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BIOSYSTEMATIC STUDY IN SOME *TAMARIX* SPECIES IN IRAN

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Sheidai M., R. Mirshekari, F. Koohdar, H. Ijbari,' S. Ghasemzadeh-Barakai (2019): *Biosystematic study in some Tamarix species in Iran*.- Genetika, Vol 51, No.3, 845-860.
The genus *Tamarix* is a medium-sized genus of the Old World with about 54 species. *Tamarix* species can play an important role in preventing deforestation in Iran. We have no detailed information on *Tamarix* species, their inter-specific hybrids and the number of intra-specific forms growing in these regions. Therefore, the present work considers morphological and molecular study of the *Tamarix* taxa growing in three provinces of 1. Chaemahal-o-Bakhteyari, 2. Kerman, and 3. Southern Khorasan for the first time to answer the above mentioned questions and also aimed to produce data on population genetics and population divergence in *T. mascatenses* that has wider distribution in the studied areas. We identified six species in these provinces based on both morphological and ITS sequences. These species were also delimited by UPGMA tree of morphological characters. Phylogenetic analyses produced three major clades with high bootstrap values (>80.00). HGT tree revealed the occurrence of gene flow between *T. meyeri* and *T. tetragyna*, and between *T. meyeri* and *T. stricta*. Population genetic study of eight populations in *T. mascatensis* by SRAP and morphological analyses separated almost all populations in distinct clusters, revealing their genetic and morphological divergence. STRUCTURE plot revealed population genetic fragmentation in *T. mascatensis*.
Keyword: ITS, Molecular markers, Population genetic, SRAP, *Tamarix*

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INTRODUCTION

Tamarix species can play an important role in preventing deforestation in Iran. They are widely grown in arid and semi-arid regions of the country. Therefore, it is of eminent importance to identify these species, their inter-specific hybrids and infra-specific forms formed in our country. The tree provinces chosen for study are among very important areas of *Tamarix* growing regions in Iran that are mainly located in the south-east to eastern parts of Iran.

Tamarix is a taxonomically very complex genus of 54 species, all native in temperate Eurasia and the Mediterranean area (SHEIDAI *et al.*, 2018; HEYWOOD *et al.*, 2007). *Tamarix* is frequently introduced outside its native distribution range: as an ornamental in gardens or public plantations or, more often, as a windbreak or for erosion control. They are easily grown and tolerant of poor soils (IJBARI *et al.*, 2014).

The genus is thought to have been named after the Tamaris River in Spain. *Tamarix* plants are shrubs, semi-shrubs and tall trees that can grow up to 18 m in height. They are adaptable halophytic or xerophytic plants mostly with multiple stems and slender branches (ARIANMANESH *et al.*, 2015).

Leaves of *Tamarix* are taxonomically useful as their shape and attachment modes vary according to species, e.g. sessile vs. vaginate (SHEIDAI *et al.*, 2018). They are scale-like, about 3 mm in length (SHEIDAI *et al.*, 2018) and usually contain salt glands (BREDENKAMP and PHEPHO, 2008). This explains Tamarix's common name 'Saltcedar'. The fact that *Tamarix* exudes salts enables it to grow in and tolerate soils with high salt concentrations (ranging from 650 to 36000 ppm) (ARIANMANESH *et al.*,2015).

Tamarix taxa have various uses, for example, *Tamarix usneoides* is mostly used for phytoremediation on the mines in southern Africa. The trees remove pollutants from the environment (MAYONDE *et al.*, 2015). *T. gallica*, *T. africana*, *T. ramosissima* are frequently used as ornamental plants for their feathery appearance and their catkin-like inflorescences (SHEIDAI *et al.*,2019). Their aesthetic value is so appealing that these plants have been brought into many countries and planted in the gardens; as a result, their introduction and subsequent spread has rendered some of the alien species (especially the pink flowering species) invasive in certain places (SHEIDAI *et al.*,2019). These species are also used for deforestation purpose in many countries like Iran (IJBARI *et al.*, 2014).

Inter-specific hybridization is known to occur frequently in the genus *Tamarix* (IJBARI *et al.*, 2014). Hybridization event followed by introgression is among the reasons that cause systematic of the genus difficult. For example, many workers consider *T. pentandra*, *T. tetranda*, *T. gallica*, *T. chinensis*, *T. ramosissima*, and *T. parvifolia* to be one variable species or hybridizing group best designated by the single name *T. pentandra* (SHEIDAI *et al.*, 2019). Similarly, *T. chinensis* and *T. ramosissima* are morphologically and genetically distinct in Asia, the North American population is dominated by their hybrids (GASKIN and SCHAAL, 2002). FRIEDMAN *et al.* (2008) referred to the complex of *T. ramosissima*, *T. chinensis*, and their hybrids as salt cedar.

