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PHYLOGENETIC RELATIONSHIPS WITHIN FERULA SECT. MERWIA (APIACEAE- FERULINAE) INFERRED FROM nrDNA AND cpDNA MARKERS

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Ferula L., as a large genus in the Umbelliferae with 180-185 species, is distributed only in the Old World. There are 25 mostly medicinal species within the *Ferula* sect. *Merwia*, of which 14 species are distributed in Iran. A molecular phylogenetic study of the Iranian species using two nrDNA (ITS and ETS) and two cpDNA (*rpL32—trnL*$^{(UAG)}$ and *rps16— trnK*$^{(UUU)}$) markers was undertaken to determine the phylogenetic relationships among species and evaluate their potential value in taxonomic treatment of the genus. The Maximum Likelihood and Bayesian Inferences trees revealed that the section contained three geographically separated subclades. Also, *F. assa-foetida* has appeared as two geographic ecotypes in Iran; one group contains the populations distributed in lower altitudes in the Centre of Iran and the other includes the populations from higher altitudes in Zagros Mt. in the south of Iran. *Ferula alliacea* is placed close to the eastern elements of the sect. *Merwia*. Similarly, the position of *F. lutensis* as well as *F. latisecta* is confirmed within the section. The used cpDNA regions could not define the boundaries of infraspecific taxa of *Ferula* species such as *F. assa-foetida* within the sect. *Merwia*, while they contributed to separating different species of other sections of the genus and also allied genera (*Leutea*). Accordingly, the most informative region was the ETS nrDNA which may contain more nucleotide substitution. In our analyses, *Leutea turcomanica* is located close to the other *Leutea* species.

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Keywords: ETS; *Ferula*; ITS; Iran; Sect. *Merwia*; Medicinal plants; Phylogeny

تعیین روابط فیلوژنتیک درون بخش Merwia از جنس Ferula (Apiaceae- Ferulinae) توسط نشانگرهای مولکولی هسته‌ای و کلروپلاستی

مهرنوش پناهی: استادیار پژوهش، موسسه تحقیقات جنگلها و مراتع کشور، سازمان تحقیقات، آموزش و ترویج کشاورزی، تهران، ایران

جنس کما (Ferula) یکی از بزرگترین جنس‌های تیره چتریان، با حدود ۱۸۰–۱۸۵ گونه منتشر شده در دنیای قدیم است. بخش Merwia در جنس Ferula طبق مطالعات اخیر، با ۲۵ گونه به عنوان یک بخش حاوی گونه‌های مهم دارویی معرفی شده است. ۱۴ گونه از این بخش در ایران گسترش دارد که توسط داده‌های مولکولی هسته‌ای (دو ناحیه ITS و ETS) و کلروپلاستی (دو ناحیه فضای بین ژنی rpL32—trnL$^{(UAG)}$ و trnK$^{(UUU)}$—rps16) به منظور تعیین موقعیت دقیق تاکسونومیک و روابط فیلوژنتیکی گونه‌های درون بخش مورد بررسی قرار گرفت. درختان حاصل از آنالیز حداکثر شباهت (ML) و بایزین (BI) نشان داد که این بخش دربرگیرنده سه گروه متمایز جغرافیایی است که از نظر خواص دارویی نیز تا حدودی متفاوتند. همچنین گونه F. assa-foetida به صورت دو اکوتیپ در ایران ظاهر می‌شود؛ یک گروه جمعیت‌های بخش مرکز ایران پراکنده در ارتفاعات پایین، و گروهی دیگر جمعیت‌های مربوط به ارتفاعات بالاتر در ناحیه زاگرس (جنوب ایران) می‌باشد. گونه F. alliacea در گروه عناصر شرقی بخش Merwia قرار می‌گیرد. جایگاه دو گونه F. lutensis و F. latisecta درون بخش Merwia تعیین می‌گردد. در این مطالعه نواحی کلروپلاستی

نتوانست تفکیک افراد فروگونه‌ای بطور مثال در گونه F. assa-foetida در این بخش را مشخص نماید در حالی که برای تفکیک سایر گونه‌های این جنس و جنس نزدیک (Leutea) موثر بودند. علاوه بر این، ناحیه هسته‌ای ETS به عنوان بهترین ناحیه حاوی اطلاعات مولکولی مشخص می‌گردد. موقعیت تاکسونومیک گونه Leutea turcomanica از نظر فیلوژنی نیز در جنس مذکور تایید می‌گردد.

INTRODUCTION

The Eurasian genus, *Ferula* L. with about 180-185 species, has a vast distribution pattern from the west of the Mediterranean region, Central Asia to the East in west China (Kadereit & Bittrich 2018). In the Flora Iranica area, it is represented by 53 species with 33 endemics of which, 34 species and 15 endemics occurred in Iran, mostly distributed in the mountainous regions such as the Alborz, Zagros, and Khorassan Mts. (Chamberlain & Rechinger 1987; Mozaffarian 2007).

Conventionally, the genus *Ferula* is illustrated through morphological aspects in the literature and characterized as monocarpic or polycarpic herbaceous plants often with onion scent. Due to the large size of most species and the unavailability of the complete herbarium specimens that often contain only some lateral branches and lateral divisions of basal leaves (Chamberlain & Rechinger 1987), the determination of *Ferula* species is difficult and sometimes may result in misidentification. However, substantial morphological variation in the genus has resulted in the description of multiple nearly indistinguishable species, which are sometimes represented in herbarium collections by a few, poorly preserved specimens.

This genus was traditionally classified in the tribe Peucedaneae Dumort. (Pimenov & Leonov 1993). However, recent phylogenetic studies using nrDNA ITS sequence variation revealed that *Ferula* is placed with allied genera; *Dorema* D.Don and *Leutea* Pimenov in the tribe Scandiceae Spreng., subtribe Ferulinae Engl. (Kurzyna-Młynik & al. 2008). Later with three non-coding plastid DNA regions, *Leutea* was determined in a sister position with *Ferula*, whereas *Dorema* was nested within it and consequently was merged with *Ferula* (Panahi & al. 2015).

Korovin (1947) subdivided *Ferula* into six subgenera and nine sections mainly based on habit and vegetative features. However, in a subsequent revision of *Ferula* for the flora of Kazakhstan, Safina and Pimenov (1984) rejected Korovin's subgenera and recognized 12 sections instead. A recent molecular phylogenetic study of *Ferula* has not supported the previous treatments and instead proposed a new classification framework with four subgenera and ten sections (Panahi & al. 2018).

Ferula subgenus *Merwia* (B. Fedtsch.) Korov., which was established with three sections (sect. *Saprosmia* Korovin, sect. *Phacocarpa* Korovin, and sect. *Discicarpa* Korovin) introduced with 12 species distributed in the deserts and dry mountain belt of SW Asia (Middle Asia, Iran, Afghanistan), (Korovin 1951). In the Flora Iranica, Chamberlain and Rechinger (1987) considered only Korovin's subgenera of *Ferula* but not the sections within the subgenera. They introduced the subgenus *Merwia* with 21 species (Chamberlain and Rechinger 1987). *Ferula litwiniwiana* Koso-Pol. (= *Merwia androssowii* B. Fedtsch.) was determined as the type of *Ferula* subgen. *Merwia* (Korovin 1947). Safina & al. (2014) divided this subgenus into six carpological groups. She indicated that *F*. subgen. *Merwia* is not a monophyletic taxon and appears to be an artificial aggregate of several small groups of species (probably sections) with few common characters. The new proposed classification of *Ferula* (Panahi & al. 2018) is not in agreement with her divisions. For example, two species *F. persica* Willd. and *F. foetida* (Bunge) Regel, which had been recognized within the *F*. subgen. *Merwia* (by resemblance of the fruits), belong to *F*. sect. *Merwia* and sect. *Scrodosma*, respectively (Panahi & al. 2018). Hence, the homoplastic features in the morphology and anatomy of fruits could not separate the infrageneric divisions. Additionally, the chromosome number studies have not determined any essential differences among various taxa within *Ferula* (with 2n =22, Pimenov & al. 2003).

