Braz. J. Bot (2015) 38(1):119–129
DOI 10.1007/s40415-014-0103-0

Application of EST-SSR marker in detection of genetic variation among purslane (*Portulaca oleracea* L.) accessions

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Received: 9 September 2014 / Accepted: 30 September 2014 / Published online: 25 October 2014

Abstract Express sequence tag (EST)-simple sequence repeat (SSR) was employed to assess the genetic diversity in elite germplasm collections of purslane (*Portulaca oleracea* L.) which is a saline and drought tolerant annual herb containing high amount of vitamins, minerals, and antioxidant properties. The 10 primers gave a total of ninety five bands among which 95 were polymorphic (100 %). The genetic diversity as estimated by Shannon's information index was 1.85, revealing a high level of genetic diversity in the germplasm. The average numbers of observed allele, effective allele, expected heterozygosity, polymorphic information content (PIC), and Nei's information index were 9.5, 5.14, 0.81, 0.33, 0.77, and 0.79, respectively. The UPGMA dendrogram based on Nei's genetic distance grouped the whole germplasm into eight distinct clusters. The analysis of molecular variance (AMOVA) revealed that 71.87 % of total variation occurred within population while 28.13 % from among populations. Considering all these criteria and results from marker-assisted diversity analysis, accessions that are far apart based on their genetic coefficient (like Ac11 and Ac17; Ac4 and Ac42; Ac16 and Ac31; Ac38 and Ac17; Ac21 and Ac31; Ac30 and Ac10) could be selected as parents for further breeding programs.

Keywords AMOVA · Dendrogram · EST SSR markers · Genetic diversity · Purslane (*Portulaca oleracea* L.)

Electronic supplementary material The online version of this article (doi:10.1007/s40415-014-0103-0) contains supplementary material, which is available to authorized users.

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Introduction

Purslane (*Portulaca oleracea* L.) is listed in the World Health Organization as one of the most used medicinal plants and it has been given the term "Global Panacea" (Dweck 2001; Samy et al. 2004). Purslane has recently been identified as the richest vegetable source of α-linolenic acid, an essential omega-3 and six fatty acids (Simopoulos et al. 2005), which is five times higher than spinach (Uddin et al. 2014). The lack of dietary sources of omega-3 and six fatty acids has resulted in a growing level of interest to introduce *P. oleracea* as a new cultivated vegetable (Yazici et al. 2007). Scientifically, purslane provides a rich plant source of nutritional benefits with high antioxidant properties (Alam et al. 2014b). *P. oleracea* is not only high in nutritional value but is also highly adaptable to many different environments (Alam et al. 2014c, d). Uddin et al. (2012) showed that *P. oleracea* flourishes in numerous biogeographical locations worldwide and is highly adaptable to many drought, saline, and nutrient-deficient conditions. These characteristics give *P. oleracea* a competitive advantage over many other cultivated crops and have led some to consider *P. oleracea* as the 'power food for the future' (Simopoulos et al. 1995).

Many research findings have been published worldwide regarding nutritional quality, medicinal properties, drought, and salinity effect on morpho-physiological attributes. but research on genetic diversity among diverse cultivars and collected germplasms are scanty. Availability of depth information on the genetic diversity and relationship among available germplasms are vital before initiating any plant breeding program. Genetic diversity in crop species can be determined using morphological (phenotypic), biochemical, and molecular (DNA) markers (Rao 2004). However, the morphological markers are strongly affected by environmental factors compared to biochemical markers and therefore are dependent on environmental conditions during cultivation. The limitations associated with morphological and biochemical markers are overcome by molecular markers (Rao 2004). Molecular diversity assessed using molecular markers is independent of the influence of environment and estimated using DNA from any growth stage (Tatikonda et al. 2009). Among the various molecular markers, polymerase chain reaction (PCR)-based markers, such as random amplified polymorphic DNA (RAPD; Williams et al. 1990), inter-simple-sequence-repeat (ISSR; Zietkiewiez et al. 1994), amplified fragment-length polymorphism (AFLP; Vos et al. 1995), and sequence-related amplified polymorphism (SRAP; Li and Quiros 2001) have become popular for genetic diversity analyses; their application does not require any prior sequence information. However, DNA microsatellites, or SSR markers often used for genetic analysis, are excellent genetic markers because they are hyper variable, co-dominant, and therefore highly informative (Du et al. 2012; Blair et al. 2010). On the other hand, express sequence tag (EST)-derived microsatellites are such type of markers which are easily obtained by electronic search of EST databases. EST-microsatellite (eSSR) polymorphism is associated with transcribed regions of the genome and reflects the genetic diversity inside or adjacent to the genes (Varshney et al. 2005), which may be functionally more informative than genomic SSR (gSSR) that are more widely used (Hu et al. 2011).

In purslane, AFLP-based drought tolerance and diversity analysis of ten collected accessions from six countries are the first and only available reports (Ren et al. 2011). Whereas, Lokhande et al. (2009) described the genetic diversity analysis of 14 collected clones of sea purslane (*Sesuvium portulacastrum* L.) using RAPD markers. On the other hand, development of EST-SSR primers to assess genetic diversity has been reported in many crops, e.g., in celery (Fu et al. 2014), sorghum (Ramu et al. 2013), in soybean (Zhang et al. 2013), in cotton (Jena et al. 2012), cucumber (Hu et al. 2011), pepper (Kong et al. 2012), and in jatropha (Wen et al. 2010a, b).

