

## MOLECULAR PHYLOGENY OF TWO *AMELANCHIER* (ROSACEAE) SPECIES BASED ON PLASTID DNA SEQUENCES

ZÜBEYDE UĞURLU AYDIN\* AND ALİ A. DÖNMEZ

*Molecular Plant Systematic Laboratory (MOBIS), Department of Biology,  
Faculty of Science, Hacettepe University, Türkiye.*

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### Abstract

*Amelanchier* is a taxonomically complex genus because of its reproductive biology and variation in morphological characters. In Türkiye, two Old World *Amelanchier* species, *A. ovalis* and *A. parviflora*, occur with intraspecific taxa recognized at the subspecies and varietal levels, respectively. To assess phylogenetic relationships, we investigated seven *Amelanchier* species covering different geographic ranges and reconstructed phylogenetic trees using *rpl20-rps12*, *psbA-trnH*, and *trnH-rpl2* markers. For this study, more than 30 sequences representing *A. ovalis* and *A. parviflora* were newly generated. Phylogenies were constructed using Maximum Likelihood and Bayesian inference. Our findings support a biogeographic disjunction among *Amelanchier* taxa rather than clearly resolved species-level phylogenetic relationships.

### Introduction

Rosaceae is a moderately large family, comprising 88-100 genera and approximately 3,100 species (Hummer and Janick, 2009; Phipps, 2014), and is the 19th largest plant family by number of taxa (Hummer and Janick, 2009). The family has a worldwide distribution and is classified into three subfamilies, Dryadoideae, Rosoideae, and Spiraeoideae, based on molecular data (Morgan *et al.*, 1994; Potter *et al.*, 2007; Xiang *et al.*, 2017). The tribe Maleae (formerly subfamily Maloideae) belongs to Amygdaloideae and contains about 30 genera and 500-1000 species (Campbell *et al.*, 2007; Lo and Donoghue, 2012; Wang *et al.*, 2023), some of which are widely cultivated and economically important, including apple (*Malus* Mill.), chokeberry (*Aronia* Medik.), loquat (*Eriobotrya* Lindl.), pear (*Pyrus* L.), quince (*Cydonia* Mill.), and serviceberry (*Amelanchier*). Numerous attempts have been made to resolve intergeneric relationships within Maleae using non-molecular data (Phipps *et al.*, 1991; Robertson *et al.*, 1991; Rohrer *et al.*, 1991, 1994) or molecular data (Campbell *et al.*, 1995, 2007; Verbylaitė *et al.*, 2006; Lo and Donoghue, 2012; Sun *et al.*, 2024), providing valuable insights into generic delimitation. More recently, large-scale phylogenomic studies based on chloroplast and nuclear genome sequences have reconstructed well-supported phylogenies (Xiang *et al.*, 2017; Zhang *et al.*, 2023).

The genus *Amelanchier* includes more than 30 species (Tutin *et al.*, 1968; Phipps *et al.*, 1990; Campbell *et al.*, 2014). Most taxa inhabit North America, whereas only seven occur in the Old World (Phipps *et al.* 1990). The genus also has edible fruits that are used for food in the Mediterranean region (Tardio *et al.* 2006). Among Old World taxa, *A. parviflora* has a narrow distribution, including the East Aegean Islands, Greece, Lebanon, and Türkiye. *Amelanchier ovalis* is broadly distributed in Europe, Türkiye, and Transcaucasia (Valev and Assenov, 1973; Indreica *et al.*, 2016) and is divided into three subspecies. The remaining two species, *A. asiatica* (Siebold & Zuccarini) Endlicher ex Walpers and *A. sinica* (C. K. Schneider) Chun, are distributed in eastern Asia (Gu and Spongberg, 2003).

