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KARYOLOGICAL STUDIES IN DIFFERENT POPULATIONS OF BUXUS HYRCANA (BUXACEAE) IN IRAN

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Karyological studies of seven populations of *Buxus hyrcana* Pojark. from different habitats are presented. We found one usual basic chromosome number in this species x=14. All populations were diploid (2n = 2x = 28). The results of kryotypic analysis made it possible to categorize different populations of a species and make a logical comparison among them.

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Key words: Buxaceae; *Buxus hyrcana*; basic chromosome; chromosome numbers; karyology

بررسی کاریوتیپی جمعیت‌های مختلف Buxus hyrcana در ایران

الهه قاسمی: دانشجوی کارشناسی ارشد دانشکده علوم باغبانی دانشگاه آزاد اسلامی واحد علوم تحقیقات

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در مطالعه حاضر بررسی کاریولوژیکی هفت جمعیت مختلف گونه .Buxus hyrcana Pojark که از مناطق مختلف کشور جمع‌آوری شده بودند ارائه شده است. نتایج نشان داد که پایه کروموزومی این گونه x =۱۴ می‌باشد. تمام جمعیت‌های مورد مطالعه دیپلوئید با ۲n=۲x =۲۸ می‌باشند. نتایج تجزیه و تحلیل کاریوتیپی امکان دسته‌بندی جمعیت‌های مختلف گونه را از مناطق مختلف رویشگاهی و همچنین مقایسه منطقی بین آنها را فراهم نمود.

INTRODUCTION

Buxaceae is a small family of mostly monoecious evergreen shrubs, and comprises five genera (*Buxus*, *notobuxus*, *sarcococca*, *pachysandra*, *styloceras*) with the largest genus, *Buxus* containing 156 species (Nixon 2006). They are mostly woody, rarely herbaceous plants. *Buxus hyrcana* is an endemic species of Hyrcanian Forests, growing as compact colonies in the forests of northern part of Iran. It is a deciduous, slow growing, evergreen plant with thick shiny leaves.

The genus Buxus is divided into three distinct sections, with the Eurasian species in one section, the Madagascan and African (without northwest Africa) species in the second, and the American species in the third. The American and African species are genetically closer to each other than to the Eurasian species.

In some taxonomic treatments, *B. hyrcana* is treated as a synonymy of *B. sempervierence*, but there are difference between *Buxus hyrcana* and *Buxus sempervirens* such as: *Buxus sempervirens* is an small tree growing to 1–9 m tall, but *Buxus hyrcana* is a tall tree growing to 1-37 m; leaf color; number of stamen surrounded the pistil and etc. But genetically *B. hyrcana* is close to *B. sempervirens*. The genetic

relationships and diversity within the European and Asiatic *Buxus* species were analyzed (Van Laere, & al., 2011). The results showed that, basic chromosome numbers are x=14 and two major clusters could be defined. One cluster contained *B. sempervirens* and *B. balearica*, the European species and *B. colchica*, an Asiatic species. Species in this cluster were characterized by a chromosome number of 2n = 2x = 28 (diploid). Just four *B. sempervirens* cultivars within this cluster were 2n=3x=42. The second cluster contained the Asiatic *Buxus* species such as: *B. microphylla, B. harlandii, B. hyrcana, B. myrica, B. henryi, B. bodinieri and B. wallichiana.* Within this cluster three different ploidy levels were observed.

The chromosome numbers are reported for *B. sempervirens* (2n=28, 0.81 pg $1C^{-1}$), *B. balearica* (2n=28) and *B. papillosa* (2n=28, 1.42 pg $1C^{-1}$) (Darlington and Wylie, 1955; Hanson & al., 2003; Bennett and Leitch, 2005).

The main aims of this research are: to clarify the chromosome numbers, ploidy level and karyological study of seven populations of *Buxus hyrcana* from different geographic regions in Iran.

MATERIALS AND METHODS

In this study, we used root tip meristems from rooted cuttings, collected from seven different habitats of *Buxus hyrcana*. The data of the collected materials is given in table 1.

Table 1. The different collection sites of *Buxus hyrcana*.

