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Karyotype Analysis among 10 populations of *Thymus eriocalyx* (Ronniger) Jalas species in Iran

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ABSTRACT

The Karyotypes asymmetry / symmetry of one taxon (10 populations) from different geographic sites of the genus Thymus are presented. The ploidy level varied between populations belonging to T. eriocalyx (2x and 4x), According to intrachromosomal asymmetry T. eriocalyx (T8: Population No.8 and T9: Population No.9) had the most asymmetrical and evolutionary karyotype and T. eriocalyx (T2, T4 and T10) had the most symmetrical karyotype in all of the populations. Based on interchromosomal asymmetry, among diploid populations, T. eriocalyx (T2) and among tetraploid populations T. eriocalyx (T7) had the most asymmetrical karyotype. In terms of the Stebbins' system, the karyotype of populations can be classified in 1A and 1B classes. The results of analysis of variance revealed significant differences between the populations based on all karyotypic characteristics (P<0.01 and 0.05). The results of cluster analysis showed that the populations of T. eriocalyx have been grouped in separate clusters. The results seemed to provide enough genetic evidence to identify the populations and useful data to clarify the interspecific relationships. Results of karyotype analysis allow us to group the different populations and to specify their relationships.

Key words: Chromosome, Diversity, Karyotype, Ploidy, Principle Components Analysis, *Thyme.*

INTRODUCTION

Thymus L. is the most important genus of Lamiaceae family and the most famous genus of aromatic plants. This family includes approximately 279 genera [13]. This genus belongs to the tribe *Mentheae* within the subfamily *Nepetoideae* [5]. It from the view of phylogenic affinity includes *Origanum*, *Zataria* and *Micromeria* genera. The genus *Thymus* L. with English and Persian common name respectively "Thyme" and ''Azorbe/Avishan'' consists of about 928 species of herbaceous, perennial and sub shrubs or shrubs distributed mainly over Mediterranean countries, northern part of Africa and Southern Greece [19],[13]. This genus is represented in Iranian flora by 18 species, four of which (*T. lancifolius*, *T. daenensis*, *T. persicus* and *T. marandensis*) are endemic for the country [6]. Among the species of the *Thymus* genus growing in Iran, *T. eriocalyx* is wide spread and more widely used as spices, herbal tea, insecticide , flavoring agents. Also, have been most frequently used in traditional herbal medicine,

due to its antiseptic, carminative, expectorant, antispasmodic, anti-inflammatory properties and antifungal, antiviral, antibacterial, antiparasitic, spasmolytic , antioxidant activities [19].

The *T. eriocalyx* occurs in the provinces of Kurdistan, Kermanshah, Markazi, Hamadan and Lorestan and outside Iran was reported in northern Iraq. [8],[20]. This species is perennial herb and according to Flora Iranica belong to the *Serphyllum* section and *kotschiani* subsection [14].

Thyme is one of the most important species which is highly variable within and between the species and it is considered taxonomically problematic group and views concerning the taxonomic definition of the forms are extremely varied. Chromosomal information is an important key for taxonomy, phylogeny, evolution, genetics and breeding in thyme. However, because of the small chromosome size and the similarity in chromosome morphology, the identification of chromosomes is difficult in thyme [7],[21],[22].

The chromosome numbers in genus *Thymus* are known as 2n=24, 26, 28, 30, 32, 42, 48, 50, 52, 54, 56, 58, 60, 84 and 90, corresponding to the diploid, tetraploid and hexaploid levels with the basic chromosome numbers x= 6,7,9,10,15. [7],[22].

There are a lot of interesting cases of different ploidy levels within the same species [17],[12]. The researches showed that the morphology and different components of essential oils in different species of *Thymus* are variable due to hybridization and polyploidization [10]. The main purpose of this research was a new investigation on the ploidy levels and chromosome number of *T. eriocalyx* from different regions and provinces (10 regions and five provinces) of Iran for the first time. Also the results of this research would be useful for a better understanding of its taxonomy and breeding purposes such as intraspecific hybridization and genetic variation induction.

MATERIALS AND METHODS

Plants:
In this study after identify the normal botanical taxonomy of the plants we examined 10 populations, representing of *Thymus eriocalyx*. The names of the populations, location, latitudes and longitudes, and codes are listed in Table 1 and illustrated in Iran's map using GIS Microsoft (Figure 1).