According to recent phylogenetic studies, *Ferula* was classified phylogenetically into four subgenera and ten sections, of which *F*. subgen. *Narthex* (Falc.) Drude was divided into eight sections (Panahi & al. 2018). *Ferula* sect. *Merwia* (B. Fedtsch.) Koso-Pol. in the subgen. *Narthex* is designated with 25 mostly medicinal species. However, the geographical distribution pattern of the section revealed its heterogeneity within the Flora Iranica area (Panahi & Mahmoodi 2021). This heterogeneity was already indicated by phytochemical studies (Pimenov & Skljar 1988) and recently confirmed that organosulfur components (thiophenes, disulfides, and trisulfides) are characteristics for both sections *Merwia* and *Scorodosma* in *F*. subgen. *Narthex* (Panahi & al. 2020). For example, the volatile oil of *Ferula behboudiana* (Rech.f. & Esfand.) D.F. Chamb which is restricted to western Iran, contains two disulphide derivatives

(Yousefi & al. 2011) that phytochemically is closely related to Asafoetida species group.

This study was conducted using four molecular markers (two nrDNA spacers and two intergenic spacers of cpDNA), that are the most popular among phylogenetic studies of angiosperms (Shaw & al. 2014), to reach a new classification and taxonomic delineation of members of the genus *Ferula* sect. *Merwia*. Two informative noncoding cpDNA regions (*rpL32—trnL*$^{(UAG)}$ and *rps16—trnK*$^{(UUU)}$ intergenic spacers) plus two nrDNAs (*ITS*; Internal transcribed spacer and *ETS*; External transcribed spacer) were selected. Shaw & al. (2014) determined these cpDNA regions among the top 10 regions as the most informative regions observed across 25 species of major clades in the angiosperms. These regions (*rpL32—trnL*$^{(UAG)}$ and *rps16 — trnK*$^{(UUU)}$) were highly variable for inter- and intraspecific studies (Calvino & Downie 2007; Shaw & al. 2014; Downie & Jansen 2015). In Apiaceae, these regions have been proven to be useful as well that were more variable and provided more parsimony informative characters than ITS region (Spalik & al. 2009; Liao & al. 2021; Downie & Jansen 2015). Although the internal transcribed spacer (ITS) region of nrDNA is the most commonly used molecular marker in phylogenetics, especially among closely related taxa at intergeneric and interspecific levels (Soltis & Soltis 1998), the ETS region could help to identify the closely related species as well (Logacheva & al. 2010; Puchałka & al. 2023). Therefore, we decided to employ these intergenic spacers to understand the species relationships within *F*. sect. *Merwia* in Iran, especially among the medicinal species of Asafoetida group (*F. assa-foetida* L., *F. alliacea* Boiss. and *F. gabrielii* Rech. f.) and determine the true related species within *F*. sect. *Merwia*.

MATERIAL AND METHODS

Taxon sampling

A total of 14 species (37 accessions) of *F.* sect. *Merwia* and three species *F. diversivittata* Regel & Schmalh., *F. foetida* (Bunge) Regel and *F. ovina* Boiss. from other sections of *Ferula*, plus three *Leutea* species including *L. cuparis* (Boiss.) Pimenov, *L. polyscias* (Boiss.) Pimenov and *L. turcomanica* (Schischk.) Mozaff. as well as two outgroups of the subtribe Daucinae (*Polylophium involucratum* (Pall.) Boiss. and *Laserpitium carduchorum* Hedge & Lamond) were selected for this aim. The plant materials were collected from the field and also herbarium specimens (voucher information and references were given in Appendix 1). The collected samples were identified through Flora Iranica (Chamberlain & Rechinger 1987) and Flora of Iran (Mozaffarian 2007).

Laboratory procedures

Genomic DNA was extracted from c. 20 mg of dried plant material. The tissue was submerged in liquid nitrogen while disrupting with a mortar and pestle, and then the prepared fine powder was used with DNA extraction kit (Sinaclon Co., Iran) based on CTAB protocol following the manufacturer's instructions. The extracted DNA was used for PCR amplification for nuclear and chloroplast regions. The PCR was performed using ready-to-use MasterMix buffer and QIAGEN PCR Kit following the protocol. Each reaction (100 µL) contained 47.60 µL of distilled water, 20 µL of Q Solution 5x, 10 µL of 10x Taq polymerase reaction buffer, 1 µL of 200 µmol/L of each dNTP solution, 1.5 mmol/L of $MgCl_2$ (optimized for different DNA samples and primers), 1.6 Units of Taq DNA polymerase (5 units/µL), 1.0 µmol/L of each primer and 1.0 µL DNA template. Sometimes to get results, it was necessary to modify the reaction conditions (e.g., increasing the $MgCl_2$ concentration, and diluting in the proportion 1:10 the template DNA concentration).

The ITS region was amplified using primers '*N-nc18S10*' and '*C26A*' (Wen & Zimmer 1996). For the amplification of the ETS region, two forward and reverse primes: '*18S-ETS*' and '*Umb-ETS*' according to Logacheva & al. (2010) protocol were used respectively. The cpDNA regions including two intergenic spacer regions: *rpL32—trnL* and *rps16—trnK* were amplified with the forward and reverse primes: '*rpL32-F*'/ '*trnL*$^{(UAG)}$' and '*rps16 x2F2*'/ '*trnK x1*' respectively (Shaw & al. 2007; Shaw & al. 2014). The PCR conditions for each region are provided in Table 2. To check the PCR products, an aliquot of the reaction sample was electrophoresed in a 1% agarose gel based on a TAE buffer and stained with the DNA-safe stain (Sinaclon Co., Iran).

Sequencing was performed using Big Dye terminators (Applied Biosystems, Foster City, CA, USA) in the laboratories of Pishgam Biotechnology Inc. in Tehran. Both DNA strands across the entire long cpDNA regions were sequenced to avoid ambiguity in base determination. The chromatographs were assembled and edited using SeqMan Pro ver. 12 (Dnastar, Madison, WI, USA). Boundaries of the exon and intron regions were determined by comparisons to the corresponding boundaries in another species of Apiaceae. The flanking and presumably more conserved exon regions of the spacers were not included.

Table 1. The primers and PCR conditions used for nrDNA and cpDNA markers.

Marker	Primers	Sequence positions of forward/ reverse primers (5´-3´)	PCR conditions	Reference
ITS	*N-nc18S10*/ *C26A*	5´ AGG AGA AGT CGT AAC AAG 3´/ 5´ GTT TCT TTT CCT CCG CT 3´	95˚C - 1 min; 94˚C – 1 min; 50-53˚C – 1 min; 72˚C – 1 min; 72˚C – 10 min	Wen and Zimmer, 1996
ETS	*18S-ETS*/ *Umb-ETS*	5´ ACT TAC ACA TGC ATG GCT TAA TCT 3´/ 5´ GCG CAT GAG TGG TGA WTK GTA 3´	94˚C - 3 min; 94˚C – 30 s; 57-60˚C – 30 s; 68˚C – 6 min; 68˚C – 10 min	Logacheva & al. 2010
rpL32—trnL	*rpL32-F*/ *trnL*$^{(UAG)}$	5´ CA GTT CCA AAA AAA CGT ACT TC 3´/ 5´ CTG CTT CCT AAG AGC AGC GT 3´	94˚C - 3 min; 94˚C – 1 min; 51-52˚C – 1 min; 72˚C – 1 min; 72˚C – 10 min	Shaw & al. 2007
rps16—trnK	*rps16 x2F2*/ *trnK x1*	5´ AAA GTG GGT TTT TAT GAT CC 3´/ 5´ TTA AAA GCC GAG TAC TCT ACC 3´	95˚C - 3 min; 94˚C – 1 min; 52˚C – 1 min; 72˚C – 1 min; 72˚C – 10 min	Shaw & al. 2007

Phylogenetic analyses

DNA sequences were initially aligned in MAFFT (Katoh & al. 2019) and edited manually using Mesquite 3.6 (Maddison and Maddison, 2017) if necessary. In the phylogenetic analyses, all gaps were treated as missing data. In cpDNA regions many indels made the alignment problematic, these ambiguous regions such as long autapomorphic indels excluded from subsequent analyses. Finally, all data matrices were trimmed in trimA1 using the PhyloSuite program (Xiang & al. 2023).

