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Molecular phylogeny of the genus *Amygdalus* (Rosaceae) based on nrDNA ITS and cpDNA *trnS-trn*G sequences

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Abstract: With over 40 species, almonds (*Amygdalus* L.) are among the most economically important Rosaceae fruit crops distributed in the Irano-Turanian region of southwestern and Central Asia and southeastern Europe. While *Amygdalus* is considered a separate genus in floristic treatments of Asian countries it is a subgenus or a section of *Prunus* L. s.l. in other treatments. Phylogenetic relationships of the Iranian wild almonds based on data from 2 nuclear and chloroplast spacers (nrDNA ITS and cpDNA *trn*S-*trn*G) were constructed using the maximum parsimony and maximum likelihood, Bayesian inference, and NeighborNet methods. Data from 2 nuclear and chloroplast spacers were congruent. All of the Iranian almonds formed a well-established monophyletic clade, and the subgenus *Cerasus* was recovered as sister to *Amygdalus*. *Amygdalus spinosissima* Bge. was sister to all other *Amygdalus* species included in this study. Most of the *Amygdalus* species were grouped in a monophyletic clade that consisted of 2 subclades. The taxonomic status of 2 traditional subgenera of *Amygdalus*, *Amygdalus* and *Dodecandra (*Spach) Browicz, did not agree with the phylogenetic relationships revealed here. Among the studied species of *Amygdalus*, species of the section *Spartioides* Spach form a monophyletic clade (BV = 83%). *Amygdalus mira* Koehne, *Amygdalus davidiana* (Carriere) Franch., *Amygdalus triloba* Ltdl., and *Amygdalus nana* L. were recovered outside the main clade *Amygdalus*, indicating that these species should be excluded from *Amygdalus.* Similar to the previous phylogenetic studies in *Prunus* s.l., phylogenetic analysis did not fully resolve relationships of the studied *Amygdalus*. NeighborNet analysis of the nrDNA ITS dataset of Iranian almonds supported reticulate relationships for all *Amygdalus* hybrids as previously reported.

Key words: *Amygdalus*, molecular phylogeny, nrDNA ITS, cpDNA *trn*S-*trn*G, Iran

1. Introduction

Rosaceae is a large family in the order Rosales, comprising about 90 genera and 3000 species mainly distributed in the northern hemisphere, especially in temperate zones (Potter et al., 2007). This family includes 29 genera and 243 species with 58 endemic taxa in Iran (Khatamsaz, 1993; Ghahreman and Attar, 1999). The members of this family can be easily identified with respect to their habit, flower, and fruit features. The traditional classification of Rosaceae in terms of subfamilial subdivisions was controversial. The type of fruit and basic chromosome number are the main features used for the identification. Schulze-Menz (1964) classified the members of this family into 4 subfamilies, namely Amygdaloideae, Maloideae, Rosoideae, and Spiraeoideae, based on fruit type. However, Takhtajan (1997) later reclassified the family into 12 subfamilies. According to phylogenetic studies by Potter et al. (2007), Rosaceae currently consists of 3 subfamilies, namely Spiraeoideae, Dryadoideae, and Rosoideae, with the 2 traditionally recognized subfamilies, Amygdaloideae and Maloideae, transferred into Spiraeoideae.

Amygdalus L. (almond) includes economically important fruit crops and consists of about 40 species worldwide. These species are phytogeographically distributed in the Irano-Turanian region in southwest and Central Asia and southeastern Europe (Browicz and Zohary, 1996). In Iran, almond in the form of trees or shrubs can be found in rocky and mountainous areas (about 400 m to 3800 m), steppes, and semiarid to arid habitats. They may grow in a wide range of habitats including stony to sandy slopes, dry valleys, woodlands, or steppe-forests at the margin of oak–pistachio parklands in western Iran.

Taxonomic status and circumscription of this group have always been controversial. In floristic treatments of Asian countries (*Flora Iranica*, *Flora of Iran*, *Flora of Turkey*, *Flora of the USSR*, *Flora of Armenia*, *Flora of Iraq*, *Flora of Palestine*, *Flora Orientalis*, and *Flora of China*) *Amygdalus*

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was considered a separate, distinct genus in the family Rosaceae based on sessile or subsessile flower, pubescent drupes, drying and splitting mesocarp, and pitted or grooved stones. *Amygdalus* consists of 2 subgenera, *Amygdalus* and *Dodecandra* (Spach) Browicz, according to Browicz (1969) in *Flora Iranica* and Khatamsaz (1993) in *Flora of Iran*. The former subgenus includes 2 sections, *Amygdalus* and *Spartioides* Spach, and the second lacks sectional classification.

According to *Flora Iranica*, 15 species and 2 hybrids of *Amygdalus* are distributed in Iran, while 21 species and 6 hybrids of this genus were reported in *Flora of Iran*. Among these, 7 species and all the hybrids are endemic to Iran (Khatamsaz, 1993). Additionally, 2 new species, *Amygdalus kurdistanica* Attar, Maroofi & Vafadar and *Amygdalus orazii* Maroofi, Attar & Vafadar, were later described as 2 introduced *Amygdalus* species in *Flora of Iran* (Attar et al., 2009).

Contrary to its classification in floristic treatments of Asian countries, *Amygdalus* has primarily been recognized as a subgenus or section in the genus *Prunus* L. by most European taxonomists. According to the most widely accepted classification of *Prunus*, this genus consisted of 5 subgenera including the subgenera *Amygdalus* (L.) Focke, *Prunus*, *Cerasus* Pers., *Laurocerasus* Koehne, and *Padus* (Moench) Koehne (Rehder, 1940).

Few phylogenetic studies have addressed relationships within and between species in the genus *Amygdalus* and the rest of *Prunus*; thus, the phylogenetic status of *Amygdalus* is still not clear. Almonds were recognized as monophyletic, based on the studies by Bortiri et al. (2001), using nrDNA ITS and cpDNA *trn*L-*trn*F data. According to the phylogenetic studies by Lee and Wen (2001), which used ITS sequences of nuclear ribosomal DNA, the subgenus *Amygdalus* (sensu Rehder, 1940) was revealed within the subgenus *Prunus* (sensu Rehder, 1940), and the relationships between the 2 sampled taxa of the subgenus *Amygdalus* remained unresolved. These results also agreed with those published by Bortiri et al. (2001).

Furthermore, *Amygdalus* was later revealed as a polyphyletic group by Wen et al. (2008), using data from nrDNA ITS, and *ndh*F showed the subgenus *Amygdalus* (sensu Rehder, 1940) as polyphyletic.

Based on recent molecular phylogenetic studies by Yazbek and Oh (2013) on the subgenus *Amygdalus* (almond and peach), which include 22 species and use 6 chloroplast gene regions (*trn*L-*trn*F, *trn*S-*trn*G, *trn*H-*psb*A, *rp*L16, *ndh*F-*rpl*32, and *trn*Q-5*rps*16) and 1 nuclear gene (*s6pdh*), a very strongly supported clade of *Prunus* subgenus *Amygdalus*, including almonds and peaches, was recovered. Within the clade *Amygdalus* s.s., a strongly supported resolution was lacking.

Almost all the former taxonomic classifications of *Amygdalus* based on morphological characters revealed no clear relationships between species. Taxonomic circumscriptions within Rosaceae have been associated with many difficulties due to high variation in morphological characters (Khatamsaz, 1993), self-incompatibility, interspecific gene transfer, and high rates of hybridization (Judd et al., 2002). In addition, micromorphological studies (pollen and drupe ultrastructural studies) and leaf anatomical studies in *Amygdalus* by Vafadar et al. (2008, 2010a, 2010b) indicated variation insufficient to resolve the taxonomic relationships of *Amygdalus*.