GASKIN and SHAFROTH, (2005) reported relatively rare and localized hybrids between *T. aphylla* (L.) Karst. and both *Tamarix ramosissima* and *T. chinensis*. *Tamarix* flowers are mainly bisexual and are cross-pollinated by wind and insect visitation (GASKIN and SHAFROTH, 2005). This brings about high genetic diversity and leads to the interspecific hybrid formation in *Tamarix*. The high intraspecific genetic variation in the studied *Tamarix* species even within the

limited area of investigation (60 km^2) may be used for local adaptation and also prevents homozygosity and genetic extinction of the studied *Tamarix* taxa (IJBARI *et al.,* 2014).

Morphological identification is sometimes unable to distinguish the inter- and intra-specific variations, whereas molecular systematic can reliably, rapidly and accurately reveal variants and cryptic species (LE ROUX and WIECZOREK, 2008).

Taxonomic confusion among plants occurs because of high hybridization rates between and within species, introgression among closely related species and regular instances of phenotypic plasticity (LE ROUX and WIECZOREK, 2008). Thus, the importance of molecular techniques when dealing with alien invasive species as taxonomic identification is the first step towards making effective management decisions.

In Iran, 35 *Tamarix* species have been reported to occur (SHEIDAI *et al.,* 2019). However, there have been very limited studies on taxonomy and genetic structure of *Tamarix* species in the country (ARIANMANESH *et al.*, 2015; IJBARI *et al.*, 2014) and almost no exclusive report exists on gene flow and introgression among the species that occur together in a single area. Therefore, the present study was performed to identify *Tamarix* species growing in 3 provinces of Iran with the aim to provide morphological and molecular evidence for their correct identification and also to identify potential hybrids, their putative parental species and to report the occurrence of bellow specific rank taxa.

For molecular confirmation of the studied species and for revealing inter-specific reticulation, we used ITS (Internal transcribed sequences) of nrDNA. Internal transcribed spacer (ITS) regions are located between the small subunit (16S–18S) and the 5.8S rRNA coding regions (ITS1), and between the 5.8S and large subunit (23S–28S) rRNA coding regions (ITS2) of the nuclear ribosomal DNA (nrDNA). The total length of the ITS1, ITS2 and the 5.8S regions of the nrDNA (rDNA) are about 900 base pairs (bp) including the flanking ubunits. The spacer regions are evolving rapidly and show intraspecific variation, whereas the 5.8S regions are usually conserved (TIPPERY and LES, 1995).

ITS sequences have been widely used to construct phylogenies of angiosperms at lower taxonomic levels (TIPPERY and LES, 1995). Polymorphisms in some individuals can occur because concerted evolution is not fast enough to homogenize repeats of mutations among the multiple copies in the genome, and/or because of recent hybridization events (WAN *et al.*, 2014).

Moreover, since *Tamarix* species form many geographical populations, a population genetic study of one of the studied species as done to produce data on their genetic structure and possible genetic fragmentation within a single species. For population genetic study we used SRAP (Sequence related amplified polymorphism) molecular markers which are used to amplify coding regions of DNA with primers targeting open reading frames. They are very useful markers in population genetics as well as molecular phylogeny studies, as they are codominant markers and produce a large number of reproducible polymorphic bands (ABEDIAN *et al.*, 2012; MOKHTARI *et al.*, 2016).

MATERIALS AND METHODS

Plant material

For species study, plant specimens were randomly collected from three provinces of 1. Sistan and Baluchestan, 2. Kerman and, 3. Southern Khorasan. Details of localities are provided in Figures 1.

