




Species delimitation and inter-specific gene flow in *Tamarix* L. (Tamaricaceae)

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Abstract

Tamarix L. play important role in preventing deforestation in Iran. *Tamarix* species exhibit wide range of morphological variation therefore, the species delimitation become difficult. This is further complicated due to similarity of morphological characters in closely related species and the occurrence of inter-specific hybridization. The present study was performed to identify *Tamarix* species and their potential hybrids in Semnan Province of Iran. We used ITS and ISSR and 42 morphological characters for our investigation. Molecular phylogeny of the studied species and their relationship was not in agreement with the species tree of morphological characters and with taxonomic treatment of the genus. HGT tree of ITS and morphological data obtained revealed the occurrence of inter-specific hybridization or introgression between *Tamarix* species.

Izvleček

Vrste rodu *Tamarix* so v Iranu pomembne za preprečevanje krčenja gozdov. Zanje je značilna široka morfološka variabilnost, s katero so sposobne preživeti v različnih ekoloških razmerah, zato je razmejitev vrst težavna. Dodatne težave predstavljajo podobni morfološki znaki pri ozko sorodnih vrstah in prisotnost medvrstnega križanja. V članku želimo določiti vrste rodu *Tamarix* in njihove potencialne križance iz province Semnan v Iranu. V raziskavi smo uporabili ITS in ISSR molekulske markerje in 42 morfoloških znakov. Molekularna filogenija obravnavanih vrst in njihova razmerja niso bili v skladu z dendrogramom morfoloških znakov in s taksonomsko členitvijo rodu. Z HGT dendrogramom podatkov iz ITS in morfološko analizo smo pokazali obstoj medvrstnega križanja oziroma introgresije med vrstami rodu *Tamarix*.

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1. Introduction

The genus *Tamarix* L. contains about 54 species that mainly grow in saline areas of deserts and semi-deserts in Asia, Europe, North-east and South-west of Africa. *Tamarix* species play important role in preventing deforestation in Iran (Sheidai et al. 2018). *Tamarix* species have limited benefits to human but have been used as ornamental plant in gardens or public plantations. For example, *T. gallica* L., *T. chinensis* Lour., *T. ramosissima* Ledeb. are frequently used as ornamental plants for their feathery appearance and their catkin-like inflorescences (Gaskin 2003). *Tamarix* species are good for windbreak (for example *T. aphylla* (L.) Karst. and *T. Africana* Poir.) or for erosion control and easily grow in poor soils (Baum 1967, Gaskin and Schaal 2002, Ijbari et al. 2014).

Tamarix species hybridize and may form different taxonomic forms due to inter-specific hybridization and introgression (Gaskin & Schaal 2003, Gaskin & Kazmer 2019, Mayonde et al. 2019). Due to different degree of gene flow among *Tamarix* species, plants with variable morphological characters occur in the same area. Therefore, high gene flow in *Tamarix* species caused by inter-specific hybridization resulting in phenotypic variations have rendered taxonomic classification of the genus problematic (Baum 1978, Ijbari et al. 2014). *T. tetrandra* Pall. ex M.Bieb., *T. gallica* L., *T. chinensis*, *T. ramosissima*, and *T. parviflora* DC. are considered to be one variable species or hybridizing group, designated by the hybrid name *T. pentandra* Pall. (Sudbrock 1993). *T. chinensis* and *T. ramosissima* are morphologically alike and differ only in some microscopic characters and genetically distinct in Asia (Mayonde et al. 2016). However,



Figure 1: Distribution map of *Tamarix* species in Iran. Slika 1: Karta razširjenosti vrst rodu *Tamarix* v Iranu.

they have been proven to be genetically different and are known to hybridize in North America and South Africa (Gaskin & Schaal 2002, Gaskin & Kazmer 2009, Mayonde et al. 2016).

Morphological characteristics are important in *Tamarix* species delimitation. *Tamarix* leaves are taxonomically useful and they show variation in shape and attachment modes in different species (Baum 1978). *Tamarix* flowers are bisexual, rarely unisexual and plants are either monoecious or dioecious. The flowers either have five or four sepals with corresponding number of petals. Flowers have five or numerous stamens that are free or fused and are inserted into a fleshy, glandular, hypogynous disc (Obermeyer 1976, Baum 1978). The presence of bisexual flowers and cross-pollination in *Tamarix* lead to the occurrence of high genetic diversity and hybrid formation in these species (Gaskin & Schaal 2002, Gaskin & Kazmer 2009, Gaskin et al. 2012, Mayonde et al. 2015, 2016). The intraspecific genetic variability may be used for local adaptation and also prevents homozygosity and genetic extinction of the studied *Tamarix* taxa (Ijbari et al. 2014).

