Cape, Eastern Cape, and North-Eastern Cape (Fig. 1). Early blight infected plants from various regions and cultivars were obtained from potato growers or collected during field trips from 1999 to 2002. Material was surface-sterilized in 1% active NaOCl for 3 min and then washed twice in sterile, distilled water. Squares with sides of approximately 5 mm were excised from the lesion edges and plated on V8 juice agar or potato dextrose agar (PDA) (Biolab, Midrand, South Africa) and incubated at 25°C. *A. solani* cultures were identified on the basis of morphological characteristics and spore size and shape. Single-spore cultures were made from pure cultures of the fungus. The Plant Protection Research Institute (PPRI) in Pretoria, South Africa, confirmed the identity of selected isolates. These isolates were allocated PPRI numbers (Table 1). Representative isolates were deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria, Pretoria, South Africa (Table 1).

Virulence assays. Tissue culture plants. Due to the difficulties involved in evaluating the virulence of pathogens (8), a virulence assay technique using clonal tissue

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culture plants was designed in an attempt to eliminate most of the host variation that normally causes errors in such assays. We used a BP1 clone. (BP1 refers to Buffelspoort, the research station where J. E. van der Plank did most of the potato breeding during the 1950s. BP1 was registered as a new variety in South Africa during the early 1960s, that is, before the International Potato Centre in Peru [CIP] came into being.) Eight-week-old tissue culture plants from the clone, which is moderately susceptible to early blight (33), were inoculated in test tubes by placing a fully colonized 5-mm-diameter V8 juice agar disk, mycelium side down, on the adaxial side of the first fully expanded leaf of each plantlet. The control plantlet received a sterilized, uncolonized block of agar. Twenty isolates from five potato production regions, representative of different climatic areas in South Africa, were used in the trial. Five plantlets were inoculated per isolate, and the experiment was repeated. Plantlets were incubated at 25°C with a light regime of 12 h near-UV/12 h dark. After 1 week, plantlets were analyzed and rated for disease severity using the following rating scale: 0 = no symptoms, 1 = slight necrosis, 2 = whole leaf necrotic, and 3 = leaf, petiole, and other plant parts necrotic.

Detached leaves. Detached leaves of BP1 plants grown in glasshouses were used in this assay. The same 20 isolates

used for the tissue culture plantlet inoculations were used in this study. For each isolate, five fully expanded, unwounded leaves were inoculated, and the experiment was repeated. Inoculation and incubation were as described for the tissue culture plant assays. Leaves were rated for disease severity after 1 week as follows: 0 = no symptoms, 1 = 0 to 30% of leaf area necrotic and chlorotic, 2 = 31 to 60% of leaf area necrotic and chlorotic, and 3 = 61 to 100% of leaf area necrotic and chlorotic.

VC. Preliminary studies were conducted using different media and different numbers of isolates per petri dish (90 mm diameter) to optimize the incompatibility reaction. The reactions were most distinct on PDA when six different isolates per petri dish were incubated in the dark at 25°C for approximately 2 weeks. Cubes of PDA, with sides of approximately 3 mm, were cut from actively growing cultures and placed approximately 20 mm apart on PDA in the "dice number six" pattern. Reactions were scored after 14 days in the dark. Pairings were repeated to confirm results. Rayner (26) stressed the need for controlled self-pairings to distinguish between true incompatibility and mutual staling or nutrient depletion. Reactions between paired isolates were, therefore, always scored alongside control reactions where isolates were paired against themselves (positive control) and against isolates known to be incompatible from initial

trial experiments (negative control). Isolates were scored as vegetatively incompatible when they formed a brown line, visible from underneath, at the interface where the mycelia of the two isolates met. Vegetatively compatible isolates produced no detectable interaction zone, and the mycelia mixed freely.

RAMS. For preparation of chromosomal DNA, mycelium from actively growing single spore isolate cultures on PDA was used to inoculate liquid malt extract medium (ME) (Biolab, Midrand, South Africa). These cultures were incubated at 25°C on a shaker for approximately 2 weeks. The biomass was harvested, freeze-dried, and the mycelium ground in a mortar under liquid nitrogen. DNA was extracted using a modification of the method of Raeder and Broda (25).

