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Utilization of ISTR, ISSR and SRAP molecular markers to reveal and classify Egyptian pomegranates (*Punica granatum* L.)

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Abstract

This study offers the comprehensive analysis of the genetic diversity among six local varieties (Manfalouti; Nab El-Gamal; Hegazy; Wardi; Assuity; Araby) and three international varieties (Wonderful; Marked Bani Rafie; Red Bani Rafie) of pomegranate in Egypt, based on the behavior of ISTR, ISSR and SRAP markers profiles. A set of 24 ISTR, 16 ISSR and 30 SRAP primers combination were compared, concerning exposed the degree of resolution and efficiency of the discriminating capacity technique alongside with the level of genetic polymorphism, to determine which varieties evolved from the others. Considering the results, SRAP assay had superior, more sensitive, higher discrimination capacity and gives much more evidence about the total number of effective alleles (1411), number of polymorphic amplicons (312), PIC (0.94), assay efficiency index (47.04), effective multiples ratio (10.04) and marker index (9.74). Unexpectedly, the ISTR profile demonstrated a significant moderate level of polymorphism among tested genotypes. To further determine the genetic relationships and the distance among varieties, a graphic demonstration of combined UPGMA tree and the PCA analysis did assemble with clear resolution and accurate along with three categories.Our findings confirm that combining different marker system were greatly better and more effective considered an important priority step toward diversity study and characterization. We can point out that, the two-widespread varieties in Egypt Manfalouti, and Nab El-Gamal, formed a high homogeneity in genetic similarity with the Wonderful, as an essential global species for breeding programs, efforts widening the genetic base of pomegranates and the introduction of new genotypes in Egypt.

Keywords: Discriminating capacity, Genetic diversity, Pomegranate (*Punica granatum* L.), PCA, UPGMA tree; UPGMA_ Unweighted Pair Group Method with Arithmetic Mean.
Abbreviations: ISSR_Inter Simple Sequence Repeat; ISTR_Inverse Sequence Tagged Repeat; PCA_Principal Coordinates Analysis; SRAP_Sequence Related Amplified Polymorphism.

Introduction

Pomegranate belongs to the family *Lythraceae* (formerly, Punicaceae), and the most commonly cultivated species, *Punica granatum* L. (Alsadi et al., 2015). It is one of the oldest known edible fruit tree species, with a large-scale series geographical global distribution (Silva et al., 2013). *Punica granatum* L. considered that it originated in Central Asia, it is native to the area occupying from Iran to the Himalayas in northern India (Chandra et al., 2010; Chauhan et al., 2012; Silva et al., 2013). Worldwide there are three mega and five macro-centers of origin and genetic diversity of pomegranate (Levin 2006a, b; Chandra et al., 2010). Mediterranean countries are the main center for commercial cultivation, it has been cultivated and naturalized over the whole Mediterranean region since ancient times including Egypt and North Africa (Silva et al., 2013). Globally, the genetic diversity of pomegranate is demonstrated by an excess of 500 globally-distributed varieties, approximately fifty of which are identified to be commercially cultivated (IPGRI, 2001). Apparently, classification of pomegranate genotypes based on morphological characteristics such as fruit size, color and acidity were associated with the environmental changes, making classifications based on morphological traits unreliable (Hajiahmadi et al., 2013). Hence, with the aid of molecular identification of pomegranate, it is becoming easier and faster to characterize germplasm and identify genotypes with desirable traits in breeding programs. Consequently, it is vital to conserve the gene pool alongside with the behavior of genes in pomegranate germplasm, to maintain a broad genetic base for the future aspect of breeding strategy in pomegranate (Rana et al., 2007). Numerous molecular marker investigations have been verified in *P. granatum*, such as RAPD (Talebi et al., 2003); ISSR (Talebi et al., 2005; Narzary et al., 2009); F-AFLP (Yuan et al., 2007); RFLP (Melgarejo et al., 2009); AFLP (Moslemi et al., 2010); SRAP (Soleimani et al., 2012); SSR (Rania et al., 2012; Hasnaoui et al., 2012); REMAP (Zhao et al., 2013) and chloroplast DNA (Hajiahmadi et al., 2013). In fact, the choice of most appropriate technology for a specific study is not obvious and depends primarily on purpose of the research as well as the degree of polymorphism and genetic assembly of the species. ISTR is a retrotransposon-based marker (Rhode 1996) which has been seen in every type of organism, are ubiquitous, dynamic and abundant in eukaryotic genomes (Torresmorán et al., 2012; Amar and Abd El Salam 2013). Hence, ISTR markers would be expected to be co-dominant and involve a various level of genetic variability, i.e. transposition results, then arbitrary markers systems such as RAPD or AFLPs, which sense polymorphism from simple nucleotide changes to genomic rearrangements (Kalendar, 2011). ISSR profile is a PCR-based method which uses microsatellites as primers in a single

