Assessing the Genetic Diversity of *Alternaria Bataticola* in South Africa using Molecular Markers

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ABSTRACT--- ***Sweetpotato (Ipomoea batatas) an economically important food crop is affected by viral and fungal diseases. The most important fungal disease is Alternaria blight caused by Alternaria bataticola. Alternaria blight can be controlled using fungicides and cultural practices in the short-term. A sustainable control measure is the development of resistant sweetpotato cultivars. A prerequisite to this approach is the knowledge of the genetic diversity of this fungal pathogen. This study assessed the genetic diversity of A. bataticola in South Africa using RAPD analysis, DNA sequencing and secondary structures of the ITS2 region. Samples were collected from 25 different localities and the pathogen was identified in the laboratory. Both RAPD and ITS2 sequence data showed that there is high levels of genetic diversity among the isolates of A. bataticola. Although the dendrograms generated from the RAPD and ITS2 sequences clustered some isolates according to their place of origin, the majority of isolates did not group according to their geographic origins. The predicted ITS2 secondary structure models were variable ranging from simple to complex. The unique secondary structures of each isolate can be used to identify and distinguish each of the isolates used in this study.***

Keywords--- *Alternaria bataticola*, sweetpotato, genetic diversity, secondary structure prediction

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1. INTRODUCTION

Sweetpotato (*Ipomoea batatas)* is an economically important food crop grown in many countries [1]. The root tubers and leaves are edible. Sweetpotato is a cash crop and is also processed into products such as starch, snacks, liquor and flour [2]. Sweetpotato is a good source of β-carotene, vitamin B6, vitamin C, vitamin E, fiber, protein and calcium. There has been a 24% decrease in the world's sweetpotato production [3]. The major production constraints are viral and fungal diseases [4].

One of the most threatening diseases of sweetpotato production especially in developing countries is caused by *Alternaria bataticola* that affects the whole vine throughout the life cycle of the crop [5]. The disease first appears as lesions on leaves, petioles and stems and eventually inhibits the photosynthetic capacity of the plant. The crop eventually wilts and the yield of the root tuber is affected [6]. The extent of damage caused by this disease depends on the plant's growth stage as well as environmental conditions [7]. The presence of *Alternaria* blight in sweetpotato in South Africa was first reported by Narayanin *et al*. [8].

Many approaches including the use of fungicides, planting with pathogen free stock, phytosanitation techniques and crop rotation are used to combat diseases in plants. However, these approaches are not sustainable. For example, reliance on fungicides may develop resistant mutants while planting with pathogen free material can lead to re-infection.

The use of resistant cultivars is an important method for reducing the proliferation of plant pathogens [9]. Resistance breeding depends on understanding the genetic diversity of the pathogen in the area of production [8]. Genetic diversity studies of pathogens like *Alternaria* is vital in understanding how hosts and pathogens interact in the development of diseases [10]. Despite the importance of *A.bataticola* in sweet potato production there is little or no information on the genetic diversity of the pathogen. Molecular markers have been used to assess the genetic diversity of several fungal pathogens [11, 12]. This information facilitates the development of resistant plant cultivars by plant breeders.

The objectives of this study were: (1) to analyse the genetic diversity of *A. bataticola* using RAPD and ITS2 sequence analysis, and (2) assess the potential of the ITS2 secondary structures as a taxonomic marker for the isolates of *A. bataticola.* RAPD and ITS2 sequence markers have been used in genetic diversity studies of other fungal pathogens

affecting plants [13, 14, 15, 16]. In addition, researchers have reported the possibilities of using the ITS2 region as a barcoding marker in taxonomic studies to identify various organisms [17, 18].

2. MATERIALS AND METHODS

Sample collection

Infected sweetpotato samples were collected from the Agricultural Research Council -Vegetable and Ornamental Plant Institute (ARC-VOPI) near Pretoria, The Free State (Sasolburg: Harry Gwala township), Vanderbijlpark (Bophelong and Tshepiso), Kwa-Zulu-Natal (Empangeni) and Limpopo (Dan village). The plant material was placed in paper bags, labeled and transported to the laboratory where it was assigned a numerical code (Table 1).