However, studies about the comparison between the collected purslane germplasms and the genetic relationships obtained using molecular markers like EST-SSRs are yet to be reported. The present study surveys the molecular diversity in forty-five accessions of purslane collected from seven states of Western Peninsular Malaysia, using ten EST-SSR primer combinations. The genotyping data were used to understand the relationships among forty-five germplasms and identify genetically diverse lines for using them in genetic improvement of purslane.

Materials and methods

Plant materials

A total of 45 purslane accessions were used in this study collected from seven states of Western Peninsular Malaysia (Fig. 1). Among 45 samples, 11 samples were collected from Selangor, 5 samples from Melaka, 5 samples from Negeri Sembilan, 9 samples from Kedah, 5 samples from Perak, 5 samples from Penang, and rest 5 samples from Perlis. The collected purslane samples were transplanted in glass house, field-2, Faculty of Agriculture, Universiti Putra Malaysia, and all the molecular study was done in Plant Breeding and Molecular Biology Laboratory, Agro-Bio Complex, Faculty of Agriculture, Universiti Putra Malaysia. Brief morphological descriptions and collection details of purslane accessions have already been published in our previous article (Alam et al. 2014a).

Genomic DNA extraction

To extract genomic DNA, young and fresh leaves were collected from all 45 purslane accessions. Genomic DNA from each accession was isolated from fresh leaf tissue of 20-day-old plants using the CTAB (cetyltrimethylammonium bromide) method modified from Doyle and Doyle (1990). About 0.5 g of fresh leaf tissue was ground in liquid nitrogen and taken into a 2-ml micro centrifuge tube and then combined with 1,000 µl of CTAB buffer [100 mM Tris–HCl pH 8.0, 20 mM EDTA (ethylenediaminetetraacetate) pH 8.0, 1.4 mM NaCl, 2 % (w/v) CTAB, 2 % (w/v) PVP (polyvinylepyrrolidone) and ß-mercaptaphenol] and 3 µl mercapethanol. The solution was then allowed to centrifuge at 500 rpm at 65 °C for one hour (60 min) by gently shaking the tubes at 10-minutes interval to mix appropriately. Finally, the whole samples were centrifuged at 13,000 rpm for 5 min to precipitate solid parts of the cell. Afterwards, the total amount of supernatant was transferred into a new 2-ml eppendorf tube. Then equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added in each sample, and the tube was gently inverted to homogenize the mixture and centrifuged at 13,000 rpm for 5 min to precipitate polysaccharides. The upper phase

Fig. 1 Map showing the seven states (*white arrow*) of Western Peninsular Malaysia from where purslane samples were collected

was then transferred into a new 2-ml eppendorf tube excluding green and white lower phase and again incorporated with 600 µl cold isopropanol and mixed gently by inverting the tube at least 50 times. All samples were then incubated at −20 °C for 30 min. After that, it was centrifuged for 10 min at 13,000 rpm to get DNA pellet. Carefully pipetted off the supernatant without losing the pellet and let the pellet air-dried. The DNA pellet was then washed and dry out 1–2 times adding 600 µl of 4 °C 75 % ethanol until white DNA fiber appeared. Finally, 50 µl of TE buffer (10 mM tris–HCl pH 8.0, 1 mM EDTA pH 8.0) and 1 µl of RNase (DNase free) were added and mixed by finger vortexing to dissolve DNA completely and incubated at 37 °C for 1 h to break down RNA if any was present in the isolated DNA.

DNA identification and quantification

One µl of each DNA sample was put on NanoDrop spectrophotometry (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA), and relative purity with concentration of the extracted DNA was estimated from the computer-displayed value. The final concentration of each DNA sample was diluted with 1× TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) to get required concentration and kept in a refrigerator of −20 °C for PCR analysis.

EST-SSR primer selection and prescreening

A total of sixty EST-SSR primers were screened with *P. oleracea* DNA out of these; only ten primers with clear and

Table 1 List of EST-SSR primers used for diversity analysis in purslane

Marker	Motif	Forward	Reverse	Tm (°C)
EST-SSR9	(ACA)11	CAATCATCATGCAGTGCTCT	GTTGTAGGTCACTCCATTCC	48
EST-SSR13	(AAG)8	GTTTGGCGGTGGATTGAGA	ATGGTGAGGAGGATGTGGG	55
EST-SSR15	(CAG)8	GTCAACAACCACAACCAAAT	GTTGATGGCATTGTTGATCT	53
EST-SSR19	(ACA)4	ATCATCATGCAGTGCTCTCT	AACTGGTTGTTCACGTTGTT	58.5
EST-SSR20	(TAA)15	TAAAGTAGGCCAACTGCAAA	ACTTTGATTGGAGGAAGAGG	53
EST-SSR22	(GAT)10	TCAACCTGCCAAAGTACAAT	TTTTTCTTGGCTGTAGCTTG	50.4
EST-SSR23	(GAT)6	ACTGACACAAATGGAAATGC	GCTCGTCAATAGTGAGATCG	50
EST-SSR26	(CGA)6	GAGAGACGACTGGCATACTG	CCACCTGTTGACATCCTATG	55.8
EST-SSR41	(GGA)5	TAACCCAGCTATGCCTTACA	TCTCCTTCCTCTTTGACCTC	55.6
EST-SSR46	(TAA)4	AGTATCTGGGATTCCTTTCG	ACTGATGTTGGATGAGCAAC	53

reproducible polymorphic bands were chosen and used for analysis of genetic diversity. Primers sequence with annealing temperature of those ten EST-SSR markers has been presented in Table 1.