Since all studied taxa were allogamous taxa, and gene transfer was a fairly common process among these species, bifurcating trees could not be employed to represent phylogenetic relationships. Hybridization/gene transfer is sometimes quite specific, and networks may be useful only for studying certain types of evolution (Lemey et al., 2009). Phylogenetic networks are important and powerful tools for studying complex patterns in molecular sequence data and have been used to study intraspecific DNA sequence variation (Winkworth et al., 2005). The NeighborNet method produces more resolved split networks for large datasets than the split decomposition method.

The main objectives of the present work were to construct molecular phylogeny among *Amygdalus* and *Prunus*, to elucidate phylogenetic relationships within *Amygdalus*, and to evaluate the taxonomic status of *Amygdalus* based on the sequence data from nrDNA ITS and *trn*S-*trn*G intergenic spacers.

2. Materials and methods

2.1. Selection of taxa

The data matrix consisted of 47 taxa (52 accessions, including 31 taxa of Iranian *Amygdalus*) for nrDNA ITS, 43 taxa (44 accessions) for cpDNA *trn*S-*trn*G, and 39 taxa as ingroups for combined analyses. In addition, sequences belonging to different subgenera of *Prunus* were also obtained from GenBank and analyzed here. *Prunus laurocerasus* L. and *P. padus* L. were chosen as outgroups following previous molecular phylogenetic studies in *Prunus* (Lee and Wen, 2001; Wen et al., 2008). The nrDNA ITS for 24 species and 4 hybrid species of *Amygdalus* and the cpDNA *trn*S-*trn*G for 13 species and 4 hybrid species of *Amygdalus* collected in Iran are published here for the first time. The taxa analyzed and voucher information are presented in Table 1.

2.2. DNA extraction

DNA extraction was performed from either fresh collected leaves or dried herbarium leaf specimens from the Tehran University Herbarium (TUH) using a modified CTAB method of Doyle and Doyle (1987). The collected specimens were deposited in the TUH after DNA extraction.

Table 1. Sampled taxa used in this study with their GenBank accession numbers (nrDNA ITS and cpDNA *trn*S-*trn*G). A hyphen shows that the sequence is not accessible.

Species	Source and voucher	GenBank accession no.
		ITS/*trn*S-*trn*G
Amygdalus communis L.	Iran: Kurdistan, Attar, Maroofi & Zamani, 36333-TUH	AB890354 / AB890323
Amygdalus orazii Maroofi, Attar & Vafadar	Iran: Kurdistan, Maroofi, Attar & Vafadar, 37225-TUH	AB890353 / AB890327
Amygdalus trichamygdalus (Hand.-Mazz.) Woronow	Iran: Kurdistan, Attar, Maroofi & Zamani, 36331-TUH	AB890355 / AB890335
Amygdalus wendelboi Freitag	Iran: Hormozgan, Ghahreman & Mozaffarian, 5420-TUH	AB890364 / AB890306
Amygdalus korshinskyi (Hand.-Mazz.) Bornm.	Iran: Kurdistan, Attar, Maroofi & Zamani, 36337-TUH	AB890346 / AB890334
Amygdalus sp.	Iran: Kurdistan, Maroofi, Attar & Vafadar, 37325-TUH	AB890344 / AB890325
Amygdalus fenzliana (Fritsch) Lipsky	Iran: east Azerbayjan, Attar & Zamani, 37212-TUH	AB890367 / AB890339
Amygdalus nairica Fed. et Takht.	Iran: east Azerbayjan, Attar & Zamani, 37219-TUH	AB890366 / AB890340
Amygdalus haussknechtii (C.K.Schneider) Bornm.	Iran: Kurdistan, Attar, Maroofi & Zamani, 36330-TUH	AB890359 / AB890337
Amygdalus kurdistanica Attar, Maroofi & Vafadar	Iran: Kurdistan, Maroofi & Mohammadi, 6588-Kurdistan Herbarium	AB890357 / AB890321
Amygdalus orientalis Duh.	Iran: Kermanshah, Attar, Vafadar & Zamani, 37231-TUH	AB890365 / AB890313
Amygdalus kotschyi Boiss. & Hohen.	Iran: Kurdistan, Attar, Maroofi & Zamani, 36029-TUH	AB890350 / AB890333
Amygdalus carduchorum Bornm.	Iran: Kurdistan, Attar, Vafadar & Maroofi, 37235-TUH	AB890349 / AB890322
Amygdalus pabotii Browicz	Iran: west Azerbayjan, Attar, Vafadar & Maroofi, 37224-TUH	AB890351 / AB890314
Amygdalus elaeagnifolia Spach subsp. *elaeagnifolia*	Iran: Esfahan, Attar & Zamani, 36186-TUH	AB890318 / -
Amygdalus elaeagnifolia Spach subsp. *leiocarpa* (Boiss.) Browicz	Iran: Kohgiluyeh and Boyer Ahmad, Attar & Zamani, 36275-TUH	AB890319 / -
Amygdalus reticulata Runemark ex Khatamsaz	Iran: Fars, Attar, Khatamsaz & Sheikh, 20390-TUH	AB890368 / AB890315
Amygdalus arabica Olivier	Iran: Kurdistan, Attar, Maroofi & Zamani, 36335-TUH	AB890356 / AB890316
Amygdalus arabica	Iran: Tehran, Vafadar & Kazemi, 37349-TUH	AB890317 / -
Amygdalus glauca Browicz	Iran: Fars, Attar & Zamani, 36299-TUH	AB890361 / AB890320
Amygdalus scoparia Spach	Iran: Esfahan, Attar & Zamani, 36106-TUH	AB890308 / -
Amygdalus scoparia	Iran: Fars, Attar & Zamani, 36285-TUH	AB890360 / AB890309
Amygdalus scoparia	Iran: Boushehr, Attar, 36382-TUH	AB890310 / -
Amygdalus scoparia	Iran: Tehran, Ghahreman & Mozaffarian, 6283-TUH	AB890307 / -
Amygdalus spinosissima Bge. subsp. *spinosissima*	Iran: Khorassan, Ghahreman, Attar, Okhovvat & Mahdigholi, 27289- TUH	AB890328 / -
Amygdalus spinosissima Bge. subsp. *turcomanica*	Iran: Khorassan, Attar & Zamani, 37181-TUH	AB890342 / AB890329
Amygdalus eburnea Spach	Iran: Kerman, Mirtadzadini, 23465-TUH	AB890363 / AB890338
Amygdalus lycioides Spach var. *horrida* (Spach) Browicz	Iran: Alborz, Vafadar & Kazemi, 37184-TUH	AB890358 / AB890332
Amygdalus lycioides var. *lycioides*	Iran: Kurdistan, Attar, Maroofi & Zamani, 36024-TUH	AB890331 / -
Amygdalus lycioides var. *lycioides*	Iran: Esfahan, Attar & Zamani, 36319-TUH	AB890330 / -
Amygdalus sp.	Iran: Hamadan, Attar & Zamani, 36318-TUH	AB890343 / AB890324
Amygdalus sp.	Iran: Chahar Mahal-e Bakhtiari, Mozaffarian, 54543-TUH	AB890345 / AB890326
Amygdalus × *keredjensis* Browicz	Iran: Alborz, Vafadar & Kazemi, 37351-TUH	AB890352 / AB890341
Amygdalus × *kamiaranensis* Khatamsaz & Assadi	Iran: Kurdistan, Attar, Maroofi & Zamani, 36313-TUH	AB890336 / AB890347
Amygdalus × *iranshahrii* Khatamsaz	Iran: Fars, Vafadar & Kazemi, 37123-TUH	AB890311 / AB890348
Amygdalus × *yasujensis* Khatamsaz	Iran: Kerman, Mirtadzadini, 23470 -TUH	AB890312 / AB890362
Subgen. *Padus*, *Prunus serotina* Ehrh.	USA: Illinois, Wen, 7229-US	NA / AM950176

Table 1. (continued).

