International Journal of Bioassays 7.6 (2018) pp. 5662-5667

Research Article

Open Access
CODEN: IJBNHY
ISSN: 2278-778X
International Journal of Bioassays

Analysis of genetic diversity of *Cercospora beticola* isolates from Swiss chard in Southern Botswana

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Received: 10/09/2018; **Revised:** 10/22/2018; **Accepted:** 10/24/2018
Available online: 31st October 2018

Abstract: Cercospora Leaf Spot (CLS) of Swiss chard (*Beta vulgaris* L. var. cicla) remains an important foliar disease worldwide, yet the causative agent *Cercospora beticola* genetic variability remains incompletely understood in Botswana. To assess the diversity of *C. beticola* from diseased Swiss chard in southern Botswana, 78 isolates from two farms were analyzed. *C. beticola* was isolated from Swiss chard at very high frequencies from both Bokaa (91%) and Glen Valley (86%) farms. However, statistical analyses indicated that the isolation rate of the pathogen was not affected by the sampling location ($p=0.01$, $p>0.01$). Phylogenetic analyses revealed that the 18 sequenced *C. beticola* isolates clustered into four major classes, which could not be differentiated by the sampling location. Similarly, genetic analysis revealed high genetic diversity of *C. beticola* strains from the two farms, accounted for by within population diversity (greatest pairwise distance=0.004). The results presented herein underscore the importance of assessment of genetic diversity of pathogens which may be important in targeted control and management of plant diseases.

Keywords: *Cercospora beticola*, ITS PCR (Internal Transcribed Spacer Polymerase Chain Reaction), genetic diversity, Swiss chard, Cercospora leaf spot (CLS)

Introduction

Swiss chard (*Beta vulgaris* L. var. cicla) is an important green leaf vegetable grown throughout Botswana [1, 2]. It is highly nutritive and its fresh leaves are rich in Vitamin C, K and A, minerals, dietary fibre and folic acid [3].

It therefore forms an important part of dishes in Botswana, both as a relish and salad.

In Botswana, Swiss chard cultivation meant for the local market is carried out at a commercial scale in open fields and greenhouses [1]. Recent efforts aimed at poverty eradication by the government have seen an upsurge in backyard gardens, especially in rural areas [4], which meet subsistence demands of poor rural households. Nonetheless, at all levels of production Swiss chard cultivation is subjected to a plethora of pest and microbial diseases [5, 6].

Leaf spot of Swiss chard is an important foliar disease in Botswana, having been reported as the major limiting factor in the production of Swiss chard due to favourable environmental conditions that are characterized by high temperature and humid conditions during the summer. Khare and Moeng [7] established *Cercospora beticola* as one of the fungal agents responsible for low spinach seed germination in Botswana while Utlwang et al. [8] studied the efficacy of locally available fungicides against Cercospora Leaf Spot (CLS) of Swiss chard under field conditions in the country.

Lesions of CLS are normally 3-5 mm in diameter and appear as light to dark brown circular spots on spinach leaves [9]. Progression of the disease eventually results in the blighted leaf falling to the ground and phytotoxins produced by *C. beticola* causing yellowing and death of the leaves [10]. Cercospora leaf spot consequently leads to economic losses due to decrease in yields [9]. Since fungicides are used to control the disease, additional losses are incurred through their procurement.

McDonald and Linde [11] having stated that understanding the origin of genetic variability in *C. beticola* is very important since it may affect the pathogen's ability to evolve in response to control measures such as deployment of resistant varieties or the application of fungicides. Therefore, this

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DOI: http://dx.doi.org/10.21746/ijbio.2018.7.6.3

study aimed to isolate *Cercospora beticola* from Swiss chard at two fields in Gaborone, Botswana. Sequencing of the Internal Transcribed Spacer (ITS) regions of the ribosomal RNA was thereafter utilized to compare the populations of *Cercospora beticola* thus obtained.

Materials and Methods

Sample collection

This study was carried out at two Swiss chard farms in southern Botswana; one located at Glen Valley (24° 35' 55" S | 25° 57' 46" E) and another located in Bokaa (24° 42' 79" S | 26° 02' 37" E). Fourty three and fourty five Symptomatic Swiss chard leaves showing CLS were collected from Glen Valley farm and Bokaa farm respectively. The leaves were placed into separately labelled sterile sample bags (Lab-Loc® Specimen) and the bags were then transported to the Mycology Laboratory (University of Botswana) in a cooler box containing ice packs for mycological analysis. All samples were processed within 4 h of collection.

