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Phylogeny of the Southwest Asian *Pimpinella* and related genera based on nuclear and plastid sequences

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ABSTRACT. *Pimpinella* L. is a large genus and arguably one of the most complex genera in the family Apiaceae. In this study, the infra-generic relationship between Southwest Asian *Pimpinella* species and their generic allies in the tribe Pimpinelleae Spreng were investigated using sequence data from the cpDNA (chloroplast DNA) *rps16* exon and *rpL16* intron and nuclear ribosomal DNA internal transcribed spacer regions. In total, 185 accessions representing 52 species of *Pimpinella*, 8 species of *Aegopodium*, and the monotypic *Opsicarpium* Mozaff. were analyzed using maximum parsimony and Bayesian methods. In our phylogenetic study, *Pimpinella* and *Opsicarpium* were considered together as a monophyletic group within the tribe Pimpinelleae. As a result, *Opsicarpium insignis* Mozaff has been formally transferred to *Pimpinella*. Our results indicate that the genera *Pimpinella* and *Reutera* Boiss formed a monophyletic group and also supported merging the

genus *Reutera* with *Pimpinella*. This study confirms the transfer of the Southwest Asian *Pimpinella anthriscoides* (Boiss.) F. Ghahrem., Khajepiri & Mozaff to the genus *Aegopodium* as *Aegopodium tribracteolatum* Schmalh.

Key words: cpDNA *rps16* exon; cpDNA *rpL16* intron; nrDNA ITS; Phylogeny; *Pimpinella*; Umbelliferae

INTRODUCTION

Downie et al. (2010) provided a general phylogenetic framework within the subfamily Apioideae based on the phylogenetic analysis of nrDNA internal transcribed spacer (ITS) sequences. These analyses revealed the evolutionary relationships of many genera and clades of this subfamily more clearly compared to a pre-existing phylogenetic classification for the group based on the methods used by Downie et al. (2001).

According to Downie et al. (2010), the Pimpinelleae tribe is recognized as a paraphyletic or polyphyletic group within Apioideae. This is because the main species-rich genus in this tribe, *Pimpinella*, is also assigned to a minimum of seven other major clades of this subfamily.

The genus *Pimpinella* is one of the largest genera of the subfamily Apioideae within Apiaceae, with approximately 150 species distributed in Asia, Europe, and Africa. A few species can also be found in South America and one can be found in the western part of North America (Pimenov and Leonov, 1993).

The derived conditions (traits) of the family in which *Pimpinella* is included are: generally herbaceous habit; presence of compound leaves; small and inconspicuous flowers, with few floral parts arranged in whorls and grouped in umbel-shaped inflorescences as the result of prolonged co-evolution with insects. The presence of inferior ovaries composed of sealed carpels is a further indication of the degree of evolution. All *Pimpinella* species can be annual, biennial, or perennial and are generally characterized by the presence of fibrous collars on the top of the rootstock and compound umbels. These plants usually grow on dry rocky places, rocky crevices, fields, meadows, mountain pastures, and grasslands (Engstrand, 1987; Velayos, 2003).

In the genus *Pimpinella*, the basic chromosome number is x = 8 to 11. Some variations in ploidy level are exhibited in this genus (2n = 2x = 18 and 2n = 4x = 36). In addition, some aneuploid species have been recorded (Constance et al., 1976; Daushkevich et al., 1995; Shner et al., 2004; Ghahremaninejad et al., 2013).

In some cases, intraspecies and even intrapopulation variation has been found in the ploidy level (Rostovtseva, 1982; De Montmollin, 1986; Ghahremaninejad et al., 2013). Many *Pimpinella* species are narrowly distributed and habitat-specific (Mozaffarian, 2007). Therefore, this indicates that the process of speciation in this genus is tightly associated with polyploidy and adaptation, and the evolutionary history of *Pimpinella* is not been well understood. Marked variations in the *Pimpinella* genus include fruit shape (ovoid-oblong to elliptic), fruit surface (glabrous to tuberous hair), fruit anatomy, leaf division, flowers and inflorescences, which result in the recognition of numerous and often hardly discernible taxa (Engstrand, 1987; Matthews, 1972). Basal leaves and mature fruits are two important factors required for accurate identification; however, they are associated with some limitations. For

example, the previous fruits on the plant are usually dried out whereas the next are not yet fully ripe; in addition, only lateral divisions of the basal leaves are preserved. Therefore, it is not surprising that both the infrageneric classification and the phylogenetic position of *Pimpinella* remain disputed.

Pimpinella is traditionally classified in the tribe Apieae (Pimenov and Leonov, 1993). De Candole (1827) divided the genus *Pimpinella* into three sections: *Tragoselinum*, characterized by its glabrous fruits and perennial roots; *Tragium*, with hairy fruits, perennial (rarely biennial) roots, and pinnate to bipinnatus radical leaves with ovate segments; and *Anisum*, which included species with down-covered annual fruits. One of the most important reviews was that of Bentham and Hooker (1867), who included 65-70 species under the name *Pimpinella*, classified into six sections according to the habitat of the plant, leaf and fruit morphology, and petal color.