PCR amplification for EST-SSR markers

Polymerase chain reaction for amplification of EST-SSR fragments was performed in 15 µl of reaction volume containing 1 µl of 70 ng template DNA, 1 µl of each forward and reverse EST-SSR primers, 7.4 µl master mix (Dream Taq green PCR master mix-2× containing green buffer, dNTPs and 4 mM $MgCl_2$, Supplied by Fermentas-Fisher Scientific, UK), and 4.6 µl nuclease free water. PCR amplification was carried out in a thermocycler (T100TM, Bio-Rad) following three phases Touchdown PCR protocol (Don et al. 1991) with slight modifications. In the first phase, initial temperature was adjusted to 95 °C for 3 min and the initial denaturation temperature was set at 95 °C for 30 s, annealing at Tm 62 °C for 60 s, and the elongation temperature was set at 72 °C for 30 s. In the second phase, denaturation temperature was set at 95 °C for 30 s, annealing at Tm 52 °C for 60 s and the elongation at 72 °C for 2 min, and finally, the elongation temperature was set at 72 °C for 10 min in the third phase. The 1st and 2nd phases were repeated 9 and 30 times, respectively, followed by cooling and infinitive hold at 4 °C. For electrophoresis, 3.0 % MetaPhor™ agarose (Lonza, USA) gel was prepared containing 1 µl Midori green in 1× TBE buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.0). The gel was run at constant voltage of 80 V for 80 min and visualized Molecular Imager® (GelDocTM XR, Bio-Rad). However, most of the patterns with extremely good polymorphism and useful information were often accompanied with a background smear. To reduce this smear, 2 % formamide was used in the reaction. All the patterns generated were repeated at least three times in order to obtain reproducible data.

Data scoring

Molecular weight for each band was measured using Alfa Imager software version 5.5. Each amplification product position was considered a particular locus. The amplified products for EST-SSR marker alleles at a specific position in a gel were scored visually as "1" for present and "0" for absent of a band to generate a binary data matrix. A 50 bp DNA ladder was added with the first load of the gel to score of the bands in each gel. The only bands that were scored are those that are >50 bp in length and reproducible. The scoring of the visual bands was done according to the size of the DNA ladder bands defined by the producers (www.genedirex.com).

Data analysis

Amplified bands were visually scored as present (1) or absent (0) for each EST-SSR marker. With the marker data, binary matrices were established and then used to calculate Jaccard's similarity coefficients (Jaccard 1908). Cluster analysis was performed to construct dendrogram based on the similarity matrix data using the unweighted pair group method using arithmetic averages (UPGMA) and the *SHAN* clustering program. All analyses were performed with the *NTSYS-pc 2.10* software (Rohlf 2002). Polymorphism information content (PIC) values were calculated to evaluate diverse level of EST-SSR marker according to Anderson et al. (1993) using the formula: $PIC = 1-\Sigma P_{ij}^2$, where P_{ij} is the frequency of the *j*th allele (marker) for the *i*th EST-SSR locus. The binary data were also subjected to PCA (principal component analysis) to investigate the structure of our collection. The PCA of the 45 purslane accessions were calculated by EIGEN module of NTSYS-pc 2.10 (Rohlf 2002) software. The genetic diversity parameters of each geographic group, which included the percentage of polymorphic loci (PLP), observed number of alleles per locus (n_a), effective number of alleles per locus

