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Tracing the Evolutionary and Migration Pathways of Economically Important Turkish *Vicia* L. Species: A Molecular and Biogeographic Perspective on Sustainable Agro-Biodiversity

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Abstract

Understanding the evolutionary and geographic trajectories of crop wild relatives is vital for enhancing agro-biodiversity and advancing climate-resilient agriculture. This study focuses on ten *Vicia* L. taxa—comprising five species, four varieties, and one subspecies—of significant agricultural importance in Türkiye. An integrative molecular framework was applied, incorporating nuclear ITS sequence data, ITS2 secondary structure modeling, phylogenetic network analysis, and time-calibrated biogeographic reconstruction. This approach revealed well-supported clades, conserved secondary structural elements, and signatures of reticulate evolution, particularly within the *Vicia sativa* L. and *V. villosa* Roth. complexes, where high genetic similarity suggests recent divergence and possible hybridization. Anatolia was identified as both a center of origin and a dispersal corridor, with divergence events estimated to have occurred during the Late Miocene–Pliocene epochs. Inferred migration routes extended toward the Balkans, the Caucasus, and Central Asia, corresponding to paleoenvironmental events such as the uplift of the Anatolian Plateau and the Messinian Salinity Crisis. Phylogeographic patterns indicated genetic affiliations between Turkish taxa and drought-adapted Irano-Turanian lineages, offering valuable potential for climate-resilient breeding strategies. The results establish a molecularly informed foundation for conservation and varietal development, supporting sustainability-oriented innovation in forage crop systems and contributing to regional food security.

Keywords: hybridization; molecular phylogenetics; reticulate evolution; sustainable agriculture; *Vicia*

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1. Introduction

The genus *Vicia* L. is one of the most widely distributed genera in the Fabaceae family, divided into two subgenera: subgenus *Vicia* and subgenus *Vicilla*, based on the morphological and phylogenetic framework proposed by Kupicha [1] and updated through taxonomic databases such as the World Checklist of *Vicia* [2]. Initial findings indicate the presence of 64 species, 22 subspecies, and 18 variants, which encompass 5 species and 3 subspecies identified as endemic [3]. *Vicia* ranks as one of the earliest cultivated forage taxa, with its domestication traced back to around 7000 BC [4]. The primary cultivated species of *Vicia* are native to Asia and Europe, particularly the Mediterranean area. In addition, Turkish

Vicia species represent an important group of crop wild relatives (CWRs) with direct relevance for gene pool conservation networks and global food security, as emphasized in FAO frameworks and in the introgressiomics concept that advocates systematic use of wild germplasm in breeding [5,6]. The genus *Vicia* comprises approximately 334 taxa globally, with 59 species found naturally in Türkiye [7]. In the Eastern and Southeastern Anatolia regions of Türkiye, *Vicia* species hold the 3rd position globally regarding their genetic differentiation potential [8]. Moreover, Davis and Plitmann [9] documented 59 species of *Vicia* in Türkiye, including six endemics, in 1970. However, recent information from the "Bizim Bitkiler" database (2024) indicates the presence of 63 species, 22 subspecies, 17 variations, and 11 endemic species [10]. These differences reflect updates in taxonomic treatments and database revisions rather than inconsistencies, as summarized in Appendix A Table A1.

Approximately 14 species of *Vicia* are extensively cultivated worldwide [2]. In Türkiye, the cultivation of *V. sativa* L. (common vetch), *V. pannonica* Crantz (Hungarian vetch), and *V. faba* L. (broad bean vetch) is widespread due to their considerable significance as animal fodder. These species are widely employed as pasture crops, green manure, and soil amendments within crop rotation systems. They are used in the food and feed industries, act as cover crops for fallow land, are employed in pastures and silage, serve as ornamental plants in parks and gardens, contribute to seed production, and function as green fertilizers [7]. *V. cracca* L. (bird vetch) exhibits considerable nectar potential for both wild and honeybee populations [11]. As a result, *Vicia* species utilized in crop rotations significantly enhance soil fertility and organic matter content by mitigating diseases, pests, and weeds. Furthermore, their capacity to symbiotically fix atmospheric nitrogen in their roots renders them significant in organic agriculture [12].

Due to their notable physical similarity, closely related *Vicia* species present challenges in differentiation when relying solely on morphological criteria. Recent DNA barcoding studies, especially those using ITS2, matK, rbcL, and psbA-trnH loci, demonstrate significantly improved species resolution [13,14]. In recent years, molecular methodologies have been essential for accurately differentiating morphologically similar taxa and understanding their evolutionary relationships [15]. The prevalence of *Vicia* species in Anatolia, situated at significant biogeographic junctions between Asia and Europe, provides an optimal context for investigating speciation and migration dynamics [16].

One useful tool for species identification is DNA barcoding using conventional DNA markers [17]. These markers have been widely employed for species discrimination [18–20]. The nuclear ribosomal DNA (nrDNA) internal transcribed spacer region (ITS) has been widely employed for these purposes. ITS regions are genomic regions situated between the conserved gene segments of 18S, 5.8S, and 26S, utilized for the comparative analysis of closely related species and taxa. ITS is commonly utilized in phylogenetic research due to its attributes, including sufficient length for data adequacy, ease of amplification through PCR, a higher nucleotide substitution rate relative to rDNA regions, significant explanatory power in phylogenetic analyses at both genus and species levels, and a high copy number. Additionally, the secondary structure of the ITS2 region alone, a segment of the ITS region, is evident in the RNA functions within cells. The eukaryotic ITS2 region typically displays characteristics of four helices and motifs [18]. Conserved motifs and regions, along with helices, enhance the reliability of secondary structure in molecular systematics. Systematic characteristics in the plant kingdom are predominantly morphological, with recent validation through molecular data. The secondary structure of the ITS2 region, illustrated by its two-dimensional representation derived from molecular data, serves as a reliable morphological attribute. The assessment of base interactions within this secondary structure at various taxonomic levels, along with its application in enhancing phylogenetic connection estimations in recent years, has gained significance [21–24]. Consequently, ITS and ITS2

have consistently demonstrated superior phylogenetic resolution among closely related legume species in comparison to most chloroplast markers, which frequently display little variability at the intrageneric level. In this context, the present study offers an integrative perspective by combining ITS and ITS2 secondary structure analyses with phylogenetic and biogeographic approaches, a framework rarely applied to *Vicia* and not yet systematically explored in Turkish taxa.

Despite global DNA sequence analysis of *Vicia* species [13,25], there is a significant lack of comprehensive molecular research in Türkiye, particularly studies that integrate phylogenetic data with biogeographic context. This disparity is particularly evident despite Türkiye being one of the five micro gene centers for *Vicia* and other legumes [2,4]. Conducting molecular research to elucidate the genetic origins, diversification patterns, and evolutionary relationships of commercially significant *Vicia* taxa is essential for understanding speciation processes, identifying beneficial wild relatives, and enhancing breeding strategies. Molecular phylogenetics can aid in selecting genetically diverse and stress-resistant genotypes, guide conservation strategies, and ultimately improve sustainable agricultural practices in response to changing climatic conditions [26,27].

This study aims to elucidate the phylogenetic relationships, evolutionary history, and migration patterns of economically important Turkish *Vicia* species, particularly those used in agriculture, through the analysis of the ITS and the secondary structure of the ITS2 region. These markers are widely employed in plant molecular systematics because of their high variability and proven effectiveness in resolving relationships at the genus and species level, which makes them more suitable than many chloroplast markers for intrageneric studies. By integrating molecular phylogenetics, biogeographic modeling, and molecular dating, this study seeks to clarify possible migration routes between Asia and Europe, while also advocating for sustainable agro-biodiversity and conservation in vetch production and consumption.