Localities	Herbarium Code	Longitude	Latitude	Altitude (m, a.b.s.)
Road of Chalous	TARI 103850	36 35 16.3	51 23 10.7	10
Tehran- cultivated in National Botanical garden	TARI 103849	51 19 0.0	35 41 0.0	1320
Pilambaran	TARI 103847	37 35 28.2	49 05 25	20
Kelarabad	HNBG 1597	36 54 81	51 31 02	-3
Behshahr ,pechat vilage	TARI 103851	36 35 42.8	53 44 25.4	909
Behshahr,emam zade hassan noor	TARI 103848	36 35 06.8	53 44 24.5	1130
Afrachal	HNBG 1529	36 14 09	53 15 04	1000

Root tip meristems were pretreated with 0.5 % saturated α-Bromo naphthalene at 3°C for 3 h, fixed in 10 % formaldehyde and 1% chromic acid (1:1) for at least 24 h at room temperature, then root tips were rinsed for 2 h in tap water. Hydrolysis was conducted with 1 N NaOH at 60°C for 20 min and was stained with Aceto-Iron hematoxylin for 16-24, then hand squashed in a droplet of mixture of 45 % acetic acid: lactic acid (10:1) (Hesamzadeh Hejazi and Rasuli 2006). The slides were observed with an optical microscope (BH2 Olympus supplemented digital color video camera) at a magnification of 2000×.The best plates of metaphase stage were selected and measured by Micro measure 3.3 software (Reeves 2001). In each mitotic metaphase (at least 10 plates) the value of arm's length of each chromosome was measured.

Some parameters such as total length (TL), long arm (LA), short arm (SA), arm ratio (AR), relative length percentage (RL %), value of relative chromatin (VRC) and centromeric index (CI) were estimated in each metaphase plate to numerically characterize the value of karyotypes (Bazzichelli 1967), (Hesamzadeh and Ziaie 2009), (Martinoli & Ogliotti 1970).

Karyotype asymmetry was calculated by three different methods namely: intra and inter-chromosomal asymmetry index (A_1 and $A_{2)}$ Romero Zarco (1986); total form percentage (TF %) Huziwara (1962) and difference of relative length (DRL). Also, we calculated Dispersion Index (DI) as the adequate measure of centromeric gradient to the coefficient of variation for chromosome length. (Lavania and Srivastava 1992).

Using the symmetry classes of Stebbins (SC), karyotypic evolution has been measured (Stebbins

1971). According to classification of Levan, chromosome morphology (K.F.) based on centromere position was determined (Levan & al. 1964). Karyograms were drawn for each population based on length of chromosome. In order to calculate the variation between populations, one-way balanced ANOVA was used on normal data and parameter means were compared by Duncan's test at $P<0.05$.

In order to evaluate the contribution of each karyotypic parameter to the ordination of species we applied principal components analysis (PCA) (data not shown).

After calculation of Cophenetic correlation coefficient (*r*) to examine karyotype identity among populations, clustering was carried out using the Ward's method.

By using of SAS ver. 6.12 (1996), JMP ver. 3.1.2 (1995) and Statisti*XL* ver. 1.7 (2007), software's we performed the numerical analysis for all populations.

RESULTS

The results showed that the basic chromosome number is x=14. The somatic chromosome numbers (2n), karyotype formals and parameters for the studied populations are summarized in table 2. The pictures of the mitotic metaphase of the populations were put in order according to their karyotypes presented in fig.1.

The mean value of chromosome's long arm was varied from 1.27µm in Behshahr (TARI 103848) to 1.55 µm in Chalous (TARI 103850). Averages of chromosome's short arm were different from 0.78µm in Behshahr (TARI 103851) to 1.01µm in Chalous (TARI 103850). The mean value of chromosome's total length was varied from 2.08µm in Behshahr (TARI 103848) to 2.56 µm in Chalous specimen and finally the mean value of chromosome's arm ratio varied from 1.46 in Kelarabad specimen (HNBG 1597) to 1.81 Tehran- Botanical garden (TARI 103849) (table 2). The chromosomes were mostly metacentric (m) or sub-metacentric (sm) in all populations (table 2).

Asymmetry indices of Romero-Zarco (1986) and symmetry types of Stebbins (SC) (1971) are shown in table 2. According to the (SC) system, the karyotype of all populations is in "A" class, which are investigated majorly initial classes in this system.

Three populations are in 1A class and remainder of populations are classified as 2A category (table 2).