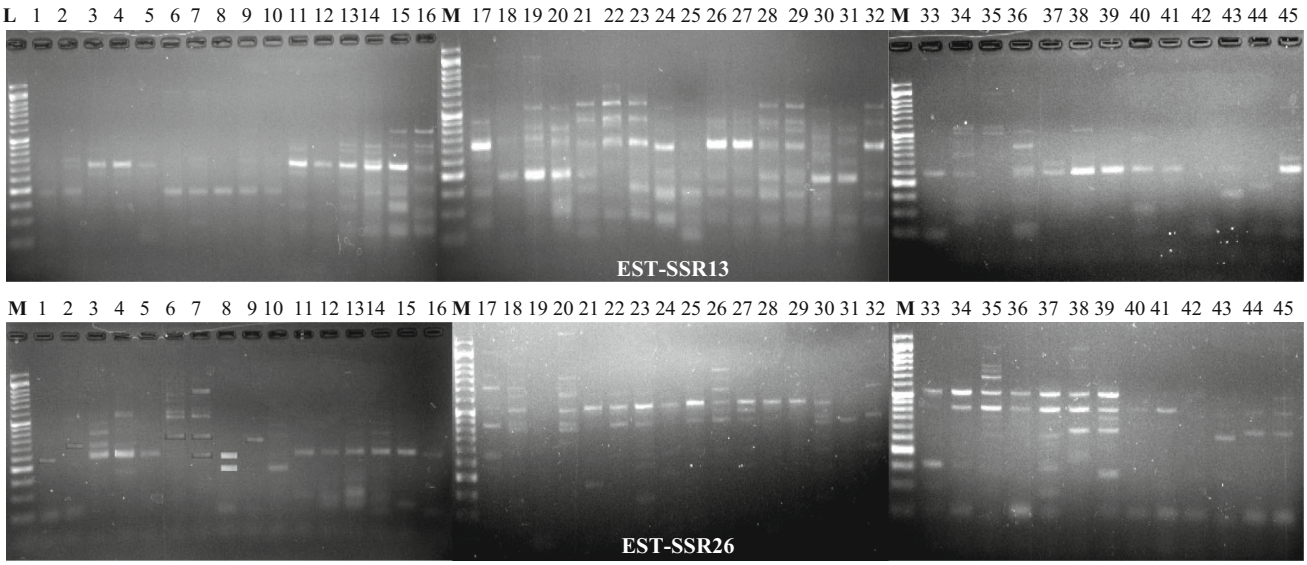

Fig. 2 Polymorphic banding patterns of EST-SSR13 and EST-SSR26 among all 45 genotypes. *Lane M* 50 bp DNA ladder (Genedirex). *1* Ac1; *2* Ac2; *3* Ac3; *4* Ac4; *5* Ac5; *6* Ac6; *7* Ac7; *8* Ac8; *9* Ac9; *10* Ac10; *11* Ac11; *12* Ac12; *13* Ac13; *14* Ac14; *15* Ac15; *16* Ac16; *17* Ac17; *18* Ac8, *19* Ac19; *20* Ac20; *21* Ac21; *22* Ac22; *23* Ac23; *24* Ac24; *25* Ac25; *26* Ac26; *27* Ac27; *28* Ac28; *29* Ac29; *30* Ac30; *31* Ac31; *32* Ac32; *33* Ac33; *34* Ac34; *35* Ac35; *36* Ac36; *37* Ac37; *38* Ac38; *39* Ac39; *40* Ac40; *41* Ac41; *42* Ac42; *43* Ac43; *44* Ac44 and *45* Ac45)

Table 2 Genetic diversity parameters among 10 EST-SSR markers for all 45 accessions of *P. oleracea*

Locus	Observed number of alleles	Effective number of alleles	Expected heterozygosity	Observed heterozygosity	PIC	Nei's index	Shannon's information index
EST-SSR9	12	3.9862	0.7576	0.3556	0.7229	0.7491	1.7643
EST-SSR13	7	5.0311	0.8102	0.4889	0.7747	0.8012	1.7437
EST-SSR15	6	3.2478	0.6999	0.8000	0.6526	0.6921	1.4219
EST-SSR19	8	4.2276	0.7720	0.3111	0.7337	0.7635	1.7017
EST-SSR20	10	6.0994	0.8454	0.6000	0.8175	0.8360	2.0078
EST-SSR22	9	6.0448	0.8439	0.0000	0.8159	0.8346	1.9771
EST-SSR23	10	4.7872	0.8000	0.0667	0.7650	0.7911	1.8673
EST-SSR26	9	5.4144	0.8245	0.3333	0.7937	0.8153	1.8951
EST-SSR41	12	5.8866	0.8395	0.1778	0.8104	0.8301	2.0082
EST-SSR46	12	6.6832	0.8599	0.2000	0.8340	0.8504	2.1089
Mean	9.5	5.1408	0.8053	0.3333	0.7721	0.7963	1.8496

(n_e), Nei's gene diversity (*h*), and Shannon's information index (I) were calculated by POPGENE software (version 3.2) (Yeh et al. 2000).

Analysis of molecular variance (AMOVA)

After obtaining the optimum number of subpopulations, an analysis of molecular variance (AMOVA) was performed using Arlequin software version 3.1 (Excoffier et al. 2005) to estimate the genetic variance components within populations and between populations.

Results

Allelic variation among the accessions using EST-SSRs

Ten EST-SSRs were used to estimate the genetic diversity of 45 accessions of *P. oleracea*. The sizes of the amplicons ranged from 100–650 bp. The 10 EST-SSRs produced 95 alleles of which all 95 (100 %) were polymorphic (Fig. 2). The number of alleles for each EST-SSR ranged from six (EST-SSR15) to twelve (EST-SSR9, EST-SSR41 and EST-SSR46) with a mean of 9.5. The PIC values for the

