Divergence times and morphological evolution of the subtribe Eritrichiinae (Boraginaceae-Rochelieae) with special reference to *Lappula*

Mahboubeh Sherafati[1], Maryam Khoshsokhan-Mozaffar[2], Shahrokh Kazempour-Osaloo[1],*

[1] *Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran 14115-154, Iran*

[2] *Department of Biology, Qom branch, Islamic Azad University, Qom, Iran*

ABSTRACT

The subtribe Eritrichiinae belongs to tribe Rochelieae (Borginaceae; Cynoglossoideae) which is composed of about 200 species in five genera including *Eritrichium*, *Lappula*, *Hackelia*, *Lepechiniella*, and *Rochelia*. The majority of the species are annual and grow in xeric habitats. The genus *Lappula* as an arid adapted and the second biggest genus of Eritrichiinae with over 50 species is distributed predominantly in Irano-Turanian region. Here, we employed BEAST Bayesian inference for divergence time estimation based on nrDNA ITS, plastid *trnL-F* and *rpl32-trnL* (UAG) sequence data of 46 species of Eritrichiinae extracted from GenBank. The results obtained from molecular dating analyses indicated that the date of Eritrichiinae crown group back to late Oligocene. Diversification events took place for *Lappula* in Asia from the late Miocene to the late Pleistocene. The North American species have been derived from the Asian relatives at the late Miocene.

Keywords: Ancestral state reconstruction; Divergence time estimation; Eritrichiinae; *Lappula*

Introduction

Eritrichiinae Benth. & Hook.f. contains approximately 200 species in five genera, i.e. *Eritrichium* Schrad. ex Gaudin, *Lappula* Moench, *Hackelia* Opiz., *Lepechiniella* M.Popov. and *Rochelia* Rchb. Together with subtribe Heterocaryinae H.Riedl they belong to the tribe Rochelieae A.DC. (1). Eritrichiinae species are small to medium-sized annual or perennial plants distributed in Eurasia, America, Africa and Australia (1-3). Morphologically, the members of this subtribe are characterized by hispid or sometimes pubescent to sericeous indumentum, calyx divided almost at the base, funnel-form to campanulate corolla blue or blue-white rarely blue-purple in color, and pyramidal to subulate gynobase. The nutlets are 1–4, oblique on pyramidal gynobase, and in dorsal view they are ovate to subcircular, with a thickened and glochidiate rarely only verrucose or nearly smooth margin (1,4-5).

* Corresponding author: skosaloo@modares.ac.ir

Lappula with 50–60 species shows a cosmopolitan distribution, with species in Eurasia, Western North America, North Africa and Australia (1-3,6). These annual or rarely biannual/perennial plants have centers of diversity in the Irano-Turanian region of Central Asia (1,3,7-8). Central Asia is characterized by an arid climate with low annual precipitation, arid-hot summer and cold winter (9). Therefore, it is expected that annual life strategy is found as an adaptive response to seasonal drought and aridity in this region, because the annuals are able to survive unfavourable conditions as seeds (10-11). The genus *Lappula* is morphologically characterized by basal and cauline leaves, blue to white corolla bearing five prominent appendages at the apex of the corolla tube. The gynobase is pyramidal to subulate with four nutlets being triangular-ovoid to ovoid in dorsal view with one or more rows of marginal spines or glochidia (3-5,7-8,12-14).

Several phylogenetic studies addressed the relationships within Rochelieae and Eritrichiinae (1,7-8,15-18). Huang *et al.* (7) and Rolfsmeier (8) employing nuclear ribosomal ITS (nrITS), cpDNA and morphological data presented the phylogenetic relationships among Eurasian and North American species. Recently, Khoshsokhan-Mozaffar *et al.* (13) using nrITS and two plastid regions (*trnL_{UAA}-F* and *rpl32-trnL_{UAG}*) evaluated the relationships among West Asian taxa of subtribe Eritrichiinae and represented a taxonomic treatment for *Lappula*. Despite all taxonomic and phylogenetic studies within Eritrichiinae, only a few studies have investigated the morphology (2,7-8) and divergence time (19-20) of Eritrichiinae.

