DNA-Based Identification of Eurasian Vicia Species Using Chloroplast and Nuclear DNA Barcodes

Irene Bosmali 1,2, Georgios Lagiotis 1,*, Nadia Haider 3, Maslin Osathanunkul 4,5, Costas Biliaderis 2,† and Panagiotis Madesis 1,6,†

1 Centre for Research and Technology Hellas, Institute of Applied Biosciences, 57001 Thessaloniki, Greece; eirinimposmali@certh.gr (I.B.); glagiotis@certh.gr (G.L.)
2 Laboratory of Food Chemistry and Biochemistry, Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; biliader@agro.auth.gr
3 The Department of Molecular Biology and Biotechnology, Atomic Energy Commission (AEC), Damascus 6091, Syria; ascientific1@aec.org.sy
4 Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand; maslin.cmu@gmail.com
5 Research Center in Bioresources for Agriculture, Industry and Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
6 Laboratory of Molecular Biology of Plants, School of Agricultural Sciences, University of Thessaly, 38446 Volos, Greece
* Correspondence: pmadesis@uth.gr

Abstract: Many legume species of the Vicia L. genus (Fabaceae Lindl.) are key components of the Mediterranean diet and have an integral role in sustainable agriculture. Given the importance of the Vicia species for Eurasian culture, it is necessary to implement methodologies, such as DNA barcoding, that can enable the effective authentication and identification of species in the genus. In this study, we analysed the chloroplast trnL and rpoC1, as well as the nuclear ITS2 DNA barcoding regions, to identify 71 Vicia specimens of Eurasian descent. Both the trnL and ITS2 regions were highly effective in discriminating the analysed taxa, while the more conserved rpoC1 region could not identify all of the selected species due to high sequence conservation or non-annotated or absent rpoC1 species sequences in GenBank. A dendrographic representation of the generated trnL data showed sufficient clustering for most of the analysed taxa, while some topological discrepancies were observed. ITS2 and rpoC1 reconstructions were also used for resolving the topological discrepancies observed in the trnL tree. Our analysis suggests that a combination of DNA barcoding regions is essential for accurate species discrimination within the Vicia genus, while single-locus analyses do not provide the necessary resolution.

Keywords: DNA barcoding; ITS2; rpoC1; trnL; Vicia

1. Introduction

Legumes are the second most important crop after cereals in terms of agricultural production, human nutrition, and animal feed [1]. Many leguminous species have been an integral part of the human diet since ancient times due to their low fat and high protein content, richness in fibre and other nutrients, as well as their anticarcinogenic and prebiotic potential [1–5]. Furthermore, legumes are a nutrient-rich feed for domesticated animals. Apart from the use of legumes for human or animal consumption, many species are also utilized in agricultural practices, such as crop rotation, given many species form symbiotic relationships with nitrogen-fixing bacteria, thus replenishing soil nitrogen levels. Finally, legumes are also an invaluable source of nutraceuticals and pharmaceuticals [4,6,7], and are important in the production of other industrial products [8]. Legume seeds can also be fractionated into high protein-, fibre-, and starch-based raw ingredients for the food,
pet, and paper-making industries due to their physicochemical properties and nutritional characteristics [9–11].

Leguminous plants belong to the Fabaceae (Leguminosae Jussieu) family, which is one of the largest plant families, including more than 10% of the known eudicots [12]. The family includes 670 genera and approximately 20,000 species [13]. Fabaceae has been traditionally divided into three subfamilies (Papilionoideae L., Mimosoideae DC., and Caesalpinioideae DC.). Legumes of the tribe Fabaeae Rchb. of the subfamily Papilionoideae are distributed worldwide throughout Eurasia, Africa, and America. The tribe Fabaeae is composed of the Vicia L., Lathyrus L., Lens Mill., Pisum L., and Vavilovia Al. Fed. genera [13], and likely originated in the eastern Mediterranean region during the Miocene, dispersing several times into Eurasia, and a few times to the American and African continents [14,15].

Amongst the various Fabaeae genera with wide distribution in the Mediterranean region, Vicia shows high levels of species diversity [16,17]. Although the number of total species has not yet been conclusively defined [18], it is estimated to be approximately 200 species. The Vicia species show a widespread distribution in the temperate zones of the northern hemisphere and the tropical regions of South America [18]. Although the genus does not largely include any threatened species, there are many rare species restricted to particular habitats [19]. Given that human activity has degraded many of the major centres of Vicia species diversity, local diversity conservation is important in preserving the genetic variation of the genus for breeding purposes, especially for wild species. For example, several wild Vicia species in Syria were threatened with local extinction, and more recent surveys show that other species are also under threat [20]. With the scope of preserving the invaluable species diversity of the genus, especially for endemic species, it is important to correctly and effectively discriminate the closely related Vicia species.

Several morphological and DNA-based descriptors (e.g., consensus chloroplast simple sequence repeats (ccSSRs) [21]) of diversity have been used to discriminate amongst species in the Vicia genus. Morphological characteristics, such as flower traits, pollen morphology, leaf structure, and legume shape, have been considered to be important taxonomic traits for correct species identification in the genus [17,22–25]. Nevertheless, phenotypic identifications require specialized taxonomic expertise and a detailed description of species morphology at various developmental stages. There are many limitations on morphology-based species characterization, and it can be impossible to describe the underlying genetic variation. These limitations become even more pronounced for closely related species with very similar morphologies and species with intermediate phenotypes [26], as well as for recently diverging species or species generated by hybridization [27]. Given the large-scale karyotype structure variation observed amongst several Vicia species, karyological and cytological studies have also been carried out for Vicia taxonomy [28,29]. However, the varying results from earlier studies in resolving species discrimination necessitates the application of more reliable and effective methods to secure the invaluable Vicia species diversity.