reaction targeting comprise a few highly informative multi-allelic loci. (Kumar et al., 2013). As for SRAP, sequence-related amplified polymorphism is a novel molecular marker system which is based on open reading frames (ORFs) developed from genome sequence data of *Arabidopsis*. As PCR-based markers, SRAP have many advantages, including the requirement for a relatively small amount of template genomic DNA, and good levels of polymorphism in many plant species (Aneja et al., 2012; Robarts and Wolfe 2014). The three molecular markers listed above have the facility to detect different parts of the genome, they have a dominant or co-dominant inheritance and the usage together may be more effectiveness (Velascoramirez et al., 2014). In this sense, comparisons are required to decide which system is most appropriate for the issue being examined (Scariot et al., 2007). In the current investigation, we sought to determine a new basis for the ongoing discussion about; first, conducted comparison on the performance and the discrimination capacity of three molecular markers including ISTR, ISSR and SRAP; secondly, to assess the taxonomic uncertainties and varieties limits concerning the genetic diversity of Egyptian *P. granatum* germplasm. To our knowledge, till now there has been no report about the comparison of discriminating capacity and efficiency of ISSR, SRAP and ISTR marker system in Egyptian *P. granatum* germplasm.

Results

Comparison of polymorphic levels and informativeness obtained with ISTR, ISSR and SRAP markers

The levels of polymorphism detected with each marker system (ISTR, ISSR, and SRAP) and the index comparing their informativeness are represented in (Table 1) and (Fig 3). The three marker systems examined turned out to be valuable tools for detection of polymorphism and assessing genetic diversity in pomegranate germplasm, but the degree of resolution depended on the technique applied.

We initially tested 70 combinations of ISTR primers, 20 of ISSR primers and 64 combinations of SRAP primers between the nine Egyptian cultivars of pomegranate. Amongst all, 24 ISTR, 16 ISSR and 30 SRAP primers presented various levels of polymorphism as exposed in (Figures 1a–c and 2) and (Table1). The total number of allele scored was 365 for SRAP with relatively high, and with an intermediate value of 219 for ISTR, while ISSR showed the lowest value (175). On behalf to, the total numbers of polymorphic amplicons (*np*) was varied from 118 for ISSR, 143 for ISTR to 312 for SRAP markers. However, the total number of effective alleles (*Ne*) was correlate positively with the average number of polymorphic amplicons per assay unit (*np/U*) and the number of allele /assay unit (*nu*). With the view of the effective number of patterns assay unit (p-value) were obtained from the marker SRAP (14.17), ISSR (7.5) and ISTR (6.33), respectively. The effective number of patterns referee to the size of an ideal population in which, given the frequencies of the patterns obtained with a marker system, all the individuals can be notable. With the view of the average of PIC for ISTR, ISSR and SRAP markers system were nearly similar and relatively high, 0.93, 0.93 and 0.94, respectively. Herein, the above result exposed that SRAP markers were the most suitable marker in all observed parameter and PIC values.

Comparison of the discriminating capacity of ISTR, ISSR and SRAP markers

A comparative scenario of the discriminating capacity of ISTR, ISSR and SRAP markers are summarized in (Table 2) and (Fig. 3). The Effective number of alleles per locus (*ne*) for ISTR, ISSR and SRAP were a nearly similar value (14.7, 15.15 and 16.12) respectively. This was reflected by the higher values of the expected heterozygosity (*Hep*) of the polymorphic loci with a percentage of 0.93 for ISTR, ISSR and SRAP markers. The three parameters, assay efficiency index, effective multiples ratio and marker index were observed more highly from SRAP marker highlights the distinctive nature of these markers compared to ISSR and ISTR (1.3×, 2.4×, respectively). Herein the highest assay efficiency index and marker index value for SRAP marker as result of greater effective multiples ratio component, suggesting that SRAP has a higher discriminating capacity for quantifying the genetic diversity and can simultaneously detect numerous polymorphic markers per reaction.

Genetic diversity and phylogenetic relationship

The data from all ISTR, ISSR and SRAP amplification amplicons were used in the similarity evaluation. The genetic similarity matrix among all materials used in the present work was obtained (Table 3, a, b, c). Based on 759 alleles a similarity matrix was calculated according to Dice's coefficient. The similarity values among the studied genotypes ranged from 0.31 to 0.83. The highest similarity was between the three pairs, Hegazy & Marked Bani Rafie (0.83), Wonderful & Red Bani Rafie (0.80) and Wardi & Marked Bani Rafie (0.76) for SRAP, ISSR, and ISTR, respectively. However, the lowest similarity was recorded between, Assuity & Red Bani Rafie (0.31), Manfalouti & Nab El-Gamal (0.37) and Wardi & Araby (0.56) for ISTR, ISSR, and SRAP, respectively. Based on data obtain from ISTR, ISSR and SRAP tree, a combined UPGMA tree was constructed as illustrated in (Fig 4). Herein, a little modification in the positioning of some genotypes was observed in the sub-clade tree formed, using different markers systems and the phylogenetic tree from SRAP data was most compatible with a combined tree.

