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INTRA-SPECIFIC GENETIC DIFFERENTIATION SHAPING THREE *PORTULACA OLERACEA* L. MICRO-SPECIES

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Abstract

Three *Portulaca oleracea* L. taxa were used as pure and mixed populations. The pure taxa are geographically separated and identified as the micro species *P. granulatostellulata*, *P. oleracea* and *P. nitida*. DNA tetraploids were recorded in all *P. nitida*, *P. granulatostellul at a*individuals and surprisingly for *P. oleracea* which is known as DNA hexaploid. It was found that the Nei's gene diversity (*h*) in all pure taxa were lower than the same taxa at the mixed case. The observed heterozygosity (H_o) of pure populations were significantly different from the mixed populations of the taxa (T = -3.14 $p<0.05$). Mean inbreeding coefficient (F_{IS}) was significantly higher in mixed populations than in pure populations. The mean G + C content differed by 1% between the pure and mixed populations of all *trnL-F* alignments studied here. The mean nucleotide diversity (*Pi*) value of mixed populations was higher than of pure populations. Hierarchical analyses under AMOVA indicated that the highest value of genetic variation occurred within populations in both maternal (cpDNA) and biparental (isozymes) markers. Gene flow among populations based on cpDNA data (N_m= 5.48) was higher than the one based on isozymes (N_m = 0.302).The phylogentic tree based on the *trnL-F* sequence comprised all the pure and the mixed populations of *P. olereacea*s L., in one clade with high bootstrap support (100%), except for one individual of *P. oleracea* growing sympatrically with *P. granulatostellulata* population. The study suggested that a higher rate of gene flow occurs between these taxa than expected and *P. oleracea* taxa are kept distinct in the mixed case by the slight difference in selfingrate, which could be one of the ambient conditions shaping the *P. oleracea* L. phenotype.

Key words: *Portulaca oleracea*, Genetic differentiation, Isozyme, cpDNA.

Introduction

Intra-specific variation provides the material for long term evolutionary adaptation and short term adaptation to environmental factors, because loss of variation may largely limit the adaptability of populations to changing environments (Ge*et al.,* 2005). The genetic variation within and between populations is the outcome of several factors, such as mutation rate, breeding size of the population, breeding strategy, migration and selection. The total genetic diversity differs widely between species and groups of species and consider as a key factor in its persistence (Rauch& Bar-Yam, 2005). Several mechanisms are involved in maintaining a genetic variation. One such mechanism is balanced polymorphism based on a higher viability of heterozygotes as compared to homozygotes. Maintenance of a minimum level of intra and interpopulation genetic diversity is important for the genetic fitness of a species (Barrett & Kohn, 1991). Increased within-individual genetic variation is provided by fixed heterozygosity (Soltis&Soltis, 2000; Soltis*et al.,* 2004; Meimberg *et al.,* 2009), which may act as a defence against deleterious recessive alleles and hence facilitate the change to inbreeding observed in manypolyploids (Vogel *et al.,* 1999; Guggisberg *et al.,* 2006; Barringer, 2007; Husband *et al.,* 2008).Genetic diversity, origin and transfer of adaptations, origin of new species or ecotypes, and breakdown or reinforcement of isolating barriers, all area number of potential evolutionary consequences of hybridization (Abbott, 1992; Rieseberg & Ellstrand, 1993; Rieseberg & Wendel, 1993; Arnold, 1997; Rieseberg, 1997).

P. oleracea is characterized by a sequence of polyploids from a base of X= 9. There are diploid races (2n = 18) (Hagerup, 1932; Danin *et al.,* 1978); tetraploid races (2n = 36) (Khullar & Dutta, 1973; Danin *et al.,* 1978) and hexaploidraces (2n = 54) (Khullar & Dutta, 1973; Sanjappa,1978; Hagerup, 1932; Bouharmont, 1965; Nyananyo & Okoli 1987; Walters, 1964; Bouharmont, 1965; Cooper, 1935; Heiser & Whittaker, 1948; Mulligan, 1961; Danin*et al.,* 1978; Kim & Carr, 1990). In addition, Sharma & Bhattacharyya (1956) reported 2n = 45, Sugiura (1936) reported 2n = 52 and Danin (2012) reported 2n= 48 for new taxa *Portulacasardoa* from Italy. Danin *et al.* (2006) reported 2n= 36 for *P. granulatostellulata* and *P. nitida* and 2n= 54 for *P. oleracea*. The most recent count of *P. oleracea* was provided by Arrigoni (2006), who reported 2n= 54 for *P. oleracea*, 2n= 36 for *P. granulatostellulata* and *P. nitida*.

The taxonomy of *Portulacaoleracea* has been considered as being complex since the aggregate is composed of many subspecies (Danin *et al.,* 1978) or a group of micro-species based on seed-coat characters, seed size, and chromosome numbers (Danin & Raus, 2012; Danin & Reyes-Betancort, 2006; Danin *et al.,* 2008; Ricceri & Arrigoni, 2000). A previous morphological and molecular study on the same taxa suggested that morphological differences did not reflect the genetic variation in *P. oleracea* taxa, and phylogenetic relationships are positively correlated with patterns of genetic variation. The study concluded that *P. oleracea* exists as a polymorphic species and is not divisible into micro-species that are restricted to a limited geographical area (El-Bakatoushi *et al.,* 2013).

In the current study, isoenzymatic genetic markers were used to assess the genetic variability of populations with pure and mixed taxa of *P. oleracea* and the possible effect of the selfingrate in genetic structure and their evolution. Isozymes, the use of which was proposed by Hunter & Market (1957), have many advantages: they are direct products of the action of genes, they segregate according to Mendel's laws, and they usually are co-dominant. However, the environment can cause direct

effects on the gene activity of a particular isozyme e.g. Werner & Sink 1977. Isozymemarkers have been applied in a wide range of research on a variety of plant species (Weeden, 1989; Yu *et al.,* 2005; Krzymińska *et al.,* 2008; Ouji *et al.,* 2011).

