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Genetic diversity of *Fusarium solani* f.sp. *cucurbitae* the causal agent of crown and root rot of watermelon in Tunisia using ISSR markers

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Abstract

Fusarium solani f.sp. *cucurbitae* (*F.s.c.*) Snyder and Hansen is one of the most important fungi causing serious damages to the cucurbits production areas in Tunisia. The goal of this investigation was to study the genetic characterization of *F. solani* f.sp. *cucurbitae* Tunisian population. Ten isolates of F.s.c. (*F.s.c.*1 to *F.s.c.*10) collected from infected watermelon plants in numerous regions of Tunisia; were subjected to PCR identification using ITS1-ITS4 primers pair, and Inter-simple sequence repeat (ISSR) technique. Results obtained confirmed the presence of *F. solani* f.sp. *cucurbitae* race 1 in Tunisia. ISSR dendrogram of the ten *F.s.c.* isolates generated by bi- and trinucleotide primers; and conducted by the unweight pair grouping using mathematical averaging (UPGMA) method from Nei's genetic distance, revealed the presence of similarities between the isolates that ranged from 31 to 84%. The isolates *F.s.c.*8 (collected from Gabes) and *F.s.c.*4 (collected from Sidi Bouzid), were genetically dissimilar presenting the lowest similarity coefficient. In contrast; isolates *F.s.c.*3 (collected from Sfax) and *F.s.c.*1 (collected from Beja) were similar. Cluster analysis based on UPGMA of the ISSR markers data assembled *F.s.c.* isolates into two major groups. These results are helpful to develop integrated management and future breeding programs for plant resistance.

Keywords: *Fusarium solani* f.sp. *cucurbitae*, molecular detection, genetic diversity, ISSR, watermelon

1. Introduction

Fusarium crown root rot caused by *F. solani*, was considered as one of the most devastating cucurbits soil-borne disease, affecting cultivations in many countries around the world (Champaco *et al*., 1993). This pathogen was responsible for cortical rot at the stem base and at the upper portion of the tap roots; causing yellowing and wilting of leaves, soft-circular lesions developed for fruits in contact with

soil (Martyn, 1996; Armengol *et al*., 2000; Boughalleb *et al*., 2005; Hussein and Juber, 2015).

F. solani species complexes were frequently isolated from different environmental sources such as; the soil, air and plants (Debourgogne *et al*., 2010). Hawthorne *et al*. (1992) reported the presence of two races of *F.s.c*. causing fruit, crown and foot rots in cucurbit plants. *F.s.c*. race 1 was responsible for crown root rots of cucurbits; whereas, *F.s.c*. race 2 caused fruit rot only. Several investigations indicated that races 1 and 2 were not easily distinguished morphologically, but their identification required mating, pathogenicity tests and molecular tools (Hawthorne *et al*., 1994; Boughalleb *et al*., 2005; Mehl and Epstein, 2007). Universally; *F. solani* species complex was recognized with a very high diversity morphological level (Brasileiro *et al*., 2004).

Nevertheless; the classification system based only on morphology did not provide an accurate tool for the identification of *F. solani* species complex, and the relationship between isolates within species. The molecular approach was promising in the establishment of a convenient characterization involving; sequence typing, restriction fragment length polymorphism, microsatellite analysis and multi-locus sequence typing (MLST) (Godoy *et al*., 2004; Zhang *et al*., 2006; Mehl and Epstein, 2007; O'Donnell *et al*., 2008; Debourgogne *et al*., 2010; Chehri *et al*., 2011). The ISSR technique; originally described by Zietkiewicz *et al*. (1994) to analyze genetic diversity in plants and animals, had proven to be a powerful tool and applicable to fungi (Leung *et al*., 1993; Hantula *et al*., 1996; Zhou *et al*., 2001). Many authors (Wunsch and Hormaza, 2002; Cohen *et al*., 2003; Mishra *et al*., 2003; Borja *et al*., 2006; Shafiquzzaman *et al*., 2012), confirmed this technique to be useful in generating genetic diversity in several groups of fungi. In Tunisia, *F.s.c*. race 1 and race 2 were detected in several watermelon production areas (Boughalleb *et al*., 2005; Boughalleb and El Mahjoub, 2006). Thus, the determination of both host specificity and genetic diversity in *F.s.c*. populations are important in developing a plant breeding program for resistance. Therefore; the objectives of this study were to identify the *F.s.c*. isolates using ITS primers, and to investigate the genetic diversity of Tunisian populations based on the ISSR technique.