By using the Romero-Zarco asymmetry indices (A_1 and $A_{2)}$ and (SC) classes, we specified the more asymmetric karyotype among the populations which have the similar Stebbins classes. For example, according to (SC), in the populations with 2A class, Tehran (TARI 103849) and Behshar (TARI 103851) populations possessed the highest A_1 value (0.41 and 0.40) respectively, therefore they have a more asymmetric karyotype (table 2). Similarly, in the populations with 1A symmetry class, Kelarabad (HNBG 1597), possessed the lowest value for A_1 (0.29) and the highest symmetric karyotype (13m+1sm). Also, among the populations with 1A symmetry class, Pilambara (TARI 103847) had the highest value for A_2 (0.20) and the highest DRL value (4.66), (table 2).

The population which is classified as 2A group showed the lowest value of A_2 (0.14), DRL (3.70), and also the lowest value of % TF (36.4).

The results of calculated (DI) showed that the highest value of DI was found in Afrachal (HNBG 1529), (7.58) and the lowest value of DI was found in Behshahr (TARI 103851), (5.08) species (table 2) (Javadi, & al. 2009).

The total karyotype mean length, measured from at least 10 metaphase plates, roughly indicates the chromatin content amongst the studied diploid taxon with x=14 was in range of 29.21 µm in Behshahr (TARI 103848), (table 2; fig. 1) to 35.96 µm in Chalous (TARI 103850), (table 2; fig. 1).

The highest VRC (value of relative chromatin) amongst all populations was obtained for Chalous (TARI 103850), which was 2.56 and the lowest were obtained for Behshahr (TARI 103848) which was 2.08 (table 2).

A statistical measurement based on balanced completely randomized design (CRD) illustrates that there are significant differences among the populations for just TL, LA and SA measured traits ($P<0.01$ and $P< 0.05$) (table 3).

Grouping of the populations are investigated based on their relative karyotypic as well as mitotic characteristics (table 4, fig. 2).

By cutting the dendrogram resulted from cluster analysis, in metric distance (2.97) and cophenetic correlation coefficient (r=0.86), the populations classified under three groups which surely the first and the second components had the most significant role to split classes.

The highest metric distance value was obtained in Chalous (TARI 103850) and Tehran botanic garden (TARI 103849). The lowest metric distance value was obtained in Chalous (TARI 103850) and Pilambara (TARI 103847), (fig. 2).