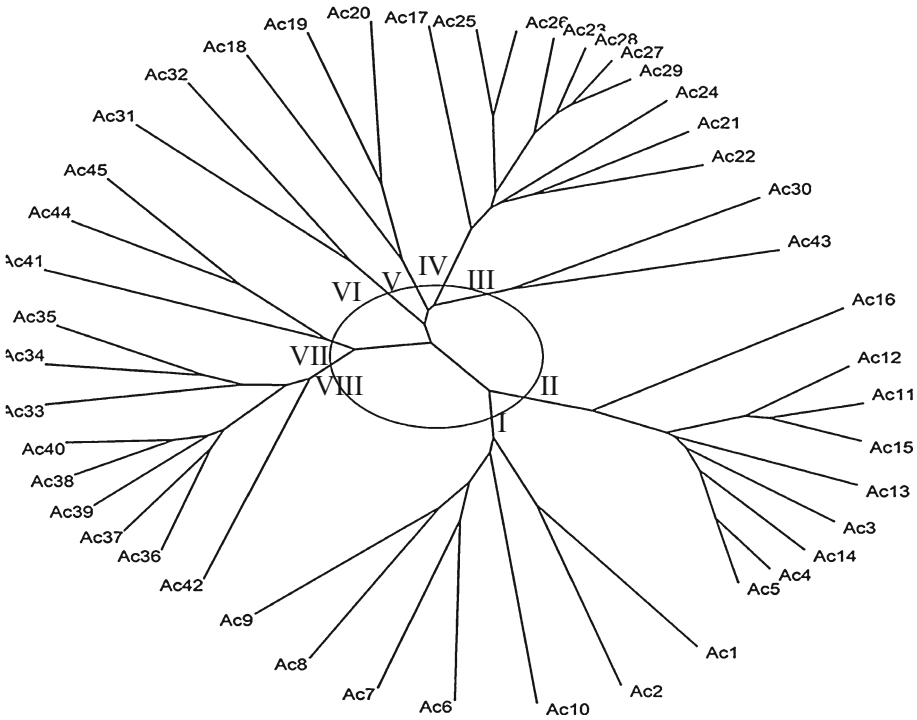

Fig. 3 Dendrogram based on UPGMA, depicting the genetic relationship among the *P. oleracea* accessions using EST-SSR markers among 45 *P. oleracea* accessions

Table 3 Accessions comprising in various clusters as shown in the dengrogram based on UPGMA method using EST-SSR markers

Clusters	Accessions
Cluster I	Ac1, Ac2, Ac6, Ac7, Ac8, Ac9, Ac10 = 7 accessions
Cluster II	Ac3, Ac4, Ac5, Ac11, Ac12, Ac13, Ac14, Ac15, Ac16 = 9 accessions
Cluster III	Ac30 and Ac43 = 2 accessions
Cluster IV	Ac17, Ac21, Ac22, Ac23, Ac24, Ac5, Ac26, Ac27, Ac28 and Ac29 = 10 accessions
Cluster V	Ac18, Ac19 and Ac20 = 3 accessions
Cluster VI	Ac31 and Ac32 = 2 accessions
Cluster VII	Ac33, Ac34, Ac35, Ac36, Ac37, Ac38, Ac39, Ac40 and Ac42, = 9 accessions
Cluster VIII	Ac41, Ac44 and Ac45 = 3 accessions

evaluated 10 EST-SSR primers were relatively high (0.65–0.83) with a mean value of 0.77, which indicate the effectiveness, polymorphism, and validity of the selected primers (Table 2).

The maximum numbers of effective alleles (6.683) were found in EST-SSR46 and the lowest (3.248) were seen in EST-SSR15 (Table 2). Furthermore, the highest expected heterozygosity was estimated in EST-SSR46 (0.859), and the lowest was recorded in EST-SSR15 (0.699), respectively, with the mean value of 0.81 (Table 2). The Nei's varied from 0.6921 to 0.8504 with the highest in EST-SSR46 and lowest in EST-SSR15 with the mean value of 0.7963 and the Shannon's information index varied from 1.4219 to 2.1089, respectively, with the highest in EST-SSR46 and the lowest in EST-SSR15 and their mean value was 1.8496 (Table 2).

Cluster analysis

The UPGMA and neighbor-joining dendrogram from EST-SSR data were constructed using Power marker software. The tree from cluster analysis showed the genetic relationships among 45 purslane accessions, and 8 distinct groups were generated from the analysis (Fig. 3; Table 3). Here, cluster IV constructed with the highest number (10) of accessions followed by cluster II and VII, both were composed of 9 accessions, cluster I had 7 accessions, clusters V and VIII were comprised with 3 accessions, and both the clusters III and VI contained 2 accessions, respectively (Table 3). The genetic differences among 45 *P. oleracea* accessions ranges from 0.90 to 0.27 (Supplementary table) where the highest distance observed in

Table 4 Pairwise population matrix of Nei's genetic distance among seven states using EST-SSR markers

States	Selangor	Melaka	Nigeri Sembilan	Kedah	Perak	Penang	Perlis
Selangor	0.00						
Melaka	0.13	0.00					
Negeri Sembilan	0.21	0.30	0.00				
Kedah	0.28	0.39	0.14	0.00			
Perak	0.24	0.32	0.15	0.23	0.00		
Penang	0.35	0.47	0.36	0.40	0.19	0.00	
Perlis	0.29	0.41	0.26	0.32	0.16	0.22	0.00

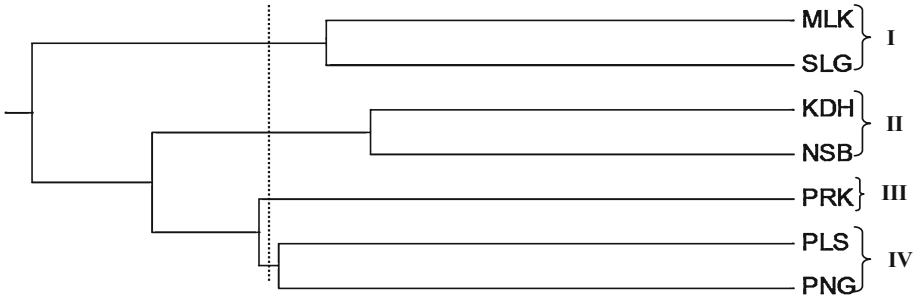

Fig. 4 The figure describes the pattern of clustering which divided the seven states into four groups based on molecular data analysis using EST-SSR markers (*MLK* Melaka, *SLG* Selangor, *KDH* Kedah, *NSB* Nigeri Sembilan, *PRK* Perak, *PLS* Perlis and *PNG* Penang)

several accessions and the least distances was found among both in Ac27 and Ac28, and Ac28 and Ac29.

