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TD-DAMD and TU-DAMD polymorphism in *Arthrocnemum macrostachyum* Moris species

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Abstract

Arthrocnemum macrostachyum (Moric.) Moris. & Delponte perennial succulent halophyte shrub suffered from taxonomic confusion. Thereby, the present study focused on phylogenetic relationships investigation among three *A. macrostachyum* genotypes grown along the Syrian coast. DNA polymorphism of *A. macrostachyum* has been investigated based on touch-down directed amplification of minisatellite DNA (Td-DAMD) and touch up-directed amplification of minisatellite DNA (TU-DAMD) markers in comparative study. Seventeen DAMD primers were tested for each TD-DAMD and TU-DAMD assesses. Comparative study revealed that TU-DAMD assay was more potent than TD-DAMD one by exhibiting the highest polymorphism level (P%) 92.035% & 87.805% of and polymorphic information content (PIC) values of and 0.411 and 0.386, for TU-DAMD and TD-DAMD, respectively. Clustering profile constructed based on the both examined assesses gave similar pattern; where, *A. macrostachyum2* genotype was genetically so far from the other two genotypes. Thereby, the actual data confirmed previous reports suggesting the existence of two subspecies belonged to *A. macrostachyum* species. Otherwise, Due to efficacy of TU-DAMD for genetic variation revealing of *A. macrostachyum* species; it is advice to employ this technique as a new assay in phylogenetic studies of other plant species.

Keywords: *Arthrocnemum macrostachyum*, polymorphism,TD-DAMD, TU-DAMD.

Introduction

Arthrocnemum macrostachyum (Moric.) Moris. & Delponte is one of the 51 species belongs to *Arthrocnemum* genus. It belongs to Chenopodiaceae family, Salicornioideae subfamily included 14-16 genera belonged to 90 species (Kunget al., 1979; Saleh, 2015). This halophytic shrub formed pure stands typically in coastal regions and salt Mediterranean marshes (Vicente et al., 2007; Saleh, 2011).

It has been demonstrated that the name *Arthrocnemum* is a synonym of *Salicornia;* of which *Arthrocnemum glaucum* Ung.-Sternb., *Arthrocnemum indicum* (Willd.) Moq. and *Salicornia macrostachya* Moric are synonyms of *Arthrocnemum macrostachyum* (Moric.) Moris. & Delponte. To earlier, Scott (1977) however, reported that the three genera of *Salicornia, Salicornia* L. and *Arthrocnemum* Moqwere distinguished each to other. In this regards, *Salicornia* L. and *Arthrocnemum* Moq genera were separated from *Salicornia* based on their morphological criteria. Whereas, Kadereit et al. (2006) reported similar results using internal transcribed spacer (ITS) and atpB–rbcL spacer sequences.

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Moreover, many studies focused on *Salicornia* phylogenetic using RAPD marker (Luque et al., 1995; Milic et al., 2011); external transcribed spacer (ETS) sequence (Kadereit et al., 2007); expressed sequence tag-simple sequence repeat (EST-SSR) marker (Xu et al., 2011);external transcribed spacer (ETS) and internal transcribed spacer (ITS) and atpB-rbcL spacer sequences (Costa et al., 2019). Otherwise, Steffen et al. (2015) reported *Sarcocornia*/*Salicornia* lineage phylogenetic status based on nuclear ribosomal DNA (external transcribed spacer) and chloroplast DNA (atpB-rbcL, rpl32-trnL) sequences. Whereas, Contreraset al. (2018) applied EST marker for the same target. Otherwise, Papiniet al. (2004) reported that *Salicornia* (annual genus) is a sister group to *Arthrocnemum*, *Halocnemum* and *Sarcocornia* (perennial genera). Indeed, *Arthrocnemum* and *Sarcocornia* closely related based on ITS DNA sequences.

