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SEASONAL AND SPATIAL VARIATIONS IN THE GENETIC DIVERSITY OF ZILLA SPINOSA (L.) INHABITING WADI HAGUL AS REVEALED BY RAPD MARKERS

ABSTRACT:
Future warming and changes in the climate have dramatic effects on natural resources and biodiversity of the desert. Plants in the desert are frequently subjected to abiotic stress that affects adversely their growth and development as well as canopy level. Drought and heat stresses are among the factors which are generally expected to have significant effects on genetic diversity of natural plant populations. In this study, random amplified polymorphic DNA (RAPD) technique was used to reveal the genetic diversity and assess differences among and between six Zilla spinosa (L.) populations grown in different habitats along Wadi Hagul during spring and summer seasons and to determine the geographical differences at the population level by examining molecular characteristics. Eight informative and reproducible primers were chosen for RAPD analysis. These random primers generated a total of 204 RAPD fragments including 199 polymorphic bands with 97.5% polymorphism. OPA-10, OPA-11, OPD-15, and OPC-03 primers gave very high ratios (100%) of polymorphic bands in PCR reactions. However, OPB-06, OPB-07, OPD-14 and OPC-01 displayed polymorphism between 92.6% and 96.9%. The lowest value of polymorphism (92.6%) was achieved by OPD-14 primer. The relationships between the genetic diversity indexes on one hand and the geographic and climatic factors on the other hand were estimated by the Pearson correlation with SPSS 11.0 software. The results of the correlation analysis show that there were significant (P<0.05) or highly significant (P<0.01) correlations between each of the genetic diversity indexes and the different temperature of spring and summer seasons and water scarcity which were mainly caused by the different populations habitats.

KEY WORDS:
Zilla spinosa (L.), Environmental stress; Genetic diversity; Random amplified polymorphic DNA (RAPD)

INTRODUCTION:
A desert is a barren area of land where little precipitation occurs and consequently living conditions are hostile for plant and animal life. The lack of vegetation exposes the unprotected surface of the ground to the processes of denudation. About one third of the land surface of the world is arid or semi-arid. Deserts can be classified by the amount of precipitation that falls, by the temperature that prevails, by the causes of desertification or by their geographical location. Wadis are the most widespread ecosystems in the mountainous desert of the world (Fossati et al., 1999). The wadi system is an extreme case of a temporary inundated ecosystem in
which the duration of flooding is shorter than the dry period (Evenari et al., 1985). Various habitats can be identified in a wadi: channels, bars, banks, etc. (Abdel Rahman and Batanouny, 1965). Wadi vegetation in general varies from year to year depending upon moisture levels (Siddiqui and Al-Harbi, 1995). Establishment, growth, regeneration, and distribution of the plant communities in the wadis are controlled by many factors such as geographical position, physiographic features, and human impact (Kürschner and Neef, 2011; Alatar et al., 2012; Korkmaz and Özçelik, 2013). Considerable efforts have been made to interpret vegetation–environmental relationships in wadi ecosystems (Kassas and Imam, 1954 & 1959; Batanouny, 1979; Shaltout and Mady, 1996; Al-Farhan, 2001; Alatar et al., 2012; Salama et al., 2013). The vegetation in the Wadis is exposed to water stress due to extreme soil water deficits, high temperature and light radiation stress. Water stress may range from moderate, and of short duration, to extremely severe and prolonged summer drought that has strongly influenced evolution and plant life (Bottner et al., 1995; Pereira and Chaves, 1995). Plants are unable to dislocate in their own environmental therefore they have to cope with various stresses with internal mechanism(s) for the “tolerance” and/or “avoidance”. Certainly, most land plants are exposed to short or long term water stress at some times in their life cycle and have tended to develop some adaptive mechanisms for adapting to changing environmental conditions. Some desert perennials, such as Zilla spinosa (L.) Prantl, survive by becoming dormant during dry periods, then springing to life when water becomes available. After rain falls, Z. spinosa quickly grows a new suit of leaves to photosynthesize food. Flowers bloom throughout most of the year, and when under the less favourable conditions it enters dormancy (Zahran, 2010). Plants adaptations to dry environments can be expressed at four levels: phenological or developmental, morphological, physiological, and metabolic or biochemical adaptations (Hanson and Hitz, 1982). Along with different physiological and biochemical mechanisms, molecular approaches are boosting to understand the concept of stress tolerance in plants very clearly. DNA is the starting point of all molecular evidences related to stress tolerance in plants and contains several stress responsive genes in their genome. A large number of genes with potential roles in heat stress responses have been identified using genetic screens and genome wide expression studies (Yeh et al., 2012). Plants tolerate such stresses by modulating multiple genes and by coordinating the expression of genes in different pathways (Vinocur and Altman, 2005).