The phylogenetic analysis revealed numerous well-supported clades with great bootstrap values. In total, we observed three strongly supported clades, which were clearly distinguishable among the local and the international varieties of pomegranate in Egypt. The first clade was clustered jointly Wonderful, Manfalouti and Nab El-Gamal. In context, the second clade represents Hegazy is closely related to Wardi, at the same time being sister to Araby. Meanwhile, the two varieties Assuity and Red Bani Rafie were closed together as a sister to Marked Bani Rafie.

To further determine the genetic relationships among the nine Egyptian varieties of pomegranate, a graphic demonstration of the principal coordinate analysis (PCA) was presented (Fig 5). The results indicated that it could be divided into three major categories. Category I compressed the two international varieties Marked Bani Rafie, Red Bani Rafie and Assuity as one of the local varieties. Category II assembled Araby, Wardi and Hegazy. Meanwhile, the local varieties Manfalouti and Nab El-Gamal are grouped jointly with the international variety Wonderful in the category III. These results showed that most of the international varieties were isolated independently with nesting to the local variety

Assuity. In contrast, the local variety was formed in two categories with a high homogeneity in genotypes correlation with nested to Wonderful, as one of the essential international varieties.

Discussion

The systematic behavior and phylogenetic affinities of the Egyptian pomegranate varieties are still obscured, and unsolved problems concerning their biology and taxonomy need further verification and confirmation. Thus, investigation of levels of genetic diversity is an important precursor for the study of plant species and will provide insights into the evolution of the species. In fact, the taxonomic ambiguities persisting with the systematic position of the genus *Punica* also needs to be resolved and the molecular methods will be the best possible approach for this (Ranade et al., 2009). This study offers the comprehensive analysis of the genetic diversity among six local varieties (Manfalouti; Nab El-Gamal; Hegazy; Wardi; Assuity; Araby) and three international varieties (Wonderful; Marked Bani Rafie; Red Bani Rafie) of pomegranate under North Sinai conditions, Egypt, based on the behavior of the three molecular markers profiles ISTR, ISSR and SRAP. In detail, the purpose of the present study was to explore the efficiency of the discriminating capacity technique alongside with the level of genetic polymorphism to determine which varieties evolved from the others. In the framework, Ferrao et al., (2012) indicated that the efficiency of a molecular marker is balanced among the level of polymorphism it can distinguish and its capacity to identify numerous polymorphisms; deciding which technique is the most suitable based on several aspects, counting the objective of the research, genetic structure and the resources presented. Interestingly, our results of the molecular diversity data can obtain desirable differentiation among the tested pomegranate varieties. Regarding the level of polymorphism, the highest value was obtained from the SRAP analysis. This is due to the superior value of the total number of effective alleles, the effective number of patterns/ assay unit and the average number of polymorphic amplicons /assay unit. This finding agrees with previous studies where SRAPs proved to be high effective tools were compared to other marker systems that used each technique separately in pomegranate germplasm, such as, RAPD (Sarkhosh et al., 2006; Narzary et al., 2009), ISSR (Talebi et al., 2005; Narzary et al., 2010), SSR (Koohi-Dehkordi et al., 2007; Pirseyedi et al., 2010) and SRAP (Soleimani et al., 2012). Other studies in several plants have also confirmed that SRAP is superior information than other methods, for example, RAPD (Liu et al., 2007), ISSR (Li-Wang et al., 2008) and SSR (Amar et al., 2011). Recently, Robarts and Wolfe (2014) propose that SRAP markers should be employed for research addressing hypotheses in plant systematics and these markers have verified to be robust and highly variable, on par with AFLP. On behalf of the behavior of ISSR profile, the level of polymorphism is relatively high; this is probably due to the value of the effective number of patterns per assay units. This is very substantial for the management of germplasm banks where several cultivars are complex need to be accurately and classification (Belaj et al., 2003). Parallel results were reported in pomegranate by Sarkhosh et al., (2006) evaluate Iranian genotypes, while that Ghobadi et al., (2005) pointed to a high level of genetic similarity (65%) between the 24 Iranian pomegranate genotypes across six ISSR markers. In subsequent studies, (Talebi t al., 2011) described that ISSR markers exhibited higher levels of polymorphisms for revealing molecular diversity among pomegranate cultivars. Additionally, Pitsiouni et al., (2012), confirmed that the uses of ISSR among the 40 Greek accessions of pomegranate genotypes gave an adequate number of polymorphic level among wild and cultivated Pomegranate. In the case of ISTR, unexpectedly, this profile demonstrated a moderate level of polymorphism among tested genotypes. This level of polymorphism, associated with ISTR markers, is to be certainly because of the correlation with the total number of effective alleles and the number of allele assay unit, responsible for generating ISTR allelic diversity, which is in concurrence with earlier reports in many plant species (Du et al., 2009; Biswas et al., 2011; Velascoramirez et al., 2014). This finding also agrees with the results of Aga and Bryngelsson (2005), confirmed that ISTR revealed the mild level of genetic variation within a gene pool of *C. arabica* in Ethiopia. By this criterion, the activity of ISTR as a retrotransposon are affected by the transcriptionally inactive, or silent in somatic tissues but active through particular stages of plant development and under the effect of stressful conditions (Torresmorán et al., 2012). Among the discrimination capacity of marker systems evaluated SRAP> ISSR > IRAP were found to be more effectiveness in the estimate of molecular diversity among the Egyptian pomegranate varieties; SRAP was evident superior through the large values of the average number of polymorphic amplicons assay unit, PIC, assay efficiency index, effective multiples ratio and marker index. Mainly, the marker index (MI) is a convenient estimate for marker efficiency (Milbourne et al., 1997; Li and Quiros 2001; Li et al., 2010; Amar and Abd El Wahab, 2013). By this criterion, arithmetically 1.3 fold and 2.4 fold higher MI calculated for SRAP in comparison to ISSR and ISTR, highlights the distinctive nature of the SRAP assay. Rather, the result of the SRAP profile was ORF-based marker system targeting functional regions of the pomegranate genome, and resulting adequate polymorphism, furnished sufficient information for influential the genetic diversity and population genetic structure of the pomegranate varieties (Soleimani et al., 2012). Recently, Li et al., (2013) recommended that SRAP has advantages with a wide range of applications in plant breeding over other molecular detection techniques in gene tagging and cloning and allows screening thousands of loci shortly to pinpoint the genetic position underlying the trait of interest. Our results approved with several research article confirmed that an SRAP marker had the greatest discrimination capacity of polymorphism, and results in a moderate number of co-dominant markers with efficient in marker assisted selection in plant breeding and genetic diversity of many plant species (Agarwal et al., 2008; Dong et al., 2010; Li et al., 2010; Aneja et al., 2012; Robarts and Wolfe 2014).