Three concatenated data sets (I, II, III) were prepared as I: nrDNA data contain ITS and ETS sequences; II: plastid DNA contain two intergenic spacer sequences (*rpL32—trnL* and *rps16— trnK*) and III: combined nrDNA and cpDNA sequences. In addition, to compare and determine the species relationships, a matrix of ITS data with our previously released data (Panahi & al. 2015; Panahi & al. 2018) was prepared to analyze, simultaneously. The Congruence of the datasets (cpDNA and nrDNA) was assessed using a hierarchical likelihood ratio test (hLTR) implemented in Concaterpillar ver. 1.7.2 (Leigh & al., 2008). Phylogenetic analyses were performed using the Maximum Likelihood (ML) and Bayesian inference (BI) methods using the PhyloSuite program (Xiang & al. 2023). Furthermore, ML analyses were performed on the IQ-tree website (Nguyen & al. 2015). The used method was performed as default (1000 Bootstrap with Ultrafast in 100 searches) and the inferred trees were compared. ML analyses using PhyloSuite included 5000 Bootstrap numbers in Ultrafast and branch support (BS) was evaluated based on 1000 rapid bootstrap replicates. Substitution models were inferred with ModelFinder implemented in PhyloSuite for nrDNA and cpDNA markers separately using the corrected Akaike information criterion (AICc) (Kalyaanamoorthy & al. 2017). The analysis of nrDNA data was performed with SYM +G +I substitution models as selected by ModelFinder and GTR +G for plastid and combined data as implemented for ML analyses. All analyses were run with branch lengths Edge-Linked among partitions. Ultrafast bootstrap approximation results were presented in the consensus tree.

In BI analyses, two independent runs were executed simultaneously, with four Monte Carlo Markov chains with 10000000 generations and a sampling frequency of 1000 generations with state frequency as fixed (empirical). The initial 25% of saved trees were discarded as burn-in and the results were summarized on the 50% majority rule consensus tree. GTR+G model was selected for the Bayesian method. The convergence of the independent runs and effective sample size (ESS) for estimated parameters were checked using Tracer v.1.7.1 (Rambaut & al. 2018).

RESULTS

Sequences and matrices obtained

The GenBank accession numbers of all obtained sequences are represented in Appendix 1. For the ITS region all the samples (45 accessions) were sequenced. For the ETS region, 3 accessions failed to get sequences (no. 9847, 9804, and 9817 without PCR results even after trying several times). Overall, the two cpDNA markers; *rpL32—trnL* and *rps16—trnK* intergenic spacers were sequenced for 41 and 43 accessions respectively, and the remaining ones failed to get results. In both ITS and cpDNA datasets, multiple accessions of most taxa were considered to ascertain their precise taxonomic position (for example *F. assa-foetita*, *F. pseudalliacea* Rech.f., *F. alliacea* and *F. gummosa* Boiss. as represented in Appendix 1). For those accessions that we failed to sequence ETS and plastid markers, we excluded them from the final analyses.

Sequence characteristics

Sequence characteristics of the nrDNA and cpDNA regions are provided in Table 2. The length of the entire ITS region among 45 accessions ranged from 597 to 610 bp. The total number of aligned positions was 628 bp, with 25 unambiguous gaps (24 mononucleotide repeat (1 bp) and 1 deletion (3 bp) in *Leutea* and outgroup taxa). The number of parsimony-informative positions was 57 (Table 2). Sequence characteristic of the ETS region is provided for 43 sequences with 457 bp in length (Table 2). This region contains 11 gaps as one and two bp repeat nucleotides and one bp indels that are frequently observed within the data matrix.

The *rpL32—trn*L intergenic spacer ranges from 982 to 1061 bp in length. This region contains 85 parsimony informative positions and long indels and repeats that were trimmed and excluded from the matrix (998 bp length after trimming). A 36 bp gap (long indel) was detected in three *Leutea* species and the remaining ones comprising autapomorphies and long uninformative repeats were 1-22 bp among the specimens (for example, the deletion 22 bp only in accession No. 9834 or one repeat 6 bp in three *Leutea* species and two outgroups).

The length of *rps16—trnK* intergenic spacer among 43 samples was 819 bp. The number of indels in this region was less than *rpL32—trnL* region and the number of constant positions was more (89.6 % of all sites) than *rpL32—trnL* (83.9 % of all sites). The longest insertion was detected in sample 9820 (19 bp in length), and the longest deletion (16 and 26 bp) was observed in outgroup samples (No. 9823 and 9824). The number of mononucleotide gaps (repeat) within plastid sequences was less than nrDNA and specifically ETS, and in contrast, the number of transition/transversion substitution sites was more frequent within the ETS region (with 71 parsimony informative positions, Table 2). Only the sequences of 40 accessions are included in the combined matrix.

Table 2. Sequence characteristics of the nrDNA and cpDNA regions, separately and combined. The number in parentheses refers to the trimmed data sets.

Sequence characteristic	ITS	ETS	rpL32—trnL spacer	rps16—trnK spacer	rpL32—trnL+ rps16—trnK	Combined
Length variation (in bp)	628 (603)	457 (443)	1131 (998)	819 (733)	1950 (1714)	3039 (2713)
No. of constant positions	464	247	838	657	1498	2279
No. of parsimony informative positions	57	71	85	38	118	229
Number of distinct site patterns	123	177	206	104	258	395

Phylogenetic analyses

The concatenation test of plastid DNA and nrDNA data sets was rejected with $P < 1 \times 10^{-6}$. Trees obtained from ML and Bayesian analyses were similar in topology, so the consensus tree inferred from ML analysis was shown here. Two outgroups of subtribe Daucinae comprising *Laserpitium carduchorum* and *Polylophium involucratum* have a sister position to Ferulinae (Fig. 1). Recently, through phylogenetic studies on subtribe Daucinae, the two mentioned species were transferred to the genus *Laser* Borkh. and named under *Laser carduchorum* (Hedge & Lamond) Wojew. & Spalik and *Laser involucratum* (Pall. ex Schult.) Spalik & Wojew., respectively (Banasiak & al. 2016).

In the phylogenetic combined trees, the *Leutea* group has a sister position with *Ferula* as indicated in the last studies. *Leutea turcomanica* (Schischk.) Mozaff., which was reported from Shah Jahan Mt. (Khorassan) is sequenced here for the first time and is placed closely with the two other *Leutea* species with high support (Bp=100/pp=1 in Fig. 1).