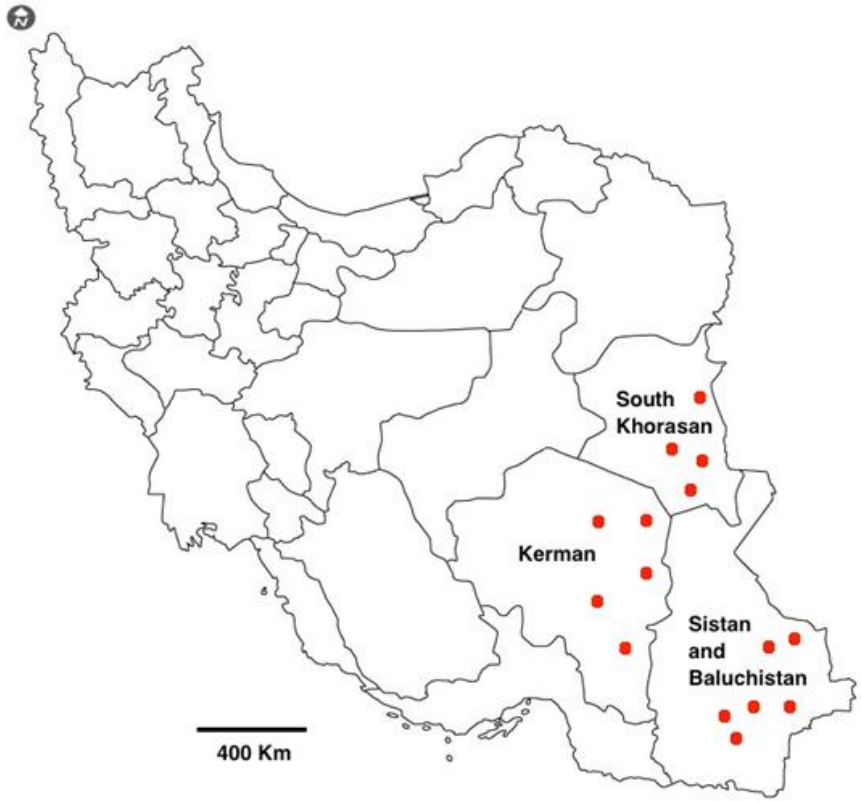

Figure1. Geographical distribution of the studied species

For population diversity within *T. Mascatansis*, we collected 24 plant specimens in 8 geographical populations (10 tree was collected in each region, but 3 to 5 specimens were *T. Mascatansis*). These plants were used for morphological and molecular investigations. Details of geographical populations are provided in Table 1.

Table1. Populations studied their locality in T. Mascatansis

	Population		Locality
T. mascatansis	1	Sistan and Baluchestan	Zabol, Nimrooz
	2	South Khorasan	Bandan
	3	South Khorasan	Pichbandan
	4	Sistan and Baluchestan	Iranshahr
	5	Sistan and Baluchestan	Nikshahr, Tange
	6	South Khorasan	Nahbandan
	7	Sistan and Baluchestan	Nikshahr, Fanooj
	8	Sistan and Baluchestan	Nikshahr,Mohtaram- abad

Morphological studies

Morphological characters used are according to IJBARI *et al*. (2014). Data were standardized (Mean = 0, Variance = 1) and used to estimate Euclidean distance, followed by clustering methods. Principal coordinate analyses (PCA) were performed to identify the most variable morphological characters (PODANI, 2000). Grouping of the *Tamarix* species and population in *T. Mascatansis* was done by UPGMA (Unweighted paired group using average method) clustering. These analyses were done by PAST ver. 2.17 (HAMMER *et al*., 2012).

Molecular studies

DNA extraction and PCR reaction

Fresh leaves were used randomly from 5-10 plants in each of the studied populations. Leaves were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (KRIZMAN *et al.*, 2006). The quality of extracted DNA was examined by running on 0.8% agarose gel. PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 1 U of *Taq* DNA polymerase (Bioron, Germany).

ITS sequencing

ITS1, 5.8S and ITS2 region DNA was amplified with 0.2 µM primer ITS4 (5′-TCCGTAGGTGAACCTGCGG-3 and ITS5 (ITS5) 5′- GGA AGT AAA AGTCGT AAC AAG G- 3′ (CHEN *et al.*, 2010). The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5Min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 52-57°C and 2 min at 72°C. The reaction was completed by the final extension step of 7-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany). To be sure about ITS sequences, we performed BLAST and found perfect match.

ITS sequences obtained was aligned with MUSCLE implemented in MEGA 5. The molecular clock test was performed as implemented in MEGA 5 (TAMURA *et al.*, 2011). The test was done by comparing the ML value for the given topology with and without the molecular clock constraints under the TAMURA and NEI, (1993) model. ML (Maximum Likelihood) tree was obtained from ITS data. Hundred times bootstrapping was used for final trees. HGT tree obtained by T-Rex after 999 permutations.

SRAP assay

Five sequences related amplified polymorphism (SRAP) primer pairs including forward primers: Me1, Me2, Me3, Me4, Me5 and reverse primers: Em1, Em2, Em3, Em4, Em5 were used (FENG et al. 2014). The amplification, reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step at 94°C, followed by five cycles of 94°C for 1min, 35°C for 45 sec, and 72°C for 1 min; followed by 35 cycles of 94°C for 1min, 55°C for 45 sec, and 72 for 1 min; followed by 7 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

SRAP bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Nei's genetic distance among populations was used for

Neighbor-Joining (NJ) clustering. These analyses were done by PAST ver. 2.17 (HAMMER *et al.*, 2012). K-Means clustering, AMOVA (Analysis of Molecular Variance), and STRUCTURE analysis were used to determine population genetic differentiation and fragmentation (SHEIDAI *et al.*, 2016).