In 1875, Boissier named specimens with emarginate or bilobate petals as *Pimpinella*. That author recognized two sections within *Pimpinella*, *P* sect *Tragium* Spreng and sect *Tragoselinum* Tourn distinguished by pubescent versus glabrous fruit. However, it was not until 1910, when Wolff used petal color, morphology, and indumentums of fruits to undertake a thorough taxonomic revision, that the status of the genus was somewhat clarified. The only taxonomic analysis covering all species in the genus is that of Wolff (19100, who divided the genus into three sections: *Reutera*, including species with yellow flowers and glabrous or hairy fruits; *Tragium*, generally with white flowers and bristly or hairy fruits, granular or tubercle, sometimes nearly glabrous or almost completely smooth; and *Tragoselinum*, also with white flowers and glabrous or totally glabrous fruits. Wolff (1927) reduced the genus *Reutera* to section rank within the genus *Pimpinella* as sect. *Reutera*. Recent phylogenetic analyses strongly suggest that the infrageneric classification of *Pimpinella* proposed by Wolff (1910) does not correspond to any of the recovered clades, and as such, it is clearly artificial. Engstrand (1987), in his treatment of species from the Iranian plateau, did not propose any infrageneric classifications for the 25 species of the area. He recorded 19 species of *Pimpinella* in the flora of Iran. Recent taxonomic studies on representatives of the Iranian *Pimpinella* revealed some new species. Today, about 22 species are generally recognized in the flora of Iran (Mozaffarian, 2007). While Ghahremaninejad and Khajepiri (2010) separated *Pimpinella anthriscoides* Boiss into the monotypic genus *Pseudopimpinella* based on fruit anatomical characters, both molecular analysis and morphological data support merging *P. anthriscoides* with the genus *Aegopodium* as *A. tribracteolatum* Schmalh. (Zakharova et al., 2012).

Matthews (1972) recognized 23 species of *Pimpinella* in the flora of Turkey, without sectional affiliations. The *Pimpinella* species of China have been divided into two groups: *P.* sect. *Tragium* (Spreng.) DC., containing those species with hairy, or distinctly roughened fruits and obsolete calyx teeth, and *P*. sect. *Tragoselinum* (Miller) DC., containing those species with glabrous fruits and obsolete or conspicuous calyx teeth (Pu and Watson, 2005).

Pimpinella has been rarely studied from a morphological or phytochemical viewpoint (Baser et al., 1996; Khajepiri et al., 2010). The most comprehensive phytochemical analysis of the genus was published by Tabanca et al. (2005), who also described the phylogenetic relationships among species.

Similar to the most traditional identifying of higher level taxa within Apiaceae, molecular systematic studies have revealed that the genus *Pimpinella* is polyphyletic, and taxonomic delimitation of the genus has not yet been resolved (Downie and Katz-Downie, 1999; Zhou et al., 2008, 2009; Downie et al., 2010). A recent molecular phylogenetic study

of *Pimpinella* clearly showed it to be a non-monophyletic taxon (Downie et al., 2010; Wang et al., 2014). Yet the monophyly of a *Pimpinella* 'core group' in the tribe Pimpinelleae is strongly supported (Magee et al., 2010; Wang et al., 2014). Most and the African and Malagasy *Pimpinella* species are placed with their Eurasian counterparts in Pimpinelleae (Magee et al., 2010; Wang et al., 2014). Chromosome base number was found to be consistent with the groupings recovered in the molecular analyses (Magee et al., 2010; Wang et al., 2014).

African and Malagasy *Pimpinella* species with a chromosome base number of x = 11 form the earliest diverging clade. The remaining African species ally with several Eurasian species of *Pimpinella* and share a chromosome base number of x = 9 (Magee et al., 2010).

Spalik and Downie (2006) showed that the African members of tribe Pimpinelleae form a monophyletic branch derived from a common ancestor of Middle Eastern origin. Recent molecular studies have suggested a close affinity between the newly described genus *Opsicarpium* and *Pimpinella* (Valiejo-Roman et al., 2006a; Wang et al., 2014).

To date, relatively few molecular systematic studies have included substantial sampling of *Pimpinella* across its distributional range, and no published study has included most of the species of *Pimpinella* from Southwest Asia. Therefore, the present study was carried out to: 1) investigate the relationships between and generic allies in the tribe Pimpinelleae for a broader analysis of *Pimpinella* evolution; 2) evaluate the monophyly of *Opsicarpium* and *Reutera* with respect to other genera in the tribe Pimpinelleae; and 3) detect polyploidy events in *Pimpinella* for the first time.

To achieve those objectives, we conducted a phylogenetic study using DNA sequences of the nuclear ribosomal ITS and two chloroplast DNA (cpDNA) loci (*rps16* exon and *rpL16* intron), because numerous studies incorporating *Pimpinella* and some of its putative allies have demonstrated the utility of these loci in resolving intra and intergeneric relationships within Apiaceae (Spalik and Downie, 2007; Downie et al., 2001, 2010; Wang et al., 2014).

MATERIAL AND METHODS

A total of 31 new sequences consisting of 15 nuclear ribosomal ITS sequences and 16 cpDNA loci sequences (rps16 and rpL16) were generated for this study. Leaf material for DNA extraction was obtained primarily from herbarium specimens (TARI, Research Institute of Forests and Rangelands, Iran; Kharazmi University; Yüzüncü Yil University, Turkey). Through fieldwork in Iran. In total, 14 species of *Pimpinella* were selected to more completely represent the morphological range of the genus in West Asia. Multiple accessions were sampled for *Aegopodium tribracteolatum* Schmalh, and *Pimpinella corymbosa* Boiss, *Pimpinella eriocarpa* Banks & Sol, *Pimpinella kotschyana* Boiss, *Pimpinella peucedanifolia* Fischer ex Ledeb, *Pimpinella puberula* (DC.) Boiss, *Pimpinella saxifrage* L., and *Pimpinella tragium* subsp *lithophila* (Schischk). Tutin to assess possible infraspecific variation in these widespread species.

Furthermore, an accession of the Iranian endemic species *Opsicarpium insignis* was included to estimate the systematic position. Taxa, voucher information, and GenBank accession Nos. for all rps16, rpL 16 and ITS are listed in the **Table S1**.

