

MOLECULAR SYSTEMATIC STUDY IN THE GENUS *LINUM* (LINACEAE) IN IRAN

M. SHEIDAI^{1*}, S. DARINI¹, S. M. TALEBI², F. KOOHDAR¹, and S. GHASEMZADEH-BARAKI¹

¹Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, Tehran, Iran
E-mail: *msheidai@yahoo.com

²Department of Biology, Faculty of Sciences, Arak University, Arak 38156-8-8349, Iran

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The genus *Linum* L. is an important plant genus as it contains the species with economic values and particularly *Linum usitatissimum* L. that is source of fibre and linseed oil. This genus contains 230 species throughout the world and has about 22 species in Iran. Little is known about *Linum* species relationship and phylogeny. Therefore, the aim of present study was molecular phylogenetic investigation of the *Linum* species growing in Iran and to present data on their biogeography. We used both ITS and chloroplast DNA sequences (psbA-trnHGUG region) for inferring the species phylogeny and relationship. We also used cpDNA for inferring the species time of divergence and with ISSR markers to identify the path of species distribution in the country. The phylogenetic trees obtained for both ITS and cpDNA sequences were almost congruent. NeighborNet diagram and BEAST tree based on Bayesian method separated the outgroup species *Hugonia* and *Anisadenia* from the other species studied. The subspecies studied in *Linum macronicum* were placed close to each other and along with *L. corymbulosum* comprised a separate clade. The clades obtained showed divergence time between 5–20 mya. The present study revealed that the species of the sect. *Linum* are monophyletic, while members of the sections *Linastrum* and *Syllinum* are intermixed and seem to be paraphyletic.

Key words: cpDNA, ITS, *Linum*, molecular phylogeny, time of divergence

INTRODUCTION

The family Linaceae is composed of 22 genera and approximately 300 species (Heywood 1993, Hickey 1988). The genus *Linum* is the most important in this family, and contains about 230 species (Heywood 1993) with world-wide distribution. About 22 *Linum* species grow wild in Iran (Rechinger 1974, Sharifnia and Assadi 2001). The genus is comprised of five sections: *Linum*, *Linastrum*, *Cathartolinum*, *Dasylinum*, and *Syllinum* (Winkler 1931). Section *Linopsis* is often referred to as sect. *Linastrum*, but *Linopsis* is the prior name at the rank of section (Rogers 1982).

Linum species are important to mankind as the source of fibre and oil, and also are considered as important ornamental and medicinal plants. For example, flax (*L. usitatissimum*) is the source of fibre and linseed oil, and its cultivation dates back to 9,000 B. C. (Habibollahi *et al.* 2015).

Linum bienne Mill. (= *L. angustifolium* Huds.) is being considered as the oldest flax wild form cultivated and its cultivation for fibres and seeds is believed to have led to the development of *L. usitatissimum* L., the modern cultivated flax (McDill *et al.* 2009). The medicinal value of *Linum* comes from lignans and α -linolenic acids used in treatments for cardiovascular diseases and breast cancer (Sheidai *et al.* 2015). These medicinal applications have promoted the interest in the studies related to the genetic and phylogenetic relationships among *Linum* species. The molecular phylogenetic investigation of the group was performed using chloroplast markers, the nuclear ribosomal internal transcribed spacer (ITS), and retrotransposon sequences (Fu and Allaby 2010, McDill *et al.* 2009, Smýkal *et al.* 2011). However, molecular studies within the Linoideae or *Linum* itself have been few, and generally limited in scope (McDill *et al.* 2009). The aim of present study was molecular phylogenetic investigation of the *Linum* species growing in Iran and to present data on their biogeography and route of distribution in the country. Moreover, molecular phylogeny will provide a test of the monophyly of the sections of *Linum* and improve our knowledge on the relationships among the sections.