Here we investigate the evolution of morphological characters in Eritrichiinae using nrITS and two plastid (*trnL_{UAA}-F* and *rpl32-trnL_{UAG}*) sequence data obtained from a previously generated data matrix by Khoshsokhan-Mozaffar *et al.* (13). The main objectives of this study are: (1) to examine the character history of selected morphological characters, (2) to estimate divergence times and evolution within Eritrichiinae, and (3) to plot the radiation of Eritrichiinae using an lineage-through-time (LTT) approach.

Materials and methods

Taxon sampling

The dating analyses were conducted for a concatenate dataset (nrDNA ITS +*trnL-F*+*rpl32-trnL_{(UAG)}*) consisting of 46 species (one accession per species) of Eritrichiinae-Rochelieae that used in our pervious study (13). Four outgroup taxa including *Heterocaryum subsessile* Vatke, *H. szovitsianum* (Fisch. & C.A.Mey.) A.DC., *H. laevigatum* A.DC. and *Suchtelenia calycina* (C.A.Mey.) A.DC. as members of subtribe Heterocaryinae-Rochelieae were selected according to previous phylogenetic results (1,13).

Divergence time estimation

Molecular dating analyses were carried out in a Bayesian framework by employing an uncorrelated lognormal relaxed clock model in BEAST ver. 1.8.0 (21) in the CIPRES Science Gateway (http://www.phylo.org/; 22). BEAST allows for rate variation between lineages and infers tree topology, branch lengths, and nodal ages by using Bayesian inference and a Markov Chain Monte Carlo (MCMC) analysis. The divergence time was estimated based on secondary calibration from a previous study (19). Calibrations using the two previous published nodal ages modelled with a normal distribution, where 95% of the prior weight fell within the highest posterior density (HPD) interval in which each node was discovered in the original study. According to Chacon et al. (19), the crown node of Eritrichiinae was calibrated using a normal distribution prior with median=20.2 Mya, and the crown node of Rochelieae (i.e. root of the tree) was calibrated using a normal distribution with median = 27.8 Mya. After selecting a GTR+I+G substitution model for the concatenated data matrix, with four gamma categories, an uncorrelated relaxed-clock model and a Yule prior were applied to the specific Bayesian starting tree used as the tree prior. Four independent runs of 50×10^6 generations each were performed, sampling every 1000th generation. Optimal convergence between four runs and the amount of burn-in were verified in Tracer version 1.4 (23) by checking effective sample size (ESS) scores and consistency of the results between multiple runs. After discarding the initial 10% as burn-in, using Logcombiner 1.8.4 (21) the four runs were combined, and a maximum clade-credibility tree with a posterior probability limit of 0.5 was calculated using Tree Annotator v.1.8.4 (21), and the tree was visualized using FigTree v.1.3.1 (24). Moreover, the radiation lineage-through-time (LTT)

plots was carried out using the BEAST posterior tree implemented in R packages 'ape' (25).

Ancestral character state reconstruction

Ancestral states were reconstructed for the four discrete morphological characters, using maximum likelihood (ML) approach. Character selection followed several relevant literatures (4,5,12,14) that highlighted the importance of the characters in supporting or diagnosing taxonomic groups in Eritrichiinae, but avoiding characters showing high levels of homoplasy. A matrix was constructed for four discrete phenotypic characters scored using herbarium material (FUMH, TARI, TMUH): life form, corolla colour, stigma position, and nutlet size.

For subsequent ancestral character state reconstruction analyses, the dataset of 50 accessions (Fig. 1) was reduced to 31, including 29 Eurasian, and two North American species. Three possible rate models of discrete character evolution implemented in 'ace' function of the R package 'ape' (25) were applied: (1) equal-rates (ER), that implies one single rate model, (2) symmetrical rates (SYM) model, in which forward and reverse transition rates are equal, and (3) all rates different (ARD) model, in which backward and forward rates between states are permitted to have different values. To determine whether the use of the most parameterized model is appropriate, a likelihood ratio test (LRT) was performed, which recognized ARD as the best fit model in all reconstructions (Table 1). The best fit model for each trait and tree was then used to reconstruct ML ancestral states, using the default setting of 'anc' function phytools (26). The most likely character state of each node was collected across 100 trees, and were presented as pie charts at the relevant nodes. All analyses were performed in R package phytools (26, 27).