We performed a BLAST search in GenBank to detect all genera that have close affinities to species that are currently classified in *Pimpinella*. In this way, some representatives of the tribes Pimpinelleae Spreng., Komarovieae J. Zhou & S.R. Downie, Oenantheae Dumort, Pleurospermeae M.F. Watson & S.R. Downie, Selineae Spreng., and of the *Acronema* clade

according to the classification by Downie et al. (2010) were also added. All newly obtained sequences have been deposited in GenBank.

As outgroups, we included representatives of closely related genera of the Apioideae tribe Pyramidoptereae Boiss. (*Bunium luristanicum* Rech.f.) and two distantly related genera of the Apioideae tribe Tordylieae W.D.J. Koch (*Heracleum persicum* Desf., *Zosima absinthifolia* Link) based on previous phylogenetic studies of the subfamily Apioideae (Downie et al., 2010).The final sampling included 115 accessions of 112 species from 37 genera for large ITS, and 85 accessions of 46 species from 25 genera for a concatenated data matrix (see **Table S1**).

DNA extraction, amplification, and sequencing

Total genomic DNA was isolated from 12-22 mg dried leaf tissues using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

Nuclear and plastid regions were amplified using specific primers and PCR conditions were as follows (Table 1). The ITS region of nrDNA was amplified using the primers ITS_4 and ITS_5. The thermal reactions were initiated for 1 min 30 s at 94°C to ensure denaturation of double-stranded template DNA, followed by 34 cycles with the following cycling profile: 1 min at 94°C (denaturation), 1 min at 53°C (annealing), and 1 min at 72°C. This was followed by a final extension of 10 min at 72°C. Amplification of chloroplast regions of rps16 and rpL 16 was performed using the primers rps16 F and Exon-CR for rps16 and rpl16, and R1516 for rpL 16. The same thermal cycling profile was used for both chloroplast primers as follows: 3 min at 94°C to ensure denaturation of double-stranded template DNA, followed by 30 cycles with the following cycling profile: 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 2 min at 72°C. This was followed by a final extension of 7 min at 72°C. PCR products were purified with *Escherichia coli* exonuclease I and Fast AP Thermo sensitive Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) following the manufacturer recommendations. Cycle sequencing reactions were performed using the purified PCR product, AmpliTaq DNA polymerase (Roche Molecular Systems, Alameda, CA, USA), and fluorescent Big Dye terminators (Applied Biosystems, Foster City, CA, USA). The products were resolved by electrophoresis using an ABI 377A automated DNA sequencer (Applied Biosystems). The resulting chromatograms were analyzed using the program ChromasPro 1.41 (TechnelysiumPty Ltd., Australia).

Table 1. Primers used for PCR and sequencing.

Region	Name	F/R	Sequence (5'-3')	Mode
ITS	ITS_4	F	TCCTCCGCTTATTGATATGC	P, S
	ITS_5	R	GGAAGTAAAAGTCGTAACAAGG	P
rps	rps16F	F	TTTGAAACGATGTGGTAGA	P, S
	Exon CR	R	ACCCACGTTGCGAAGAT	P, S
rpL	rpl16	F	GCTATGCTTAGTGTGTGACTCGTTG	P, S
	R1516	R	CCCTTCATTCTTCCTCTAYGTTG	P

F/R, forward and reverse, respectively. Mode refers to PCR amplification (P) and sequencing (S).

Sequence and phylogenetic analyses

Nucleotide sequences of the four data matrices [large ITS, reduced ITS, cpDNA (*rps16* exon plus *rpl16* intron), and concatenated (reduced ITS and cpDNA)] were each aligned

initially using MUSCLE (Edgar, 2004) with default parameters for gap penalty and extension. The alignment was then edited where necessary using Mesquite version 1.12 (Maddison and Maddison, 2001). Indels were coded as independent, single, binary characters following the methodology of Simmons and Ochoterena (2000) and appended to the sequence matrix for phylogenetic analysis.

Three data matrices including those for the large ITS, reduced ITS and cpDNA (*rps16* exon plus *rpl16* intron), were used for phylogenetic analyses of the individual loci. The large ITS data matrix included representatives of all genera detected as close affinities to species currently classified in the genus *Pimpinella* (Zhou et al., 2009; Wang et al., 2014). We generated a reduced ITS data matrix, which included those species with cpDNA sequence information available in GenBank. For the concatenated data, only reduced ITS and cpDNA (*rps16* exon plus *rpl16* intron) data were applied. Congruence in the phylogenetic signal of the reduced ITS and cpDNA data sets was examined by a visual comparison of tree topologies and branch support, and a partition homogeneity (or ILD) test was performed with PAUP* 4.0b10 (Farris et al., 1994; Swofford, 2002) using a heuristic search with 1000 replicates, a maximum tree limit of 1000, and tree-bisection-reconnection (TBR) branch swapping. Sequence divergence was calculated using the Kimura two parameter distance model (Kimura, 1980). Phylogenetic analyses included the Bayesian inference (BI) method using MrBayes ver. 3.1 (Ronquist and Huelsenbeck, 2003), and maximum parsimony (MP) and maximum likelihood (ML) methods implemented using PAUP* ver. 4.0b10 (Swofford, 2002). MP and BI analyses were conducted on both separate and combined (total evidence analysis) datasets. For MP analysis, heuristic searches were performed with 1000 random stepwise addition replicates and TBR branch swapping using the MULTREES option. In order to prevent PAUP* from crashing, the options CHUCK and CHUCKSCORE were used. The substitution model for the Bayesian analysis was selected using the program model test ver. 3.6 (Posada and Crandall, 1998) and the Akaike information criterion. The analyses were carried out for 5,000,000 generations with four Monte Carlo Markov Chains initiated and a sampling frequency of 100 generations. The initial 10,000 saved trees were discarded, and the consensus and posterior probabilities (PP) of particular clades were calculated based on the remaining trees. Following completion, sampled trees were plotted against their likelihood in order to recognize the point where the likelihoods converged on a maximum value, and all trees prior to this convergence were discarded as the ‘‘burn-in’’ phase. The remaining trees were combined in a majority rule consensus.