DNA barcoding, which involves the sequencing of small nuclear and chloroplast genome regions (DNA barcodes) amongst diverse samples, results in an easily comparable dataset that enables species discrimination, and it has been proposed to be an effective methodology for the taxonomic identification, description, revision, and reordering of species [30,31]. Although there has been extensive research into the application of single and multi-loci analyses in plant species identification [32–36], there is still no consensus on barcodes that are universally ideal for all plant species, especially in the Vicia genus. In several plant species, the variable chloroplast DNA regions, such as trnL, rbcL, and matK, are usually ideal and have been used effectively in species discrimination [33,35,37–39]. Apart from the various chloroplast regions, the nuclear ITS region has also been proposed to be an ideal barcode for species discrimination, given that it has high PCR amplification efficiency and is highly variable in many plant species [30]. The ITS region was previously used to effectively discriminate amongst several medicinal plants in the Fabaceae family [40]. Within the Vicia genus, several studies have demonstrated the effectiveness of this region in species identification and phylogenetic reconstructions [15,41–43]. However, it becomes
increasingly apparent that there are some limitations in single loci analyses, and the combination of multiple barcodes may provide higher discrimination accuracies, especially for closely related species [44].

The combination of the chloroplast regions *trnL* and *rpoC1*, as well as the nuclear ITS2 region, has been previously used for the discrimination of major Mediterranean legume species, with *trnL* and ITS2 showing the highest level of sensitivity [39]. More specifically for *Vicia*, the ITS2 and several regions of the chloroplast genome were previously used for the taxonomic classification of several species in the genus [45–47]. More recently, the combination of the barcoding regions ITS2, *matK*, and *rbcL* was also evaluated along with morphological taxonomic traits for their efficacy in discriminating among South Korean *Vicia* species [48].

Apart from species discrimination, DNA barcoding data have also been used for inferring and resolving phylogenetic relationships in several genera in the Fabaceae family, including *Vicia*. Phylogenetic analyses based on both morphological descriptors of diversity and DNA data showed that the Eurasian species are likely to have originated in the eastern Mediterranean or the western Asiatic regions and the New World species were derived from lineages that invaded America [1,41]. The divergence of the two groups occurred relatively recently [41] and was likely the result of three independent invasion events [12,49]. Nevertheless, *Vicia* species phylogeny is still inconclusive, owing to the taxonomic complexities that emerge from incongruent phylogenetic analyses and the high intraspecific genetic diversity observed in the genus [1], as well as the classification of several taxa as subspecies and races [50] or aggregates [50–52].

To verify the species identity of several *Vicia* specimens of Eurasian descent in local seed banks, we implemented DNA barcoding analyses using the *trnL* and *rpoC1* plastid regions, as well as the nuclear ITS2 region. Species discrimination was carried out in terms of sequence similarity (>90%) with indicative GenBank entries, and Maximum Likelihood (ML) dendrograms were utilized for visualizing the sequence differences amongst the studied specimens. Our analysis indicates that the combination of both sequence similarity assessments and dendrographic representations allows for more accurate species discrimination in the *Vicia* genus.

### 2. Results

To support species identification for 71 indicative Eurasian *Vicia* specimens from local seed banks, DNA barcoding analysis was implemented using, initially, the *trnL* region. The *trnL* DNA barcoding region was successfully amplified and sequenced for all 71 specimens, generating reads of approximately 362 bp in length. A Basic Local Alignment Search Tool (BLAST) analysis of the generated *trnL* sequences against GenBank entries enabled us to confidently identify the *Vicia* specimens at the species level with high-percentage identity values above 90% for most of the specimens (Table 1).