Subgen. *Padus*, *Prunus padus* L.	USA: Colorado, cult. CS TS82097, Lee & Wen, 4027-CS	AF318726 / AY871259
Subgen. *Laurocerasus*, *Prunus ilicifolia* (Nutt.) Walp.	USA: Santa Barbara, Young s.n., CS	AF179543 / AY871258
Subgen. *Laurocerasus*, *Prunus laurocerasus* L.	Cult. AA 889-72-D, Lee & Wen, 5001-CS	AF318724 / AY500740
Amygdalus argentea (Lam.) Rehd.	DPRU 194	AF318749 / AY871254
Amygdalus nana L.	USA: Colorado, cult. CS TS93054, Lee & Wen, 4011-CS	AF179560, AF179561 / AY500734
Amygdalus mira Koehne	DPRU 0953. EB 93	DQ003551 / AY500732
Amygdalus davidiana (Carriere) Franch.	DPRU 581	AF318744 / AY500731
Amygdalus triloba Lidl.	USA: Colorado, cult. CS s.n.: Berggren s.n., CS	EU669088 / NA
Amygdalus bucharica (Korsh.) Hand.-Mazz.	DPRU 192.2	AF318719 / NA
Prunus dulcis (Mill.) Webb.	USA: Missouri, cult. MBG1983-0585: Davis s.n., CS	EU669085 / EU669146
Prunus persica (L.) Batsch.	China: Zhejiang Prov., Wen, 3017-CS	AF318741 / AY500733
Subgen. *Cerasus*, *Prunus pumila* L.	DPRU 389.1	NA / AY871255
Subgen. *Cerasus*, *Prunus avium* (L.) L.	Cultivar: Van, no voucher	AF318737 / AY871252
Subgen. *Cerasus*, *Prunus tomentosa* Thunb.	USA: Colorado, cult. CS TS81261, Lee & Wen, 4010-CS	AF179500 / AY500729
Subgen. *Cerasus*, *Prunus mahaleb* (Dougl.) L.	USA: Colorado, cult. CS TS83156, Lee & Wen, 4015-CS	AF318747 / AY500736
Subgen. *Cerasus*, *Prunus pensylvanica* L.	USA: Wisconsin, Wen, 7298-US	EU669090 / AY500737
Subgen. *Cerasus*, *Prunus emarginata* (Hook.) Walp.	DPRU 2214	AF318717 / AY871260
Subgen. *Cerasus*, *Prunus fruticosa* Pall.	DPRU 385.11	AF318738 / AY871257
Subgen. *Cerasus*, *Prunus glandulosa* Thunb.	USA: Colorado, cult. Ft. Collins, Berggren s.n., CS	AF318727 / AY500727
Subgen. *Cerasus*, *Prunus bifrons* Fritsch	DPRU 1213.1	AF318757 / AY871246
Subgen. *Cerasus*, *Prunus microcarpa* (C.A.Mey.) Boiss.	DPRU 165.4	AF492416 / AY871248

Abbreviations: TUH: Tehran University Herbarium, Cs: Colorado State University Arboretum, MBG: Missouri Botanical Garden, DPRU: USDA National Clonal Germplasm Repository.

2.3. Amplification, sequencing, and alignment of target regions

The nrDNA ITS spacer was amplified using primers ITS4 published by White et al. (1990) and ITS5m published by Sang et al. (1995). The cpDNA *trn*S-*trn*G region was amplified using primers trnS and trnG published by Hamilton (1999). PCR amplification of the selected markers was performed in a 20 µL volume for both fragments containing 7.2 µL of deionized water, 10 µL of 2x Taq DNA polymerase master mix Red [Amplicon, cat. no. 180301; 150 mM Tris-HCl, pH 8.5; 40 mM $(NH_4)_2SO_4$; 3.0 mM $MgCl_2$; 0.4 mM dNTPs; 0.05 units μL^{-1}Amplicon Taq DNA polymerase; inert red dye; and stabilizer], 0.5 µL of each primer (5 pmol/µL), 1 µL of DMSO, and 0.8 µL of template DNA (20 ng/µL).

The PCR profile for nrDNA ITS consisted of an initial 2.5 min premelt at 94 °C and 26–35 cycles of 1 min denaturation at 94 °C, annealing at 54 °C for 50 s, and a 55 s extension at 72 °C followed by a final extension of 55 s at 72 °C. The PCR profile for *trn*S-*trn*G consisted of an initial 4 min premelt at 95 °C and 28–35 cycles of 1 min denaturation at 95 °C, annealing at 62 °C for 1 min, and a 1 min extension at 72 °C followed by a final extension of 7 min at 72 °C. PCR products were sequenced using the BigDye terminator cycle sequencing ready kit with the same primers in an ABI Prism 3730x1DNA Analyzer (Applied Biosystems, USA).

The sequences were edited using BioEdit version 7.0.9.0 (Hall, 1999). The sequence alignment was carried out using ClustalX (Larkin et al., 2007) and adjusted manually. Although frequent single and multiple-base indels (insertions/deletions) were observed in the data matrix, positions of indels were treated as missing data for all datasets. The preliminary analyses of datasets, including the indels, produced similar results.

2.4. Phylogenetic analyses

2.4.1. Parsimony method

Maximum parsimony (MP) analyses were conducted using PAUP* version 4.0b10 (Swofford, 2002). The heuristic search option was employed for each of the 2 single datasets using tree bisection-reconnection (TBR) branch swapping with 1000 replications of random addition sequence and an automatic increase in the maximum number of trees. Uninformative characters were excluded from analyses. Branch support values were calculated using a full heuristic search with 1000 bootstrap replicates

(Felsenstein, 1985), each with simple addition sequence. The combinability of these 2 datasets was assessed using the partition homogeneity test (incongruence length difference test; Farris et al., 1995) as implemented in PAUP*. The test was conducted with invariant characters excluded (Cunningham, 1997) using the heuristic search option and including 100 replications of random addition sequence and TBR branch swapping with 1000 homogeneity replicates. An automatic increase in the maximum number of trees (by 100 trees) was selected.

2.4.2. Bayesian method

The general time-reversible model, with a parameter for invariant sites and gamma distribution (GTR+I+G), was chosen as the best fitting model of sequence evolution for the combined dataset using MrModeltest version 2.3 (Nylander, 2004) based on the Akaike information criterion (AIC) (Posada and Buckley, 2004). Both datasets were analyzed using the GTR+I+G model. The combined dataset was analyzed as a single partition with the GTR+I+G model using MrBayes (Ronquist and Huelsenbeck, 2003). Posteriors on the model parameters were estimated from the data using the default priors.

The analysis was carried out with 5 million generations for ITS and combined data matrices and 2 million generations for the *trn*S-*trn*G data matrix using Markov chain Monte Carlo search. Using MrBayes, 2 simultaneous analyses were performed starting from different random trees (nruns = 2), each with 4 Markov chains and trees sampled every 100 generations. The first 25% of trees were discarded as the burn-in. The remaining trees were then used to build a 50% majority rule consensus tree accompanied by posterior probability (PP) values. Tree visualization was carried out using TreeView version 1.6.6 (Page, 2001).

2.4.3. Maximum likelihood method

The maximum likelihood (ML) analyses were performed using SeaView4 (Gouy et al., 2010). The model of evolution employed for each dataset is the same used in Bayesian analyses.

2.4.4. Network analysis

The analysis was performed with the program Splits Tree 4.0 (Huson and Bryant, 2006). Analyses were carried out for all examined taxa for phylogenetic studies of the nrDNA ITS dataset.