Nowadays, molecular marker, have become an important tool to evaluate genetic diversity in plants more efficiently. Among various molecular markers, the random amplified polymorphism DNA (RAPD) is most widely used. It does not require prior genomic information and are simpler and more economical than other DNA marker techniques (Lattoo et al., 2008; Khan et al., 2009; Nejatzadeh-Barandozi, 2013). RAPDs have also been successfully used by Waugh et al. (1992) to fingerprint individual accessions of Theobroma cacao (Cocoa). It was showed that RAPDs can be used to study the systematic relationship among closely related species and sub-specific taxa (Mehria et al., 2005). Similar type of work has been done on Podophyllum hexandrum collected from high altitude regions of North Western Himalayas. RAPD analysis revealed a high degree of genetic diversity among the 12 collected accessions, attributed to their geographical and climatic conditions. It was demonstrated that RAPD markers are very useful tools to compare the genetic relationship and pattern of variation among prioritized and endangered medicinal plants (Sultan et al., 2010).

The objectives of this study were to assess the genetic diversity and estimate the differences among and between the six Z. spinosa populations inhabiting Wadi Hagul and determine the geoclimatic differences by examining molecular characteristics.

**MATERIAL AND METHODS:**

**Plant materials:**

Z. spinosa is one of the common plants which grow along Wadi Hagul. Plant sample with similar size and age collected from three different locations along Wadi Hagul (upstream, midstream and downstream) during two seasons. The first spring-collected plants during flowering season (April) and the second fruiting dry season plants (September) were used for estimating the genetic diversity of Z. spinosa. At each location, the geographical, ecological and climatic threats parameters were recorded.

**Determination of moisture content of soil:**

The soil moisture content was measured according to the method described by Black (1965). This is easily done by determining the mass of the moist soil sample, then drying the sample in an oven to remove moisture, and then the mass of the remaining oven-dried sample was measured. The difference between the mass of the remaining oven-dried sample (M2) and the mass of the original moist sample (M1) is the mass of water in the original sample. The percentage of soil moisture content (W) calculated by: W = M1/M2 x100

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Extraction and Purification of Genomic DNA:

A modified CTAB (hexadecyltrimethyl ammonium bromide) procedure based on the protocol of Porebski et al. (1997) was adopted for obtaining good quality total DNA.

A half-gram of young plant tissues was collected from individual herb and quickly frozen in liquid nitrogen and then ground using mortar and pestle. Five ml of CTAB extraction buffer (60°C), 50 mg PVP (polyvinyl pyrrolidone) and 15 μl β-Mercaptoethanol (0.3%) were added. The tubes were mixed by inversion and incubated at 65°C for one hour. Then, 6 ml of chloroform: isooamyl alcohol (24:1) was added. The tubes were centrifuged at 5000 rpm for 20 min at room temperature. The top aqueous layer was further centrifuged at 5000 rpm after addition of 6 ml of chloroform: isooamyl (24:1). Half-volume of 5 M NaCl and two volumes of cold absolute ethanol were added to the supernatant and mixed well. The tubes were incubated at −20°C overnight, then centrifuged at 8000 rpm for 15 min. The supernatant was discarded, the pellet washed with 70% cold ethanol, and dried in speed vacuum for 10 min. The pellet was dissolved in 300 μl TE buffer (pH 8.0) overnight at 4°C, then transferred to 1.5 ml centrifuge tube. To remove RNA contamination, 4 μl (10 mg/ml) RNaseA (Sigma Co., USA) were added to the DNA solution and incubated at 37°C for 2 hours. The extracted DNA was deproteinized by adding 4 μl (1 mg/ml) proteinase K (Sigma Co., USA) and incubating at 37°C for 2 hours. Three hundred μl of Tris-saturated phenol-chloroform were added, and mixed by inversion. Tubes were centrifuged at 14000 rpm for 15 min in a microfuge (Eppendorf, USA). The upper layer was transferred to new tubes using wide bore pipette tip and 150 μl of TE buffer was added to the phenol phase, mixed, spun for 10 min, then the upper layer containing the DNA was removed and added to the sample. DNA was precipitated overnight at −20°C using 0.1 volume 3 M sodium acetate (pH 8.0) and two volumes of chilled absolute ethanol. The samples were centrifuged at 14000 rpm at 4°C for 15 min, the DNA was washed with 70% ethanol, briefly air-dried and re-dissolved in TE buffer.