According to Mouterde, this species did not present in Syrian flora (Mouterde, 1966). However, its occurrence was firstly reported by Saleh (2011) in the coastal region of Syria, forming pure or mixed populations with other halophytic species such as *Tamarix* sp., *Halimione portulacoides* (L.) Aellen, *Juncus acutus* L. and *Inula chritmoides* L.

Taxonomically, *A. macrostachyum* Moris species has been morphologically characterized and little attention has been given to its genetic diversity based on PCR-based markers, in spite of its importance as a valuable genetic resource for genes salinity tolerance (Saleh, 2015) and its application in industrial and pharmacological activities.

In this regards, Saleh (2011) reported its genetic diversity in Syria based on random amplified polymorphic DNA (RAPD) and Inter-simple sequence repeat (ISSR) molecular markers. Moreover, Saleh (2015) applied random amplified microsatellite polymorphism (RAMP) molecular marker for the same target.

To earlier, an assay named directed amplification of minisatellite region DNA (DAMD) to direct the PCR based amplification of minisatellite regions has been firstly used by Heath et al. (1993). This marker has been then successfully employed in phylogenetic studies of many plant species (Saleh, 2019).

However, in 2011, Ince and Karaca were the first who introduced a changes including, deceasing in annealing PCR temperature during the first ten PCR cycles amplifications to investigate genetic diversity in common bean landraces (Ince and Karaca, 2011). Theses alterations will allow to improve its reproducibility and to eliminate artifact PCR profile and finally named as touch-down directed amplification of minisatellite DNA (Td-DAMD) marker. Then this marker has been successfully used in phylogenetic studies of others plant crops; *e.g.* in *Salvia* species (Ince and Karaca, 2012); *Allium* sp. (Deniz et al., 2013); carnation cultivars (Ince and Karaca, 2015) and in commercial cotton (Gocer and Karaca, 2016).

This alteration in term of annealing PCR temperature encouraged us to go so far, meaning to increase annealing PCR temperature and called touch up-directed amplification of minisatellite DNA (TU-DAMD) marker; as a new assay provides us with new information in phylogenetic studies. The advantageous of TU-DAMD techniques over DAMD one, could be attributed to the fact that higher annealing temperature may decrease the PCR artifacts occurrence, leading consequently to augment the DNA markers reliability and reproducibility.

Thereby, the current work focused on TD-DAMD and TU-DAMD application to investigate DNA genetic diversity of *A. macrostachyum* species in comparative study, highlighting the genetic relationships among three *A. macrostachyum* naturally grown along the Syrian coastline.

Material and Methods

Plant materials

Three *A. macrostachyum* genotypes were found, identified and collected (bulk of 5 plants/genotype) from the North of Lattakia – Syria coastline (longitude of 35°35′22″N and Latitude of 35°44′10″E) at 12 km to North of Lattakia with EC of 70 ds/m, and annual rainfall of700 mm. Collection site descriptive and sampling were as reported by Saleh (2011).

DNA isolation

Total genomic DNA was isolated from the three *A. macrostachyum* studies genotypes by a cetyltrimethylammonium bromide (CTAB) protocol as reported by Doyle and Doyle (1987). DNA concentration was finally quantified by DNA fluorimeter and stored at -80 °C until use.

TD-DAMD and TU-DAMD tests

Seventeen DAMD primers were employed in TD-DAMD and TU-DAMD tests for genetic relationships investigation among three *A. macrostachyum* naturally grown along the Syrian coastline.TD-DAMD amplification reactions were performed in 25 µl total volume according to Ince and Karaca (2012) using T-gradient thermal cycler (Bio-Rad, Hercules, USA) programmed as following: 1 cycle for 4 min at 94 ºC, followed by ten cycle of pre-PCR involving of 30 s at 94°C for denaturation, 45 s at 60°C for annealing, and 3 min at 72°C for extension. Annealing temperature was reduced 0.5°C/ cycle for the first 10 cycles. Then 30 cycles at a constant 55°C as annealing temperature; followed by final extension at 72°C for 10 min. Whereas, for TU-DAMD assay, similar PCR steps were done except that the annealing temperature (55°C)was increased 0.5°C/ cycle for the first 10 cycles. Final PCR products were separated on a 2 % ethidium bromide-stained agarose (Bio-Rad) in 0.5× Tris-borate-EDTA (TBE) buffer. Electrophoresis was carried out at 100 V for 2.5 h and visualized with a UV transilluminator. A VC 100bp Plus DNA Ladder (Vivantis) ladder standard was used to determine molecular weight of DAMD-PCR amplification products.