Recent efforts to reconstruct robust phylogenetic relationships among genera and clarify the placement of *Amelanchier* have used whole chloroplast genome and nuclear genome sequences

\*Corresponding author, Email: [zubeydeugurlu@hacettepe.edu.tr](mailto:zubeydeugurlu@hacettepe.edu.tr)

generated by high-throughput DNA sequencing methods (Liu *et al.*, 2020; Zhang *et al.*, 2023). In addition, Lo and Donoghue (2012) showed the monophyly of *Amelanchier* based on 11 chloroplast regions and nuclear ribosomal ITS sequences. Burgess *et al.* (2015) used five chloroplast regions and sequences from ETS, ITS, and the second intron of LEAFY in 431 diploid accessions of Old World *Amelanchier* taxa to evaluate diploid diversity and the relationships of polyploid apomicts. However, these studies included limited sampling of *A. ovalis* and *A. parviflora* for evaluating phylogenetic relationships within the genus. Indeed, none has addressed the intraspecific phylogeny of these two Old World *Amelanchier* species.

This phylogenetic study analyzed five North American *Amelanchier* species and 12 specimens representing two Eurasian species collected from Türkiye. We aimed to determine whether multiple chloroplast markers are useful for inferring phylogenetic relationships and biogeographic patterns in these two Old World *Amelanchier* taxa.

### Materials and Methods

Leaf material from 12 individual specimens representing four taxa was collected in the field, and the voucher specimens were deposited in the Hacettepe University Herbarium (HUB).

Three non-coding regions of the chloroplast genome were selected for the phylogenetic analyses. Primer information for *rpl20-rps12*, *psbA-trnH*, and *trnH-rpl2*, as well as PCR conditions, is available in Shaw *et al.* (2005). Chloroplast sequences were obtained by direct sequencing in both forward and reverse directions. In total, 33 new sequences were generated from the three plastid regions for this study. These sequences were combined with sequences retrieved from GenBank, yielding a total alignment of 1079 bp. GenBank accession numbers for each gene region are presented in Table 1.

**Table 1. GenBank accession numbers for *Amelanchier* taxa used in molecular analyses. \*newly generated sequence for this study.**

Taxa	<i>rpl20-rps12</i>	<i>psbA-trnH</i>	<i>trnH-rpl2</i>
<b>Western North America</b>			
<i>A. alnifolia</i> (Nutt.) Nutt.	KC206967	-	KC206880
<b>Eastern North America</b>			
<i>A. arborea</i> (F.Michx.) Fernald	EF127226 JQ390786	EF127152 -	EF127189 JQ392066
<i>A. bartramiana</i> (Tausch) M.Roem	JQ390784 EU500331	JQ390641 EU500285	JQ392060 EU500408
<b>Europe and Western Asia</b>			
<i>A. ovalis</i> subsp. <i>ovalis</i>	*PQ541615 *PQ541623 *PQ541613	*PQ541606 *PQ541607 *PQ541602	*PQ781219 *PQ810560 -
<i>A. ovalis</i> subsp. <i>integrifolia</i>	*PQ541622 *PQ541621 *PQ541614	*PQ541604 *PQ541603 *PQ541605	*PQ810567 *PQ810566 *PQ810568
<i>A. parviflora</i> var. <i>parviflora</i>	*PQ541619 *PQ541618	*PQ541611	*PQ810564
<i>A. parviflora</i> var. <i>dentata</i>	*PQ541620 *PQ541617 *PQ541624 *PQ541616	PQ541610 PQ541609 PQ541608	*PQ810563 *PQ810562 *PQ810561
Outgroups	*PQ541625	PQ541612	*PQ810565

The best-fit nucleotide substitution model for the combined alignment was estimated using MEGA11 (Tamura *et al.*, 2021). Phylogenetic trees were inferred using IQ-TREE and MrBayes through the CIPRES Science Gateway (Miller *et al.* 2010), as implemented in Geneious Prime 2023, under the General Time Reversible model. For the MrBayes analysis, two independent runs, each with four Markov chain Monte Carlo chains, were run for 1 million generations and sampled every 1000 generations to ensure that all effective sample size (ESS) values were above 200. The first 25% of the sampled trees were discarded as burn-in, and the remaining trees were used to build a 50% majority-rule consensus tree. Branch support values are shown as Bayesian posterior probabilities and Maximum Likelihood bootstrap values. Maximum Likelihood support was calculated using 100 bootstrap replicates.

