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Evaluation of the evolutionary process within *Populus caspica* species from Hyrcanian forests by karyotype analysis

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Abstract. Caspian poplar (*Populus caspica* Bornm.) is distributed exclusively in the Hyrcanian forests. Hyrcanian forests are the final remnants of Tertiary temperate deciduous forests in Western Eurasia and worldwide. This species plays a significant ecological role in the protection of the natural environment in Hyrcanian forests. In this research, chromosome number and karyotype details of 11 populations of the species were investigated for the first time, using fresh root cuttings collected from mature trees in different parts of the forest, located in the northern parts of Iran. Pretreatment, fixation, hydrolyzing, and staining were conducted by α-bromonaphthalene, carnoy's solution, 1N HCl, and hematoxylin agent, respectively. Chromosomal data were analyzed according to a nested model based on a completely randomized design. Chromosome numbers of all of the populations were the same as $2n = 38$, which mostly were medium region and sub-metacentric types. Significant differences were observed between the provinces and populations, based on chromosome length grand means, arm ratios and centromere indices. The results demonstrated that structural rearrangement has occurred within the studied populations and indicated an active evolutionary process within and between populations of the species due to natural hybridization. Also, these results showed that artificial inter-specific hybridization between the *P. caspica* and its relative species can be employed to broaden the ecological zone of the species.

Keywords: asymmetry indices, chromosome number, karyotype, *Populus caspica.*

INTRODUCTION

Hyrcanian forests inscribed on the UNESCO World Heritage List contain the final remains of tertiary wide-leaved forests in western Eurasia and worldwide (UNESCO World Heritage Centre 2019; Bayranvand et al. 2017; Alipour et al. 2023). Hyrcanian forests are located between the north of the Alborz Mountain chains and the Caspian sea (Iran and Caucasus) (Sabeti, 1994). In Iran, which accounts for most of the hotspots of Iranian-Anatolian biodiversity, 30

percent of vascular plant species are endemic (Noroozi et al. 2018). In flora of Iran only two species, *Populus caspica* Bornm., and *P. euphratica*, are native and the others (*P. alba*, *P. deltoides*, *P. nigra* and hybrid species (*P. canadensis*)) are widely cultivated at different parts of Iran (Maassoumi et al. 2011). Caspian poplar (*P. caspica*), a member of the Salicaceae family, is classified as endangered in Iran (Jalili and Jamzad, 1999; Alipour et al. 2021). Many species including *P. caspica* have survived periods of glaciation in the Hyrcanian forests (Qin et al. 2017; Mohammadi et al. 2019). Poplars are so noteworthy in agroforestry industry because of their fast growth, desirable figure, as well as in providing wood, fiber, fuel-wood, and other forest products (Stettler, 2009; Evans, 2014). Natural habitats of *P. caspica* have largely been destroyed through environmental conditions and human activities like planting non-indigenous species, and agricultural usage (Khoshravesh et al. 2009). Habitat degradation has impacted the restoration of *P. capsica* due to unsuitable seedbed conditions (Asadi and Mirzaie-Nodoushan, 2011).

Comparative genomics and sequencing have been done in several poplar species that demonstrated high genetic diversity and frequent interspecific hybridization among the species (Li et al. 2023). Other karyological studies showed that in *Populus* genus, with a basic haploid chromosome number of 19, diploidy is predominant. Due to the small chromosomes, karyotype information has been reported for several *Populus* species (Islam-Faridi et al. 2009; Liu et al. 2021). Hence, in recent years fluorescence *in situ* hybridization (FISH) has been employed for identifying chromosomes in poplars to compare the karyotype and similarity of chromosome structure among different species (Xin et al. 2020; Kim et al. 2020). Apart from *P. euphratica*, there is no information on the number of chromosomes and karyotypic indices of the *Populus* species, including *P. caspica,* in Iran. Therefore, this study was undertaken to provide cytological information on the species based on conventional methods that can be useful as a guide in future breeding programs and evaluation of evolutionary process.