In Table (4), the results for the correlation coefficients (*r*) among similarity matrices are presented. Values of (*r*) were non-significant correlations were observed when comparing the ISSR and ISTR (-0.111) markers and between SRAP and ISTR (-0.031). While the significant correlations to be found among the ISSR and SRAP with a value of 0.43. The low correlations among different molecular marker systems show the importance of using different markers for estimating diversity and genetic similarity. Because this issue, Velascoramirez et al., (2014) recommended that the information that is generated by various markers had a valuable capacity for genetic studies involving both, diversity and relationships. By this criterion, the combining different marker system was critically better for diversity study as recommended through numerous reports such as (Jhang et al.,

Table 1. Levels of polymorphism and PIC value generated by ISTR, ISSR and SRAP and assays among nine Egyptian varieties of pomegranate.

Index with their abbreviations	Number of markers	Number of allele	Number of allele /assay unit	Total Banding pattern	Effective number of patterns/ assay unit	Total number of effective alleles	Number of monomorphic amplicons	Number of polymorphic amplicons	Average number of polymorphic amplicons /assay unit	PIC Value
Markers Name	*U*	*L*	n_u	*Bp*	*p*	*Ne*	n_{np}	n_p	n_p/U	*%*
ISTR	24	219	9.13	152	6.33	464	76	143	5.96	0.93
ISSR	16	175	10.94	120	7.50	578	57	118	7.38	0.93
SRAP	30	365	12.17	425	14.17	1411	53	312	10.40	0.94

Table 2. Comparison of information obtained with and discriminating capacity of ISSR, SRAP and ISTR markers among nine Egyptian varieties of pomegranate.

Index with their abbreviations	Average of the allele frequency	Effective number of alleles per locus	Expected heterozygosity of the polymorphic loci	Fraction of polymorphic loci	Assay efficiency index	Effective multiples ratio	Marker Index
	(pi2)	*(ne)*	*(He)*	*(β)*	*(Ai)*	*(EMR)*	*(MI)*
ISTR	0.068	14.7	0.932	0.65	19.37	5.96	5.55
ISSR	0.066	15.15	0.934	0.67	36.18	7.38	6.88
SRAP	0.062	16.12	0.938	0.85	47.04	10.04	9.74

Table 3. a, b and c. Similarity matrix resulting from ISTR, ISSR and SRAP data for the nine pomegranate varieties.