Analysis of the genetic relationships of haplotypes of alleles may provide important information about the extent of similarity and gene flow among populations (Nei & Kumar, 2000). In this study, the *trnL–F* non-coding sequences of cpDNA were sequenced and utilized as markers to examine the intra-specific phylogeny and population genetic structure of seven populations. Chloroplast DNA (cpDNA) *trnL–F* non-coding sequence has been frequently used to survey phylogeographical pattern, plant intra-specific phylogeny and population genetic structure (e.g., Chiang *et al.,* 2003; Dobeš *et al.,* 2004; Grivet & Petit, 2002; Larena *et al.,*2002; Trewick *et al.,* 2002). Its uniparental inheritance, nearly neutral, fast evolution and is well suited to reconstructedintra-specific phylogeography of plants (Huang *et al.,* 2001). Chloroplast DNA (cpDNA)*trnL–F* non-coding sequence (Taberlet *et al*., 1991) has been found often to exhibit differences between closely related species (Bo¨hle *et al.,* 1994; Gielly & Taberlet, 1994a, b; Petit *et al.,* 2003) , as well as being variable within species (McCauley *et al.,* 1996; Wares & Cunningham, 2001).

By investigating the pattern of allozyme shared alleles and patterns of within- and among mixed populations cpDNA diversity we are able to test the hypothesis that the taxa are kept distinct by selection pressures despite countering gene flow. Our assumption in this study is; the amount of variation may be explained by the breeding strategy of taxa and selfingrate one of the ambient conditions shaping the *P. oleracea* phenotype.

Material and Methods

Plant sampling: Fourteen individuals well morphologically defined were selected from previous study including 63 individuals of 16 populations and 6 localities (El-Bakatoushi *et al.,* 2013). Seven populations were selected representing three taxa of *P. oleracea* aggregates; *P. granulatostellulata*, *P. oleracea* and *P. nitida*. Three pure populations were used for comparison and as standards. They were from districts where only one taxon occurs and 4 populations from 2 localities where 2 taxa mixed together were selected (Table 1).

Chromosome counts: Seeds of 14 individuals representing pure and mixed populations were germinated at room temperature. Root tips of 1-2cm long were treated with a solution of 0.002M 8-hydroxyquinoline for three hours at 4ºC before fixation in ethanol-glacial acetic acid (3:1 V/V) for 24 hrs. Five to six roots of each individual were stained by modified carbolfuchsin in (Millerd *et al.,* 1971). Semi-permanent slides were examined by light microscopy. Five metaphase chromosome cells per root were counted visually and by using Micro-Measure computer program (Reeves, 2001).

Isozyme electrophoresis: Four Enzymes (Peroxidase (PX), Amylase (AMY), Esterase (EST) and α Esterase (EST) for 14 individuals were resolved on Agar-starch-polyvinyl pyrolidine (P.V.P) and gel electrophoresis was carried out according to the procedures described by Shaw &Kaen (1967) and Sabrah& El-Metainy (1985).As Enzymes with multiple loci migrating toward the cathode and others migrating to the anode, the most cathodly migrating isozyme was designated locus-1with negative charge. Bands on agarose gels were scored as present or absent and a pair wise similarity matrix was constructed using Nei coefficient. Dendrograms were constructed using the un-weighted pair-group method analysis (UPGMA) with SAHN module of NTSYS-pc (Rohlf, 2002).The indices of genetic diversity were calculated using POPGENE 3.2 software (Yeh *et al.,* 1999) on the basis of gene frequencies. Hierarchical analysis of molecular variance (AMOVA) within and among populations was done using allele frequencies using Arlequin version 3.0 (Excoffier *et al.,* 2005).

Chloroplast DNA: Two cpDNA non-coding regions, *rpl32-trnL* and *trnL-F*, for 12 accessions including pure and mixed taxa were analysed. Total genomic DNA was extracted using theBiospin Plant Genomic DNA Extraction Kit (BioFlux, China). Primers used for amplification and sequencing were *rpl32-trnL* (Shaw *et al.,* 2007) and *trnL-F* (Taberlet *et al*., 1991). PCR was performed in reaction volume of 25µl using 20ng of DNA of each sample, 25 pmol of each primer, 10X Taq DNA polymerase buffer (Fermentas) including MgCl2, 0.2 mMd NTPs and 0.5 unit/µlTaq DNA polymerase (Fermentas). Thermal cycling (Gen Amp PCR system 9700) was carried by initial denaturation at 95°C for 5 min, followed by 35 cycle of 1 min. at 95°C, 1 min. at 59°C, 1 min at 72°C and a final extension at 10 min. at 72°C. PCR products were then separated electrophoretically on agarose gel using 2% (w/v) agarose in 0.5×TBE buffer. The gel was stained thereafter with 0.5 µg/mL ethidium bromide. Amplifications of *rpl32-trnL* repeatedly failed in most of individuals. Therefore, PCR products of only *trnL-F*marker were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada), following the manufacturer's instruction. The DNA sequences were determined by Macrogene Company (Korea).The DNA sequences are deposited in European Molecular Biology Laboratory (EMBL) Bank under the accession numbers from LN559088 to LN559099.

Pair-wise and multiple DNA sequence alignment and nucleotide composition were carried out using CLUSTAL W version 1.81 (http://seqtool.sdsc.edu/CGI/BW.cgi; Thompson *et al*., 1997). The coefficient of differentiation and transition/transversion (ns/nv) ratio were computed by Molecular Evolutionary Genetics Analysis MEGA 4 (Tamura *et al*., 2008). Nucleotide diversity (*Pi*) and Gene flow (N_m) (Hudson *et al*., 1992) among populations were quantified using DnaSP (Rozas & Rozas, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.,* 2008). Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) and Maximum Parsimony (MP) algorithms were employed to construct phylogenetic relationships. The reliability of the branches wasassessed by bootstrapping the data with 1000 replicates. Phylogenetic comparisons included *Echinocereuscinerascens* (HM041260), *Opuntiapalmadora* (HM041326) and *Pereskiaaculeata* (HM041338) from family Cacteceae out-groups. Analyses of molecular variance (AMOVA) were performed to assess the genetic differentiation among and within groups of populations, using Arlequin version 3.0 (Excoffier *et al.,* 2005).