Ferula diversivittata forms an isolated branch in all analyses as a member of the previously represented section (*Pachycarpa*, Panahi & al. 2018), (Figs. 1 & 2). *Ferula foetida* indicated as representer of sect. *Scrodosma* was placed in a sister position with *F*. sect. *Merwia*. It is striking that *F. ovina* as a member of sect *Peucednoides* placed as a sister clade with specimen 9844 of *F. alliacea* (Figs. 1 & 2). The latter specimen has distinct linear leaf lobs, distributed in Kashmar (Chalpu village) and phylogenetically represents close affinity to the members of the previous genus *Dorema* (close to *Ferula hyrcana* (Koso-Pol.) Puchałka, Spalik, Panahi & Piwczyński based on ITS analysis). The sample 9844 with linear to lanceolate leaf lobs shows a similarity in leaves with *Ferula ammoniacum* (D.Don) Spalik, M.Panahi, Piwczyński & Puchałka, but the inflorescence structure is different (compound umbel with hermaphrodite flowers). It seems that this specimen may be a new species. To investigate its independent position, more sampling for completing morphological studies is necessary.

Within *F*. sect. *Merwia* three subclades (A, B, C) are represented that contain three groups based on nuclear and combined data sets (I, III). Plastid data could not separate these groups and just separated the genus *Leutea* from *Ferula*, so the tree is not shown. The first subclade "**A**" contains eastern members of *F*. sect. *Merwia* such as *F. karakalensis* Korovin, *F. flabelliloba* Rech.f. & Aellen, *F. gabrielii*, *F. hirtella* Boiss., *F. szowitsiana* DC., *F. alliacea* and some accessions of *F. assa-foetida* from the lower altitudes growing in desertic condition. *Ferula lutensis* Rech.f. placed closely with them with high support (Fig. 1) that confirmed its position within *F*. sect. *Merwia*.

Ferula szowitsiana with three accessions from different locations in Iran (Khorasan, Azerbaijan, and Golastan Provinces) placed in this subclade. For this species, unfortunately, accession No. 9817 failed to sequence for ETS and plastid DNA regions so in the combined tree only two remaining specimens were included that were placed close to *F. hirtella* (Fig. 1). *Ferula szowitsiana* as an Irano-Turanian species, represents diversity in its populations based on nrDNA tree (Figs. 2 & 3). Different specimens of *F. szowitsiana* have not been placed closely (Fig. 3). This event could be affected by the vast geographic distribution of its populations from west to east of the Irano-Turanian region (Panahi & Mahmoodi 2021).

F. alliacea with three accessions reported from different locations (No. 9813 from Neyshabour to Kashmar, No. 9839 from Bezgh village, and No. 9844 from Chalpu) of Khorasan Province shows different positions. The accession No. 9844 indicated here as a new species or local hybrid, is related to the previous genus *Dorema* and *F*. sect. *Peucedanoides* members (Figs. 1 & 2). Two remaining accessions are placed in subclade A close to Khorasanian elements such as *F. gabrielii* and *F. karakalensis* (Fig. 1) that confirmed their position within the subclade A of *F*. sect. *Merwia*. In subclade A, the four accessions of *F. assa-foetida* were observed (No. 9832, 9833, 9834 and No. 9851 as cultivated sample) that separated from the other populations and grouped with khorasanian elements such as *F. flabelliloba* (Fig. 1).

The second subclade "**B**" includes several species from the south of Iran (Zagrossian elements), several *F. assa-foetida* accessions from the south of Iran, and closely allied *F. pseudalliacea* accompanied by *F. behboudiana* and one accession of *F. persica* (Bp=96/pp=1, Fig. 1). *Ferula sharifii* Rech.f. & Esfand. is also placed within this subclade (Fig. 2). *F. pseudalliacea* as Zagrossian element with more western distribution than *F. assa-foetida*, has a distinct position using nrDNA data (Figs. 2 & 3). The concatenated data set III could define the populations of *F. assa-foetida* from south of Iran (Hormozgan, Fars Provinces) and west Iran (Chaharmahal & Bakhtiari Province), that are distributed in higher altitudes (mountainous area) from the populations of Central Iran (Yazd, Kerman and Naien of Isfahan Provinces) distributed in lower altitudes which grouped with the eastern species (*F. flabelliloba*) in subclade A (Figs. 1 & 3). It seems that *F. assa-foetida* is differentiated into two ecotypes growing in different ecological conditions with variability in the morphology of leaves (from small to large leaf lobes). It is suggested that these ecotypes should be considered as two subspecies. Central ecotypes from lower altitudes with longer leaf lobes differ from the southern ecotypes (Zagrossian) of higher altitudes with shorter leaf lobs. After checking the nrDNA sequences of these specimens, the genetic differences were observed, and specimens No. 9832, 9834, and 9833 represented the same nucleotide substitutions while the other specimens No. 9830, 9831, 9806, and 9807 have similar nucleotide substitutions with the type specimen (No. 0359 from Lar, Fars Province). In this study, two reported varieties of *F. persica* (*F. persica* var. *persica* No. 9850 and *F. persica* var. *latisecta* No. 9849) have separated phylogenetically. The accession No. 9849 grouped closely with subclade B, while the accession No. 9850 has a distinct position without affinity to any subclades (Fig. 1). Therefore, their differentiation with respect to their disjunct distribution (Panahi & Mahmoodi, 2021) should be studied more.

Subclade "**C**" mostly comprises *F. gummosa* and its allies that contain Galbanum (oleo-gum) resin (see Fig. 3). *F. latisecta* Rech.f. & Aellen with two accessions placed in subclade C (Figs. 1 & 2) and its position within *F*. sect. *Merwia* is confirmed.