Thirty-five *Tamarix* species occur in Iran as reported by Schiman-Czeika (1980). These species have been used in plantation to prevent deforestation in Iran. However, our general survey and extensive collections in different provinces may suggest the occurrence of more number of species/ sub-species in the country. The correct identity of our *Tamarix* collections can be verified at population level through detailed taxonomic investigations using both morphological and molecular approaches (Arianmanesh et al. 2014, Ijbari et al. 2014).

Tamarix species occur in 21 provinces of Iran (Figure 1). Ecological and climatic differences may influence the morphological appearance of *Tamarix* but will not affect the identity of the species (Sheidai et al. 2018). Despite the phenotypic differences in our plant collection and the previous studies observed in different localities, we hypothesized that the identities are most likely to be the same. Due to co-occurrence of two or three *Tamarix* species in overlapping areas, it is very important to identify and delimit these species. Moreover, due to frequent gene exchange between species in the same area there is high probability of hybrid formation. Therefore, it is necessary to highlight gene flow among the species growing within each locality (Ijbari et al. 2014, Sheidai et al. 2018).

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 introgression patterns between closely related species (Le Roux & Wiczorek

2008). ITS sequences are useful to construct phylogenies of angiosperms at lower taxonomic levels (Baldwin et al. 1995) and reveal polymorphisms (double base readings) within plant individuals (Campbell et al. 1997). 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 (Campbell et al. 1997). ISSR molecular markers were shown to be informative for genetic diversity and population structure studies (see for example, Sheidai et al. 2012, 2013, Azizi et al. 2014).

Our study was conducted in the Semnan Province of Iran because species of this area have not been identified. After morphological identification of *Tamarix* species, their identification was also checked by BLAST using ITS (Internal transcribed sequences) of the nuclear DNA (nrDNA). Furthermore, the morphological and ITS analyses of identified species were carried to reveal the species delimitation and relationship (Sheidai et al. 2013, Minaeifar et al. 2016) and we carried out introgression patterns in populations of in *T. szowitsiana* Bge. and *T. androssowii* Litv. species by using Inter-simple sequence repeats (ISSR) and in *T. Androssowii*, *T. Meyerii* Boiss. and *T. Szowitsiana* by ITS molecular markers.

2. Materials and methods

2.1 Morphological investigation

Eighty plants were randomly collected from 22 geographically areas in Semnan Province in Iran and used for morphological investigations. The voucher specimens were deposited in Herbarium of Shahid Beheshti University (HSBU) (Table 1).

Morphological characters (Table 2) used are according to Ijbari et al. (2014). Morphological data were standardized (Mean = 0, Variance = 1) and used to estimate Euclidean distance. Grouping of the species was done by UPGMA (Unweighted paired group using average method) clustering and principal coordinate analysis (PCoA) (Podani 2000). These analyses were done using PAST ver. 2.17 (Hammer et al. 2012).

2.3 Molecular investigations

For molecular analyses, we used both the multilocus genome-wide markers (i.e. ISSR) and the single locus (i.e. ITS 1, 5.8S, ITS2) regions. Both markers were used for species diversity analysis and phylogeny (Weising et al. 2005, Sheidai et al. 2014).

Table 1: Geographic areas studied and ecological features.

Tabela 1: Obravnavana geografska območja in njihove ekološke značilnosti.