Table 1. Model testing (equal-rates model – ER, symmetrical rates model – SYM and all-rates-different model – ARD) for ancestral state reconstruction under the ML approach by means of Likelihood Ratio Test (LRT).

Trait	Trait Type	Models			LRT
		ER	SYM	ARD	
Life-Form	multistate	-42.17628	-32.96743	-25.81417	18.4177[1] 14.30652[2] 32.72422[3]
Corolla color	multistate	-25.29527	-22.80176	-16.68684	4.98702[1] 12.22984[2] 17.21686[3]
Stigma Position	binary	-20.50203	-20.50203	-19.88033	1.2434
Nutlets Size	binary	-18.35764	-18.35764	-18.12004	0.4752

[1]LRT (ER vs. SYM); [2]LRT (SYM vs. ARD); [3]LRT (ER vs. ARD).

Results

Divergence time estimation

The BEAST chronogram is consistent with those resulting from Bayesian analyses in our pervious study (13; Fig. 3). The former is represented in Fig. 1A with 95% highest posterior density (HPD) intervals associated with nodal heights for the nodes of interest (nodes A–L; Fig. 1A; see also Table 2).

The overall estimated mean ages and mean rates of evolution are presented in Table 2. The trace of the ESS and the parameters confirmed that the four runs were converged within 50 million generations, and the rate of evolution in Eritrichiinae node was 0.0013 substitutions per site per million years (s/s/Myr; 95% HPD: 0.0001-0.0036). The molecular dating analyses indicated that the split between Eritrichiinae and the Heterocaryinae clade occurred ca. 26.96 million years

Table 2. Analyses results of the of molecular dating (BEAST) of the tribe Rochelieae.