#### Table 1. *trnL* BLAST analysis of the 71 *Vicia* specimens.

| Species/Subspecies Name | Sample ID | GenBank Accession Number | Bit Score | E-Value | % Identity |
|--------------------------|-----------|--------------------------|-----------|---------|------------|
| *V. aintabensis* Boiss.  | 3         | MZ334891                 | 737       | 0       | 100%       |
| *V. aintabensis* Boiss.  | 13        | MZ334892                 | 702       | 0       | 100%       |
| *V. aintabensis* Boiss.  | 20        | MZ334893                 | 697       | 0       | 100%       |
| *V. anatolica* Turrill.  | 5         | MZ334894                 | 713       | 0       | 99.24%     |
| *V. anatolica* Turrill.  | 50        | MZ334895                 | 845       | 0       | 99.57%     |
| *V. anatolica* Turrill.  | 54        | MZ334896                 | 704       | 0       | 99.48%     |
| *V. anatolica* Turrill.  | 65        | MZ334897                 | 630       | 0       | 99.43%     |
| *V. bithynica* L.        | 23        | MZ334898                 | 501       | 1E-137  | 100%       |
| *V. bithynica* L.        | 43        | MZ334899                 | 488       | 3E-141  | 100%       |
| *V. bithynica* L.        | 68        | MZ334900                 | 466       | 4E-127  | 100%       |
| *V. bithynica* L.        | 72        | MZ334901                 | 516       | 4E-142  | 100%       |
| *V. dionysiensis*        | 12        | MZ334902                 | ‡         | ‡       | ‡          |
| Species/Subspecies Name | Sample ID | GenBank Accession Number | Bit Score | E-Value | % Identity |
|-------------------------|-----------|--------------------------|-----------|---------|------------|
| V. dionysiensis         | 16        | MZ334903                 | ‡         | ‡       | ‡          |
| V. dionysiensis         | 52        | MZ334904                 | ‡         | ‡       | ‡          |
| V. ervilia L.           | 8         | MZ334905                 | 736       | 0       | 100%       |
| V. ervilia L.           | 17        | MZ334906                 | 734       | 0       | 100%       |
| V. ervilia L.           | 34        | MZ334907                 | 652       | 0       | 100%       |
| V. ervilia L.           | 44        | MZ334908                 | 693       | 0       | 100%       |
| V. ervilia L.           | 78        | MZ334909                 | 739       | 0       | 100%       |
| V. faha L.              | 37        | MZ334911                 | 198       | 7E-52   | 90.13%     |
| V. faha L.              | 61        | MZ334910                 | 307       | 2E-84   | 79.41%     |
| V. faha L.              | 22        | MZ334912                 | 412       | 3E-116  | 89.05%     |
| V. grandiflora Scop.    | 55        | MZ334913                 | 377       | 3E-107  | 100%       |
| V. grandiflora Scop.    | 57        | MZ334914                 | 433       | 3E-124  | 100%       |
| V. grandiflora Scop.    | 71        | MZ334916                 | 518       | 9E-150  | 100%       |
| V. grandiflora Scop.    | 70        | MZ334915                 | 481       | 2E-138  | 100%       |
| V. lathyroides L.       | 36        | MZ334923                 | 449       | 4E-122  | 99.59%     |
| V. lathyroides L.       | 38        | MZ334924                 | 178       | 4E-50   | 89.36%     |
| V. lathyroides L.       | 46        | MZ334925                 | 472       | 8E-129  | 99.61%     |
| V. lathyroides L.       | 49        | MZ334926                 | 459       | 6E-125  | 99.60%     |
| V. lutea L.             | 2         | MZ334927                 | 761       | 0       | 98.76%     |
| V. lutea L.             | 45        | MZ334928                 | 704       | 0       | 99.23%     |
| V. lutea L.             | 26        | MZ334929                 | 739       | 0       | 99.30%     |
| V. lutea L.             | 48        | MZ334930                 | 730       | 0       | 98.31%     |
| V. michauxii Spreng.    | 11        | MZ334931                 | 717       | 0       | 99.74%     |
| V. michauxii Spreng.    | 19        | MZ334932                 | 612       | 7E-171  | 99.70%     |
| V. monantha Retz.       | 21        | MZ334933                 | 628       | 0       | 100%       |
| V. narbonensis L.       | 1         | MZ334934                 | 390       | 2E-104  | 99.53%     |
| V. narbonensis L.       | 4         | MZ334935                 | 392       | 5E-105  | 100%       |
| V. narbonensis L.       | 30        | MZ334936                 | 514       | 1E-141  | 99.65%     |
| V. narbonensis L.       | 14        | MZ334937                 | 377       | 1E-100  | 100%       |
| V. narbonensis L.       | 47        | MZ334938                 | 444       | 2E-120  | 99.59%     |
| V. noeana Boiss.        | 7         | MZ334939                 | 767       | 0       | 99.76%     |
| V. noeana Boiss.        | 9         | MZ334940                 | 760       | 0       | 100%       |
| V. noeana Boiss.        | 29        | MZ334941                 | 773       | 0       | 100%       |
| V. pannonica Crantz     | 85        | MZ334945                 | 741       | 2E-179  | 95.91%     |
| V. pannonica Crantz     | 64        | MZ334942                 | 835       | 0       | 100%       |
| V. pannonica Crantz     | 76        | MZ334943                 | 758       | 0       | 100%       |
| V. pannonica Crantz     | 15        | MZ334944                 | 719       | 0       | 99.74%     |
| V. pannonica Crantz     | 77        | MZ334946                 | 765       | 0       | 99.52%     |
| V. peregrina L.         | 35        | MZ334947                 | 693       | 0       | 99.47%     |
| V. peregrina L.         | 51        | MZ334948                 | 846       | 0       | 100%       |
| V. peregrina L.         | 63        | MZ334949                 | 791       | 0       | 99.77%     |
| V. peregrina L.         | 73        | MZ334950                 | 785       | 0       | 99.77%     |
| V. sativa L.            | 66        | MZ334952                 | 455       | 9E-124  | 94.24%     |
| V. sativa L.            | 59        | MZ334951                 | 483       | 4E-132  | 99.25%     |
| V. sativa L.            | 67        | MZ334953                 | 488       | 8E-134  | 99.63%     |
| V. sativa L.            | 80        | MZ334956                 | 503       | 3E-138  | 100%       |
| V. sativa L.            | 69        | MZ334954                 | 523       | 4E-144  | 99.31%     |
| V. sativa L.            | 79        | MZ334955                 | 420       | 3E-113  | 100%       |
| V. sativa L.            | 82        | MZ334957                 | 453       | 3E-123  | 99.60%     |
| V. sericocarpa Fenzl     | 32        | MZ334958                 | 628       | 7E-176  | 96.06%     |
| V. sericocarpa Fenzl     | 40        | MZ334959                 | 597       | 2E-166  | 97.97%     |
| V. sericocarpa Fenzl     | 53        | MZ334960                 | 656       | 0       | 99.72%     |
| V. villosa Roth         | 24        | MZ334961                 | 667       | 0       | 99.46%     |

‡ Species sequences missing from the GenBank database.