MATERIALS AND METHODS

Plant materials were obtained from trees in 11 different parts of two provinces, Gilan and Mazandaran, located in north part of Iran. Root tip meristems, collected from cutting grown under hydroponic conditions, were pre-treated with 0.5% α-bromonaphthalene for 1 hour in refrigerator, then fixed in a mixture of ethanol alcohol and glacial acetic acid (3:1 v/v) for 16 hours. The fixed samples were washed 2–3 times and preserved in 70% ethanol. Root tips were hydrolyzed with 1N HCl solution at 60°C for 6 minutes, stained in hematoxylin reagent for 2 hours at 60°C, and finally squashed in 45% acetic acid (v/v) (Mirzaie-Nodoushan and Asadi-Corom, 2002). Somatic chromosomes were photographed using digital camera and the chromosomes were measured via Ideokar 1.2 software (Ghader Mirzaghaderi and Karim Marzangi, 2015). Based on the centromere position nomenclature of chromosomes was described (Levan et al. 1964). Along with chromosomal dimensions several chromosomal parameters, such as Arm ratio, r-value, Relative length of chromosome, Form percentage of chromosome, centromeric index (CI=S/TL) were calculated. As well as, asymmetry indices were calculated using Intra-chromosomal asymmetry index (A1) (Zarco, 1986), Inter-chromosomal asymmetry index (A2) (Zarco, 1986), Symmetry index (S%) (Watanabe et al. 1999), Total form percentage (TF%) (Huziwara, 1962), difference of range relative length (DRL) and Stebbins class asymmetry index (Stebbins, 1971).

Chromosomal data were analyzed using a nested model based on a completely randomized design , regarding the provinces, populations and chromosomes as the three nested factors with three replications of well-spread metaphasic plates. In this case provinces are considered as factor A, populations as factor B, nested within factor A, which is shown in statistical point of view as, populations (A), and chromosomes as factor C, nested within factor B, (chromosomes (B A)). Duncan multiple range test was carried out for classifying the populations by SAS 9.4 software. Cluster analysis was performed in order to classify the populations based on chromosomal measures and karyotypic indices using Ward method, by JMP 13.2.0 software.

RESULTS

The chromosome counting revealed that all of the studied populations were diploid, containing a total of 38 chromosomes ($2n$ = 38) with a single pair carrying the satellites, located on the short arms of the chromosomes (Fig. 1). Chromosome length grand mean and arm ratio (AR) showed a significant difference ($p \leq 0.05$) between the provinces, while arm ratios and centromere indices were different ($p \leq 0.01$) between the populations (Table 1). Size of the chromosomes among the studied populations varied from 0.65 μm (Tash12) to 2.90 μm (Tash2) and from 0.67 μm to 2.32 μm in Mazandaran and Gilan populations, respectively (Table 2).

The karyotype formula of the studied populations is presented in Table 1. In both provinces, medium region