DNA was amplified by the RAMS technique (6,37) using primers CAC₅, GACA₄, and CA₈ (Table 2). Reactions were also done using two primer combinations, namely GACA₄-CAC₅ and CA₈-CAC₅. PCR reactions were carried out in total volumes of 20 µl under reaction conditions recommended by the manufacturer of BI-OTAQ DNA Polymerase (Bioline, GmbH, Luckenwalde, Germany). A final concentration of 4 µM was used for all RAMS primers. DNA was amplified in an Eppendorf Mastercycler PCR machine (Eppendorf, Hamburg, Germany) with the following program: initial denaturation cycle of

- A Sandveld
- B Ceres
- C South Western Cape
- D Northern Cape
- E Eastern Cape
- F Western Free State
- G Eastern Free State
- H Kwazulu-Natal
- I Mpumalanga
- J Northern Province
- K North West
- L Southern Cape
- M Gauteng
- N North Eastern Cape

Fig. 1. Map of South Africa showing potato production regions.

95°C for 2 min; 40 cycles of 95°C for 30 s, 44°C for 45 s, 72°C for 1 min; 1 cycle of 72°C for 10 min; and a hold at 4°C.

The PCR products were separated on 2% agarose gels (Roche Diagnostics, Mannheim, Germany) in TAE buffer (1 mM Tris-acetate, 1 mM EDTA, pH 8.0). Gel electrophoresis was 50 min at 80 V cm⁻¹. Gels were stained with ethidium bromide and photographed under ultraviolet illumination. All reactions were repeated at least twice to confirm results.

Statistical analysis. Analysis of results from the virulence assays was performed using nonhierarchical classification (5). It is often found that nonhierarchical methods provide a more acceptable classification into fewer major groups. The groupings obtained are also more robust to any aberrant similarities between individual pairs of units (5).

Scoring of RAMS amplification products between 280 and 1,400 bp was performed visually from photographic prints with no correction for band intensity. Isolates were scored for the presence [1] or absence [0] of a given RAMS band, and a binary matrix was constructed. Only reproducible bands with the same molecular weight on a single gel were considered to be identical. Calculation of Nei's genetic diversity (21 in 12) and construction of a dendrogram was performed using POPGENE Version 1.31 (36). Construction of the dendrogram was based on Nei's genetic distances (22) using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis. Isolates were also grouped according to geographic origin into six subpopulations, and genetic diversity was calculated for each subpopulation.

RESULTS

Virulence assays. Similar results for all isolates were obtained from both virulence assays. Nonhierarchical grouping of results from virulence assays on detached leaves and tissue culture plants showed that isolates could be divided into two main groups (Table 3). Isolates in group 1 had average virulence ratings of either 0 or 1, while those in group 2 had average ratings of either 2 or 3. Most isolates fell into group 1, showing low levels of virulence. Geographical region of isolation had little correlation with the relative virulence of isolates, although isolates from the Eastern Free State and North West Province production regions grouped only into group 1 (low virulence) (Table 3).

VC. Forty of the 53 *A. solani* isolates included in this study could be divided into six distinct multimember vegetative compatibility groups (VCGs) (Table 4). The remaining 13 isolates, however, could not be assigned to any of the six multimember VCGs and thus formed numerous single-member VCGs.

RAMS. A total of 47 amplification products were obtained for all RAMS

Table 1. Description of the *Alternaria solani* isolates used in this study, all isolated from potatoes (*Solanum tuberosum*) in South Africa