Another dendrogram constructed based on the source of sample collection from seven states in Western Peninsular Malaysia where broadly divided into two major groups: with Melaka (MLK) and Selangor (SLG) in one cluster while other states belongs to the second cluster (Fig. 4). Individually, Melaka and Selangor comprised cluster I, Kedah and Negeri Sembilan formed cluster II, Perak separately consisted cluster III, and finally cluster IV was composed with Perlis and Penang (Fig. 4). The pairwise population matrix of Nei's genetic distance has been presented in Table 4.

Principal component analysis

The patterns of cluster analysis were also confirmed by the Principal Component Analysis (Figs. 5, 6). The principal component analysis (PCA) results derived from EST-SSR markers indicated 81.9 % of total variation came from the first three principal components with PC1, PC2, and PC3 having 63.4, 11.9, and 6.6 %, respectively (Table 5). From PC1, the highest value was 0.259 followed by 0.219 and 0.193, while the least were −0.439, −0.396, and −0.359, respectively. The highest (0.259) was found in Ac44, while the least was found in Ac40 (Table 5).

Analysis of molecular variance (AMOVA)

The analysis of molecular variance unveiled that variation among the population was found to be low (28.13 %), while variation among the population accounted for 71.87 % of the total variation of *P. oleracea* accessions (Table 6).

Discussion

Any crop improvement program starts with identification of variability among the genotypes. Study of genetic diversity is a critical component of applied plant breeding for optimizing the choice of parents in a crop-breeding program. An effective germplasm assessment provides the scientific basis for selection of parents/donors for recombination breeding or hybrid breeding, and to breed for specific agro-ecological conditions and situations (Kumar et al. 2012). Diversity analysis at the molecular level using PCR-based markers is the cheapest and rapid method of identifying the relationships and/or differences among the genotypes. Formerly, genetic diversity was mostly measured based on the morphological and physiological variations of quantitative and economically significant traits, but these methods have several weaknesses, such as time consuming and laborious, and this method cannot define the exact level of genetic diversity among germplasm because the trait appears by interaction between genes and environment (Schulman 2007). The genetic uniqueness of each accession can be determined and quantified by the use of DNA profiling which employing various molecular markers such as RFLPs, DAFs, RAPDs, and microsatellites (Brown and Kresovich 1996); however, PCR-based

Table 5 Principal component analysis and percentage variation in first three principal components for 45 purslane accessions by EST-SSR markers

Accessions	Eigenvector		
	PC1	PC2	PC3
Eigen value	28.54	5.35	2.99
Percent	63.4	11.9	6.6
Cumulative	63.4	75.3	81.9
Ac1	0.055	0.061	−0.332
Ac2	0.009	−0.155	0.012
Ac3	0.078	−0.087	−0.011
Ac4	−0.396	0.038	0.159
Ac5	0.064	0.103	−0.120
Ac6	0.043	−0.383	−0.120
Ac7	0.069	0.001	0.225
Ac8	−0.130	−0.048	−0.122
Ac9	−0.033	0.240	−0.075
Ac10	−0.009	0.159	0.415
Ac11	−0.040	−0.010	0.031
Ac12	0.107	0.245	−0.130
Ac13	0.122	0.002	−0.133
Ac14	−0.055	−0.084	−0.106
Ac15	−0.138	0.030	0.302
Ac16	0.148	0.330	−0.073
Ac17	0.090	0.239	0.265
Ac18	−0.168	0.064	−0.059
Ac19	0.074	−0.060	0.092
Ac20	−0.173	−0.234	−0.060
Ac21	0.039	0.015	−0.081
Ac22	−0.342	0.097	−0.043
Ac23	0.064	−0.122	0.095
Ac24	0	0.027	−0.017
Ac25	−0.040	0.063	0.058
Ac26	0.061	0.018	−0.192
Ac27	−0.009	−0.030	−0.025
Ac28	0.049	−0.227	0.096
Ac29	0.059	−0.045	−0.025
Ac30	−0.040	−0.078	0.055
Ac31	0.193	−0.148	0.073
Ac32	−0.007	−0.088	0.099
Ac33	0.219	0.145	0.052
Ac34	0.077	0.007	−0.061
Ac35	−0.349	0.108	−0.093
Ac36	0.042	−0.126	−0.254
Ac37	−0.021	−0.107	−0.162
Ac38	0.083	−0.071	−0.222
Ac39	0.069	−0.040	0.017
Ac40	−0.439	−0.067	0.019
Ac41	0.171	−0.125	0.302
Ac42	−0.003	−0.300	0.148
Ac43	−0.006	0.318	−0.037
Ac44	0.259	−0.058	0.072
Ac45	0.098	0.152	−0.026