Both ITS and chloroplast DNA sequences have been major sources of data for inferring plant phylogenies including *Linum* (Fu and Allaby 2010, Ruiz-Martín *et al.* 2015). Therefore, for revealing species relationship and studying the monophyly of the *Linum* sections we used both ITS (Internal transcribed sequences) of the nuclear DNA (nrDNA) and chloroplast psbA-trnHGUG region.

The total length of the ITS1, ITS2 and the 5.8S regions of the nrDNA (rDNA) is about 900 base pairs (bp) including the flanking subunits. The spacer regions are evolving rapidly and show intraspecific variation, whereas the 5.8S region is relatively more conserved (Baldwin *et al.* 1995). 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 homogenise repeats of mutations among the multiple copies in the genome, and/or because of recent hybridisation events (Campbell *et al.* 1997).

Similarly, the non-coding regions of chloroplast DNA provide a high level of variation for phylogenetic inferences and are widely used to infer the species time of divergence (Minaeifar *et al.* 2016).

MATERIAL AND METHODS

Plant materials

We studied 14 *Linum* taxa growing in Iran, with *Hugonia busseana* (Linaceae subf. Hugonioideae) and *Anisadenia pubescens* (Linaceae) as outgroup species (McDill *et al.* 2009). The name of the studied taxa, their respective section and geographical distribution have been provided in Table 1.

DNA extraction

For genetic analyses, genomic DNA was extracted from silica gel dried leaves by using CTAB activated charcoal protocol (Križman *et al.* 2006). The

Table 1
The studied *Linum* taxa and their sections

| Nr | Taxon | Section | Geographical distribution in Iran |
|----|--|------------------|-----------------------------------|
| 1 | <i>L. album</i> Ky. ex Boiss. | <i>Syllinum</i> | C, D, E, J, P, N |
| 2 | <i>L. austriacum</i> var. <i>austriacum</i> L. | <i>Linum</i> | A, B, C, D, E, F, G, H, I |
| 3 | <i>L. mucronatum</i> subsp. <i>orientale</i> (Bordzil.) P. H. Davis | <i>Syllinum</i> | C, I, O |
| 4 | <i>L. glaucum</i> Boiss. et Noë | <i>Linum</i> | D, J, O |
| 5 | <i>L. usitatissimum</i> L. var. <i>usitatissimum</i> | <i>Linum</i> | B, C, K, R |
| 6 | <i>L. mucronatum</i> subsp. <i>armenum</i> (Bordzil.) P. H. Davis | <i>Syllinum</i> | C |
| 7 | <i>L. mucronatum</i> subsp. <i>assyriacum</i> (Bordzil.) P. H. Davis | <i>Syllinum</i> | C, J, M, P, O |
| 8 | <i>L. nervosum</i> var. <i>bungei</i> Waldst. et Kit. | <i>Linum</i> | A |
| 9 | <i>L. nervosum</i> var. <i>nervosum</i> Waldst. et Kit. | <i>Linum</i> | A, C |
| 10 | <i>L. corymbulosum</i> Reichenb. | <i>Linastrum</i> | A, B, C, H, M, P, Q |
| 11 | <i>L. strictum</i> L. var. <i>strictum</i> | <i>Linastrum</i> | K, M, N, O |
| 12 | <i>L. strictum</i> L. var. <i>spicatum</i> | <i>Linastrum</i> | J, K, L, M |
| 13 | <i>L. mucronatum</i> subsp. <i>mucronatum</i> (Bordzil.) P. H. Davis | <i>Syllinum</i> | D, J, S |
| 14 | <i>L. austriacum</i> L. var. <i>album</i> | <i>Linum</i> | A, B, C, D, E, F, G, H, I |

A = Mazandaran, B = Gilan, C = Azarbayejan, D = Kordestan, E = Esfahan, F = Hamedan, G = Bakhteyari, H = Khorasan, I = Tehran, J = Kermanshah, K = Hormozgan, L = Booshehr, M = Khoozestan, N = Kohgiluyeh-Boyerahmad, O = Fars, P = Lorestan, Q = Gorgan, R = Baloochestan, S = Ilam

Table 2
Primers for ITS sequences

| Nr | Primer |
|----|--|
| 1 | ITS5: 5'- GGA AGT AAA AGTCGT AAC AAG G- 3' |
| 2 | ITS4: 5'- TCC GCT TATTGA TAT GC- 3' |

quality and quantity of extracted DNA were assessed by running on 0.8% agarose gel and NanoDrop spectrometer, respectively.