| Nodes	Description	BEAST analysis, mean nodal age (95% HPD)
A	Split of Eritrichiinae from its sister	26.69 Mya (22.99-34.54)
B	Initial diversification of Eritrichiinae	23.96 Mya (22.61-25.35)
C	Split of *Pseudolappula sinaica* from other species of Eritrichiinae	20.76 Mya (16.68-23.8)
D	Split of Eritrichium clade from other species of Eritrichiinae	17.18 Mya (12.99-21.02)
E	Split of *Lappula* s.s from Rochelia clade	14.98 Mya (10.87-18.91)
F	Origin of *Rochelia*	11.3 Mya (7.55-15.29)
G	Initial diversification of *Lappula* s.s.	10.68 Mya (7.09-14.64)
H	Diversification of *Lappula*	8.22 Mya (3.9-12.58)
I	Initial diversification North American *Lappula*	2.96 Mya (0.96-5.77)
J	Diversification of *Lappula*	4.81 Mya (2.69-7.41)
K	Diversification of *Lappula*	3.02 Mya (1.39-5.2)
L	Diversification of *Lappula*	3.79 Mya (1.86-6.14)
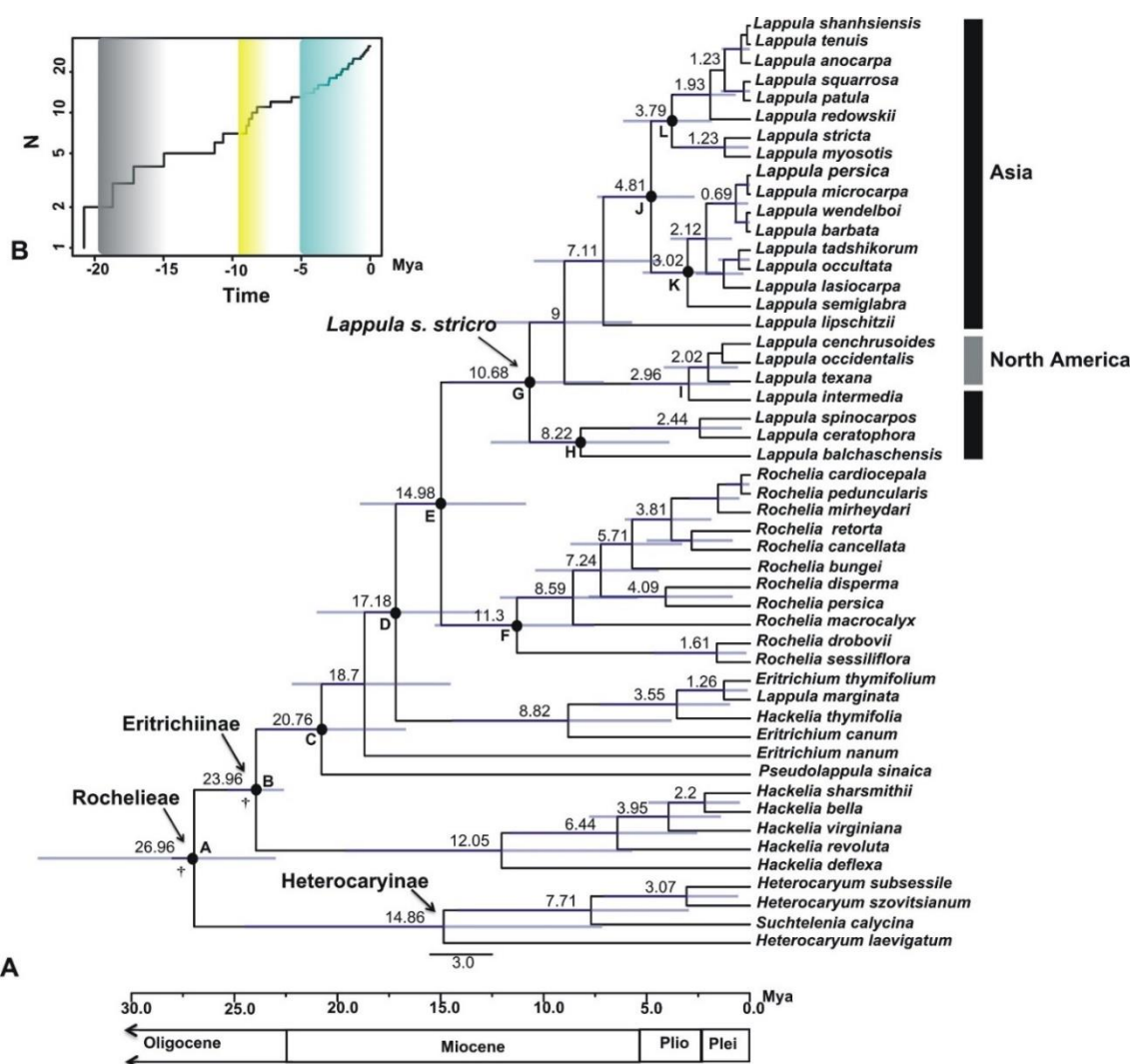
Figure 1. A. Chronogram inferred from Bayesian dating analysis (BEAST) of the 56 taxa data set including 46 accessions from Eritrichiinae and four accessions from Heterocaryinae. Blue bars represent the 95% highest posterior density intervals around mean nodal ages. The letters indicate nodes as used in Table 2. '†' indicates the secondary calibration points. B. Lineage-through-time (LTT) plot for the Eritrichiinae radiation in Asia. The sharp increase in branching rate, are indicated by gray, yellow and blue gradients.

ago (95% HPD 22.99–34.54Mya) in the middle Oligocene (node A; Fig. 1A; Table 2). The crown group of Eritrichiinae was estimated to date back to late Oligocene ~ 23.96 Mya (95% HPD 22.60–25.35 Mya) (node B; Fig. 1A, Table 2).

