Acta Botanica Hungarica 61(3–4), pp. 421–434, 2019
DOI: 10.1556/034.61.2019.3-4.11

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

M. Sheidai[1*], S. Darini[1], S. M. Talebi[2], F. Koohdar[1], and S. Ghasemzadeh-Baraki[1]

[1]*Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, Tehran, Iran*
*E-mail: *msheidai@yahoo.com*
[2]*Department of Biology, Faculty of Sciences, Arak University, Arak 38156-8-8349, Iran*

(Received: 11 June, 2017; Accepted: 22 February, 2019)

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 worldwide 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	*L. album* Ky. ex Boiss.	*Syllinum*	C, D, E, J, P, N
2	*L. austriacum* var. *austriacum* L.	*Linum*	A, B, C, D, E, F, G, H, I
3	*L. mucronatum* subsp. *orientale* (Bordzil.) P. H. Davis	*Syllinum*	C, I, O
4	*L. glaucum* Boiss. et Noë	*Linum*	D, J, O
5	*L. usitatissimum* L. var. *usitatissimum*	*Linum*	B, C, K, R
6	*L. mucronatum* subsp. *armenum* (Bordzil.) P. H. Davis	*Syllinum*	C
7	*L. mucronatum* subsp. *assyriacum* (Bordzil.) P. H. Davis	*Syllinum*	C, J, M, P, O
8	*L. nervosum* var. *bungei* Waldst. et Kit.	*Linum*	A
9	*L. nervosum* var. *nervosum* Waldst. et Kit.	*Linum*	A, C
10	*L. corymbulosum* Reichenb.	*Linastrum*	A, B, C, H, M, P, Q
11	*L. strictum* L. var. *strictum*	*Linastrum*	K, M, N, O
12	*L. strictum* L. var. *spicatum*	*Linastrum*	J, K, L, M
13	*L. mucronatum* subsp. *mucronatum* (Bordzil.) P. H. Davis	*Syllinum*	D, J, S
14	*L. austricum* L. var. *album*	*Linum*	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 = Kohgilooyeh-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 $MgCl_2$; 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 2_ 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.* 2012*a*, *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.* 2012*a*, *b*) was used for these analyses. The initial files were made by BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond *et al.* 2012*a*, *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 ($u = 1.0 \times 10^{-9}$ s s^{-1} $year^{-1}$) (Zurawski *et al.* 1984) and ITS sequence ($u = 3.4 \times 10^{-9}$ s s^{-1} $year^{-1}$) (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.* 2012*a*, *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 Jury 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.* 2014*a*, *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.

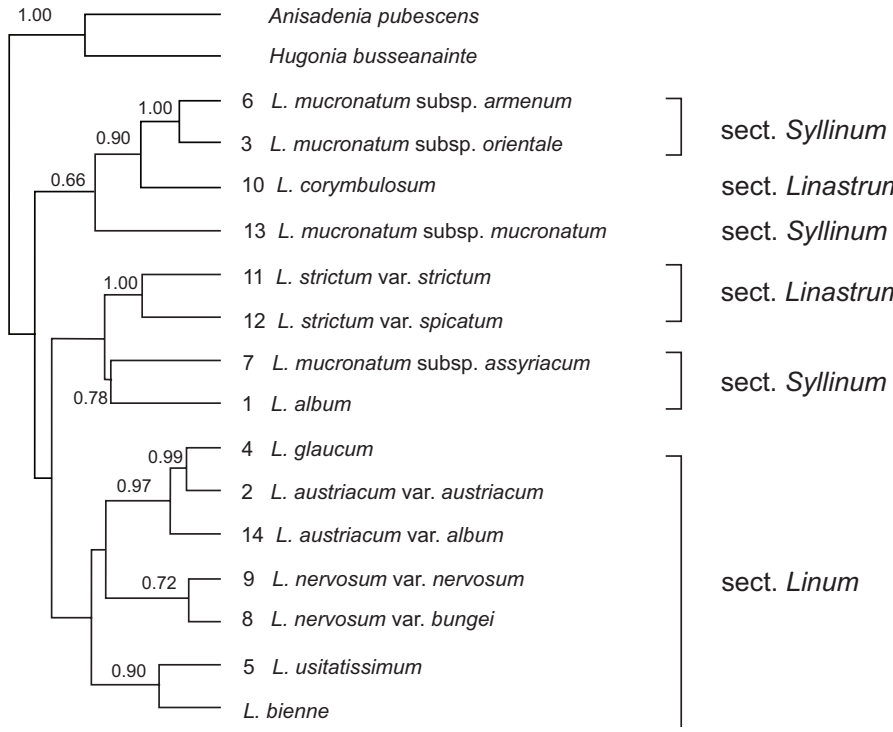

Fig. 1. MP diagram based on ITS and cpDNA sequences

Table 3
ITS accession numbers (GenBank, NCBI)