RESULTS

Species identification based on morphological characters and ITS marker

Tamarix species were identified morphologically based on descriptions provided in Flora Iranica and BAUM, (1967). Our preliminary identification based on selected morphological characters resulted in six distinct species (*T. aucheriana*, *T. korolkowii*, *T. mascatensis*, *T. stricta*. *T. teragyna* and *T. meyeri*) .

One sample of any species ITS sequences were obtained and compared with available sequences in *Tamarix* species. All identified species had at least 95% homology with the reported ITS sequence for the same taxa in NCBI (National Center for Biotechnology Information) (Table2).

Table2. Tamarix species identified and their ITS sequence homology to the reported species

Species	Homology %	Accession No.
T. mascatensis	98	KT809493.1
T. korolkowii	95	KT809491.1
T. tetragyna	98	KT809497.1
T. meyeri	96	KJ729661.1
T. aucheriana	98	AF484762.1

Description of species and relationship between species based on morphological studies

Different clustering methods based on morphological characters studied (quantitative and qualitative characters) produced similar results. Therefore, only UPGMA dendrogram is presented (Figures 2). Plants of each species were almost grouped together and formed a distinct cluster. The species studied were separated from each other due to their morphological differences and were delimited morphologically.

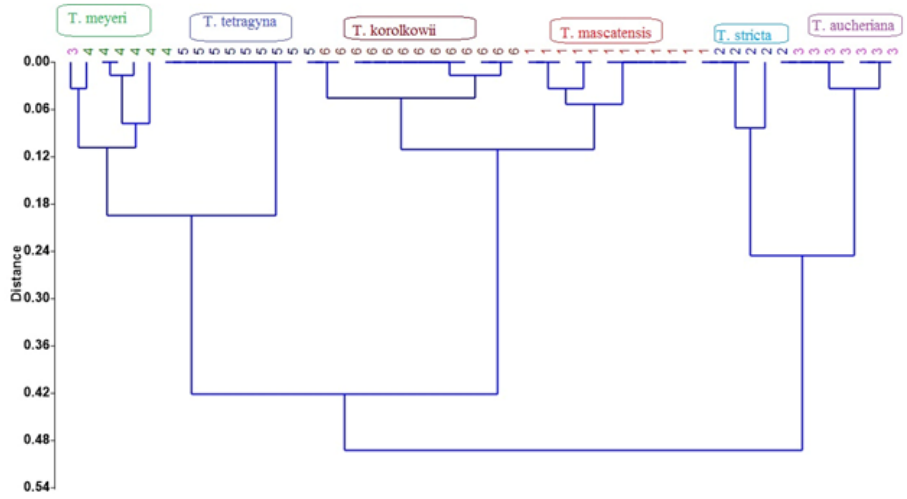

Figure 2. UPGMA dendrogram of *Tamarix* species based on morphological characters

Comparisons of the leaf and flower disk in the studied species are provided in Figs. 3 and 4. The leaves were amplexicaul in *T. aucheriana*, *T. korolkowii* and *T. mascatensis*, while the leaves were covering the stem in *T. stricta*. T. teragyna and T. meyeri had the leaves with narrowing at the base (Figures 3). In *T. tetragyna* and *T. meyeri*, the flower disk contained four stamens and these stamens were attached on the lobes, while *T. aucheriana* and *T. stricta* had five or more stamens that were attached either on the lobes or in between the lobes. *T. korolkowii* and *T. mascatensis* contained five stamens attached to the lobes (Figures 4).