2. Materials and Methods

In this study, seeds representing five economically important and widely cultivated *Vicia* species—*V. sativa* L. (common vetch), *V. pannonica* Crantz (Hungarian vetch), *V. villosa* Roth (hairy vetch), *V. narbonensis* L. (large vetch), and *V. faba* L.—were utilized. In addition, again economically important and widely cultivated, one subspecies, *V. villosa* subsp. *dasycarpa* Cav. (hairy-fruited vetch), and four horticultural variations were included: *V. pannonica* Crantz (pink-flowered Hungarian vetch), *V. villosa* Roth (Munzur ecotype), *V. pannonica* Crantz (white-flowered Hungarian vetch), and *V. sativa* L. (Alinoğlu common vetch). All seeds of these genotypes, selected for their economic and agricultural importance, were obtained from the Directorate of the Field Crops Central Research Institute, operating under the Ministry of Agriculture and Forestry of Türkiye (Figure 1). The seeds were germinated in containers under controlled laboratory conditions (16 h light/8 h dark photoperiod at 25 °C) to obtain fresh leaf tissues for genomic DNA extraction.

Genomic DNA was extracted from fresh leaf tissue using the GeneMATRIX Plant & Fungi DNA Purification Kit (Cat. no. E3595; EURx Ltd., Gdańsk, Poland) following the manufacturer's protocol. The quality and quantity of the extracted DNA were evaluated through gel electrophoresis, employing Thermo Scientific™ DNA Gel Loading Dye (6X) at a 4:1 ratio. The forward primer ITSL (5′-TCGTAACAAGGTTTCCGTAGGTG-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were employed, as they have been extensively assessed for plant applications in the study by Hisao et al. [28] (ITS1–5.8S–ITS2). As a result, several attempts were made to optimize annealing temperatures and solutions to determine the appropriate amplification for the samples (Appendix A Table A1). The PCR amplicons, verified via agarose gel electrophoresis, were

submitted to a commercial provider (BM Labosis, Ankara, Türkiye, 2024) for bidirectional sequencing using the capillary-electrophoresis-based Sanger dideoxy chain-termination method. The sequencing results provided by the company were presented as chromatograms using Finch TV 1.4 software (Version 1.4.0 manufactured by Geopiza Research Team, https://digitalworldbiology.com/FinchTV (accessed on 31 May 2025)) [29], enabling the assessment of sequence quality to eliminate ambiguous base calls before data analyses. Sequences were aligned by the Multiple Sequence Comparison by Log Expectation (MUSCLE) tool of Molecular Evolutionary Genetics Analysis (MEGA) 11 software [30]. Molecular diversity statistics such as GC contents (%), nucleotide deletions and insertions, conserved and variable sites, parsimony informative sites, transition/transversion (tr/tv) ratio, and nucleotide diversity were calculated via MEGA 11 (Table 1).

Figure 1. (**a**) *Vicia pannonica* (White Flower) flower; (**b**) seeds of *Vicia* samples (Photos were taken by Dr. Ates).

The optimal substitution model for reconstructing the species' phylogeny was identified using the Model Test program within MEGA 11 software. The computer program recommended the use of the general time reversible (GTR) model with gamma distribution for sequence analysis, as indicated by AIC values [31]. Phylogenetic trees were constructed using the maximum likelihood (ML) method based on the GTR+G model, along with bootstrap test analysis (with 1000 replicates). Moreover, the Bayesian evolutionary analysis by sampling trees (BEAST) software [32] utilizes the GTR+G substitution model with uniform rates across data partitions. The Yule tree was employed, and a preliminary tree was generated through 10,000,000 iterations of Markov chain Monte Carlo (MCMC) analysis. The phylogenetic trees were condensed and consolidated using TreeAnnotator software (https://beast.community/treeannotator (accessed on 31 May 2025)), with a posterior probability threshold [33]. The trees were illustrated using Fig Tree V 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 31 May 2025)) [34]. The phylogenetic trees generated independently by MEGA and BEAST software, utilizing the same model (GTR+G), had their bootstrap and posterior probability values manually integrated into a single tree for comparative and integrative analysis. In this study, taxa from the Fabaceae family, specifically *Astragalus mongholicus* Bunge, *Lupinus luteus* L., and *Lathyrus aureus* Steven ex Fisch. & C. A. Mey. D. Brândza, were utilized as outgroups to assess the accuracy of the phylogenetic tree. Sequence data for these taxa were sourced from the National Center for Biotechnology Information (NCBI) database. Additionally, to assess the placement of the examined *Vicia* species within a broader molecular context, various species from the *Vicia* genus, naturally occurring in Asia, Europe, and Anatolia, were incorporated into the phylogenetic trees. All accession numbers for the ITS gene region sourced from the NCBI database, along with the studied sequences, are provided in Appendix A, Table A2.

Table 1. Molecular diversity statistics for ITS gene regions of *Vicia* at the MEGA 11 program (Kimura-2 statistical parameters).

	ITS (ITS1 + 5.8S + ITS2)
Number of taxa	10
Total length (bp)	628
GC content (%)	50
Conserved sites (bp)	440
Variable sites (bp)	171
Parsimony informative sites (bp)	45
Transition/transversion (tr/tv) (R) ratio	1.02
Molecular diversity (overall)	0.12

The ITS2 region was extracted from each sequence individually using the ITS2 Database web application (https://its2.bioapps.biozentrum.uni-wuerzburg.de/ (accessed on 31 May 2025)), with automated annotation applied for precise delimitation. The annotated sequences were subsequently used to predict RNA secondary structures through mFOLD, available on the UNAFold Web Server (http://www.unafold.org/mfold/applications/rna-folding-form.php (accessed on 31 May 2025)). The resulting structural models were visualized and recorded as graphical outputs. In addition, the minimum free energy (MFE) values associated with each predicted structure were calculated via the same server.

To investigate potential reticulate evolutionary patterns and visualize conflicting phylogenetic signals among *Vicia* species, a split network analysis was performed using SplitsTree5 (version 5.5.1) [35]. The multiple sequence alignment of the ITS regions was generated using MUSCLE and exported in FASTA format. The alignment was imported

into SplitsTree5, and a Neighbor Net algorithm was applied to construct the split network under an uncorrected P-distance model. Bootstrap support values were calculated based on 1000 replicates to assess the robustness of the network topology. This approach enabled the detection of possible hybridization events, incomplete lineage sorting, or unresolved phylogenetic signals among closely related taxa.

Additionally, historical biogeographical reconstructions were performed using a phylogenetic framework based on the nuclear ribosomal ITS region. A time-calibrated ultrametric tree was inferred using BEAST [32], employing a GTR substitution model, a strict molecular clock, and a Yule speciation prior. Fossil-based calibration points were incorporated to estimate divergence times among lineages. A normal prior distribution (mean = 14.4 Mya) was assigned to the crown node of *Astragalus* based on divergence time estimates for sect. *Hymenostegis* [36]. Additionally, a lognormal prior distribution (M = 35, S = 5) was applied to the most recent common ancestor of *Vicia* species, reflecting estimated divergence within the *Vicieae* clade [37]. The resulting posterior distribution of trees was summarized using TreeAnnotator to generate a maximum clade credibility (MCC) tree with mean node heights. This ultrametric topology was subsequently pruned and reformatted for downstream biogeographic analysis. Reconstruct Ancestral State in Phylogenies (RASP) v4.2 [38] was then employed to infer ancestral range distributions and historical migration patterns based on this calibrated phylogeny, thereby enhancing the temporal resolution of inferred dispersal events. Biogeographic inferences were performed using three complementary approaches: Statistical Dispersal–Vicariance Analysis (S-DIVA). Each internal node was assigned probabilistic ancestral ranges across predefined biogeographic regions, based on the native distribution patterns documented in the online flora databases [10,39,40].