2. Materials and methods

2.1. Fungal isolates

F.s.c. pathogen was isolated from watermelon plants showing typical crown root rot symptoms sampled from different regions of Tunisia.

2.2. DNA extraction

DNA of the ten Tunisian isolates of *F. solani* f.sp. *cucurbitae* was extracted. The isolates were grown in 250 ml of potato dextrose broth (PDB) (Difco) in a rotary shaker at 180 rpm, for 48 h at 28°C. Then; the genomic DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA, USA), following manufacturer's instructions.

2.3. PCR amplification

The ITS rDNA regions containing partial ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') sequences were amplified with PCR (White *et al*., 1990). Each PCR reaction mixture contained 1.25×PCR buffer, 1.25 Mm $MgCl_2$, 1 µM each dNTP, 0.5 µM of each primer, 0.1 U of DNA Taq polymerase (Dominion MBL, Córdoba, Spain), and 1 µl of template DNA. The PCR reaction mix was adjusted to a final volume of 13 µl with water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). DNA amplification was performed using PCR with a Peltier Thermal Cycler-200 (MJ Research). The program consisted of an initial step of 5 min. at 94°C; followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 56°C for 1 min., and an elongation at 72°C for 2 min. A final extension was

performed at 72°C for 7 min. Five microliters of PCR products were subjected to electrophoresis in 0.7% agarose gels (agarose D-1 Low EEO; Conda). The amplification products were examined under UV light after ethidium bromide staining; and photographed using Alpha digidoc 1000 gel documentation system (Alpha Innotech Corporation, USA), for bands scoring. The 100 bp DNA ladder (Biotools, Madrid, Spain) was used as molecular size marker. Amplification from each DNA sample was repeated at least twice. The PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics). Finally; these PCR products were visualized in 1.5% agarose gels (agarose D-1 Low EEO, Conda, Madrid, Spain), and molecular weights were estimated using the GeneRuler 100 bp Plus DNA Ladder (Fermentas, Carlsbad, CA).

2.4. Genetic diversity

The same 10 isolates that have been identified as *F. solani* f.sp. *cucurbitae* were used in this study. A total of six ISSR primers were evaluated for their capacity to produce polymorphic, scored and reproducible DNA fingerprint patterns. The primers used were two dinucleotide, and four trinucleotide repeats with or without 5' anchors: 5'DVD (CT) 7C (Mahuku *et al*., 2002), 5'YHY (GT) 7G, 5'DDB (CCA) 5 (Hantula *et al*., 1997), 5' (GAC) 5, 5' (GTG) 5 (Pina *et al*., 2005) (Tib Molbiol, Berlin). Each PCR reaction contained 1 PCR buffer, 2.5 mM MgCl2, 100 mM each dNTPs, 0.4 mM of each primer, 0.5 U DNA Taq polymerase (Dominion MBL, Cordoba), and 0.5–5 ng template DNA were quantified spectrophotometrically. The PCR reaction mix was adjusted to a final volume of 25 ml with sterile distilled water (Chromasolv Plus, Sigma-Aldrich, Steinheim). PCR amplifications were performed on a Peltier Thermal Cycler-200 (MJ Research, Waltham, Massachusetts). The program consisted of an initial step of 5 min. at 95°C; followed by 34 cycles of denaturation at 95°C for 1 min., annealing at 41°C (CT) 7, 58°C (GT) 7, 64°C (CCA) 5, 61°C (CGA) 5, 46°C (GAC) 5, 56°C (GTG) 5 for 1 min., and an elongation at 72°C for 2 min. The final extension was performed at 72°C for 10 min. Electrophoresis was conducted at 80V, 500 mA for 160 min.; after which the PCR products were electrophoretically separated in a 1.5% agarose gel in Tris-Acetate-EDTA buffer, visualized by ethidium bromide and then photographed under UV light (Eagle Eye II, Still Video System). Gene Ruler 100 bp DNA Ladder Plus was used as a molecular weight marker (MBI Fermentas). All PCR amplifications were performed at least twice for each isolate. ISSR assay was repeated three times; whereas, only clear and reproducible bands were considered.