Table 1. Collection localities of *P. oleracea*s L., taxa.

Populations	Location	Longitude and latitude
Pure *P. nitida*	Alexandria – Abiese	30º0.0´09.61´´and 31º08´51.1´´
Pure *P. granulatostellulata*	Alexandria, Bourg Al-Arab	29º36´37.68´´and 30º55´8.56´´
Pure *P. oleracea*	Al-Fayoum	34º19´04.29´´ and 27º55´10.3´´
P. oleracea mixed with *P. granulatostellulata*	El-Behiera	30º31´01.86´´and 31º11´39.57´´
P. granulatostellulata mixed with *P. oleracea*	El-Behiera	30º31´01.86´´and 31º11´39.57´´
P. nitida mixed with *P. granulatostellulata*	El-Gharbia	31º15´46.59´´and30º41´03.98´´
P. granulatostellulata mixed with *P. nitida*	El-Gharbia	31º15´46.59´´and30º41´03.98´´

Results

C-metaphase chromosome counts: Within the area studied, DNA tetraploids were recorded in all *P. nitida*, *P. granulatostellulata* individuals and surprisingly for *P. oleracea* individuals which is known as DNA hexaploid. Populations either being mixed or pure comprised a single cytotype, and different ploidy levels within the same population, or even within the same plant, were not detected (S1). The chromosomes are tiny, forming clusters; many connections link chromosomes with each other (Fig. 1).

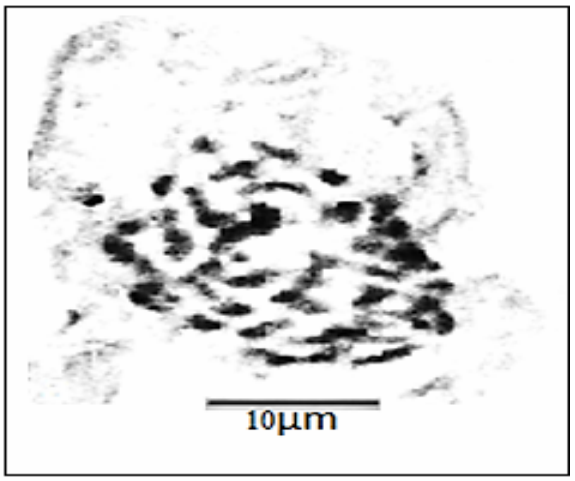

Fig. 1. Somatic metaphase chromosomes of the *P. oleracea*s L. taxa.

Isozyme analysis

Genetic diversity in pure and mixed populations: Four isozymes (Table 3) generated 13 polymorphic loci. The number of polymorphic loci varied from one locus in Amylaseto 6 loci in Esterase (Fig. 1).There were 6 new allele loci in the 4 mixed populations and 8 diagnostic alleles from in the purepopulations. The maximum number (4) of invasive diagnostic alleles was from *P. oleracea* toward *P. granulatostellulata* (Table 2).

The electrophoretic pattern of Amylase (AMY) isozyme showed one cathodal locus and one anodal locus. AMY-1A showed fixed heterozygous phenotypes in all examined individuals. Locus AMY-1C had one allele in 4 individuals; 2 from pure populations and 2 from mixed ones (Fig. 2a).

The electrophoretic pattern of peroxidase (Prx) enzyme showed one cathodal locus and 2 anodal loci. Locus Prx-1C was polymorphic in all pure and mixed populations. Locus Prx-1A was absent in most of pure populations except one individual from *P. oleracea*. All individuals of pure and mixed populations were heterozygous with two alleles for Prx-2A locus except 2 pure individuals from *P. oleracea* and *P. granulatostellulata* (Fig. 2b).

The electrophoretic pattern of α Esterase (α EST) showed one cathodal locus and two anodal loci. Pure and mixed populations were monomorphic with one allele for α EST-1A locus (Fig. 2c). Locus α EST-2A had no activity in all individuals except 3 individuals from mixed taxa.

The electrophoretic pattern of Esterase (EST) enzyme from the pure and mixed populations showed 3cathodl loci and 9 anodal loci (Fig. 2d). The locus EST-2C showed higher activities than the other 5 loci in all individuals. Mixed individuals were polymorphic with 2 allelesat most of loci (EST-1A, EST-3A and EST-4A), while monomorphic loci with one allele were common in pure individuals.

Four diagnostic alleles of *P. oleracea* were detected in sympatrically growing *P. granulatostellulata*, two of *P. granulatostellulata* in *P. oleracea*, two of *P. nitida* in *P. granulatostellulata* and one in *P. nitida* growing sympatrically with *P. granulatostellulata*. Three new alleles were found in all mixed populations except *P. granulatostellulata* growing sympatrically with *P. oleracea* (Table 2).

The genetic diversity of *P. oleracea* taxa, including percentage of polymorphic loci (*PPL*), index of diversity (*I*) and Nei's gene diversity (*h*) was 66.67%, 0.5269 and 0.343, respectively (Table 3). The genetic diversity among pure and mixed populations of *P. oleracea* taxa showed obvious differences, where the genetic parameters (*PPL*%, *I*, *h*, N_a, N_e) at mean population level were *PPL*= 36.76, *I*= 0.2257, *h*= 0.298, N_a= 1.674, N_e= 1.604, respectively (Table 3).It was found that the Nei'sgene diversity (*h*) in all puretaxa were lower than the same taxa at mixed case (Table 3). Gene flow (N_m) among populations based on isozymes data was 0.302.