3. Results

Analysis of the sequence data from the ITS regions of *Vicia* revealed that the total lengths of the regions were around 628 base pairs (bp). A total of 171 variable sites were identified, of which 45 were deemed parsimony informative. The estimated overall molecular diversity among the examined taxa was 0.12 (Table 1) (detailed pairwise genetic distance values among the studied Turkish *Vicia* taxa, calculated using P-distance with uniform rates, are provided in Supplementary Table S1).

The phylogenetic tree constructed using ITS sequencing data demonstrated robust links among the ten taxa analyzed within *Vicia* and its allied genera (Figure 2). The research confirmed the monophyly of *Vicia*, exhibiting robust bootstrap support at many internal nodes. The species *L. luteus* received the designation as the outgroup with the greatest support (bootstrap = 100), while *A. mongholicus* and *L. aureus* appeared as successive sister lineages to the core *Vicia* clade, indicating their remote evolutionary position within Fabaceae. Accessions of *V. villosa*, including the Munzur population, along with the white-flowered variant of *V. pannonica*, were resolved within a single, robustly supported subclade (bootstrap = 93–99, pp = 1), indicating a close genetic relationship despite their phenotypic diversity. Likewise, *V. sativa* and its locally named "Alinoglu" variety clustered closely with robust support (bootstrap = 94), indicating minimal divergence and a potential recent common ancestry. The two varieties of *V. pannonica*, pink- and white-flowered, were recognized as different taxa, potentially indicating subspecific divergence. Furthermore, *V. faba* and *V. narbonensis* clustered together (bootstrap = 57, pp = 1), signifying a moderate degree of common ancestry. These results indicate that, despite the presence of morphologically diverse taxa, the ITS-based phylogeny generated stable clades with significant taxonomic resolution. Bootstrap values for most nodes were moderate to high, supporting the inferred evolutionary relationships among the examined taxa.

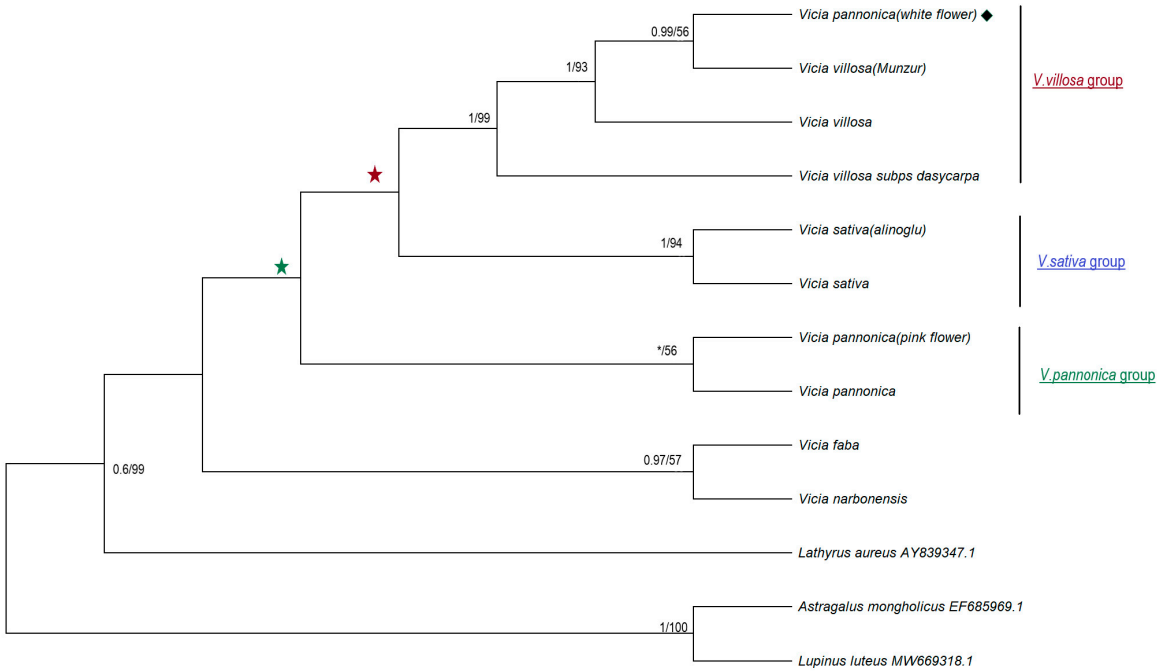

Figure 2. Phylogenetic tree of the studied *Vicia* taxa constructed under the GTR+Gamma model of the nrDNA ITS region using MEGA 11 and BEAST. Bootstrap support values (based on 1000 replicates) and Bayesian posterior probabilities are shown next to the nodes (values below 0.50 for pp and 50 for bootstrap are indicated with asterisk (*)). Key clades discussed in the text are highlighted with stars and labeled as *V. villosa* group, *V. sativa* group, and *V. pannonica* group for clarity. The distinct variety *V. pannonica* (white flower) is additionally marked with a diamond symbol.

The ITS-based ML phylogeny, comprising 44 taxa of *Vicia* from neighboring countries and provinces around Anatolia, together with outgroup taxa (*A. mongholicus*, *L. aureus*, *L. luteus*), demonstrated well-resolved clades and substantial bootstrap support across the majority of internal branches (Figure 3). *L. luteus* was consistently identified as the outgroup with maximum support (bootstrap = 100), while *A. mongholicus* and *L. aureus* appeared as early diverging lineages within Papilionoideae, establishing unique and well-supported clades (bootstrap = 99, pp = 1). The *Vicia* clade was monophyletic and demonstrated distinct internal structure. A substantial basal divergence within *Vicia* distinguished a western clade, including *V. pannonica*, *V. villosa*, and variants of *V. villosa* (white-flowered, Munzur), from an eastern clade containing *V. cracca*, *V. cassubica*, *V. benghalensis*, and *V. leucantha*. The support for these subclades varied from moderate to strong (bootstrap = 81–99, pp = 1). A strong cluster (bootstrap = 94–98, pp = 0.85–1) comprised *V. sativa*, *V. sativa* subsp. *nigra*, *V. sativa* subsp. *angustifolia*, and the cultivated "Alinoglu" variant, showing recent divergence and local diversification. Likewise, *V. villosa* and its subspecies (subsp. *dasycarpa* and subsp. *varia*) clustered closely (bootstrap = 85–98, pp = 0.99–1), emphasizing their genetic similarity. A robustly supported clade (bootstrap = 91–98) included *V. ervillia*, *V. galilaea*, *V. narbonensis*, and *V. assyriaca*, indicating a common ancestry in the Levantine and Central Asian areas. Moderate support was noted for the clade combining *V. faba* with this group, although more basal lineages such as *V. tigridis*, *V. noeana*, and *V. balansae* showed bootstrap values of 91 (pp = 1), indicating early diversification in Middle Eastern or Mediterranean refugia. The tree topology and bootstrap values provide strong evidence for both regionally structured clades and taxonomically unified groups within *Vicia*. The incorporation of both wild and cultivated accessions, as well as subspecies and ecotypes, enhances the comprehension of intrageneric relationships and illustrates historical dispersal and domestication occurrences.