To graphically represent the sequence differences amongst the studied specimens, a multiple sequence alignment was assembled for the generated *trnL* sequences of the 71 *Vicia*
specimens, along with trnL reference sequences from GenBank. The resulting alignment generated a matrix of 492 sites in total, with 207 variable and 130 parsimony-informative sites (Table S1). When studying the generated trnL ML tree, most specimens of the same Vicia species showed distinct clustering (Figure 1).

Figure 1. Dendrogram based on the trnL alignment matrix generated for 71 specimens across 20 Vicia species. The tree was generated using the Maximum Likelihood method and the Tamura-Nei model with +G = 0.693. The tree with the highest log likelihood (−908.72) is shown. Branch labelling represents the percentage of replicate trees in which the corresponding taxa clustered together (1000 bootstrap replicates). The Trifolium repens trnL sequence (GenBank acc. number: JN617179) was used as an outgroup to root the tree. The taxa are colour-coded at the species level for visualization purposes. The Vicia species analysed in this work are labelled with circle markers. Numbers in species labelling correspond to sample ID (Table 1). GenBank-derived reference sequences are indicated by square-shaped markers and accession numbers present in the taxon labelling.

However, there were some discrepancies observed in the topology of several species, especially for the V. faba samples, which were scattered throughout the tree (Figure 1). Furthermore, individual specimens, such as V. sericocarpa (specimen 53) and V. lathyroides
Plants 2022, 11, 947 (specimen 38), did not cluster with other specimens of the same species (Figure 1). Despite the apparent clustering of specimens in the clade formed by the V. aintabensis, V. hybrida, V. noeana, and V. peregrina species, there were very few differences in the trnL sequence in these species that could allow further sub-clustering; hence, bootstrap support is very low in this clade (Figure 1). Similar cases for topology were also observed for the V. sativa and V. grandiflora species (Figure 1). Despite some taxon misplacements, broader patterns can be clearly observed. According to the trnL tree, V. dionysiensis shows an early divergence, followed by a clade that radiates to species with very similar trnL sequences (V. hybrida, V. aintabensis, V. noeana, and V. peregrina), a clade that splits to the V. anatolica and V. pannonica species, a clade that primarily contains the V. lutea, V. michauxii, and V. eravilia, and finally, a clade that diverges into the V. narbonensis, V. bithynica, V. lathyroides, and V. sativa/V. grandiflora species (Figure 1).

To further verify broad tree topologies and resolve taxon discrepancies, the ITS2 and partial rpoC1 regions were also analysed for selected taxa. For these analyses, specimens were primarily selected for their misplacement on the trnL tree, especially those that did not form any apparent clustering, such as V. faba (Figure 1). Individual specimens with an unsuccessful amplification of the ITS2 and rpoC1 regions were excluded from the subsequent analyses, although alternative representatives of the species were used in this case. A BLAST analysis of the generated ITS2 sequences for the selected taxa showed high levels of similarity with the corresponding reference sequences from GenBank (Table 2). The alignment of those sequences with reference sequences generated a matrix of 436 bp, with 200 variable and 37 parsimony-informative sites (Table S1). In contrast to trnL, the ITS2 tree resolved the topological discrepancies observed in the V. faba and V. lathyroides clades, with all the specimens of the same species displaying distinct clustering (Figure 2). Despite the lack of strict clustering for the V. sericocarpa species, the ITS2 sequences in these specimens are still more similar with each other than those from the other specimens; hence, the V. sericocarpa branches did not interject the well-defined clusters on the ITS2 tree, in contrast to what has been observed in the trnL tree (Figures 1 and 2).

Table 2. ITS2 BLAST analysis of selected Vicia specimens.