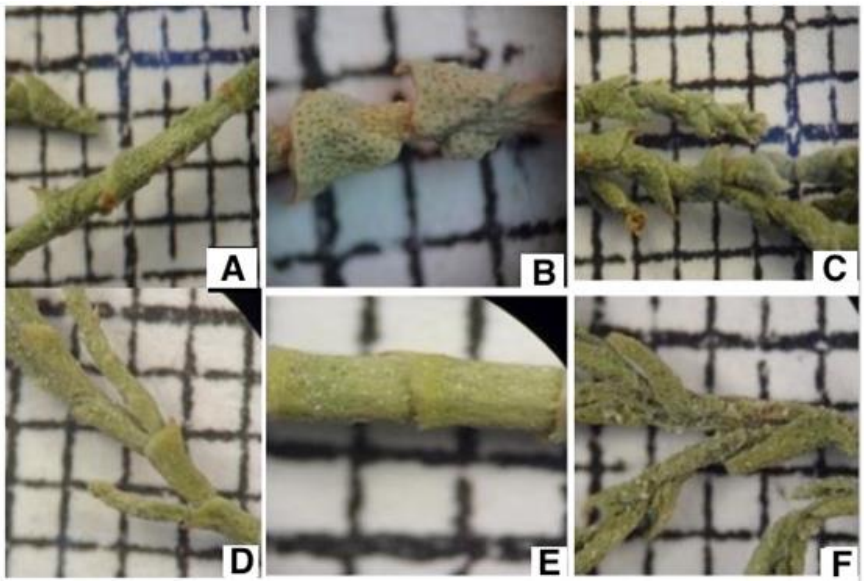

Figure 3. Leaves in *Tamarix* species studied. A-F = *T. mascatensis*, *T. aucheriana*, *T. meyeri*, *T. korolkowii*, *T. tetragena* and *T. stricta*, respectively

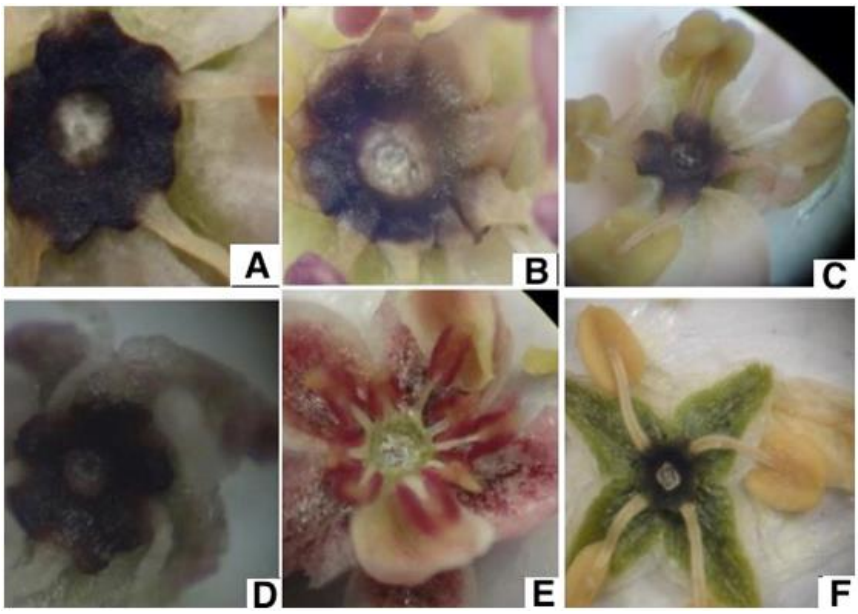

Figure 4. Flower disk in *Tamarix* species studied. A-F = *T. mascatensis*, *T. aucheriana*, *T. meyeri*, *T. korolkowii*, *T. stricta* and *T. tetragena*, respectively

PCA analysis of morphological data revealed that the first three PCA components comprised about 83% of the total variance. In the first component with about 47% of total variability, morphological characters like the leaf shape, flower density, ratio of pedicel length to the calyx, and the number of stamens are the most variable characters with r >0.80. The number of sepals and petals are important morphological characters of the second PCA component with about 22% of the total variability.

ITS sequences based phylogeny

Different phylogenetic analyses of ITS data of the studied species by UPGMA, NJ, and maximum likelihood (ML) method produced similar results therefore only ML tree is presented (Figures 5). Three major clades were observed with high bootstrap values (>80.00). *T. korolkwii*, *T. tetragyna* and *T. mascatensis* formed the first major clade with the last two species showing closer genetic affinity. *T. aucheriana* joined this clade with some distance, followed by *T. meyeri*. Two samples of *T. stricta* that had morphological difference also differed to some degree in ITS sequences and were placed with some distance.

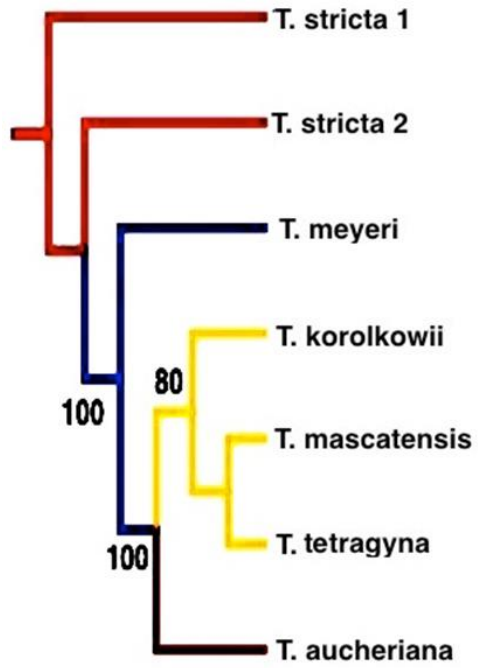

Figure 5. ML tree of *Tamarix* species based on ITS data.