Table 1: Code, isolates of *A. bataticola*, and collection sites of samples used in the study

Code	Isolate Name	Collection Site
A1	Excel/A/5	Pretoria (ARC-VOPI)
A2	2004/3/1	Pretoria (ARC-VOPI)
A3	2003/23/6	Pretoria (ARC-VOPI)
A4	Beauregard	Pretoria (ARC-VOPI)
A5	2004/3/9	Pretoria (ARC-VOPI)
A6	W-119	Pretoria (ARC-VOPI)
A7	2004/9/1	Pretoria (ARC-VOPI)
A8	2004/9/2	Pretoria (ARC-VOPI)
A9	Sasolburg	Free State
A10	Harry Gwala1	Free State
A11	Harry Gwala2	Free State
A12	Harry Gwala3	Free State
A13	Bophelong	Vanderbiljpark
A14	Tshepiso1	Vanderbiljpark
A15	Tshepiso2	Vanderbiljpark
A16	2010/12/2	Pretoria (ARC-VOPI)
A17	2010/4/4	Pretoria (ARC-VOPI)
A18	W-119(2)	Pretoria (ARC-VOPI)
A19	University of Zululand1	KwaZulu-Natal (Empangeni)
A20	University of Zululand2	KwaZulu-Natal (Empangeni)
A21	University of Zululand3	KwaZulu-Natal (Empangeni)
A22	Pretoria	Pretoria
A23	Dan Village	Limpopo (Mopani District)
A24	Mohlaba cross	Limpopo
A25	2005/1/11	Pretoria (ARC-VOPI)

Isolation of *A. bataticola* from infected plants

Alternaria bataticola was isolated from decontaminated sweetpotato leaves and stems using a modified version of the technique described by Lopes & Boiteux [6]. All the isolates were subjected to pathogenicity tests (isolation and re-isolation) as described by Osiru *et al.* [19] with modifications in order to confirm that the causal agent was *A. bataticola*. The isolates were grown for sporulation as described by Shahin & Shepard [20]. Spore suspensions for each isolate were then prepared and adjusted to 5 X 10^4 conidia per milliliter. Susceptible cultivars 'W119' and 'Excel' as well as tolerant cultivars 'Impilo' and 'Khano' that had been grown in a greenhouse for 25 days from disease free stocks were used for this test [6]. The upper and lower leaf surfaces of these cultivars were atomized with the fungal inoculum using a hand sprayer. The plants were inspected daily for symptom development for a period of 30 days prior to re-isolation.

DNA extraction
DNA from freeze dried mycelia was extracted using a modified method described by Gonzalez-Mendoza *et al*. [21]. The DNA was quantified using the NanoDrop ® 2000 (NanoDrop Technologies, Wilmington, DE).

RAPD Analysis
Twenty RAPD primers from Operon Technologies (Alameda, CA) were used to amplify the genomic DNA of the samples. The PCR reaction and thermal cycling conditions were carried out as described by Nguluta *et al*. [22]. Gel electrophoresis of the amplicons were done as described in Nguluta *et al*. [22]. The amplicons were scored as (1) presence and (0) absence of bands. The data matrix was used to construct a consensus dendrogram based on Jaccard's similarity coefficient using the NTSYS-pc programme version 2.2 [23] following the unweighted pair group method (UPGMA).

ITS2 sequence analysis
The rDNA ITS2 region was amplified following a modified protocol described by Gomes *et al.* [24] using the two primers (5'-TCCGTAGGTGAACCTGCGG-3'; '5'-GCTGCGTTCTTCATCGATGC-3') [25]. The PCR products were sequenced at Inqaba Biotech (Pretoria, South Africa). The phylogenetic relationships among the 25 *A. bataticola* sequences were determined using the Molecular Evolutionary Genetics Analysis version 5 software (MEGA5) [26]. The tree was inferred using the maximum likelihood based on the Tamura-Nei model [27]. The bootstrap consensus trees were inferred from 1000 replicates [28]. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 175 positions in the final dataset. Evolutionary analysis was also conducted in MEGA5 [26].

Secondary structure prediction
The 25 *A. bataticola* sequences were used to generate ITS2 secondary structures using the Sfold web server. Sfold predicts a secondary structure based on statistical sampling paradigm to fold nucleic acids (http://sfold.wadsworth.org/cgi-bin/sirna.pl) [29]. Default settings were used to derive the models.

