# Genetic and morphological analyses of distyly in *Linum mucronatum* (*Linaceae*)

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**Abstract.** The present study was aimed at researching the morphological and genetic variability in the long-styled and short-styled plant forms in four subspecies of *L. mucronatum*. ANOVA and PCA analysis of morphological characters revealed significant difference among the long-styled and short-styled plants in all four studied subspecies. Similarly, AMOVA and Gst test, as well as Hickory test have shown significant molecular difference among these subspecies, while PCoA plot of molecular data after 99 permutations has disclosed genetic divergence of the long-styled plants versus short-styled plants in these subspecies. STRUCTURE analysis indicated a far greater genetic differentiation between the two plant forms of *L. mucronatum* subsp. *mucronatum*. Some degree of gene exchange was identified between all four subspecies, indicating that they are not completely isolated from each other.

**Key words:** Genetic variation, heterostyly, *Linum*, reticulation

## Introduction

*Linum* is the largest genus of *Linaceae*. It contains about 180 species that mainly grow in temperate and subtropical regions of the world (Rogers 1982, 2008; Muir & Westcott 2003). *Linum* species are used as a source of fiber (*L. usitatissimum*), seed oils, fodder, and as ornamentals.

*Linum mucronatum* Bertol. is member of the section *Syllinum* Griseb. It is a heterostylous species, with four subspecies in Iran, namely *L. mucronatum* subsp. *armenum* (Bordzil) P.H. Davis, *L. mucronatum* subsp. *assyriacum* P.H. Davis, *L. mucronatum* Bertol. subsp. *mucronatum*, and *L. mucronatum* subsp. *orientale* (Boiss.) P.H. Davis., (Sharifnia & Assadi 2001). *Linum mucronatum* was reported as a very variable species (Özcan & Zorlu 2009) and different studies, such as palynological investigation, have confirmed these interpretations (Talebi & al. 2012a).

Heterostyly is sexual polymorphism in which the plant populations have two (distyly) or three (tristyly) floral morphs. They show reciprocal arrangements of anthers and stigmas (reciprocal heterogamy) (Talebi & al. 2012 b). Distylous plant species produce either only long-styled (LS or Pin) or only short-styled (SS or Thrum) flowers. Flowers with LS morphology have stigma(s) positioned above the anthers, whereas flowers with SS morphology have their anthers above the stigma(s).

Distyly is widespread and very common in the genus *Linum* (about 40 % of the *Linum* species are distylous) (Rogers 1979). Some such species are *Linum pubescens*, *L. grandiflorum* and *L. mucronatum* (Dulberger 1973, 1981), *L. perenne*, *L. grandiflorum* and

*L. alpinum* (Dulberger 1981), *L. aretioides* (Güvensen & al. 2013), *L. austriacum*, *L. album,* and *L. glaucum* (Talebi & al. 2012 b).

A common feature associated with heterostyly is the presence of a self- and intramorph incompatibility system that allows only legitimate (between anthers and stigmas of the same level) pollination to set fruit (Güvensen & al. 2013). The floral morphs differ reciprocally in stamen and style length, which in turn reduces the pollen wastage by increasing legitimate pollination (Güvensen & al. 2013). Moreover, the supergene that determines floral morphology also controls a diallelic sporophytic self- incompatibility system. Therefore, only pollinations between morphs are compatible (Güvensen & al. 2013).

Different investigations have shown that the long-styled plants differ in various characteristics from the short-styled plants, such as style and stamen arrangements, number and size of the pollen grains, shape of the stamen, shape and color of the stigma, stigma surface papillae (Dulberger 1981), exine sculpturing (Dulberger 1981; Talebi & al. 2014), and morphological and nuclear genome size (Talebi et al. 2012b)

The present study considers inter-population morphological and genetic variability in the long-styled plants versus the short-styled plants in four subspecies of *L. mucronatum.*

## Material and methods

### Plant material

Extensive field visits and collection were undertaken during 2010–2013 across the country and several geographical populations were identified for different Wild Flax species, including *Linum mucronatum* Bertol. (Table 1). These populations were selected from different habitats with varied ecological factors. Although both short-styled and long-styled flowers were present in each population, each individual had one form of flowers, either short-styled or long-styled. In the present study, four subspecies of *L. mucronatum* were studied: *L. mucronatum* subsp. *mucronatum, L. mucronatum* subsp. *assyriacum*, *L. mucronatum* subsp. *armenum,* and *L. mucronatum* subsp. *orientale*.