2.5. Statistical analysis

Fragments amplified by the ISSR primers were visually scored as present (1) or absent (0). However, fragments with the same size were considered equal. The similarity matrix was used to construct dendrogram by the help of the unweight pair grouping using mathematical averaging (UPGMA) method, and the Nei's coefficient.

3. Results

3.1. Molecular identification of *F. solani* f.sp. *cucurbitae*

Results showed that ten *F.s.c.* isolates were recovered from watermelon plants of different origins (Table 1). They were amplified positively using ITS1-ITS4 primers pair of 400 bp fragments each (Fig.1).

3.2. ISSR analysis

Six ISSR primers were tested individually using DNA of *F.s.c*. isolates; to determine their results in profile of light bands on agarose gels; however, they showed polymorphisms between different isolates (Fig. 2). Seventy-four bands were amplified; where the scorable bands ranged in size between 200-2000 bp.

Table 1. Origins of the ten *F. solani* f. sp. *cucurbitae* (*F.s.c.*) isolates used in this study

Isolates	Regions	Hosts
*F.s.c.*1	Testour (Beja)	Watermelon
*F.s.c.*2	Chebika (Kairouan)	Watermelon
*F.s.c.*3	Jbeniana (Sfax)	Watermelon
*F.s.c.*4	Regueb (SidiBouzid)	Watermelon
*F.s.c.*5		Watermelon
*F.s.c.*6	Metouia (Gabes)	Watermelon
*F.s.c.*7		Watermelon
*F.s.c.*8		Watermelon
*F.s.c.*9	Mareth (Gabes)	Watermelon
*F.s.c.*10	Skira (Gabes)	Watermelon

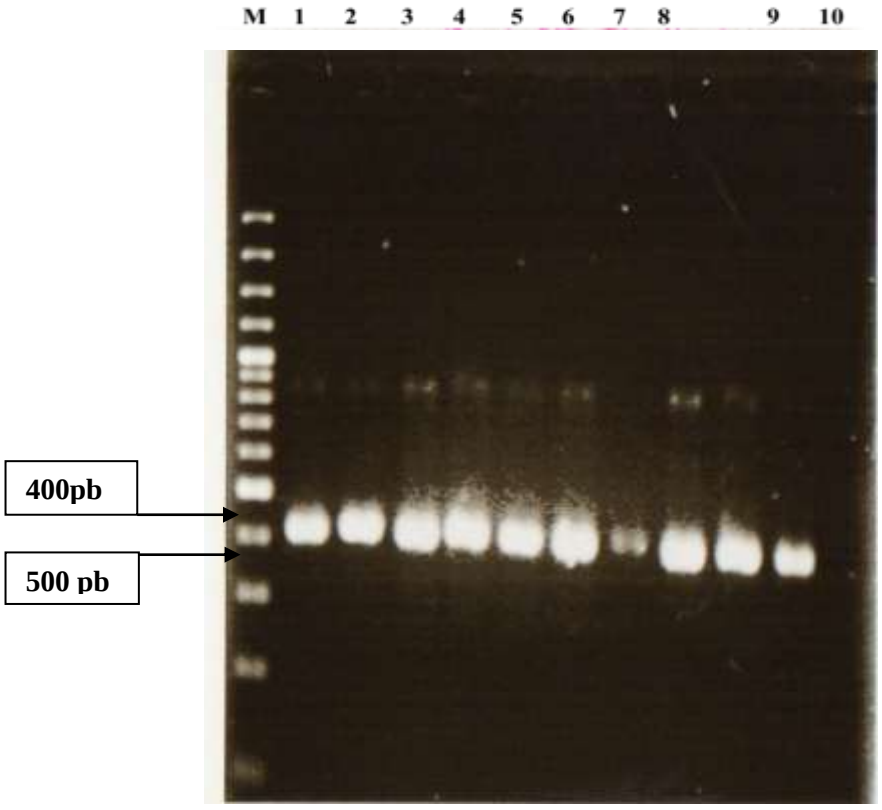

Fig. 1: ITS1 and ITS4 primers; produced band on agarose gel 0.7% (M: Size marker); 1: *F.s.c.*8, 2: *F.s.c.*10, 3: *F.s.c.*6, 4: *F.s.c.*2, 5: *F.s.c.*3, 6: *F.s.c.*7, 7: *F.s.c.*5, 8: *F.s.c.*1, 9: *F.s.c.*4, and 10: *F.s.c.*9