### Results and Discussion

A total of 18 concatenated cpDNA sequences representing 10 taxa were analyzed. The concatenated dataset included 13 newly generated sequences and five sequences retrieved from NCBI (Table 1). The phylogenetic tree of *Amelanchier* taxa was reconstructed based on ML and BI analyses, with *Pyrus syriaca* included as the outgroup (Fig. 1).

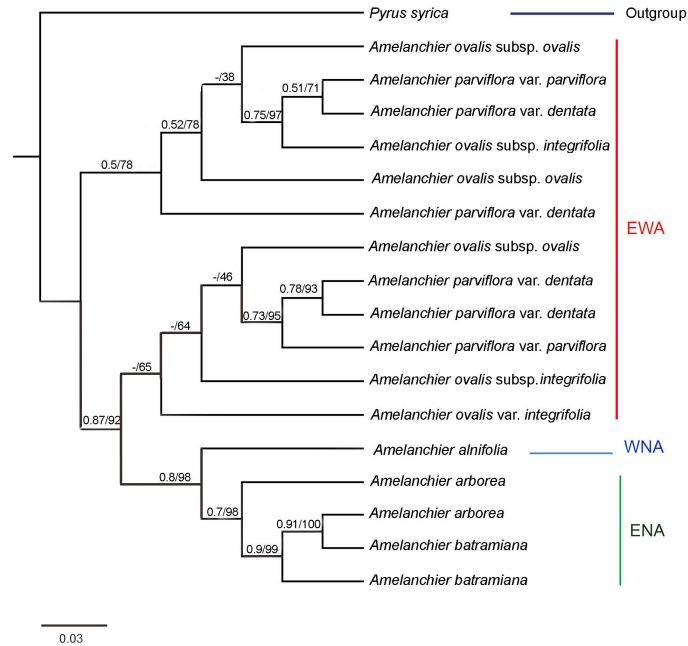


Fig. 1. Phylogenetic tree of *Amelanchier* taxa based on cpDNA data. Numbers on branches indicate posterior probabilities (left)/bootstrap values (>0.5, right). EWA: European and western Asian taxa; WNA: western North American taxa; ENA: eastern North American taxa.

The ML and BI analyses recovered largely similar topologies, with the exception of a few poorly resolved clades. The phylogenetic tree revealed two major clades within *Amelanchier*, and the North American taxa formed a well-supported monophyletic group (PP: 0.8, BS: 98). The two eastern North American species of *Amelanchier* (*A. arborea*, *A. batramiana*) formed a well-supported clade (PP: 0.7, BS: 98) that was sister to the only western North American representative, *A. alnifolia* (PP: 0.8, BS: 98). The two Old World taxa, *A. ovalis* and *A. parviflora*,

did not form a single clade but were nested in two separate groups. Some specimens of these two species were sister to all North American taxa with high support (PP: 0.87, BS: 92). However, the relationships among subspecies of *A. ovalis* were poorly resolved, and they appeared in a polytomy with *A. ovalis* and *A. parviflora*.

Since its description as a genus (Medikus, 1789), *Amelanchier* has undergone various taxonomic treatments, and some of its taxa were previously placed in different genera, such as *Mespilus* L. and *Chionanthus* Royen. One European *Amelanchier* species, *A. ovalis*, is treated as comprising three subspecies; two of them, *A. ovalis* subsp. *ovalis* and *A. ovalis* subsp. *integrifolia* (Boiss. & Hohen.) Bornm., are widespread in the South Caucasus, Türkiye, and Europe, whereas *A. ovalis* subsp. *cretica* (Willd.) Maire & Petitm. is found in Greece and Italy (Browicz, 1972; Kurtto, 2009). Morphologically, these two subspecies are very closely related, with leaf margin as the only distinguishing character. Our results indicate that multiple chloroplast markers are not sufficient to discriminate between them, and that additional taxonomic studies are required for delimiting taxa within *A. ovalis*.