The lineages-though-time (LTT) plot gained from the BEAST chronograms, for the members of the subtribe Eritrichiinae is illustrated in Fig. 1B. The expected number of lineages versus time was used to characterise clade diversification as a function of time (28). The gradient of the curve of the LTT plot increases between about 20 and 15 Mya, 8 and 9 Mya, and an increase of the gradient in the curve is observed in the last 5 Mya (Fig. 1B).

Ancestral character state reconstruction
Most of diagnostic morphological features originated multiple times within Eritrichiinae. The character states of life form evolved ten times in Eritrichiinae. The most likely ancestral life form for Eritrichiinae was reconstructed as annual herb (21 species; Fig. 2A). The patterns of evolution of two characters, corolla colour and stigma position during fruiting are shown in Figs. 2B and 2C. The state 'stigma inserted' is evolved at least seven times in this subtribe whereas the 'white-blue' state of the corolla colour is evolved five times and the 'blue-purple' state twice. The nutlet character state, 'big-sized nutlets' appears at least three times (18 species; Fig. 2D) in Eritrichiinae.
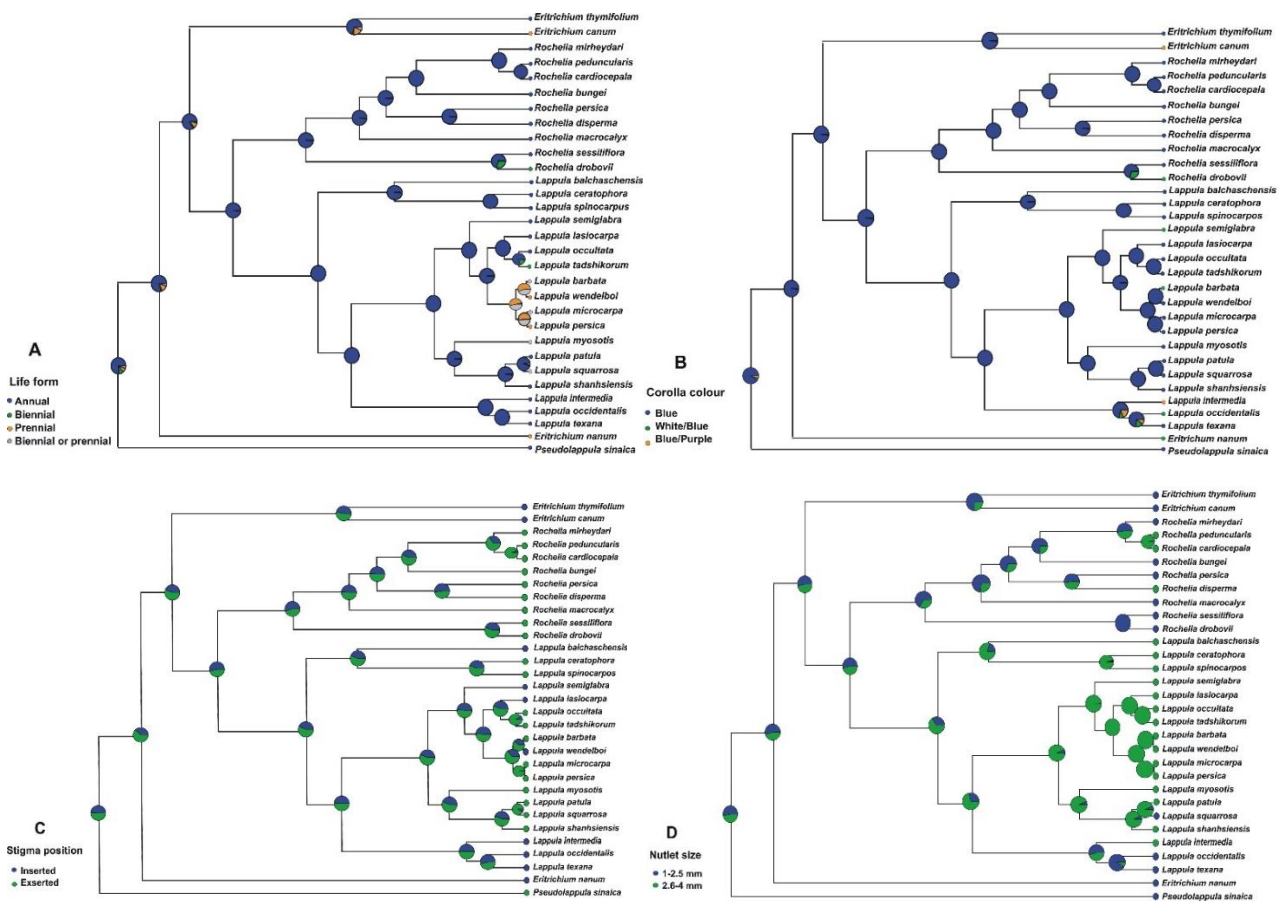
Figure 2. Ancestral state reconstruction for four key phenotypic traits of Eritrichiinae. Morphological character evolution of: A, Life form; B, Corolla colour; C, Stigma position; D, Nutlet size; optimised on a chronogram inferred from Bayesian dating analysis with one sample per species. Pie charts at internal nodes indicate the proportion of likelihood associated with the ancestral states of different morphological characters, based on the ARD model.