3. Results

3.1. Size and structure of molecular datasets

The aligned nrDNA ITS dataset consisted of 636 nucleotide sites and of these sites 58 were parsimony-informative. The length of nrDNA ITS varies from 570 bp in *Amygdalus triloba* Lindl. to 610 bp in *Amygdalus argentea* (Lam.) Rehd. In ITS sequences of Iranian almond, many polymorphic sites were observed (Table 2). A total of 30 accessions of *Amygdalus* including 17 species, 3 undetermined species, and 4 studied hybrids possessed nucleotide site polymorphisms for nrDNA ITS sequences. Among the taxa studied, *Amygdalus orientalis* Duh., *Amygdalus lycioides* Spach var. *lycioides* (Esfahan population), and *Amygdalus* × *yasujensis* Khatamsaz had the highest number of polymorphic sites (9, 9, and 13, respectively) (Table 2).

The aligned *trn*S-*trn*G dataset consisted of 797 sites; among these, 3 sites were parsimony-informative. The length of the *trn*S-*trn*G dataset varied from 584 bp in *Prunus bifrons* Fritsch to 709 bp in *Amygdalus mira* Koehne and *Prunus persica* (L.) Batsch. These datasets differed in their taxon sampling, with 56 accessions for nrDNA ITS and 48 for *trn*S-*trn*G. Large gaps throughout the *trn*S-*trn*G aligned matrix were introduced. The aligned, combined nrDNA ITS-*trn*S-*trn*G dataset for 46 taxa was 1372 bp long, and 302 sites were parsimony-informative.

3.2. Analysis of the nrDNA ITS dataset

MP analysis of the dataset resulted in 226 shortest trees of length (L) = 119 steps, CI = 0.605, and RI = 0.804. The strict consensus tree of these trees is shown in Figure 1. Bayesian and ML trees are topologically similar to the MP tree (trees not shown). The ITS tree exhibits several polytomies. Taxa of the subgenus *Cerasus* (sensu Rehder, 1940) were recovered as sister to *Amygdalus*. All Iranian *Amygdalus* with *Amygdalus bucharica* (Korsh.) Hand.-Mazz. and *A. argentea* were grouped in a monophyletic clade with BV = 100 and 77 (parsimony and likelihood analyses, respectively), and PP = 1.00. Three Chinese almond species, namely *Amygdalus mira* Koehne, *A. davidiana* (Carriere) Franch., and *A. triloba*, in addition to *Amygdalus nana* L. (with distribution in China, Russia, and southeastern Europe) were recovered from the clade *Amygdalus*. The clade *Amygdalus* comprises 3 main subclades. Subclade A consists of members of the section *Spartioides*. Most species in subclade B belong to the subgenus *Amygdalus*, section *Amygdalus*. In this subclade, *Amygdalus communis* L. and *A. orazii* Maroofi, Attar & Vafadar (2 tree almonds) formed a monophyletic group, although these relationships were poorly supported. Subclade C contained various species from the 2 subgenera *Amygdalus* and *Dodecandra*.

3.3. Analysis of the chloroplast *trnS-trn*G dataset

Parsimony analysis of the dataset resulted in 15 shortest trees of length (L) = 249 steps, CI = 0.927, and RI = 0.984 (tree not shown). Bayesian and ML trees are topologically similar to the MP tree (trees not shown). All trees showed a high degree of polytomy, and the relationships of the studied species were poorly resolved, perhaps due to the low number of parsimonious sites. As seen in the nrDNA ITS tree, progressing upward from the base, the subgenus

Table 2. Variable nucleotide sites for ITS region in 30 *Amygdalus* taxa. Sequence symbols: Y = T/C, M = C/A, R = G/A, K = T/G, S = G/C, W = T/A.

Taxa	Nucleotide number																																											
	13	38	39	57	70	81	84	113	116	121	122	124	125	126	129	133	187	195	197	202	203	219	227	228	235	238	241	244	247	268	456	497	539	621	627	628	53	206	164	108	450	426	569	599	59
Amygdalus pabotii	Y	Y			Y																																								
Amygdalus carduchorum	Y																														Y														
Amygdalus sp.	Y																																												
Amygdalus eburnea			Y																																										R
Amygdalus fenzliana																																													
Amygdalus elaeagnifolia subsp. *elaeagnifolia*				K																											Y														
Amygdalus elaeagnifolia subsp. *leiocarpa*									Y						Y	Y															S										Y				
Amygdalus × *iranshahrii*				R	Y																										S			Y											
Amygdalus × *yasujensis*					Y										Y				Y							Y	Y	Y	Y	Y	S			Y				Y				Y		R	
Amygdalus orientalis					Y				Y					Y					Y						Y	Y	Y	Y														Y			
Amygdalus sp.					Y				Y						Y	Y																													
Amygdalus communis						Y	Y																	M																			R		
Amygdalus sp.						Y																																							
Amygdalus kotschyi								Y																		Y	Y																		
Amygdalus reticulata									Y																										Y										
Amygdalus arabica (Karaj)									Y						Y	Y																		Y											
Amygdalus wendelboi												Y																																	
Amygdalus spinosissima subsp. *turcomanica*										W	M												R									Y			M					R					
Amygdalus spinosissima subsp. *spinosissima*													R									Y										Y			M										
Amygdalus nairica														Y																															
Amygdalus × *kamiaranenensis*														S																			Y	Y		R									
Amygdalus haussknechtii																	M		Y						Y		Y	Y											M						
Amygdalus arabica (Kurdistan)																		Y																											
Amygdalus scoparia (Esfahan)																				Y																									
Amygdalus scoparia (Fars)																															K														
Amygdalus scoparia (Bushehr)					Y				Y																																				
Amygdalus lycioides (Karaj)																															Y				M		M								
Amygdalus lycioides (Kurdistan)																															Y			Y											
Amygdalus lycioides (Esfahan)								Y						Y			M		Y		Y				Y		Y								M							Y			
Amygdalus × *keredjensis*																																			M										