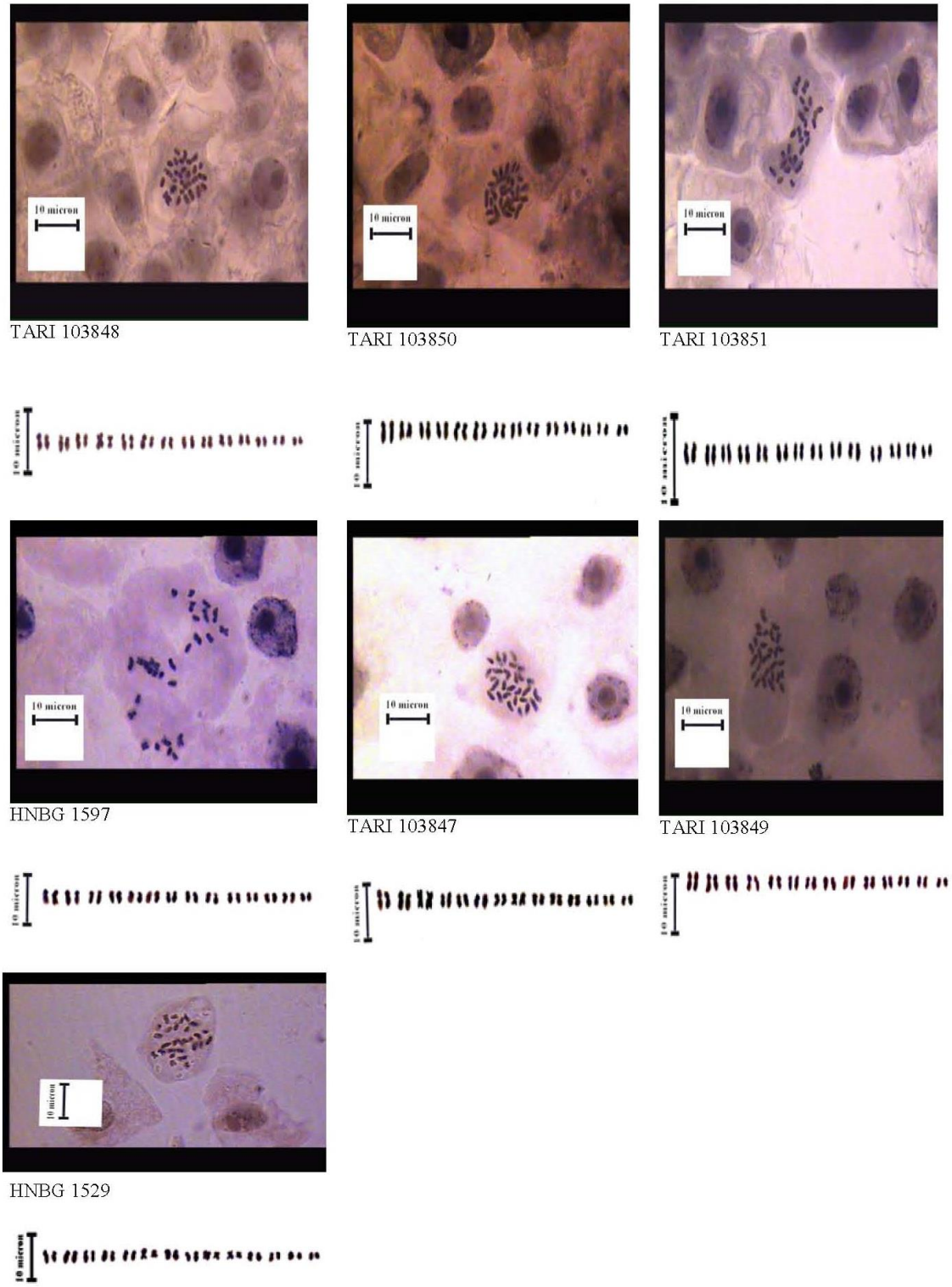

Fig. 1. Mitotic metaphase and karyotypes of diploid *Buxus hyrcana* in seven populations with GPS code. Bar = 10µm.

Table 2. Karyotype characteristics of seven populations of *Buxus hyrcana*. TL- total length, LA- long arm, SA-short arm, AR- arm ratio, CI- centromeric Index, Long arm percentage (LA%), Short arm percentage (SA%),), total form percentage (TF%), difference of range relative length (DRL), value of relative chromatin (VRC), asymmetry indices (A1, A2) of Romero Zarco, dispersion index(DI), symmetry classes (SC) of Stebbins and karyotype formula (K.F.).

Herbarium Code	2n	TL	LA	SA	AR	CI	%LA	%SA	%TF	DRL	VRC	A_1	A_2	DI	SC	K.F.
TARI 103850	28	2.56	1.55	1.01	1.58	0.39	4.31	2.83	39.5	4.65	2.56	0.33	0.19	7.09	1A	**12m+2sm**
HNBG 1597	28	2.42	1.42	0.99	1.46	0.41	4.19	2.94	41.3	3.78	2.42	0.29	0.15	6.28	1A	**13m+1sm**
TARI 103847	28	2.47	1.49	0.97	1.57	0.39	4.31	2.82	39.7	4.66	2.47	0.33	0.20	7.34	1A	**12m+2sm**
TARI 103849	28	2.34	1.49	0.85	1.81	0.36	4.54	2.59	36.4	3.70	2.34	0.41	0.14	5.64	2A	**6m+8sm**
TARI 103851	28	2.17	1.39	0.78	1.78	0.36	4.57	2.56	36.9	3.96	2.17	0.40	0.16	5.08	2A	**7m+7sm**
TARI 103848	28	2.08	1.27	0.81	1.59	0.39	4.34	2.79	41.7	3.82	2.08	0.28	0.15	6.35	2A	**11m+3sm**
HNBG 1529	28	2.14	1.31	0.84	1.57	0.40	4.34	2.79	39.2	4.61	2.14	0.34	0.19	7.58	2A	**9m+5sm**

DISCUSSION

In this study, chromosome numbers of different populations of *Buxus hyrcana* were counted for the first time in Iran. The results of this study reveal a detailed picture of the chromosome features in *Buxus hyrcana*. The knowledge of chromosome numbers, karyotype evolution, ploidy level and genome size can prepare additional information that not only gives extra insight in to the functioning of the genome, but also have notable predictive powers.