Discussion

Our molecular dating analyses suggest that Eritrichiinae diverged from its sister group Heterocaryinae in the middle Oligocene ~ 26.96 Mya (95% HPD 22.99–34.54 Mya) (node A; Fig. 1A; Table 2), experienced at least three major diversification events (Fig. 1B; the gray, yellow and blue gradients), and its initial

diversification took place 23.96 Mya in late Oligocene to Early Miocene (node B in Fig. 1A; Fig. 1B gray gradient; Table 2). This date is older than the previous reports (19) estimating this age by 20.2 Mya (95% HPD= 14.2–26.7). This incongruence is likely due to the rich sampling of Eritrichiinae in the present study. The first diverging within this subtribe is represented by the genus *Hackelia* and its diversification extended from the middle-late Miocene to Pliocene and early Pleistocene (Fig. 1A). Then *Lappula sinaica* (A.DC.) Asch. & Schweinf. that was treated as *Pseudolappula sinaica* in our previous study (13) is evolved 20.76 Mya in the early Miocene (node C; Fig. 1A).

Concerning paleoclimates, the origin and diversification of Eritrichiinae could have been affected by some significant events including global aridification and cooling, as well as deposition of hundreds of meters of dust sediments under influence of the dry winter Asian monsoon, beginning at the Oligocene-Miocene boundary and reinforcement thereafter (29, 30). Also, the retreat of the Paratethys Sea in western Central Asia during the Late Oligocene to Early Miocene and the uplift of the Tibetan-Himalayan plateau, have played a major role in the change of the Central Asian climate from oceanic to continental (31), and would have been as the source of increasing level of aridity (30). These events occurred concomitantly with the origin and initial diversification within Eritrichiinae (nodes A, B, C, D; Fig. 1A, B gray gradient). Probably, species of Eritrichiinae were distributed in other latitudes by animal-dispersed nutlets (Epizoochory) (19).

Likewise, annual life strategy may have evolved in common ancestor of the Eritrichiinae in Asia in response to increasing aridity (30,32). With increasing continentalization at the Middle-late Miocene, the formation of the Aegean Sea, and the tectonic uplift and variation in the amount of aridity due to the Mountains uplift (e.g. Carpathians, Alborz and Zagros) close to the Mediterranean area as well as Iranian plateau or between the Iranian plateau and Central Asia (Pamirs and Hindu Kush) caused the climate in Central Asia drying to an increasing extent (30,33-37). These events caused a new geological/climatic configuration in the area in the late Miocene (38-39). These events occurred concomitantly with the diversification within Eritrichiinae in