3. RESULTS AND DISCUSSION

Isolation and characterization of *A. bataticola* from infected plants
The colony texture, colour and the type of conidia of the isolates are listed in Table 2. The conidia had long beaks, muriform, obclavate and solitary (Fig 1).

Table 2: Morphological characteristics of *A. bataticola* isolates

Code	Isolate	Colony texture	Colony colour	Conidia
A1	Excel/A/5	felty to woolly	Brown	solitary, obclavate, muriform with long beaks
A2	2004/3/1		Brown	
A3	2003/23/6		Brown	
A4	Beauregard		Brown	
A5	2004/3/9		Brown and maroon pigmented	
A6	W-119		Brown	
A7	2004/9/1		Brown and maroon pigmented	
A8	2004/9/2		Brown and maroon pigmented	
A9	Sasolburg		Brown	
A10	Harry Gwala1		Brown	
A11	Harry Gwala2		Brown	
A12	Harry Gwala3		Brown	
A13	Bophelong		Brown	
A14	Tshepiso1		Brown	
A15	Tshepiso2		Brown	
A16	2010/12/2		Brown	
A17	2010/4/4		Brown and olive green pigmented	
A18	W-119(2)		Brown	
A19	University of Zululand1		Brown	
A20	University of Zululand2		Brown	

A21	University of Zululand3		Brown	
A22	Pretoria		Brown	
A23	Dan Village (Nkowankowa)		Brown	
A24	Mohlaba cross		Brown	
A25	2005/1/11		Brown and olive green pigmented	

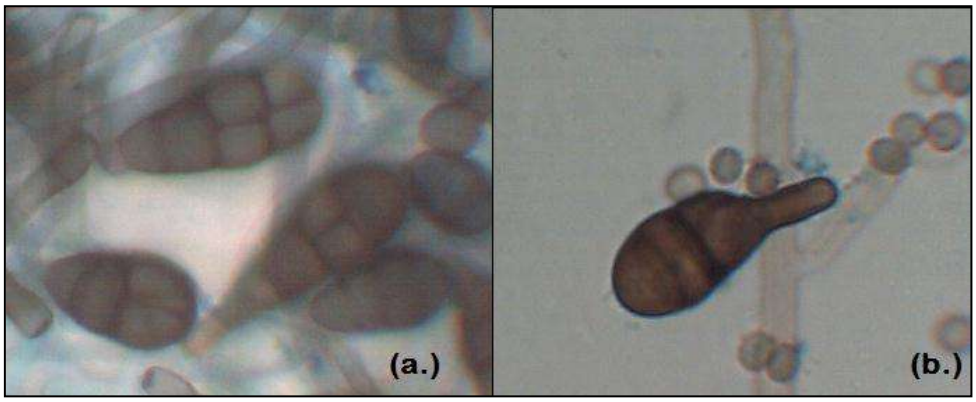

Figure 1: *A. bataticola* conidia from isolates (a.) A16_2010/12/2 and (b.) A17_2010/4/4

The conidia of the isolates were solitary, brown, obclavate (club-shaped), muriform (patterned like a brick wall) with long beaks (Fig 1). This is consistent with the description of *A. bataticola* by David [30] and Lopes & Boiteux [6]. While most colonies were similar in colour, a few isolates produced a red maroon pigment while others were produced an olive green pigment. These findings correspond with those of Alajo [31] who reported the same pigmentation in *Alternaria* isolates (*A. bataticola and A. alternata)* from sweetpotato.

RAPD Analysis

The twenty RAPD primers generated variable banding patterns for the *A. bataticola* isolates as shown in Fig 2.