| Species/Subspecies Name | Sample ID | GenBank Accession Number | Bit Score | E-Value | % Identity |
|--------------------------|-----------|--------------------------|-----------|---------|------------|
| V. anatolica Turill.     | 65        | MZ338313                 | 475       | 2E-137  | 99.61      |
| V. bithynica L.          | 23        | MZ338299                 | 743       | 0       | 100%       |
| V. faba L.               | 37        | MZ338302                 | 763       | 0       | 100%       |
| V. faba L.               | 61        | MZ338311                 | 763       | 0       | 100%       |
| V. grandiflora Scop.     | 55        | MZ338309                 | 621       | 0       | 99.71%     |
| V. grandiflora Scop.     | 57        | MZ338310                 | 621       | 0       | 99.71%     |
| V. grandiflora Scop.     | 71        | MZ338318                 | 580       | 2E-168  | 99.41%     |
| V. grandiflora Scop.     | 70        | MZ338317                 | 627       | 0       | 100%       |
| V. lathyroides L.         | 36        | MZ338301                 | 737       | 0       | 99.75%     |
| V. lathyroides L.         | 38        | MZ338303                 | 737       | 0       | 99.75%     |
| V. lathyroides L.         | 46        | MZ338306                 | 752       | 0       | 99.76%     |
| V. lathyroides L.         | 49        | MZ338308                 | 758       | 0       | 100%       |
| V. lutea L.               | 2         | MZ338297                 | 743       | 0       | 100%       |
| V. lutea L.               | 45        | MZ338305                 | 743       | 0       | 100%       |
| V. lutea L.               | 48        | MZ338307                 | 743       | 0       | 100%       |
| V. pannonica Crantz      | 64        | MZ338312                 | 739       | 0       | 99.75%     |
| V. pannonica Crantz      | 76        | MZ338319                 | 743       | 0       | 100%       |
| V. pannonica Crantz      | 15        | MZ338298                 | 743       | 0       | 100%       |
| V. pannonica Crantz      | 77        | MZ338320                 | 743       | 0       | 100%       |
| V. sativa L.              | 66        | MZ338314                 | 763       | 0       | 99.52%     |
| V. sativa L.              | 67        | MZ338315                 | 780       | 0       | 100%       |
| V. sativa L.              | 80        | MZ338322                 | 778       | 0       | 100%       |
| V. sativa L.              | 69        | MZ338316                 | 771       | 0       | 99.76%     |
| V. sativa L.              | 79        | MZ338321                 | 771       | 0       | 99.76%     |
| V. sativa L.              | 82        | MZ338323                 | 774       | 0       | 100%       |
| V. sericocarpa Fenzl      | 32        | MZ338300                 | 353       | 5E-93   | 98.03%     |
| V. sericocarpa Fenzl      | 40        | MZ338304                 | 353       | 5E-93   | 98.03%     |
Figure 2. Dendrogram based on the ITS2 alignment matrix generated for 27 specimens across 9 *Vicia* species. The tree was generated based on the Maximum Likelihood method and the Kimura 2-parameter model with +G = 2.119. The tree with the highest log likelihood (∼1112.59) is shown. Branch labelling represents the percentage of replicate trees in which the corresponding taxa clustered together (1000 bootstrap replicates). The *Trifolium repens* ITS2 sequence (GenBank acc. number: BSYJ31) was used as an outgroup to root the tree. Newly generated *Vicia* species sequences are labelled with circular markers. Number annotations in species labelling correspond to sample IDs (Table 2). Reference sequences from GenBank with the corresponding accession numbers are marked with square-shaped markers.

Overall, in the context of the studied species, the overall ITS2 tree topology retains the broad pattern observed in the *trnL* tree, with an earlier divergence of the *V. sericocarpa* species followed by *V. anatolica*, a clade diverging to *V. lutea* and *V. pannonica*, as well as a clade comprised of the rest of the species (Figure 2). It is noteworthy that the divergence of *V. faba*, according to the ITS2 dendrogram, is placed before that of *V. bithynica*, as well as those of *V. sativa* and *V. grandiflora* clades (Figure 2).

To further resolve the taxon discrepancies observed in the *trnL* tree with a more conserved region, BLAST analysis and dendrographic reconstruction were also performed using part of the *rpoC1* region, which encodes for the beta subunit of the DNA-dependent RNA polymerase. Despite the successful amplification and sequencing of the *rpoC1* region for all of the *Vicia* specimens examined (Table 3), there were no available data in the GenBank repository for most of the tested species. As such, verification of species identity by sequence similarity using this region was not feasible. Nevertheless, the two species for
which data exist, namely *V. faba* and *V. sativa*, showed 100% similarity with high bit score values, as expected for a conserved DNA barcoding region (Table 3).

**Table 3. rpoC1 BLAST analysis of selected *Vicia* specimens.**

| Species/Subspecies Name | Sample ID | GenBank Accession Number | Bit Score | E-Value | % Identity |
|-------------------------|-----------|--------------------------|-----------|---------|------------|
| *V. anatolica* Turill.  | 5         | MZ285764                 | ‡         | ‡       | ‡          |
| *V. anatolica* Turill.  | 65        | MZ285781                 | ‡         | ‡       | ‡          |
| *V. dionysiensis*       | 12        | MZ285766                 | ‡         | ‡       | ‡          |
| *V. dionysiensis*       | 16        | MZ285767                 | ‡         | ‡       | ‡          |
| *V. dionysiensis*       | 52        | MZ285775                 | ‡         | ‡       | ‡          |
| *V. faba* L.            | 37        | MZ285772                 | 857       | 0       | 100%       |
| *V. faba* L.            | 61        | MZ285780                 | 857       | 0       | 100%       |
| *V. faba* L.            | 22        | MZ285769                 | 857       | 0       | 100%       |
| *V. grandiflora* Scop.  | 55        | MZ285777                 | ‡         | ‡       | ‡          |
| *V. grandiflora* Scop.  | 57        | MZ285778                 | ‡         | ‡       | ‡          |
| *V. grandiflora* Scop.  | 71        | MZ285786                 | ‡         | ‡       | ‡          |
| *V. grandiflora* Scop.  | 70        | MZ285785                 | ‡         | ‡       | ‡          |
| *V. lathyroides* L.     | 38        | MZ285773                 | ‡         | ‡       | ‡          |
| *V. michauxii* Spreng.  | 11        | MZ285765                 | ‡         | ‡       | ‡          |
| *V. michauxii* Spreng.  | 19        | MZ285768                 | ‡         | ‡       | ‡          |
| *V. pannonica* Crantz   | 85        | MZ285790                 | ‡         | ‡       | ‡          |
| *V. peregrina* L.       | 35        | MZ285771                 | ‡         | ‡       | ‡          |
| *V. sativa* L.          | 66        | MZ285782                 | 857       | 0       | 100%       |
| *V. sativa* L.          | 59        | MZ285779                 | 857       | 0       | 100%       |
| *V. sativa* L.          | 67        | MZ285783                 | 857       | 0       | 100%       |
| *V. sativa* L.          | 80        | MZ285788                 | 857       | 0       | 100%       |
| *V. sativa* L.          | 69        | MZ285784                 | 857       | 0       | 100%       |
| *V. sativa* L.          | 79        | MZ285787                 | 857       | 0       | 100%       |
| *V. sativa* L.          | 82        | MZ285789                 | 857       | 0       | 100%       |
| *V. sericocarpa* Fenzl  | 32        | MZ285770                 | ‡         | ‡       | ‡          |
| *V. sericocarpa* Fenzl  | 40        | MZ285774                 | ‡         | ‡       | ‡          |
| *V. sericocarpa* Fenzl  | 53        | MZ285776                 | ‡         | ‡       | ‡          |

‡ Species sequences missing from the GenBank database.