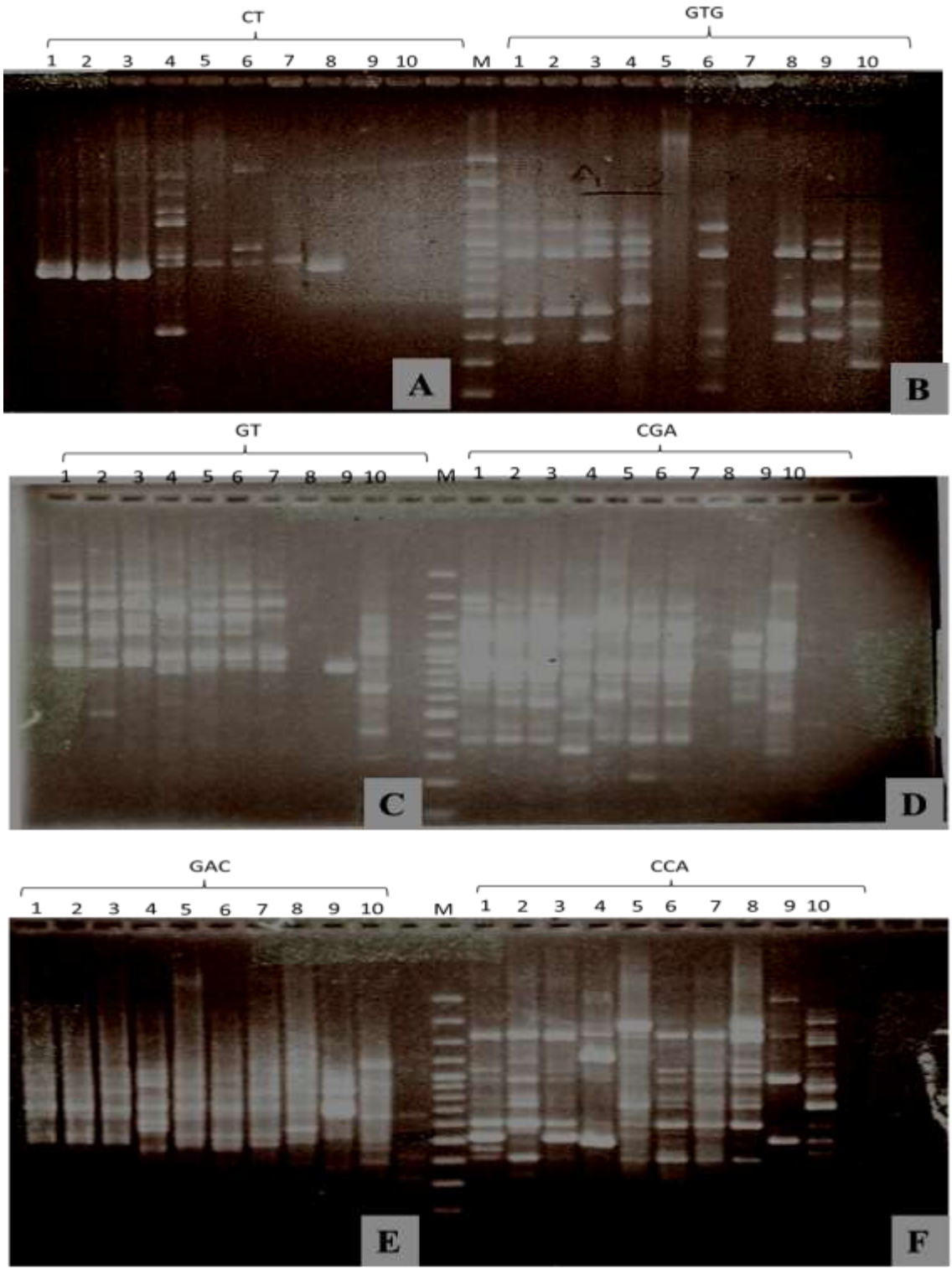

Fig. 2: Agarose gels showing ISSR banding patterns generated by primers CT (a), GTG (b), GT (c), CGA (d), GAC (e) and CCA (f), of *F.s.c.* isolates (*F.s.c.*1 to *F.s.c.*10); M: 3000-bp DNA ladder marker.