| Number of locality | Province | Locality | Altitude (m) | Longitude | Latitude | Voucher number |
|--------------------|----------|------------------------------|--------------|-----------|----------|----------------|
| 1 | Semnan | Bagh village | 1109 | 36.12507 | 54.26741 | 1294 |
| 2 | Semnan | 10 km to Garmsar | 1033 | 35.19760 | 52.60075 | 1394 |
| 3 | Semnan | 5 km to Damghan | 1146 | 36.7152 | 54.15927 | 1494 |
| 4 | Semnan | Sorkheh | 1149 | 35.27208 | 53.10930 | 1594 |
| 5 | Semnan | 10 km to Semnan | 1165 | 35.30488 | 53.17239 | 1694 |
| 6 | Semnan | Bagh village | 1109 | 36.12507 | 54.26741 | 1794 |
| 7 | Semnan | 5 km to Damghan | 1146 | 36.7152 | 54.15927 | 1894 |
| 8 | Semnan | 50 km to Chesameh ali villag | 1395 | 36.15214 | 54.9535 | 1994 |
| 9 | Semnan | 20 km to Chesameh ali villag | 1400 | 36.15169 | 54.9289 | 2194 |
| 10 | Semnan | Chesameh ali villag | 1376 | 36.14671 | 54.10539 | 2294 |
| 11 | Semnan | 25 km to Garmsar | 1033 | 35.19760 | 52.6075 | 2394 |
| 12 | Semnan | 20 km to Garmsar | 995 | 35.18984 | 52.7327 | 2594 |
| 13 | Semnan | 10 km to Garmsar | 844 | 35.14412 | 52.17773 | 2694 |
| 14 | Semnan | 20 km to Garmsar | 995 | 35.18984 | 52.7327 | 2794 |
| 15 | Semnan | Bagh village | 1109 | 36.12507 | 54.26741 | 2894 |
| 16 | Semnan | 5 km to Damghan | 1146 | 36.7152 | 54.15927 | 2994 |
| 17 | Semnan | Amiriyeh villag | 1144 | 36.6482 | 54.14472 | 3294 |
| 18 | Semnan | Turan Protected Area | 1007 | 36.28135 | 55.42987 | 3394 |
| 19 | Semnan | Hadad village | 1124 | 36.16662 | 54.44510 | 3494 |
| 20 | Semnan | 20 km to Shahrood | 1114 | 36.12805 | 54.29208 | 3694 |
| 21 | Semnan | 10 km to Semnan | 909 | 35.14338 | 52.24440 | 3794 |
| 22 | Semnan | 10 km to Sorkheh | 903 | 35.14463 | 52.24709 | 3894 |

Table 2: Morphology characteristics in *Tamarix*.

Tabela 2: Morfološke značilnosti vrst rodu *Tamarix*.

| No Characters | |
|---------------------------------------|---------------------------------|
| 1 leaf length | 21 leaf shape |
| 2 length of inflorescence | 22 shape of leaf margin |
| 3 width of inflorescence | 23 leaf pile |
| 4 ratio of leaflet size/ pedicel size | 24 inflorescence |
| 5 ratio of leaflet size/ calyx size | 25 flower density |
| 6 length of leaflet | 26 leaflet attachment |
| 7 width of leaflet | 27 shape of leaflet |
| 8 ratio of pedicel size/ calyx size | 28 shape of leaf top |
| 9 calyx segments | 29 shape of internal calyx |
| 10 length of internal calyx | 30 shape of external calyx |
| 11 length of external calyx | 31 tip of internal calyx |
| 12 width of internal calyx | 32 tip of external calyx |
| 13 width of external calyx | 33 internal calyx naviculate |
| 14 corolla segments | 34 external calyx naviculate |
| 15 corolla length | 35 calyx pile corolla symmetry |
| 16 corolla width | 36 base of filament |
| 17 stamen number | 37 attachment of stamen to lobe |
| 18 anther length | 38 place of stamen extrusion |
| 19 anther width | 39 anther tip, anther |
| 20 disc diameter stem pile | |

2.3.1 DNA extraction

Fresh leaves were randomly collected from 5-10 *Tamarix* trees in each population. CTAB activated charcoal protocol was used to extract genomic DNA (Križman et al. 2006). The quality of extracted DNA was examined by running on 0.8% agarose gel.

2.3.3 ITS analysis

ITS region DNA was amplified with 0.2 μM primer ITS1 (5' TCCGTAGGTGAACCTGCGG-3', Bioron, Germany), and primer ITS4 (5'- TCC GCT TATTGA TAT GC -3') (Chen et al. 2010). PCR reactions were performed 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), 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany). The amplification reaction was performed in a Techne thermocycler (Germany) using the following parameters: 2 min initial denaturation step at 94 °C, followed by 35 cycles of 5 min at 94 °C; 1.30 min at 56 °C and 2 min at 72 °C. The reaction was completed by a final extension step of 7 min at 72 °C. PCR products were visualized on 2.5% agarose gels with GelRed™ Nucleic Acid Gel Staining.

Fragment sizes were estimated using a 100 bp size ladder (Thermo- Fisher Scientific, Waltham, MA USA).

ITS sequences obtained were aligned with MUSCLE (Robert 2004) 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. Different phylogenetic trees were obtained from ITS data like UPGMA (Unweighted paired group using average), Neighbor Joining (NJ) and Maximum likelihood (ML) methods. Hundred times bootstrapping was used for final trees.