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

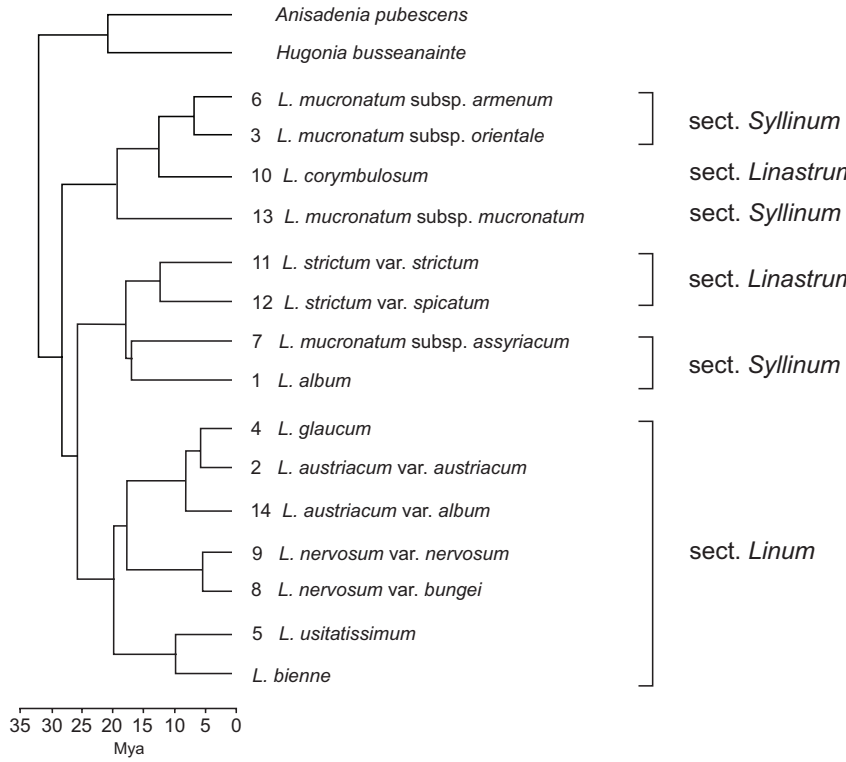

Fig. 2. BEAST time tree based on ITS sequences

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.

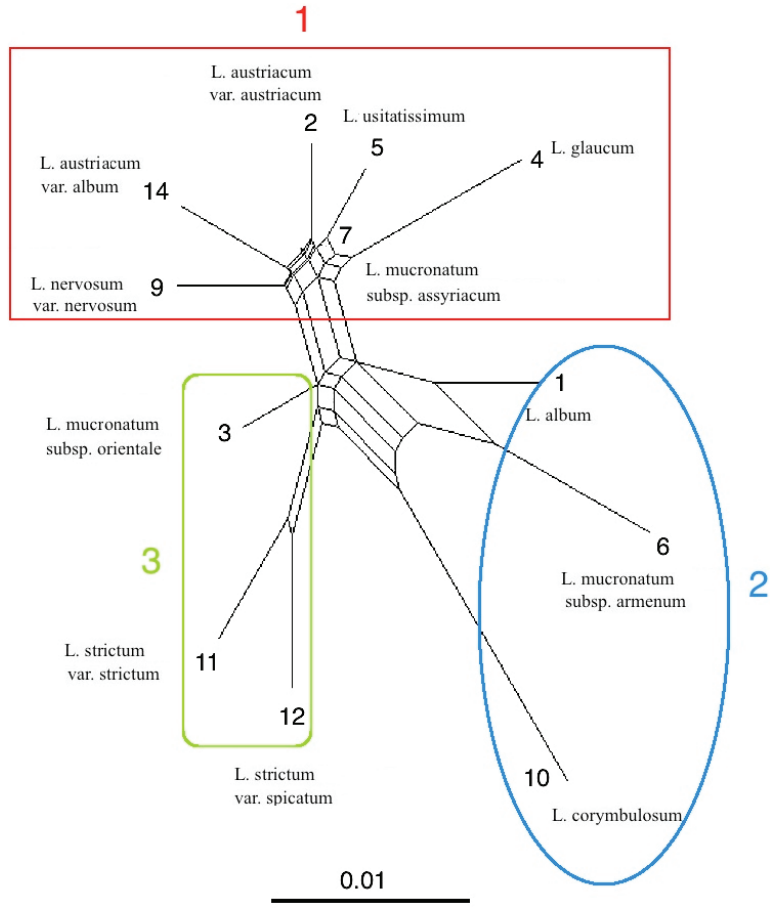

Fig. 3. Cp-haplotype network of the studied species in *Linum*

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 Azarbayajan 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.