Chromosome preparation:
Mitotic metaphase chromosomes were studied in meristematic cells of root tips obtained from rooted cuttings at 20°C. Root tip meristems (1cm) were pretreated with 0.5% saturated α-Bromonaphthalene at 4°C for 4 h, fixed in 10% formaldehyde and 1% chromic acid (1:1) for at least 16 h at room temperature. The root tips were then rinsed for 3 h in dishwater and were hydrolyzed in 1 M NaOH at 60°C for 7min and stained with hematoxylin for 3-4 h at room temperature. The roots were then gently squashed in mixture of 45% acetic acid: lactic acid (10:1). For the cytological investigation, images were captured with a BH_2 Olympus supplemented digital color video camera at a magnification of about 2000x. The best metaphase plates were selected (at least 3 plates) and measured by Micromeasure 3.3 software [15]for each specimen.

Statistical Analyses
In each mitotic metaphase the arm's length of each chromosome was measured according to the previous studies [1],[2],[3]. The following parameters were estimated in each metaphase plate to characterize the karyotypes numerically: long arm (LA), short arm (SA), total length (TL) [LA+SA], arm ratio (AR) [LA/SA], centromeric index (CI) [SA/(LA+SA)]. Karyotype asymmetry was estimated using the total form percentage (TF %) [(ΣSA/ΣTL)*100] [4], difference of range relative length (DRL) [$Max_{RL\%}$-$Min_{RL\%}$], intrachromosomal asymmetry index (A_1) $\left[1-\sum(\overline{SA}/\overline{LA})/n\right]$ and interchromosomal asymmetry index (A_2) [$S_{\bar{d}} / \overline{X}$] where *n* is the number of homologues, $\bar{Sd}$ is the average of standard deviation, and $\overline{X}$ is the mean chromosome length [16]. Karyotypic characteristics have been determined using the symmetry classes of Stebbins (SC) [18]. Karyotype formula was determined from chromosome morphology based on centromere position in accordance with the classification of Levan [9]. For each population, karyograms were drawn based on length of chromosome size (arranged according to diminishing size). In order to determine the variation between populations, one-way ANOVA was performed on normal data and mean of parameters were compared by Duncan's multiple range test. The principal components analysis (PCA) was performed to determine the most important variables on the variation between populations. A

cluster analysis of the karyotype data performed using the Average method to examine karyotype similarity among populations. Numerical analysis were performed using SAS ver. 6.12 (1996); JMP ver. 3.1.2 (1995),

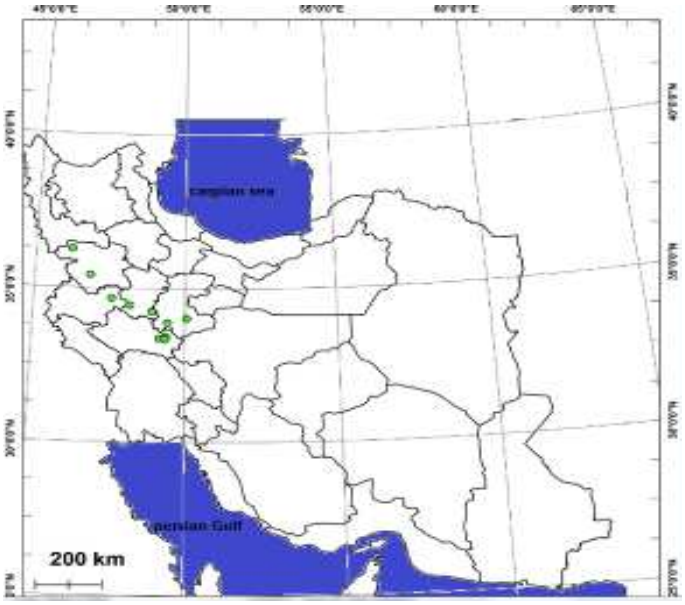


Fig 1. Collection sites of *Thymus eriocalyx* populations on the map of Iran designed using GIS Microsoft

Table 1. Geographical data of the collections on *Thymus eriocalyx* from different populations