CT primer generated intraspecific polymorphism among different isolates with 19 bands which ranged between 500-2300 bp (Fig. 2.a). Similarly, GTG primer produced 18 bands with sizes varied between 400-1200 bp (Fig. 2.b) followed by; GT, CGA, GAC and CCA primers, having discriminate 12 bands with values between 350-1800 bp (Fig. 2.c), 300-2000 bp (Fig. 2.d), 500-1500 bp (Fig. 2.e), and from 350-2000 bp (Fig. 2.f), respectively.

Reproducibility of amplified bands was confirmed with GT and CCA primers. GT primer however was chosen to analyze the genetic diversity of *F.s.c*. population. In some cases, no DNA bands were produced with certain primers. The genetic distance matrix separated the *F.s.c*. isolates into distinct groups (Table 2). Results showed high genetic diversity, and *F.s.c*. isolates were distributed at a similarity percentage comprised between 33-84%. ISSR analysis data set among isolates provided a genetic homology between *F.s.c*.1, *F.s.c*.2 and *F.s.c*.3 (83%), and between F.s.c.6 and F.s.c.7 (82%). Moreover, *F.s.c*.6 and *F.s.c*.7 isolates shared a similarity of 60 and 50% with *F.s.c*.5 and *F.s.c*.10 isolates, respectively.

UPGMA and Nei's genetic distance data revealed low genetic polymorphism level within the Tunisian *F.s.c*. populations. The 10 genotypes were divided into two major groups designed as I and II. Cluster I comprised a single genotype *F.s.c*.10 which could be considered as out grouping sample. However, Cluster II which was the abundant one consisted of the rest of the *F.s.c*. isolates. Cluster II was divided into three subgroups joined with 40% of genetic similarity namely; IIa, IIb and IIc. The subgroup IIa included seven isolates with relatively similar coefficient of 71%. A genetic similarity over 80% was registered between *F.s.c*.1 (Beja), *F.s.c*.2 (Kairouan), *F.s.c*.3 (Sfax); and between *F.s.c*.6, *F.s.c*.7 (Gabes), which however, generated a genetic dissimilarity with *F.s.c*.8 (Gabes), *F.s.c*.5 (Sidi Bouzid). Subgroup IIb and IIc comprised one isolate each; *F.s.c.* 4 (Sidi Bouzid) and *F.s.c*. 9 (Gabes), respectively (Fig. 3).

4.Discussion

In this study, molecular identification of a Tunisian *F.s.c*. population was established using the two primers ITS1 and ITS4. The obtained molecular data supported the morphology identification of the same *F.s.c*. population established in previous study of Boughalleb and El Mahjoub, (2006). Current results were in agreement with the findings of Chehri *et al*., (2011), who mentioned that molecular approach by PCR-ITS analyses strongly supported the existence of two distinct clades among *F. solani* isolates. This technique was reported by Crowhurst *et al*., (1991) to be a rapid method for producing species- or race-specific probes for mold fungi. Therefore, this PCR method provided a simple procedure to distinguish between *Fusarium* strains. Hussein and Juber, (2015) used PCR technique with species-specific primers to identify thirty isolates of *F.s.c*.; and found that nineteen isolates belonged to race 1 and four isolates to race 2, whereas, seven isolates were not amplified with primers of both races. Similar results were also reported by several authors in many countries such as Mehl and Epstein, (2007) in California, and Hong *et al*., (2010) in Korea. In the same sense, Konstantinova and Yli-Mattila, (2004) earlier established PCR–RFLP of ribosomal intergenic spacer region to analyze *Fusarium* species. In our study, ISSR analysis showed similarities between the populations of *F.s.c*. in Tunisia. Thus; ISSR markers can be a quick and reliable alternative to separate isolates of *Fusarium* spp. into their respective pathogenicity groups, which supported the results of our current study. Usefulness of such technique in differentiating between closely related strains was supported by many authors in the field of molecular biology such

Table 2. Genetic distance values calculated from ISSR patterns of the 10 isolates of *F. solani* f.sp. *cucurbitae* (*F.s.c.*) used in this study