The observed heterozygosity (H_o) over all loci was higher than those expectedby Hardy-Weinberg model in all pure and mixed taxa populations. *P. granulatostellulata* population growing sympatrically with *P. oleracea* had the highest observed heterozygosity (H_o) value. The observed heterozygosity (H_o) of pure populations were significantly different from mixed populations (T = -3.14 $p<$ 0.05).Estimates of inbreeding coefficient (F_{IS}) for all populations indicated a general excess of heterozygosity as they were significantly negative for all loci. Mean inbreeding coefficient (F_{IS}) was significantly higher in mixed populations than pure ones. *P. granulatostellulata* population growing sympatrically with *P. nitida* had significantly higher inbreeding coefficient (F_{IS}) value (-0.343) than *P. granulatostellulata* population growing sympatrically with *P. oleracea* (-0.279) (Table 4).

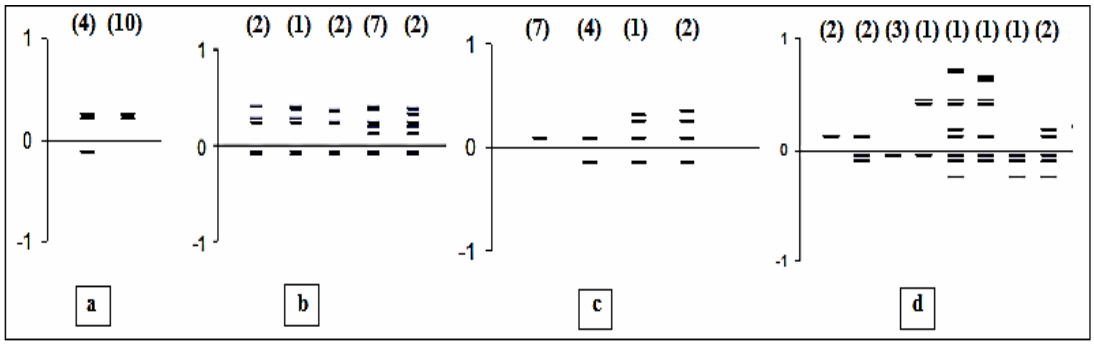

Fig. 2. Zymogram for four enzymes; a: Amaylase (AMY), b: Peroxidase (PX) c: α□Esterase (α□Est), d: □Esterase (□Est).

Table 2. Characterized isozyme alleles in pure populations, their Rm and distribution of introgressive isozyme alleles in the examined mixed populations. The presence of allele is indicated with+ and absence with -.n, g, o means likeness with pure P. *nitida*, *P. granulatostellulata* or *P. oleracea*.

Allele locus	Allele	Pure populations						Mixed populations							
	Rm	*n*	*n*	*g*	*g*	*o*	*o*	*oxg*	*oxg*	*gxo*	*gxo*	*nxg*	*nxg*	*gxn*	*gxn*
□EST-1C	-0.25	-	-	-	-	-	-	-	*?*	-	-	*?*	*?*	*?*	*?*
□EST-2C	-0.10	-	+	-	-	-	+	-	+	*o*	-	+	+	*n*	-
	-0.06	+	+	+	+	+	+	-	*g,o*	*o,g*	*o,g*	*g,n*	*g,n*	*n,g*	*n,g*
□EST-1A	0.12	+	+	-	-	-	+	-	+	*o*	-	-	+	+	+
	0.18	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	0.20	-	-	-	-	-	-	-	-	-	-	-	-	-	+
□EST-2A	0.41	-	-	-	+	-	-	*g*	*g*	+	-	-	-	-	-
	0.44	-	-	-	+	-	-	-	*g*	+	-	-	-	-	-
□EST-3A	0.61	-	-	-	-	-	-	-	-	*?*	-	-	-	-	-
	0.65	-	-	-	-	-	-	-	-	*?*	-	-	-	-	-
□EST-4A	0.68	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	0.72	-	-	-	-	-	-	-	+	-	-	-	-	-	-
PX-1A	0.12	-	-	-	-	-	+	*?*	+	*o*	*o*	+	+	+	+
PX-2A	0.20	-	-	-	-	+	+	*?*	+	*o*	*o*	+	+	+	-
	0.24	+	+	+	+	-	+	*g,o*	*g,o*	*o,g*	*o,g*	*g,n*	*g,n*	*n,g*	*n,g*
	0.28	+	+	+	-	-	-	-	-	-	-	-	-	-	*n,g*
PX-3A	0.33	-	-	-	+	+	-	*g,o*	*g,o*	-	-	-	-	-	-
	0.37	-	+	+	-	-	+	*g,o*	*g,o*	*o,g*	*o,g*	*g,n*	*g,n*	*n,g*	*n,g*
	0.41	+	-	-	-	-	+	-	-	*o*	*o*	+	+	*n*	*n*
α□EST-1C	-0.15	-	-	+	+	+	-	-	*g,o*	-	-	*g*	*g*	+	+
α□EST-2A	0.25	-	-	-	-	-	-	-	-	-	-	-	*?*	*?*	*?*
	0.31	-	-	-	-	-	-	-	-	-	-	-	*?*	-	-
	0.35	-	-	-	-	-	-	-	-	-	-	-	-	*?*	*?*
AMY-1C	-0.12	+	-	-	+	-	-	-	-	-	-	-	*g,n*	-	*n,g*
	Number of introgressive diagnostic alleles							g=2		o=4		g= 1		n= 2	
	Number of new alleles							3		0		3		3	

Table 3. Genetic diversity parameters of three pure and mixed *P. oleraceas* L. taxa.