TD-DAMD and TU-DAMD data analysis

TD-DAMD and TU-DAMD bands were manually scored and the presence or absence of each band size was recorded as 1 or 0, respectively. The Unweighted Pair Group Mean Arithmetic average (UPGMA) using Statistica program (Statistica, 2003), was constructed based on percent disagreement values (PDV). Genetic similarity among the three *A. macrostachyum* studied genotypes was determined according to Jaccard (1908). Moreover, polymorphic information content (PIC) was calculated according to the formula:

$$PIC = 1 - \Sigma(Pij)^2$$

Where Pij is the frequency of the i^{th} pattern revealed by the jth primer summed across all patterns revealed by the primers (Botestein et al., 1980).

Results and Discussion

Seventeen DAMD primers were tested for each TD-DAMD and TU-DAMD assesses. PCR amplification size ranged between 100-800 bp. Comparative study between TD-DAMD and TU-DAMD polymorphism profile yielded by URP1F, HBV5 and URP17R DAMD primers was presented in Figure 1.TD-DAMD marker gave 108 (P% = 87.805) polymorphic bands among a total bands of 123 bands (Table 2). Total bands/primer varied from 4 (URP2R) to 11 (URP1F & URP17R) with a mean average of 7.235 total bands/primer; whereas, polymorphic bands/primer varied from 4 (URP2R, YNZ22, 14C2 & URP13R) to 10 (URP1F & URP17R) with a mean average of 6.353 polymorphic bands/primer (Table 2). As for TU-DAMD, this marker gave 104 (P% = 92.035) polymorphic bands among a total bands of 113 bands (Table 2). Total bands/primer varied from 4 (14C2) to 11 (URP1F) with a mean average of 6.647 total bands/primer; whereas, polymorphic bands/primer varied from 3 (URP13R) to 11 (URP1F) with a mean average of 6.118 polymorphic bands/primer (Table 2). Moreover, PIC value was recorded to be 0.386 and 0.411 for TD-DAMD and TU-DAMD markers, respectively.

Genetic relationships among three *A. macrostachyum* studies genotypes were investigated using 17 DAMD primers for each TD-DAMD and TU-DAMD assesses in comparative study. The current study revealed that TD-DAMD assay produced total bands number of 123 bands and 108 polymorphic bands, representing polymorphism level of 87.805% with a mean PIC average of 0.386. Whereas, in TU-DAMD assay, 113 total bands and 104 polymorphic bands were produced representing polymorphism level of 92.035 % with a mean PIC average of a 0.411.

Saleh (2011) applied RAPD and ISSR markers to investigate genetic relationships among the same three *A. macrostachyum* studies genotypes. This study revealed a total band of 185 bands, of which 160 (86.486%) were polymorphic with a mean PIC average of 0.385 using 20 RAPD primers. Whereas, ISSR gave a total band of 88 bands, of which 80 (90.909%) were polymorphic with a mean PIC average of 0.395 using 7 ISSR primers. Moreover, Saleh (2015) reported a total band of 143 bands, of which 139 (95.862%) were polymorphic with a mean PIC average values of 0.431 using 21 RAPM PCs primer combinations.

UPGMA clustering analysis has been employed to construct relationships among the three *A. macrostachyum* studies genotypes. Cluster analysis has been constructed based on TD-DAMD (Figure 2a) and TU-DAMD (Figure 2b) data separately. Moreover, to give an overview image about the relationships among the three *A. macrostachyum* studies genotypes, cluster analysis has been performed also based on TD-DAMD and TU-DAMD data combination (Figure 2c).