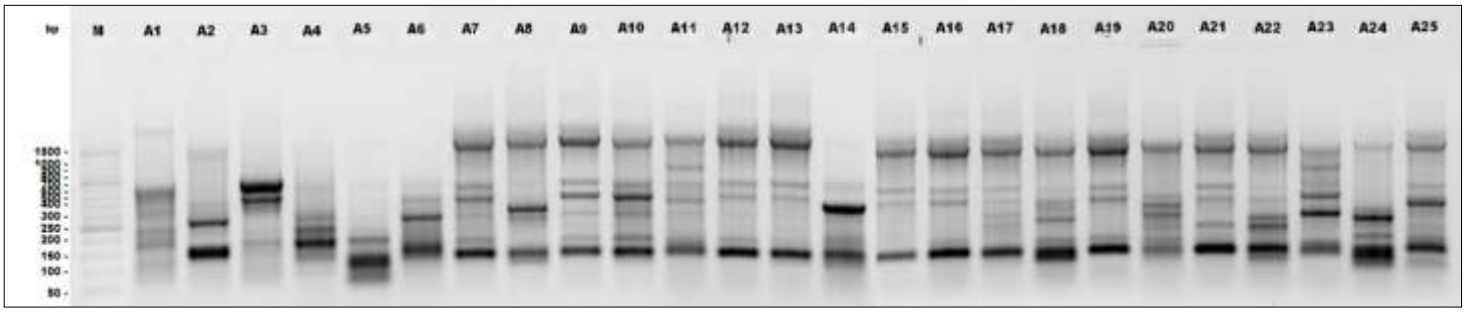

Figure 2: Banding patterns produced by RAPD Primer OPA-1. (bp): base pairs, (M): Kapa Biosystems universal molecular weight marker. A1- A25 corresponds to the names in Table 2.

The dendrogram derived from the RAPD analysis grouped the 25 isolates into 4 main clusters (Fig 3). Amongst the four clusters, only cluster-I was further divided into three subclusters i.e. I_A, I_B and I_C. Most of the isolates (13) fell into the third subcluster (I_C) in two branches: Sasolburg, Harry Gwala1 and Harry Gwala2 (sm = 0.778), 2010/12/2 and University of Zululand3 (sm = 0.817), University of Zululand1 and University of Zululand2 (0.824), Tshepiso1, Harry Gwala3 and Bophelong (sm = 0.791) as well as Mohlaba cross were South African isolates except W-1192 from the USA which shared a sister relationship with Pretoria (sm = 0.738). The isolate from Dan Village and 2004/3/1 occurred independently.