Populations	Sample size	Polymorphic loci	Percentage population level (*PPL* %)	Observed number of alleles (N_a)	Number of effective alleles (N_e)	Shannon's index of diversity (I)	Nei's gene diversity (h)
Pure *P. nitida*	2	5	33.33	1.625	1.511	0.370	0.264
Pure *P. granulatostellulata*	2	5	33.33	1.666	1.585	0.409	0.278
Pure *P. oleracea*	2	5	33.33	1.667	1.541	0.395	0.264
P. oleracea mixed with *P. granulatostellulata*	2	8	53.33	1.727	1.691	0.492	0.352
P. granulatostellulata mixed with *P. oleracea*	2	7	46.67	1700	1.660	0.472	0.337
P. nitida mixed with *P. granulatostellulata*	2	7	46.67	1.583	1.583	0.404	0.292
P. granulatostellulata mixed with *P. nitida*	2	7	46.67	1.750	1.660	0.451	0.302
Mean	2	6.286	41.904	1.674	1.604	0.428	0.298
Over all loci		10	66.670	2.00	1.744	0.527	0.343

The UPGMA tree divided *P. oleracea* populations into two clusters; A and B. Cluster A comprised two individuals; one from pure *P. oleracea* population and the other from Pure *P. granulatostellulata* at the dissimilarity distance 0.44 (Fig. 3). Cluster B further divided into two groups B1 and B2. Cluster B1 comprised individuals of *P. oleracea* growing sympatrically with *P. granulatostellulata* population. Cluster B2further divided into two groups B2a and B2b. Group B2a comprised three individuals; two from pure population of *P. nitida* and one individual from pure *P. granulatostellulata* population. Cluster B2b comprised the rest of individuals from populations of mixed taxa except one individual from pure *P. oleracea* population.

Chloroplast DNA: Sequence sizes showing high level of length polymorphism with a maximum length of intergenic spacer *trnL-F*primer 1508bp. Within *P. oleracea* populations, lengths of nucleotide sequences polymorphic sites were 97, Singleton variable sites were 88 and Parsimony informative sites were 9. Nucleotides G and C are uncommon in the chloroplast sequence, with contents of 27.80–32.20%, which is consistent with the nucleotide composition of most non-coding region. The small GC content observed in the cpDNA alignment is mainly due to polyA or polyT regions. Mean G+C content differed by 1% between the pure and mixed populations of all *trnL-F* alignments studied here (Table 6).

The coefficient of differentiation between pure and mixed taxa was 0.185; while between taxa (*P. nitida*, *P. granulatostellulata* and *P. oleracea*) was 0.145. The transition/transversion rate ratios (mutation rate) were lower within the pure taxa (k1 = 1.23 (purines) and k2= 1.524 (pyrimidines) (R=0.449)) than within the mixed taxa (k1 = 3.918 (purines) and k2 = 3.054 (pyrimidines) (R= 0.803)). Gene flow among populations based on cpDNA data was higher than that based on isozymes (N_m = 5.48).

The nucleotide diversity *Pi* among the populations was low, ranging from 0.00507 to 0.15203 (Table 7). The *Pi* (t) value was nearly the same within *P. oleracea* populations except between *P. oleracea* population growing sympatrically with *P. granulatostellulata* (0.152). The mean nucleotide diversity *Pi* value (0.047±0.069) of mixed populations was higher than the pure populations (0.009±0.0035).

The phylogenetic tree based on UPGMA analyses with 1000 bootstrap replications revealed that the pure and mixed populations of *P. olereacea* taxa were clustered into two clades (Fig. 4a). Clade I included all pure and mixed populations of *P. olereacea* taxa with high boots trap support (100%). Clade II with also highly significant bootstrap support (100%) comprised one individual of *P. oleracea* growing sympatrically with *P. granulatostellulata* (the sequence repeated twice). Both clades were paraphyletic to *Opuntiapalmadora* and *Echinocereuscinerascens* and all were paraphyletic to *Persikaaculata* outgroup taxa.The maximum parsimony tree illustrated in Fig. 4b showed the same results of UPGMA analyses with moderate bootstrap support for clade I (44%) and high bootstrap support for clade II (100%).