As can be seen in Figure 1, TD-DAMD and TU-DAMD DNA polymorphism profile generated by URP1F, HBV5 and URP17R DAMD primers.

Overall, cluster analysis revealed that the three *A. macrostachyum* studies genotypes were clustered into two main groups (Figure 2) based on TD-DAMD and TU-DAMD data separately or/and in combination together.

This result was consisting with Saleh (2011) using RAPD and ISSR markers and also with Saleh (2015) using RAMP marker.

Percent disagreement values (PDV) (Table 3) and Jaccard similarity index (Table 4) have been calculated based on TD-DAMD, TU-DAMD and TD-DAMD+TU-DAMD combination. According to TD-DAMD, TU-DAMD and TD-DAMD+TU-DAMD combination, similar clustering pattern has been recorded. Cluster analysis revealed that the three *A. macrostachyum* studied genotypes were split into two main distinguished groups. The first group involves *A. macrostachyum* genotype 2, which is genetically distinct from the two others. Whereas, the second one involves *A. macrostachyum* genotypes 1 & 3 which were closely related at PDV = 0.16 (Jaccard index = 0.762), PDV = 0.11 (Jaccard index = 0.818) and PDV=0.14 (Jaccard index = 0.787) for TD-DAMD, TU-DAMD and TD-DAMD+TU-DAMD combination, respectively (Tables 3 & 4).

Similarly, Saleh (2011) reported that the *A. macrostachyum* species includes two distinguished supspecies; and that *A. macrostachyum* genotypes 1 & 3 were closely related closely related at PDV = 0.29 (Jaccard index = 0.56) and PDV = 0.39 (Jaccard index = 0.45)using RAPD and ISSR markers, respectively. Moreover, the results obtained herein were coherent with Saleh (2015) findings, where *A. macrostachyum* genotypes 1 & 3 were closely related closely related at PDV = 0.37 (Jaccard index = 0.47) using RAMP markers.

Kadereit et al. (2007) successfully investigated halophyte *Salicornia* L. genus phylogenetic based on External Transcribed Spacer (ETS) sequence data. Whereas, Steffen *et al*. (2015) reported *Sarcocornia*/*Salicornia* lineage phylogenetic status based on nuclear ribosomal DNA (external transcribed spacer) and chloroplast DNA (atpB-rbcL, rpl32-trnL) sequences. More recently, Costa et al. (2019) reported phylogenetic study of *Salicornia* genus using External Transcribed Spacer (ETS), Internal transcribed spacer (ITS) and atpB-rbcL spacer sequences.

Whereas, Milic et al. (2011) reported DNA polymorphism among 4 *Salicornia* populations using 7 RAPD primers. They reported that polymorphic bands ranged between 1-11 polymorphic bands/primer with Nei and Li genetic distance ranged between 0.469-0.750. Whereas, Xu et al. (2011) applied expressed sequence tag-simple sequence repeat (EST-SSR) marker to investigate DNA polymorphism in two *Salicornia* populations. They reported that six EST-SSR loci gave 27 alleles, ranged between 3-5 alleles/locus with an average number of alleles/locus ranging between 4.33- 4.17 combined with a PIC average ranging between 0.520- 0.563.

Previously, Saleh (2015) reported that the found differences among the three *A. macrostachyum* studies genotypes were genetically related to phenologically once. Where, *A. macrostachyum* genotype 2 was characterized by its inflorescence occurrence, whereas, no inflorescences were observed in *A. macrostachyum* genotypes 1 and 3. In plants taxonomy studies, this criteria is considered as a primary criteria for plant taxonomy. This observation was coherent with others studies (Dalby, 1962; Wilson, 1980; Kühn et al., 1993); where, these researches reported that the high reduced flowers inflorescence is considered as a diagnostic criteria for Salicornioideae family. Moreover, Tolken (1967) reported to earlier the occurrence of naturally hybrids in the *Arthrocnemum* and *Salicornia* genera based on an anatomy study including sterility and pollen irregularities.