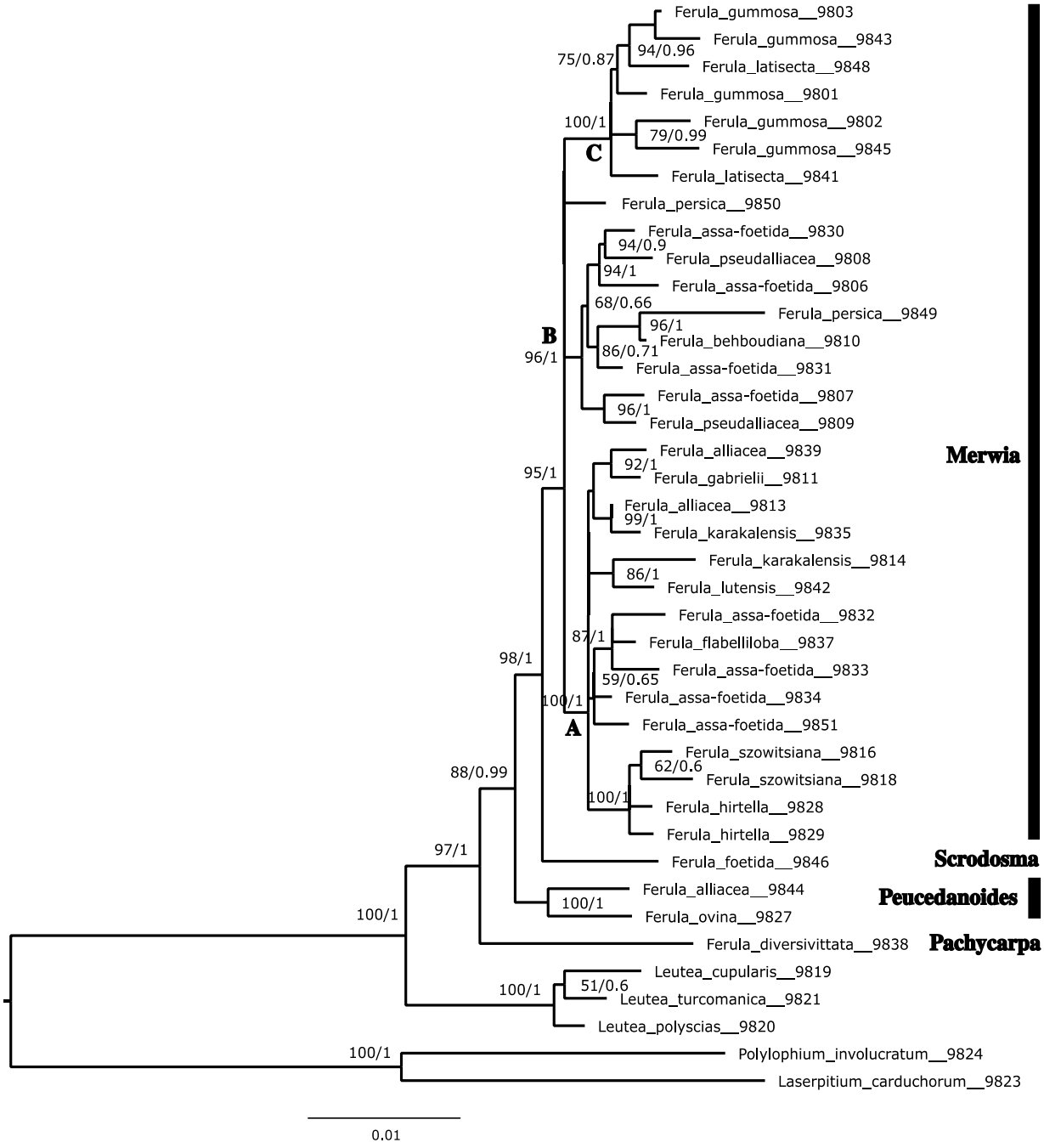

Fig. 1. The maximum likelihood tree of 40 representatives of *F*. sect. *Merwia* with *Leutea* and 2 outgroups inferred from analysis of combined data (set III). Bootstrap support (left) and posterior probability (right) of the Bayesian 50% majority-rule consensus tree are given along branches.

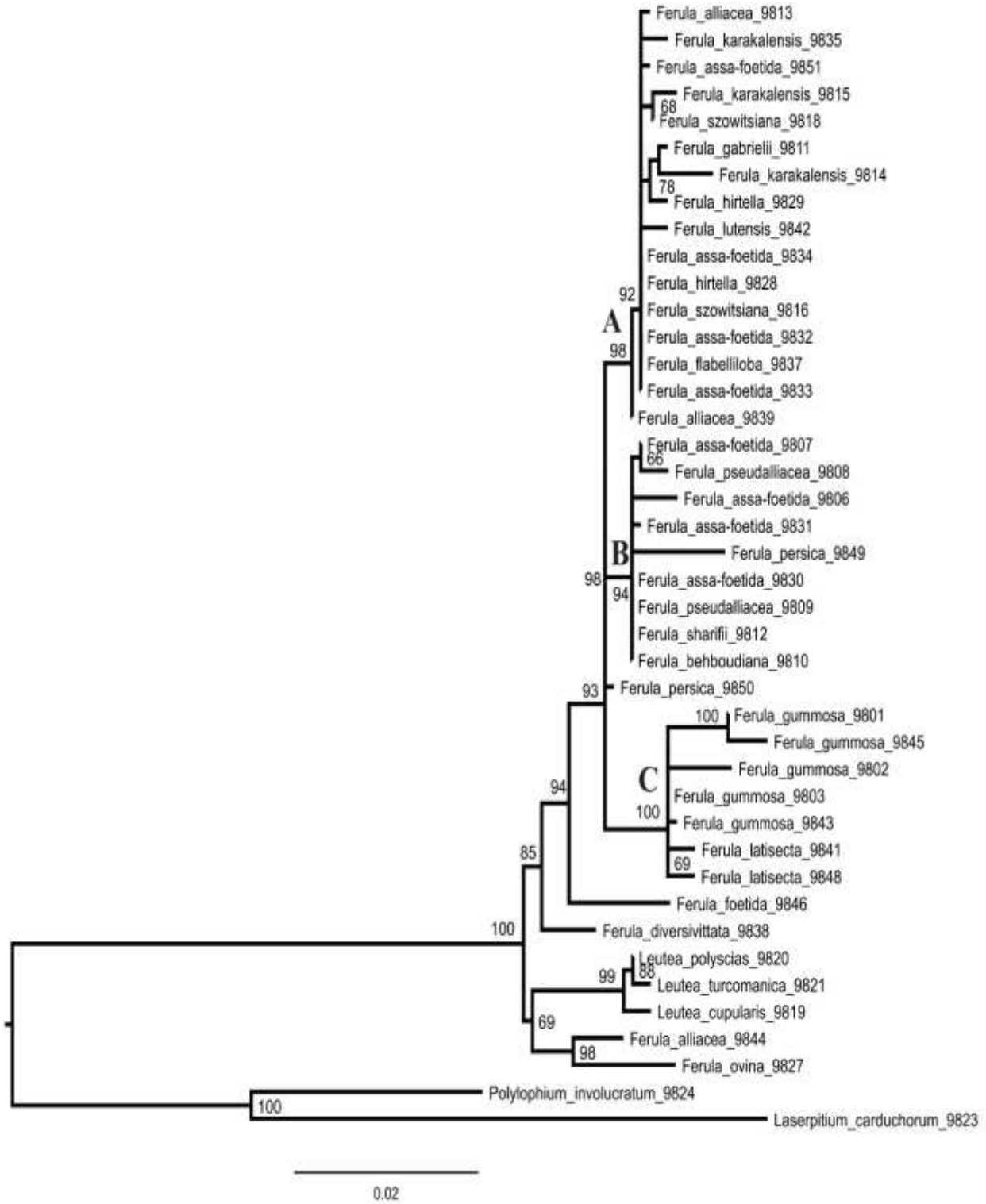

Fig. 2. The consensus tree inferred from ML analysis of 42 representatives of *F*. sect. *Merwia* with *Leutea* and 2 outgroups using nrDNA (ITS and ETS, data set I). Bootstrap support of consensus tree is given along branches.