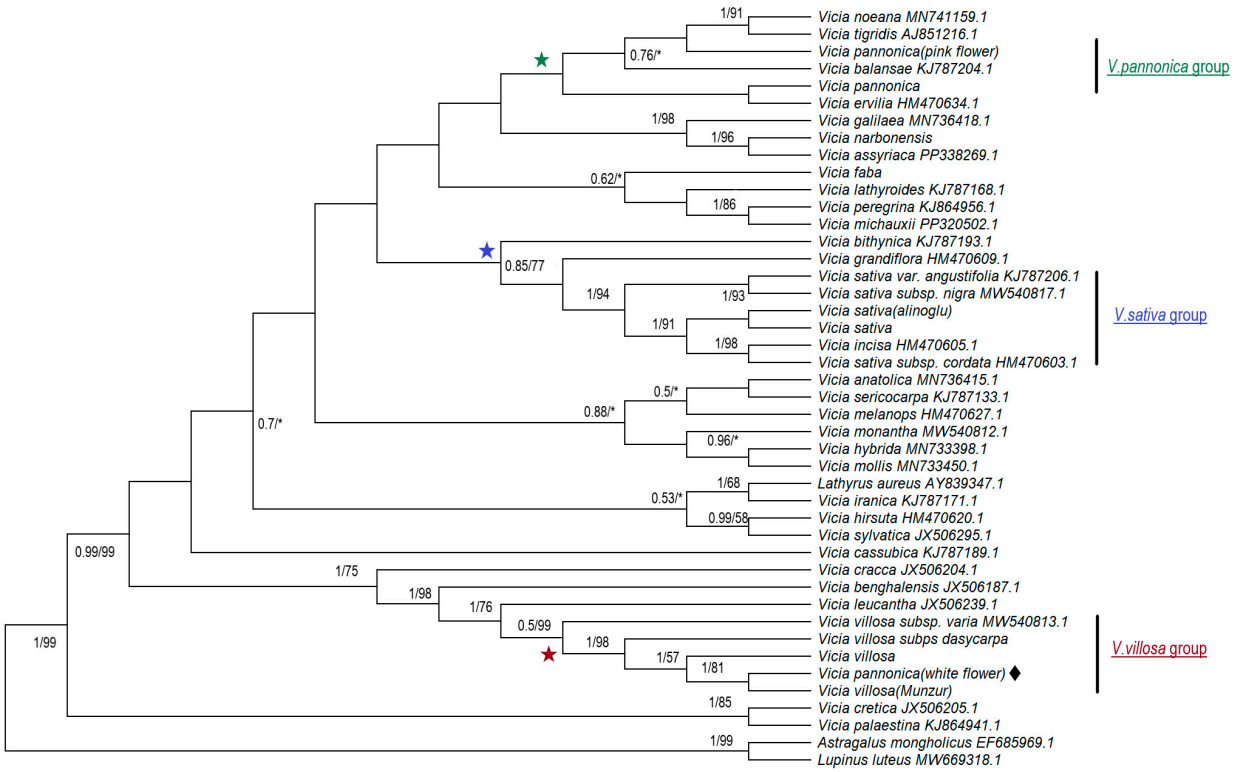

Figure 3. Phylogenetic tree of the studied *Vicia* taxa and selected species from neighboring countries, constructed under the GTR+Gamma model of the nrDNA ITS region using MEGA 11 and BEAST. Bootstrap support values (based on 1000 replicates) and Bayesian posterior probabilities are shown next to the nodes (values below 0.50 for pp and 50 for bootstrap are indicated with asterisk (*)). Key clades emphasized in the manuscript are highlighted with stars and labeled for clarity. The distinct variety *V. pannonica* (white flower) is additionally marked with a diamond symbol.

The predicted ITS2 secondary structures of the examined *Vicia* taxa displayed a predominantly conserved core topology, defined by three to four principal helices emanating from a central multi-branched loop—an arrangement characteristic of eukaryotic ITS2 regions (Figure 4). Among the species investigated, *V. faba*, *V. sativa*, and *V. narbonensis* exhibited the highest thermodynamic stability, with minimum free energy (MFE) values of −68.90, −68.10, and −66.50 kcal/mol, respectively. The significantly low MFE values signify robust intramolecular base pairing and a compact secondary structure, indicating a high level of structural stability. The helices of these species were remarkably well-structured, with significant contiguous base pairing and terminal hairpin loops, particularly prominent in helix III, which is frequently regarded as phylogenetically informative in ITS2-based research. The core internal loop had a consistent size and shape across different taxa, indicating a conserved functional or evolutionary constraint. Conversely, *V. pannonica* showed enhanced structural heterogeneity among its forms. Although the pink- and white-flowered morphotypes maintained a standard ITS2 structure characterized by three principal helices and moderate MFE values (−64.40 and −62.60 kcal/mol, respectively), one specimen (*V. pannonica* unqualified form) exhibited a significant deviation, presenting an unusual, elongated configuration with dispersed helices and a considerably elevated MFE of –50.58 kcal/mol. This alteration likely indicates nucleotide changes or insertions/deletions that impact pairing potential, resulting in diminished stability and modified folding dynamics. Likewise, *V. villosa* and its associated species (*V. villosa* subsp. *dasycarpa* and the Munzur population) exhibited well-conserved ITS2 structures, each preserving the characteristic multi-helical arrangement with MFE values ranging from −62.00 to −62.60 kcal/mol. This morphological consistency, despite variations in taxonomic rank,

may indicate recent divergence or continuous gene flow. The secondary structure profiles indicate both interspecific uniqueness and intraspecific consistency across the examined *Vicia* taxa. The persistent occurrence of three predominant helices and a stable central loop in most species underscores the evolutionary significance of ITS2 structural motifs. Furthermore, thermodynamic characteristics like MFE augment the interpretability of structural variations and may assist in species delimitation among morphologically analogous taxa.