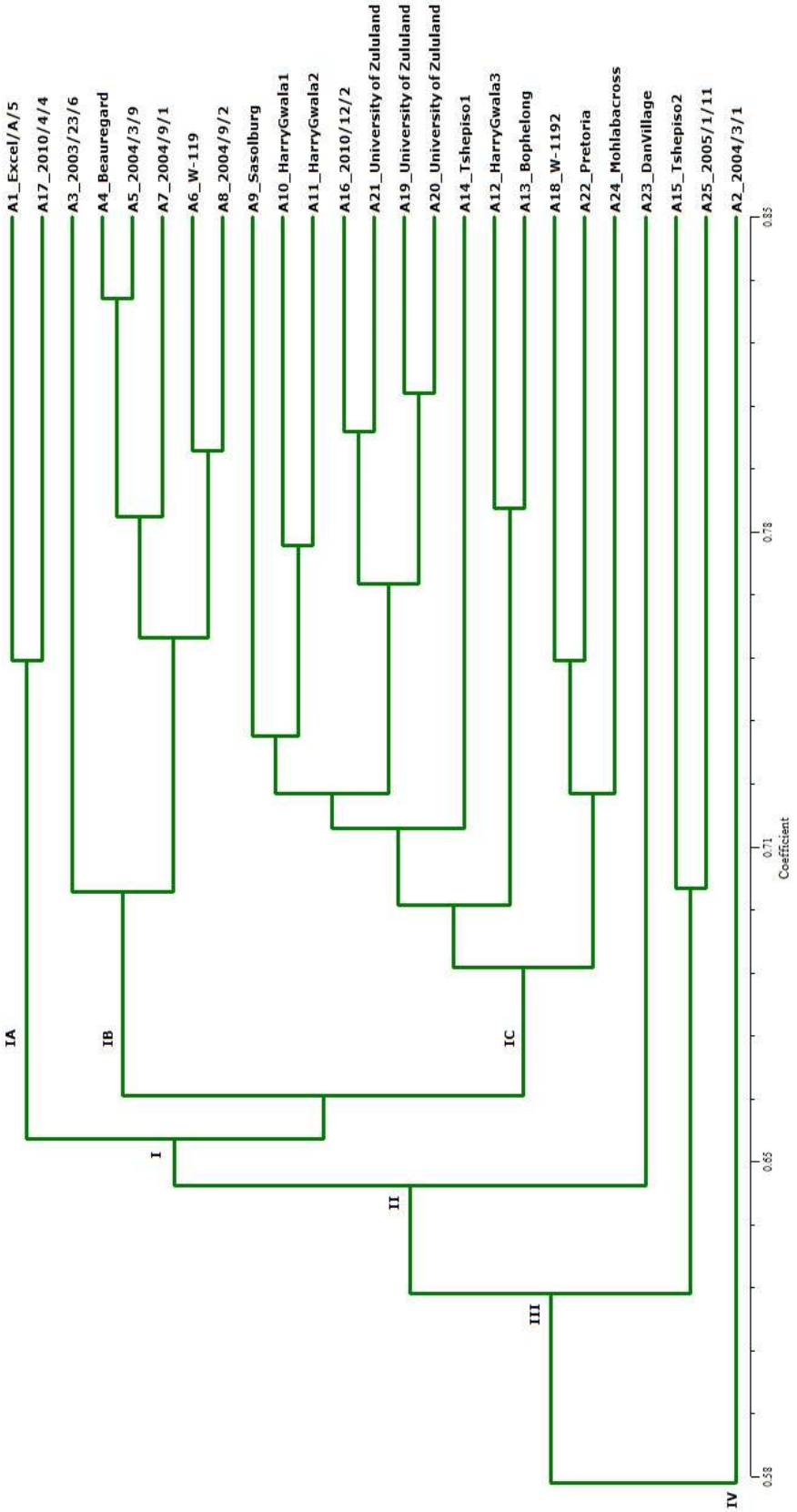

Figure 3: Unweighted pair group method analysis (UPGMA) dendrogram showing genetic diversity of 25 *A. bataticola* isolates based on pooled RAPD data

RAPD analysis revealed high levels of genetic diversity among the 25 isolates of *A. bataticola* (Fig 3). Various reports have also indicated high levels of genetic variability in other *Alternaria* species using RAPD primers [14, 15, 16, 32, 33]. Significant levels of diversity in *Alternaria* species have also been reported using other molecular techniques such as microsatellites [34] and amplified fragment length polymorphism markers (AFLP) [35]. In addition to this, high levels of variation in *Alternaria* species have also been observed using other techniques like morphometric characters [36], structures of host-selective toxins (HSTs) [37] and protein analysis [38].

The isolates from the three sweetpotato cultivars (Excel, Beauregard and W-119) grouped together with some breeding lines in the subclusters of cluster I. Excel, Beauregard and W-119 originated from the USA and are used in the sweetpotato breeding programme at ARC-VOPI. The close genetic similarity of the *Alternaria* isolates from these cultivars and those from the breeding lines is perhaps due to fact that the material was collected from the same locality. These isolates are closely related but not identical implying they are evolving at a rapid rate. The differentiation of the isolates can be attributed to mutations and recombination which occur during the sexual, asexual or parasexual reproductive cycle [15].

The mixed cultivar plantings of sweetpotato at ARC-VOPI may be an additional reason for the variation [39].

The isolates from Sasolburg and Harry Gwala clustered together which was expected due to the close proximity of these areas. A similar grouping was observed in the isolates from the University of Zululand due to the fact that the samples were obtained from the same locality. The isolates from Harry Gwala3 and Bophelong clustered together. A possible explanation might be the transfer of plant material between the two localities. The two isolates collected from Limpopo (Mohlabacross and Dan Village) appeared in separate clusters suggesting genetic differentiation. It was interesting to observe that the dendrogram (Fig 3) did not group some of the isolates according to their origins. The findings from this study are consistent with those reported by Kumar *et al.* [40] who observed that isolates of *A. solani* did not group according to their points of origin. This is perhaps due to the rapid rates of evolution in the isolates.