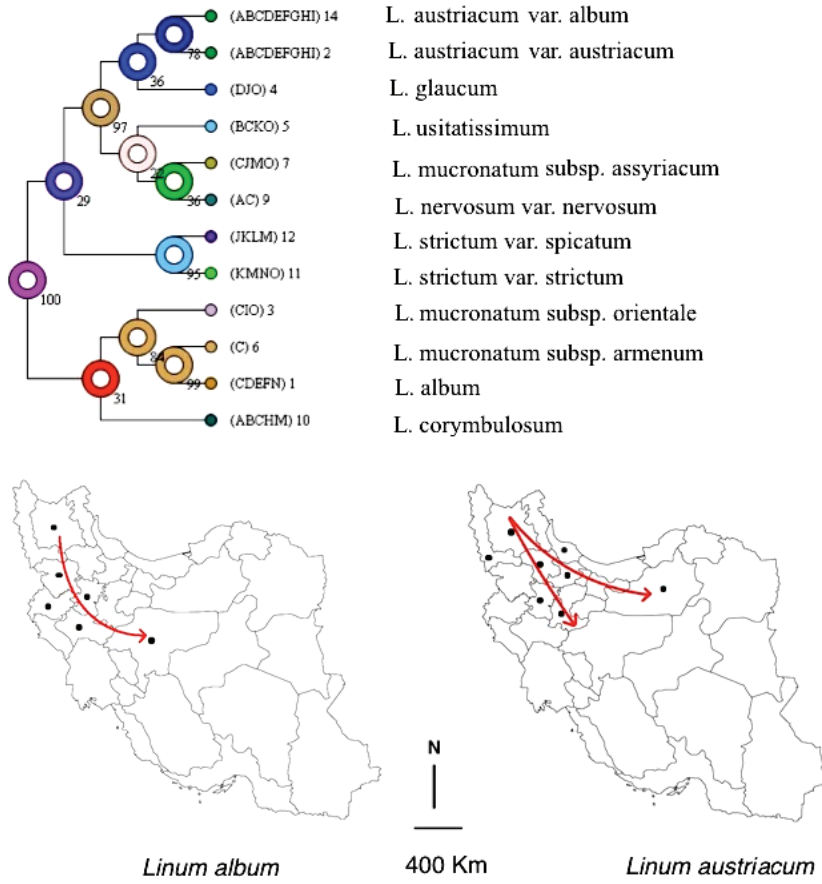

Fig. 4. RASP results by Bayesian binary method (MCMC) (Top: *Linum* species studied based on Cp-DNA. – Bottom: Representative geographical distribution of the species based on ISSR data. – Locality abbreviations: A = Mazandaran, B = Gilan, C = Azarbayejan, D = Kordestan, E = Esfahan, F = Hamedan, G =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

REFERENCES

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. and Donoghue, M. J. (1995): The ITS region of nuclear ribosomal DNA-A valuable source of evidence on Angiosperm phylogeny. – *Ann. Mo. Bot. Gard.* **82**: 247–277. https://doi.org/10.2307/2399880

Campbell, J. Y., Lo, A. and MacKinlay, A. C. (1997): *The econometrics of financial markets.* – Princeton University Press, Princeton, N. J.

Clausing, G., Vicker, K. and Kadereit, J. W. (2000): Historical biogeography in a linear system: Genetic variation of Sea Rocket (Cakile maritima) and Sea Holly (Eryngium maritimum) along European coasts. – *Mol. Ecol.* **9**: 1823–1833. https://doi.org/10.1046/j.1365-294x.2000.01083.x

Dercourt, J., Zonenshain, L. P. E., Ricou, L., Kazmin, V. G., Le Pichon, X., Knipper, A. L., Grandjacquet, C., Sbortshikov, I. M., Geyssant, J., Lepvrier, C., Pechersky, D. H., Boulin, J., Sibuet, J. C., Savostin, L. A., Sorokhtin, O., Westphal, M., Bazhenov, M. L., Lauer, J. P. and Biju-Duval, B. (1986): Geological evolution of the Tethys belt from the Atlantic to the Pamirs since the LIAS. – *Tectonophysics* **123**: 241–315. https://doi.org/10.1016/0040-1951(86)90199-x