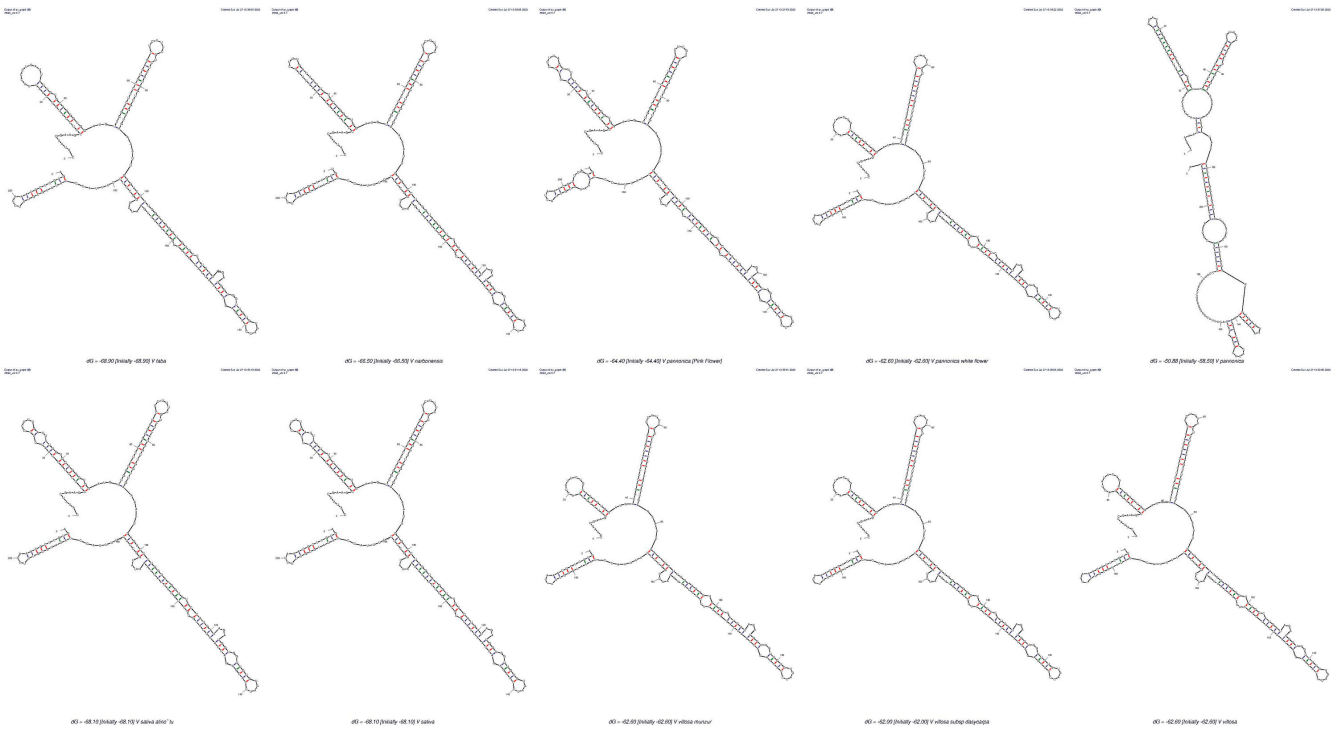

Figure 4. Predicted ITS2 secondary structures of ten examined *Vicia* taxa. Structures were determined using Mfold based on minimum free energy (MFE) predictions. All taxa display the distinctive eukaryotic ITS2 structure, with three principal helices and a central loop, despite minor differences in helix length and loop shape. At the top from the left to the right (a) *V. faba*, (b) *V. narbonensis*, (c) *V. pannonica (pink flower)*, (d) *V. pannonica (white flower)*, (e) *V. pannonica*; at the bottom from the left to the right (f) *V. sativa (Alinoğlu)*, (g) *V. sativa*, (h) *V. villosa (Munzur)*, (i) *V. villosa* subsp. *dasycarpa*, (j) *V. villosa*.

To investigate putative reticulate evolution and illustrate contradicting phylogenetic signals among the *Vicia* taxa, split decomposition and consensus network analyses were conducted utilizing SplitsTree. The resultant split network (Figure 5a) exhibited a predominantly tree-like structure with distinctly resolved clusters corresponding to the morphotypes of *V. faba*, *V. narbonensis*, *V. sativa*, and *V. pannonica*. The close clustering of *V. sativa* and *V. sativa* (Alinoğlu) indicates their negligible sequencing divergence and recent common ancestry. The consensus network (Figure 5b) further demonstrated inconsistent signal patterns, especially for the positioning of *V. villosa* and its associated species. The existence of parallel edges and web-like structures in this area indicates potential historical hybridization or insufficient lineage sorting. The remarkable separation of the *L. aureus*, *A. mongholicus*, and *L. luteus* outgroups reinforces the uniqueness of the *Vicia* clade. The network-based results confirm the key clades identified in the ITS phylogeny and also uncover subtle evolutionary complexities that are hidden when strictly dividing into two branches.

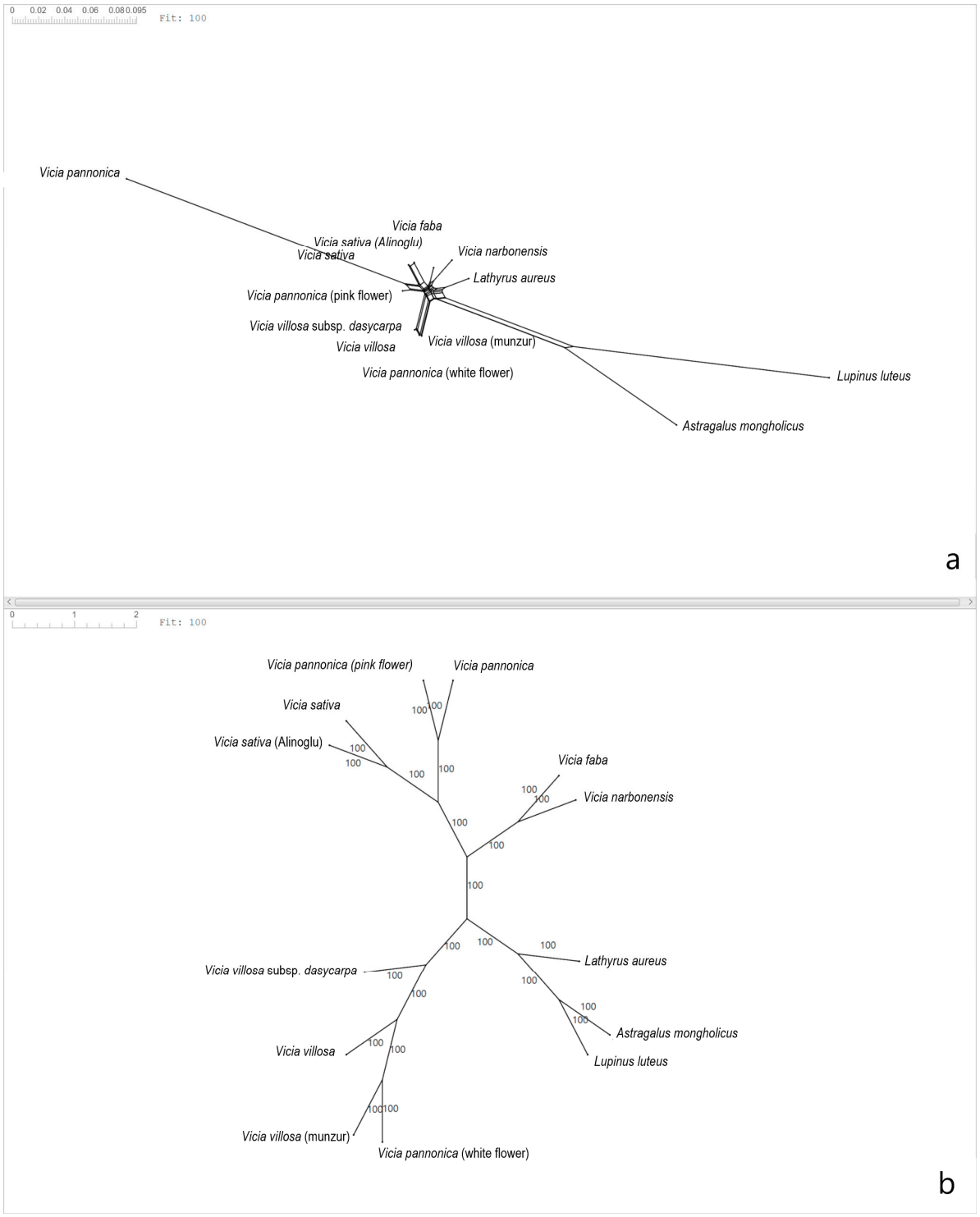

Figure 5. Network-based analysis of studied *Vicia* taxa utilizing ITS sequences. (**a**) Split decomposition network illustrating inferred linkages and potential reticulation among taxa. (**b**) Consensus network illustrating regions of discordant phylogenetic signals (with bootstrap values).