In this study, the basic chromosome number was x=14 for diploid *Buxus hyrcana* populations in Iran. Based on the results of other researcher such as Van Laere, & al. (2011) that analyzed genetic relationships and diversity within the European and Asiatic *Buxus* species, they found *B. harlandii, B. hyrcana* and nine *B. microphylla* cultivars were tetraploid (2n = 4x = 56) with a genome size of >2.5 pg $2C^{-1}$. Fifteen other *B. microphylla* cultivars were triploid (2n = 3x = 42). The other Asiatic *Buxus* species, *B. henryi, B. bodinieri* and eight *B. microphylla* cultivars, were diploid with a genome size of ca. 1.5 pg $2C^{-1}$. This result for *B. hyrcana* is adverse with our results that we explained in this paper. The present study reports the existence of 2n=2x=28 for different populations of *Buxus hyrcana* in Iran.

Results obtained from this research allow us to compare for the first time the karyotypes of diploid species of *Buxus hyrcana* species in Iran. Analysis of karyotype formula generally showed that, in all populations of *B. hyrcana*, the number of "m" chromosomes is more than "sm" chromosomes. This means that all populations in Iran have karyotypes in the early stage of evolution. Difference in the karyotypic formula of the same species and populations studied may indicate the occurrence of chromosomes structural changes like translocations in metaphase of meiosis-1. Considering the interchromosomal asymmetry index (A_2) changes among populations showed that the variation in the size of chromosomes is dependent on geographical area. Also, with increase of A_2 value we found increase of dispersion index value in almost all populations.

The Duncan's test applied to the chromosome morphometric traits (TL, LA and SA) showed a highly significant difference among all examined populations belonging to different area (table 4). In general, cytological studies of the *Buxus hyrcana* growing in Iran indicate the importance of ploidy, chromosome structural changes, presumably quantitative changes in the amount of DNA and probably the role of habitat in species diversification and suggest that such data may be used in the taxonomy and phylogenetic consideration of the genus. *Buxuus sempervirens*, the European species, with leaf morphology almost similar to *B. hyrcana*, with different leaf color and height also have the same ploidy level with *Buxus hyrcana* in Iran with chromosome number of 2n=2x=28. This study indicates the role of environment in evolution of *Buxus* species through structural change of chromosomes. These genomic differences within populations, can be used for breeding purposes.

Table 3. The results of analysis of variance for karyotypic data based on balanced CRD design.

		Mean of squares											
Source of variation	df	TL	LA	SA	AR	CI	%LA	%SA	%TF	DRL	A1	A2	DI
Population	6	0.1009**	0.0316*	0.0287*	0.0142	0.001	0.055	0.055	14.74	0.0349	0.006	0.0022	0.065
Error	14	0.0124	0.0088	0.0065	0.0085	0.0006	0.036	0.036	10.90	0.0298	0.0036	0.0012	0.050
CV%		4.81	6.64	8.977	7.31	6.45	4.34	6.87	8.44	8.14	10.24	8.41	8.83

** and * significant at 1% and 5% levels of probability respectively.

Hierarchical Clustering, Method = Ward

Clustering History

Number of Clusters	Distance	Leader	Joiner
6	0.81179998	340	376
5	1.508943258	310	355
4	2.1175574498	357	382
3	2.9667132978	340	322
2	4.3875751691	340	357
1	6.5213355862	340	310