Hypothesis testing and alternative topology

We used the SH test (Shimodaira and Hasegawa, 1999) to compare the best ML trees recovered from analyses of the combined molecular data with the constraint topologies based on existing hypotheses of the monophyly of the genus *Opsicarpium* constructed in Treeview version 1.6.6 (RDM. Page, 2001). The trees were loaded as a backbone into PAUP*. Heuristic searches were conducted using the outlined ML parameters to find the shortest trees compatible with the constraint. The likelihood score for the best ML tree was then compared with the score of the best ML tree using one-tailed non-parametric SH tests. The SH test, which was performed on the combined data set to test the monophyly of the genus *Opsicarpium*, is significantly favored over the phylogenetic hypothesis suggested by other evidence.

RESULTS

Comparison of the divergence and phylogenetic utility of chloroplast and nuclear DNA data sets

The main characteristics of the large ITS, reduced ITS, cpDNA, and combined data sets, along with the corresponding tree statistics, are summarized in Table 2.

Table 2. Data set and tree statistics from separate and combined analyses of the nuclear and two chloroplast regions.

	Large ITS	Reduced ITS	cpDNA (*rpl16* intron + *rps16*)	Combined (reduced ITS + cpDNA)
Number of sequences	115	24	24	24
Length range	530-610	535-650	1520–1670	2055-2320
Aligned length (including informative indels)	661	657	2110	2767
GC content mean (%) – ingroup	35.1	45.6	23.1	29.9
GC content mean (%) – outgroup included	34.6	45.5	23.3	29.8
Sequence divergence (%) – ingroup	0.10-23.5	0.00-21.54	0.00-27.73	0.00-16.24
Sequence divergence (%) – outgroup included	1.12-25	0.00-23.14	4.76-32.12	5.79-35.55
Number of variable sites – ingroup	123	59	251	345
Number of variable sites – outgroup included	117	146	232	378
Potentially informative characters – ingroup (%)	400	121	198	294
Potentially informative characters – outgroup (%)	415	124	107	231
Number of unambiguously coded indels	7	3	2	11
Coded indel size range	2-6	2-4	1-3	2-5
of MPTs	0.83	0.88	0.84	0.81
CI of MPTs (excluding uninformative characters)	0.72	0.71	0.71	0.71
of MPTs	0.78	0.84	0.85	0.78
Number of MPTs	100	3	1500	9
Length of MPTs	2235	402	157	848

CI = consistency index; RI = retention index; MPTs = most-parsimonious trees.

The combined cpDNA data set contained 2110 characters, of which 251 were variable and showed the lowest percentage of informative characters (5.07%), whereas nrDNA sequences contained 657 characters, of which 59 were variable and showed the highest percentage of informative characters (18.8%).

The combined cpDNA sequences were 1.26-times more variable than the average in-group pairwise divergence, and 3.7-times less variable than the reduced nrDNA sequences with regard to the absolute number of informative characters. Therefore, the use of the ITS sequences seemed to be an appropriate strategy for phylogenetic reconstruction in terms of the number of informative characters.

Genetic distances between reduced ITS sequences in the sample were computed using Kimura's two-parameter model (Table 2) and ranged from 0.0014 to 0.2154 among the species of *Pimpinella*, and from 0.0021 to 0.2314 between taxa of *Pimpinella* and other genera in the tribe Pimpinelleae. No infraspecific variation in the ITS sequences was found in the two accessions of *P. eriocarpa* and *P. kotschyana*, whereas the accessions of *A. tribracteolatum*, and *P. corymbosa*, *P. peucedanifolia*, *P. puberula*, *P. saxifrage*, and *P. tragium* subsp *lithophila* exhibited a distance of 0.01488 to 0.09407.

Phylogenetic analyses

Large ITS sequence analyses

Two large ITS methods of phylogenetic reconstruction produced congruent trees

without any major differences; therefore, Figure 1 shows the BI tree with the addition of the parsimony bootstrap percentages.