For the morphological studies, five randomly collected plants were investigated, while for the molecular study, fresh leaves were collected from five randomly selected plants. These leaves were mixed together and used for DNA extraction. Plant specimens were identified on the basis of descriptions provided in the *Flora of Iran* (Sharifnia & Assadi 2001). The voucher specimens were deposited in the Herbarium of Shahid Beheshti University (HSBU).

**Table 1.** *L. mucronatum* **subspecies, their localities and ecological features.**

| Subspecies | Locality | Longitude | Altitude | Latitude (m) | Voucher No. |
|---|---|---|---|---|---|
| Subsp. *mucronatum* long-style | Hamedan, Avaj. | 35°41' | 49°31' | 1800 | HSBU2011196 |
| Subsp. *mucronatum* short-style | Hamedan, Avaj. | 35°41' | 49°31' | 1800 | HSBU2011296 |
| Subsp. *orientale* long-style | Zanjan. | 36°24' | 48°55' | 1839 | HSBU2011132 |
| Subsp. *orientale* short-style | Zanjan. | 36°24' | 48°55' | 1839 | HSBU2011232 |
| Subsp. *armenum* long-style | Azerbaijan, Ghoshchi. | 38°02' | 44°57' | 1577 | HSBU2011140 |
| Subsp. *armenum* short-style | Azerbaijan, Ghoshchi. | 38°02' | 44°57' | 1577 | HSBU2011240 |
| Subsp. *assyriacum* long-style | Khuzestan, Izeh. | 31°45' | 49°48' | 867 | HSBU2011164 |
| Subsp. *assyriacum* short-style | Khuzestan, Izeh. | 31°45' | 49°48' | 867 | HSBU2011264 |

### Morphological study

The following morphological characters were studied: stem height, basal leaf shape, floral leaf shape, width and length of the basal and floral leaves, length/width ratio of the basal and floral leaves, shape of the leaf apex, size of the calyx width and calyx length, calyx width/length ratio, size of the sepal width, size of the corolla width and length, corolla width/length ratio, size of the petal width and length, petal width/length ratio, style length, and length of the anther and stamens, filament. The mean value of quantitative characters was measured in each population and different forms of qualitative characters were recorded when encountered.

### DNA extraction and ISSR assay

For the molecular studies, fresh leaves were collected randomly from 10 randomly selected plants in each population (five individuals per each form) and dried protocol in silica gel powder. Genomic DNA was extracted using CTAB activated charcoal (Križman & al. 2006). The extraction procedure was based on activated charcoal and polyvenylpyrrolidone (PVP) for binding the polyphenolics during extraction. The mild ex-

traction and precipitation conditions promoted the high-molecular weight DNA isolation without interfering contaminants. The quality of extracted DNA was examined by running on 0.8 % agarose gel.

Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, UBC810, (CA)7AT, (GA)9C, UBC807, UBC811, (GA)9A, and (GT)7CA commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8; 5Fig. 3. PCoA plot of ISSR data based on distyly in *L. mucronatum* subspecies. Plant numbers = 1 & 2: *L. mucronatum* subsp. *mucronatum*, 3 & 4: *L. mucronatum* subsp. *assyriacum*, 5 & 6 = *L. mucronatum* subsp. *armenum* and 7 & 8 = *L. mucronatum* subsp. *orientale*. (The first number in each subspecies is a short-styled plant, while the second number is a long-styled plant).

KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplification, reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94 °C, 30 s at 94 °C; 1 min at 50 °C, and 1 min at 72 °C. The reaction was completed by a final extension step of 7 min at 72 °C. The amplification products were visualized by running on 2 % agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

## Data analysis

The analysis of variance (ANOVA) test was performed for quantitative morphological characters to indicate the significant difference among populations. Principal coordinate analysis (PCoA), principal correspondence analysis (PCA), as well as multidimensional scaling (MDS) were performed to group the plant specimens on the basis of standardized (mean = 0, variance = 1) morphological characters.

The obtained ISSR bands were treated as binary characters and coded accordingly (presence = 1, absence = 0). Genetic diversity parameters were determined in each population. These parameters represented percentage of allelic polymorphism, allele diversity (Weising & al. 2005), Nei's gene diversity (H), Shannon information index (I), number of the effective alleles, and percentage of polymorphism (Weising & al. 2005; Freeland & al. 2011).

Nei's genetic distance (Weising & al. 2005; Freeland & al. 2011) was determined among the studied populations and used for neighbor joining (NJ) clustering after 100 times bootstrapping (Freeland & al. 2011), by using PAST ver. 2.17 (Hamer & al. 2012) and DARwin ver. 5 (2012).

Genetic affinity of the populations was determined by principal coordinate analysis plot (PCoA) after 99 permutations (Podani 2000), as performed in GenAlex 6.4 (Peakall & Smouse 2006), and by distance-based NeighborNet (Bryant & Moulton 2004) as implemented in SplitsTree4 (Huson & Bryant 2006).

The Mantel test was performed to check correlation between geographical distance and genetic distance of the studied subspecies and populations (Podani 2000).

In order to investigate the significant genetic difference among populations (provinces), different methods were used: 1. AMOVA (analysis of molecular variance) test (with 1000 permutations) as performed in GenAlex 6.4 (Peakall & Smouse 2006), and 2. Nei's Gst analysis of GenoDive ver.2 (2013) which was originally written by Meirmans & van Tienderen (2004). Furthermore, new parameters of genetic differentiation were determined, such as G'ST est = standardized measure of genetic differentiation ((Gst est_(n-1+Hs est))((n-1).(1-Hs est)) (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008). Moreover, in order to overcome the potential problems caused by dominance of the ISSR markers, a Bayesian program, Hickory (ver. 1.0) (Holsinger & al. 2003) was used to estimate the parameters related to genetic structure (Theta B value). Three runs were conducted with default sampling parameters (burn-in = 50,000, sample= 250,000, thin = 50) to ensure consistency of results (Tero & al. 2003).

Genetic continuity versus population stratification was checked by two methods. First, we carried out structure analysis (Pritchard & al. 2000). For this, data were scored as dominant markers and analysis followed the method suggested by Falush & al. (2007). Second, we performed K-means clustering as done in GenoDive ver. 2. (2013).

In K-means clustering, optimal clustering is the one with the smallest amount of variation within clusters, calculated using the within-clusters sum of squares. Minimization of the within-groups sum of squares used in K-means clustering is, within the context of a hierarchical AMOVA, equivalent to minimizing the among-populations-within-groups sum

of squares, SSDAP/WG. The hierarchical population structure in AMOVA then consists of different hierarchical levels: individuals, populations, and clusters of populations. Different F-statistics can be calculated on the basis of variance components for the different hierarchical levels. In terms of F-statistics, minimization of SSDAP/WG is reduced to maximization of FCT, the variance among clusters (C) relative to the total variance (T) (Meirmans 2012).

We used two summary statistics to present the K-means clustering: 1. pseudo-F (Caliński & Harabasz 1974 ) and 2. Bayesian information criterion (Schwarz 1978 ). Pseudo-F (Caliński & Harabasz 1974) relates r2, the fraction of total variance that is explained by the clustering, to the number of clusters k and the number of populations n: $F_k = r^2 / (1–r^2)(n–k)$, where $r^2$ = (SSDT – SSDAP/WG)/(SSDT – SSDWP). The clustering with the highest value for pseudo-F is considered as providing the best fit. The Bayesian information criterion (BIC) is calculated as:

$$BIC_k = n \cdot \ln (SSE) + k \cdot \ln (n).$$

Reticulation analysis was performed to show the gene exchange or presence of shared genes among the distylous plants. The analysis was performed by DARwin ver. 5 (2000) which infers the reticulogram from a distance matrix. Therefore, we have first built a supporting phylogenetic tree by neighbor joining (NJ), which was then followed by a reticulation branch that minimizes the least-squares at each step of the algorithm (Legendre & Makarenkov 2002).