2.3.2 ISSR analysis

7 ISSR (inter simple sequence repeat) primers UBC810, UBC849, (CA) 7AC, (GA) 9T, (GA) 9A and (AGC) 5GG were used according to Ijbari et al. (2014) and were purchased from University of British Columbia, Canada. The polymerase chain reaction (PCR) reactions were performed 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 each primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The polymerase chain reaction was performed in a Techne thermocycler (Germany) with the following parameters: 5 min initial denaturation step at 94 °C, followed by 40 cycles of 45s at 94 °C; 1 min at 55 °C and 1min at 72 °C. The reaction was completed with a 7 min extension step at 72 °C. The amplification products were visualized by running on 2% agarose gels. The fragment size was estimated using a 100 bp molecular size ladder (Fermentas, Germany). In order to identify reproducible bands, the experiment was replicated 3 times.

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). Grouping of the plant specimens was done by different clustering and ordination methods such as UPGMA (Unweighted paired group using average), and MDS (Multidimensional scaling) (Podani 2000). These analyses were done in PAST ver. 2.17 (Hammer et al. 2012).

3. Results

3.1 Species identification based on morphological characters and its marker

Our preliminary identification based on selected morphological characters resulted in nine distinct species (Table 3).

Table 3: Identified species based on morphological characters.

Table 3: Vrste, določene na osnovi morfoloških znakov.

| No. | Species identified with morphological characters | Localities in Table 1 |
|-----|---|-----------------------|
| 1 | <i>Tamarix arceuthoides</i> Bge. | 11 |
| 2 | <i>T. ramosissima</i> Ledeb. | 8, 9, 10 |
| 3 | <i>T. karkalensis</i> Lour. | 19 |
| 4 | <i>T. szowitsiana</i> Bge. | 12, 13 |
| 5 | <i>T. meyeri</i> Boiss., | 4,5,6, 7 |
| 6 | <i>T. androssowii</i> Litw. | 14, 15, 16, 17, 18 |
| 7 | <i>T. androssowii</i> var. <i>transcaucasica</i> (Bunge) Qaiser | 20 |
| 8 | <i>T. aucheriana</i> (Decne. ex Walp.) B. R. Baum. | 1, 2, 3 |
| 9 | <i>T. mascatensis</i> Bge | 21, 22 |

One sample of any species ITS sequences were obtained and compared with available sequences in *Tamarix* species. The results are provided in Table 4. All identified species had at least 95% homology with the reported ITS sequence for the same taxa in NCBI (National Center for Biotechnology Information).

Table 4: *Tamarix* species identified and their ITS sequence homology to the reported species.

Tabela 4: Določene vrste rodu *Tamarix* in istorodnost njihovih ITS sekvenc z obravnavanimi vrstami.

| Species | Homology % | Accession No. |
|--|------------|---------------|
| <i>T. aucheriana</i> | 100 | AF484762 |
| <i>T. arceuthoides</i> | 95 | AY452028 |
| <i>T. mascatensis</i> | 99 | KT809493 |
| <i>T. ramosissima</i> | 98 | KM657148 |
| <i>T. karkalensis</i> | 97 | KJ377278 |
| <i>T. meyeri</i> | 96 | KJ729661 |
| <i>T. androssowii</i> | 99 | KT377273 |
| <i>T. androssowii</i> var. <i>transcaucasica</i> | – | – |
| <i>T. szowitsiana</i> | – | – |

3.2 Relationship between species based on morphological studies

Different clustering methods (WARD, NJ and UPGMA dendrograms) based on 42 morphological characters in identical species produced similar results. Therefore, only UPGMA dendrogram is presented (Figure 2). Plants of

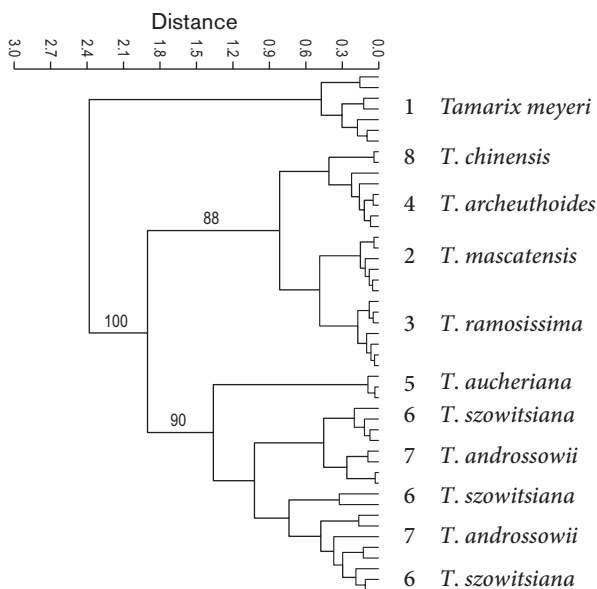


Figure 2: UPGMA dendrogram of *Tamarix* species based on morphological data.