Culture and morphological identification of *Cercospora beticola*

Isolation of *C. beticola* was performed according to a method described previously [12]. Two discs containing lesions were excised from each Swiss chard leaves. The discs were moistened by dipping into sterile water for 2 h to facilitate the release of pseudothecia. The discs were then plated on the surface of 2% malt extract agar (Merck, Darmstadt, Germany) and incubated for 24 h in the dark for the release and germination of canidia. The single canidia cultures thus germinated were examined for typical *Cercospora beticola* morphology (Figure 1), picked and transferred to fresh malt extract agar and then incubated at 25°C for 5-7 days.

DNA extraction and PCR amplification

Genomic DNA from fungal mycelia of each isolate was done using the Master Pure yeast DNA purification kit (Epicetre Biotechnologies, Madison, WI, USA) according to instructions from the manufacturer. PCR amplification utilized ITS1

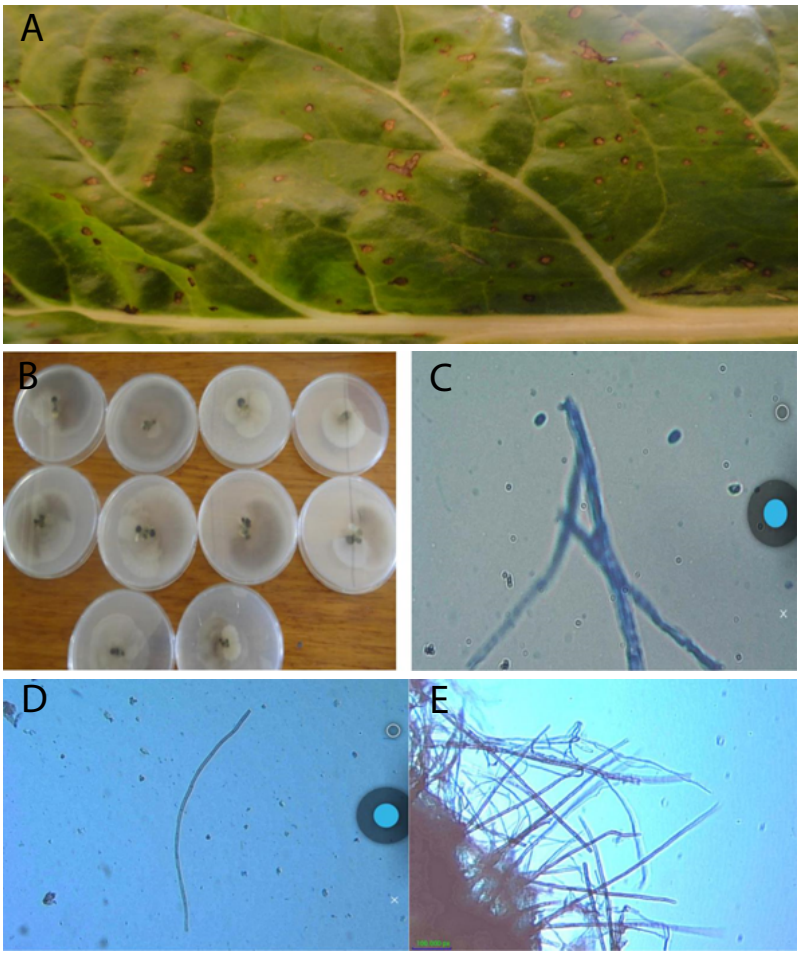

Figure 1: A. Shows symptomatic Swiss chard leaf collected at Bokaa farms; B. Cultures grown from the symptomatic leaves; C. Weakly developed stroma with conidiophores ; D. Filliform conidium of *C. beticola*, short and conically truncate at the tip; E. Loosely fasciculate conidiophores positioned on a stroma.

(5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [13] in a Techne thermocycler (Cole-Parmer, Staffordshire, UK). Each PCR reaction mixture consisted of 12.5 µL of 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 1 µL each of reverse and forward primers, 2 µL of genomic DNA and the mixture made up to 25 µL with sterile nuclease-free water.