## Results and discussion

### Morphometry

The ANOVA test performed on quantitative morphological characters showed significant difference among the studied subspecies for the stem length, width of the basal leaves, width and length of the floral leaves, and width and length of the calyx and corolla (data with $p<0.05$ are not provided for conciseness). These results showed that magnitudes of the morphological changes differ in the studied subspecies (see, for example, Fig. 1). The highest mean value of stem length (38.00 cm) has occurred in the long-styled plants of *L. mucronatum* subsp. *orientale*, while the lowest mean value of the same character (90.00 cm) has occurred in the short-styled plants of *L. mucronatum* subsp. *assyriacum*.

The long-styled plants had greater stem length as compared to the short-styled plants in *L. mucronatum* subsp. *assyriacum* and *L. mucronatum* subsp. *orientale*. The reverse situation was observed in the *L. mucronatum* subsp. *armenum*. The greatest basal leaf length (2.25 cm) has occurred in the short-styled plants of *L. mucronatum* subsp. *orientale*, while the smallest one (1 cm) has occurred in the long-styled plants of *L. mucronatum* subsp. *mucronatum*. Similarly, the long-styled plants of *L. mucronatum* subsp. *assyriacum* have had larger basal leaves. In the other studied subspecies, the short-styled plants have had the largest basal leaves.

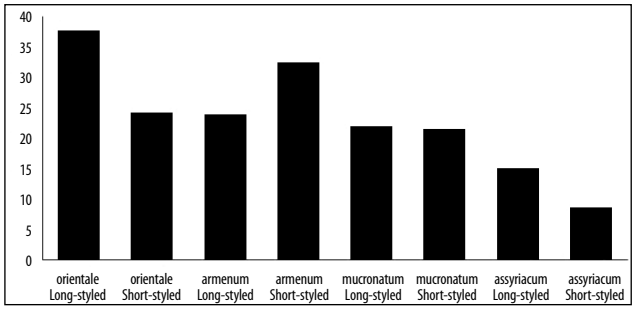


**Fig. 1.** Mean stem length in *L. mucronatum* subspecies based on the long-styled and short-styled plants.

Grouping of the studied subspecies of *L. mucronatum* on the basis of morphological characters by PCA, PCoA and MDS plots has produced similar results. Therefore, only the PCA plot is presented and discussed here (Fig. 2). The long-styled and the short-styled plants in each subspecies were positioned closer to each other than to the plant forms of the other subspecies. Moreover, the long-styled plants were positioned at some distance from the short-styled plants in each subspecies, which indicates their morphological differentiation.

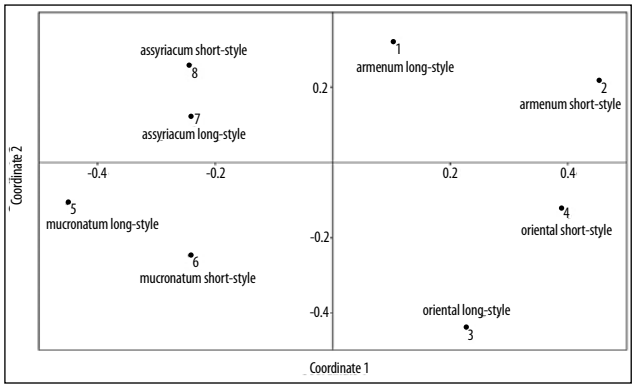


**Fig. 2.** PCA plot of morphological data in *L. mucronatum* subspecies based on distyly.

Dulberger (1973) studied distyly in *Linum pubescens* and *L. mucronatum* and had reported differences of the two plant forms in the style length, stamen length and size of stigmatic papillae. The attempts at performing artificial self- and intramorph cross-pollinations were incompatible in both species, while intermorph pollination resulted in seed production. In a similar study, Talebi & al. (2012b) reported morphological differences between the short-styled and the long-styled plants in *L. austriacum* L., *L. album* Kotschy ex Boiss. and *L. glaucum* Boiss. & Nöe. These plant forms also differed in their genome size (C-value content). The heterostylous plants in *L. aretioides* (Güvensen & al. 2013) differed in the size of petal width and sepal, pistil, and stamen lengths.