The alignment of the corresponding sequences with reference species sequences from GenBank generated an alignment matrix of 442 bp with 25 parsimony-informative and 37 variable sites (Table S1), which indicates very low variation amongst the analysed sequences, as expected for a rather conserved region. On the basis of the generated rpoC1 dendrogram, most of the recently diverged species, such as *V. sativa*, *V. grandiflora*, and *V. faba*, were clearly separated into distinct clades, showing topological agreement with the ITS2 tree (Figures 2 and 3).

However, the *V. lathyroides*, *V. peregrina*, and *V. michauxii* species showed much earlier divergence than that observed in the *trnL* and ITS2 trees (Figures 1 and 2). Especially for *V. michauxii* and *V. peregrina*, the rpoC1 tree shows an even earlier divergence than that of the *V. dionysiensis* clade (Figure 3), which was shown to have the earliest divergence amongst the analysed *Vicia* species in the *trnL* tree (Figure 1).
Species sequences missing from the GenBank database. The alignment of the corresponding sequences with reference species sequences from GenBank generated an alignment matrix of 442 bp with 25 parsimony-informative and 37 variable sites (Table S1), which indicates very low variation amongst the analysed sequences, as expected for a rather conserved region. On the basis of the generated rpoC1 dendrogram, most of the recently diverged species, such as *V. sativa*, *V. grandiflora*, and *V. faba*, were clearly separated into distinct clades, showing topological agreement with the ITS2 tree (Figures 2 and 3). However, the *V. lathyroides*, *V. peregrina*, and *V. michauxii* species showed much earlier divergence than that observed in the trnL and ITS2 trees (Figures 1 and 2). Especially for *V. michauxii* and *V. peregrina*, the rpoC1 tree shows an even earlier divergence than that of the *V. dionysiensis* clade (Figure 3), which was shown to have the earliest divergence amongst the analysed *Vicia* species in the trnL tree (Figure 1).

Figure 3. Dendrogram based on the rpoC1 alignment matrix generated for 27 specimens across 10 *Vicia* species. The tree was generated using the Maximum Likelihood method and the Jukes–Cantor model with +G = 0.05. The tree with the highest log likelihood (−927.88) is shown. Branch labelling represents the percentage of replicate trees in which the corresponding taxa clustered together (1000 bootstrap replicates). The *Pisum sativum* rpoC1 sequence (GenBank acc. number: NC014057) was used as an outgroup to root the tree. The *Vicia* species sequences generated in this work are labelled with circular markers. Number annotations in species labelling correspond to sample IDs (Table 3). Reference sequences from GenBank with the corresponding accession numbers are labelled with square-shaped markers.

3. Discussion

Legume crops and their products are important elements of the human diet, an essential animal feed, as well as an integral component of sustainable agriculture. Many leguminous species of the *Vicia* genus have been domesticated and are commonly used in various Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) products [53], which necessitates the application of methodologies that will enable the effective authentication and identification of the product’s composition. Furthermore, a comparative analysis of morphological, biochemical, karyotypic, and genetic characteristics of several *Vicia* species revealed inconsistencies in the resulting classifications within the genus [54–58], which indicates that more reliable methodologies using genetic markers with general taxon specificity are required for species identification.

DNA barcoding analysis was proven to be one of those methods capable of discriminating among species on the basis of the sequence of small DNA region(s) [30,37,38]. This
methodology was successfully used not only for species identification and forensic analyses, but also for biodiversity and phylogenetic studies [59,60]. DNA barcoding in plants most commonly uses chloroplast sequences, which are species-specific [37,38]. Unlike the nuclear genome that follows Mendelian inheritance, chloroplasts do not undergo meiotic recombination and show maternal inheritance in the majority of the angiosperms [61]. Furthermore, the chloroplast genome is sufficiently rich in polymorphic sequences, which facilitates the conduct of evolution studies and species discrimination in plants [62–64]. Nevertheless, given the lack of a single universal barcode that can be effectively used for all plant species [65], the application of multi-loci approaches, utilizing both nuclear and chloroplast DNA barcodes, has significantly increased the identification accuracies, especially for closely related species [44].

To verify the accuracy of species identification for indicative Eurasian *Vicia* specimens deposited in local seed banks, we implemented DNA barcoding analyses using the highly variable *trnL* and ITS2 DNA barcoding regions. Both DNA barcodes have rich representation in the GenBank repository for many plant species, including species in the *Vicia* genus, thus allowing for a more accurate species discrimination using sequence similarity (>90%). The rather conserved chloroplastic *rpoC1* region was also used, although it is not the most optimal barcode for species discrimination in the Fabaceae [39], given that it can facilitate a more optimal interspecific alignment and tree topology than the highly variable *trnL* and ITS2 regions.

