PRELIMINARY STUDIES ON THE IMPACT OF ENVIRONMENTAL STRESSES ON POLYPLOID FORMATION IN PLANTS: CASE STUDY IN *Aeluropus littoralis* (Poaceae)

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Abstract. It is generally thought that stressful environments e.g. salinity and doughtiness can trigger polyploid formation in plants and that frequency of polyploids is higher under these environments, and consequently polyploids have higher geographical distribution ranges in harsh environments. We studied the frequency of polyploids in natural diploid populations of *Aeluropus littoralis* (Poaceae) in regions having soils of high salinity using somatic metaphase of root tip. Moreover, the variations of some chromosomal parameters such as whole chromosomes lengths, relative chromosomal lengths (e.g. large/short arm (q/p) and short arm/whole length (p/wl) ratios) were analyzed among different karyotypes to determine the stable chromosomal parameters. The eight out of total 100 plants (8%) sampled from two populations were tetraploids with 2n=4×=40, while the rest (92%) were diploids with 2n=2×=20. The absolute whole chromosomes lengths was significantly different among tetraploids ($P < 0.0002$), but not relative chromosomal lengths (q/p: $P > 0.21963$; p/wl: $P > 0.664$). The study showed that environmental stresses e.g. soil salinity may trigger polyploid formation in plants, and that polyploidy is ongoing process in *Aeluropus* evolution. The results also indicated that the values of relative chromosomal lengths are reliable criteria than absolute chromosome size, and that to detect ploidy in any diploid populations of plant species it is necessary to sample higher number of plants.

Key words: *Aeluropus littoralis*; chromosome size; environmental stresses; karyotype; polyploidy.

INTRODUCTION

Polyploidy, whole genome duplication, is a common mode of speciation in plants and plays important role in plant evolution and distribution [16, 25-28], so that on average 50% of angiosperm species have polyploid ancestry [8, 18, 25, 27, 28]. Polyploidy has occurred multiple times over the past 200 million years of angiosperm evolution [25, 26].

The polyploids have several advantages over diploid progenitors including gaining asexual reproduction and heterosis, enabling polyploids to reproduce in the absence of sexual mates, and being adapted to stressful environments [5, 9, 18, 20, 26-28].

The genus *Aeluropus* (Poaceae) includes five species in the world, and is distributed through the Central Asia, the tropical Asia, Europe and Africa, Europe-Siberia, the Mediterranean, Irano-Turanian and Sahara-Sandi regions. Three out of five species occur in Iran, of which *A. littoralis* is a rhizomatous C_4 perennial, widely distributed across the country including deserts and regions with high salinity [22].

In addition, it is commonly thought that environmental stresses can prompt polyploid formation in plants [2, 4, 7, 14, 24, 28-31]. Therefore, it is expected that the polyploids occur among ancestral diploid populations at high frequency in stressful environments. To test this hypothesis, we studied the frequency of polyploids among diploid populations of *Aeluropus littoralis* (Gouan) Parl. (Poaceae) in regions of high salinity soils.

The chromosomal and karyotypic variations in plants impact on the ecological differentiation and adaptation, and consequently on speciation. In addition, chromosomal features are useful in elicitation relationship between taxa [11, 30]. The various karyotypic indices including variation in chromosome number, morphology, karyotype symmetry, ploidy, symmetry and total haploid length have been widely used plants for interpreting evolutionary relationship among angiosperms [30, 34].

MATERIAL AND METHODS

Sampling and study regions: 100 plants of *Aeluropus littoralis* were randomly sampled from diploid populations in two regions of highly saline soils in East Azerbaijan Province, Iran (50 samples from each region) (Bonab town: at geographical position of 37° 20′ 25″ N 46° 03′ 22″E; and Jazireyeh Islami with geographical location of N 30 ° 50' 43 " E45 ° 29'41" located on the shores of highly saline lake of Urmia Lake). The distance between two populations was approximately 75Km. The ripen seeds were collected from the plants in order for germination and preparing karyotypes. In each seed (each seed representing one individual plant), the ploidy level and chromosomal parameters were studied in minimum number of three cells at somatic metaphase.

Seed germination and karyotyping: The seeds were germinated over wet filter paper on a Petri dish under 27 or 28°C, allowing the roots containing actively dividing cells to grow to a height of 4 to 6 mm. Samples of root tips were stored in a refrigerator for pre-treatment in aqueous solution of alpha-bromonthalin at 4°C for 2 hours. The samples was fixed in Levitsky solution [12] for 24 hours, and were then hydrolyzed in a NaOH normal for 8 minutes at 60°C, and consequently stored in hematoxylin iron solution for 22 hours under 30°C. The stained root tips containing actively growing cells were mounted on the slides. The slides with well-spread somatic metaphase chromosomes were screened under Olympus optical microscope. The karyotypes were prepared using Micromeasure software. The all steps of procedures for all samples were constantly conducted in order to avoid any effects of treatment variations on the results.