## Genetic diversity analysis

All 10 ISSR primers used have produced polymorphic bands in the studied *L. mucronatum* subspecies. The highest percentage of polymorphism occurred in *L. mucronatum* subsp. *mucronatum* (55 %), while the lowest value occurred in *L. mucronatum* subsp. *armenum* (17.86 %, Table 2). Similarly, the highest values for the effective number of alleles (Ne), Shanon information index (I) and gene diversity (H) occurred in *L. mucronatum* subsp. *mucronatum*. The lowest values of the same parameters were observed in *L. mucronatum* subsp. *armenum* (Table 2).

**Table 2. Genetic diversity parameters in *L. mucronatum* subspecies based on distyly.**

| Subspecies | Na | Ne | IS | H | UHe | %P |
|---|---|---|---|---|---|---|
| Subsp. *mucronatum* | 1.236 | 1.389 | 0.333 | 0.228 | 0.304 | 55.00 % |
| Subsp. *assyriacum* | 1.107 | 1.146 | 0.125 | 0.086 | 0.114 | 20.71 % |
| Subsp. *armenum* | 1.093 | 1.126 | 0.108 | 0.074 | 0.099 | 17.86 % |
| Subsp. *orientale* | 1.064 | 1.136 | 0.117 | 0.080 | 0.107 | 19.29 % |
| Total | 1.125 | 1.200 | 0.171 | 0.117 | 0.156 | 28.21 % |

**Abbreviations**: **Na** = number of different alleles, **Ne** = number of effective alleles = 1 / (p^2 + q^2), **IS** = Shannon's Information Index = –1* (p * Ln (p) + q * Ln(q)), **H** = gene diversity = 2 * p * q, **UHe** = unbiased gene diversity = (2N / (2N–1)) * He, **%P** = percentage of polymorphism.

The AMOVA test has produced Fst value (0.22) of almost significant (p =0.07) genetic difference among the studied populations. The same was true for G'st (Nei) = 0.22 (p =0.07) and D_est = 0.11 (p = 0.07). The Hickory test also produced a Theta B value of 0.20, which is a moderate one.

The Hst values of these subspecies were 0.550 (Hst/Htotal = 0.48), 0.207 (Hst/Htotal = 0.180), 0.179 (Hst/Htotal = 0.158), and 0.179 (Hst/Htotal = 0.158), respectively. Therefore, *L. mucronatum* subsp. *mucronatum* manifested the highest contribution to total genetic diversity. This was also evidenced by the PCoA plot of genetic data (Fig. 3), which showed that the long-styled and the short-styled plants of *L. mucronatum* subsp. *mucronatum* (plant numbers 1 and 2 in Fig. 3) were quite separated from the others.

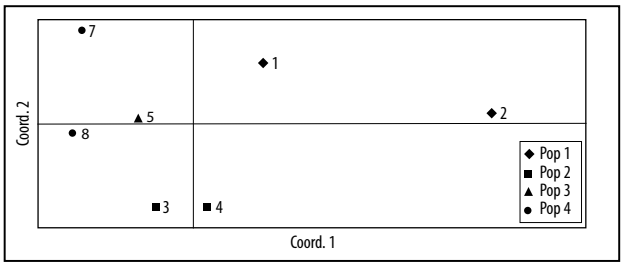


**Fig. 3.** PCoA plot of ISSR data based on distyly in *L. mucronatum* subspecies.
Plant numbers = 1 & 2: *L. mucronatum* subsp. *mucronatum*, 3 & 4: *L. mucronatum* subsp. *assyriacum*, 5 & 6 = *L. mucronatum* subsp. *armenum* and 7 & 8 = *L. mucronatum* subsp. *orientale*. (The first number in each subspecies is a short-styled plant, while the second number is a long-styled plant).