ISTR	Wonderful	Manfalouti	Nab El-Gamal	Hegazy	Wardi	Araby	Assuity	Red Bani Rafie
Wonderful								
Manfalouti	0.527							
Nab El-Gamal	0.573	0.402						
Hegazy	0.625	0.538	0.444					
Wardi	0.688	0.744	0.723	0.649				
Araby	0.719	0.663	0.707	0.742	0.756			
Assuity	0.634	0.544	0.612	0.573	0.728	0.61		
Red Bani Rafie	0.612	0.505	0.604	0.594	0.708	0.644	0.311	
Marked Bani Rafie	0.602	0.578	0.64	0.647	0.766	0.685	0.533	0.434

ISSR	Wonderful	Manfalouti	Nab El-Gamal	Hegazy	Wardi	Araby	Assuity	Red Bani Rafie
Wonderful								
Manfalouti	0.62							
Nab El-Gamal	0.652	0.375						
Hegazy	0.709	0.507	0.477					
Wardi	0.795	0.568	0.449	0.442				
Araby	0.709	0.468	0.462	0.38	0.388			
Assuity	0.643	0.488	0.396	0.439	0.41	0.425		
Red Bani Rafie	0.802	0.663	0.598	0.63	0.678	0.621	0.633	
Marked Bani Rafie	0.731	0.687	0.591	0.588	0.519	0.581	0.438	0.729

SRAP	Wonderful	Manfalouti	Nab El-Gamal	Hegazy	Wardi	Araby	Assuity	Red Bani Rafie
Wonderful								
Manfalouti	0.745							
Nab El-Gamal	0.806	0.757						
Hegazy	0.792	0.743	0.749					
Wardi	0.771	0.729	0.714	0.655				
Araby	0.764	0.749	0.754	0.655	0.561			
Assuity	0.758	0.75	0.723	0.702	0.589	0.582		
Red Bani Rafie	0.758	0.77	0.769	0.732	0.692	0.67	0.599	
Marked Bani Rafie	0.752	0.787	0.807	0.83	0.78	0.727	0.715	0.682

Table 4. Correlation among similarity matrices derived from ISTR, ISSR and SRAP markers.

Variables	ISSR	SRAP	ISTR
ISSR	**1**	0.0089	0.51
SRAP	0.43	**1**	0.878
ISTR	-0.111	-0.031	**1**

Values in bold are different from 0 with a significance level alpha=0.05

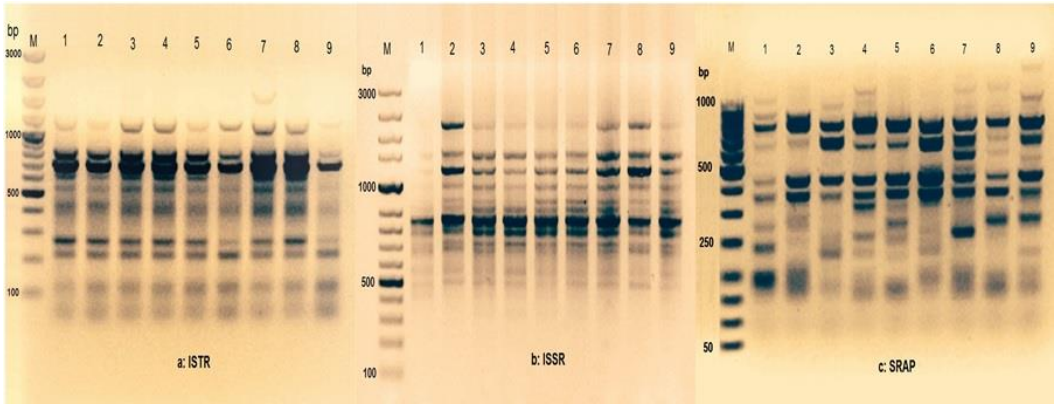

Fig 1. ISTR (A), ISSR(B) and SRAP (C) profiles of nine Egyptian pomegranate varieties.