Ploidy levels and chromosomal parameters: The ploidy levels were investigated in total 100 plants (50 plants from each population). The various chromosomes parameters were measured using somatic metaphase karyotypes in some randomly selected numbers of karyotypes. These parameters for each chromosome were as follow: whole chromosome length (wl), the long arm length (q), the short arm length (p), the q/p and p/wl ratios (p/wl: defined as centromeric index =CI). Consequently, the variations in three parameters were analyzed among karyotypes using ANOVA test. The stable and reliable chromosomal parameter was based on the assumption that the variation among karyotypes indicates no significant differences.

RESULTS

Two different ploidy levels were detected among the samples randomly taken from both study regions (Figures. 1 and 2). In total, eight out of total 100 plants sampled from two regions (four plants from each region) were found to be tetraploid (8%) with a chromosome number of 2n=4×=40 (Figures 1 and 2; A and B). However, the majority of studied plants (92%) showed the chromosome number of 2n=2×=20, indicating the diploidy (Figures 1 and 2; C and D).

The whole chromosomes lengths of somatic metaphase karyotypes of eight tetraploid individuals of *Aeluropus littoralis* detected in two populations are

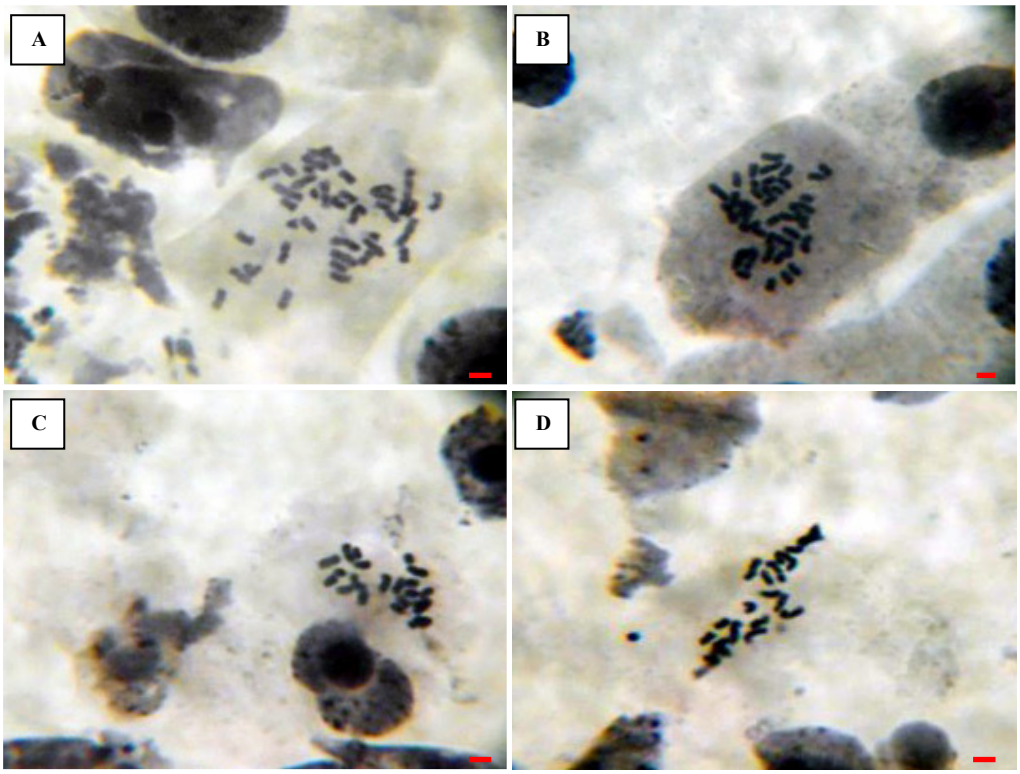

Figure 1. The somatic metaphase karyotypes of root tip cells in *Aeluropus littoralis*: A and B: The tetraploid samples (2n=4×=40) of Bonab and Jazireyeh Islami populations, respectively. C and D: The diploids (2n=2×=20) of Bonab and Jazireyeh Islami populations, respectively (The scale bar in red = 5*μm*).