Isolate code	CMW ^a number	PPRI ^b number	Town and region isolated from	Date
1	9515	6846	Reitz, EFS ^c	Apr. 99
2	9516		Reitz, EFS	Apr. 99
3	9517		Reitz, EFS	Apr. 99
4	9518		Reitz, EFS	Apr. 99
5			Lichtenburg, NW ^d	Mar. 99
6		6845	Christiana, NC ^e	Mar. 00
7			Lichtenburg, NW	Mar. 99
8	9519		Christiana, NC	Mar. 00
9			Roodeplaat, Gauteng	Mar. 00
10			Uitvlug, NC	Oct. 99
11	9520	6844	Cedara, KZN ^f	Mar. 99
12			Lichtenburg, NW	Mar. 99
13			Lichtenburg, NW	Mar. 99
14	9521		Lichtenburg, NW	Mar. 99
16	9522		Lichtenburg, NW	Mar. 99
17	9523	6844	Lichtenburg, NW	Mar. 99
18	9524		Christiana, NC	Mar. 00
19	9525		Reitz, EFS	Apr. 99
20			Reitz, EFS	Apr. 99
21	9526		Reitz, EFS	Apr. 99
22	9527	6844	Reitz, EFS	Apr. 99
23	9528		Reitz, EFS	Apr. 99
24	9529		Reitz, EFS	Apr. 99
25			Roodeplaat, Gauteng	Dec. 00
26	9530		Kamberg, KZN	Dec. 00
28		6844	Roodeplaat, Gauteng	Dec. 00
29			Roodeplaat, Gauteng	Dec. 00
30			Lion River, KZN	Jan. 01
31			Lion River, KZN	Mar. 01
34			Dargle, KZN	Jan. 01
35	9533	6844	Cedara, KZN	Jan. 01
36			Cedara, KZN	Jan. 01
37	9534		Cedara, KZN	Jan. 01
38			Lion River, KZN	Mar. 01
39	9539		Lion River, KZN	Jan. 01
41	9535	6844	Cedara, KZN	Jan. 01
42	9536		Cedara, KZN	Jan. 01
51			Christiana, NC	Apr. 01
53			Christiana, NC	Apr. 01
54	9537		Kimberley, NC	Apr. 01
55		6844	Kimberley, NC	Apr. 01
58			Kimberley, NC	Apr. 01
59			Kimberley, NC	Apr. 01
60			Kimberley, NC	Apr. 01
61			Kimberley, NC	Apr. 01
62	9540	6844	Leipoldtville, SV ^g	May 01
63	9541		Leipoldtville, SV	May 01
64	9542		Leipoldtville, SV	May 01
65	9543		Leipoldtville, SV	May 01
66	9544		Leipoldtville, SV	Sep. 01
67	9545	6844	Leipoldtville, SV	Sep. 01
68			Leipoldtville, SV	Sep. 01
71	9546		Roodeplaat, Gauteng	Jan. 02
72	9547		Roodeplaat, Gauteng	Jan. 02
74	9548	6844	Roodeplaat, Gauteng	Jan. 02
77	9549		Cedara, KZN	Jan. 02
78	9550		Cedara, KZN	Jan. 02
79	9551		Cedara, KZN	Jan. 02
80	9552		Cedara, KZN	Jan. 02
81	9553	6844	Cedara, KZN	Jan. 02
82	9554		Cedara, KZN	Jan. 02

^a Culture Collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

^b Plant Protection Research Institute, Pretoria, South Africa.

^c Eastern Free State.

^d North West.

^e Northern Cape.

^f KwaZulu-Natal.

^g Sandveld.

primers and primer combinations. These products ranged in size from 200 to 1,500 bp. Only fragments between 280 and 1,400 bp were used for analyses due to the difficulty in reproducing fragments outside this range. Due to the high number of amplification products, the relationship between loci and alleles was not determined and the variation observed was considered an on/off type polymorphism. Among the 46 isolates examined, 44 unique genotypes were found. The gene diversity for all 46 isolates was 0.27 (21 in 12). The genetic diversity calculated for each population is presented in Table 5. The dendrogram showed no apparent clustering of isolates according to geographic origin (Fig. 2).

DISCUSSION

This study has shown that the genetic diversity among *A. solani* isolates from South Africa is relatively high for an asexually reproducing fungus. Furthermore, there is no clear clustering of isolates according to geographical origin.

The virulence assays in this study showed a moderate degree of variation in virulence of different isolates. This is indicated by the fact that the largest number of isolates (75%) was not virulent or showed low virulence. Although virulence assays are often used to detect variation in fungal populations, this technique has many disadvantages: it is laborious and time-consuming; scoring is subjective and influenced by environmental factors; and genes involved in virulence represent a small fraction of the genes in the pathogen genome, and may be subject to strong selection by the host (12,16). In addition, it has

been shown for *Alternaria alternata* (Fr.:Fr.) Keissl. that virulence determinants are unstable and virulence may be lost during repeated subculturing (23). These factors may have contributed to the low levels of virulence and variation thereof recorded for isolates in this study, and hampered the use of these results to characterize the populations of these fungi.