Reconstructions of ancestral ranges based on the calibrated ITS phylogeny revealed complex biogeographic patterns among *Vicia* species and their close relatives (Figure 6). The most recent common ancestor of the ingroup likely originated within a broad ancestral region encompassing the Mediterranean Basin, Southwest Asia, the Caucasus, and surrounding areas (zones B, F, G, H). Temporal estimates indicate that the diversification of *Vicia* lineages primarily occurred between 12 and 3 million years ago, a period characterized by significant paleoclimatic shifts and geotectonic events. Node-level ancestral range analyses suggest that early diversification events were predominantly driven by dispersal rather than vicariance. For instance, Node 45—marking the divergence between *A. mongholicus* and the remainder of the ingroup—reflected a dispersal expansion from the BFGH ancestral zone into Anatolia (D) and the Levant (H). Similarly, Node 49 and Node 53 showed directed migrations from the BFGH region into Eastern Europe (C) and Türkiye (D), potentially facilitated by Late Miocene topographic corridors. In contrast, vicariance events were detected at Node 56 and Node 76, involving splits between the Levant (H), Central-Eastern Europe (C), and Anatolia (D), likely shaped by habitat fragmentation during the Pliocene. Additional dispersal bursts were evident at Node 66, where various *V. sativa* subspecies radiated into nearly all predefined regions (A–H), indicating a rapid ecological expansion. Complex migration paths were also inferred at Node 69 and Node 79, suggesting connections between Central and Southeastern Europe, with the latter implying an Aegean–Eastern vicariant pattern. Finally, Node 86, which distinguishes *Lathyrus* from *Vicia*, displayed a vicariant split between regions B and C, aligning with early divergence in Papilionoideae. These findings collectively support a model of *Vicia* evolution involving a widespread ancestral range followed by repeated dispersal into Anatolia, the Balkans, and Eastern Europe, and punctuated by occasional vicariance events. High rates of within-region speciation in zones F, G, and H further highlight these areas as diversification hotspots. Bidirectional migration across the Iranian Plateau and Levant is also inferred, contributing to the genus's reticulate biogeographic history.

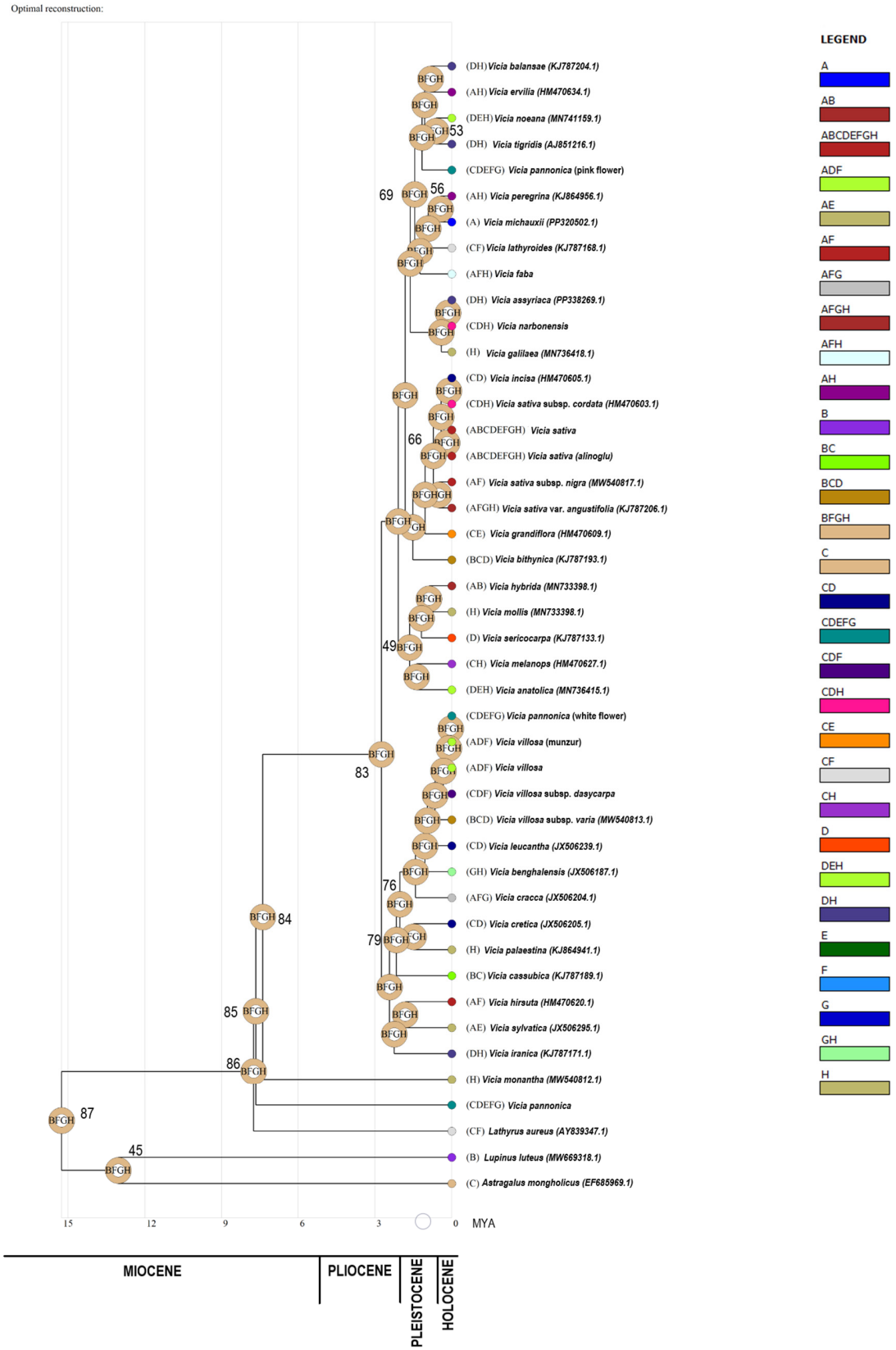

Figure 6. Ancestral Range Reconstruction of *Vicia* and related taxa based on ITS sequences using RASP v4.2. Maximum clade credibility tree based on ITS sequences, calibrated with fossil constraints. Ancestral areas were inferred using S-DIVA analysis in RASP v4.2. Pie charts at nodes represent probabilities of ancestral regions (numbers at the nodes indicate important node numbers for event route explanations in the text). Region codes are: A = Europe, B = Mediterranean, C = Eastern Europe, D = Türkiye, E = Caucasus, F = Western Asia, G = Asia, H = Iran–Levant.