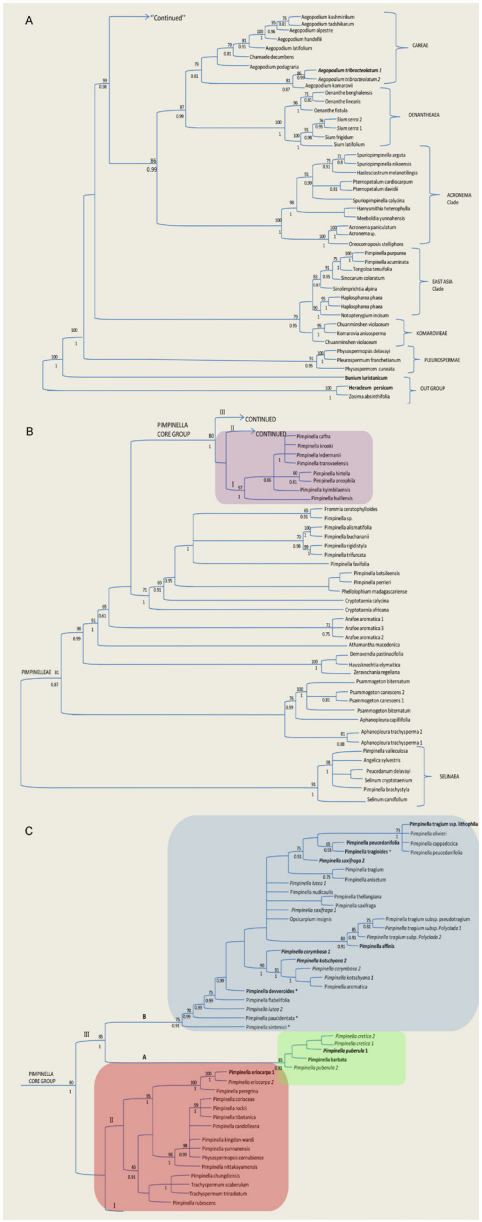

Figure 1. Bayesian majority-rule consensus tree derived from the large ITS dataset. Posterior probability and bootstrap support values are shown on the branches. The species involved in this study are boldfaced. The species with more than one accession are indicated by numbers. Species names in bold and italics indicate that those species were involved in this study and that the accessions were obtained from GenBank, respectively. Asterisks indicate species that are confirmed as *Reutera* group.

The monophyly of *Pimpinella* is not supported statistically by the large ITS data sets. Parsimony analysis recovered a topology that is consistent with that of Wang et al. (2014). There are six clades and three subclades of "major importance" discussed by those authors on our cladogram. The following tribes were strongly supported by ITS sequences: the Pimpinelleae (PP = 0.87, BS = 81%), Selineae (PP = 1.00, BS = 91%), Oenantheae (PP = 1.00, BS = 100%), and Komarovieae (PP = 0.95, BS = 79%), and the *Acronema* (PP = 1.00, BS = 100%) and East Asia (PP = 0.97, BS = 75%) clades, and *Pimpinella* 'core group' comprising three subclades, subclade I (PP = 1.00, BS = 97%), subclade II (PP = 0.87, BS = 74%), and subclade III (PP = 1.00, BS = 85%).

In the BI tree (Figure 1), all West Asian *Pimpinella* species fell into the *Pimpinella* 'core group', which were mostly in subclade III (PP = 1.00, BS = 85%). Taxa in subclade III shared a primarily Eurasian distribution; however, relationships within this subclade were largely unresolved. Within subclade III, we identified two additional major subgroups, termed subgroup A and B (Figure 1).

DNA combined analysis

In total, 48 accessions representing 41 taxa were used in the phylogenetic analyses of combined data [reduced ITS and cpDNA (*rps16* exon plus *rpl16* intron)]. Separate analyses of each cpDNA and nuclear DNA region were shown to be significantly incongruent according to the ILD test ($P < 0.01$). However, results from each individual analysis did not show any incongruence supported by BP. Because no hard incongruence was found, the inclusion of all DNA regions in a single analysis should maximize the explanatory power regardless of the level of character incongruence between data sets detected with the ILD (Hipp et al., 2004). When each data set was analyzed separately (data not shown), the results were similar to those obtained by the combined analyses, but both BP and resolution were greater in the combined analysis. Statistics for all single and combined analyses are given in Table 2. The BI trees resulting from analyses of the combined data matrices are presented in Figure 2. In general, relationships common to the BI tree derived from the large ITS dataset with overlapping taxon sets were found in the combined data set BI tree (Figures 1 and 2). Although the BI tree of the combined analysis offered greater resolution of relationships than the BI tree of the large ITS dataset, a high level of congruence was apparent between them, with many nodes supported by high PP and BS support values, That is, two major groups found in previous phylogenetic studies (Wang et al., 2014) were recovered (subclade II, subclade III) within the *Pimpinella* core group. While subclade II encompassed two species of Chinese *Trachyspermum* and *P. eriocarpa*, which is an annual species widespread in Southwest Asia, subclade III comprised almost all annual and perennial Eurasian species of *Pimpinella* and the Iranian endemic species *O. insignis*.

Within subclade III, we identified two additional major subgroups (A and B) comprising species distributed in Europe, and Western, Central, and Southern Asia. However, some differences in relationships can be observed in the relative positions of the *Acronema* cade and tribe Oenantheae, which were placed as a sister group to the remaining clades (Figure 2).

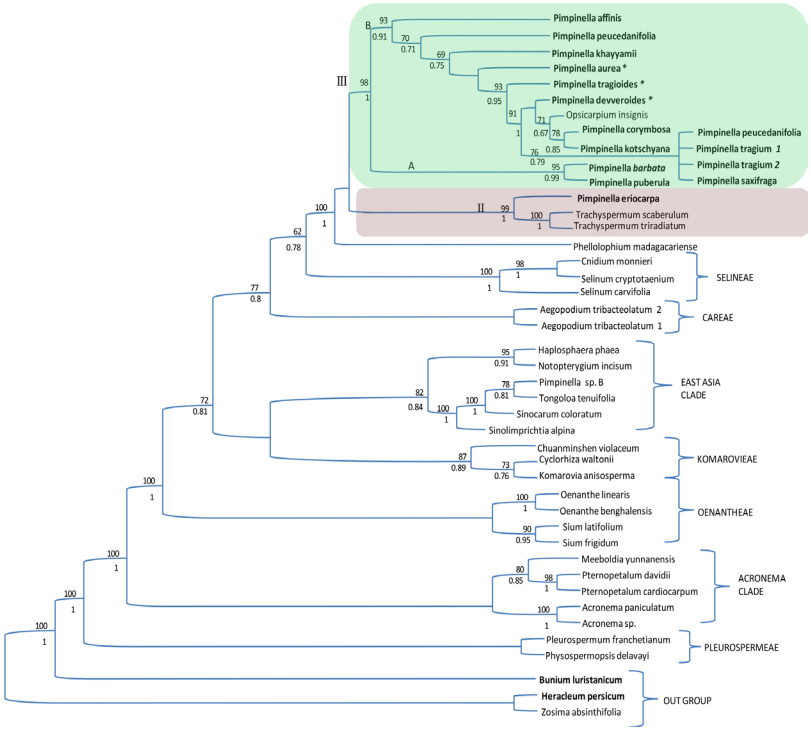

Figure 2. Bayesian majority-rule consensus trees derived from reduced ITS and cpDNA datasets. Posterior probability and bootstrap support values are shown on branches. The species involved in this study are boldfaced; the accessions which are obtained from GenBank are indicated in normal text. Bold and italic indicate species with multiple accessions. Asterisks indicate species that are confirmed as *Reutera* group.