ITS sequences

The primers used for ITS region are provided in Table 2.

PCR reactions were carried out in a 25 µl 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). The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min 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 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).

CP-DNA

The intergenic spacer of chloroplast genome *psbA-trnHGUG* was amplified and sequenced with universal primers following the methodology of Shaw and Small (2005) and Timmer *et al.* (2007). The *psbA-trnHGUG* forward primer was (*trnHGUG*) 5'-CGCGCATGGTGGATTCACAATCC-3' and, the reverse primer was (*psbA*) 5'-GTTATGCATGAACGTAATGCTC-3'. Each 20 ml PCR mixture contained 10 ml of 2₁ PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 unit of *Taq* DNA polymerase (Bioron, Ludwigschafen, Germany), and 1 ml of template genomic DNA at 20 ng ml⁻¹. The PCR amplification program was 5 min at 95 °C, followed by 35 cycles of 60 sec at 95 °C, 60 sec at 56 °C, and 60 sec at 72 °C, with a final extension step of 5 min at 72 °C.

Data analysis

Species relationship by ITS and Cp-DNA sequences – Different phylogenetic methods were used to study the species relationship like maximum parsimony (MP), maximum likelihood (ML), networking and Bayesian approach. For

these, the ITS and cpDNA sequences were first aligned and used to test the proper nucleotide substitution model as implemented in MEGA 7. Program (Tamura *et al.* 2012). Networking was done by Splits Tree4 program (Huson and Bryant 2006, Bayesian analysis by BEAST software v1.6.1 (Drummond *et al.* 2012a, b). Bootstrap and clade credibility values were determined after 100 reiteration for maximum parsimony and Bayesian tree.

Estimation of species time of divergence – Both ITS and cpDNA sequences were used for estimating time of divergence in the studied species. BEAST v1.6.1 (Drummond *et al.* 2012a, b) was used for these analyses. The initial files were made by BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond *et al.* 2012a, b) and used in BEAST.

A Yule process of speciation ('a pure birth' process) was used as a tree prior for all the tree model analyses (Nee 2006). For the MCMC posterior analyses, the length of chain was 10,000,000. After 100 trees burn-in processing, 10,000 trees were used for the analyses.

The maximum clade credibility (MCC) chain generations were repeated five times for each molecular clock model with independent runs to ensure suitable convergence and adequate mixing. The MCC tree was made under the relaxed clock model with HKY substitution. We used a rate of evolution of the plastid sequence ($u = 1.0 \times 10^{-9} \text{ s s}^{-1} \text{ year}^{-1}$) (Zurawski *et al.* 1984) and ITS sequence ($u = 3.4 \times 10^{-9} \text{ s s}^{-1} \text{ year}^{-1}$) (Rambaut and Bromham 1998).

To examine the output of model parameters and convergence results of BEAST, we used Tracer v1.5 software (Drummond and Rambaut 2007), while TreeAnnotator v1.6.1 software (Drummond *et al.* 2012a, b) was used to annotate the phylogenetic results as a form of single 'target' tree.

FigTree v1.3.1 (Rambaut 2009) program was used to produce the tree with the posterior probability 0.5, which is equivalent to the bootstrapping value in PAUP (Phylogenetic Analysis Using Parsimony analysis) analyses (Hong and July 2011).

Biogeography – For biogeography analysis we used cpDNA data of the present study and also ISSR data obtained in our previous studies (Sheidai *et al.* 2014a, b, Talebi *et al.* 2015). Bio- and phylogeographic markers used are preferentially DNA sequences, however, recently fingerprinting techniques, like SSRs, AFLPs, RAPDs and ISSRs, are also frequently used for phylogeographic studies, due to the generally high levels of variability retrieved (Clausing *et al.* 2000, Hess *et al.* 2000, Pleines *et al.* 2009).