Slika 2: Dendrogram UPGMA vrst rodu *Tamarix* na osnovi morfoloških podatkov.

each species were grouped together and formed a distinct cluster. Therefore, the studied species were delimited based on morphological characters.

In PCoA plot of the morphological characters (Figure 3), the species in the sect. *Tamarix* viz. *T. archeuthoides*, *T. mascatensis* and *T. ramosissima* and *T. karkalensis* were grouped in one cluster. Similarly, the species of the sect. *Oligadenia* viz. *T. meyerii*, *T. androssowii*, *T. szowitsiana* and were grouped together, while *T. auscheriana* of the

sect. *polyadenia* was placed far from the other species. In addition *T. aucheriana* was also placed far from the other study species within the sec. *Polyadenia*. This is due to stamen number, width of inflorescence and disc diameter.

Within the sec. *Oligadenia*, *T. androssowii* and *T. szowitsiana* show close affinity due to its ratio of pedicel size/ calyx size, inflorescence, length of inflorescence, length of external calyx and leaf length. While, *T. meyeri* is placed far from of them due to leaflet length, leaflet width, width of external and internal calyx.

3.2 Relationship between species based on its studies

UPGMA, NJ, and maximum likelihood (ML) methods in identical species produced similar results for ITS data. Therefore, only the NJ tree is presented (Figure 4). In general, the studied species from different sections were placed intermixed. Therefore, ITS data could not delimit the species according to the presumed sections in genus *Tamarix*. All the obtained clades had high bootstrap value (>80%). *Tamarix karkalensis* differed the most from the other species and formed a single clade. This was followed by *T. archeuthoides*. The samples identified as *T. androssowii* were placed close to each other.

3.3 Introgression evidenced

Molecular phylogeny of the studied species and their relationship was not in agreement with the species tree of morphological characters and with taxonomic classification of the genus.

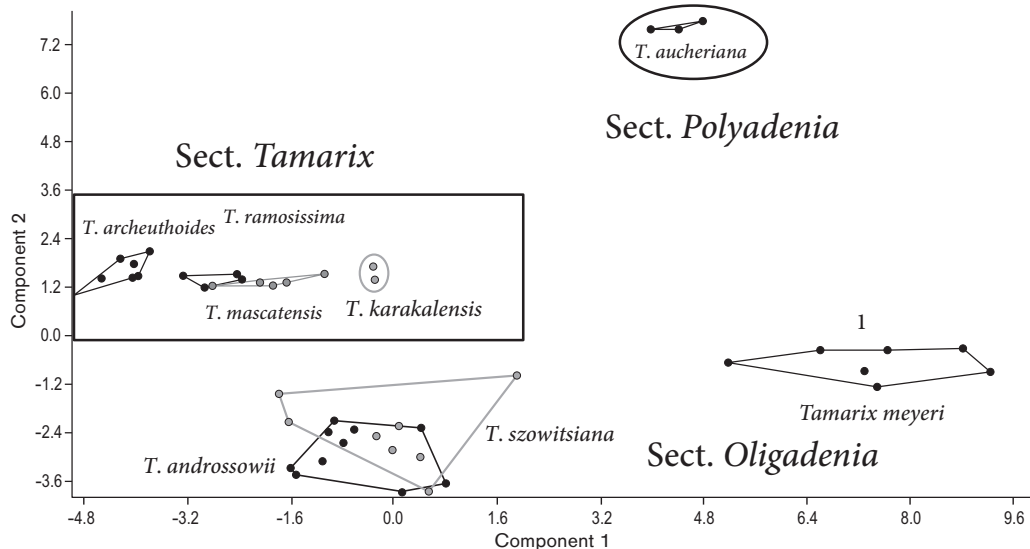


Figure 3: PCoA plot of *Tamarix* species based on morphological character.

Slika 3: Graf PCoA vrst rodu *Tamarix* na osnovi morfoloških podatkov.