Population Code	Place of Collection	Altitude (m)	Latitude and Longitude
T1	Lorestan Province, Azna, Sefid kooh	2245	N=33□□ 26' 51.6″ E=49□□ 22' 13.2″
T2	Lorestan Province, Azna, Dare Takht, Oshtoran kooh	2053	N=33□□ 20' 45.3″ E=49□□ 22' 02.7″
T3	Lorestan Province, Doroud, Gahar Road, Saravand village	1907	N=33□□ 22' 42.5″ E=49□□ 09 53.2″
T4	Markazi Province, Arak Shazand, Souraneh village, Rasvand mountain	2362	N=33□ 52' 57.3″ E=49□ 25' 59.20″
T5	Markazi Province, Arak, Qom Road Latehdar village, Absar mountain	2200-2500	N=34□□ 01' 40.1″ E=50□□ 03' 35.5″
T6	Hamadan Province, Malayer, Conservation area of Lashkardar	1942-1970	N=34□□ 14' 51.9″ E=48□□ 54' 51.1″
T7	Hamadan Province, Toysderkan, Tormeyanak village, Khangormaz mountain	1863	N=34□□ 26' 50.5″ E=48□□ 10' 58.3″
T8	Kermanshah Province, 11 km Riad of Songhor to Bistoon, Ahmadabad village, Dalakhani mountain	1930-1950	N=34□□ 40' 19.8″ E=47□□ 34' 44.1″
T9	Kurdestan Province, Sanandaj Marivan old Road, Ariz gorge	2035-2070	N=35□□ 24' 55.8″ E=46□□ 50' 46.1″
T10	Kurdestan Province, Saghez, The road of Malgharani village	1825	N=36□□ 15' 15.8″ E=46□□ 12' 28.5″

RESULTS AND DISCUSSION

This study reveals a detailed picture of the chromosome features in *Thymus eriocalyx*. The pictures of the mitotic metaphases and their karyograms of the populations are presented in figure 2. The somatic chromosome numbers (2n), ploidy levels, ranges of chromosome length, symmetry index percentage, intra- and inter-asymmetry indices, difference of range relative length, total form percentage, symmetry classes, total karyotype length and karyotype formula of the taxa and populations investigated are summarized in Table 2. The somatic chromosome number and details of the karyotypes of the studied populations, revealed that *T. eriocalyx* populations possessed two ploidy levels, diploid (2n=2x=30) and tetraploid (2n=4x=60).

Mahdavi and Karimzadeh [11] has reported that one populations of *T. eriocalyx* located in Hamadan province in Iran is diploid (2n=2x=30). Our study indicated that the chromosome numbers in different populations of the Hamadan province (populations T6 and T7) are diploid (2x) and tetraploid (4x) respectively. The pollination system in the genus is OP, and Aneuploidy has been an important phenomenon during the evolution of this genus and it is responsible for the other numbers.

The chromosome numbers of T1, T2, T3, T4, T5 and T8 populations were 2n=2x=30, and the chromosome numbers in the other studied populations such as T9 and T10 were 2n=4x=60. This result was carried out for the first time.

Total karyotype length, roughly indicative of the DNA content, ranges from 38.17 to 71.04 µm in diploid taxa and from 61.02 to 83.60 µm in tetraploid taxa. Also size of the chromosomes among the populations varied from 0.72 µm in population T7 to 3.59 µm in population T8.

All of the populations had mainly ‘m’ type chromosomes (centromers at median region). However, two populations T6 and T9 each possessed two ‘M’ type chromosomes (Median).

Among the studied populations the highest TF% value (46.96) was estimated in the population T2 and the lowest TF% value (45.03) was estimated in the population T8 that is one of the main reasons to make its karyotype asymmetric. In view of the fact that, fewer DRL value indicated more symmetry of karyotype, population T9 and population T2 respectively with DRL 1.75 and 5.68 values had the most symmetric and asymmetric karyotypes. Intrachromosomal asymmetry index (A_1) showed sharp differences between the chromosome arms in the different populations. In general, based on intrachromosomal asymmetry (A1 and TF %), population T8 had the most asymmetric karyotype and population T2 had the most symmetrical karyotype in all of the populations. According to interchromosomal asymmetry (A2 and DRL), among diploid populations, population T2 and among tetraploid populations population T7 had the most asymmetrical karyotype.