RASP program (Reconstruction Ancestral State in Phylogenies) (Yu *et al.* 2010) programs was used for biogeography analyses. RASP is a tool for inferring ancestral state using S-DIVA. We used both Binary MCMC and S-Diva methods of RASP for our analyses, and based on ancestral areas of species distribution, the route of these species in the country was reconstructed.

RESULTS

Species relationship and their time of divergence

Phylogenetic trees obtained for both ITS and cpDNA sequences were almost congruent and therefore, phylogenetic trees of maximum parsimony and BEAST (based on Bayesian method) methods (Figs 1–2, Table 3) are presented and discussed.

In both analyses, the outgroup species *Hugonia* and *Anisadenia* were separated from the other species studied. The subspecies studied in *Linum mucronatum* were placed close to each other and along with *L. corymbulosum* comprised a separate clade. The members of this cluster diverged sometime about 8–18 mya.

Linum album and *L. strictum* showed close affinity and formed a separate clade, with divergence time about 18–20 mya. Similarly, *L. austriacum*, *L. glaucum* and *L. nervosum* showed close affinity and were placed together in a single clade with divergence time about 5 mya. Finally, *Linum bienne* and *L. usitatissimum* formed a single clade with divergence time about 9 mya.

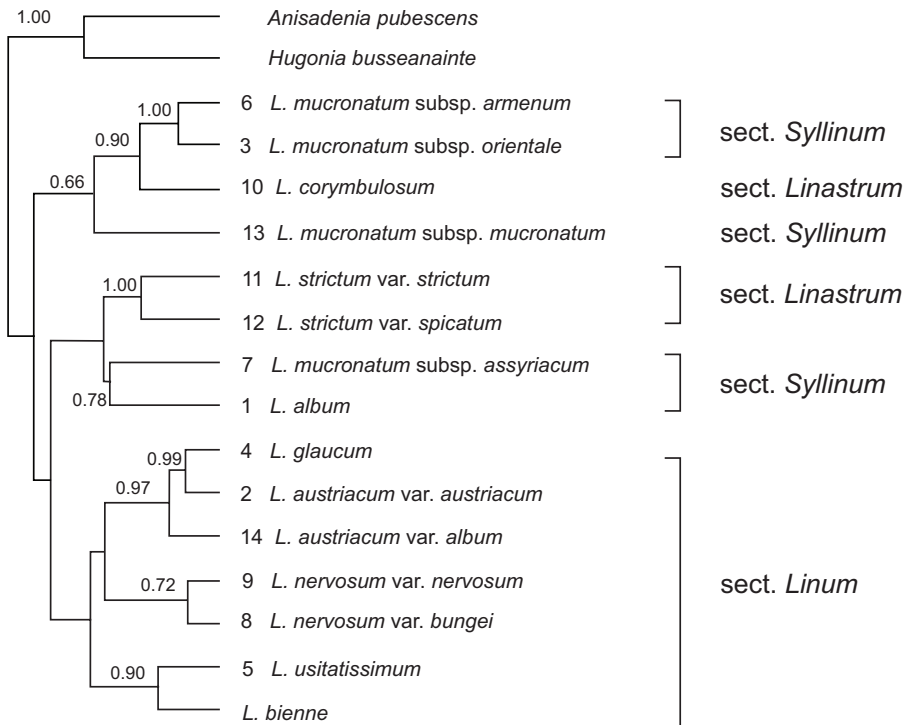


Fig. 1. MP diagram based on ITS and cpDNA sequences

Table 3
ITS accession numbers (GenBank, NCBI)