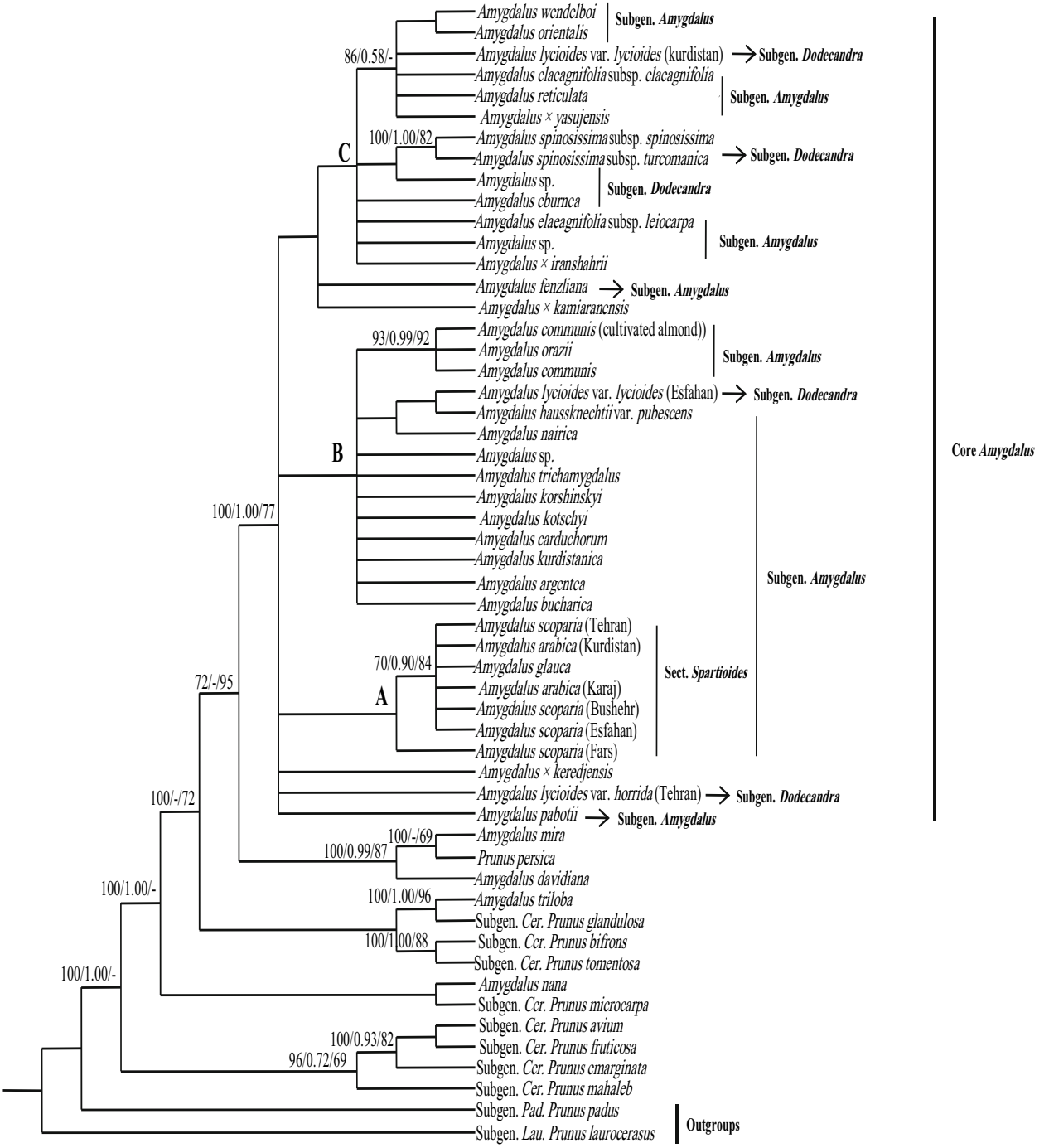

Figure 1. Strict consensus tree of the 226 shortest trees produced by the MP analysis of nrDNA ITS sequence data. Branch support values are shown above the branches as bootstrap values (BV) of MP/posterior probabilities (PP)/BV of ML.

Cerasus (sensu Rehder, 1940) was recovered as sister to *Amygdalus*. Iranian *Amygdalus* revealed a monophyletic, well-supported clade (BV = 100 and 80; PP = 0.94). However, within the clade *Amygdalus*, 3 monophyletic subclades with unresolved relationships were recovered.

3.4. The combined ITS-*trnS*-*trn*G dataset

The ILD test suggested that the *trn*S-*trn*G and nrDNA ITS datasets were congruent (P = 0.07). The combined dataset consisted of 1372 nucleotide sites, and 302 of these were parsimony-informative.

MP analysis of the combined dataset of the aligned, combined nrDNA ITS- *trn*S-*trn*G dataset resulted in 140 shortest trees, each consisting of 415 steps (CI = 0.809 and RI = 0.944). The combined tree is presented in Figure 2. The topology of the Bayesian and ML trees of the combined dataset was compatible with parsimony analysis, with a few exceptions. The tree from the combined dataset is better resolved and supported than both the nrDNA ITS and *trn*S-*trn*G trees. Taxa of the subgenus *Cerasus* were recovered as sister to the clade *Amygdalus*, similar to the individual trees (Figure 2).

With the exceptions of *Amygdalus mira* and *A. nana*, all *A.* species were grouped in a monophyletic clade (BV = 100 and 57; PP = 0.97) (Figure 2). *A. spinosissima* Bge. was recovered as sister to all other species. The main clade of almond consisted of subclade (A) (BV = 74 and 73; PP = 0.64), which comprises 2 subgroups (A_1 and A_2). Subclade A_1 comprised 2 groups, 1 from species of the section *Spartioides* and 1 with 5 species (*A. orientalis*, *A. wendelboi* Freitag, *A. reticulata* Runemark ex Khatamsaz, *A.* × *yasujensis*, and *A.* sp.).

In subclade A_2, 2 groups were observed, 1 from tree almonds including *A. communis*, *A. orazii*, and *A. trichamygdalus* (Hand.-Mazz.) Woronow as well as 1 unknown shrubby species, *A.* sp. Another group consists of some species belonging to the subgenus *A.*, section *A.* with morphological similarities including *A. nairica* Fed. et Takht., *A. fenzliana* (Fritsch) Lipsky, *A. haussknechtii* (C.K.Schneider) Bornm., *A. argentea*, and *A. kurdistanica*. The relationship of *A. kotschyi* Boiss. & Hohen. and *A. carduchorum* Bornm. to their relatives is unknown in this group. *A. nairica* and *A. fenzliana* formed a well-supported monophyletic clade (in combined and *trn*S-*trn*G trees).

Among the hybrids, *Amygdalus* × *iranshahrii* Khatamsaz was grouped with only one of its parents (*Amygdalus eburnea* Spach). The other hybrids did not form a clade with their parents (Figure 2).

3.5. Phylogenetic network

NeighborNet method analysis of the nrDNA ITS dataset of *Prunus* and *A.* recovered 2 main groups, *Prunus* and *Amygdalus* (Figure 3). *Amygdalus* taxa were grouped with each other and have not mixed with species of *Prunus* s.l. other than *Amygdalus mira*, *A. davidiana*, *A. nana*, and *A. triloba* (Figure 3). In the *Prunus* group, 3 lineages were distinguished in a split graph compatible with the clades in the *Prunus* ITS MP tree (Figure 1) including (1) *Prunus avium* (L.) L., *P. fruticosa* Pall., *P. emarginata* (Hook.) Walp., *P. mahaleb* (Dougl.) L., as well as *P. laurocerasus* and *P. padus*; (2) *P. tomentosa* Thunb., *P. bifrons* Fritsch, *P. glandulosa* Thunb., *Amygdalus triloba*, and *Prunus microcarpa* (C.A.Mey.) Boiss.; and (3) *Amygdalus mira*, *A. davidiana*, *Prunus persica*, and *Amygdalus nana*.

There was a difference between this split graph and the *Prunus* ITS MP tree. While in the tree *Amygdalus nana* and *Prunus microcarpa* formed a clade, in the split graph these 2 species were located far from each other (Figure 3). NeighborNet analysis of the nrDNA ITS dataset of Iranian almonds confirmed reticulate relationships for all *Amygdalus* hybrids, as previously suggested (Figure 4). Iranian *Amygdalus* hybrids including *Amygdalus* × *iranshahrii* (*Amygdalus scoparia* Spach × *Amygdalus eburnea*), *Amygdalus* × *kamiaranensis* Khatamsaz & Assadi (*Amygdalus lycioides* × *Amygdalus arabica* Olivier), *Amygdalus* × *yasujensis* (*Amygdalus scoparia* × *Amygdalus elaeagnifolia* Spach), and *Amygdalus* × *keredjensis* (*Amygdalus lycioides* × *Amygdalus scoparia*) produced networks with their parents (both parents or one parent). In addition, *Amygdalus pabotii* Browicz, *A. kurdistanica*, *A. fenzliana*, and one unknown species represented reticulation.

In addition to reticulation, 3 lineages are compatible with the clades in the ITS MP tree with a few exceptions (Figure 1). Respectively, the 3 lineages consist of: (1) *Amygdalus nairica, Amygdalus lycioides* (Esfahan population), *A. haussknechtii*, *A. korshinskyi* (Hand.-Mazz.) Bornm., *A. carduchorum*, *A. communis*, *A. orazii*, *A. trichamygdalus-Amygdalus* sp., and *A. kotschyi*; (2) all populations of *A. scoparia*, *A. glauca* Browicz, *A. arabica* (Kurdistan population), as well as both subspecies of *A. spinosissima* and 1 unknown species; and (3) *A. orientalis*, *A. wendelboi*, *A. reticulata*, and *A. elaeagnifolia* subsp. *elaeagnifolia*.