Similarly, high DRL value leads to more changes in the structure of chromosomes, but it is mentioned that the DRL is the dependent to ploidy levels and chromosome numbers. Therefore, it is not a good criterion for comparing various species with different ploidy levels, because the DRL values are lesser at upper ploidy levels than lower ploidy levels. So, this parameter will be useful for comparison of species with the same ploidy levels.

In terms of the Stebbins’ system, the karyotype of populations seizes 1A and 1B classes which are considered mainly primitive classes in this system. By using A_1 and A_2 parameters we can determine the more asymmetric karyotype among the populations which have the similar Stebbins classes of symmetry. The populations which are classified as 1A group also showed the lowest value of A_2 in range of 0.12- 0.17 and the highest value of TF% ranged from 45.14 to 46.84.

To analyze the variability of the karyotypes among populations, the length of chromosome, the long and short arms of chromosome, the arm ratio values, the difference of range relative length, the total form percentage and asymmetry indexes (A_1, A_2) were compared by one-way analysis of variance (CRD). Also, Duncan test was carried out to test differences between each pair of means. The results of variance analysis revealed significant differences between the populations based on all karyotypic characteristics ($P<0.01$ and $P<0.05$). This indicated the occurrence of quantitative changes in chromosome size of the studied populations (Table 3).

Significant effect of chromosomal traits proved karyotypic variation between populations. It lets us to know the importance of chromosome study to distinguish the state of evolution and affinity between different populations.

The Duncan's test applied to the chromosome morphometric traits showed a highly significant difference among the all examined populations (Table 4). So, mean value of chromosomes total length varied from 1.02 µm in populations T9 to 2.37 µm in populations T8. The results showed that the average length of chromosomes in diploid populations of T1, T3, T4 and T8 is much greater than tetraploid populations. But chromosomal data in this study reflect the smaller size of chromosomes in diploid populations of T2, T5 and T6 than tetraploid population of T7. This has been caused many problems associated with karyotype studies.

The mean value of chromosomes long arm varied from 0.56 µm in population T9 to 1.30 µm in population T8. Also the mean value of chromosomes short arm was different from 0.46 µm in population T9 to 1.07 µm in population T8.

Using principal components analysis (PCA), the first two independent components accounted about 90% of total variation. The first component indicated that arm ratio, total form percentage, centromeric index and intrachromosomal asymmetry index were important characters for classification of populations with about 53% of total variation. Total length of chromosome, long arm length of chromosome, interchromosome asymmetry index, difference of relative length and short arm length of chromosome were important traits in the second component (37.41%) (Table 5).

Grouping of studied populations was based on their karyotypic traits (Fig.4). By cutting dendrogram resulted from cluster analysis by average method with cophenetic correlation coefficient ($r_{=}$0.80) in metric distance 2.98, the populations classified under four groups which certainly the first and the second components had the most significant role in separated classes. The results showed that populations of *T. eriocalyx* have been grouped in separate cluster. This indicates that populations of a specific species in their within will show variety. Thus, these studies could greatly help us in the classification and taxonomic studies.

The first cluster includes the populations of T1, T3, T6, T7, T5 and T10. The population of T9 with 2n=4x=60 through the difference of DRL value separately classified as an apart group. Populations of T2 and T4 with 2n=2x=30, grouped together in same cluster (third cluster) that it seems the factors of similarity were A1, AR and TF values. (Table 4 and Fig.3). The populations of T8 with chromosome number (2n=2x=30) and with the highest value of LA, TL, TF% and A_1, classified as a fourth group.

The highest metric distance (6.26) was obtained between populations of T1 and T8 which imply the least affinity between them. The lowest metric distance (1.270) was obtained between two populations of T1 and T3 which imply the least karyotypic difference between them (Fig. 4).

The diagram of populations' dispersion, based on two first components, showed that the populations separated in four groups, which completely fits with results obtained through the grouping analysis by average method (Fig. 3).
The present study shows the change in the chromosomal traits as one of the mechanism of inter and intra-species diversification in the *Thymus* genus as well as the earlier cytological reports [1],[2],[3].