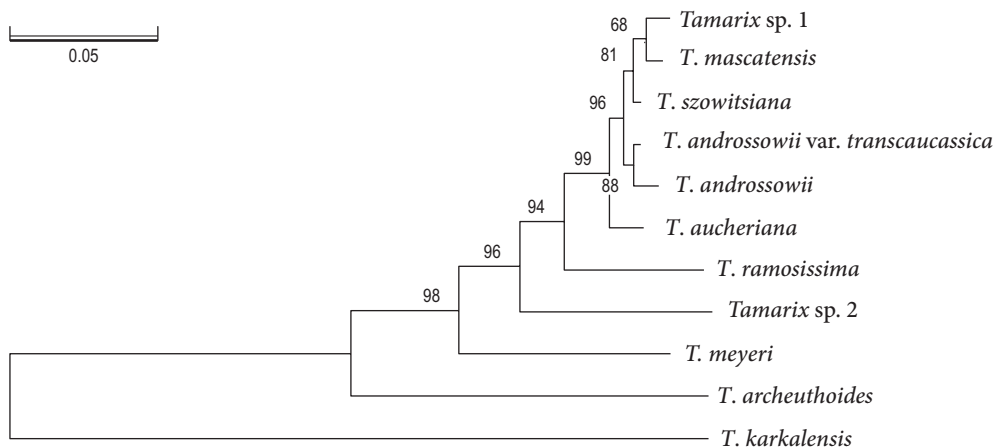


Figure 4: NJ tree of *Tamarix* species based on ITS sequences. Numbers above branches are bootstrap value.

Slika 4: Drevo združevanja NJ vrst rodu *Tamarix* na osnovi ITS sekvenc. Številke nad vejami prikazujejo število bootstrap ponovitev.

Therefore, the potential gene flow among the studied species was investigated by HGT (Horizontal Gene Transfer) analysis with the help of T-REX program (Figure 5). The HGT tree is based on both morphological and ITS tree of the studied species. The results revealed some degree of gene flow between *T. meyerii* and almost all the other studied species in the region. Moreover, *T.*

karkalensis had gene exchange with *T. archeuthoides*, while, *T. ramosissima* exchanged gene with *T. archeuthoides*. The plant named *Tamarix* sp1, was therefore, considered to be *T. androssowii* that was produced by gene flow between this species and *T. meyerii*. However, *Tamarix* sp2 was considered to be new variety of *T. szowitsiana*, formed by introgression between *T. szowitsiana* and *T. meyerii*.