| Taxa | Id number in NCBI | Accession number | Source area |
|---|-------------------|------------------|-------------|
| <i>L. album</i> | SUB4227033 seq1 | MH547116 | Azarbajejan |
| <i>L. austriacum</i> var. <i>album</i> | SUB4245303 seq12 | MH592586 | Arak |
| <i>L. mucronatum</i> subsp. <i>orientale</i> | SUB4245303 seq1 | MH592578 | Zanjan |
| <i>L. glaucum</i> | SUB4245303 seq2 | MH592579 | Kurdistan |
| <i>L. usitatissimum</i> var. <i>usitatissimum</i> | SUB4245303 seq3 | MH592580 | Arak |
| <i>L. mucronatum</i> subsp. <i>armenum</i> | SUB4245303 seq4 | MH592581 | Azerbaijan |
| <i>L. nervosum</i> var. <i>bungei</i> | SUB4245303 seq6 | MH592582 | Mazandaran |
| <i>L. nervosum</i> var. <i>nervosum</i> | SUB4245303 seq7 | MH592583 | Mazandaran |
| <i>L. corymbulosum</i> | SUB4245303 seq8 | MH592584 | Gilan |
| <i>L. mucronatum</i> subsp. <i>mucronatum</i> | SUB4245303 seq11 | MH592585 | Hamedan |

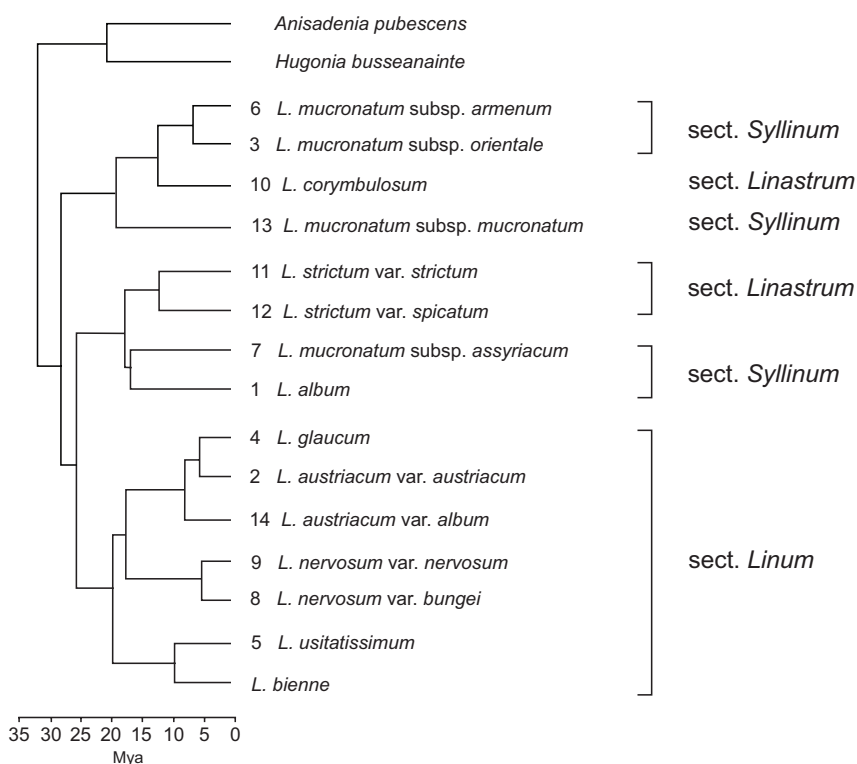


Fig. 2. BEAST time tree based on ITS sequences

The present study revealed that the species of the sect. *Linum* are monophyletic, while members of the sections *Linastrum* and *Syllinum* are intermixed and seem to be paraphyletic.

Cp-haplotypes and ancestral biogeography

The network diagram of cpDNA haplotypes are provided in Figure 3. In general 3 major haplotypes were identified. The species *L. austriacum*, *L. glaucum*, *L. nervosum*, *L. usitatissimum*, and *L. mucronatum* subsp. *assyriacum* comprised the first haplotype group. *Linum album*, *L. mucronatum* subsp. *armenum* and *L. corymbulosum* formed the second haplotype group, while *Linum mucronatum* subsp. *orientale* and *L. strictum* formed the third haplotype group.