Conclusion

In the current study, TU-DAMD technique was employed as a new assay for the first time in plant phylogenetic studies. Molecular characterization of *A. macrostachyum* species has been investigated through TD-DAMD and TU-DAMD markers in comparison study. The current study revealed that TU-DAMD marker was more potent than TD-DAMD marker by showing the highest P% and PIC estimated values. In this regards, these values were recorded to be 92.035% & 87.805% for P% 0.411 and 0.386 for PIC value using TU-DAMD and TD-DAMD, respectively. Moreover, TU-DAMD technique could produce high DNA polymorphism profile with high resolution and reducibility. Thereby, it is advice to use it as a new technique for other plants genetic screening.

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Table 1: Selected DAMD primers employed in the current study.

Primer Number	Primer name	Primer sequence 5'-3'
1	URP1F	ATCCAAGGTCCGAGACAACC
2	URP2R	CCCAGCAACTGATCGCACAC
3	URP4R	AGGACTCGATAACAGGCTCC
4	URP25F	GATGTGTTCTTGGAGCCTGT
5	URP30F	GGACAAGAAGAGGATGTGGA
6	FVIIex8	ATGCACACACACAGG
7	HBV3	GGTGAAGCACAGGTG
8	HBV5	GGTGTAGAGAGGGGT
9	YNZ22	CTCTGGGTGTGGTGC
10	14C2	GGCAGGATTGAAGC
11	M13	GAGGGTGGCGGCTCT
12	HBVb	GGTGTAGAGAGAGGGGT
13	URP13R	TACATCGCAAGTGACACAGG
14	URP17R	AATGTGGGCAAGCTGGTGGT
15	M13	GAGGGTGGCGGTTCCT
16	HVA	AGGATGGAAAGGAGGC
17	HVV	GGTGTAGAGAGGGGT

Table 2: TD-DAMD and TU-DAMD banding pattern of amplified bands scored.

TD-DAMD					
Primer name	**Primer sequence 5'-3'**	TB	PB	P%	PIC
URP1F	ATCCAAGGTCCGAGACAACC	11	10	90.909	0.404
URP2R	CCCAGCAACTGATCGCACAC	4	4	100.000	0.445
URP4R	AGGACTCGATAACAGGCTCC	9	8	88.889	0.395
URP25F	GATGTGTTCTTGGAGCCTGT	8	7	87.500	0.389
URP30F	GGACAAGAAGAGGATGTGGA	5	5	100.000	0.445
FVIIex8	ATGCACACACACAGG	7	6	85.714	0.381
HBV3	GGTGAAGCACAGGTG	8	7	87.500	0.381
HBV5	GGTGTAGAGAGGGGT	7	6	85.714	0.381
YNZ22	CTCTGGGTGTGGTGC	5	4	80.000	0.356
14C2	GGCAGGATTGAAGC	6	4	66.667	0.296
M13	GAGGGTGGCGGCTCT	7	6	85.714	0.370
HBVb	GGTGTAGAGAGAGGGGT	6	5	83.333	0.356
URP13R	TACATCGCAAGTGACACAGG	5	4	80.000	0.356
URP17R	AATGTGGGCAAGCTGGTGGT	11	10	90.909	0.404
M13	GAGGGTGGCGGTTCCT	9	8	88.889	0.395
HVA	AGGATGGAAAGGAGGC	7	7	100.000	0.445
HVV	GGTGTAGAGAGGGGT	8	7	87.500	0.370
Totale		123	108		
Average		7.235	6.353	87.602	0.386
TU-DAMD					
Primer name	**Primer sequence 5'-3'**	TB	PB	P%	PIC
URP1F	ATCCAAGGTCCGAGACAACC	11	11	100.000	0.445
URP2R	CCCAGCAACTGATCGCACAC	5	5	100.000	0.445
URP4R	AGGACTCGATAACAGGCTCC	5	4	80.000	0.356
URP25F	GATGTGTTCTTGGAGCCTGT	5	4	80.000	0.356
URP30F	GGACAAGAAGAGGATGTGGA	5	5	100.000	0.445
FVIIex8	ATGCACACACACAGG	9	6	66.667	0.296
HBV3	GGTGAAGCACAGGTG	8	7	87.500	0.389
HBV5	GGTGTAGAGAGGGGT	8	7	87.500	0.389
YNZ22	CTCTGGGTGTGGTGC	8	7	87.500	0.389
14C2	GGCAGGATTGAAGC	4	4	100.000	0.445
M13	GAGGGTGGCGGCTCT	7	7	100.000	0.445
HBVb	GGTGTAGAGAGAGGGGT	7	6	85.714	0.381
URP13R	TACATCGCAAGTGACACAGG	3	3	100.000	0.445
URP17R	AATGTGGGCAAGCTGGTGGT	5	5	100.000	0.455
M13	GAGGGTGGCGGTTCCT	6	6	100.000	0.455
HVA	AGGATGGAAAGGAGGC	7	7	100.000	0.455
HVV	GGTGTAGAGAGGGGT	10	9	90.000	0.400
Totale		113	104		
Average		6.647	6.118	92.052	0.411