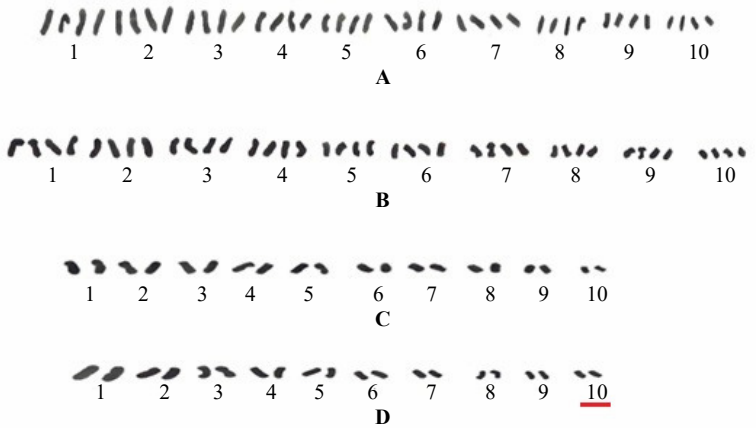

Figure 2. The somatic metaphase karyotypes of root tip cells in samples taken from the two populations of *Aeluropus littoralis*: From up tp down: The tetraploid of Bonab, tetraploid of Jazireyeh, diploid of Bonab, diploid of Jazireyeh populations (The scale bar in red = 5*μm*).

indicated in Table 1. The chromosomes lengths in the tetraploids ranged from 1.40μ to 4.78μ. The average of the shortest and longest chromosomes lengths for eight tetraploids were 1.93μ (stDev: ±0.46) and 3.97μ (stDev: ±0.66), respectively.

The population-level of average values of the shortest and longest chromosomes lengths were 2.03 (stDev: ±0.57) and 3.98 (stDev: ±0.82) for Bonab, and 1.84 (±0.39) and 3.97 (±0.58) for Jazireyeh. The variations of whole chromosomes length (wl) were significantly different among all eight karyotypes of tetraploid plants (ANOVA test: F-statistic value = 4.7169, $P \leq 0.0002$) (Table 2). However, the whole chromosomes length (wl) variations between two sets of tetraploids was not significant at 0.05 level (T-Test, $P \leq 0.45595$; Table 3).

The chromosomes' centromeric index (CI) defined as short arm length/whole chromosome length ratio, are displayed in Table 4. The CI values among Bonab population ranged from 0.37 to 0.53 with average of 0.443 (StDev: ±0.0339), while this range for Jazireyeh population was 0.38 - 0.48 with average of 0.438 (StDev: ±0.0264). The ANOVA analysis of CI values showed no significant difference across all eight tetraploid karyotypes (F-statistic value = 0.7096, $P \geq 0.664$; Table 5). Similarly, the variation of CI values was not significat between two tetraploid sets at 0.05 level (T-Test, $P \geq 0.2433$; Table 6). The CI values also indicate that all chromosomes were methacentric.

The smallest and largest chromosomes' long arm/short arm ratios (q/p) among all eihgt tetraploid karyotypes were 1.00 and 1.68, respectivley. On average the q/p ratio ranged from 1.23 (StDev: ±0.012) to 1.36 (StDev: ±0.10) (Table 6). ANOVA analysis indicated no significant differences of q/p ratios among all eihgt karyotypes (ANOVA test: F-statistic value = 1.3977; $P \geq 0.21963$; Table 7).

Table 1. Whole chromosomes length (wl) variations among eight karyotypes of tetraploid of *Aeluropus littoralis* in two different populations. The 'wl' values were significantly different among karyotypes of eight tetraploid plants ($P < 0.0002$).

Ch. No.	Bonab: Individual plant No.				Jazireyeh: Individual plant No.				Aveg.	stDev
	1	2	3	4	1	2	3	4		
1	4.56	4.61	2.85	3.88	3.41	3.84	4.78	3.83	3.97	±0.66
2	4.16	3.97	2.57	3.4	2.89	3.48	4.07	3.47	3.50	±0.56
3	3.18	3.55	2.42	2.98	2.68	3.13	3.7	3.3	3.12	±0.42
4	3.67	3.35	2.28	2.77	2.45	2.92	3.45	3.09	3.00	±0.49
5	3.39	3.09	2.17	2.69	2.25	2.66	3.29	3.85	2.92	±0.58
6	3.04	2.78	2.01	2.54	2.12	2.46	3.05	2.75	2.59	±0.39
7	2.8	2.63	1.79	2.44	1.87	2.41	2.97	2.51	2.43	±0.41
8	2.68	2.49	1.68	2.28	1.76	2.36	2.85	2.37	2.31	±0.41
9	2.53	2.28	1.56	2.02	1.63	2.15	2.63	2.17	2.12	±0.38
10	2.78	2.1	1.46	1.76	1.40	1.73	2.34	1.88	1.93	±0.46

Table 2. The one way ANOVA analysis of whole chromosomes lengths among eight karyotypes indicating significant differences in whole chromosomes lengths among eight karyotypes.