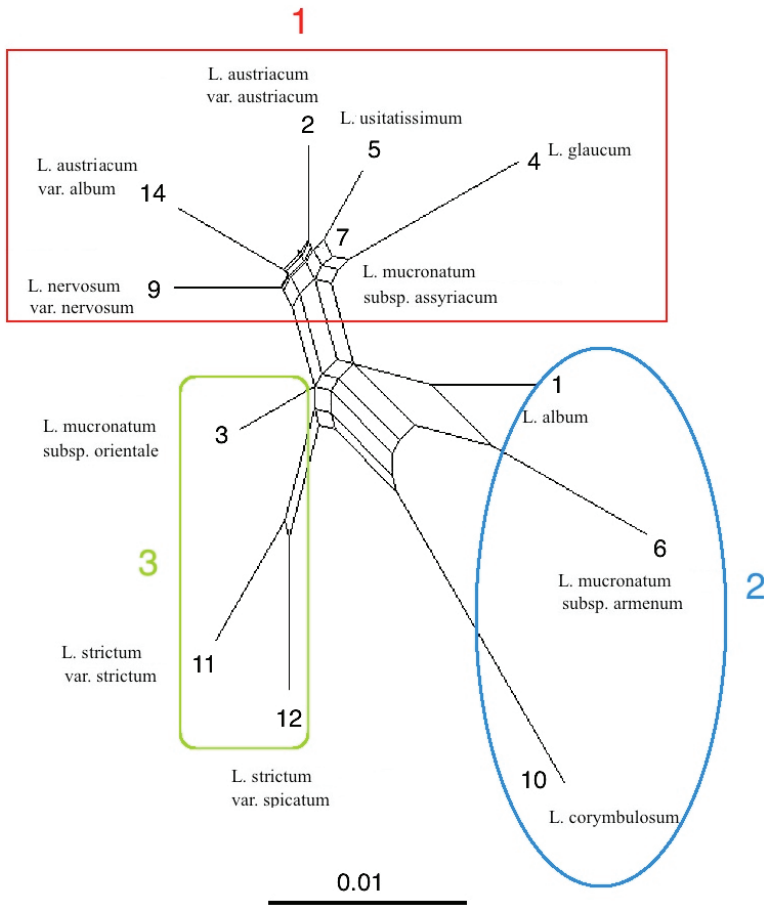


Fig. 3. Cp-haplotype network of the studied species in *Linum*

RASP tree and reconstruction of the ancestral areas with regard to the species studied is presented in Figure 4. A separate ancestral node was observed for *Linum album*, *L. mucronatum* and *L. corymbulosum* with Azarbayegan province as the main ancestral area. This province is located in the northeast region of the country.

The second ancestral node was formed for *Linum strictum* with Hormozgan and Khoozestan provinces as the ancestral area. These provinces are located in south of Iran. The third major ancestral area node contained *L. austriacum*, *L. glaucum*, *L. nervosum*, *L. usitatissimum*, and *L. mucronatum* subsp.