DISCUSSION

The phylogenetic trees developed in the present study are compatible with the division of Apioideae proposed by Downie et al. (2010) and Zhou et al. (2009), and with the results of previous investigations on Pimpinelleae (Wang et al., 2014). Our discussion of the phylogenetic relationships in subclades I and II of the *Pimpinella* core group was based on the large ITS dataset BI tree, which included more representative sampling (Figure 1), with emphasis on Southwest Asian species.

Phylogenetic placement of *Pimpinella* species

Phylogenetic reconstructions using three different data sets consistently showed that *Pimpinella* is polyphyletic with the majority of its members contained within the *Pimpinella* core group with two major well-supported subclades (II, III; Figure 2).

The affinity of some members of *Pimpinella* with the tribes Oenantheae, Selineae, and the clades *Acronema* and East Asia, and the polyphyly of the genus, were inferred in earlier phylogenetic studies (Figure 1) (Downie and Katz-Downie, 1996; Zhou et al., 2008, 2009; Downie et al., 2010; Magee et al., 2010; Wang et al., 2014).

Consistent with previous data (Magee et al., 2010; Wang et al., 2014), a close correlation was observed between geography and the phylogenetic tree in our analysis (Figure 1). Within the *Pimpinella* core group, all of the African species of *Pimpinella* constituted an early diverging branch and formed a sister group to Eurasian *Pimpinella*. It has been suggested that southern Africa is the origin of the predominantly herbaceous Apiaceae subfamily Apioideae, and that the woody habit is plesiomorphic (Downie and Katz-Downie, 1999). Those species share a chromosome base number of x = 9 for many species. The subclades (II and III) identified are consistent with those previously reported (Magee et al., 2010; Wang et al., 2014). All species of *Pimpinella* from Southwest Asia fall within subclades II and III, which show affinity to other *Pimpinella* species. Based on molecular evidence within the context of the genus *Pimpinella*, Wang et al. (2014) placed some Chinese species in four other tribes within the subfamily (Figure 1).

Interspecific relationships within subclade II were well resolved and strongly supported (BS = 85%, PP = 93) (Figure 1). Two more clades were present, one comprising *P. eriocarpa* and *P. peregrina* in the large ITS dataset BI tree (Figure 1), and one comprising *P. eriocarpa* in the BI tree of the combined analysis (Figure 2) with a wide distribution in Eurasia, sharing the chromosome base number of x = 8 (Al-Eisawi, 1989; Pimenov et al., 1996). Another clade was represented by all species native to China and shared a chromosome base number of x = 9. An anatomical character possibly associated with the results of the molecular phylogenies is the number of vallecular vittae between each rib. Based on the fruit anatomy, *P. eriocarpa* can be distinguished from other Southwest Asian *Pimpinella* by the presence of two vallecular vittae between each rib, while most of the Southwest Asian species have more than four vallecular vittae between the ribs (Khajepiri et al., 2010).

Subclade III encompasses species with a wide distribution in Eurasia, and is divided into two major subgroups (A and B). *P. barbata* (DC.) Boiss., *P. puberula* and *P. cretica* Poir formed subgroup A and occupy a basal position in subclade III of both trees (Figures 1 and 2). In addition to the molecular support, the relationship between *P. barbata*, *P. puberula*, and *P. cretica* is strongly supported by several morphological characters, including pubescent annual herb, cauline leaf with linear lobe, white petal, and depressed stylopodium. *P. cretica* differs from *P. puberula* by being glabrous or sometimes minutely puberulent, with glabrous rays, and fruit minutely setulose.

Subgroup B contained all other taxa insubclade III (BS = 75%, PP = 0.91). This subgroup was resolved by the combined large ITS dataset (Figures 1 and 2).

The basal portion of subgroup B in the large ITS dataset BI tree was a ladder composed of *P. sintenisii* H. Wolff., *P. paucidentata* V.A. Matthews, *P. lutea* Desf., *P. flabellifolia* (Boiss.) Benth. ex Drud, and *P. deverroides* Boiss. (Figure 1). This relationship was strongly supported by morphology; however, these species are very similar and are described as perennial herbs with yellow, not emarginated, and acute apex petals, and simple, oblong, or cylindrical basal leaves (Matthews, 1972). They show a wide distribution from Lebanon, extending into Turkey. The remaining *Pimpinella* species and the monotypic genus, *O. insignis* form a strongly supported clade (Figure 1, PP = 0.99). Most of the relationships in this clade are unresolved. This clade consists of two well-supported groups (Figure 1) in the molecular analysis. In one of those groups, one accession of *P. corymbosa* was sister to all members of the clade (Figure 1, BS = 90%, PP = 1) and the other accession of *P. corymbosa* is the sister taxon to one accession of *P. kotschyana* and *P. aromatica* (Figure 1, BS = 91, PP = 1). This relationship is strongly supported by morphology, and these species are very similar with the exception

of *P. kotschyana*, which differs from *P. corymbosa* with the presence of 1.5-4.0-cm long leaf segments, umbels with 10-22 rayes, and fruit with very long dense hairs (Matthews, 1972).