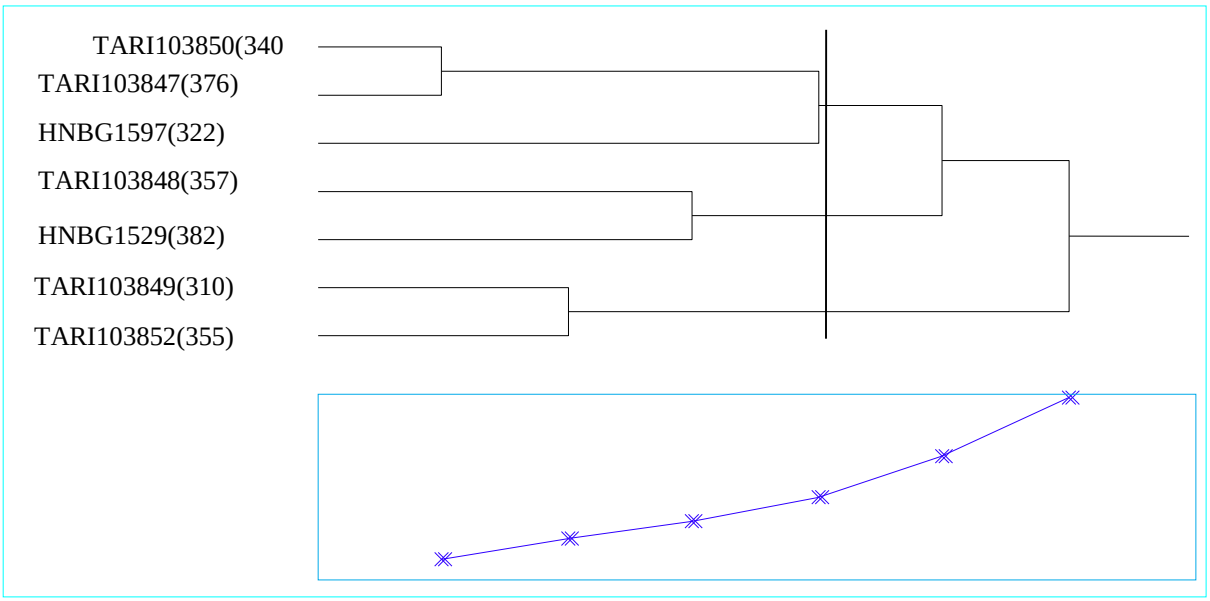

Fig. 2. Dendrogram of seven populations of *Buxus hyrcana* by analyzing 12 karyotypic parameters using Ward cluster analysis method. Cophenetic correlation r=0.86.

Table 4. Mean of parameters of chromosomes analysis of *Buxus hyrcana* populations. TL- total length, LA- long arm, SA-short arm, AR- arm ratio, CI- centromeric index, Long arm percentage (LA%), Short arm percentage (SA%), total form percentage (TF%), difference of range relative length (DRL), asymmetry indices (A_1, A_2) of Romero Zarco, dispersion index (DI). a,b,c,d: Common alphabets do not have a significant difference at the probability level of 0.05.

Population	TL	LA	SA	AR	CI	%LA	%SA	%TF	DRL	A_1	A_2	DI
TARI 103847	2.48^{ab}	1.49^{a}	0.97^{abc}	1.57^{ab}	0.39^{a}	4.30^{ab}	2.83^{ab}	39.68^{a}	4.66^{a}	0.33^{a}	0.19^{a}	7.35^{a}
TARI 103850	2.57^{a}	1.55^{a}	1.01^{a}	1.58^{ab}	0.39^{a}	4.31^{ab}	2.82^{ab}	39.53^{a}	4.65^{a}	0.32^{a}	0.18^{a}	7.09^{a}
HNBG 1597	2.42^{ab}	1.42^{ab}	0.99^{ab}	1.46^{b}	0.41^{a}	4.19^{b}	2.94^{a}	41.25^{a}	3.79^{a}	0.29^{a}	0.15^{a}	6.28^{a}
TARI 103849	2.34^{bc}	1.49^{a}	0.85^{bcd}	1.81^{a}	0.36^{a}	4.54^{ab}	2.59^{ab}	38.36^{a}	3.70^{a}	0.41^{a}	0.14^{a}	5.65^{a}
TARI 103851	2.17^{dc}	1.39^{ab}	0.78^{d}	1.81^{a}	0.36^{a}	4.57^{a}	2.56^{b}	35.91^{a}	3.96^{a}	0.40^{a}	0.16^{a}	5.09^{a}
TARI 103848	2.09^{d}	1.27^{b}	0.82^{d}	1.59^{ab}	0.39^{a}	4.34^{ab}	2.79^{ab}	41.68^{a}	3.81^{a}	0.28^{a}	0.15^{a}	6.35^{a}
HNBG 1529	2.15^{dc}	1.30^{b}	0.84^{cd}	1.57^{ab}	0.39^{a}	4.32^{ab}	2.77^{ab}	39.20^{a}	4.61^{a}	0.34^{a}	0.19^{a}	7.58^{a}

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