4. Discussion

4.1. Phylogenetic status of *Amygdalus* with regard to *Prunus*

All *Amygdalus* taxa growing in west and Central Asia were recovered in a well-supported clade in both combined and individual dataset analyses (Figures 1 and 2). However, 4 almond species outside Iran and Central Asia, namely *Amygdalus mira*, *A. davidiana*, *A. triloba*, and *A. nana*, were placed outside the clade *Amygdalus* (Figures 1 and 2). These findings were confirmed with our split graph of the nrDNA ITS dataset of *Prunus* and *Amygdalus* in which these 4 *Amygdalus* species were grouped with *Prunus* species outside of the clade *Amygdalus* (Figure 3).

The results suggest that these species belong to *Prunus* s.l. and should be excluded from *Amygdalus*. Yazbek and Oh (2013) indicated that *Amygdalus nana* (*Prunus tenella*) and *Amygdalus triloba* should be excluded from the subgenus *Amygdalus*. In the phylogenetic study of *Prunus* by Shaw and Small (2004), *Amygdalus nana* was revealed as sister to other studied almonds.

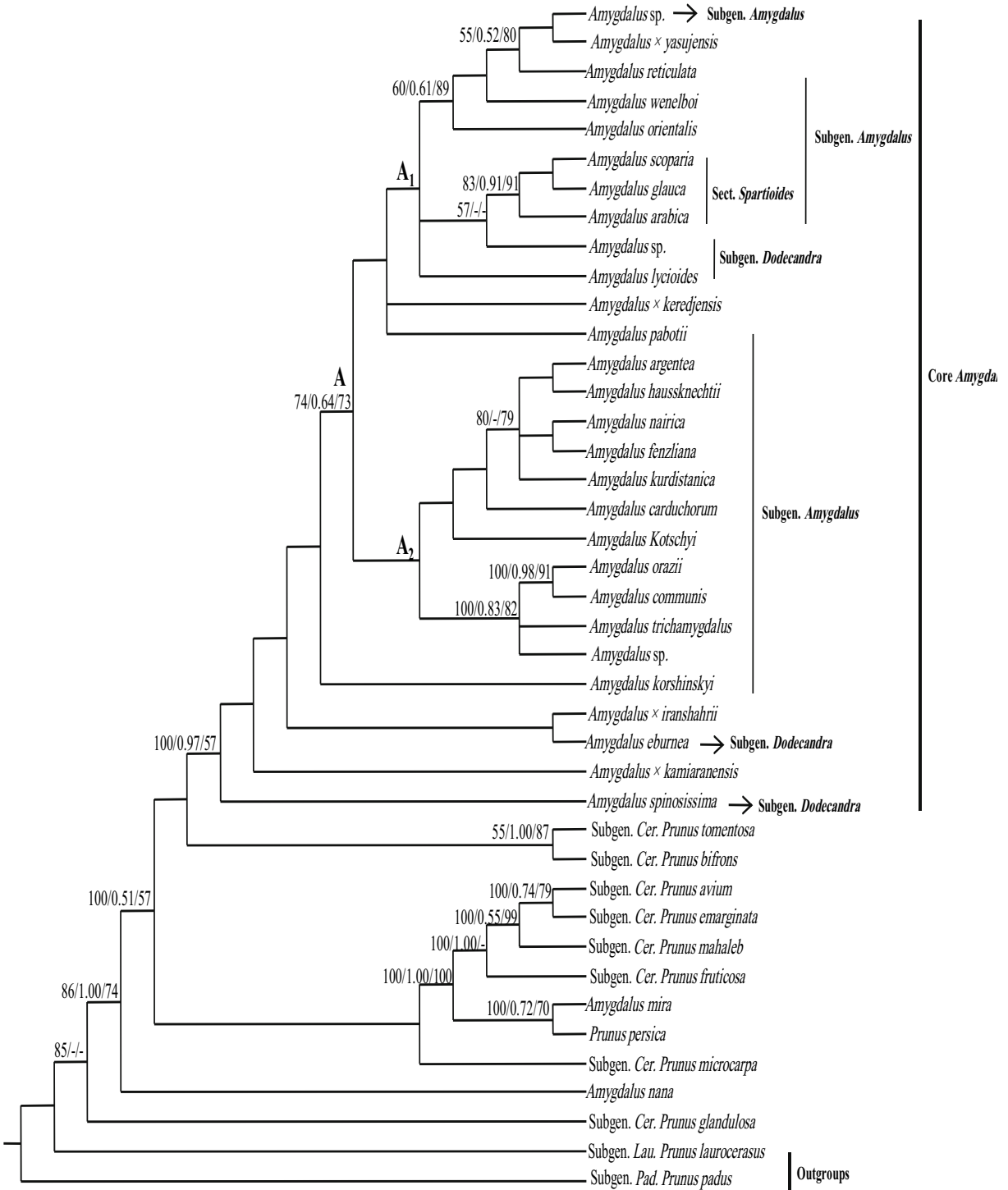

Figure 2. Strict consensus tree of the 140 shortest trees produced by the MP analysis of the combined nrDNA ITS-*trn*S-*trn*G dataset. Branch supports were shown above the branches as bootstrap values (BV) of MP/posterior probabilities (PP)/BV of ML.

In addition, in Yazbek and Oh's (2013) study, *Amygdalus mira* and *A. davidiana* clustered with peach taxa in a separate subclade. *A. mira* was recovered as sister to *Prunus persica* in a highly supported clade based on nrDNA ITS and combined datasets (Figures 1 and 2), and *Amygdalus davidiana* was recovered as sister to *A. mira* and *Prunus persica*, based on analyzing ITS sequences (Figure 1). These results agreed with the previous studies in *Prunus* by Bortiri et al. (2001) and Wen et al. (2008) that identified *Amygdalus davidiana* as sister to *Prunus persica*.