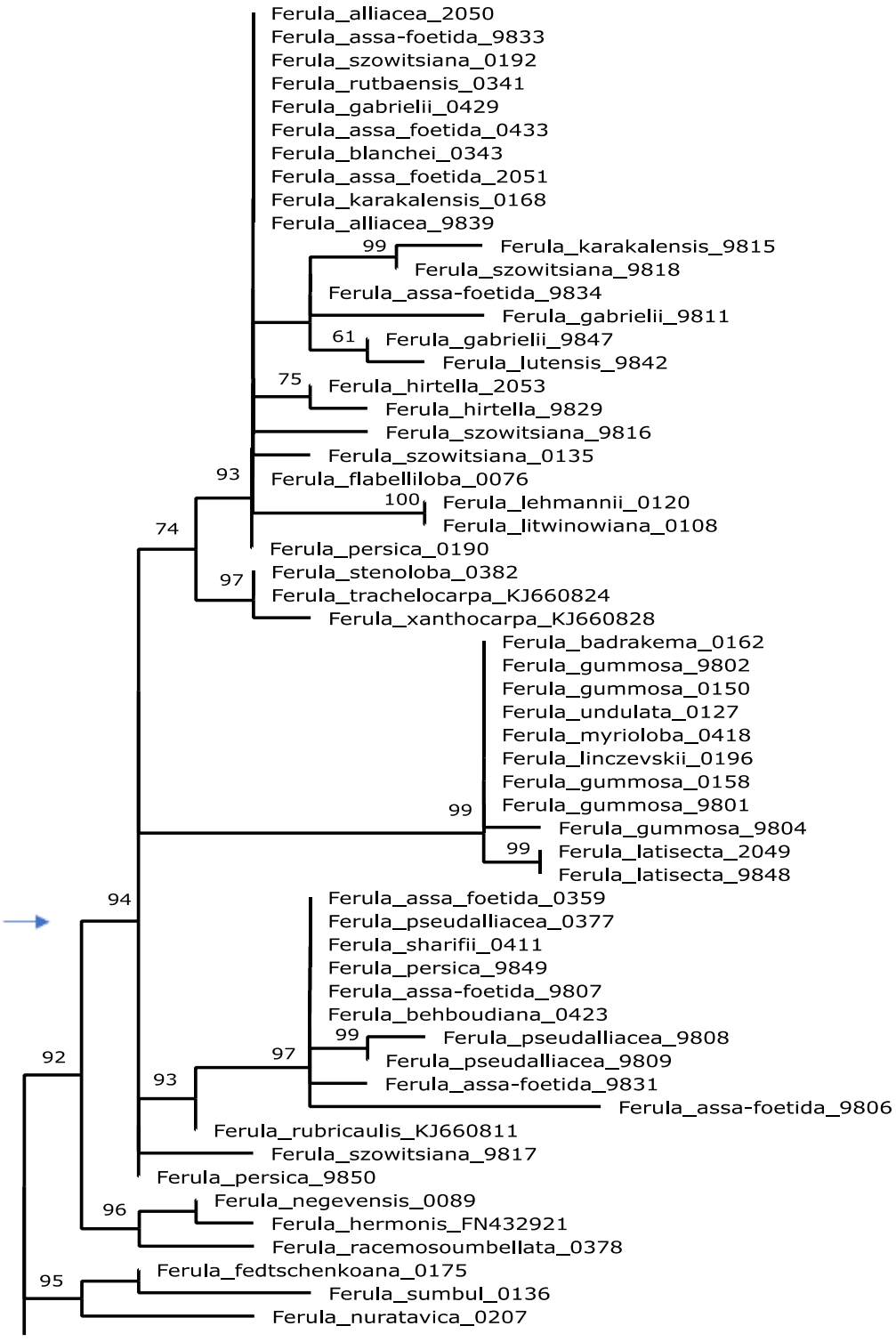

Fig. 3. The consensus tree inferred from nrDNA ITS data of Ferulinae (new accessions accompanied with previous data in Panahi & al., 2018). The clade of *Ferula* sect. *Merwia* (arrow) are represented with bootstrap support.

DISCUSSIONS

According to Xie & al. (2022), highly obvious diversification in Apiaceae explained the exhibited higher differentiation than other families and this event not only reflects the great variation in genome size but also represents the higher nucleotide diversity of apiaceous species. This means that the species of Apiaceae underwent more complicated evolutionary processes and have a high species diversification. For example, two closely related species, *Ferula turcica* Akalın, Miski, & Tuncay, and *F. latialata* Akalın, Miski, & Tuncay have recently been described from Turkey (Central Anatolia), from saline soils as new species. Phytochemical and morphological characters exhibit the close affinity of the two mentioned species to *F. szowitsiana* and *F. persica* and phylogenetically placed them within *F.* sect. *Merwia* (Tuncay & al. 2023). These species with narrow distributions indicate the complicated status within the genus and specifically in the section. As indicated here the populations of *F. szowitsiana* and *F. persica* represent geographical heterogeneity in Iran, so the two mentioned species would be assigned as the complex species. It seems that the exceptionally high number of species recognized in the genus is an artifact resulting both from hybridization and from taxonomic splitting. Hybridization and introgression diffuse boundaries among species and confound phylogenetic reconstructions, particularly when distant species exchange genetic material. However, these newly described species from Turkey may represent local hybrids of *F. szowitsiana* or *F. persica*. With the aim of avoiding misidentification and delimitation of a taxon precisely, using informative molecular markers is appropriate with adequate sampling.

Shadrin & al. (2023) revealed the variability of the intergenic spacers *rpL32—trnL* (with more and longer indels) and *rps16—trnK* (with single nucleotide insertions and deletions) than *rps16* intron and *trnQ—rps16* intergenic spacer within the *Heracleum* L. In *F.* sect. *Merwia*, the cpDNA loci (*rpL32—trnL*, *rps16—trnK*) presented a few informative characters to define boundaries among the intraspecific taxa of *F. assa-foetida* within *F.* sect. *Merwia* but among different species with distant position and the genus *Leutea*, the variability in nucleotide sites were observed more. As expected, based on other molecular systematic studies of Apiaceae, the most variable region appears to be the first ETS and ITS of nuclear DNA, which has 128 parsimony informative positions more than plastid loci (118 parsimony informative positions). Nevertheless, the greatest resolution of relationships was obtained from the analyses of combined data (nrDNA and cpDNA). The problem with estimating phylogeny occurs when the distances between sequences are very small and the data have a low variability within and between the populations.

The present and previous studies have shown that there is no single cpDNA marker that can provide a comparable amount of phylogenetic information. However, the ITS alone does not provide enough phylogenetic information to resolve terminal clades in groups with rapid diversification, such as *Ferula*. Using the external transcribed spacer (ETS) more informative positions have occurred than the other markers to resolve the relationships among the closely related species. In this region, the number of transition/transversion substitution sites was more frequent. It is recommended that the introduction of a new species in *Ferula* should be based on the ETS plus ITS nrDNA sequences to determine the relationships with close relatives. According to used plastid markers, the *rpL32—trnL* intergenic spacer provides the greatest number of parsimony informative characters and is more variable at the species level than introns which were used before (Shaw & al. 2014; Panahi & al. 2015; Danderson & al. 2018). Although this region could define the boundaries between related species and genera, it could not display infraspecific variations. In fact, nucleotide differences in such non-coding regions were generally too low to differentiate these infraspecific taxa unequivocally.

The inferred trees represented the clade of *Merwia* section comprises three subclades; the subclade "A" includes the species distributed in eastern Iran such as *F. karakalensis* and *F. szowitsiana* followed by central Iranian plateau elements, viz., *F. hirtella*, *F. gabrielli*, and *F. lutensis*. The species *F. gabriellii*, endemic to central Iran and Kavir deserts, along with *F. lutensis* which is the endemic of E and SE Iran (Chamberlain and Rechinger, 1987), and *F. hirtella*, are distributed in desert and warmer plains and can be considered as a geographical group (Khorassanian elements). Two allied species *F. flabelliloba*, endemic to E Iran with a distribution in Khorassan province, and *F. karakalensis* from NE Iran placed in this group and despite their molecular proximity are morphologically differentiated (Chamberlain & Rechinger 1987). They were classified as Kopet-Dagh elements before (Panahi & Mahmoodi 2021). Within this subclade, *F. alliacea* is nested and accompanied by these Kopet-Dagh elements. Furthermore, the central ecotypes of *F. assa-foetida* from the lower altitudes are grouped in one clade of subclade A with compatibility in desertic conditions.