Drummond, A. J. and Rambaut, A. (2007): BEAST: Bayesian evolutionary analysis by sampling trees. – *BMC Evol. Biol.* **7**: 214. https://doi.org/10.1186/1471-2148-7-214

Drummond, A. J., Rambaut, A. and Xie, W. (2012*a*): *BEAUti version 1.6.1* [computer program]. – http://beast.bio.ed.ac.uk

Drummond, A. J., Rambaut, A. and Suchard, M. A. (2012*b*): *BEAST version 1.6.1* [computer program]. – http://beast.bio.ed.ac.uk

Fu, Y. B. and Allaby, R. G. (2010): Phylogenetic network of Linum species as revealed by non-coding chloroplast DNA sequences. – *Genet. Resour. Crop Evol.* **57**: 667–677. https://doi.org/10.1007/s10722-009-9502-7

Habibollahi, H., Noormohammadi, Z., Sheidai, M. and Farahani, F. (2015): Genetic structure of cultivated flax (Linum usitatissimum L.) based on retrotransposon-based markers. – *Genetika* **47**: 1111–1122. https://doi.org/10.2298/gensr1503111h

Hess, J., Kadereit, J. W. and Vargas, P. (2000): The colonization history of Olea europaea L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and intersimple sequence repeats (ISSR). – *Mol. Ecol.* **9**: 857–868. https://doi.org/10.1046/j.1365-294x.2000.00942.x

Heywood, V. H. (1993): *Flowering plants of the world*. – Oxford University Press, Oxford, UK.

Hickey, M. (1988): *100 families of flowering plants*. – Cambridge Univ. Press, Cambridge, UK.

Hong, S. W. P. and Jury, S. L. (2011): Phylogeny and divergence times inferred from rps16 sequence data analyses for Tricyrtis (Liliaceae), and endemic genus of north-east Asia. – *AoB PLANTS* **2011**: plr025. https://doi.org/10.1093/aobpla/plr025

Huson, D. H. and Bryant, D. (2006): Application of phylogenetic networks in evolutionary studies. – *Mol. Biol. Evol.* **23**: 254–267. https://doi.org/10.1093/molbev/msj030 (the software is available from www.splitstree.org)

Križman, M., Jakše, J., Baričevič, D., Javornik, B. and Prošek, M. (2006): Robust CTAB-activated charcoal protocol for plant DNA extraction. – *Acta Agric. Sloven.* **87**: 427–433.

McDill, J., Repplinger, M., Simpson, B. B. and Kadereit, J. W. (2009): The phylogeny of Linum and Linaceae subfamily Linoideae, with implications for their systematics, biogeography, and evolution of heterostyly. – *Syst Bot.* **34**: 386–405. https://doi.org/10.1600/036364409788606244

Minaeifar, A. A., Sheidai, M., Attar, F., Noormohammadi, Z. and Ghasemzadeh-baraki, S. (2016): Biosystematic study in the genus Cousinia Cass. (Asteraceae), section Cousinia. – *Biochem. Syst. Ecol.* **69**: 252–260. https://doi.org/10.1016/j.bse.2016.10.008

Nee, S. (2006): Birth-death models in macroevolution. – *Annu. Rev. Ecol. Evol. Syst.* **37**: 1–17. https://doi.org/10.1146/annurev.ecolsys.37.091305.110035

Noormohammadi, Z., Sakhaee, M., Sheidai, M. and Talebi, S. M. (2017): Assessment of genetic variation in Linum L. using SPAR markers. – *Biologija* **63**(1): 49–57. https://doi.org/10.6001/biologija.v63i1.3474

Pleines, T., Jakob, S. S. and Blattner, F. R. (2009): Application of non-coding DNA regions in intraspecific analyses. – *Plant Syst. Evol.* **282**: 281–294. https://doi.org/10.1007/s00606-008-0036-9

Rambaut, A. (2009): *FigTree (v1.3.1)* [computer program]. – http://tree.bio.ed.ac.uk/ software/figtree

Rambaut, A. and Bromham, L. (1998): Estimating divergence dates from molecular sequences. – *Mol. Biol. Evol.* **15**(4): 442–448. https://doi.org/10.1093/oxfordjournals.molbev.a025940

Rechinger, K. H. (1974): *Flora Iranica. No. 106, Linaceae*. – Akad. Druck- u. Verlagsanstalt, Graz.