The PCoA plot of ISSR data after 99 permutations has revealed genetic differences of the studied subspecies as they were placed in different groups (Fig. 3). This plot also showed that the long-styled and the short-styled plants of *L. mucronatum* subsp. *mucronatum* differed the strongest from each other, as compared with the other studied subspecies. This has been further supported by the neighbor-net diagram (Fig. 4), which clearly showed that the long-styled

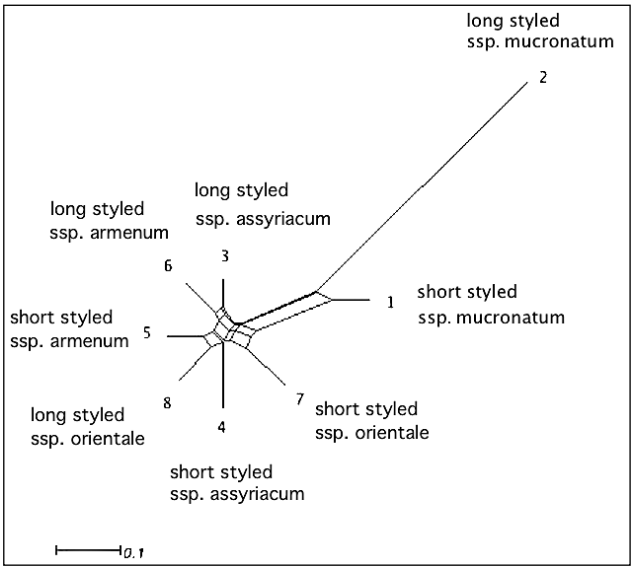


**Fig. 4.** Neighbor-net diagram of *L. mucronatum* subspecies based on ISSR data.

plants of *L. mucronatum* subsp. *mucronatum* are very much differentiated genetically from the short-styled plants, as well as from the other studied subspecies. The neighbor-net diagram also indicated genetic differences between the long-styled and short-styled plants of the other studied subspecies, as these plant forms were positioned far from each other.

STRUCTURE analysis (Fig. 5) supported PCoA and neighbor-net results and revealed an extensive allelic difference (different colored segments) between the long-styled and short-styled plants of *L. mucronatum* subsp. *mucronatum* (plant numbers 1 and 2 in Fig. 5). The STRUCTURE plot also showed a higher genetic similarity among the three studied subspecies of *assyriacum*, *orientale* and *armenum*. The K-means clustering results (Table 3) indicated that the best clustering of populations according to the Caliński & Harabasz (1974) pseudo-F value was k =2, while the best clustering according to the Bayesian information criterion index was k = 4. The value of k = 4 corresponds to the overall genetic differences of the four studied subspecies, while k = 2 is in agreement with the PCoA result, which separated these subspecies into two major groups.

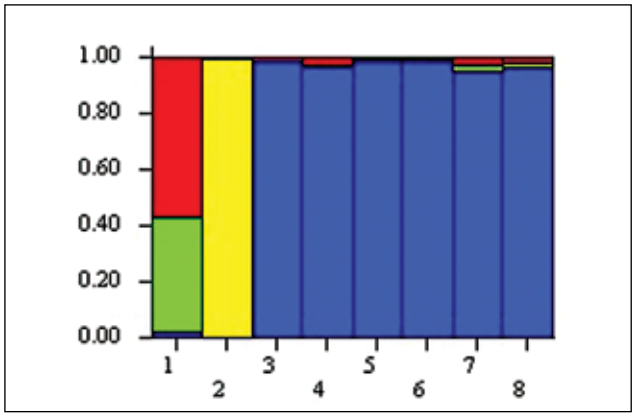


**Fig. 5.** STRUCTURE plot based on k = 4 in *L. mucronatum* subspecies on the basis of distyly.
Plant numbers: 1 & 2 = *L. mucronatum* subsp. *mucronatum*, 3 & 4 = *L. mucronatum* subsp. *assyriacum*, 5 & 6 = *L. mucronatum* subsp. *armenum*, and 7 & 8 = *L. mucronatum* subsp. *orientale* (The first number in each subspices is a short-styled plant and the second number is a long-styled plant).