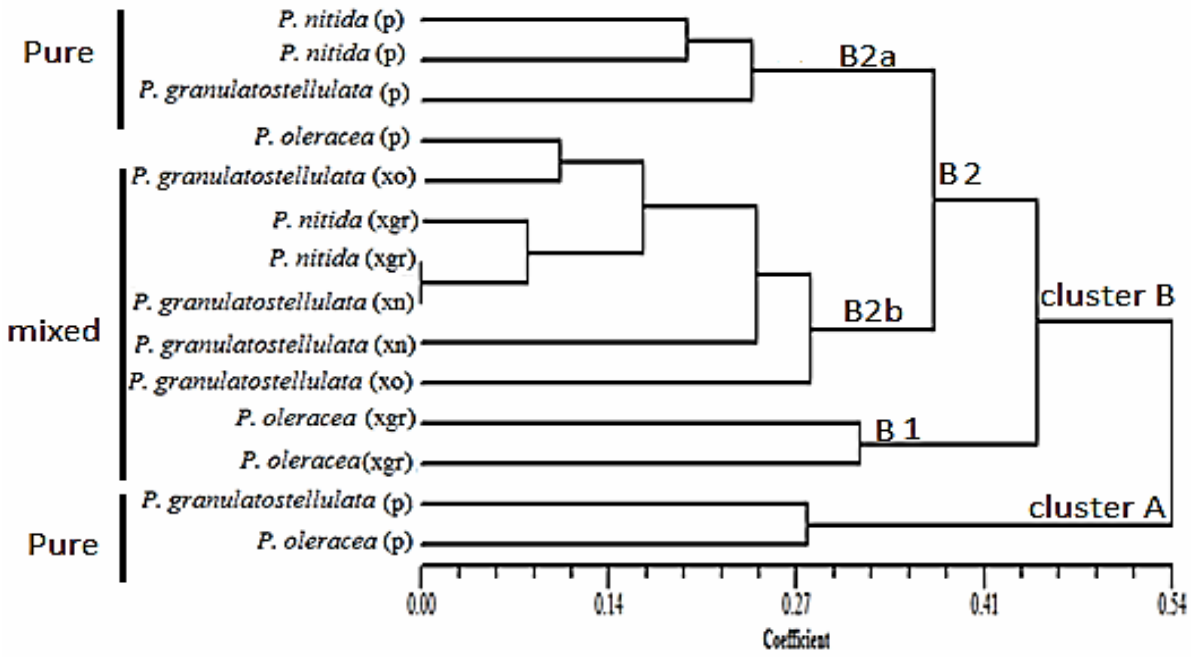

Fig. 3. dendrogram based on genetic distances and Nei coefficient computed from isozyme data using algorithm of unweighted pair group method with arithmetic averages (UPGMA) in the studied populations.
P = pure, X = mixed, gr = *P. granulatostellulata*, o = *P. oleracea* and n = *P. nitida*.

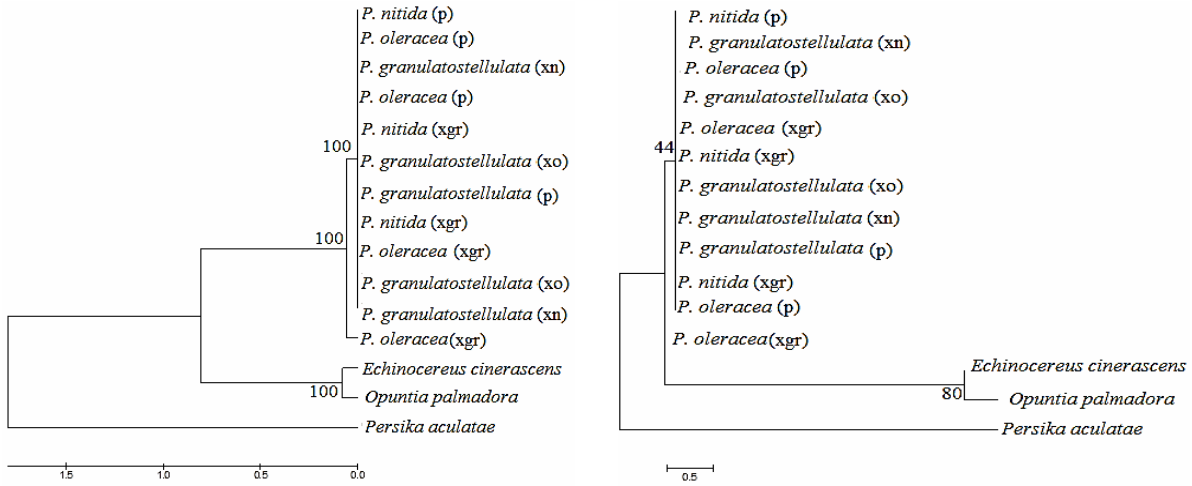

Fig. 4. Phylogenetic tree based on the nucleotide sequences of cpDNA of three *P. oleracea* taxa: (A) UPGMA method with 1000 bootstrap replications; (B) Maximum parsimony method with 1000 bootstrap replications.

Population structure and differentiation: The estimate of genetic structure (F_{st}= 0.269) based on sequence variation of cpDNA *trnL–F* non-coding region is non- significantly different from zero (p =0.239).Hierarchical analyses of AMOVA indicated that 11.76% of the cytoplasmic variation occurred among populations and most of the variation (88.23%) occurred within populations (Table 6). AMOVA indicated that the highest value of cytoplasmic variation (60.18%) was recorded between pure *P. granulatostellulata* population and *P. granulatostellulata* mixed with *P. oleracea* population (Table 6), while the lowest value was between populations of *P. nitida* and *P. granulatostellulata* growing sympatrically. With respect to DNA divergence, among taxa, origin and regions the genetic variations were non- significantly differ and were nearly the same -5.619, -3.102 and 4.061 respectively (Table 6).The cytoplasmic variation between populations of *P. granulatostellulata* and *P. oleracea* growing sympatrically was higher than *P. granulatostellulata* and *P. nitida* growing sympatrically. Using the variance component value for isozymes and cpDNA value of the populations, pollen/seed migration ratio (*r*) was calculated as 0.437 indicating low level of pollen flow when compared with seed flow.

Table 4. Estimates of observed heterozygosity (H_o) and expected heterozygosity (H_E) and in breeding coefficient (F_{IS}) of three pure and mixed *P. oleracea*s L. taxa.

Populations	H_o	H_E	F_{IS}
Pure *P. nitida*	0.438	0.396	-0.105
Pure *P. granulatostellulata*	0.389	0.390	-0.026
Pure *P. oleracea*	0.389	0.351	-0.105
P. oleracea mixed with *P. granulatostellulata*	0.439	0.352	-0.247
P. granulatostellulata mixed with *P. oleracea*	0.650	0.517	-0.279
P. nitida mixed with *P. granulatostellulata*	0.583	0.440	-0.325
P. granulatostellulata mixed with *P. nitida*	0.541	0.403	-0.343
For all loci	0.553	0.343	-0.743

Discussion

Our chromosome counts of *P. granulatostellulata* and *P. nitida* (tetraploids) agree with Danin & Raus (2012). Contrary, the chromosome counts of *P. oleracea* presented here are not in agreement with Danin & Raus (2012). Although *P. oleracea* has a hexaploid seed size, it is actually tetraploid. Seed diameter is normally related to the ploidy level, with 0.85 mm being the threshold between the hexaploids (diameter above the threshold) and the tetraploids and diploids (diameter below the threshold) (Danin *et al.,* 1978). An exception to this rule was found also in *P. sicula*, which has a ''hexaploid seed size'' but is actually a tetraploid (Danin *et al.,* 2008).