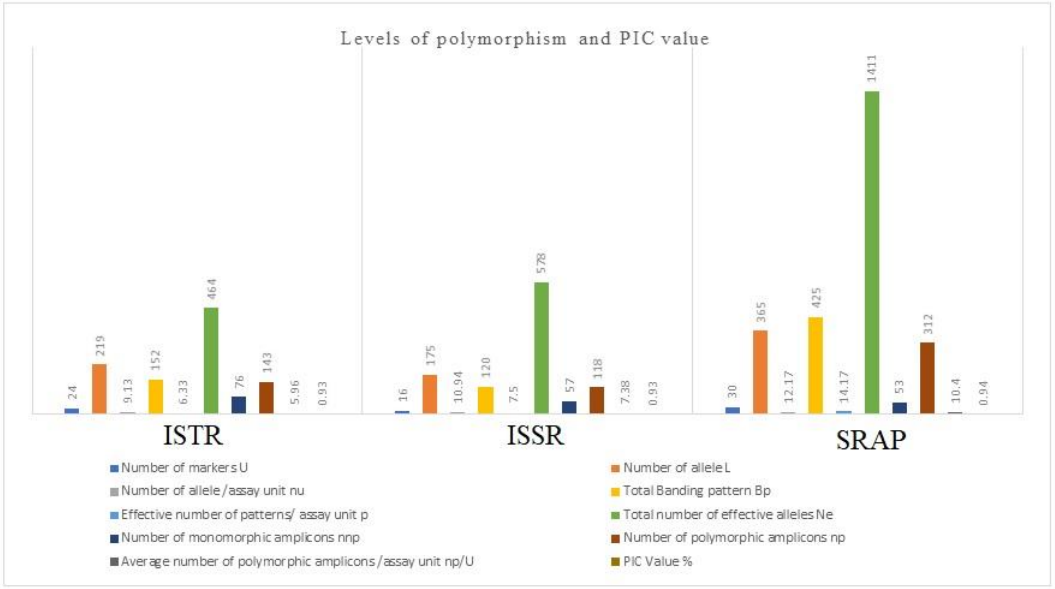

Fig 2. Schematic representation of the Levels of polymorphism and PIC value via SRAP, ISSR and ISTR markers in nine Egyptian varieties of pomegranate.

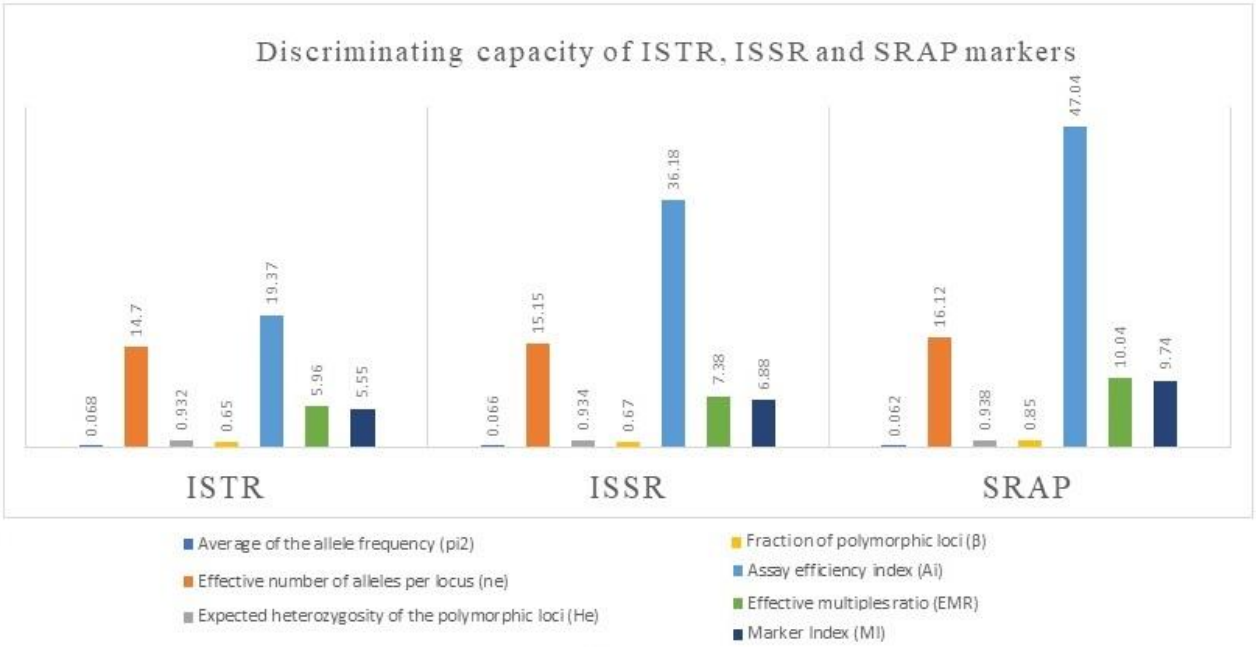

Fig 3. Schematic representation the comparison information obtained and the discriminating capacity of ISTR, ISSR and SRAP markers among nine Egyptian varieties of pomegranate.