The PCR was run with the following thermocycling conditions: initial denaturation at 94°C for 8 min; followed by 35 cycles for 1 min at 94°C, 55°C for 1 min and 2 min at 72°C and final extension at 72°C for 12 min. The PCR products were resolved on 1% agarose gel (Sigma Aldrich, Missouri, USA) for 1 h at 80 V. 5 µL of molecular weight marker (New England Biolabs) was loaded alongside 4 µL of PCR products. The gels were then visualized on a gel documentation system (Bio-Rad, Carlifornia, USA).). The PCR products were cleaned with a Clean-Up Kit (Zymo Research, Irvine, CA, USA) following instructions from the manufacturer. After purification, the products were sequenced in both directions using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in an automated ABI 3500XL sequencer (Applied Biosystems), following instructions from the manufacturer.

Statistical analyses

Graphpad Prism 7 (GraphPad Software Company, LaJolla, CA, USA) was used to analyze the statistical significance of the data. One-way ANOVA was employed to separate the means of occurrence of *Cercospora beticola* on Swiss chard in the two farms.

Phylogenetic analyses

Alignment of sequences was done using consistency-based algorithms implemented in Multiple Alignment using Fast Fourier Transform (MAFFT) version 7.307 [14] and assembly and editing of sequence data was performed using BioEdit [15]. The phylogenetic tree of ITS rDNA was constructed using MEGA (Molecular Evolutionary Genetics Analysis program) version 6.06 [16]. The tree was inferred using the Neighbour-Joining method based on the Kimura-2 model. Bootstrap analysis involved 1000 replicates for the tree. Furthermore, the genetic congruence among *C. beticola* isolates was analyzed using pairwise distances based on the Jukes-Cantor model [17] implemented in MEGA6.

Results

The present study comparatively analyzed 78 *Cercospora beticola* isolates from Swiss chard (*Beta vulgaris* L. var. cicla). *C. beticola* was isolated from Swiss chard in both Bokaa (91%) and Glen Valley (86%) farms (Table 1). However, statistical analyses revealed that the rate of isolation of the pathogen was not affected by the sampling location (p=0.01, p>0.01).

Isolates that were putatively identified as *C. beticola* using morphological and microscopic characteristics (Figure 1) were further identified by amplification of the Internal Transcribed Spacer (ITS) spacer region of rDNA utilizing ITS1 and ITS4 primers. All the 78 isolates that were amplified yielded bands that were approximately 550 base pairs (Figure 2).

Table 1: Incidence of *C. beticola* from Swiss chard at two farms in Southern Botswana.

Host species	No. (%) positive from	
	Glen Valley farm	Bokaa farm
Beta vulgaris (Swiss chard)	37 (86)*a	41 (91)

*=Numbers in parentheses indicate percentages of positive isolates.
a=The data are not significantly different (p=0.01, p>0.01).

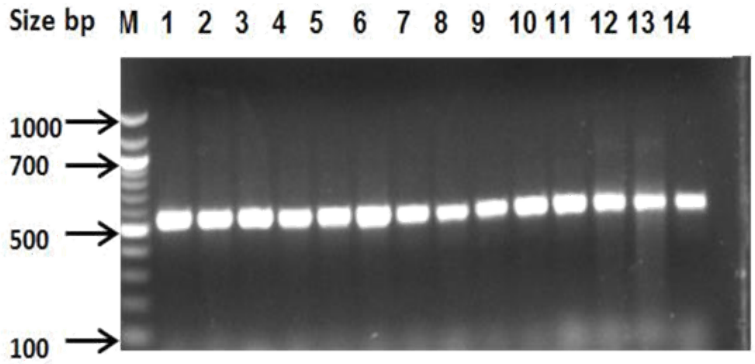

Figure 2: Gel electrophoresis of the amplified rDNA Internal Transcribed Sequence (ITS) region of *Cercospora beticola* isolates with ITS1 and ITS4 primers pairs. (M) 100 bp DNA ladder (New England Biolabs). *Cercospora beticola* isolates from; Glen valley Farms, (Lanes 1-7) and Bokaa farms, (Lanes 8-14).

The present study amplified the 78 strains from Bokaa and Glen Valley farms. Thereafter, 18 PCR products were randomly selected for sequencing. The samples consisted of 9 *C. beticola* strains isolated from Swiss chard from Glen valley farms and 9 other samples from Bokaa farms. The letters Gv and Bk in the phylogenetic tree (Figure 3) above refer to Glen Valley and Bokaa farms, respectively. Figure 3 shows that phylogenetic analysis of *C. beticola* isolates resulted in 4 clusters. In cluster 2, isolate Gv24 and Bk7 dispersed from the other four isolates within the cluster and can therefore be considered a sub-population within a cluster. Furthermore, it is important to note that there were representatives of *C. beticola* isolates from both farms within the four clusters.