**Table. 3. K-means clustering of *L. mucranatum* subspecies.**

| k | SSD(T) | SSD(AC) | SSD(WC) | r-squared | pseudo-F | BIC | Rho |
|---|---|---|---|---|---|---|---|
| 2* | 172.500 | 73.071 | 99.429 | 0.424 | 4.409 | 40.954 | 0.661 |
| 3 | 172.500 | 103.000 | 69.500 | 0.597 | 3.705 | 40.169 | 0. 625 |
| 4& | 172.500 | 122.000 | 50.500 | 0.707 | 3.221 | 39.694 | 0.559 |

* Best clustering, according to Caliński & Harabasz' pseudo-F: k = 2
& Best clustering, according to Bayesian information criterion: k = 4
Best BIC clustering has been stored as clones.

The reticulogram obtained showed a certain degree of gene exchange among the populations of the four subspecies (Fig. 6), indicating that these subspecies are not completely isolated from each other. The gene exchange occurred between *L. mucronatum* subsp. *mucronatum* and subsp. *Assyriacum*, and between *L. mucronatum* subsp. *armenum* and subsp. *orientale*, as well as between *L. mucronatum* subsp. *armenum* and *L. mucronatum* subsp. *assyriacum*. However, the Mantel test has shown a significant positive correlation between the subspecies genetic distance and their geographical distance (p = 0.01). This indicates that the subspecies which are geographically closer to each other exchange genes more readily.

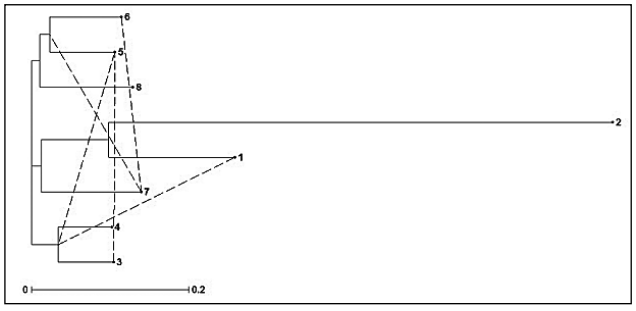


**Fig. 6.** Reticulogram of *L. mucronatum* subspecies.
Plants' codes are: 1 & 2 = the short-styled and the long-styled plants of *L. mucronatum* subsp. *mucronatum*, 3 & 4 = the short-styled and long-styled plants of *L. mucronatum* subsp. *assyriacum*, 5 & 6 = the short-styled and the long-styled plants of *L. mucronatum* subsp. *armenum*, and 7 & 8 = the short-styled and the long-styled plants *L. mucronatum* subsp. *orientale* (dashed lines indicate the gene exchange).

Since we failed to find any report on genetic diversity analysis of distyly in literature, we could not compare our results with any kindred studies. All these findings showed the genetic and morphological divergence of the short-styled and long-styled plants in *L. mucronatum*, but whether these differences are directly related to pollination behavior of these plant forms is not known to us.

## Conclusion

Although heterostyly is widespread in this genus, this phenomenon exceeds the simple variations in stamen and style length between the long-styled and short-styled flowers. Heterostyly has strong effects on the different features of these plants. Different types of analyses, such as ANOVA and PCA, have confirmed a significant difference in the morphological traits between the long-styled and short-styled plants in the studied subspecies,

while genetic structure of the studied subspecies also varied between the long-styled and short-styled plants. AMOVA, Gst test and Hickory test have shown a significant molecular difference between them too. There have been many discussions about taxonomy of this species and the genus *Linum* and different ideas about classifications of the *Linum* taxa, but most of them faced different challenges. It is possible that heterostyly is one of the main reasons for these challenges which create high infrageneric and infraspecific variations in the genus.

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