Results of isozyme analysis showed obvious differences in the genetic diversity among populations of *P. oleracea* taxa from different locations. The total gene diversity (h_t) and the genetic parameters (*PPL*%, *I*, *h*, N_a, N_e) at population level of the investigated *P. oleracea* taxa were lower in pure populations than in mixed populations. This result is equivalent to F_{IS} value which implies a low degree of selfing among pure populations. In the present study, the mean value of Nei's gene diversity index (*h*) was 0.298, near to the maximum *h* value (0.174–0.328) of nine out-crossing plants summarized by Schoen & Brown (1991).Previous study on the same taxa comprised a large number of populations from four *P. oleracea* taxa using ISSR marker indicated that the mean value of Nei's gene diversity index (*h*) (0.15) was near to the minimum value of out-crossing plants. Self -incompatibility measure as gene diversity significantly different between pure and mixed taxa (T = -3.49, p<0.05). All populations showed relatively high values of observed heterozygosity (H_O = 0.553) for the isozyme marker and the mean observed heterozygosity of mixed taxa (H_O = 0.650-0.389) was higher than pure taxa (H_O = 0.389-0.438).The high estimates of observed heterozygosity may be explained by life history attributes such as the presence of self-incompatibility and may be this pattern is enhanced by natural selection favoring heterozygosity (Hedrick, 2000) and perhaps for some loci by linkage between allozyme loci and the loci that control the self-incompatibility (Leach, 1988). If observed heterozygosity is higher than expected we might suspect an isolate-breaking effect (the mixing of two previously isolated populations) might be occurred. The percentage of selfing in mixed taxa significantly higher than pure taxa (T = -6.36, p< 0.005), which may allow mixed taxa to retain its own morphological characters and the selection may work to isolate the taxa maintain their independence. Establishment and spread of polyploid populations can be expected to require reproductive isolation from parental taxa (Coyne & Orr, 2004; Whitton, 2004), for example by a change in breeding system to reduce the tendency for back-crossing. Zohary (1999) postulated that self-pollination is one mechanism causing sympatric speciation which might explain also the high diversity of annual species within the families Gramineae, Papilionaceae, and Asteraceae in the East Mediterranean, on basis that self-pollination is the mechanismby which sympatric speciation takes place.

The analysis by AMOVA based on isozyme data implied that most of the variation occurred within the populations (73.08%). The presence of such percentage of variation (61.9%), were found in an earlier study of four *P. oleracea* L. taxa using ISSR marker (El-Bakatoushi*et al.,* 2013). Previous using of DNA marker confirms the independence of the used isozymes from the environment. It was worth noting that *P. granulatostellulata* shares nearly the same number of alleles with *P. nitida* and *P. oleracea*. The diagnostic introgressive alleles from *P. oleracea* to *P. granulatostellulata* were the highest (4 alleles).The AMOVA showed that the nuclear (isozyme) and the cytoplasmic (cpDNA) gene flow between *P. granulatostellulata* growing sympatrically with *P. nitida* were slightly lower than between populations of *P. granulatostellulata* and *P. olercea*. The percentage of variation among populations is negative. Negative percentages can arise in AMOVA (Excoffier *et al.,* 1992) but usually coincide with non-significant *P* values which is coincide with non-significant value of F_{st}, which means genes from different population can be more related to each other than genes from the same populations. The data support the idea of significant amounts of backcrossing from the *P. granulatostellulata* to both taxa. The result coincides with the higher crossing rate of *P. granulatostellulata* among taxa. It was concluded before that *P. granulatostellulata* has the highest gene flow estimates among four taxa *of P. oleracea* aggregates based on ISSR marker and that may cause that this taxon has retained many genetic features of the other taxa (El-Bakatoushi, 2013). The wide distribution of *P. granulatostellulata* was inferred already earlier from the random sample of Euro-Mediterranean collections of the *P. oleracea* aggregate from 28 territories (Greuter & Raab-Straube, 2011).Taxon frequency may be a key component in determining the selection pressure in favouring taxa and asymmetric bidirectional introgression may be density dependent(El-Bakatoushi *et al.,*2007; El-Bakatoushi, 2012).

Table 5. Hierarchical analysis of molecular variance (AMOVA) within and among populations of *P. oleracea* as estimated by using allele frequencies with ARLEQUIN version 1.1. The *P* values are the probabilities of having a greater variance component than the observed values by chance alone and are based on 1023 random permutations of the data matrix.

Source of variation	Isozymes			cpDNA		
	Variance components	Percentage variation	*P*	Variance components	Percentage variation	*P*
Among populations	1.131	26.910	*	32.000	11.761	NS
Within populations	3.071	73.080	**	240.070	88.238	***
Fst= 0.269	*			*Fst*= 0.112		NS
Between populations of *P. nitida* and *P. granulatostellulata* growing sympatrically	0.000	0.000	**	-41.000	-25.390	NS
within populations of *P. nitida* and *P. granulatostellulata* growing sympatrically	2.750	100.00	NS	202.500	125.390	NS
Between populations of *P. oleracea* and *P. granulatostellulata* growing sympatrically	0.250	6.670	NS	9.875	3.220	NS
within populations of *P. oleracea* and *P. granulatostellulata* growing sympatrically	3.500	93.330	NS	297.000	96.780	NS
Between pure *P. nitida* and *P. nitida* mixed with *P. granulatostellulata* populations	-0.250	-18.000	NS	170.500	54.169	**
within pure *P. nitida* and *P. nitida* mixed with *P. granulatostellulata* populations	1.625	118.180	NS	144.250	45.830	NS
Between pure *P. granulatostellulata* and *P. granulatostellulata* mixed with *P. nitida* populations	-0.177	-13.820	NS	-8.750	-4.750	NS
within pure *P. granulatostellulata* and *P. granulatostellulata* mixed with *P. nitida* populations	1.458	113.820	NS	141.250	104.790	NS
Between pure *P. oleracea*and *P. oleracea* mixed with *P. granulatostellulata* populations	-0.271	-25.490	NS	-77.250	-23.714	NS
Within pure *P. oleracea*and *P. oleracea* mixed with *P. granulatostellulata* populations	1.333	125.490	NS	403.000	123.714	NS
Between pure *P. granulatostellulata* and *P. granulatostellulata* mixed with *P. oleracea* populations	-0.104	-7.940	NS	179.870	60.184	***
Within pure *P. granulatostellulata* and *P. granulatostellulata* mixed with *P. oleracea*populations	1.416	107.940	*	119.000	39.810	NS
Among taxa	-0.136	-3.278	*	-15.087	-5.619	NS
Among population within taxa	1.235	29.620	*	43.495	16.200	NS
Within populations	3.071	73.658	NS	240.070	89.410	NS
Among origin	0.619	13.870	**	-8.330	-3.102	NS
Among population within origin	0.776	17.390	*	36.760	13.690	NS
Within origin	3.071	68.740	*	240.070	89.411	NS
Among regions	1.082	25.136	*	11.092	4.061	NS
Among population within regions	0.151	3.525	NS	21.964	8.041	NS
Within regions	3.071	71.330	*	240.071	87.890	NS