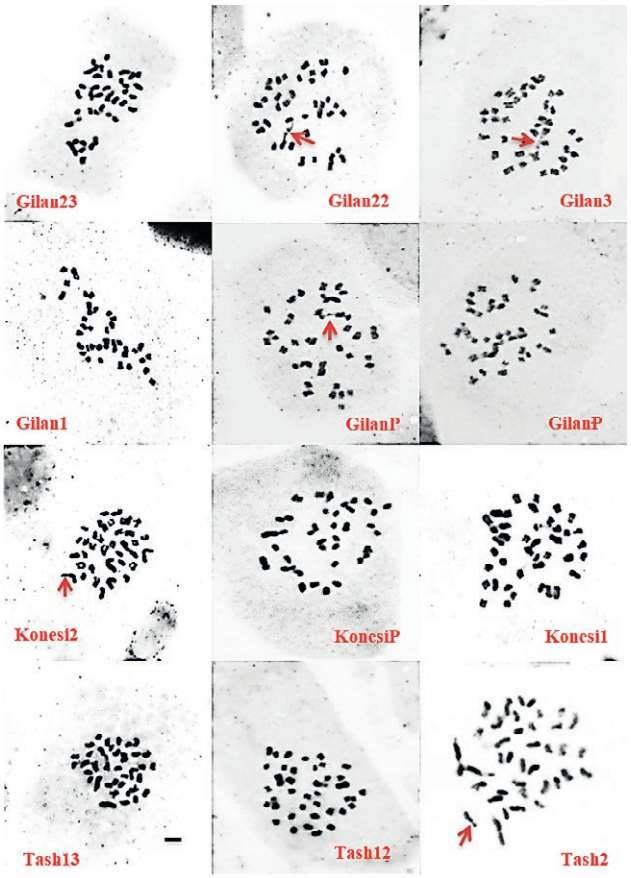

Figure 1. Mitotic metaphase chromosomes of the studied populations of *Populus caspica* in Iran (Arrows are pointing to satellites; Bar=100 μm).

(m) type chromosomes were the dominant type, especially in Gil22 and Tash13. That's why their karyotypes are symmetrical. All populations possessed one to seven chromosomes of sub-metacentric (sm) type. Sub-terminal region (st) type was observed only in two populations of Mazandaran. The most asymmetrical karyotype was found in the Konesi2 population of the same province (21m+14sm+3st). In this population, medium point and terminal point types of chromosomes were also found in several single plants (23m+4sm+9st+2T+sat; 1M+34m+1sm+2st) (Table 3). Also based on chromosome characteristics and karyotypic indices, the plant populations were clustered in three groups. The konesi2 population, with the most asymmetrical karyotype, was clustered into a single group. (Fig. 2).

DISCUSSION

In most modern poplars, the cell nucleus typically contains two sets of 19 ($2n$ = 38) chromosomes (Chen et al. 2005; Shou-Gong et al. 2005) that agree with the results obtained by this research. Triploids with three sets of chromosomes ($2n$ = 57) (Peto, 1983) and tetraploids ($2n$ = 76) have also been identified in section *Populus* (Einspahr et al. 1964; Every and Wiens 1971). According to IPCN and literature surveys, triploidy in *P. nigra* and *P. canadensis* (Shou-Gong et al. 2005; Chen et al. 2005) and aneuploidy in two varieties of *P. alba* have been reported (IPCN, http://www.tropicos.org/Project/IPCN). The presence of only one couple carrying one pair of satellite, represents the basic profile of this species same as other species of poplars.

Chromosome numbers and chromosome rearrangements are the major source of karyotype evolution and closely related species maintain a similar chromosome number. Despite the similar chromosome number in the studied populations, a structural diversification was observed in the studied populations. Difference in the karyotypic formula within the species indicates that chromosome structural changes have occurred. Pericentric inversions is one of the most common mechanisms related to karyotypic variation (Molina and de Freitas Bacurau, 2006; Carbone et al. 2014). They can shift the position of the centromere within a chromosome and cause the arm ratio to change. Inverted chromosomes

Table 1. Mean squares resulted from nested model analysis of variance of chromosome parameters of *Populus caspica* populations.

Source of variation	DF	L (μm)	S (μm)	TL^{M} (μm)	AR	r-Value	RL%	F%	CI
A: provinces	1	0.37*	0.004^{ns}	0.0.07*	0.97*	0.002^{ns}	0.00^{ns}	0.006^{ns}	0.001^{ns}
B: populations (A)	9	0.16^{ns}	0.11^{ns}	0.48^{ns}	1.31**	0.06**	1.68^{ns}	0.06^{ns}	0.009**
C: Chromosomes (B A)	198	0.13**	0.07**	0.39**	0.14^{ns}	0.01^{ns}	0.1**	0.4**	0.002^{ns}
Error	418	0.007	0.004	0.01	0.21	0.02	0.02	0.02	0.002
CV%		13.65	14.46	10.16	31.98	17.04	5.84	12.52	11.27

L= length of the longest chromosome, S= length of the shortest chromosome, TLM = grand mean of chromosome length, AR= L/S, r-Value= S/L, RL%= relative length of chromosome, F%= form percentage of chromosome, CI= centromeric index.
**: significant difference at 1% level, *: significant difference at 1% level, ns: no significant difference.