Ferula alliacea as one of the asafoetida sources, with similar medicinal applications to *F. assa-foetida*, has been investigated phytochemically. The results showed that it possesses volatile sulfur-containing

compounds causing a strong sulfurous odor (Kasaian & al. 2016). Meanwhile, our molecular studies placed this species close to *F. assa-foetida* accessions that confirm its phytochemical similarities. When Boissier (1872) introduced *F. alliacea* from three locations viz., E Iran, Jandagh-Yazd (Buhse! specimen), near Shahrud-Neyshabour-Mashhad, and Kerman (Buhse! specimen), he could not detect the true species because of complexity in leaf morphology. Later Rechinger (1987) in Flora Iranica, noted a confused status for *F. alliacea* and indicated that Boissier's specimens referred to different species. He determined the Buhse specimen from Yazd as *F. gabrielii*, the specimen collected from Neyshabour was referred to as *F. flabelliloba*, and the sample of Kerman was referred to as *F. assa-foetida*. Recently our collection from Kerman in comparison with other samples revealed the differences within *F. assa-foetida* populations that are sometimes misidentified as *F. alliacea*. Through morphological observations, the specimen No. 9833 with large leaf lobes was expected to be *F. alliacea* (based on leaf morphological similarity with the Buhse specimen from Yazd, Haussknecht Hrbarium JE), and after analysis of the ITS data it placed close to *F. alliacea* (No. 2050 from Neyshabour, Fig. 3). However, by adding plastid data it has identical sequences to *F. assa-foetida* (no. 9832) (Figs. 1 & 2), and is related to those populations of *F. assa-foetida* distributed close to desertic plains. Therefore, morphological observations could not be useful enough for determining the true species in the case of *Ferula* and the phylogenetic data could help this issue.

Ferula alliacea has rectangular to oval or linear leaf lobs with short erect corky stems (from 70 to 120 cm), the diameter of the crown of the root seldom attaining more than 5 cm, and it also contains "Asafoetida" gum. It is distributed in altitudes of 900–2100 m a.s.l., and has the same appearance as *F. assa-foetida*. Phylogenetic results revealed its position close to *F. assa-foetida* samples from Kerman and also other species such as *F. gabrielii*, whose affinity was reported before (Chamberlain & Rechinger 1987).

The second subclade "B" includes some western (Zagrosian) elements; *F. assa-foetida* (southern populations from the mountainous area as indicated by real Asafoetida), *F. pseudalliacea*, *F. behboudiana* viz., *F. rubricaulis* Boiss. and *F. sharifii* (species endemic to Makran in the South of Iran). All these species are mostly restricted to mountainous areas and show an overlapping distribution area in the Zagros region (Panahi & Mahmoodi 2021). It is proposed that the populations of *F. assa-foetida* that are distributed in lower altitudes in the Centre of Iran and those populations from higher altitudes in Zagros Mt. be considered as two subspecies taxa, but we refrain from it now and plan to further investigations to elucidate their infraspecific status. The position of *F. persica* within this subclade with disjunct distributions (from North to Centre of Iran) needs to be investigated more precisely to determine the taxonomic boundaries of species.

The subclade "C" contains *F. gummosa* and its allies; *F. badrakema* Koso-Pol., *F. latisecta*, *F. mirioloba* Rech.f., *F. linczevskii* Korovin, see Fig. 3 as demonstrated previously (Panahi & al. 2018). All the accessions of *F. gummosa* from Tehran to Khorasan are placed closely without any differentiation, so *F. galbaniflua* Boiss. & Buhse (No 9804) is merged with the others that should be synonymized based on morphological and molecular affinity as supposed by Pimenov & Kljuykov (1996).

Apioideae members with considerable morphological diversity especially in fruit characters (Lyskov & al. 2017; Wojewódzka & al. 2019), could widely distribute with winged fruits even in high-altitude areas. This opportunity would result in long-distance dispersal through flight and expand the dispersal distance by occupying more living space (Wen & al. 2020). However, in *Ferula* with winged fruits, this potential has appeared to expand the species distribution and originate the local endemics.

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Appendix 1. Species list of *Ferula* sect. *Merwia* (voucher and reference number). GenBank accession numbers of each earned genome are represented.

Taxa	Collecting data	DNA ID	GenBank accession numbers			
			ITS	ETS	rpL32_trnL	rps16_trnK
Ingroup/ *Ferula* L.						
F. alliacea Boiss.	Khorasan, 70 km from Neyshabur to Kashmar (EE2). 11 June 1981, 1550-1950 m, *Assadi & Mozaffarian 35506* (TARI)	**9813**	OR773148	OR781625	OR756676	OR781669
	Khorasan, Mountains of Bezgh village and Ataeeh village. 13 April 2012, *Iranshahi 12613*, (in Pharmacognozi Dep. Medical University of Mashhad/ MMU)	**9839**	OR773146	OR781623	OR756674	OR781667
	Kashmar, Chalpu. N: 35 37 29.9, E: 58 31 37.5, 4 May 2012, 1822 m, *F.Ashna 44740* (Ferdowsi University of Mashhad/ FUMH)	**9844**	OR773147	OR781624	OR756675	OR781668
F. assa-foetida L.	Chaharmahal & Bakhtiari, Tange Sayyad protected area from Bostanshir and Abshorshor. 5 June 2009, 2245 m, *Mozaffarian 97203* (TARI)	**9806**	OR773136	OR781613	OR756665	OR781657
	Hormozgan, Bastak, Kuh-e parzy. 10 May 1996, 1800 m, *Moradi 78261* (TARI)	**9807**	OR773135	OR781612	OR756664	OR781656
	Kerman, Kianshahr, near to coal mine Khomrud. 26 May 2019, 2396 m, *Panahi 107137* (TARI)	**9831**	OR773137	OR781614	OR756666	OR781658
	Yazd, Taft toward Dehbala, 3 km after Taft. 25 May 2019, 1560 m, *Panahi 107133* (TARI)	**9834**	OR773139	OR781616	OR756668	OR781660
	Khorassan, Chahe soukhte, plain around Sabzevar. N: 35 46 48, E: 58 00 01, 1500 m, *Rezaii* (cultivated)	**9851**	OR773138	OR781615	OR756667	OR781659
	Kerman, Kianshahr to Zarand, 33 km to Kianshahr, close to Dasht-e Khak, 26 May 2019, 2248 m, *Panahi 107135* (TARI)	**9832**	OR773140	OR781617	OR756669	OR781661
	Kerman, Zarand to Kianshahr, 38 km Kianshahr, 26 May 2019, 2255 m, *Panahi 107136* (TARI)	**9830**	OR773141	OR781618	OR756670	OR781662
	Kerman, Rafsanjan to Zarand, Darreh Jowz, 26 May 2019, 2125 m, *Panahi 107134* (TARI)	**9833**	OR773151	OR781627	OR756678	OR781671