	Degree of Freedom	Sum of Squares	Mean Square	statistic value	*P*-value
Between groups	7	14.373	2.053	4.7169	0.0002
Within groups	72	31.342	0.4353	-	-

Table 3. The whole chromosomes length (wl) variations between two sets of tetraploids at 0.05 level (T-Test, $P \leq 0.45595$).

Groups	N	Mean	Std.Dev.	P-value
Bonab population	40	2.78	0.79	0.45595
Jazireyeh population	40	2.8	0.75	

Table 4. Chromosomes' centromeric index (CI) values (defined as short/whole arms' lengths ratio) of eight karyotypes of tetraploid of *Aeluropus littoralis*. These CI values showed no significant differences ($P \geq 0.664$).

Ch. No.	Bonab: karyotype sample No.				Jazireyeh: Karyotype sample No.				Aveg.	stDev.
	1	2	3	4	1	2	3	4		
1	0.45	0.42	0.48	0.44	0.44	0.48	0.45	0.42	0.45	±0.02
2	0.42	0.39	0.42	0.42	0.43	0.41	0.46	0.42	0.42	±0.02
3	0.53	0.47	0.47	0.42	0.42	0.46	0.47	0.45	0.46	±0.03
4	0.43	0.40	0.41	0.44	0.44	0.44	0.47	0.45	0.44	±0.02
5	0.46	0.42	0.41	0.49	0.42	0.45	0.40	0.40	0.43	±0.03
6	0.46	0.48	0.44	0.43	0.40	0.41	0.43	0.48	0.44	±0.03
7	0.48	0.48	0.43	0.46	0.39	0.45	0.44	0.44	0.45	±0.03
8	0.43	0.45	0.52	0.44	0.38	0.47	0.46	0.44	0.45	±0.04
9	0.45	0.43	0.41	0.45	0.44	0.39	0.44	0.46	0.43	±0.02
10	0.37	0.42	0.49	0.41	0.47	0.47	0.43	0.46	0.44	±0.04

Table 5. The ANOVA analysis of chromosomes' centromeric index (CI) values across eight karyotypes of tetraploids showed no significant difference among ratios *(P* ≥ 0.664).

	Degree of Freedom	Sum of Squares	Mean Square	statistic value	*P*-value
Between groups	7	0.005	0.0007	0.7096	0.664
Within groups	72	0.068	0.0009	-	-

Table 6. Analyzing of the Chromosomes' centromeric index (CI) values between two sets of tetraploids showed no significant difference between two populations *(*T-test, *P* ≥ 0.491).

Groups	N	Mean	Std.Dev.	Std. Error	*P*-value
Bonab population	40	0.443	0.0339	0.0054	0.2433
Jazireyeh population	40	0.438	0.0264	0.0042	

Table 7. Chromosomes' long arm/short arm' lengths ratios (q/p) in eight karyotypes of tetraploids of *Aeluropus littoralis* in two different populations.

Ch. No.	Bonab: karyotype sample No.				Jazireyeh: Karyotype sample No.				Aveg.	stDev.
	1	2	3	4	1	2	3	4		
1	1.20	1.38	1.07	1.26	1.29	1.06	1.20	1.37	1.23	±0.12
2	1.34	1.51	1.35	1.37	1.31	1.44	1.18	1.35	1.36	±0.10
3	1.27	1.10	1.15	1.39	1.39	1.19	1.11	1.22	1.23	±0.11
4	1.35	1.47	1.43	1.30	1.24	1.29	1.14	1.19	1.30	±0.11
5	1.16	1.36	1.37	1.04	1.36	1.21	1.49	1.50	1.31	±0.16
6	1.16	1.11	1.27	1.29	1.52	1.43	1.32	1.08	1.27	±0.15
7	1.08	1.08	1.45	1.15	1.61	1.20	1.28	1.28	1.27	±0.19
8	1.34	1.23	1.06	1.31	1.68	1.14	1.18	1.25	1.27	±0.19
9	1.23	1.33	1.52	1.27	1.35	1.55	1.24	1.15	1.33	±0.14
10	1.07	1.46	1.00	1.31	1.23	1.52	1.34	1.29	1.28	±0.18

Table 8. The ANOVA analysis of chromosomes' long arm/short arm' lengths ratios (q/p) showed no significant difference among karyotypes of all tetraploids examined (*P* ≥ 0.2196).