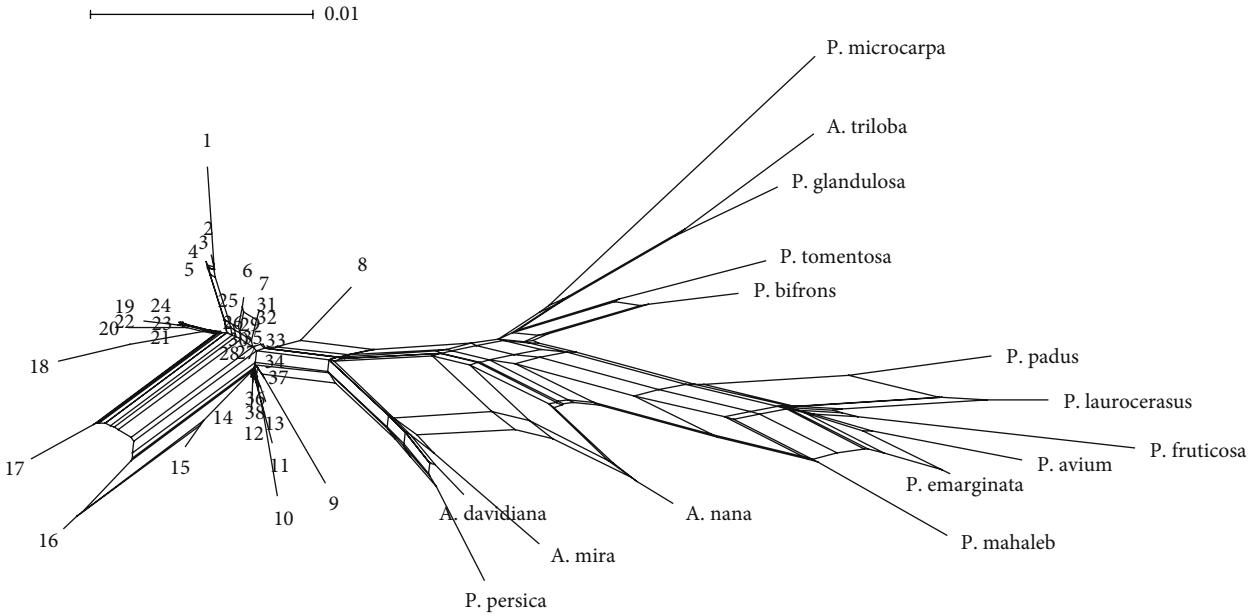

Figure 3. Split graph for nrDNA ITS sequences of *Amygdalus* and *Prunus*. Two major lineages were recovered: *Prunus* s.l. and *Amygdalus*. *Amygdalus* taxa were shown with numbers 1–38. 1. *Amygdalus orientalis*, 2. *A. wendelboi*, 3. *A. elaeagnifolia* subsp. *elaeagnifolia*, 4. *A. reticulata*, 5. *A. lycioides* (Kurdistan), 6. *A. eburnea*, 7. *A. fenzliana*, 8. *A. nairica*, 9. *A. bucharica*, 10. *A. lycioides* (Esfahan), 11. *A. haussknechtii*, 12. *A. korshinskyi*, 13. *A. argentea*, 14. *A. communis*, 15. *A. communis* (cultivated almond), 16. *A. orazii*, 17. *A.* sp., 18. *A. spinosissima* subsp. *spinosissima*, 19. *A. spinosissima* subsp. *turcomanica*, 20. *A. scoparia* (Bushehr), 21. *A. glauca*, 22. *A. arabica* (Kurdistan), 23. *A. scoparia* (Tehran), 24. *A. scoparia* (Esfahan), 25. *A.* sp., 26. *A.* × *yasujensis*, 27. *A. arabica* (Karaj), 28. *A. scoparia* (Fars), 29. *A. elaeagnifolia* subsp. *leiocarpa*, 30. *A.* × *iranshahrii*, *A.* × *kamiaranensis*, 31. *A. lycioides* (Tehran), 32. *A.* × *keredjensis*, 33. *A. pabotii*, 34. *A. carduchorum*, 35. *A. kotschyi*, 36. *A. kurdistanica*, 37. *A. trichA.*, and 38. *Amygdalus* sp.

The genus *Amygdalus* is characterized by subsessile flowers, smooth sepal margins, pubescent drupes, splitting mesocarp, and pitted or grooved stones. In this genus, flowers appear before leaves. The maximum length of stamens and petals in *Amygdalus* are 9 mm and 20 mm, respectively. The shape of the hypanthium in *Amygdalus* is campanulate to broad-campanulate, tubular, or campanulate-tubular to semispheric.

However, in *Amygdalus mira*, the mesocarp is fleshy and does not split when ripe (Lu and Bartholomew, 2003). In *A. nana* (belonging to the section *Chamaeamygdalus* in *Flora of the USSR*), leaves and flowers appear at the same time, and stamen filaments are very long. Moreover, the margin of sepals is slightly serrate with more or less sparse remote papilliform glands, the petals are very long, and the hypanthium is funneliform (Shishkin and Yuzepchuk, 1941). In *Amygdalus davidiana* (a Chinese almond), the mesocarp is dry but does not split when ripe (Lu and Bartholomew, 2003).

Amygdalus triloba is another almond species suggested for exclusion from *Amygdalus* due to sepal character, because the sepal margin in this species is sparsely serrate. Browicz (1989) suggested this exclusion.

In the present study, the subgenus *Cerasus* (sensu Rehder, 1940) was recovered as sister to *Amygdalus* based on both nrDNA ITS and the combined ITS-*trn*S-*trn*G molecular datasets (Figures 1 and 2). Two species of this subgenus, including *Prunus tomentosa* and *Prunus bifrons* (sect. *Microcerasus* Webb.), were closest to *Amygdalus*. Taxa of this section exhibited characters that placed them close to subgenera *Amygdalus* and *Prunus* (sensu Rehder, 1940), such as the presence of 3 auxiliary buds and shorter pedicels. Lersten and Horner (2000) indicated that leaf crystals in *Prunus* subgenus *Cerasus* section *Microcerasus* were similar to those of *Prunus* subgenus *Amygdalus* (sensu Rehder, 1940) and *Prunus* subgenus *Prunus*. Moreover, in a study using isozyme data on *Prunus*, Mowrey and Werner (1990) indicated that the section *Microcerasus* was grouped with the 2 above-mentioned subgenera.

4.2. Relationships within the clade *Amygdalus*

According to traditional classification of *Amygdalus* (Browicz, 1969; Khatamsaz, 1993) this genus is divided into 2 subgenera (subgen. *Amygdalus* and subgen. *Dodecandra*) based on the presence of thick spines or their absence. The findings of the current study did not confirm the infrageneric treatment of *Amygdalus,* because

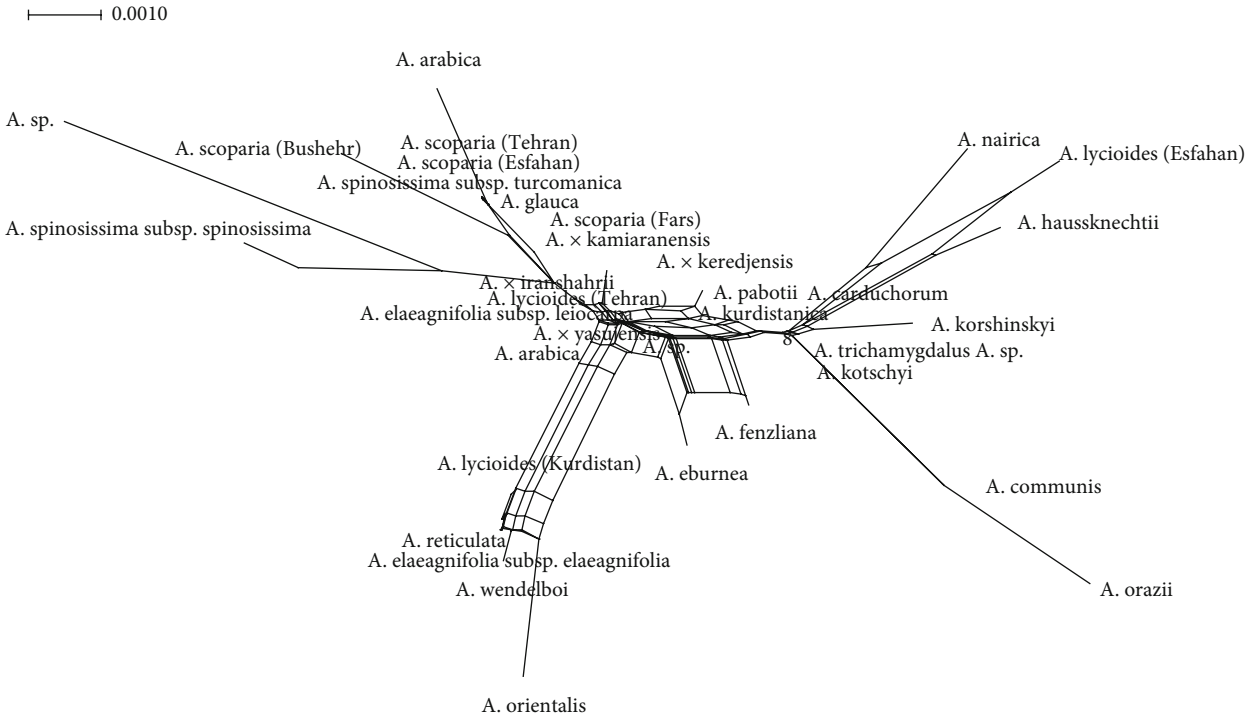

Figure 4. Split graph for nrDNA ITS sequences of *Amygdalus*. Three major lineages are more or less congruent with the clades in the ITS MP tree.