Appendix 1. Continued

Taxa	Collecting data	DNA ID	GenBank accession numbers			
			ITS	ETS	rpL32_trnL	rps16_trnK
F. behboudiana (Rech.f. & Esfand.) D.F.Chamb.	Ilam, Abdanan, Dinarkuh. 6 May 2009, 1030 m, *Mozaffarian 93166* (TARI)	**9810**	OR773142	OR781619	OR756671	OR781663
F. diversivittata Regel et Schmalh	Khorasan, Chenaran, Abshar-e Akhlamad. 25 June 2019, 1505 m, *Panahi 107131* (TARI)	**9838**	OR773166	OR781641	OR756691	OR781685
F. flabelliloba Rech.f. &Aellen	Khorasan Razavi, Mashhad, above Zoshk village. 25 June 2019, 1781 m, *Panahi 107132* (TARI)	**9837**	OR773157	OR781633	OR756683	OR781677
F. foetida (Bunge) Regel	Khorassan, Ghayen mounth., N Haji Abad, Deh nou. 10 April 1988, 1000 m, *Joharchi & Zangooei 15962* (FUMH)	**9846**	OR773165	OR781640	OR756690	OR781684
F. gabrielii Rech.f.	Khorasan, ca. 65 km from Tabas to Yazd, around Robat-e Kalmard. 7 May 1997, 1200 m, *Mozaffarian 77277* (TARI)	**9811**	OR773149	OR781626	OR756677	OR781670
	Khorasan: E Nehbandan, km 14 Hajat mine to Bazarche marzi. 30 April 1996, 1000 m, *Rafeie & Zangooie 26478* (FUMH)	**9847**	OR773150	-	-	-
F. gummosa Boiss	Tehran, Damavand, road from Chenar to daryache and around daryache tar. 4 June 2011, 2895 m, *Mozaffarian 97675* (TARI)	**9801**	OR773131	OR781609	OR756660	OR781652
	Khorasan, Esferayen, Shahjahan Mts. Region rocky. Soily Mt. Tourkan from deep gorge close to Noushirvan village. 8 June 1984, 1400-2500 m, *Mozaffarian 48590* (TARI)	**9802**	OR773130	OR781608	OR756659	OR781651
	Khorasan, between Mashhad and Sarakhs, Shoorlagh. 17 April 1985, 350 m, *Ayatollahi & Rezaie 12027* (FUMH)	**9843**	OR773133	OR781610	OR756662	OR781654
	Khorasan, W Gonabad, Yakhchi (Siah Mounth). 26 April 1995, 2500 m, *Rafeie & Zangooie 25103* (FUMH)	**9845**	OR773134	OR781611	OR756663	OR781655
	Tehran, 13 km from Firouzkuh to Semnan (XV3). 9 June 1981, 2000 m, *Assadi & Mozaffarian 35283* (TARI)	**9803**	OR773129	OR781607	OR756658	OR781650
	Tehran, Arak, Toureh, Besri, N.E. slope Kuh-e Aladagh. 11 July 1985, 2100-3100 m, *Mozaffarian* 64114 (TARI) (as ***F. galbaniflua***)	**9804**	OR773132	-	OR756661	OR781653
F. hirtella Boiss.	Semnan to Damghan, 50 km to Damghan. 23 June 2019, 1534 m, *Panahi 107127* (TARI)	**9828**	OR773153	OR781629	OR756680	OR781673
	Kerman, 28 km a Rayen from main road Kerman-Bam, 27 May 2019, 2371 m, *Panahi 107141* (TARI)	**9829**	OR773152	OR781628	OR756679	OR781672

Appendix 1. Continued

Taxa	Collecting data	DNA ID	GenBank accession numbers			
			ITS	ETS	rpL32_trnL	rps16_trnK
F. karakalensis Korovin	Semnan, ca. 5 km from Shahmirzad to Chashm. 2000 m, *Mozaffarian 72629* (TARI)	**9814**	OR773156	OR781632	OR756682	OR781676
	Gorgan, Mohammad Reza-Shah wildlife park, below Almeh. 20 June 1974, 1250-1400 m, *Wendelbo & Foroughi 12715* (Det: Chamberlain) (TARI)	**9815**	OR773154	OR781630	-	OR781674
	Semnan, Shahrud, Mt. Shahvar, above Tash village. 23 June 2019, 2701 m, *Panahi 107128* (TARI)	**9835**	OR773155	OR781631	OR756681	OR781675
F. latisecta Rech.f. & Aellen	Khorasan, Hezar Masjed mountain, Hll village. 19 May 2006 (29/02/1385), *Iranshahi 10089* (MMU)	**9841**	OR773158	OR781634	OR756684	OR781678
	Khorasan, South of Dargaz, between Rishkhar and darbandi, Kalateh Goorni. 28 May 1990, 1800 m, *Joharchi & Zangooei 18660* (FUMH)	**9848**	OR773159	OR781635	OR756685	OR781679
F. lutensis Rech.f.	Khorasan, SW Ferdows, SW Boshrouyeh, Shotori Mountains, 4 km W of Khoda-Afarid. 16 May 2017, 1770 m, *Joharchi & Memariani 46150* (FUMH)	**9842**	OR773160	OR781636	OR756686	OR781680
F. ovina Boiss.	Tehran, Firuzkuh to Sorkheh, Taren, 22 June 2019, 2348 m, *Panahi 107130* (TARI)	**9827**	OR773164	OR781639	OR756689	OR781683
F. persica Willd. Rech.f.	Tehran, Karaj valley, Sarvedar, 5 June 1974, 1500m, *Foroughi & Sanii & Amini 12322* (TARI) (as: ***F. persica*** var. ***latisecta)***	**9849**	OR773127	OR781605	OR756656	OR781648
	Mazandaran, ca. 50 km SW of Chalous, above the village Delir, 18 Aug 1984, 2800m, *Assadi & Mozaffarian 51639* (TARI) (as ***F. persica*** var. ***persica)***	**9850**	OR773128	OR781606	OR756657	OR781649
F. pseudalliacea Rech.f.	Fars, Mian jangal protected area, Tange Ahram. 27 April 2003, 1900-2200 m, *Mozaffarian 83627* (TARI)	**9808**	OR773143	OR781620	OR756672	OR781664
	Yazd, Harat, Baghe Shadi. 1900 m, Jafari & *Mozaffarian 104910* (TARI)	**9809**	OR773144	OR781621	OR756673	OR781665

Appendix 1. Continued

F. sharifii Rech.f. &Esfand.	Baluchestan, 130 km from Bampor to Iranshahr, Tange Sarhe. 14 April 1983, *Mozaffarian 43056* (TARI)	**9812**	OR773145	OR781622	-	OR781666

Taxa	**Collecting data**	**DNA ID**	**GenBank accession numbers**			
			ITS	**ETS**	**rpL32_trnL**	**rps16_trnK**
F. szowitsiana DC	Khorasan, Salehabad, border of Iran & Afghanistan. 26 April 1989, 620 m, *Mozaffarian 67603* (TARI)	**9816**	OR773161	OR781637	OR756687	OR781681
	Azarbaijan, ca. 18 km N.W. of Marand, between Kashk-Saraj and Orlan (NH1). 15 June 1988, 1500 m, *Assadi & Shahsavari 65417* (TARI)	**9817**	OR773162	-	-	-
	Gorgan, 49 km from Shahpassand on road to Shahrud, Tilabad. 17 May 1978, 1000 m, *Wendelbo & Assadi 29606* (TARI)	**9818**	OR773163	OR781638	OR756688	OR781682
Outgroups /*Leutea* Pimenov						
L. cupularis (Boiss.) Pimenov	Chaharmahal-e Bakhtiari, Road from Shahr-e Kurd to Naghan, N. of Sulegan, Kuh-e Shahpur Naz, 9 July 1986, 2100 m, *Mozaffarian 57455* (TARI)	**9819**	OR773167	OR781642	OR756692	OR781686
L. polyscias (Boiss.) Pimenov	Gilan, Rudbar, ca. 2 km from Rudbar to Manjil, 8 Aug 2013, 220 m, *Mozaffarian 102537* (TARI)	**9820**	OR773169	OR781643	OR756693	OR781687
L. turcomanica (Schischk.) Mozaff.	Khorassan, Esferayen, Shah Jahan Mts., Tourkan. from deep gorge close to Noushirvan village, 8 June 1984, 1400-2500 m, *Mozaffarian 48601* (TARI)	**9821**	OR773170	OR781644	OR756694	OR781688
Laserpitium carduchorum Hedge & Lamond (≡***Laser carduchorum*** (Hedge & Lamond) Wojew. & Spalik)	Kurdistan, 15 km N.E. of Baneh, Gardaneh-e Khan, 1 June 1989, 2450 m, *Fattahi & Tavakoli & Hatami 2454* (TARI)	**9823**	OR773172	OR781647	OR756697	OR781691
Polylophium involucratum (Pall.) Boiss. (≡ ***Laser involucratum*** (Pall. ex Schult.) Spalik & Wojew.)	Mazandaran, Ramsar, Javaherdeh. 18 Aug 2014, 3360 m, *Mozaffarian 103126* (TARI)	**9824**	OR773171	OR781646	OR756696	OR781690