ITS2 Sequences analysis

The ITS2 primer used in this study produced a clear fragment of approximately 247 bp in all the isolates. However, sequencing results showed that the length of the ITS2 region ranged from 225 to 297 bp with an average size of 248 bp. The G~C content varied from 34.7 to 49% while the A~T content ranged from 51 to 65.3%. Alignment of the sequence data produced 209 variable nucleotides, 60 were conserved and 155 were informative for parsimony analysis. The maximum likelihood tree grouped the 25 isolates into eight clusters (not shown). The isolates from Mohlaba cross and Dan village appeared as independents.

The size of the ITS2 region and the G-C content of *A. bataticola* corresponds to those reported in other studies [13, 41].

Clustering patterns produced with the RAPD data (Fig. 3) and ITS2 sequences (Fig. 4) were quite different with a few similarities. For example, the isolate WII9_2 from an established cultivar clustered with the breeding line 2003/23/6 as expected since it is used in the breeding programme at ARC-VOPI. Isolates from some of the breeding lines such as 2005/1/11, 2004/3/9 and 2004/9/2 grouped together perhaps due to the similarity of the parents from which they were derived. Similarly, isolates from Sasolburg and Harry Gwala as well as Excel and 2010/4/4 clustered together in both the RAPD and ITS2 analysis. As observed in the RAPD analysis (Fig. 3) clustering did not occur according to the geographic origins of the isolates.

The amount of genetic diversity in *A. bataticola* using RAPD analysis and ITS2 sequences is relatively high. This study supports the findings of Anginyah *et al.* [42] and Alajo [31] and confirms high levels of diversity among *A. bataticola* isolates in South Africa.