The essential oils of 10 populations' studies in this paper also have been chemically investigated revealing about 32 different volatile components in total. The amount of components was varied between populations especially between different ploidy levels (data not shown). So it seems the different components of essential oils in different populations of *Thymus* species are variable due to hybridization, polyploidization and place of growing.

the results of the molecular markers used among 10 populations, also showed a high diversity as well as the amount of components of essential Oils(data not shown).

As a rule, the variation of climate and soil in Iran provides a suitable field for plant variations. One of the genetic variations in Thymus sp., which is clearly detectable, is the chromosome numbers and structural changes of chromosomes. These genomic differences could be used for breeding purposes.

Table 2.Somatic chromosome number (2n), ploidy levels, ranges of chromosome length, asymmetry indexes (A_1, A_2) of Romero Zarco, difference of range relative length (DRL), total form percentage (TF%), and symmetry classes (SC) of Stebbins, total karyotype length (TKL) and karyotype formula (K.F.) for 10 population of *Thymus eriocalyx* investigated, metacentric (m), median (M)

Taxon (population)	2n	Ploidy level	Chromosome length range	A_1	A_2	DRL	TF%	SC	TKL (µm)	K.F.
T. eriocalyx (T1)	30	2x	1.20-2.01	0.16	0.14	3.50	45.65	1A	46.32	30m
T. eriocalyx (T2)	30	2x	0.76-1.84	0.12	0.19	5.68	46.96	1B	38.17	30m
T. eriocalyx (T3)	30	2x	1.35-2.25	0.15	0.15	3.48	45.88	1A	52.15	30m
T. eriocalyx (T4)	30	2x	1.18-2.16	0.12	0.17	4.06	46.84	1A	48.40	30m
T. eriocalyx (T5)	30	2x	1.04-1.66	0.14	0.12	3.12	46.37	1A	39.48	30m
T. eriocalyx (T6)	30	2x	1.05-1.58	0.15	0.12	2.71	45.70	1A	39.08	2M+28m
T. eriocalyx (T7)	60	4x	0.72-1.77	0.15	0.18	2.31	45.90	1B	83.60	60m
T. eriocalyx (T8)	30	2x	1.75-3.59	0.17	0.18	5.18	45.03	1B	71.04	30m
T. eriocalyx (T9)	60	4x	0.79-1.33	0.17	0.13	1.75	45.14	1A	61.02	2M+58m
T. eriocalyx (T10)	60	4x	0.74-1.48	0.12	0.17	2.22	46.58	1A	65.66	60m

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

		Mean of squares								
S.O.V	D.F	TL	LA	SA	AR	CI	DRL	TF	A1	A2
Populations	9	0.449**	0.139**	0.089**	0.003*	0.001*	4.858**	1.329*	0.041**	0.002**
Error	20	0.027	0.009	0.005	0.001	0.001	0.098	0.574	0.001	0.001
%C.V.		11.15	11.738	10.741	3.111	1.647	9.191	1.647	6.742	12.752

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

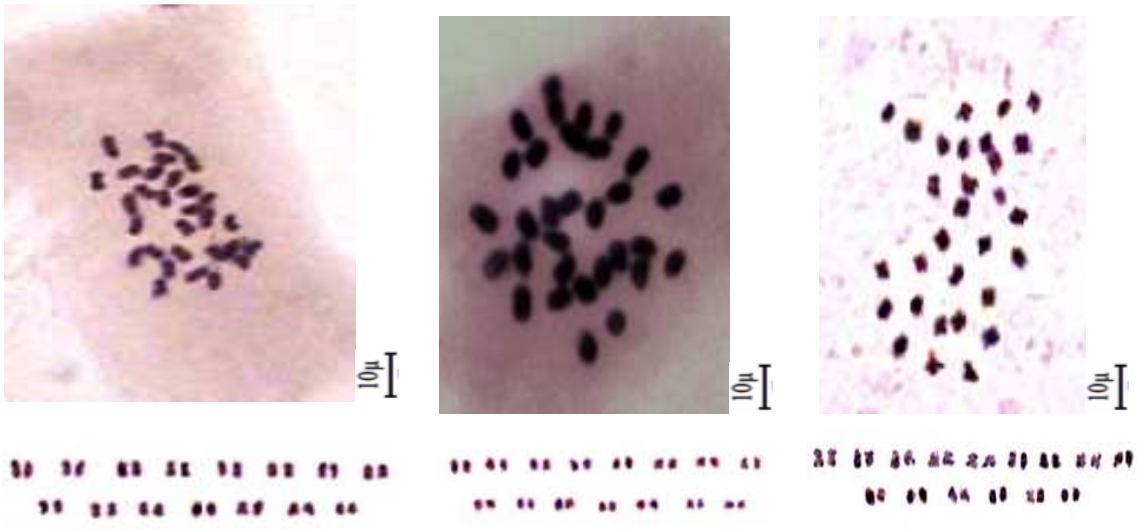
Table 4. Mean of parameters of chromosomes analysis of *Thymus eriocalyx* populations.