the region during this period (nodes E, F (*Rochelia* clade), G and H (*Lappula* s.s clade); Fig.1A, B yellow gradient). These results support several Miocene dispersals events (back and forth into the Irano-Turanian region) that were inferred for Eritrichiinae by Chacon *et al.* (19). Divergence bet-ween common ancestors of *Rochelia* and *Lappula* s.s clades took place in middle Miocene (node E; Fig. 1A). *Lappula drobovii* (Popov) Pavlov, Lipsch. & Nevski and *L. sessiliflora* Gürke which were treated as the species of *Rochelia* in our previous study (13) diverged from other nine species of *Rochelia* 11.3 Mya in the late Miocene (node F; Fig. 1A). All members of *Rochelia* clade do share the common feature: stigma exserted above the nutlets (Fig. 2C).

Diversification events within *Lappula* s.s clade in Asia (Fig. 1A; nodes G, H, J, K, and L) took place at different time periods from the late Miocene to the late Pleistocene. The origin of node H (Fig. 1A) comprising *L. balchaschensis* Popov ex Pavlov, *L. ceratophora* (Popov) Popov and *L. spinocarpos* (Forssk.) Asch. ex Kuntze dates back to 10.68 Mya (late Miocene). Then *L. balchaschensis*, as the closet species to *L. ceratophora* and *L. spinocarpos* (4,7,13) was diversified 8.22 Mya (Fig. 1A). These three species are similar in having annual life-form, blue corolla and equal-sized nutlets (Figs 2B, D). Four species *Lappula intermedia* (Ledeb.) Popov, *L. texana* (Scheele) Britton, *L. occidentalis* (S.Watson) Greene and *L. cenchrusoides* A.Nelson that share two common features: annual life-form and exerted stigma (Figs 2A, C), diverged around 9 Mya (node I; Fig. 1A). *Lappula intermedia* is a widespread (Asia, Europe, East Siberia to western North America) and transitional form between *L. stricta* (Ledeb.) Gürke (from Asia) and *L. echinata* Fritsch (from North America) (4,7,13).

The split between Asian and American clades could be the result of LDD or migration via Bering land bridges that continuously linked the continents on the northern hemisphere in the Miocene (40-42). Since Epizoochory dispersal were inferred for Eritrichiinae by Chacon *et al.* (19), birds could serve as a dispersal vector and lead to long-distance dispersal (43-44). It seems that *L. intermedia* became physically separated in the Late Miocene after establishing the Bering land bridge (40), diversified from its Asian ancestor, and

then the American species of *Lappula* were derived from it 2.96 Mya in the late Pliocene (Fig. 1A; node I). A central Asian species, *L. lipschitzii* Popov, diverged from remaining species 7.11 Mya in the late Miocene (Fig. 1A). Around 4.81 Mya, divergence between common ancestors of two distinct clades (node J; Fig. 1A) took place coincidentally with the tectonic reorganization and further uplift of mountain range in Asia. The geological and tectonic events might have caused the formation of a land corridor among the Hyrcanian, Central, eastern and western sides of the Iranian plateau that provided the routes for the expansion and diversification of Eritrichiinae (nodes J, K, L) and then the diversification extended to late Pleistocene (Fig. 1B blue gradient) followed by climate modifications and different mountain systems in the Pleistocene which caused aridification and a cooler climate in Central Asia and Central Iran during the Pleistocene (45-48). These events might have provided the ecological opportunity required for the diversification of some species within nodes K and L (Fig. 1A). Node L comprising eight Central Asian species that are similar in having exerted stigma (Fig. 2C), started to diversify 3.79 Mya (Fig. 1A). Node K with eight species sharing equal-sized nutlets (2.5-4 mm; Fig. 2D) evolved 3.02 Mya, in the middle Pliocene.

Acknowledgments

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Editorial Note

Volume 7, issue 2 of Progress in Biological Sciences was initialy scheduled to be published in December 31, 2017. However, some administrative changes leaded to a major delay in processing of the manuscripts. This issue is actually published in May 1, 2020. Editor-in-chief apologizes deeply for any inconvenience caused especially to the authors.