The other Old World taxon, *A. parviflora* (Fig. 2), has been divided into two varieties in Türkiye and was identified as *A. parviflora* var. *parviflora* and *A. parviflora* var. *dentata* by Browicz (1972). However, more recent taxonomic work treats it as *A. parviflora* subsp. *parviflora* (distributed in Türkiye) and *A. parviflora* subsp. *chelmea* (Halácsy) Ziel., distributed in the eastern Aegean islands of Greece (Kurtto, 2009). The phylogenetic tree provided low support for discriminating between these two subspecies. Genome-scale data and more comprehensive sampling are needed to clarify the taxonomic relationships among intraspecific taxa of *A. parviflora*.

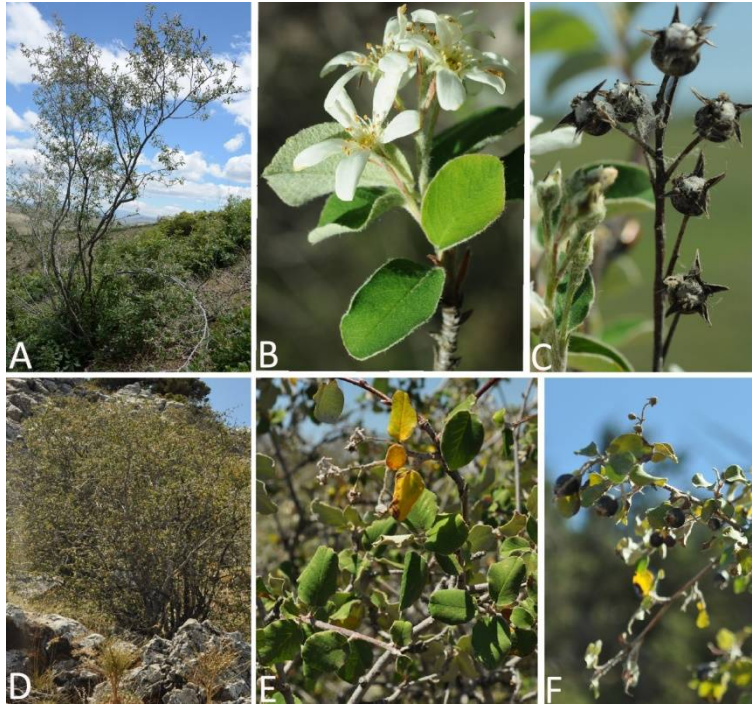


Fig. 2. *Amelanchier parviflora*. A-C: *A. parviflora* var. *parviflora*: general view (A), flower (B), fruit (C). D-F: *A. parviflora* var. *dentata*: general view (D), leaves (E), fruit (F). Photographs were taken by A.A. Dönmez.

The phylogenetic analysis, including *Amelanchier* samples collected from the three main distribution areas of the genus, showed that North American taxa are well separated from European and western Asian taxa. Liu *et al.* (2020) showed a close relationship between the western North American species of *Amelanchier* (*A. alnifolia* and one European species, *A. ovalis*); however, the broader sampling of European and western Asian taxa in this study supported a clear distinction between these groups. Nevertheless, the phylogenetic relationships between the two morphologically distinct species were not clear, and they did not form a monophyletic clade based on chloroplast molecular markers. Plastid genomes commonly reflect biogeographical ranges rather than taxonomic relationships (Liu *et al.*, 2020). Woody Rosaceae species are known to have complex reproductive biology, including hybridization and apomixis (Zhang *et al.*, 2017). These processes can lead to chloroplast capture, which involves the sharing of chloroplast genomes among hybridizing species (Stegemann *et al.*, 2012; Liu *et al.*, 2020). This may complicate the evaluation of phylogenetic relationships among taxa. Overall, the chloroplast markers used in this study were insufficient to resolve the taxonomic boundaries between these taxa, although they clearly reflected broad biogeographic patterns.

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