population	TL	LA	SA	AR	CI	A2	A1	DRL	TF%
T. eriocalyx (T1)	1.54 bcd	0.84 bcd	0.71 bcd	1.19 abc	0.46 abc	0.14 cd	0.16 a	3.49 c	45.65 abc
T. eriocalyx (T2)	1.27 def	0.67 de	0.60 de	1.13 c	0.47 a	0.19 a	0.11 c	5.66 a	46.96 a
T. eriocalyx (T3)	1.74 b	0.94 b	0.80 b	1.18 abc	0.46 abc	0.15 bcd	0.15 ab	3.48 c	45.88 abc
T. eriocalyx (T4)	1.61 bc	0.86 bc	0.76 bc	1.14 c	0.47 a	0.17 abc	0.11 c	4.06 b	46.83 a
T. eriocalyx (T5)	1.32 cdef	0.71 cde	0.61 de	1.16 abc	0.46 abc	0.12 d	0.13 b	3.11 cd	46.36 abc
T. eriocalyx (T6)	1.30 def	0.71 cde	0.60 de	1.19 abc	0.46 abc	0.12 d	0.15 ab	2.70 de	45.70 abc
T. eriocalyx (T7)	1.39 cde	0.76 cd	0.63 cde	1.18 abc	0.46 abc	0.18 ab	0.15 ab	2.31 ef	45.90 abc
T. eriocalyx (T8)	2.37 a	1.30 a	1.07 a	1.22 a	0.45 c	0.18 ab	0.17 a	5.18 a	45.03 c
T. eriocalyx (T9)	1.02 f	0.56 e	0.46 f	1.22 a	0.45 c	0.13 cd	0.17 a	1.76 f	45.14 bc
T. eriocalyx (T10)	1.09 ef	0.58 e	0.51 ef	1.15 bc	0.47 a	0.17 abc	0.12 bc	2.23 ef	46.57 ab

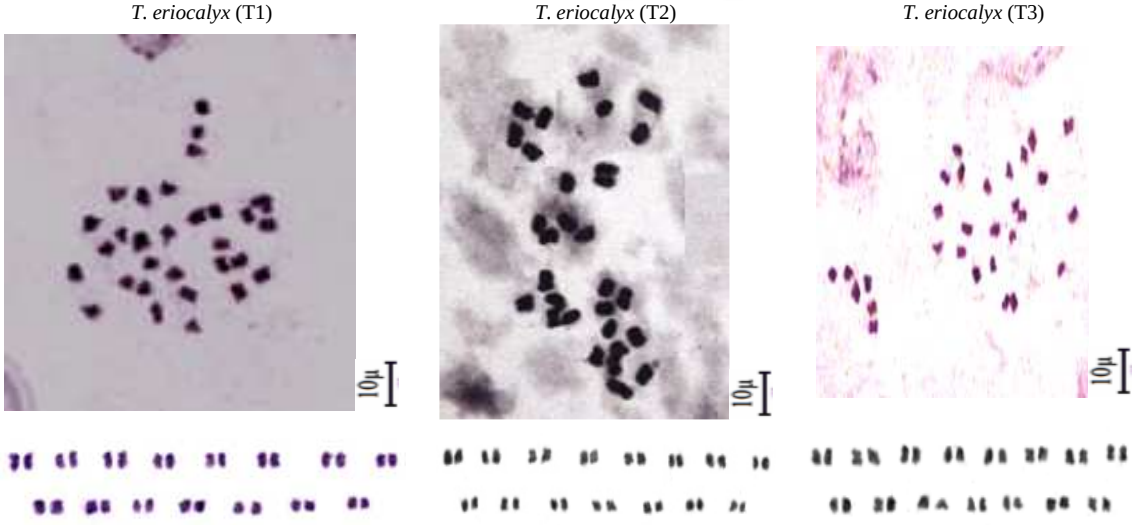
TL: total length of chromosome, LA: long arm, SA: short arm, AR: arm ratio, CI: Centromic index, A_2: interchromosome asymmetry index , A_1: intrachromosome asymmetry index, DRL: difference of relative length, TF%: total form percentage,