4. Discussion

The phylogenetic studies utilizing nuclear ITS sequences demonstrated clearly defined and taxonomically consistent clades among Turkish *Vicia* species, generally corresponding with traditional morphological classifications. Cultivated varieties such as *V. sativa* and its local variant "Alinoğlu," along with *V. villosa* and its ecotypes, demonstrated negligible sequence divergence and grouped with substantial bootstrap support, suggesting recent divergence or shared ancestry [41]. This pattern is consistent with the biological characteristics of these taxa, which are predominantly cross-pollinated and ecologically adaptable. Such life-history traits facilitate hybridization and introgression, thereby maintaining high genetic similarity despite morphological differentiation. Similar shallow divergence has been reported for *Vicia* complexes in broader phylogenetic analyses [15,41], supporting the interpretation that these lineages represent recently diversified yet ecologically resilient groups. The existence of paraphyletic and polyphyletic groupings within the *V. sativa* complex suggests possible historical hybridization or imperfect lineage sorting—evolutionary processes well-documented in Fabaceae, especially among cross-pollinating *Vicia* species. The secondary structure analysis of the ITS2 region further corroborated these phylogenetic trends by uncovering highly conserved helices—particularly in Helix III—and subtle yet informative alterations in loop regions among closely related taxa. Species like *V. faba* and *V. narbonensis* exhibited notably stable and thermodynamically advantageous ITS2 structures, marked by low minimum free energy (MFE) values and conventional multi-helix configurations. The conserved structural features, shaped by functional and evolutionary constraints, augment the trustworthiness of ITS2 as a phylogenetically informative marker [42–44]. The branching patterns observed in *V. sativa*, *V. narbonensis*, and *V. villosa* are consistent with their known morphological traits, such as leaf architecture and peduncle structure, thereby reinforcing previous systematics based on phenotypic characters [45,46]. From a breeding standpoint, the alignment of sequence-based and structure-based data promotes precise genotype identification, elucidates species boundaries in morphologically ambiguous taxa, and guides strategic decisions regarding cultivar development, hybridization potential, and germplasm conservation. In addition, this study combines ITS, ITS2 secondary structure modeling with time-calibrated BEAST dating and RASP-based ancestral range reconstructions, offering a more integrative framework for Turkish *Vicia* taxa. Unlike previous large-scale phylogenetic studies that primarily addressed global relationships in the genus (e.g., [15,47]), the analysis of the current study focuses on Turkish taxa and integrates structural and biogeographic perspectives to highlight evolutionary processes specific to Anatolia.

Split network analyses displayed substantial evidence of reticulate evolution and phylogenetic discordance among various *Vicia* taxa, notably within the *V. sativa* aggregate and the *V. villosa* complex, indicating the impact of historical hybridization, incomplete lineage sorting, or recent radiation—phenomena well-documented in Fabaceae and commonly linked to cross-pollinating and ecologically adaptable lineages [48–51]. The network topology revealed non-tree-like interactions and contradicting phylogenetic signals, especially among species with overlapping morphologies or ecological niches, like *V. villosa*, *V. sativa*, and *V. peregrina*. These patterns are essential for breeding as they may indicate fundamental gene flow and introgression, which might represent adaptive qualities such as drought tolerance, increased biomass production, or more edaphic adaptability. Amid escalating climate stress and the proliferation of arid regions, phylogenetic closeness and reticulation patterns provide valuable insights for identifying genotypes appropriate for marginal habitats [51]. Furthermore, the utilization of split networks serves as a robust adjunct to traditional phylogenetic trees, especially in taxa with intricate evolutionary backgrounds where bifurcating models may conceal essential linkages [35]. Consequently,

network-based phylogenetic analyses not only improve our comprehension of vetch diversification but also directly inform breeding techniques that fit with sustainability and resilience goals.

Historical biogeographic reconstructions using a time-calibrated BEAST phylogeny and RASP studies have determined that Anatolia and the Eastern Mediterranean served as both the origin and pathway for the diversification of Turkish *Vicia* species. The established migration pathways suggest numerous dispersals and vicariance occurrences from Central and Eastern Anatolia to the Balkans, the Caucasus, Eastern Europe, and the Iranian–Azerbaijani areas, with divergence events tracing back to the Late Miocene–Pliocene epochs—eras characterized by significant geological changes, including the uplift of the Anatolian plateau and the Messinian Salinity Crisis [52,53]. The Eastern Mediterranean's complex geological history—especially events like the closure of the Tethys Sea and the uplift of the Anatolian Plateau—acted as strong evolutionary drivers for diversification in *Vicia*. The Late Miocene–Pliocene era thus marks a critical window in shaping endemic legume flora, as corroborated by both molecular divergence estimates and ancestral range reconstructions [3,52,53]. These patterns align with earlier suggestions regarding Anatolia's biogeographic function as a phyto-corridor connecting Asia and Europe [49,50]. Furthermore, the westward expansions noted for taxa such as *V. sativa* and *V. villosa*, presumably reflect historical agricultural and trade-mediated dispersal, as previously suggested by Davis et al. [9]. The strong phylogeographic connections shown between Turkish and Irano-Turanian lineages, as indicated by the clustering of *V. narbonensis*, *V. faba*, and *V. cracca* with Irano-Turanian accessions in both BEAST and RASP analyses, highlight the potential of these gene pools in breeding initiatives designed to improve drought resilience, particularly in the context of continued climate change and desertification. Moreover, these findings align with the well-established ecological roles of *Vicia* species in nitrogen fixation and soil fertility improvement [54], further underscoring their dual importance in both ecological adaptation and agricultural sustainability. Combining molecular dating with ancestral area reconstruction provides evolutionary insights and forecasting tools for climate-adaptive breeding and the conservation of genetic resources within the legume family [55].

The genus *Vicia* exhibits diverse significance in sustainable agricultural systems, fulfilling essential functions in nitrogen fixation, cattle nutrition, soil regeneration, green manure, and human consumption [15,52]. This study's combination of molecular phylogenetics, secondary structure analysis, and calibrated biogeographic reconstruction highlights the evolutionary heterogeneity and adaptive capacity of Turkish *Vicia* germplasm, especially regarding regional endemism and ecological adaptability. The ultrametric BEAST phylogeny, combined with RASP-based ancestral range modeling, indicated that significant divergence events occurred from the Late Miocene to the Pliocene, aligning with the uplift of the Anatolian plateau and the Messinian Salinity Crisis, both crucial catalysts for plant radiation throughout the Eastern Mediterranean [52,56]. These occurrences established Anatolia as both a source and a biogeographic pathway for the dissemination of *Vicia* into the Balkans, Caucasus, and Central Europe throughout glacial and interglacial fluctuations [50,51]. This analysis reveals time-calibrated diversification patterns and associates them with functionally significant lineages, establishing a genetic framework for prioritizing conservation efforts, supporting ex situ collections, and directing varietal development. Ultimately, these findings are crucial for developing resilient agricultural systems that conform to global sustainability objectives and for ensuring food and feed security against climate change.

The study's genomic and biogeographic findings have significant implications for the conservation and strategic use of Turkish *Vicia* species, which are essential for traditional agriculture, animal feed, and soil regeneration methods. The identification of genetically distinct lineages and regionally restricted taxa, particularly in Eastern Anato-

lia and Irano-Turanian transition zones, underscores the necessity for in situ and ex situ conservation, especially as these regions confront heightened ecological vulnerability due to climate change. The robust correlation between molecular divergence patterns and ecological characteristics provides critical guidance for identifying evolutionarily independent gene pools in breeding programs focused on improving climate resilience and resource use efficiency in legumes [15,52]. Furthermore, the integrative methodology utilizing ITS phylogenetics, ITS2 structural modeling, time-calibrated divergence analysis, and biogeographic reconstruction exemplifies a reproducible framework for evaluating other underappreciated legume groups of economic and ecological importance. As agrobiodiversity diminishes due to monocultural intensification and environmental instability, these evolutionary and spatial insights offer essential tools for enhancing food and forage security, preserving marginal landscapes, and ensuring the resilience of agriculture through biodiversity-informed innovation [57]. While the current analyses relied on the widely applied ITS region, which continues to provide valuable phylogenetic resolution at the genus and species levels, we acknowledge that the field of legume systematics is rapidly advancing toward high-throughput sequencing approaches such as RADseq, genotyping-by-sequencing (GBS), and target capture phylogenomics [58,59]. These technologies enable genome-wide perspectives and finer resolution of reticulating evolutionary patterns that may remain unresolved by single-locus studies. By explicitly recognizing this methodological context, the current work establishes a molecular baseline for Turkish *Vicia* taxa that can be further complemented by future phylogenomic research. Such integration will not only enhance taxonomic resolution but also allow a deeper exploration of adaptive variation and introgression processes in the genus.