More specifically, to facilitate species identification for the 71 Eurasian *Vicia* specimens studied in this work and verify their morphological identification prior to seed bank submission, we initially analysed the chloroplast *trnL* DNA barcoding region in these specimens. The *trnL* region was chosen as the primary barcode of our analysis, given that it has the desirable characteristics for DNA barcoding, especially for sample preparations with compromised DNA quality [32,35], and was also used successfully for species discrimination in the Fabaceae family, including *Vicia* [15,39]. Using the BLAST analysis of the generated *trnL* sequences, the majority of the specimens were successfully discriminated at the species level with notably high similarity values, which were higher than 90% identity (Table 1), further indicating that the *trnL* region can be reliably used for species discrimination in the *Vicia* genus. The derived *trnL* tree showed an overall agreement in the topology of the taxa with the corresponding reference entries, although some discrepancies were noted for several taxonomic groups (Figure 1). Although the generated dendrogram is not a phylogenetic tree per se, it can still be used to facilitate species discrimination, especially for inconclusive BLAST results. As such, pertaining to the *trnL* dendrogram, the most pronounced incongruences were observed for the *V. sativa* and *V. grandiflora* clades that are not clearly defined, as well as the *V. faba* and *V. sericocarpa* species, which do not form distinct clusters, with several individual specimens being placed throughout the tree (Figure 1). Very recently, Han et al. (2021) inferred similar topologies when testing the phylogeny of species in the *Vicia* genus, with *V. sativa* and *V. faba* showing the most recent divergence and the clades radiating to *V. panonica*, *V. aintabensis*, and *V. peregrina* having an earlier divergence [48].

In an attempt to further resolve the topological incongruences observed in the *trnL* tree, especially for the *V. faba*, *V. grandiflora*, *V. lathyroides*, *V. sativa*, and *V. sericocarpa* species, and to further support species discrimination amongst the specimens, the ITS2 region was also analysed for the selected taxa. The selection of taxa for the ITS2 analysis was primarily based on specimens that did not form any apparent clustering and were spread across the *trnL* tree, such as *V. faba* and *V. sericocarpa* (Figure 1). Several other taxa were also selected from various taxonomic groups, despite their placement on the *trnL* tree, in order to preserve the overall dendrographic topology, and broad topological comparisons can be made between the two barcoding regions. Kress et al. (2005) have reported the use of the nuclear ITS2 region as a suitable barcode for flowering plants, although it has been problematic for some species [37]. Furthermore, the ITS2 region was regarded as successful for species discrimination in the Fabaceae family [39,43] and more specifically
in the *Vicia* genus [41,46,48,66]. BLAST analysis using the ITS2 region was also highly effective in discriminating the analysed taxa (Table 2), with remarkably high-percentage identity values (>98%) between the analysed taxa and GenBank reference sequences. This is consistent with previous findings that show both *trnL* and ITS2 as being equally effective in species discrimination for species in the Fabaceae family [39]. The overall topological discrepancies observed in the *trnL* tree have been resolved with high bootstrap support using the ITS2 region (Figure 2). More specifically, the *V. sativa* and *V. grandiflora* clades were sufficiently resolved in distinct clusters (Figure 2). According to the ITS2 tree, the *V. faba* samples showed a more defined clustering, which positions this species divergence in between *V. bythinica* and *V. lathyroides* (Figure 2). This placement of *V. faba* is consistent with previous phylogenetic studies [41,48,66–69], which further supports the accuracy of the ITS2 tree topology.

Although the ITS2 analysis greatly resolved the general topological incongruences observed in the *trnL* tree, despite being a highly variable region, the conserved *rpoC1* region was also used for selected species, notwithstanding the reported low discrimination efficiency [32]. We argue that a more conserved region of the chloroplast genome would be more effective in discriminating species in the genus. However, in contrast with the other barcoding regions, *rpoC1* could not identify the selected species apart from the *V. sativa* and *V. faba* species, which showed 100% identity (Table 3). This was likely the result of non-annotated or absent *rpoC1* sequences in the GenBank repository. The generated *rpoC1* tree showed consistency with the ITS2 tree pertaining to the topology of the recently diverging *V. sativa*, *V. grandiflora*, and *V. faba* taxa, whilst early diverging clades, such as *V. lathyroides*, *V. peregrina*, and *V. michauxii*, were not congruent between the two barcodes (Figures 2 and 3). Although all of the taxa show high intraspecific bootstrap support and clear intraspecies clustering, the majority of the analysed clades, except for *V. sativa*, *V. grandiflora*, and *V. faba*, show topological discrepancies compared to the rest of the trees present in this study (Figure 3). This finding may be attributed to the fact that *rpoC1* is a highly conserved region to allow for accurate species discrimination for at least some species in the *Vicia* genus. Moreover, the *rpoC1* region was shown to be the least discriminatory when used for species identification amongst several coding and non-coding barcodes within the Fabaceae family [39,40]. Hence, species discrimination within the *Vicia* genus would be difficult to achieve using only this barcode and will require more thorough investigation with more specimens in these groups.

### 4. Materials and Methods

#### 4.1. Plant Materials and DNA Extraction

Seeds of 71 specimens corresponding to 20 different species of the genus *Vicia* (Fabaeae, Fabaceae) were provided by the Genetic Resources Unit at the International Center for Agricultural Research in the Dry Areas (ICARDA) in Aleppo, Syria, and by the General Commission for Scientific Agricultural Research (GCSAR) in Damascus, Syria. Species selection was based on the availability of *Vicia* species germplasms in the corresponding seed banks. Information on species names and sources of origin, which were derived from the seed bank records, is presented in Table 4.