Rogers, C. M. (1982): The systematics of Linum sect. Linopsis (Linaceae). – *Plant Syst. Evol.* **140**: 225–234. https://doi.org/10.1007/bf02407299

Ruiz-Martín, J., Jiménez-Mejías, P., Martínez-Labarga, J. M. and Pérez-Barrales, R. (2015): Linum flos-carmini (Linaceae), a new species from Northern Morocco. – *Ann. Bot. Fennici* **52**: 383–395. https://doi.org/10.5735/085.052.0521

Savolainen, V., Chase, M. W., Hoot, S. B., Morton, C. M., Soltis, D. E., Bayer, C., Fay, M. F., De Bruijn, A. Y., Sullivan, S. and Qiu, Y.-L. (2000): Phylogenetics of flowering plants based on combined analysis of plastid atpB and rbcL gene sequences. – *Syst. Biol.* **49**: 306–362. https://doi.org/10.1093/sysbio/49.2.306

Sharifnia, F. and Assadi, M. (2001): *Flora of Iran, No. 34: Linaceae.* – Research Inst. Forests and Rangelands, Ministry of Jahad-e-Sazandegi, Iran. [in Persian]

Shaw, J. and Small, R. L. (2005): Chloroplast DNA phylogeny and phylogeography of the North American plums (Prunus subgenus Prunus section Prunocerasus, Rosaceae). – *Amer. J. Bot.* **92**: 2011–2030. https://doi.org/10.3732/ajb.92.12.2011

Sheidai, M., Afshar, F., Keshavarzi, M., Talebi, S. M., Noormohammadi, Z. and Shafaf, T. (2014*a*): Genetic diversity and genome size variability in Linum austriacum (Linaceae) populations. – *Biochem. Syst. Ecol.* **57**: 20–26. https://doi.org/10.1016/j.bse.2014.07.014

Sheidai, M., Ziaee, S., Farahani, F., Talebi, S. M. Noormohammadi, Z. and Farahani, Y. (2014*b*): Infra-specific genetic and morphological diversity in Linum album (Linaceae). – *Biologia* **69**: 32–39. https://doi.org/10.2478/s11756-013-0281-4

Sheidai, M., Farahani, F., Talebi, S. M. and Noormohammadi Z. (2015): Genetic and morphological analysis of distyly in Linum mucronatum Bertol. (Linaceae). – *Phytol. Balcan*. **21**: 13–19.

Smýkal, P., Bačová-Kerteszová, N., Kalendar, R., Corander, J., Schulman, A. H. and Pavelek, M. (2011): Genetic diversity of cultivated flax (Linum usitatissimum L.) germplasm assessed by retrotransposon-based markers. – *Theor. Appl. Genet*. **122**: 1385–1397. https://doi.org/10.1007/s00122-011-1539-2

Talebi, S. M., Sheidai, M., Atri, M., Sharifnia, F. and Noormohammadi, Z. (2015): Infraspecific morphological and genome size variations in Linum glaucum in Iran. – *Biodiversitas, J. Biol. Div.* **16**: 69–78. https://doi.org/10.13057/biodiv/d160109

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2013): MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. – *Mol. Biol. Evol.* **28**: 2731–2739. https://doi.org/10.1093/molbev/msr121

Timmer, J. C., Enoksson, M., Wildfang, E., Zhu, W., Igarashi, Y., Denault, J. B., Ma, Y., Dummitt, B., Chang, Y. H., Mast, A. E., Eroshkin, A., Smith, J. W., Tao, W. A. and Salvesen, G. S. (2007): Profiling constitutive proteolytic events in vivo. – *Biochem. J.* **407**: 41–48. https://doi.org/10.1042/bj20070775

Yu, Y., Harris, A. J. and He, X. J. (2010): S-DIVA (Statistical Dispersal-Vicariance Analysis): A tool for inferring biogeographic histories. – *Mol. Phyl. Evol.* **56**: 848–850. https://doi.org/10.1016/j.ympev.2010.04.011

Winkler, H. (1931): *Linaceae, Trib. I. 3. Linoideae-Eulineae*. – In: Engler, A. (ed.): Die natürlichen Pfanzenfamilien: nebst ihren Gattungen und wichtigeren Arten, insbesondere den Nutzpflanzen. 2nd ed. W. Engelmann, Leipzig, pp. 111–120.

Zhang, M. L. and Fritsch, P. W. (2010): Evolutionary response of Caragana (Fabaceae) to Qinghai-Tibetan Plateau uplift and Asian interior aridification. – *Plant Syst. Evol.* **288**: 191–199. https://doi.org/10.1007/s00606-010-0324-z

Zurawski, G., Clegg, M. T. and Brown, A. H. D. (1984): The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. – *Genetics* **106**: 735–749.