5. Conclusions

This study highlights the value of an integrative molecular framework in elucidating the evolutionary complexity and agricultural relevance of Turkish *Vicia* species. By combining ITS-based phylogenetic reconstruction, ITS2 secondary structure analysis, and time-calibrated biogeographic modeling, this study offers a balanced yet novel perspective that has rarely been applied to *Vicia*, particularly in the Turkish flora. Valuable insights into diversification processes, ancestral range dynamics, and adaptive potential across a key biogeographic transition zone between Asia and Europe were obtained. These findings provide a foundational reference for aligning genotype selection with environmental variability under increasing climate pressure.

The application of advanced molecular tools, including phylogenetic network analysis and ancestral area reconstruction, demonstrates the utility of evolutionary perspectives in informing breeding strategies aimed at improving drought resilience, seed quality, and ecological adaptation. Further integration with high-throughput sequencing, landscape genomics, and ecological niche modeling is encouraged to refine taxonomic resolution and uncover deeper layers of adaptive variation.

Conservation strategies should increasingly account for both evolutionary distinctiveness and regional vulnerability, particularly within ecologically sensitive zones such as Eastern Anatolia. The framework established herein offers a transferable model for biodiversity-driven innovation, bridging evolutionary biology with sustainable agricultural practices. In the context of accelerating global environmental change, leveraging evolutionary and molecular evidence will be essential for building resilient and adaptive agro-ecosystems.

Recent advances in high-throughput sequencing, genome-wide association studies (GWASs), and landscape genomics are rapidly reshaping the study of crop wild relatives. These approaches can identify adaptive loci linked to drought tolerance, disease resistance,

and yield stability at a much finer resolution. Although the current study is based on the ITS region, it establishes a phylogenetic and biogeographic framework that can be readily expanded with genome-wide data. Integrating Turkish *Vicia* taxa into such genomic initiatives will provide a more comprehensive understanding of adaptive variation and will directly inform molecular breeding strategies. This perspective aligns this work with global research trajectories and highlights how future studies can build upon our results to accelerate biodiversity-informed crop improvement. By linking molecular systematics with agro-biodiversity conservation and climate-adaptive breeding, this study contributes directly to the United Nations Sustainable Development Goals (SDGs) related to food security, sustainable agriculture, and biodiversity conservation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su17177914/s1, Table S1: Pairwise genetic distances (p-distance) among Turkish *Vicia* taxa based on nuclear ITS sequences. Values represent the number of base substitutions per site, averaged over all sequence pairs. Analyses were conducted in MEGA 11 using the p-distance model with uniform rates.

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Abbreviations

The following abbreviations are used in this manuscript:

NCBI	National Center for Biotechnology Information
MEGA	Molecular Evolutionary Genetic Analysis
RASP	Reconstruction of Ancestral State in Phylogenies
MFE	Minimum free energy
S-DIVA	Statistical DIVA

Appendix A

Table A1. PCR optimized compositions (mix and temperatures) for ITS PCR studies.

	Volumes	Steps	Temperature	Time
Solis Biodyne 5x HOT FIREPol Blend PCR Mix 15 mM $MgCl_2$	3 µL	First denaturation	95 °C	5 min

Table A1. *Cont.*

	Volumes	Steps	Temperature	Time	
Primer pairs (10 mM)	0.5 µL + 0.5 µL	Denaturation	95 °C	45 s	
dH_2O	15 µL	Annealing	54 °C	30 s	35 cycles
DNA (10 ng)	1 µL	Extension	72 °C	45 s	
Total volume	20 µL	Final extension	72 °C	10 min	

Table A2. Accession numbers of species were obtained from the NCBI database to enlarge the dataset and the studied taxa (indicated with an asterisk).

Species Name	Accession Numbers
Vicia pannonica Crantz (white_flower) *	PX049010 *
Vicia pannonica Crantz (pink_flower) *	PX049011 *
Vicia pannonica Crantz *	PX049012 *
Vicia villosa Roth *	PX049013 *
Vicia villosa Roth (Munzur) *	PX049014 *
Vicia villosa subps *dasycarpa* (Ten.) Cav.*	PX049015 *
Vicia faba L.*	PX049016 *
Vicia narbonensis L.*	PX049017 *
Vicia sativa L. *(Alinoglu)* *	PX049018 *
Vicia sativa L.*	PX049019 *
Vicia cracca L.	JX506204.1
Vicia ervilia (L.) Willd.	HM470634.1
Vicia hirsuta (L.) S.F.Gray	HM470620.1
Vicia benghalensis L.	JX506187.1
Vicia grandiflora Scop.	HM470609.1
Vicia anatolica Turrill	MN736415.1
Vicia bithynica L.	KJ787193.1
Vicia sativa var. *angustifolia*	KJ787206.1
Vicia sylvatica L.	JX506295.1
Vicia lathyroides L.	KJ787168.1
Vicia assyriaca Boiss. & Hausskn.	PP338269.1
Vicia balansae Boiss.	KJ787204.1
Vicia iranica Boiss. & Hohen.	KJ787171.1
Vicia melanops Sibth. & Sm.	HM470627.1
Vicia leucantha Biv.	JX506239.1
Vicia tigridis Boiss.	AJ851216.1
Vicia sativa subsp. *nigra* (L.) Ehrh.	MW540817.1
Vicia sativa subsp. *cordata* Wulfen ex Hoppe	HM470603.1
Vicia villosa subsp. *varia* (Host) Corb.	MW540813.1
Vicia cretica (Willd.) Willd.	JX506205.1
Vicia galilaea Plitmann	MN736418.1
Vicia hybrida L.	MN733398.1
Vicia mollis Willd.	MN733450.1
Vicia noeana Reut. ex Boiss.	MN741159.1
Vicia palaestina Boiss.	KJ864941.1
Vicia peregrina L.	KJ864956.1
Vicia sericocarpa Fenzl	KJ787133.1
Vicia monantha Retz.	MW540812.1
Vicia incisa M.Bieb.	HM470605.1
Vicia michauxii Spreng.	PP320502.1
Vicia cassubica L.	KJ787189.1
Lathyrus aureus Steven	AY839347.1
Astragalus mongholicus Bunge	EF685969.1
Lupinus luteus L.	MW669318.1

Table A3. Reported numbers of *Vicia* taxa in Türkiye according to different sources.

Source	Species (spp.)	Subspecies (subsp.)	Varieties (var.)	Endemics
Davis [3]	64	22	18	8
Other recent compilations (literature cited in text)	59	22	18	6
Bizim Bitkiler [10]	63	22	17	11

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