Polyploidy in the evolution of *Pimpinella*

Magee et al. (2010) reported the chromosome numbers of 2n = 18, 20 for Eurasian *Pimpinella*. The most common base chromosome number is x = 10. Numerous cases of aneuploidy and polyploidy have been recorded for this genus (Ghahremaninejad et al., 2013; Shner et al., 2004). However, chromosome number alone has limited taxonomic value, because high levels of variation in infraspecific chromosome number have been noted for some species (Ghahremaninejad et al., 2013; Shner et al., 2004). Hybridization, while relatively rare in Apiaceae, has been reported in *Heracleum* (Logacheva et al., 2008).

The diploid *P. flabellifolia* may have been involved in the origin of the polyploidy in *P. deverroides* (Figure 1). These species are morphologically very similar, but the diploid taxa can be distinguished from the polyploid taxa by the presence of fewer umbel rays and flowers (Engstrand, 1987; Mozaffarian, 2007). Western Eurasian *P. deverroides* and *P. flabellifolia* are very narrow endemic species occurring in western Iran and southern Turkey.

Taxonomic implications

Pimpinella tragium Vill.

P. tragium Vill. is a perennial plant widely distributed across Europe, Asia, and North Africa (Matthews, 1972; Engstrand, 1987). Morphological variability and density of leaf indumentum, as well as leaf incision and geographical pattern of those morphological characters along the whole geographic area of the *P. tragium*, make the taxonomy of the *P. tragium* group difficult.

The species *P. tragium* is divided into five subspecies: *P. tragium* subsp *lithophila* (Schischk.) Tutin, *P. tragium* Vill. subsp *polyclada* (Boiss. & Heldr.) Tutin, *P. tragium* Vill. subsp *pseudotragium* (DC.) Matthews, and *P. tragium* subsp *depressa* (DC.) Tutin. *P. tragium* subsp *titanophila* (Woronow) Tutin differs in leaf shape and structure, life history, and development (Matthews, 1972; Engstrand, 1987).

Recently, Yurtseva and Tikhomirov (1998) reassessed the classification of *P. tragium*. They believed that the recognition of infraspecific taxa is impractical and can misrepresent the complicated pattern of morphological variability within *P. tragium.* Those authors suggested that *P. tragium* subsp *lithophila* and some other subspecific taxa (*P. tragium* subsp *depressa* and *P. tragium* subsp *titanophila*) should be included in this group under the name *P. tragium*, and suggested that *P. tragium* subsp. *Polyclada* should be treated at the specific level as *P. polyclada* Boiss. et Heldr.

From a morphological point of view, *P. tragium* subsp *lithophila* has smaller leaf lobes, which are 5-10 (12)-mm long with a deeply serrate margin, while *P. tragium* subsp *polyclada* has bigger leaf lobes, which are 10-15 (20)-mm long and a margin that is not deeply serrate (Matevski, 2005).

The phylogenetic analysis united *P. affinis* with the two sampled subspecies of *P. tragium* [*P. tragium* subsp *Polyclada* and *P. tragium* subsp *pseudotragium* (DC.) Mathews], which received strong support while nested deeply within subgroup B. *P. tragium* subsp *lithophila* (Schischk.) Tutin, and the group of five species from Southwest Asia, constituted

an unresolved polytomy; these findings showed that the group of *tragium* subspecies was not monophyletic (Figure 1). The sister-group relationship between *P. tragium* subsp *polyclada* and *P. tragium* subsp *pseudotragium* reflected high sequence variation among subspecies within the *tragium* group, and confirms that the two subspecies share a common ancestor and carry the same genome. Non monophyly of the *tragium* subspecies group is not surprising, because from a morphological point of view, *P. tragium* subsp *lithophila* has smaller leaf lobes, which are 5-10 (12)-mm long with a deeply serrate margin, while *P. tragium* subsp *polyclada* has bigger leaf lobes at 10-15 (20)-mm long, and a margin that is not deeply serrate (Matevski, 2005).

The present data suggested that the character "length and margin of leaf lobe color" can be considered reliable for assessing phylogenetic relationships within the *tragium* subspecies group. This finding is inconsistent with those of Yurtseva and Tikhomirov (1998).

Opsicarpium Mozaff.

Traditionally, genera are delimited on the basis of morphological distance rather than phylogeny. However, morphological differences between two groups of closely related species, or putative genera, do not necessarily imply that they constitute sister lineages. Rather, one is nested within the other, with the former being defined based on synapomorphies and the latter on symplesiomorphies (Kurzyna-Młynik et al., 2008). The genus *Opsicarpium*, and several other segregates of *Pimpinella*, illustrated this taxonomic practice.

The current phylogenetic analyses strongly suggested that *Opsicarpium* is nested within *Pimpinella*, specifically among the taxa of subclade III (Figure 1 and 2). *Opsicarpium* consists of one narrowly distributed and endemic species, *O. insignis* Mozaff., which occurs in the northern and middle Zagros Mountains of Iran (Mozaffarian, 2007). The generic status of the monotypic *Opsicarpium* is accepted (Valiejo-Roman et al., 2006a; Wang et al., 2014) because of the close relationship with *Pimpinella*. Morphologically, *Opsicarpium* is similar to *Pimpinella* for fruit structures (Khajepiri et al., 2010) and pinnate leaves. Compared with *Pimpinella*, *Opsicarpium* has no or only highly reduced sepals (versus reduced sepals in *Pimpinella*). The most significant difference between *Opsicarpium* and *Pimpinella* was thought to be the possession of a terminal segment roughly circular in *Opsicarpium* versus a terminal segment that is roughly linear in *Pimpinella* (Mozaffarian, 2007).