The level of pairwise nucleotide variation between individual haplotypes of ITS gene were determined to be 0.000 to 0.004 (Figure 4), indicating high genetic diversity among *Cercospora beticola* isolates. The highest pairwise distance among the isolates was 0.004.

Discussion

Molecular characterization of pathogens of agricultural importance is a crucial factor in understanding epidemiology and control of these pathogens. Previous studies [18, 19] have reported that *C. beticola* caused losses estimated at US$45 million to the American Sugar Company, owing to fungicide application costs and yield losses.

Several genomic loci such as parts of the histone, actin and calmodulin genes have been employed for the molecular identification of *Cercospora* species [20]. In the present study, we employed the amplification of the Internal Transcribed Spacer Regions (ITS) and intervening 5.8S rRNA genes of *Cercospora beticola* isolates from two farms in Southern Botswana. Amplification resulted in fragments ranging between 500-600 base pairs, a finding that was consistent with previous studies [21, 22].

In this study, *Cercospora beticola* was isolated from

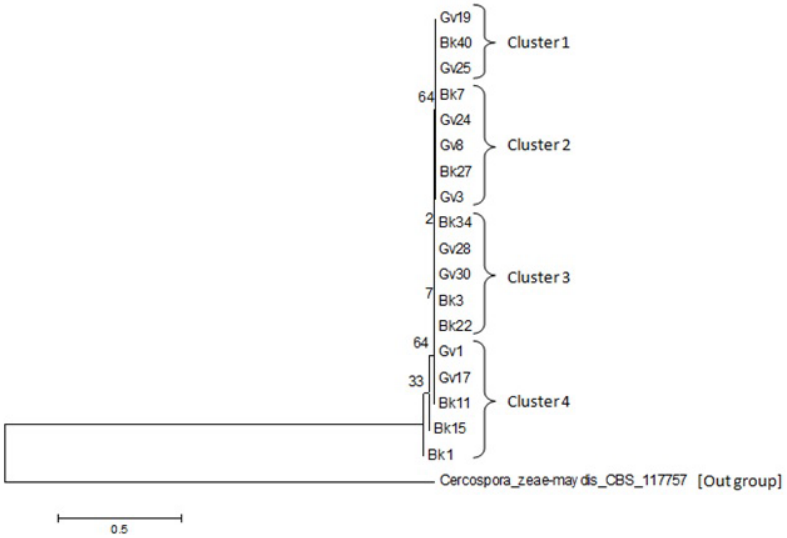

Figure 3: Evolutionary relationships among *C. beticola* isolates from Bokaa (denoted Bk) and Glen valley (Gv) farms. *Cercospora zeaemaydis* strain CBS 117757 was included as an outgroup.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Gv8																		
2. Gv17	0.004																	
3. Bk7	0.002	0.004																
4. Gv19	0.002	0.002	0.002															
5. Bk11	0.004	0.000	0.004	0.002														
6. Bk40	0.002	0.002	0.002	0.000	0.002													
7. Bk27	0.000	0.004	0.002	0.002	0.004	0.002												
8. Bk15	0.004	0.000	0.004	0.002	0.000	0.002	0.004											
9. Gv28	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002										
10. Gv24	0.002	0.004	0.000	0.002	0.004	0.002	0.002	0.004	0.004									
11. Gv30	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002	0.000	0.004								
12. Bk3	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002	0.000	0.004	0.000							
13. Bk22	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002	0.000	0.004	0.000	0.000						
14. Bk1	0.004	0.000	0.004	0.002	0.000	0.002	0.004	0.000	0.002	0.004	0.002	0.002	0.002					
15. Gv3	0.000	0.004	0.002	0.002	0.004	0.002	0.000	0.004	0.002	0.002	0.002	0.002	0.002	0.004				
16. Gv4	0.004	0.000	0.004	0.002	0.000	0.002	0.004	0.000	0.002	0.004	0.002	0.002	0.002	0.000	0.004			
17. Bk34	0.000	0.004	0.002	0.002	0.004	0.002	0.000	0.004	0.002	0.002	0.002	0.002	0.002	0.004	0.000	0.004		
18. Gv21	0.002	0.002	0.002	0.000	0.002	0.000	0.002	0.002	0.004	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.002	

Figure 4: Pairwise distances among *C. beticola* isolates infecting Swiss chard based on nucleotide sequence of ITS gene.