Molecular phylogeny of the studied species and their relationship was not in agreement with the species tree of morphological characters and with the taxonomic treatment of the genus, for example *T. tetragyna* and *T. mascatensis* were grouped together in ITS study, while in the morphological study was placed far from each other.

Therefore, the potential gene flow among the studied species was investigated by HGT (Horizontal Gene Transfer) analysis with the help of T-REX program. The HGT tree (Figures 6) is based on both morphological and ITS tree of the studied species revealed the occurrence of gene flow between *T. meyeri* and *T. tetragyna*, and between *T. meyeri* and *T. stricta*. This indicates that plants observed with a mixture of morphological characters are probably the results of such inter-specific hybridization.

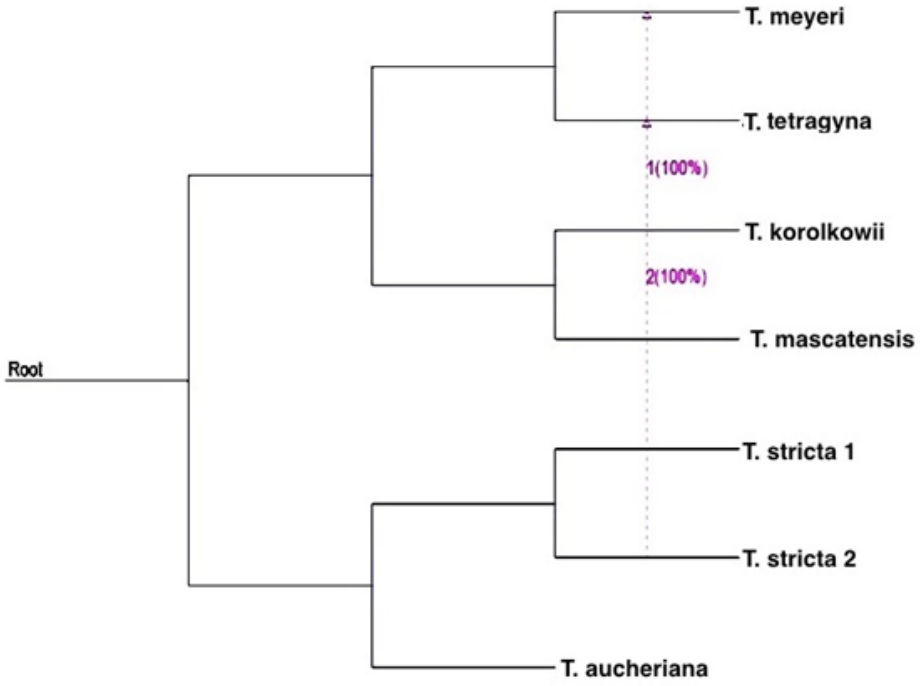

Figure 6. HGT tree showing gene flow among the studied *Tamarix* species.

Infra-specific forms in T. stricta

We encountered plants with morphological variability within *T. stricta*. These plants could be separated in two forms: 1. Plants with flowers having six stamens, the filament length <0.2 mm and anther elongated at tip. 2. Flowers with eight or more stamens, filament >0.5, and anther not pointed at tip. As stated before these plants differed in ITS sequences and were joined each other with some distance and separated from the other studied species. Therefore, we consider them as two new varieties for *T. stricta* namely, 1. *T. stricta* var. *stricta*, and 2. *T. stricta* var. *persica*, respectively (Fig.6).

Population genetic analysis in T. mascatensis

Plants randomly selected from eight populations of *T. mascatensis* were used for SRAP and morphological analyses. NJ tree of the studied populations based on SRAP molecular markers (Figures 7) separated almost all populations in distinct clusters, revealing their genetic difference.

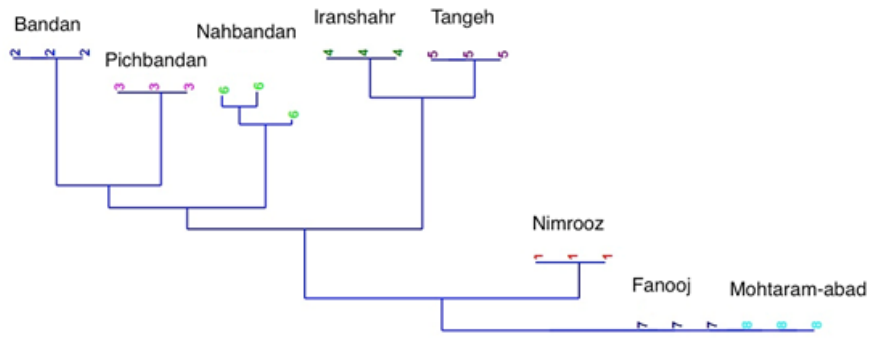

Figure 7. NJ tree of populations based on SRAP data.