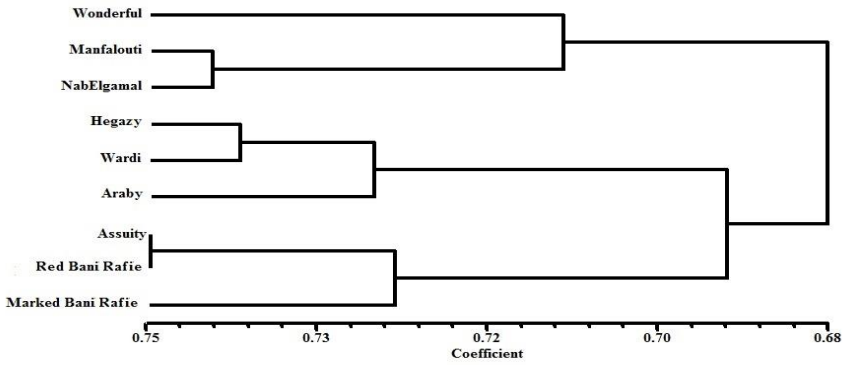

Fig 4. An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of genetic relationships among nine Egyptian varieties of pomegranate based on the Dice similarity coefficients obtained using the combined data of ISTR, ISSR and SRAP

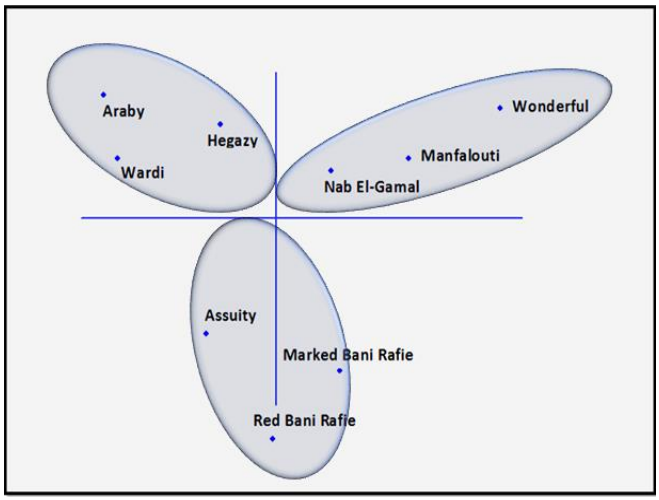

Fig 5. Schematic representation the principal components analysis among nine Egyptian varieties of pomegranate markers.

2010; Wang et al., 2012; Amar and Abd El Wahab, 2013; Velascoramirez et al., 2014; Costa et al., 2016). In view of the performance of the combined tree and the principal components analysis (PCoA) (Fig 4 and 5), formed groups consistent with the cluster analysis. Our findings demonstrated that both had been reliable sources of information. Hence, our results are clearly distinguishing among the local and the international varieties of performance in Egypt along with three separate categories. Apparently, in view with the previous revisions, our results obtained are better than expected, along with the discrimination capacity and the level of polymorphism when compared to the previous studies in Iran, China, Morocco, Oman and Egypt (Moslemi et al., 2010; Alsadi et al., 2012; Zhao et al., 2013; Ajal et al., 2014; Sinjare, 2015; Al-Sadi et al., 2015; Ismail et al., 2014). In contrast with the previous study in Egyptian pomegranate cultivars created on ISSR and AFLP by Ismail et al., (2014). The low level of polymorphism observed in this study may be due to the little number of cultivars for marker screening and the limited primer selection without the use of filtering steps. Additionally, the analysis of genetic relationship also less resolution in distinctly the differentiate among pomegranate cultivars in Egypt. In the present revised, we can point out that, the two-widespread variety in Egypt Manfalouti and Nab El-Gamal were highly nested genetically with Wonderful variety as a most important global variety for breeding genetics programs. Furthermore, we strongly proposed that Assuity variety is a sister to the pair of varieties Red Bani Rafie, Marked Bani Rafie and could be as a separate closely varieties. This finding is new information and critical for the further breeding program, efforts widening the genetic base of pomegranates and the introduction of new genotypes in Egypt. In this sense, we can point out that our PCA and the combined tree are suitable to confirm the parental relationships with clear resolution within the pomegranate varieties in Egypt along with three separates categories.

Materials and methods

Plant material

Pomegranate varieties belong to the gene bank located at the experimental field station of North Sinai Station (NSS), province of North Sinai (Egypt). Nine accessions obtained selected from the varietal groups (Manfalouti; Nab El-Gamal; Hegazy; Wardi; Assuity; Araby) local varieties, and the international varieties (Wonderful; Marked Bani Rafie; Red Bani Rafie). These samples were considered representative to evaluate the method at both the inter- and intra-varietal diversity. Apical sample leaves were randomly collected from adult trees avoiding those reddish one's rich in anthocyanins to not interfere with PCR analysis.

Isolation of genomic DNA (gDNA)

Total genomic DNA was extracted from a fresh leaf of pomegranate using plant DNA purification mini kit (Thermo Scientific GeneJET kit, K0791, USA) according to manufacturer's protocol. Three to five different DNA preparations were made for each variety. The quality and concentration of the DNA samples were checked in a Quawell Q5000 UV-Vis spectrophotometer (V2.1.4, USA). And a portion of the DNA was diluted to 50 ng/µl for use in ISTR, ISSR and SRAP analysis. Both the stock and diluted portions were stocked at -20°C.