Random Amplified Polymorphic DNA (RAPD) RAPD-PCR Reactions:

Eight random 10-mer primers were used in the detection of polymorphism among the 6 plant populations (Table 1). RAPD was carried out according to the procedure given by Williams et al. (1990). The amplification reaction was carried out in 25 μl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 μM primer, 1 U Taq DNA polymerase and 25 ng templates DNA.

Table 1. Sequence of the eight arbitrary primers assayed in RAPD-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA-11</td>
<td>TCTGTGCTGG</td>
</tr>
<tr>
<td>OPA-10</td>
<td>GAACGGGGTG</td>
</tr>
<tr>
<td>OPB-06</td>
<td>CATCCCCCTG</td>
</tr>
<tr>
<td>OPB-07</td>
<td>TGCTCTGCC</td>
</tr>
<tr>
<td>OPD-14</td>
<td>TTCCCCCGCT</td>
</tr>
<tr>
<td>OPD-15</td>
<td>TTTGCCCAG</td>
</tr>
<tr>
<td>OPC-01</td>
<td>TTTGAGCGAG</td>
</tr>
<tr>
<td>OPC-03</td>
<td>GGGGTCCTTT</td>
</tr>
</tbody>
</table>

Thermal cycling Profile and Detection of the PCR Products:

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 36°C for 1 min, and an elongation step at 72°C for 1.5 min. The primer extension segment was extended to 7 min at 72°C in the final cycle. A 100 bp DNA ladder was used as a molecular size standard.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

Data Analysis:

RAPD amplified fragments were scored ‘1’ for the presence or ‘0’ for the absence of homologous bands and a matrix of RAPD phenotypes was assembled. Each RAPD band was treated as a separate character and a rectangular binary data matrix was obtained. A similarity matrix was obtained using Jaccard’s coefficient (1908) and converted to distances (Fig. 4). Moreover, based on the binary matrix obtained in the study, genetic diversity indexes, including the percentage of polymorphic bands and the total diversity indexes analyzed with POPGENE, and Nei’s genetic differentiation index were done.

Correlation Analysis:

The relationships between each of the genetic diversity and each of the three different stands during spring and summer seasons were estimated with the Pearson correlation with SPSS 11.0 software. Meanwhile, the relationships between the Nei’s unbiased genetic distance matrices were measured by POPGENE.
RESULTS AND DISCUSSION:

Climate:

The climatic means of Suez metrological station; the nearest station to Wadi Hagul, had been recorded during the period between 2008-2012 to throw light on the climatic conditions prevailing in the surveyed Wadi (Table 2).

Table 2. The meteorological data (climatic condition) of Wadi Hagul at the six years of 2008-2013 as recorded by the Suez metrological station

<table>
<thead>
<tr>
<th>Months</th>
<th>Mean maximum Temp. °C</th>
<th>Mean minimum Temp. °C</th>
<th>Rain fall mm/month</th>
<th>Relative humidity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>20</td>
<td>10</td>
<td>3.4</td>
<td>59</td>
</tr>
<tr>
<td>February</td>
<td>21</td>
<td>11</td>
<td>3.7</td>
<td>58</td>
</tr>
<tr>
<td>March</td>
<td>25</td>
<td>13</td>
<td>3.2</td>
<td>54</td>
</tr>
<tr>
<td>April</td>
<td>28</td>
<td>16</td>
<td>17.7</td>
<td>47</td>
</tr>
<tr>
<td>May</td>
<td>32</td>
<td>19</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>June</td>
<td>36</td>
<td>22</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>July</td>
<td>37</td>
<td>24</td>
<td>0.5</td>
<td>52</td>
</tr>
<tr>
<td>August</td>
<td>37</td>
<td>24</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>September</td>
<td>34</td>
<td>22</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>October</td>
<td>30</td>
<td>19</td>
<td>0.2</td>
<td>58</td>
</tr>
<tr>
<td>November</td>
<td>26</td>
<td>15</td>
<td>1.7</td>
<td>60</td>
</tr>
<tr>
<td>December</td>
<td>22</td>
<td>11</td>
<td>3.9</td>
<td>62</td>
</tr>
</tbody>
</table>

Air temperature:

The climatic condition of the study areas was of the arid type with mild winter temperatures. January was the coldest month while July and August were the hottest ones (Table 2).

Rainfall:

The rainfall was scanty, the relatively highest amount of annual rainfall recorded during April that reached about 17.7 mm while the precipitation was nil in July and August (Table 2).