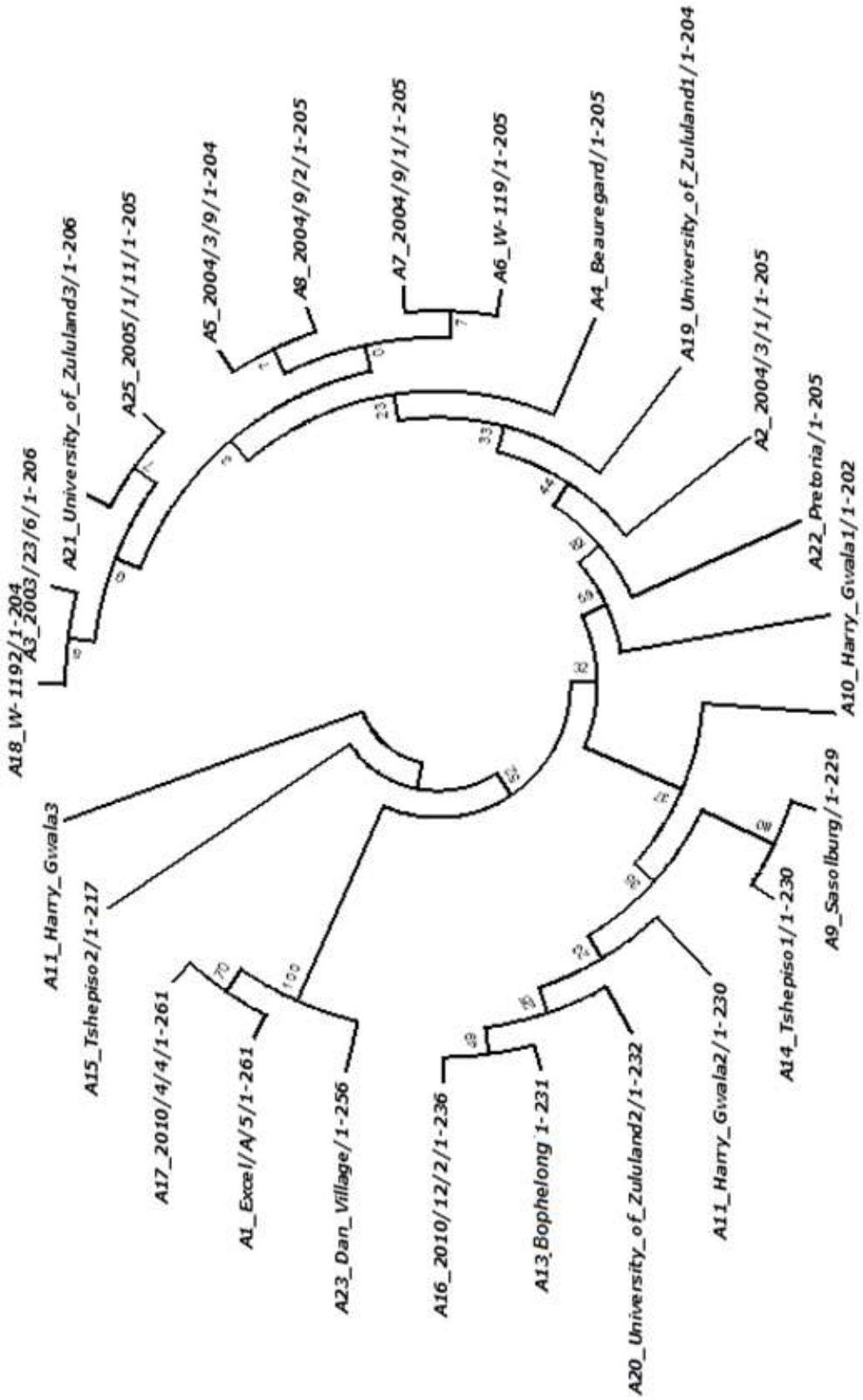

Figure 4: Maximum likelihood analysis of the ITS2 sequences of 25 *A. bataticola* isolates. Numbers on branches are bootstrap values.

Secondary structure prediction

The secondary structures of *A. bataticola* are in agreement with the proposed 4 domain ITS2 model for eukaryotes and yeast [43]. An example of the ITS2 structure for *A. bataticola* is presented in Fig. 5.