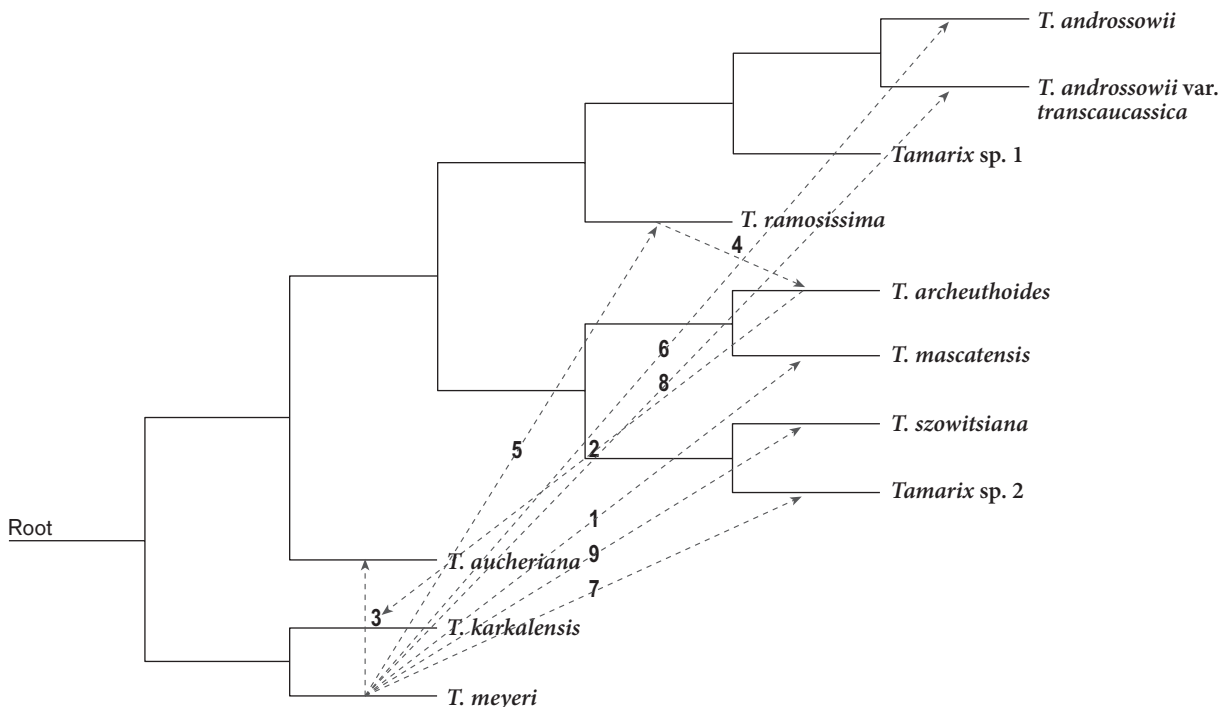


Figure 5: HGT tree of *Tamarix* species based on morphological and ITS data, showing gene flow among these taxa.

Slika 5: Drevo HGT vrst rodu *Tamarix* na osnovi morfoloških in ITS podatkov, ki prikazuje pretok genov med taksoni.

As evidenced in UPGMA tree of morphological characters (Figure 6), plants of *T. szowitsiana* and *T. androssowii* were placed intermixed due to variability and overlap in their morphological characters. Detailed morphological study of these plants in six geographical populations revealed that some plants have a two or more mixture of species characters. The same result was obtained by ISSR study.

UPGMA tree (Figure 6) and MDS plot (Figure 7) of ISSR data revealed admixture of samples in *T. szowitsiana* (coded 1), *T. androssowii* (coded 2) and the trees with mixture of characters from both species (coded 3).

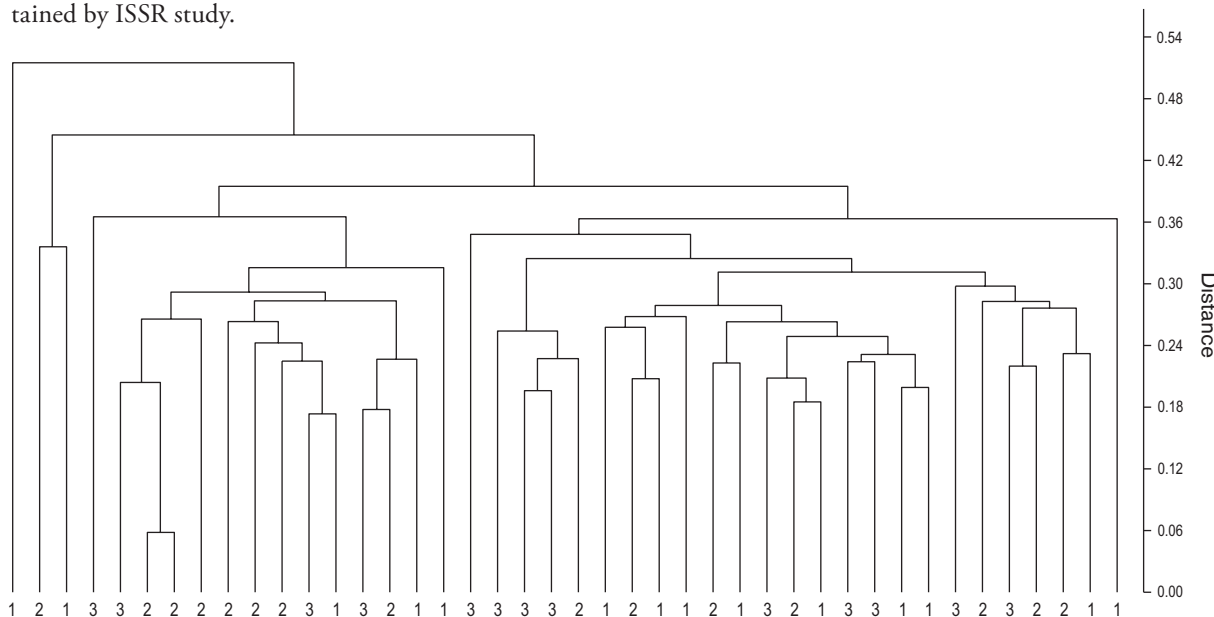


Figure 6: UPGMA tree of the studied samples based on ISSR data.

Slika 6: Drevo združevanja UPGMA preučevanih vzorcev na osnovi ISSR podatkov.