Relative humidity:

Whereas the highest relative humidity value recorded during December while, the lowest value was measured during May which varying between 45% and 62% (Table 2).

Soil water content:

The three locations studied located at successively declining contour from the downstream to the mouth of wadi Hagul near the sea shore (Fig. 1). The inclination angle of the slope of the wadi course is quite noticeable and consequent features of soil erosion and deposition occur along the Wadi course.

Fig. 1. Map of Cairo-Suez road showing the location of the studied area of Wadi Hagul

Data presented in figure 2 show that the soil moisture content was markedly increased during the spring season compared to that of the dry summer season at all studied locations. The greatest value of soil moisture content was measured in the second location (midstream) during spring and summer seasons. Such values reached about 2.90% and 2.75% during spring and summer seasons respectively. However, the minimum value of soil moisture content was measured in downstream during summer (0.36%).

Fig. 2. Percentage of soil water content of different habitats of Wadi Hagul

Polymorphism as detected by RAPD analysis:

The RAPD banding patterns of Zilla spinosa inhabiting different habitats along Wadi Hagul during the spring and summer seasons are illustrated in figure 3. The eight primers (1-8) used for RAPD-PCR were able to amplify the DNA of the six populations studied.
Fig. (3 a-h). DNA polymorphism using randomly amplified polymorphic DNA (RAPD-PCR) technique for *Zilla spinosa* inhabiting upstream, midstream and downstream (1,3,5) during Summer and (2,4, 6) Spring seasons.

The six samples of *Zilla* shoots harvested from three different habitats of Wadi Hagul in summer and spring season used in replicates for DNA isolations have similar morphology. The RAPD markers were used to assess the genetic variability among the six populations of *Z. spinosa* and genetic relationships were established. Eight RAPD markers could be identified distinguishing the studied materials. The sign of amplified DNA fragments produced by these primers varied between 100 to 1200 Pm. The random primers generated a total of 204 RAPD fragments. Each primer produced approximately 2 to 8 fragments. OPA-10, OPA-11, OPD-15, and OPC-03 primers gave very high ratios (100%) on polymorphic bands in PCR reactions. However, OPB-06, OPB-07, OPD-14 and OPC-01 displayed polymorphism between 92.6% and 96.9%. The lowest value of polymorphism (92.6%) was achieved by OPD-14 primer (Table 3).
Among all DNA amplified fragments, certain polymorphic fragments could be generated using all the primers in all studied samples.

The genetic similarity was reflected on the dendogram also, where the studied Zilla samples could be grouped into two distinct clusters (Fig. 4). In the first cluster Zilla at first stand was grouped with Zilla at third location during the wet season and in the second cluster the three locations were grouped together during the dry seasons. Moreover, in the first cluster, the populations at the first location were genetically similar to these at the third location during the wet season, however, the second and the third sites are closer during the dry season.

Dendrogram using Average Linkage (Between Groups)

<table>
<thead>
<tr>
<th>Number</th>
<th>Primers</th>
<th>Total amplified bands</th>
<th>Polymorphic bands</th>
<th>Polymorphic percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPA-11</td>
<td>31</td>
<td>31</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>OPA-10</td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>OPB-06</td>
<td>19</td>
<td>18</td>
<td>94.7</td>
</tr>
<tr>
<td>4</td>
<td>OPB-07</td>
<td>22</td>
<td>21</td>
<td>95.5</td>
</tr>
<tr>
<td>5</td>
<td>OPD-14</td>
<td>27</td>
<td>25</td>
<td>92.6</td>
</tr>
<tr>
<td>6</td>
<td>OPD-15</td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>OPC-01</td>
<td>32</td>
<td>31</td>
<td>96.9</td>
</tr>
<tr>
<td>8</td>
<td>OPC-03</td>
<td>25</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>199</td>
<td>97.5</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Dendrogram (UPGMA) showing the genetic relationships between summer and spring collected Zilla spinosa populations inhabiting three different habitats along Wadi Hagul. 1 & 2 are specific for upstream, 3 & 4 for midstream, and 5 & 6 for downstream during summer and spring seasons, respectively.