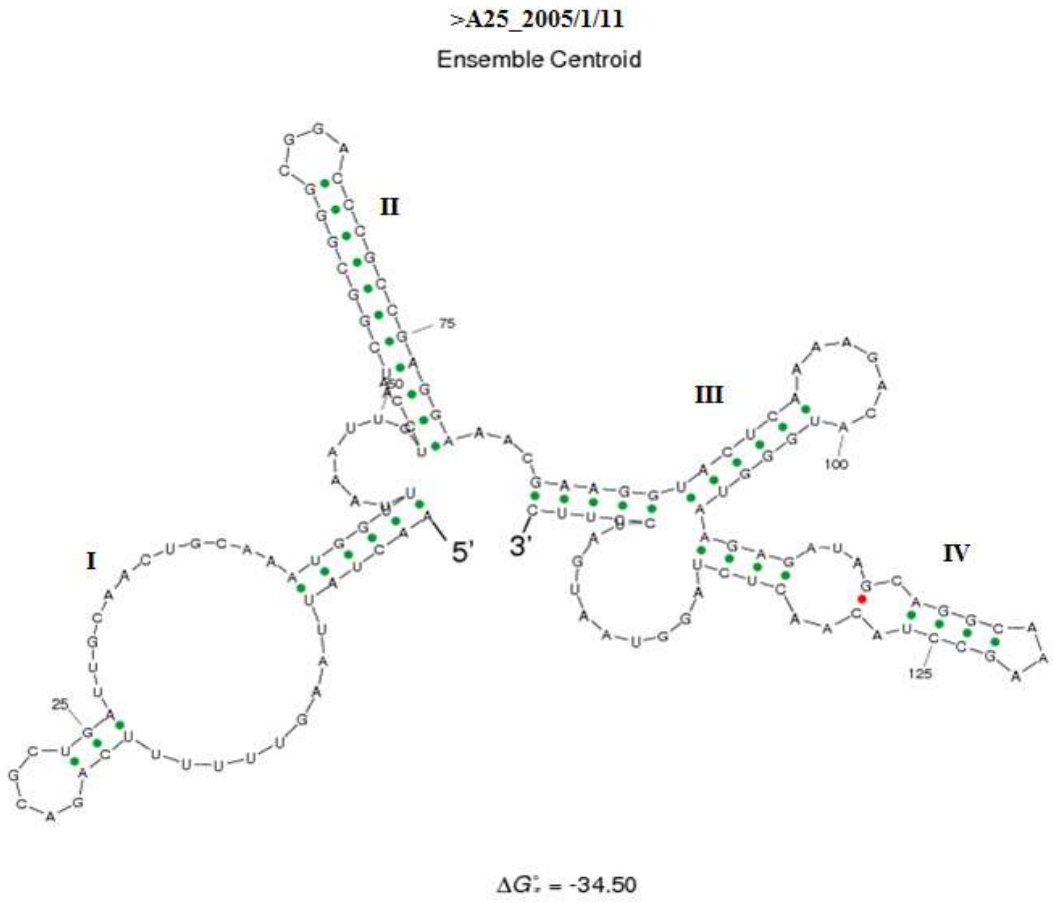

Figure 5: ITS2 secondary structure for *A. bataticola* isolate A25_2005/1/11. The red and green dots indicate hydrogen bonds between bases (A, C, G and U).

The 25 isolates of *A. bataticola* produced an assortment of models ranging from simple to complex structures. For instance, the isolate from Dan village, Limpopo, produced a unique secondary structure composed of a short helix and a very large bulge, whereas the isolate from Sasolburg had 3 multi-branched loops, 2 bulge loops and 6 helices. Similar findings in the ITS2 secondary structure of fungi was reported by Landis & Gargas [44].

There was great variation in the ITS2 secondary structures of the 25 isolates. However, there was some close similarities in a few isolates (The structures are available from the authors on request). For example, the isolate from the cultivar Beauregard and that from the breeding line 2004/9/2 generated similar structures with conserved elements (helix I, helix II and helix III). An additional interior loop in helix III of Beauregard could be used to separate the isolates. Perhaps the cultivar Beauregard was involved in the parentage of 2004/9/2. Although the ITS2 secondary structures in the isolates from Harry Gwala2 and Bophelong were very similar, there was an additional helix in the Harry Gwala2 isolate. These isolates clustered together in the dendrogram derived from the RAPD data analysis but not in the ITS2 sequence analysis. Four isolates 2004/9/1, 2005/1/11, W-119 and University of Zululand3 also produced similar secondary structures. These findings may suggest that W-119 is involved in the pedigree of the isolates 2004/9/1, 2005/1/11, and University of Zululand3. An additional interior loop was identified in the helix I of W-119 and University of Zululand3.

In spite of the variation observed in the secondary structures of the 25 isolates, a conserved helix II element was present in 16 of the isolates (2003/23/6, 2004/3/1, 2004/3/9, 2004/9/1, 2004/9/2, 2005/1/11, Beauregard, W-119, W-1192, Sasolburg, Harry Gwala1, Harry Gwala1, Bophelong, University of Zululand1, University of Zululand3 & Pretoria). Furthermore, a structural motif UAAC was present in helix II in 9 of the isolates (2004/3/1, 2004/3/9, 2004/9/1, 2004/9/2, 2005/1/11, Beauregard, W-119, W-1192 & University of Zululand3). A variant of this motif was observed in the Pretoria isolate. Conserved motifs such as UAAC in the ITS2 region are considered to have a functional significance [45] and may be involved in the processing of rRNA as well as the formation of 60S subunits [46].

Genetic diversity in *A.bataticola*

This study showed RAPD analysis, ITS sequencing and the secondary structures derived from the ITS2 sequences portray high levels of genetic variation among the isolates of *A. bataticola*. High levels of genetic variation has also been reported for other fungal pathogens [9, 47]. A high level of genetic variation in an organism is usually explained by sexual reproduction since it creates novel recombinants. However, no sexual stage of reproduction has been reported in *A. bataticola.* Bock *et al.* [48] reported high levels of genetic diversity among *A. brassicola* isolates and attributed this to an unidentified sexual stage in the life-cycle of *A. brassicola*. The genetic variation observed in *A. bataticola* in this study is perhaps due to a similar reason. Other mechanisms such as mitotic recombination, mutations, translocations, chromosomal deletions and duplications can also contribute to genetic variation in *A. bataticola.*

4. CONCLUSION

This study showed that molecular techniques such as RAPD, DNA sequencing and the secondary structures of the ITS2 regions are useful in assessing the genetic diversity of *A. bataticola*. High levels of genetic variation was present in the isolates irrespective of the geographic origins. The ITS2 secondary structures can be used as a tool to identify each of the isolates used in this study.

5. ACKNOWLEDGEMENTS

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