Our phylogenetic trees were consistent with those previously reported (Valiejo-Roman et al., 2006a; Wang et al., 2014) such that *Opsicarpium* and *Pimpinella* are considered together as a monophyletic group within Pimpinelleae (Figure 1 and 2). The secure placement of *Opsicarpium* within *Pimpinella* is also strongly supported by our data, where *Pimpinella* is considered to be monophyletic requires eight more steps, and topology of *Pimpinella* to be monophyletic have lower likelihood value (one-tailed SH test; $P = 0.016$). Hence, our results suggest that *Opsicarpium* should be included in *Pimpinella*. This conclusion is also supported by similarities in fibrous collar, pinnate leaves, compound umbel inflorescence, bracts and bracteoles, fruit structure (numerous vittae) and variable commissural vittae (2-8) shared by several species of *Pimpinella* (Khajepiri et al., 2010). Therefore, the new synonym and the necessary combination are proposed here.

Pimpinella insignis (Mozaff.) Fereid., comb. nov.

Opsicarpium insignis Mozaff. in Botanich. Zhurn 88, 2: 89 (2003). Type: [Iran],

Kordestan, Bane, ca. 10 km on the road from Sute to Sonnateh to Saghez, after haji-Mohammadan, 1950-2150 m. 17.08. 1991. *V. Mozaffarian*, *70103* (holotype: TARI).

A. tribracteolatum

A. tribracteolatum was described initially under *Pimpinella*, as *P. anthriscoides* Boiss. This perennial species is distributed in southwest and central Asia and possesses 1-2-pinnate basal leaves and glabrous fruit (Boissier, 1872). *Aegopodium* L., a genus in Careae tribe, used to contain seven perennial species distributed in the Palearctic (Europe, temperate Asia, North Africa, adventives in North America). *Aegopodium* is characterized by several synapomorphies: glabrous fruits without prominent ribs; inconspicuous vallecular and commissural vittae, sometimes invisible in mature fruits (3-5 in each furrow and 4-9 on the commissural side); big, slightly lignified endocarp cells and a broad ultimate leaf (Zakharova et al., 2012).

Recently, molecular studies grouped *P. anthriscoides* with species of *Aegopodium* based on nuclear region ITS DNA (Zakharova et al., 2012). Consequently, Zakharova et al. (2012) transferred *P. anthriscoides* to *Aegopodium*, as *A. tribracteolatum*, which is the nomenclature adopted here. On the basis of the results of a fruit anatomical study, Ghahremaninejad et al. (2010) transferred *P. anthriscoides* to the monotypic genus *Pseudopimpinella*.

The placement of *A. tribracteolatum* within *Aegopodium* is also strongly supported here. In both ITS and combined plastid trees, this is nested in a group with *Aegopodium* and Careae (Figure 1 and 2). The strongly supported position of this species in the *Aegopodium* group confirms that the submersion of *P. anthriscoides* into *Aegopodium* by Zakharova et al. (2012) was correct. This conclusion is also supported by the clear morphological similarity in glabrous fruits shared by several species of *Aegopodium* (Khajepiri et al., 2010). In IPNI (http://www.ipni.org), *Pseudopimpinella* is mentioned as a valid taxon name. Thus here, we synonymize this taxon with *Aegopodium*:

Aegopodium L. Sp. Pl. 1: 265. 1753

Pseudopimpinella F. Ghahrem., Khajepiri & Mozaff., Flora, Morphol. Distrib. Funct. Ecol. Pl. 205(5): 353. 2010.

Aegopodium tribracteolatum Schmalh. Bull. Soc. Geogr. Cauc. (1892) 22; et in Ber. Deutsch. Bot. Ges. x. (1892) 289.

Pseudopimpinella anthriscoides (Boiss.) F. Ghahrem., Khajepiri & Mozaff., Flora, Morphol.

Distrib. Funct. Ecol. Pl. 205(5): 353. 2010.

P. anthriscoides Boiss. Fl. Orient. [Boissier] 2: 874. [Dec 1872 or Jan 1873].

Reutera Boiss.

Pimpinella was established by Linnaeus (1753) in Species Plantarum, which consists of plants with bisexual flowers with five stamens and two carpels. In 1875, Boissier named specimens with entire petals as *Reutera* Boiss. and specimens with emarginate or bilobate petals as *Pimpinella*.

In the most comprehensive classification of *Pimpinella*, based mainly on morphological

characters, Wolff (1910) treated *Reutera* as a section of *Pimpinella*. This view was dismissed by subsequent systematic studies, which proposed no infrageneric classification for the genus (Engstrand, 1987; Matthews, 1972). The results of recent phylogenetic analyses strongly suggested that the infrageneric classification of *Pimpinella* as proposed by Wolff (1910) does not correspond to any of the clades recovered and is thus clearly artificial (Magee et al., 2010).

Of approximately 150 *Pimpinella* species, five were placed in the genus *Reutera* according to Boissier (1875). *Reutera* is recognized by the unique character of the petal, which has an entire or yellow petal at the rank of genus and section, respectively (Boissier, 1875; Wolff, 1910). However, when the presence of the entire petal trait is mapped onto the tree, it appears to be homoplastic in *Pimpinella* (Figure 1, character 1, 2), indicating that these characters are of limited phylogenetic value and that Wolff's submersion of *Reutera* into *Pimpinella* was correct. The phylogenies presented here show that the genus defined by Boissier (1875) is paraphyletic. Our molecular data unambiguously support the circumscription of *Pimpinella* by Wolff (1910); however, recognition of *Reutera* as a section of *Pimpinella* proposed by Wolff (1910) is not supported.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Voucher information (or reference to voucher information) and GenBank accession No. for taxa used in the present study. A dash indicates that the region was not sampled. Voucher specimens are deposited in the following herbaria: TARI, Research Institute of Forests and Rangelands, Iran; T, Kharazmi University; VANF, Yüzüncü Yil University, Turkey.