ISTR assay

ISTR assay was carried out according to Aga et al., (2005). ISTR primer combinations were initially screened using a total of 70 primer combinations from ten forward primers and seven reverse primers. Across whole primers screened only 24 ISTR combination (Supplementary Table 1) were chosen for further analysis. Each PCR contained a reaction mixture of 25µl made up of 50 ng of genomic DNA, 200 µM of dNTPs, 0.3 µM of each primer, 3.5µl of Green PCR buffer, 1 unit of taq DNA polymerase, and deionized water. All reagents and their buffers were supplied by Thermo Scientific Inc, (Germany). PCR amplification was performed in Agilent's (Sure Cycler 8800 thermal cycler, USA), consisted of: 1 cycle at 95 ◦C, 3 min; 40 cycles of 94 ◦C, 30 s; 45◦C, 30 s; 72◦C, 2 min; 1 cycle at, 72◦C, 10 min; ◦C and 4◦C for infinitive. Agarose gel electrophoresis (.8%) used for resolving the PCR amplification products. GeneRuler 100 bp plus DNA ladder (Thermo Scientific, SM0321) was used as the molecular standard to confirm the appropriate ISTR markers. Bands were detected using Bio-Rad Gel Doc™ XR+ imaging system with Image Lab™ (USA) (Fig 1a).

ISSR assay

To execute the ISSR experiment, PCR amplification was performed as described by Sankar and Moore, (2001). Across all ISSR primers screened only sixteen ISSR were selected for further analysis (Supplementary Table 1). ISSR amplifications were performed following the same procedure described for ISTR with minor modifications as follow: 1 cycle at 94 ◦C, 4 min; 35 cycles of 94 ◦C, 45 s; 45◦C, 45 s; 72◦C, 1 min; 1 cycle at, 72◦C, 10 min; ◦C and 4◦C for infinitive (Fig 1b).

SRAP assay

The SRAP analysis was performed as described by Li and Quiros (2001). SRAP primer combinations (PCs) were screened using 30 different combinations which employed using ten forward, and eleven reverse primers were used (Supplementary Table 1). All reagents and their buffers were supplied by Thermo Scientific Inc, (Germany). Each PCR contained a reaction mixture of 25µl made up of 30 ng of genomic DNA, 200 µM of dNTPs, 0.3 µM of each primer, 3.5µl of Green PCR buffer, 1 unit of taq DNA polymerase, and deionized water. PCR cycling parameters included 4 min of denaturing at 94°C, five cycles of three steps: 1 min of denaturing at 94°C, 1 min of annealing at 35°C and 1min of elongation at 72 °C. In the following 35 cycles, the annealing temperature was raised to 50 °C, and for extension, one cycle of 7min at 72 °C. GeneRuler 50 bp Plus DNA Ladder (Thermo Scientific, SM0371) was used as molecular standard to confirm the appropriate SRAP markers (Fig 1c).

Amplicon scoring and data analysis

Profiles for each variety and marker technique (ISTR, ISSR and SRAP) were constructed by scoring (0) and (1) for absence and presence of bands and assembled onto a data matrix. Comparisons of the discriminating capacity, the level of polymorphism and informativeness of each marker system of ISTR, ISSR and SRAP were calculated according to Belaj et al., (2003), to compare the efficiency of the three markers (ISTR, ISSR and SRAP) in varietals identification, genetic diversity and differentiation. The genetic similarity among the genotypes was estimated according to DICE (qualitative

data module) coefficients (Dice, 1945; Rohlf, 2000) of the NTSYS-PC software package (version 2.1). The phylogenetic analyses were obtained through clustering analysis by the unweighted pair-group method (UPGMA). To verify the adjustment between similarity matrices and respective dendrogram derived matrices, the cophenetic correlation coefficient (*r*) was estimated using the software NTSYS PC 2.1.

Principal components analysis (PCA)

Principal components analysis (PCA) was carried out to display the multidimensional genetic relationship and its partition among varieties across the binary matrix using TotalLab.TL120.v2009-NULL software (Schlüter and Harris, 2006).

Conclusion

In the ongoing revised we introduced a highlights utility of ISTR, ISSR and SRAP markers system with a good performance in explaining the genetic diversity, systematic behavior and phylogenetic affinities of the Egyptian pomegranate varieties. As far as we know, here is the first attempt to report a comparison of discriminating capacity, efficiency and the ability of ISTR, ISSR and SRAP in Egyptian pomegranate. Apparently, SRAP assay had more sensitive, higher discrimination capacity and gives much more evidence about the unique genetic background, furthermore to simultaneously detect numerous polymorphic markers per reaction. Our findings confirm that combining different marker system were greatly better and considered an important priority step toward diversity study, characterisation and a prerequisite for more effective breeding programs in pomegranate germplasm.

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Author's contribution

Mohamed Abd. S. El Zayat carried out the experiments. Amar planned the project and performed the analysis of data and writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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