#Swiss chard isolates from Bokaa are indicated as Bk while isolates from Glen valley are indicated as Gv followed by the isolate number, respectively.

diseased leaves of Swiss chard (*Beta vulgaris* L. var. cicla). *C. beticola* was isolated at high frequencies from both Bokaa (91%) and Glen valley (86%) farms. To assess the genetic diversity of the isolates, we randomly pooled 18 isolates from the 78 strains that were putatively identified as *Cercospora beticola* by PCR for sequencing. Phylogenetic analysis revealed that the 18 strains clustered into four major clusters, including a distinct sub-cluster within cluster 2. This indicates a high degree of genetic diversity of *C. beticola* isolates in the present study. Notably, clustering of the isolates could not be distinguished by their geographic origin (Bokaa or Glen valley farms). Moretti et al. [23] also found great intraspecific variability within a small population of *Cercospora beticola* infecting sugarbeet in Italy. Also in concordance with the present study, high genetic diversity was found within populations of *C. beticola* from several European countries [24]. Furthermore, we evaluated the genetic diversity of Swiss chard populations of *C. beticola* using pairwise distances. This analysis also found high genetic diversity of *C. beticola* isolates ranging from 0.000 to 0.004. Similar to results obtained utilizing phylogenetic analysis, populations of *C. beticola* could not be differentiated based on place of origin. These results are consistent with a recent study [25] which found genetically diverse *C. beticola* isolates obtained from Swiss chard and table beet that could not be discriminated based on sampling location or host in five fields at New York and Hawaii in the USA. Rousset [26] postulated that genetic diversity can be attributed to fungal populations with long distance dispersal of ascospores where a pattern of isolation by distance is detected resulting from gradual spread of the disease from its original source, hence resulting in genetic differentiation as distance increases. This study has demonstrated the genetic diversity of *Cercospora beticola* from Swiss chard at two farms of southern Botswana. The high genetic diversity is rather surprising because the pathogen is asexual, with no known sexual phase. However, mating type studies [27] have found evidence that suggested a cryptic sexual cycle in *C. beticola*. In light of the high levels of genetic diversity associated with isolates in the present study, we intend to conduct additional tests on the strains to assess the frequency of mating type genes in *C. beticola*. To ascertain this however, cross-inoculation tests will also be needed for verification [28].

Conclusion

Knowledge of genetic diversity is an important tool in optimizing control of crop pathogens. A recent study found resistance to some pesticide by *C. beticola* causing CLS in Swiss chard in Botswana, thus suggesting that chemical control alone may not prove a viable option. It remains a distinct possibility that some resistance may be linked to the genetic diversity of *C. beticola* isolates in Botswana as demonstrated in this study. It thus remains important in pathogen disease cycles, to study the plant host as well as the genetic diversity of the pathogen for enhanced efficacy of control measures that prevent yield losses in important crops such as Swiss chard.

Acknowledgements

The Office of Research and Development, University of Botswana is acknowledged for funding this research.

References:

1. Obopile M, Munthali DC, Matilo B. Farmers percerptions and management of vegetable pests and diseases in Botswana. Crop Prot. 27 (2008):1220-1224.
2. Madisa ME, Assefa Y, Obopile M. Asssesment of production constraints, crop and pest management practices in peri-urban vegetable farms of Botswana. Egypt Acad J Biol Sci. 1.1 (2010):1-11.
3. Bakry AA, Youssed ME, El-Gindy AA. Utilization of Swiss chard as functional food. Nature and Science 12.12 (2014):104-111.
4. Marumo DS, Madisa ME, Lesole T. The effectiveness of backyard gardening initiative in poverty eradication: The case of beneficiaries in Ramotswa village, Botswana. Journal of Poverty, Investment and Development. 33 (2017):59-74.
5. Baliyan SP. Improving Sustainable Vegetable Production and Income through Net Shading: A Case Study of Botswana. J Agri Susta. 5.1 (2014):70-103.
6. Daiber KC. Injurious insects, mites and nematodes on various vegetables in southern Africa. J Plant Diseases Prot. 103.3 (1996):325-332.
7. Khare KB, Moeng KO. Fungi associated with seeds of two varieties of spinach beet and their possible control in vitro by fungicide treatment. Int J Bioassays.4.11 (2015):4515-4517.
8. Utlwang R, Khonga EB, Khare KB, Segwagwe AT. Assessing the effectiveness of locally available fungicides in managing Cercospora leaf spot of Swiss chard under field conditions in Botswana. Int J Food Agri Veter Scie. 7.1 (2017):1-8.
9. Jacobsen BJ, Franc GD. Cercospora leaf spot.