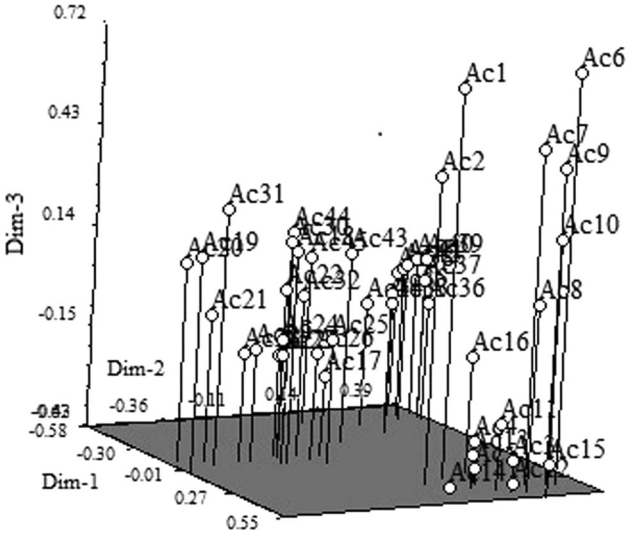

Fig. 5 Three-dimensional plots of PCA indicating relationships among 45 purslane accessions based on EST-SSR markers

markers are more suitable for large-scale analysis. Among the PCR-based markers, microsatellites are becoming more popular, both for genetic diversity and breeding research (Joshi et al. 2000). Proficient and consistent use of molecular markers such as EST-SSR for study of genetic diversity in any food crop or tree crop requires selection and application of primers which will give clear, distinct, reliable, and sufficient information required to study the divergence that occur within the crop (Arolu et al. 2012). In our research, the number of polymorphic loci detected per primer combination varies according to the primer. The EST-SSR markers amplified distinct band patterns among the 45 *P. oleracea* accessions, and each marker revealed polymorphism.

The 10 EST-SSR markers detected 95 alleles (100 %) with a mean of 9.5, showing a higher discrimination power. Also, PIC values presented a similar result (Table 2). The PIC values that ranged from 0.65 to 0.83 with a mean of 0.77. Our result is in agreement with the findings of Hu et al. (2011) where they estimated genetic diversity among cucumber germplasm collections using SSR and EST-SSR markers. Furthermore, in our study, the number of alleles

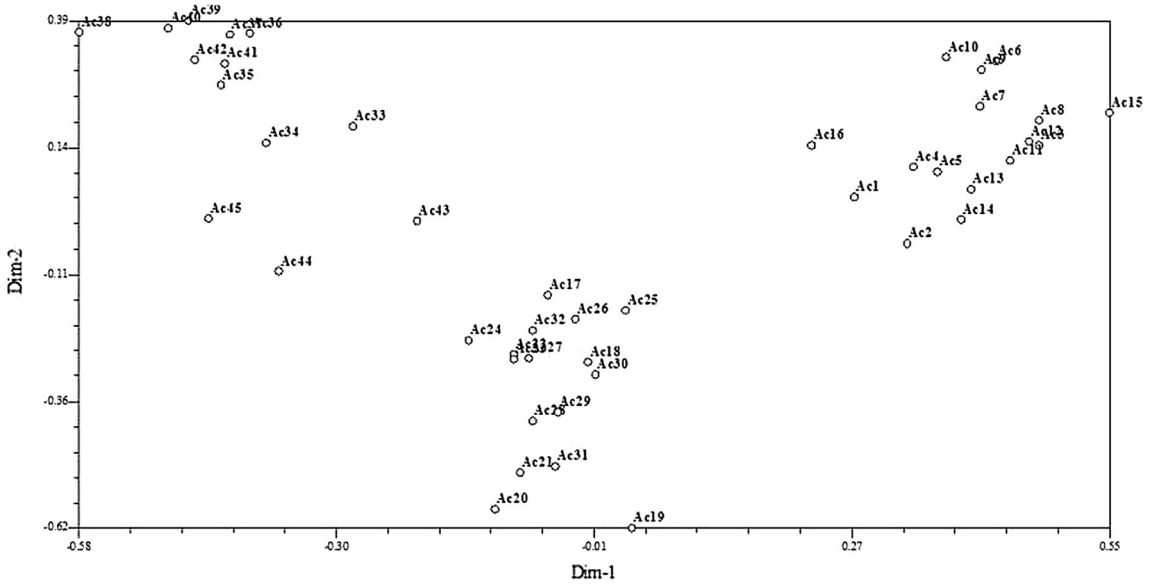

Fig. 6 Two-dimensional plots of PCA indicating relationships among 45 purslane accessions based on EST-SSR markers

Table 6 AMOVA within and between 45 purslane accessions using EST-SSR markers

Source	df	SS	MS	Est. Var.	%	F_{st}	*P* value
Among pops	6	107.435	17.91	1.18326	28.13	0.2813	<0.001
Within pops	38	250.920	3.02	3.02313	71.87		<0.001
Total	44	358.355		4.20639	100.00		

for each EST-SSR ranged from 6 to 12 with a mean of 9.5 (Table 2) which is very similar with the outcomes of watermelon diversity analysis using EST-SSR by Mujaju et al. (2013) and Hwang et al. (2011).