This was supported by K-Means clustering (MEROMANS, 2012) that produced the best clustering according to pseudo-F and Bayesian Information Criterion (BIC) as k = 8. This is in agreement with NJ tree result.

Analysis of Molecular Variance (AMOVA) on best clustering according to BIC also revealed that about 1% of total variance was due to within clusters difference, while 98% was due to among clusters (genetic groups) difference.

STRUCTURE plot followed by Evanno test produced Delta K =3. STRUCTURE plot based on k = 3 (Figures 8), showed genetic affinity among populations 1, 7 and 8 (similarly colored). The same holds true for populations 2, 3 and 6, as well as populations 4 and 5. Therefore, the studied populations in *T. mascatensis*, show genetic fragmentation.

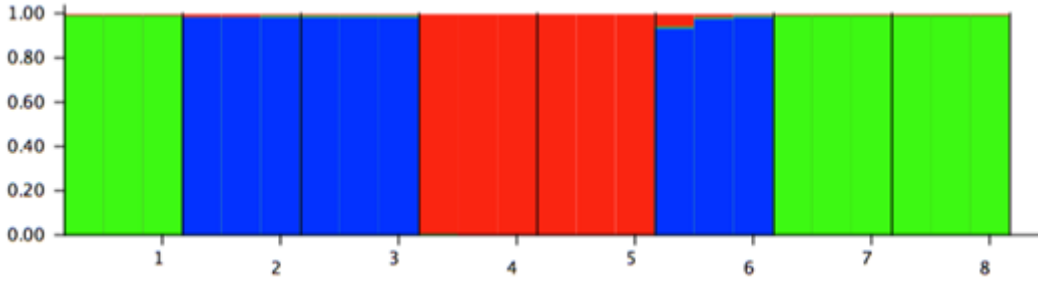

Figure 8. STRUCTURE plot of the studied populations in *T. mascatensis* based on SRAP data

Morphometric analysis of the same populations revealed morphological difference among populations as they formed distinct clusters in UPGMA dendrogram of morphological data (Fig.9). This indicates that, the studied populations are genetically and morphologically differentiated in *T. mascatensis*.

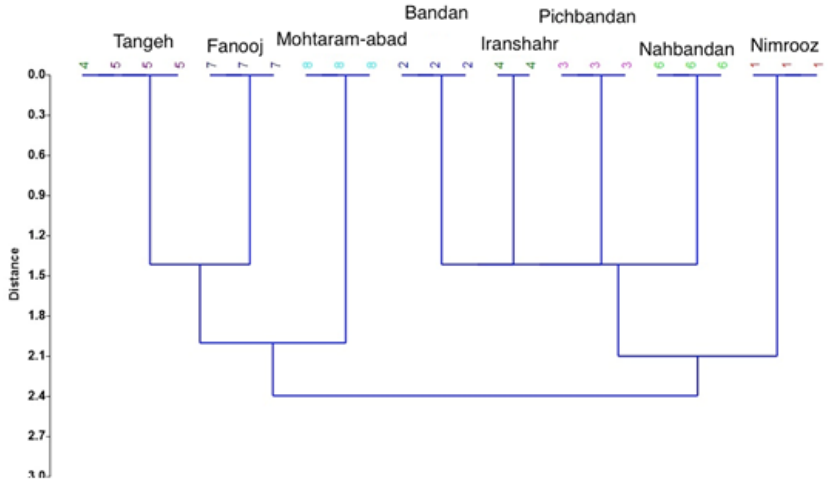

Figure 9. UPGMA dendrogram of morphological data

DISCUSSION

Identification of *Tamarix* species and their hybrids throughout the country is important for conservation and preventing deforestation in Iran. They form many geographical populations and due to their ability to adapt to different and adverse environmental conditions form may inter-specific hybrids, varieties and ecotypes.

We surveyed three provinces and identified six *Tamarix* species growing in these areas. *Tamarix* species are invasive in nature and for that they need to have a high degree of genetic variability and also potential to produce inter-specific hybrids. Hybridization as a driving force of invasion biology, when new species are introduced into a new region, they may meet closely related species or genotypes and form hybrid. These hybrid individuals have high genotypic fitness in the newly-invaded habitat (GASKIN and KAZMER, 2009).