Proximity Matrix										
	Nei's genetic distance									
	***F.s.c*.1**	***F.s.c*.2**	***F.s.c*.3**	***F.s.c*.4**	***F.s.c*.5**	***F.s.c*.6**	***F.s.c*.7**	***F.s.c*.8**	***F.s.c*.9**	***F.s.c*.10**
***F.s.c*.1**	1.00	0.836	0.842	0.393	0.510	0.731	0.642	0.678	0.560	0.508
***F.s.c*.2**	0.836	1.00	0.828	0.484	0.577	0.717	0.667	0.700	0.588	0.467
***F.s.c*.3**	0.842	0.828	1.00	0.500	0.556	0.764	0.643	0.613	0.642	0.419
***F.s.c*.4**	0.393	0.484	0.500	1.00	0.448	0.475	0.533	0.333	0.386	0.364
***F.s.c*.5**	0.510	0.577	0.556	0.448	1.00	0.735	0.680	0.536	0.383	0.357
***F.s.c*.6**	0.731	0.717	0.764	0.475	0.735	1.00	0.824	0.632	0.500	0.316
***F.s.c*.7**	0.642	0.667	0.643	0.533	0.680	0.824	1.00	0.552	0.367	0.310
***F.s.c*.8**	0.678	0.700	0.613	0.333	0.536	0.632	0.552	1.00	0.400	0.375
***F.s.c*.9**	0.560	0.588	0.642	0.386	0.383	0.500	0.367	0.400	1.00	0.364
***F.s.c*.10**	0.508	0.467	0.419	0.364	0.357	0.316	0.310	0.375	0.364	1.00

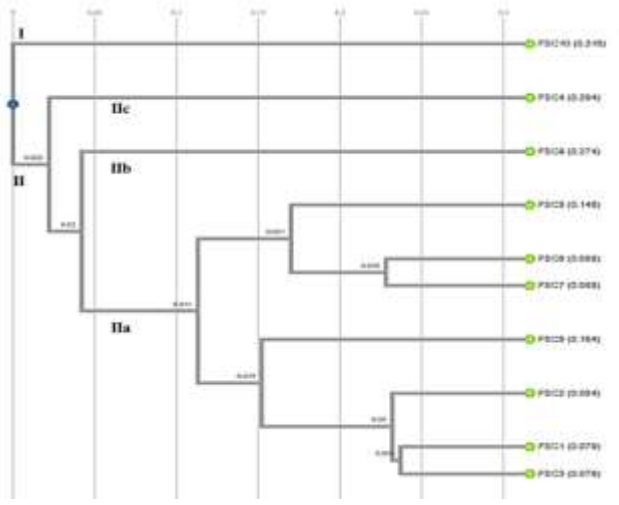

Fig. 3: Dendrogram showing relationships among the 10 isolates of *F. solani* f. sp. *cucurbitae* (*F.s.c.*), based on unweight pair grouping by mathematical averaging (UPGMA) method using the Nei's coefficient.

as; Hantula and Müller, (1997; Zhou *et al*., (1999); Hong *et al*., (2010); Kolawole *et al*., (2015), and proved to be particularly useful for resolving taxonomic conflicts within *F. solani* strains. In our investigation, the ISSR analysis showed different levels of genetic diversities among the *F.s.c*. isolates collected from different geographical areas. This might be attributed to differences in frequency of the genomic sequences; which was a reflection of difference in the genome size, and in the level of repetitive DNA of the investigated species. Moreover, results of ISSR analysis that had been obtained with individual ISSR primers between 23 and 57 nucleotides in length; was supported by the previous suggestion of Welsh and McClelland, (1990). These authors affirmed that any unique primer of any size might be useful to detect the polymorphisms between organisms. Crowhurst *et al*., (1991) revealed that advantages of using such primers were that they were readily available in most laboratories; and frequently included one or more restriction endonuclease cleavage sites as a part of the primer sequence.

Conclusion

The present study highlighted the genetic basis of these potential diagnostic markers; and provided additional insights into the molecular evolution of *F.s.c*., the causal agent of crown and root rot of watermelon in Tunisia. Both ITS and ISSR analyses were very useful in assessing the intraspecific diversity of this pathogen. In addition, current results will be useful for the development of integrated strategies for disease management and breeding programs.

Conflict of interests

No potential conflict of interests was reported by the authors.

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