	Degree of Freedom	Sum of Squares	Mean Square	statistic value	*P*-value
Between groups	7	0.1988	0.0284	1.3977	0.2196
Within groups	72	1.4628	0.0203	-	-

DISCUSSION

Mixed-ploidy populations: In the current study tetraploid individuals of *Aeluropus littoralis* were, for the first time, detected within two different diploid populations at 8% frequency in each population in the regions with saline soils. While our previous karyotyping study on *A. littoralis* sampled from normal regions along with some other studies from the other regions of the country showed only diploid populations [e.g. 10, 19]. The occurance of polyploid plants within diploid populations in our study could be attributed to the environmental stresses of soil salinity. This is consistent with general idea that harsh and stressful environmental conditions can trigger polyploid formation in plants [8, 21, 28,29, 31]. The occurrence of mixed-ploidy have been frequently reported from populations of many other plant species. In *Andropogon gerardii* (Poaceae) 47% populations were mixed-ploids, consisting of 6× and 9× [15], 21% populations of *Brachypodium distachyon* (Poaceae) had different percentage of di- and tetraploids [14], almost 60% *of Themeda triandra* (Poaceae) populations were shown to have mixed ploidy [1]. 60% populations of *Chamerion angustifolium* were found to have plants of different ploidy levels [24]. In 16 natural populations of different variety of *Actinidia chinensis*, different double-combinations of four ploidy levels i.e. diploids and tetraploids were reported [13]. Compared to the percentage of polyploids reported from within diploids populations from othter taxa, 8% tetraploid in diploid populations *A. littoralis* seems to be low. This may indicate the polyploidization has recently occurred, and by extending the salinity in the region, its level might increase by the time.

Environmental stresses and polyploidization: In comparison to the previous reports from the other regions of the country, our study indicates occurance of polyploids in *Aeluropus littoralis* under saline soils. Similar association between polyploidization and environmental stresses have been frequently reported from other plant taxa. The occurance of higher population-level of mixed-polyploid populations of *Tragopogon miscellus* [4] and *Chamerion angustifolium* [24] was shown to be correlated with latitudinal and elevation-latitude levels, respectively. In populations of *Brachypodium distachyon* the frequency of polyploids was significantly associated with the three-way interaction of among longitude, latitude and elevation [14]. Many recent studies have indicated that formation of polyploid plants corresponds with major global climatic/geologic change and major stress [2, 28, 31].

On the other hand, there are many studies, which documented that polyploidis have high addaptations to steressful environments. The natural tetraploid

populations of both *Arabidopsis thaliana* [3] and *Citrullus lanatus* showed high adaptation to salt stresses [33]. Similarly, *Oryza sativa* [32] and hybrid *Citrus sinensis* indicated high tolerance to salty and drought stresses [23]. Populations of *Themeda triandra* (Poaceae) were widely distributed in hotter climates than its diploid populations [1]. The reasons beyond the frequent occurrence of polyploids under stressful environments is that the environmental stresses e.g., doughtiness, salinity, hot and cold weather may prompt the production of unreduced gamete and consequently polyploid formation [8]. Moreover, polyploidy in plants is believed to enhance adaptations and resistance to environmental stresses especially salinity [8, 29], and consequently help to extend the geographical distribution range of the plant taxa. We predict that by increasing the soil salinity by the time in our study areas, the percentage of tetraploid *Aeluropus littoralis* will increase in the region, since polyploidy (especially autopolyploidy) will continue to be effective speciation mechanism to habitat divergence [11].

Sampling size: With respect to small percentage of tetraploid frequency (8%) of *Aeluropus littoralis* within diploid populations, it is suggested that to reveal the polyploids within plant populations, it is impotatnt to monitro higher numbers of plants samples per population, since the frequency of polyploids could be lower among some natural populations. For example, investigating on *Acacia Senegal*, Odee et al. [16] by sampling very small number of plants per population (on average 3 plants from each of 54 populations) showed that 91% of populations were of single ploidy level, and only 10% had mixed ploidy. However, in other study on the same species polyploidy were detected in all populations under investigation at very different levels of 2%, 10%, 14% and 83% [6]. All these data indicate the importance of sampling size in detecting mixed-ploid populations.

This study may indicate that environmental stresses might be one of the causes of pushing the plant populations to evolve polyploidy in order to tolerate harsh conditions and to widen their geographical distribution ranges. Moreover, the current work emphasis that to reveal the occurrence of polyploidy within plant populations, it is important to monitor enough sample size per population. It also suggests that both the chromosomes' centromeric index and chromosomal arms lengths ratios are reliable parameters in karyotyping studies rather than absolute chromosomal size.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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