Table 5. Eigen vectors from the first two principal components for 9 karyotype parameters to classify 10 populations of *Thymus eriocalyx*

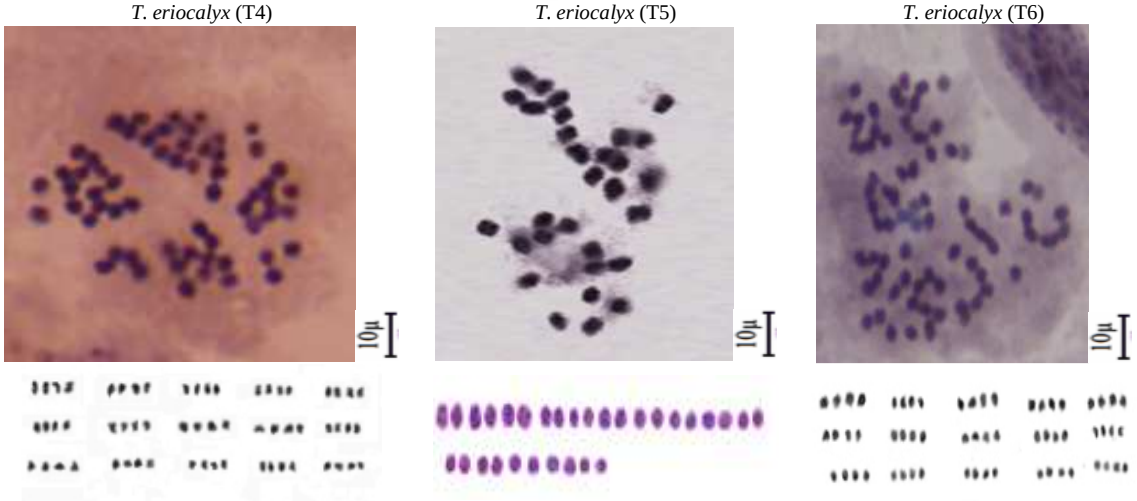
Parameters	First component	Second component
TL	0.32	**0.38**
LA	0.33	**0.37**
SA	0.30	**0.40**
AR	**0.42**	-0.20
CI	**-0.42**	0.20
A2	-0.06	**0.39**
A1	**0.41**	-0.24
DRL	0.03	**0.48**
%TF	**-0.42**	0.20
Eigen Value	4.78	3.37
Percentage of Variance	53.09	37.41
Cum Percentage of variance	53.09	90.50



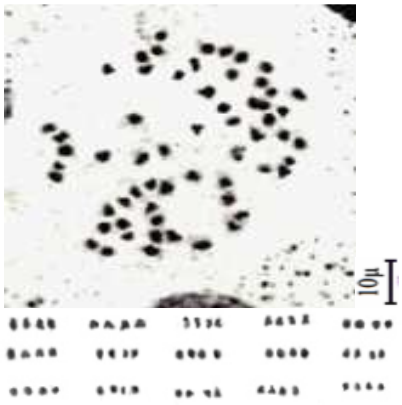
T. eriocalyx (T1) *T. eriocalyx* (T2) *T. eriocalyx* (T3)



T. eriocalyx (T4) *T. eriocalyx* (T5) *T. eriocalyx* (T6)



T. eriocalyx (T7) *T. eriocalyx* (T8) *T. eriocalyx* (T9)



T. eriocalyx (T10)

Fig 2. Mitotic metaphase of *Thymus eriocalyx* populations accompanied by karyograms. T1 (2n=2x=30); T2 (2n=2x=30); T3 (2n=2x=30); T4 (2n=2x=30); T5(2n=2x=30); T6(2n=2x=30); T7(2n=4x=60) ; T8(2n=2x=30) ; T9(2n=4x=60) ; T10(2n=4x=60).