TB – total bands, PB – polymorphic bands, P % – polymorphic % and PIC – polymorphic information content.

Table 3: Percent disagreement values (PDV) yielded by TD-DAMD, TU-DAMD and TD-DAMD+TU-DAMD combination

TD-DAMD			
Genotype	*A. macrostachyum1*	*A. macrostachyum2*	*A. macrostachyum3*
A. macrostachyum1	0.00		
A. macrostachyum2	0.81	0.00	
A. macrostachyum3	0.16	0.78	0.00
TU-DAMD			
Genotype	*A. macrostachyum1*	*A. macrostachyum2*	*A. macrostachyum3*
A. macrostachyum1	0.00		
A. macrostachyum2	0.89	0.00	
A. macrostachyum3	0.11	0.84	0.00
TD-DAMD+TU-DAMD			
Genotype	*A. macrostachyum1*	*A. macrostachyum2*	*A. macrostachyum3*
A. macrostachyum1	0.00		
A. macrostachyum2	0.85	0.00	
A. macrostachyum3	0.14	0.81	0.00

Table 4: Jaccard similarity index as yielded by TD-DAMD, TU-DAMD and TD-DAMD+TU-DAMD combination

TD-DAMD			
Genotype	*A. macrostachyum1*	*A. macrostachyum2*	*A. macrostachyum3*
A. macrostachyum1	1.000		
A. macrostachyum2	0.145	1.000	
A. macrostachyum3	0.762	0.165	1.000
TU-DAMD			
Genotype	*A. macrostachyum1*	*A. macrostachyum2*	*A. macrostachyum3*
A. macrostachyum1	1.000		
A. macrostachyum2	0.090	1.000	
A. macrostachyum3	0.818	0.144	1.000
TD-DAMD+TU-DAMD			
Genotype	*A. macrostachyum1*	*A. macrostachyum2*	*A. macrostachyum3*
A. macrostachyum1	1.000		
A. macrostachyum2	0.118	1.000	
A. macrostachyum3	0.787	0.155	1.000