The EST-SSR markers also demonstrated different levels of genetic diversity among *P. oleracea* accessions under present study. The Shannon's information index varied from 1.42 to 2.11 with the mean value of 1.85, which is about 10-fold higher that the reported mean value (0.16) by Mujaju et al. (2013) in watermelon. Greater value of Shannon's information index in the present study was another indication of presence of higher genetic diversity in the genotypes under this study. The Nei's gene diversity varied from 0.69 to 0.85 with the mean value of 0.79. This average value was higher than the value recorded by Wen et al. (2010a, b) in *Jatropha curcas* L. who found 0.38 which also indicated presence of adequate genetic diversity (Table 2). This average value was higher than the value recorded in rice by Zai-quan et al. (2012) who found 0.33.

Expected heterozygosity from this study found to vary from 0.69 to 0.86 with the mean value of 0.81. This finding is also 8-fold and 20-fold higher than the expected heterozygosity mean output (0.10 and 0.04) described in watermelon accessions (Mujaju et al. 2013) and in Sorghum (Ramu et al. 2013), respectively, using EST-SSR markers.

The genetic diversity parameters such as expected heterozygosity, Shannon's information index, Nei's gene diversity, and PIC have positive correlation to the number of allele. It was observed from the current study, that EST-SSR marker had the highest values of expected heterozygosity (0.81), Shannon's information index (1.85), Nei's gene diversity (0.79), and PIC (0.77; Table 2). Since, higher value of genetic diversity parameter implies greater diversity. So, EST-SSR maker was more effective and suitable for genetic diversity analysis in *P. oleracea* germplasm collections.

A better resolution of the relationship among the 45 purslane accessions was provided by the UPGMA cluster analysis using EST-SSR markers. EST-SSR marker produced eight major clusters among 45 *P. oleracea* accessions (Fig. 3). It is notable that EST-SSRs produced a total of eight clusters and the reason could be explained in this way that EST-SSR markers might target larger number of repeated sequences specifically in the centromeric region that might heavily influence the classification pattern (Parsons et al. 1997). These clustering patterns prove the acceptability and adaptability of EST-SSR markers for the genetic diversity analysis among purslane germplasm collections.

Additionally another dendrogram was constructed based on the germplasm collections from seven states of Western Peninsular Malaysia where Selangor and Melaka were grouped in same cluster may be due to the agro-geographical relationship and becoming neighbor states. But very interestingly, Negeri Sembilan was clustered with Kedah though they are situated far away from each other; may be any how, the primary plant materials were transferred to Kedah from N. Sembilan or vice versa. Same grouping was also observed among Penang and Perlis as grouping pattern of Selangor and Melaka. Whereas, Perak was clustered separately but broadly with the same group of Penang, Perlis and Perak leaving their neighbor state Kedah (Fig. 4).

Pairwise population matrix of Nei's genetic distance showed the maximum distance among Penang and Melaka (0.47). Both states are situated to the opposite corner from each other and the minimum (0.13) was found among Melaka and Selangor (Table 4). Yan et al. (2009) conducted AFLF marker-based genetic diversity analysis among *P. oleracea* accessions sampled worldwide regarding α-linolenic fatty acid (ALA) composition analysis. Whereas, the first based on taxonomy Danin et al. (1978) noted the high level of diversity in Australian *P. oleracea* accessions which differed to those in all other parts of the world.

It was found that cluster analysis was profoundly supported by the PCA. The genetic diversity results which were observed under this study supported by the consonance between cluster and PC analyses (Figs. 3, 5; Table 5). Principal components analysis (PCA) indicated 81.9 % of total variation among all the accessions, and the first three principal components (PC1, PC2, and PC3) from the Eigen vectors analysis were 63.4, 11.9, and 6.6 %, respectively (Table 5). Fu et al. (2014) also stated that the cluster analysis and PCA results were almost same who determined the genetic diversity among celery (*Apium graveolens* L.) cultivars using EST-SSR markers. The similar findings have also been reported by Zhang et al. (2013) using EST-SSR markers in vegetable soybean genetic diversity analysis.

The AMOVA revealed that higher percentage of variation was attributed to variation within populations within populations (71.87 %) and among populations (28.13 %) of *P. oleracea* accessions (Table 6). Li et al. (2011) opined that, assessment of genetic diversity within a population is essential to characterize germplasm and provides insights into evolutionary aspects, conservation, utilization, and establishment of breeding programs.

Conclusion

There was a high level of genetic diversity among accessions of *P. oleracea* in this study suggesting that EST-SSR markers were effective in detection of polymorphism in this species, and this is the first report on using EST-SSR markers to study the genetic diversity on *P. oleracea* germplasm collections. To broaden the genetic base and improvement of the *P. oleracea,* populations having the lowest genetic similarities could be selected as parents. Therefore, hybridization should be made between two distant populations. Results from marker-assisted diversity analysis accessions that are far apart based on their genetic coefficient (like Ac11 and Ac17; Ac4 and Ac42; Ac16 and Ac31; Ac38 and Ac17; Ac21 and Ac31; Ac30 and Ac10 from EST-SSRs respectively) could be selected as parents for further breeding programs. This will bring about greater diversity which will lead to high productive index in terms of increase in yield and overall quality.

Acknowledgments The authors sincerely acknowledge UPM Research University Grant (01-02-12-1695RU) for financial support of the project and IGRF (International Graduate Research Fellowship, UPM) for PhD Fellowship.

Conflict of interests The authors declare that there is no conflict of interests regarding the publication of this article.

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