Table 2- Means of mitotic features of the studied populations of *Populus caspica.*

Provinces	Populations	L (μm)	S (μm)	TL^M (μm)	AR	r-Value	RL%	F%	CI
Gilan	Gil13	0.59^{d}	0.43^{d}	1.02^{g}	1.43^{b}	$0.74b^{cd}$	2.63^{a}	1.11^{abc}	0.42^{ab}
Gilan	Gil23	$0.66b^{c}$	0.47^{c}	1.13^{d}	1.47^{b}	$0.74b^{cd}$	2.63^{a}	1.09^{bc}	0.42^{b}
Gilan	Gil22	0.67^{bc}	0.51^{b}	1.18^{bc}	1.34^{b}	0.78^{abc}	2.63^{a}	1.14^{ab}	0.43^{ab}
Gilan	Gil31	$0.66b^{c}$	0.52^{b}	1.18^{c}	1.31^{b}	0.80^{a}	2.63^{a}	1.16^{a}	0.44^{a}
Gilan	GilP	0.64^{c}	0.46^{c}	1.10^{de}	1.47^{b}	0.73^{cd}	2.63^{a}	1.09^{bc}	0.41^{b}
Mazandaran	Konesi1	0.61^{d}	0.44^{cd}	1.05^{fg}	1.46^{b}	0.74^{abcd}	2.63^{a}	1.11^{abc}	0.42^{ab}
Mazandaran	Konesi2	0.69^{b}	0.46^{c}	1.15^{cd}	1.87^{a}	0.70^{d}	2.63^{a}	1.05^{c}	0.40^{c}
Mazandaran	KonesiP	0.61^{d}	0.46^{c}	1.07^{ef}	1.40^{b}	0.77^{abc}	2.63^{a}	1.14^{ab}	0.43^{ab}
Mazandaran	Tash12	0.60^{d}	0.45^{cd}	1.05^{fg}	1.39^{b}	0.77^{abc}	2.63^{a}	1.13^{ab}	0.43^{ab}
Mazandaran	Tash13	0.69^{b}	0.53^{ab}	1.23^{b}	1.35^{b}	0.79^{ab}	2.63^{a}	1.15^{ab}	0.44^{ab}
Mazandaran	Tash23	0.76^{a}	0.55^{a}	1.31^{a}	1.42^{b}	0.75^{abc}	2.63^{a}	1.11^{abc}	0.42^{ab}

L= length of the longest chromosome, S= length of the shortest chromosome, TL^M =grand mean of chromosome length, AR= L/S, r-Value= S/L, RL%= relative length of chromosome, F%= form percentage of chromosome, CI= centromeric index, Similar letters within each column, indicate no significant difference between the populations at 5% level.

Table 3. Karyotypic parameters of the studied populations of *Populus caspica.*

Provinces	Populations	Stebbins	FK	A1	A2	S%	TF%	DRL%
Gilan	Gil1	1B	34m+4sm	0.26	0.09	33.77	42.05	3.34
Gilan	Gil23	2B	32m+6sm	0.26	0.05	32.09	41.55	3.59
Gilan	Gil22	1B	37m+1sm (**2sat**)	0.22	0.08	34.33	43.49	3.40
Gilan	Gil3	1B	32m+6sm (**2sat**)	0.20	0.06	32.68	43.96	3.48
Gilan	GilP	1B	34m+4sm (**2sat**)	0.27	0.06	30.68	41.58	3.78
Mazandaran	Konesi1	1B	32m+6sm	0.26	0.09	33.42	42.01	3.44
Mazandaran	Konesi2	2B	21m+14sm+3st (**2sat**)	0.30	0.13	28.28	39.93	4.08
Mazandaran	KonesiP	2B	34m+3sm+1st	0.23	0.11	32.53	43.21	3.26
Mazandaran	Tash12	1B	35m+3sm	0.23	0.11	34.26	42.88	3.25
Mazandaran	Tash13	1B	36m+2sm	0.21	0.08	32.31	43.59	3.54
Mazandaran	Tash2	1B	31m+7sm (**2sat**)	0.25	0.05	26.04	42.00	4.31