Amygdalus lycioides of *Amygdalus* subgenus *Dodecandra* was grouped with *Amygdalus wendelboi*, *A. orientalis*, *A. reticulata*, and some other species of *Amygdalus* subgenus *Amygdalus*. The subgenus *Amygdalus* consists of 2 sections: *Amygdalus* and *Spartioides*. Our results showed that the section *Amygdalus* is not monophyletic, and its members are scattered across the core clade *Amygdalus*; however, species of the section *Spartioides* were recovered in a clade (Figures 1 and 2).

In contrast to the phylogenetic results based on the combined data matrix, the relationships of the analyzed species were poorly resolved in each individual phylogenetic analysis. The unresolved relationships within *Amygdalus* species in our study were also congruent with the previous studies of the subgenus *Amygdalus* by Yazbek and Oh (2013). However, in a comparison between the present molecular phylogenetic study and the study by Yazbek and Oh (2013), more species of *Amygdalus* have been included and analyzed in the present molecular study.

Amygdalus spinosissima (subgen. *Dodecandra*) was recovered as sister to other *Amygdalus* species included here in all analyses (Figure 2). This species is found in Khorassan Province in eastern and northeastern Iran and was revealed as a distinct species based on our molecular data. Morphological characters such as spatulate leaves, a long hypanthium tube, the occurrence of thick fiber tissue around the phloem in leaf midrib anatomy, and a reticulate pollen exine sculpture type, in contrast to the common striate type, also support the separation of this species from *Amygdalus* species (Vafadar et al., 2008, 2010a).

Most of the analyzed *Amygdalus* species were recovered as a monophyletic group and consisted of 2 main subclades (A_1 and A_2) (Figure 2). In subclade A_1, species of the section *Spartioides* were recovered in a monophyletic group, and these results were in agreement with Yazbek and Oh (2013). The other group in this subclade possessed species from the section *Amygdalus* with one hybrid taxon.

Subclade A_2 consisted of 2 groups: 1 from tree almond species in the section *Amygdalus* including *A. communis*, *A. orazii*, and *A. trichamygdalus* (morphologically related to each other) and 1 shrubby unknown species; all of these formed a well-supported, monophyletic clade (BV = 100 and 82; PP = 0.83). *A. orazii*, a new almond species for *Flora of Iran* (Attar et al., 2009), was grouped with its relative (*Amygdalus communis*) in a clade. According to Yazbek and Oh (2013), *Prunus dulcis* (*Amygdalus communis*) and *Prunus trichamygdalus* were clustered in a subclade. Another group in subclade A_2 consisted of some shrubby species of subgenus *Amygdalus* section *Amygdalus* with morphological similarities, including *A. nairica*, *A.*

fenzliana, *A. haussknechtii*, *A. argentea*, and *A. kurdistanica* as well as *A. kotschyi* and *A. carduchorum*; the 2 last species were sister species to the rest in this group. Yazbek and Oh (2013) found that *A. kotschyi* and *A. haussknechtii* formed a sister group for other related species.

Amygdalus nairica (syn. *Amygdalus urumiensis* from subgen. *Amygdalus* sensu Browicz, 1969) and *A. fenzliana* formed a well-supported clade in the combined and *trn*S-*trn*G trees. Both species are found in northwestern Iran. According to Khatamsaz (1993), *A. nairica* belongs to the subgenus *Dodecandra*. However, our molecular data supported the previous conclusions of Browicz which indicated that *A. nairica* was more closely related to the subgenus *A.*, with respect to morphological characters. *A. nairica* exhibits features that differentiate it from other species in the subgenus *Dodecandra*; characters such as long petiole and leaves, comparatively large flowers, and broad tubular hypanthium lead us to suggest that its position is in subgenus *A.*, section *Amygdalus*.

The most closely related species to *Amygdalus nairica* is *A. fenzliana*. The close phylogenetic relationship between these 2 species is confirmed by other evidence such as leaf anatomical features and pericarp indumentum of drupe (Vafadar et al., 2008, 2010b).

In the combined tree, *Amygdalus argentea* (syn. *Amygdalus orientalis*) and *A. haussknechtii* were grouped in a clade. These 2 species are also morphologically similar to each other. However, *A. argentea* and *A. orientalis* had different positions in the combined MP tree (Figure 2).

Amygdalus kurdistanica was reported as a new shrubby almond from the west of Iran fairly recently by Attar et al. (2009). This species is similar to *A. haussknechtii* with respect to morphological characters; however, the phylogenetic relationship between these species was poorly resolved in our study due to the low number of informative sites or small sample size of genes.

Based on Khatamsaz (1993) in *Flora of Iran*, 6 hybrids of *Amygdalus* occur in Iran. Four hybrid species, namely *Amygdalus* × *iranshahrii*, *Amygdalus* × *kamiaranensis*, *Amygdalus* × *keredjensis*, and *Amygdalus* × *yasujensis*, were analyzed here. Among the studied hybrids, *Amygdalus* × *iranshahrii* was grouped with one of its parents, *Amygdalus eburnea*, solely in the combined MP tree (Figure 2). Among *Amygdalus* species, only *A. eburnea* shows a hairy hypanthium. Interestingly, the hypanthium in *Amygdalus* × *iranshahrii* is also hairy. This hybrid was far from its other parent, *A. scoparia*. In individual trees (Figure 1), 3 studied hybrids were relatively near to 1 of their parents but did not form a monophyletic clade with them (*Amygdalus* × *yasujensis* near *A. elaeagnifolia*, *Amygdalus* × *iranshahrii* near *A. eburnea*, and *Amygdalus* × *keredjensis* near *A. scoparia*).

This molecular phylogenetic study with unresolved relationships within *Amygdalus* showed that bifurcating trees could not help trace phylogenetic relationships within such a problematic and diverse genus as *Amygdalus* because of the high degree of inter/intraspecific variation, hybridization, and gene transfer. A split graph of the nrDNA ITS dataset of *Prunus* and *Amygdalus* (Figure 3) showed that 2 groups were distinguished: 1 from *Amygdalus* species (sensu Browicz, 1969) and 1 from *Prunus* s.l. species. Similar to our phylogenetic trees, *Amygdalus nana*, *A. davidiana*, *A. triloba*, and *A. mira* were located apart from core *Amygdalus* and included in *Prunus* s.l.

The split graph of the nrDNA ITS dataset of Iranian *Amygdalus* confirmed the idea that bifurcating trees are not appropriate tools for reconstructing phylogenetic relationships in *Amygdalus* (Figure 4). In this graph, 3 lineages as well as a network were recovered in which the lineages were more or less compatible with the clade in the ITS tree (Figure 1). *Amygdalus* hybrids were located inside the network.

In conclusion, *Amygdalus* (with its unique morphological features) was a well-supported monophyletic group. In all analyses in this study, 4 species, namely *A. davidiana*, *A. mira*, *A. nana*, and *A. triloba*, were recovered outside the main clade of *Amygdalus* species, indicating that these species should be excluded from *Amygdalus*. Molecular data in the present study were insufficient to resolve relationships within *Amygdalus*. More sequence data from other gene regions with high degrees of variation (chloroplast regions including *psb*A-*trn*H, *trn*H-*rpl2*, *rpl20-rps12*, and nuclear single copy gene, LEAFY *intron2*) and greater sampling from a larger geographic distribution range are needed to address the question of phylogenetic relationships within *Amygdalus*.

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