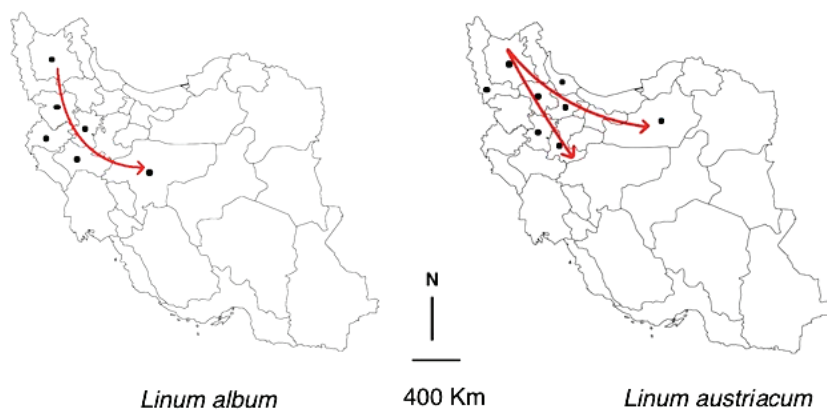
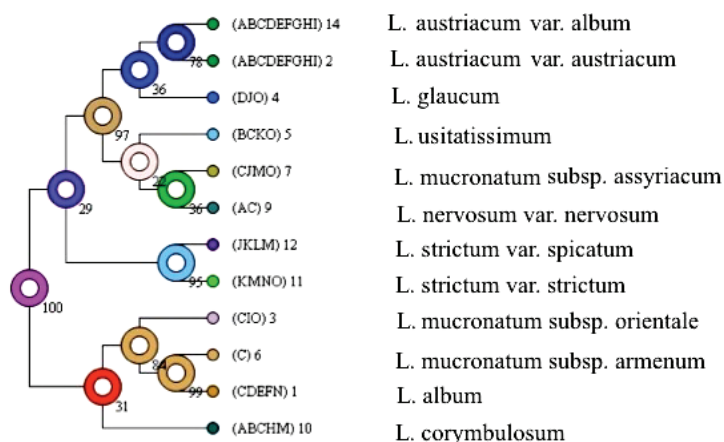


Fig. 4. RASP results by Bayesian binary method (MCMC) (Top: *Linum* species studied based on Cp-DNA. – Bottom: Representative geographical distribution of the species based on ISSR data. – Locality abbreviations: A = Mazandaran, B = Gilan, C = Azarbayegan, D = Kordestan, E = Esfahan, F = Hamedan, G = Bakhteyari, H = Khorasan, I = Tehran, J = Kermanshah, K = Hormozgan, L = Booshehr, M = Khoozestan, N = Kohgiluyeh-Boyerahmad, O = Fars, P = Lorestan, Q = Gorgan, R = Baloochestan)

assyriacum with three provinces of Mazandaran, Gilan, Azarbayejan, and Kordestan as the main distribution areas.

Detailed intraspecific phylogeographical analysis based on ISSR profiles also identified Azarbayejan province as one of the main ancestral regions of *Linum* species distribution in the country. In Figure 4, the presumed route of geographical distribution path as revealed by RASP analysis (figure not given to be brief) are provided for *Linum album* and *L. austriacum*.

DISCUSSION

Species relationship and sections monophyly

The present study based on the sampled species revealed that the section *Linum* is monophyletic, while two sections *Linastrum* and *Syllinum* are paraphyletic. Paraphyly of the sections within the genus *Linum*, was also reported by the others (Savolainen *et al.* 2000, McDill *et al.* 2009, Ruiz-Martín *et al.* 2015).

According to McDill *et al.* (2009), Linoideae are monophyletic, but *Linum* is not. They considered *Anisadenia*, *Reinwardtia*, and *Tirpitzia* as the basal members of Linoideae and that the rest of the subfamily forms two major lineages: a blue-flowered clade (*Linum* sections *Linum* and *Dasylinum*) and a yellow-flowered clade (*Linum* sects. *Linopsis*, *Syllinum*, and *Cathartolinum*, and the genera *Cliococca*, *Hesperolinon*, *Radiola*, and *Sclerolinon*). They suggest that diversification of Linoideae may have begun 46–51 mya, probably in Southeast Asia.

Based on ITS and chloroplast data (McDill *et al.* 2009), section *Linum* turned out to be paraphyletic in relation to sect. *Dasylinum* due to the position of *L. stelleroides* (sect. *Linum*), which emerged either as sister to *Dasylinum* (rbcL) or as sister to *Dasylinum* plus the remaining members of sect. *Linum* (ITS and Cp-DNA data).

Morphologically, sect. *Linum* is quite well characterised with lanceolate leaves without stipular glands. Within this section (excl. *L. stelleroides*), two well-supported groups were identified, (McDill *et al.* 2009); 1) the *L. perenne* group, and; 2) a clade containing *L. bienne* and *L. usitatissimum*.