KF= karyotypic formulae, A1= Intra-chromosomal asymmetry index, A2= Inter-chromosomal asymmetry index, S%= Symmetry index, TF%= Total form percentage, DRL%= Differences between the maximum and minimum relative length of the chromosomes; sat= satellite.

have the potential to contribute to asymmetrical bivalents (Singh, 2017). In fact, chromosomal rearrangements are often the main source of karyotypical evolution and would indicate an active evolutionary process within and between populations of the species (Mirzaie-Nodoushan et al. 2006; Xin et al. 2020). In Hyrcanian forest, the natural hybridization and large-scale interspecific hybridization between *P. caspica* and other cultivated poplar species, such as European *P. alba*, *P. nigra*, *P. deltoids* (North American poplar), is documented using cpDNA (chloroplast DNA) and ITS (Internal Transcribed Spacer) fragments (Yousefzadeh et al. 2019). Inter-chromosomal translocation is another factor for chromosomal rearrangements and diversity of karyotypic parameters in *P. caspica* as a result of cross-pollination (Fig. 2) while this result is contrary to previous study by Xin et al. (2020). By chromosome painting probes they demonstrated that no chromosomal rearrangements on any of the 19 chromosomes among some species of *Populus* including *P. euphratica* and *P. deltoids* have occurred. On the other hand, the potential for intact or largely partial chromosome transfer between poplar hybrids has been proposed by Liu et al. (2021) using labeled telomeres, rDNA, and repetitive sequences as probes, that supports the findings in the present study.

The existing variation within the species based on chromosomal parameters was remarkable. As it was mentioned earlier, sexual and clonal reproduction of *P. caspica* is limited and the species is endangered in the area of its habitation in the country. This impor-

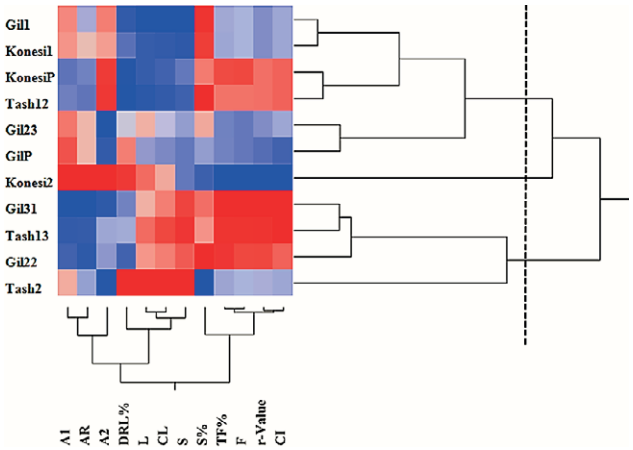

Figure 2. Diversity between the populations of *Populus caspica* based on chromosome characteristics and karyotypic indices (Ward method).

tant point should be regarded as a major restriction of the species. Along with natural hybridization, artificial inter-specific hybridization between the *Populus* species was suggested by other researchers (Mirzaie-Nodoushan et al. 2015) for inducing genetic variation and broadening the genetic basis of poplar germplasm. This suggestion can be employed on *P. caspica* and its relative species, with the same chromosome number, to broaden its genetic basis, as well as broadening the ecological zone of the species which is restricted to the northern part of Iran and Caucasus.

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