A distance matrix was also made on the basis of Nei and Li (1979) similarity indexes (Table 4) and clustered by Neighbor-Joining analysis (Fig. 4). Among this cluster analysis, the genetic distances range up 1 to 25. However, Zilla inhabiting the first location during spring and those at the second and third locations are different. The first and third locations, inhabiting plants during winter, also formed a sister group by Average Linkage analysis. Zilla, inhabiting the second location during wet season which formed a single group, was part of a group with first and the third locations during winter.
The present study, the RAPD marker was employed to estimate genetic relatedness and diversity among three medicinally important populations of *Zilla* inhabiting different habitats of Wadi Hagoul during the summer dry and spring wet seasons. The characterization of the genetic diversity and examination of the genetic relationship among *Zilla* inhabiting different stressful conditions are important for sustainable characterization of the molecular changes associated with the changes in climate and habitat. Marker systems differ in their information content, which depends on polymorphism. The concept of polymorphism is used to define genetic variation in a population, which has been extensively studied in recent years by several established scientific disciplines, for example, genetics, ecology, zoology, and microbiology (Mukherjee et al., 2010; Abdul Muneer et al., 2011; Rajkumar et al., 2011). DNA polymorphism among individuals can be due to mismatches at the primer site, appearance of a new primer site and length of the amplified region between primer sites (Kumar and Gurusubramanian, 2011). *Zilla* plants have significant genetic variation. Those strong genetic differences occurred between plants from inhabiting three different locations only 10 Km apart along Wadi Hagul. Moreover, *Zilla* populations inhabiting the third location are correlated with those of the first one during the wet spring season. However, *Zilla* populations inhabiting the second location had significant differentiation from the other groups and exhibited the highest degree of divergence. The UPGMA (unweighted pair-group method with arithmetic means) dendrogram based on Nei’s unbiased genetic distance calculated with POPGENE also, indicated that the genetic differentiation was consistent with the geographic distance between *Zilla* populations inhabiting location 1 and 3 at the dry season. The results of this investigation also, showed significant correlations among the genetic diversity and soil moisture content in the spring wet and dry summer seasons. The positive relations with environmental stress particularly the reduction in soil moisture level associated with the raising of temperature can prove that *Zilla* populations at altered environmental conditions have different expected number of alleles, the base of diversity at gene level (Kraushaar et al., 2002). The relations of genetic diversity parameters with mean temperature and soil moisture content in May and in August mean that the plant genetic diversity could be influenced by the variations in the environmental conditions particularly the extreme temperature. Such results are confirmed by many other studies (Curado et al., 2006; Ding et al., 2009; Martínez-Natarén et al., 2014). Indeed, there are correlations between the genetic indexes and the mean temperature (Warren et al., 2001; Thomas et al., 2004). The mean temperature in Wadi Hagoul has also raised with the process of global warming in recent years. The stress from environment forces the species to escape or tolerate the extreme temperature. It was postulated that when the altitude is changed, there will be difference at genetic level among groups. Currently, a number of species are showing rapid range expansion from warmer biomes to the previously colder ones (Thomas et al., 2004), and many species are currently moving to the higher altitudes (Pearson and Dawson, 2003). However, little is known about the factors that influence the future performance of species expanding in their new habitats. So, the expansion is a key adaptive feature of species in response of changes in climate, habitat availability and other limiting factors (Thomas et al., 2004). The results suggested that the natural stress which could lead to the phenomenon of genetic diversity has a very deep influence on *Zilla* plant (Falconer and Mackay, 1996).

UPGMA dendogram (Fig. 4) showed distinct separation of the collected accessions from three locations during the spring and summer seasons into two major clusters. *Zilla* inhabiting the first and the third locations at the wet season diverged from that at the three investigated sites during summer. From this we can conclude that *Zilla* inhabiting the second location is more diversified. In the dendrogram based on Nei’s genetic distance obtained (Fig. 4), the populations were highly differentiated by their own genetic distance. The clustering results of different populations suggest that *Z. spinosa* undergoes major part of genetic variation by environmental factors rather than the spatial one. Genetic diversity refers to the variation at the level of individual genes (polymorphisms), and provides a mechanism for populations to adapt to their ever-changing environment. The genetic variability in *Zilla* may be partly explained as a result of abiotic and biotic factors. It is desired to maximize the preservation of alleles. Geographical, climatic or reproductive variables explain the partitioning of the diversity observed which may aid in improving the strategies for understanding the adaptive responses of *Zilla* plants. The utility of RAPD markers in estimating genetic variability has been demonstrated in several studies. A similar study was done in *Withania somnifera* by Mir et al. (2010). Seven populations of *Withania coagulans* from the districts of Kohat and Karak in Pakistan were analyzed by Syed (2009). It was also postulated that analysis of RAPD could be useful to detect genetic differentiation of *Withania somnifera* among five different geographical locations. Each location varied with respect to environmental factors and genetic parameters (Dharmar and Britto, 2011). Finger et al. (2010) estimated the genetic diversity of 49 accessions of
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