The amount of genetic variation found in *A. solani* using VC and RAMS markers is relatively high, but not atypical, for a species that is thought to reproduce only asexually (12,18,31). A possible explanation for the high levels of genetic diversity found among isolates of *A. solani* could be natural chance mutations, combined with the fact that the fungus can produce abundant numbers of spores in a relatively short period of time (12,24). However, high levels of genetic variation are usually due to recombination, which occurs sexually through mating or asexually through the parasexual cycle. It is not known if the parasexual cycle occurs regularly in the genus *Alternaria*, and there is no known sexual cycle for *A. solani*, although teleomorphs have been found in other *Alternaria* species (4,24,29). Taylor et al. (31) reviewed various studies of variation in supposedly clonal fungi and concluded that truly asexual fungi are extremely rare,

if they exist at all. Most populations showed some influence of recombination, albeit rare events.

Analysis of the virulence data, VC groups, and RAMS profiles showed no significant difference of *A. solani* isolates according to geographical origin. In fact, two VC groups were each found in four of the five geographically isolated potato-growing regions in South Africa. Previous studies on *A. solani* populations in the United States (24,34) have also shown no geographic clustering of isolates. These results indicate that the various geographical subpopulations are not genetically isolated, which may be due to dissemination of spores by biotic and abiotic factors. We consider the distribution of potato tubers and vegetative material over a wide geographic area to be another cause of the even distribution of genetic variation of *A. solani* in South Africa. Early blight is one of the few diseases not specified in the South African Seed Potato Certification Scheme (27), thus allowing free movement of early blight infected material throughout the country.

This study is the first to investigate VCG diversity within *A. solani* isolates from potato plants in South Africa. Six multi-member and 13 unique single-member VCGs were identified among the 53 isolates tested. Many researchers have used

Table 4. Assignment of *Alternaria solani* isolates from potatoes in South Africa to vegetative compatibility groups (VCGs)

VCG	Isolate	Origin
1	1, 2, 3, 4 5, 12, 14, 16 6, 8 11	Reitz, EFS ^a Lichtenburg, NW ^b Christiana, NC ^c Cedara, KZN ^d
2	13 18 19, 21, 23, 24 26 30 35	Lichtenburg, NW Christiana, NC Reitz, EFS Kamberg, KZN Lion River, KZN Dargle, KZN
3	37, 41 39	Cedara, KZN Lion River, KZN
4	51, 53	Christiana, NC
5	59, 60 61, 62, 63, 64, 65	Kimberley, NC Lambert's Bay, SV ^e
6	71, 72, 74 77, 78, 79, 80, 82	Roodeplaat, Gauteng Cedara, KZN
7	7	Lichtenburg, NW
8	10	Uitvlug, NC
9	17	Lichtenburg, NW
10	22	Reitz, EFS
11	29	Roodeplaat, Gauteng
12	41	Cedara, KZN
13	42	Cedara, KZN
14	54	Kimberley, NC
15	55	Kimberley, NC
16	58	Kimberley, NC
17	66	Leipoldville, SV
18	67	Leipoldville, SV
19	81	Cedara, KZN

^a Eastern Free State.

^b North West.

^c Northern Cape.

^d KwaZulu-Natal.

^e Sandveld.

Table 2. Random amplified microsatellite primers used to determine genetic diversity among *Alternaria solani* isolates from potatoes in South Africa

Primer	Sequence ^a
CAC-primer	5' DBD (CAC) ₅
GACA-primer	5' HBDB (GACA) ₄
CA-primer	5' DB (CA) ₈

^a The following designations are used for degenerative sites: H (A, C, or T), B (C, G, or T), and D (A, G, or T).