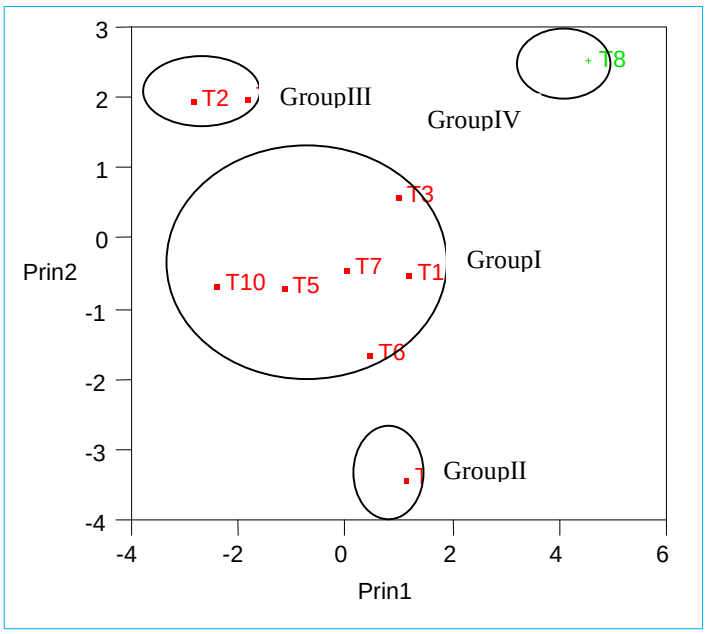


Fig 3. Scatter plot of 10 populations for the first two principals

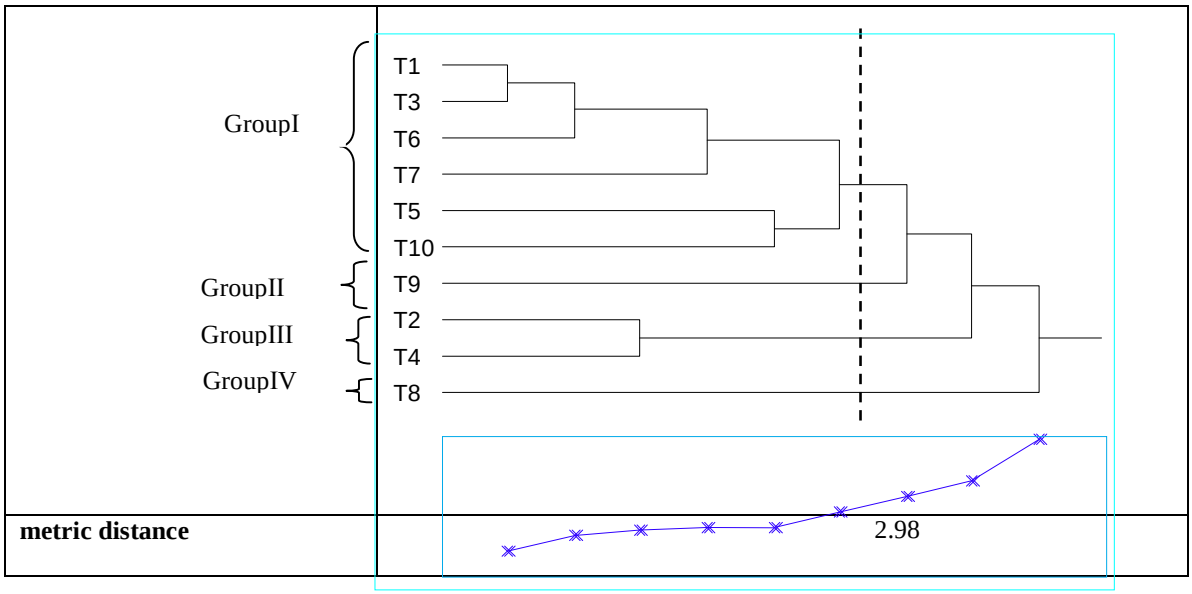


Fig4. Dendrogram of 10 populations of *Thymus eriocalyx* by analyzing 9 karyotipic parameters using average cluster analysis method. Cophenetic correlation *r*=0.80.

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