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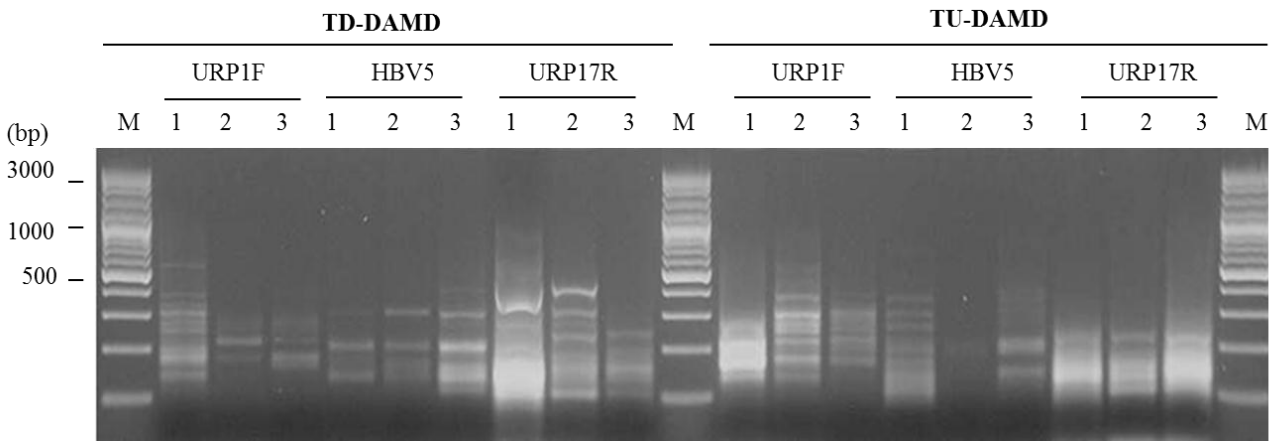

Fig. 1: TD-DAMD and TU-DAMD polymorphism profile among the three *A. macrostachyum* studied genotypes as yielded by URP1F, HBV5 and URP17R DAMD primers in comparative study, M: A VC100bp Plus DNA Ladder (Vivantis) ladder standard

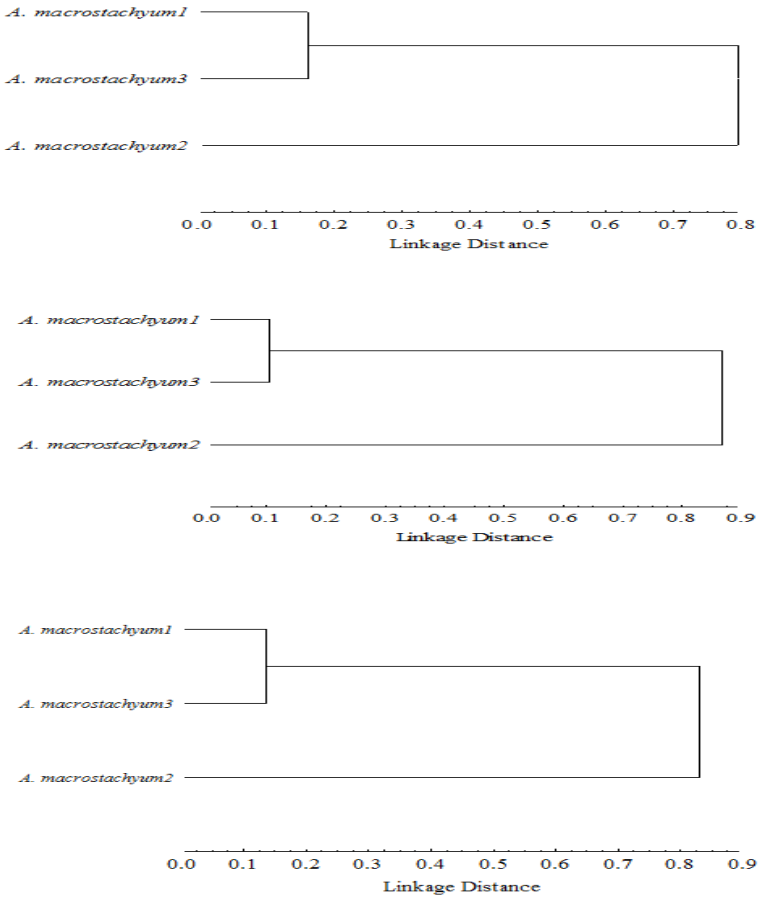

Fig. 2: Cluster analysis constructed based on TD-DAMD (Figure 2a), TU-DAMD (Figure 2b) and TD-DAMD + TU-DAMD (Figure 2c) data

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