*** $p < 0.0001$, ** $p < 0.005$, *$p < 0.05$; NS= not significant

Table 6. GC content and nucleotide diversity (Pi) characterization of *trnL-F* intergenic region within pure and mixed taxa of *Portulacaoleracea*s L.

Populations	GC content (%)	Pi
Pure *P. nitida* population	27.80	0.01014
Pure *P. granulatostellulata* population	31.50	0.00507
Pure *P. oleracea* population	31.35	0.01182
P. oleracea population mixed with *P. granulatostellulata*	30.40	0.15203
P. granulatostellulata population mixed with *P. oleracea*	31.85	0.01689
P. nitida population mixed with *P. granulatostellulata*	32.2	0.01182
P. granulatostellulata population mixed with *P. nitida*	30.15	0.01014
Pure populations	30.22 ± 2.09	0.009 ± 0.0035
Mixed populations	31.15 ± 1.03	0.047 ± 0.069

Table 7. GC content and nucleotide diversity (Pi) characterization of *trnL-F* intergenic region within pure and mixed taxa of *Portulacaoleracea*s L.

Populations	GC content (%)	Pi
Pure *P. nitida* population	27.80	0.01014
Pure *P. granulatostellulata* population	31.50	0.00507
Pure *P. oleracea* population	31.35	0.01182
*P. oleracea*po pulation mixed with *P. granulatostellulata*	30.40	0.15203
P. granulatostellulata population mixed with *P. oleracea*	31.85	0.01689
P. nitida population mixed with *P. granulatostellulata*	32.2	0.01182
P. granulatostellulata population mixed with *P. nitida*	30.15	0.01014
Pure populations	30.22 ± 2.09	0.009 ± 0.004
Mixed populations	31.15 ± 1.03	0.047 ± 0.069

The phylogenic tree based on *trnL-F* sequence comprised all pure and mixed populations of *P. olereacea* taxa in one clade with high bootstrap support (100%) regardless their geographical location, except one individual of *P. oleracea* population growing sympatrically with *P. granulatostellulata* population. Mixed distribution of haplotypes from different locations in the phylogeny trees suggested the regular occurrence of cytoplasmic gene flow among populations and the high within population's cpDNA diversity found in *P. oleracea* taxa could be explained by the ancient presence of this species, which allowed the accumulation of a significant number of mutations.

The AMOVA revealed lower percentage of cytoplasmic variation occurred among the populations (11.76%) within sites which attributed to the occurrence of cytoplasmic gene flow more than pollen gene flow (by isozyme 26.91%) among taxa growing in the same site.Our data reported gene flow among populations based on cpDNA data(N_m= 5.48) higher than that based on isozymes (N_m = 0.302). Isozyme data suggest a significant amount of population isolationfor nuclear gene flow via pollen and high gene flow via seed. The pollen to seed migration ratio (*r*)obtained for the three *P. oleracea* taxa (r = 0.437) is very lower than the corresponding average value reported for seedplant species estimated over 93 species (Petit *et al.,* 2005; Hodgins & Barrett, 2007). It is not unexpected as cpDNA is generally maternally inherited in angiosperms (Dumolin, 1995; Rajora & Dancik, 1992) and has therefore a smaller effective population size than nuclear DNA. It has been shown theoretically that, for many models of population structure, the level of genetic differentiation among populations is expected to be higher for maternally inherited cpDNA markers than for biparentally inherited nuclear genes (Birky *et al.,* 1983; Petit *et al.,* 1993a; Ennos, 1994; Hu & Ennos, 1997), which is not the case here probably because the species was able to maintain high levels of diversity for maternal genomes, suggesting rapid change of aggregates. The relatively low among-population variation observed in both genomes of *P. oleracea* consistent with high levels of gene flow via both pollen and seeds and indicates that most of the species' genetic diversity occurs within individual populations.

The study concentrates mainly on situations where two taxa among *P. oleracea*s L., aggregate coexist. A higher rate of gene flow occurs between these two taxa than expected if they are mostly self-fertilised. Our data reported gene flow among populations based on cpDNA data higher than that based on isozymes. Genetic structure significantly varies within regions for nuclear gene and non-significantly varies for chloroplast indicating a strong asymmetry between pollen and seed flow. The percentage of breeding coefficient (F_{IS}) in

mixed taxa significantly higher than pure taxa (T = -6.36, $p<0.005$), which may allow mixed taxa to retain its own morphological characters and the selection may work to isolate the taxa to maintain their independence. The presence of self-incompatibility may be this pattern is enhanced by natural selection favoring heterozygosity. Our hypothesis that the taxa are kept distinct by selection pressure, despite countering of gene flowis held. The breeding strategyis one of the conditions shaping the *P. oleracea* L. phenotype.

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