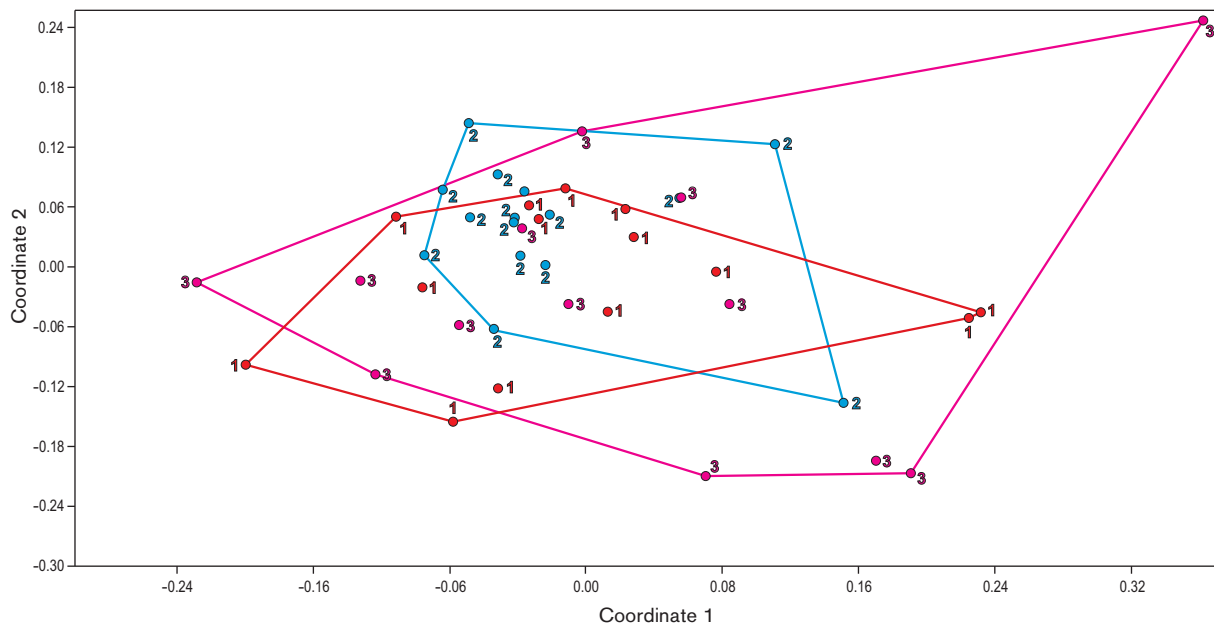


Figure 7: MDS plot of the studied samples based on ISSR data.

Slika 7: Graf MDS preučevanih vzorcev na osnovi ISSR podatkov.




4. Discussion

Tamarix species act against deforestation in Iran, therefore identification of these species and their hybrids throughout the country is crucial for conservation strategy. *Tamarix* species grow in different geographical populations in the country and face diverse environmental conditions. It is usually expected that species that grow in different geographical populations, show genetic and morphological variability (Sheidai et al. 2018). The same holds true for *Tamarix* species (Ijbari et al. 2014, Sheidai et al. 2018). Moreover, *Tamarix* species are known to form frequent inter-specific hybrids (Sheidai et al. 2018). Due to morphological overlaps in *Tamarix* species as a result of inter-specific gene flow, identification of *Tamarix* species is problematic and needs to have clear cut differentiating morphological features. The present study revealed that, it is better to start morphological identification of *Tamarix* species, first by considering 4-merous flowers versus 5-merous flowers. Secondly, characters like shape of disks and leaves should be considered for *Tamarix* identification (Obermeyer 1978, Bredenkamp & Phepo 2008).

However, we may still have some degree of overlap even in these characters, therefore, it is better to accompany morphological identification with molecular data support. Combination of both morphological and molecular results provide a more reliable and consistent method of identifying *Tamarix* species.

Our second main objective in this study was to reveal gene flow or hybridization within *Tamarix* species. Molecular tools also provide the means to investigate the genetic diversity within and among populations, and can also detect hybridization and introgression patterns between closely related species (Le Roux & Wiczorek 2008).

Hybridization is a driving force of invasion, 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 & 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 & Ellstrand 2009). These hybrids may survive in extreme habitats that are not suitable for either of the parent taxa, as was reported in *Helianthus* (Riesberg et al. 2003). The present study revealed the occurrence of inter-specific hybridization or introgression between *T. meyeri*, *T. szowitziana*, and *T. androssowii*. The ecotypes formed show the separation of a single lineage into separate lineages that act as the initial stage of genetic divergence, which in some cases may lead to speciation (Schaal et al. 2003).

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