These two groups are morphologically very similar, but differ from each other on the basis of their sepals and stigmas. Members of the *L. perenne* group have capitate stigmas and entire sepals with a smooth margin, while, in the second group, stigmas are linear and the sepals have ciliate margins (McDill *et al.* 2009). Our result is in agreement with above said suggestion as we observed close affinity between *Linum bienne* and *L. usitatissimum*. They formed a clade separated from the other species within the sect. *Linum*, viz. *L. austriacum*, *L. glaucum*, *L. nervosum*, and *L. album*.

Our recent study (Noormohammadi *et al.* 2017) performed to investigate the genetic variability in *Linum usitatissimum* subsp. *usitatissimum* L. and *L.*

bienne L. by using a combination of single primer amplification reaction markers (SPAR) including RAPD, ISSR, and RAMPO molecular markers, also revealed very close genetic similarities between these species due to common shared alleles.

Time of divergence and biogeography

Eurasia has usually been considered the ancestral area for *Linum*, as the *Linum* sections, and majority of species in most of them, occur in the Mediterranean region and western Asia (Rogers 1982).

According to McDill *et al.* (2009), the blue-flowered flaxes (sections *Dasylinum* and *Linum*) originated during the Oligocene (29–32 mya) in temperate Asia. Our ITS based divergence time also suggests that the separation of the sect. *Linum* from sections *Syllinum* and *Linastrum* occurred around 26 mya. Similarly, divergence of *L. bienne* clade occurred around 20 mya. Therefore, there is a high degree of agreement between these two studies.

Diversification date estimated for the *L. perenne* group as sampled by McDill *et al.* (2009), was around 3.3–3.8 mya. However, the date we obtained based on the studied species is between 6–9 mya during Pliocene. This may be due to difference in the species sampled in these two studies.

The yellow-flowered clade i.e. the sections *Linopsis*, *Syllinum*, and *Cathartolinum* is estimated to have originated during the early Oligocene (36–32 mya) in Europe or Southwest Asia, that subsequently spread into parts of the northern hemisphere, Africa and South America (McDill *et al.* 2009). Our result suggests early separation of the yellow-flowered group from the blue-flowered clade somewhat around 27 mya. The active species diversification within the clade occurred around 20 mya during Miocene.

The Late Miocene age of *Linum* diversification estimated here agrees well with the geological events within the Irano-Turanian phytogeographical region. The uplifting of mountain belts in the borders of the Iranian plateau during the rapid QTP uplift took place in the Late Miocene (*ca* 8 mya), following the collision of the Indian and Eurasian plates (Dercourt *et al.* 1986, Zhang and Fritsch 2010).

Fu and Allaby (2010) worked on *Linum* by using non-coding chloroplast DNA sequences, resulted in a Phylogenetic network and they concluded that the evolution of the genus *Linum* involved several periods of change that resulted in mixed characters. One likely possibility is that there was a series of fairly rapid fracturing events of a large ancestral species population into large subpopulations. On the basis of the molecular clock that population fracturing may have occurred during Oligocene–Miocene transition.

The Oligocene was a warm time with a land bridge between Europe and the New World. The Miocene was a period of global cooling with glaciations and the break of the land bridge. It is a time in which there were numerous opportunities for geologically driven, population fracturing and probable reticulation that occurred in *Linum* species. Similar, geologically driven fracturing processes could also have generated mixed characters through recombination.

Since geographical populations of this species are distributed in quite different regions of the country, we used preliminary biogeography analysis to identify the ancestral locality and possible route of migration of this species in the country. Bio- and phylogeographical markers should preferentially be DNA sequences, however, recently fingerprinting techniques, like SSRs, AFLPs, RAPDs and ISSRs, are also frequently used for phylogeographical studies, due to the generally high levels of variability retrieved (Clausing *et al.* 2000, Hess *et al.* 2000, Pleines *et al.* 2009).

Considering the route of species distribution in the country, RASP analyses revealed Azarbayejan as the main route of dispersal for most of the studied species.

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