Hybridization followed by introgression (natural back-crossing between hybrids and parental lineages) can provide necessary genetic variability for *Tamarix* species to cope with environmental condition they face (SCHIERENBECK and ELLSTRAND, 2009). These hybrids may survive in extreme habitats that are not suitable for either of the parent taxa, as was reported in *Helianthus* (RIESEBERG BERG *et al.*, 2003).

Our study also revealed the occurrence of inter-specific hybridization between *T. meyeri, T. stricta and T. tetragyna*. It has possibly resulted in formation of two different varieties in *T. stricta*. The separation of a single lineage into two separate lineages is the initial stage of genetic divergence, which in some cases it is a prelude to speciation (SCHAAL *et al.*, 2003).

Population genetic studies provide data on genetic fragmentation versus gene flow among geographical populations in a single species (SHEIDAI *et al.*, 2014) as well as ecotype formation (SHEIDAI *et al.*, 2013). The information obtained reveals how the species become diverged throughout their geographical range and illustrates the evolutionary mechanism involved in population adaptation to their local habitat.

The present population genetic investigation that was performed oneight geographical populations of *T. mascatensis*, revealed that geographical populations present in three provinces

studied become diverged both in morphological and genetic characteristics. Changes in these characteristics may play role in local adaptation and are potentially useful to the plants growing in those particular environments (SHEIDAI *et al.,* 2016).

The plant species that are distributed in various geographical regions face different ecological conditions and variable altitudes and therefore are subjected to different selection pressures and sometimes suffer from population fragmentation. In these situations, plants reveal morphological and genetic divergence among geographical populations (AZIZI *et al.,* 2014; SHEIDAI *et al.,* 2013).

Population genetics analyses produce important data on the levels of genetic variation, the partitioning of genetic variability within/between populations, gene flow, inbreeding, self-pollination versus outcrossing, effective population size and population bottleneck. These data can help in developing effective management strategies for endangered and/or invasive species (CHEN, 2000; ELLIS and BURKE, 2007). AMOVA revealed a high degree of genetic variation due to among populations in *T. mascatensis*, while STRUCTURE and morphometric analysis showed that these populations are fragmented. Moreover, IBD and limited gene flow were observed in the studied populations.

CONCLUSION

In conclusion, for *Tamarix* systematic work, we suggest starting *Tamarix* species identification by considering first, 4-merous flowers versus 5-merous flowers. Furthermore, studying characters like the shape of disks and leaves are very useful for *Tamarix* identification. However, field experience reveals high morphological variability in all of these taxonomically important characters and also the occurrence of plants with intermediate morphological characters and with mixture of different species characters, due to extensive inter-specific hybridization and introgression. Therefore, it is better to accompany morphological identification with molecular data support. The molecular method of plant species identification offers a more reliable and consistent method of identifying *Tamarix* species.

Molecular tools in systematic provide the means to investigate the identity of different plant species at the DNA level, showing genetic variation within and among populations, and can also detect hybridization and introgression patterns between closely related species.

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BIOSISTEMATSKO PROUČAVANJE NEKIH *TAMARIX* VRSTA U IRANU

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Izvod

Rod Tamarix je rod srednje veličine iz Starog sveta sa oko 54 vrste. *Tamarix* vrste mogu igrati važnu ulogu u sprečavanju krčenja šuma u Iranu. Nemamo detaljne informacije o vrstama *Tamarix*, njihovim interspecifičnim hibridima i broju intra-specifičnih oblika koji rastu u ovim regionima. Stoga, ovaj rad razmatra morfološko i molekularno istraživanje *Tamarix* taksona koje rastu u tri provincije 1. Chaemahal-o-Bakhteiari, 2. Kerman i 3. Južni Khorasan, da bi se prvi put dobili odgovori na gore pomenuta pitanja, I da bi se dobili podaci o populacionoj genetici i divergenciji populacija kod *T. mascatenses* koja ima širu distribuciju u proučavanim oblastima. Identifikovali smo šest vrsta u ovim provincijama na osnovu morfoloških i ITS sekvenci. Ove vrste su takođe bile ograničene UPGMA klasterom morfoloških karaktera. Filogenetske analize proizvele su tri glavne grane sa visokim vrednostima (> 80,00). HGT stablo otkrilo je pojavu protoka gena između *T. meieri* i *T. tetragina*, kao i između *T. meieri* i *T. stricta*. Populacijsko genetičko istraživanje osam populacija u *T. mascatensis* pomoću SRAP-a i morfoloških analiza razdvojilo je gotovo sve populacije u različite klastere, otkrivajući njihovu genetsku i morfološku divergenciju. STRUCTURE je otkrio genetsku fragmentaciju populacije kod *T. mascatensis*.

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