In: Compendium of beet diseases and pests 2nd Ed, Harveson RM Hanson LE and Hein GL Eds APS Press. St Paul Minnesota USA. (2009):7-10.

10. Steinkamp MP, Martin SS, Hoefert LL, Ruppel EG. Ultrastructure of lesions produced in leaves of Beta vulgaris by cercosporin, a toxin from Cercospora beticola. Phytopathol. 71.12 (1981):1272-1281.
11. McDonald BA, Linde C. Pathogen population genetics, evolutionary potential, and durable resistance. Annual Rev Phytopathol. 40 (2002):349-379.
12. Crous PW. Mycosphaerella spp. and their anamorphs associated with leaf spot diseases of Eucalyptus. Mycology Memoir. 21 (1998):1-170.
13. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ Eds PCR protocols. A guide to methods and applications. Academic Press. San Diego. (1990):315-322.
14. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 30.4 (2013):772-780.
15. BioEdit. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).
16. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis Version 6.0. Mol Biol Evol. 30.12 (2013):2725- 2729.
17. Jukes TH, Cantor CR. Evolution of protein molecules. In Munro HN Ed Mammalian protein metabolism Academic Press. New York. (1969):21-132.
18. Cattanach A. Managing Cercospora leafspot tolerance or resistance to fungicide. American Crystal Sugar Company. (1999).
19. Khan M, Smith LJ. Evaluating fungicides for controlling Cercospora leaf spot on sugar beet. Crop Prot. 24.1 (2005):79-86.
20. Groenewald JZ, Nakashima C, Nishikawa J, Shin HD, Park JH, et al. Species concepts in Cercospora: spotting the weeds among the roses. Studies in Mycology. 75.1 (2013):115-170.
21. Groenewald M, Groenewald JZ, Crous PW. Distinct species exist within the Cercospora apii morphotype. Phytopahol. 95.8 (2005):951-959.
22. Lartey RT, Weiland JJ, Caesar-TonThat C, Bucklin-Comiskey S. A PCR protocol for rapid detection of Cercospora beticola in sugar beet tissue. J Sugarbeet Res. 40.1-2 (2003):1-10.
23. Groenewald M, Linde CC, Groenewald JZ, Crous PW. Indirect evidence for sexual reproduction in Cercospora beticola populations from sugar beet. Plant Pathol. 57 (2008):25-32.
24. Vaghefi N, Nelson SC, Kikkert JR, Pethybridge J. Genetic structure of Cercospora beticola populations on Beta vulgaris in New York and Hawaii. Scientific Reports. 7.1 (2017):1726.
25. Rousset F. Genetic differentiation and estimation of gene flow from F statistics under isolation by distance. Genetics. 145.4 (1997):1219-1228.
26. Groenewald M, Groenewald JZ, Harrington TC, Abeln ECA, Crou PW. Mating type gene analysis in apparently asexual Cercospora species is suggestive of cryptic sex. Fung Gene Biol. 43.12 (2006):813-825.
27. Ruppel EG. Cercospora leaf spot. Compendium of beet diseases and insects. Whitney ED and Duffus JE Eds St Paul MN American Phytopathology Society. (1986).
28. Moretti M, Saracchi M, Farina G. Morphological, physiological and genetic diversity within a small population of Cercospora beticola Sacc. Annals Microbiol. 54.2 (2004):129-150.

Cite this article as:

Rebecca Utlwang, Daniel Loeto, Krishna B. Khare, Kabo R. Wale, Elenimo B Khonga et al. Analysis of genetic diversity of Cercospora beticola isolates from Swiss chard in southern Botswana. *International Journal of Bioassays 7.6 (2018) pp. 5662-5667*. DOI: http://dx.doi.org/10.21746/ijbio.2018.7.6.3