Table 3. Nonhierarchical grouping of *Alternaria solani* isolates from South Africa into two groups based on virulence on potato tissue culture plants and detached leaves of potatoes^a

Origin	Isolate	
	Group 1	Group 2
Eastern Free State	2, 3, 21	
KwaZulu-Natal	11, 34, 35, 42	26, 41
North West Province	13, 16, 17	
Northern Cape	51, 53, 54, 60	10, 58
Sandveld	64	65

^a Group 1 is least virulent (average ratings of either 0 or 1) and group 2 is most virulent (average ratings of either 2 or 3).

the natural phenomenon of non-self rejection or VC, which is known to occur in Basidiomycetes and Ascomycetes, as a method of assessing genetic diversity in various fungal species (15,16,26,32,35). Although the technique is simple to perform, it is not considered by all to be an ideal genetic marker for determining population structures in fungi (12). The reasons are that the genetics of VC-loci can be very complicated and because this trait normally lacks the level of polymorphism needed for an accurate assessment of genetic variability (12,15). Nevertheless, this technique was useful in this study for tracking levels of diversity, as well as geographic distribution of isolates sharing a VCG phenotype.

No correlation was noted between VCGs and virulence or RAMS profiles. All multimember VCGs contained isolates with different RAMS profiles. Three VCGs (VCGs 2, 3, and 5) contained isolates with more than one virulence rating. There was also no structure in the tree that correlated with multimember VCG groupings. These results indicate underlying genetic variation within VCGs. Nonclonality of VCGs has previously been demonstrated (2,9,10,30, but see 19). Variation within a VCG can result from a low number of segregating loci and alleles controlling the VCG recognition, extensive inbreeding, parasexual recombination or mutation. Varying RAMS profiles within a VCG could also be the result of rapid evolution of the microsatellite repeats to which the primers bind. However, the fact that pathogenicity also varied within VCGs indicates that this is not the only source of the observed nonclonal VCGs. Nothing is currently known regarding a possible mating system or other sources of genetic variation in *A. solani* (24). Characterization of these biological processes will greatly enhance our understanding of this fungus and its behavior as a pathogen.

This study is the first to use RAMS markers to assess genetic diversity of *A. solani* isolates from potato plants. The level of genetic variation obtained is slightly lower than that found in previous studies using other genetic markers (24,34). Petrunak and Christ (24) studied isozyme variability among 54 *A. solani* isolates from different solanaceous hosts

and geographic regions across the United States. Weir et al. (34) used RAPDs to investigate genetic variation among 35 *A. solani* isolates from potato and tomato plants, predominantly from locations in the United States, with the exception of one isolate from South Africa and three from

Costa Rica. The fact that the genetic diversity among *A. solani* isolates in this study was found to be lower than in previous studies can possibly be attributed to the fact that the *A. solani* isolates used in this study were only from potatoes and not different solanaceous hosts.

Table 5. Genetic variation within each geographical subpopulation of South African *Alternaria solani* isolates (number of isolates represented in each population is listed in parenthesis)

Population	Genetic diversity
Eastern Free State (7)	0.1388
Sandveld (6)	0.1625
North West (6)	0.1750
Gauteng (5)	0.2360
KwaZulu-Natal (13)	0.2408
Northern Cape (9)	0.2654

Fig. 2. Dendrogram of 46 *Alternaria solani* isolates from potatoes in South Africa. Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis was based on Nei's unbiased genetic distances (22). EFS = Eastern Free State, KZN = KwaZulu-Natal, NC = Northern Cape, NW = North West, and SV = Sandveld.

The amount and distribution of genetic variation within and among populations of phytopathogenic fungi gives an indication of the fitness of the pathogen to circumvent effects of natural or artificial stresses on the population, and thus counteract control measures such as fungicide applications (1,13,14). According to McDonald and Linde (13), pathogen populations with a high degree of gene flow and high genetic diversity, such as is shown for *A. solani* in this study, pose a great risk of breaking down genetic resistance in the host. This could be one of the reasons no South African potato cultivars are available with complete resistance to early blight (33). McDonald and Linde (13) suggest that in such cases, breeding efforts should concentrate on quantitative resistance, or the development of cultivar mixtures and multilines that can be used in combination with other control strategies. Resistance screening should include as many isolates from different locations as possible, in order to reflect the genetic diversity present in the pathogen. Understanding the genetic diversity of *A. solani* on potato in South Africa will thus aid in future disease management strategies of early blight in this country. Future studies to improve understanding of the influences on the population structure and levels of genetic variation observed in this species should be conducted using additional, co-dominant molecular markers and more isolates from different hosts, from as many potato-growing locations worldwide as possible.

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