A total of 5 seeds from each specimen were sown in a 0.5 L plastic pot containing turf (Euroveen B. V., Charleston, SC, USA). Seeds were allowed to germinate under laboratory conditions (21 °C) and grow for 1 month. DNA was extracted from 0.5 g leaf tissue of each specimen using the CTAB method according to Doyle and Doyle (1987) [70]. The DNA concentration was measured with standard spectrophotometric procedures using the Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). DNA quality and quantity were further assessed with gel electrophoresis on a 1% agarose gel. Samples were then diluted to 20 ng/µL working stocks.
| Species/Subspecies Name | Sample ID | Source          |
|-------------------------|-----------|-----------------|
| V. aintabensis Boiss.   | 3         | Italy           |
| V. aintabensis Boiss    | 13        | France          |
| V. aintabensis Boiss    | 20        | Syria           |
| V. aintabensis Turill.  | 5         | Turkmenistan    |
| V. anatolica Turill.    | 80        | Turkey          |
| V. anatolica Turill.    | 54        | Turkey          |
| V. anatolica Turill.    | 65        | Australia       |
| V. bithynica L.         | 23        | Syria           |
| V. bithynica L.         | 43        | Malta           |
| V. bithynica L.         | 68        | Azerbaijan      |
| V. bithynica L.         | 72        | Syria           |
| V. dionysiensis         | 12        | Syria           |
| V. dionysiensis         | 16        | Syria           |
| V. dionysiensis         | 52        | Syria           |
| V. ervilia L.           | 8         | Syria           |
| V. ervilia L.           | 17        | Syria           |
| V. ervilia L.           | 34        | Syria           |
| V. ervilia L.           | 44        | Syria           |
| V. ervilia L.           | 78        | Syria           |
| V. faba L.              | 37        | Syria           |
| V. faba L.              | 61        | Syria           |
| V. faba L.              | 22        | Syria           |
| V. grandiflora Scop.    | 55        | Armenia         |
| V. grandiflora Scop.    | 57        | Armenia         |
| V. grandiflora Scop.    | 71        | Sweden          |
| V. grandiflora Scop.    | 70        | Turkey          |
| V. hgaeniscyamus Mouterde| 41        | Syria           |
| V. hgaeniscyamus Mouterde| 86       | Syria           |
| V. hybrida L.           | 25        | Iraq            |
| V. hybrida L.           | 42        | Jordan          |
| V. hybrida L.           | 60        | Italy           |
| V. hybrida L.           | 62        | Morocco         |
| V. lathyroides L.       | 36        | Turkey          |
| V. lathyroides L.       | 38        | Turkey          |
| V. lathyroides L.       | 46        | Armenia         |
| V. lathyroides L.       | 49        | Algeria         |
| V. lutea L.             | 2         | Tunisia         |
| V. lutea L.             | 45        | Azerbaijan      |
| V. lutea L.             | 26        | Algeria         |
| V. lutea L.             | 48        | Turkey          |
| V. michauxii Spreng.    | 11        | Turkey          |
| V. michauxii Spreng.    | 19        | Tajikistan      |
| V. monantha Retz.       | 21        | Syria           |
| V. narbonensis L.       | 1         | Lebanon         |
| V. narbonensis L.       | 4         | Turkey          |
| V. narbonensis L.       | 30        | Syria           |
| V. narbonensis L.       | 14        | Syria           |
| V. narbonensis L.       | 47        | Jordan          |
| V. nocana Boiss.        | 7         | Syria           |
| V. nocana Boiss.        | 9         | Turkey          |
| V. nocana Boiss.        | 29        | Syria           |
| V. pannonica Crantz     | 85        | Uzbekistan      |
| V. pannonica Crantz     | 64        | Australia       |
| V. pannonica Crantz     | 76        | Italy           |
| V. pannonica Crantz     | 15        | Italy           |
| V. pannonica Crantz     | 77        | Turkey          |
| V. peregrina L.         | 35        | Iraq            |
4.2. PCR Amplification and Sequence Analysis

PCR amplification was performed to a final volume of 50 µL in a Veritti research thermocycler (Applied Biosystems, Waltham, MA, USA). The reaction mixture contained 20 ng genomic DNA, 1X PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs mix, 0.4 µM forward and reverse primers, and 1 U Kapa Taq DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA). The primer sequences used for the DNA barcoding analyses are shown in Table S2. PCR cycling was carried out with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30s, 55 °C for 40s, and 72 °C for 1 min, with a final extension step at 72 °C for 2 min. The PCR products were sequenced in two directions with the Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an automated ABI 3730 sequencer (PE Applied Biosystems). Sequence ambiguities were manually corrected using the CHROMAS software version 2.6.6 (Technelysium Pty Ltd., South Brisbane, Australia).

4.3. Basic Local Alignment Search Tool (BLAST) Analysis

Species identification, using the sequence similarity approach, was performed using the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 13 May 2021). The default search parameters were used, optimizing for highly similar sequences (megablast). Species identification was regarded as successful when both the species label of the specimen deposited in the local seed banks and the molecular identity according to sequence similarity agreed by >90%. The generated DNA barcoding sequences were submitted to GenBank with accession numbers MZ334891-MZ334961 for trnL, accession numbers MZ338297-MZ338323 for ITS2, and accession numbers MZ285764-MZ285790 for rpoC1.

4.4. Multiple Sequence Alignment and Dendrographic Representation

Multiple sequence alignments were generated for the trnL, ITS2, and rpoC1 sequences of the samples used herein, along with representative reference sequences from GenBank for representative taxa. The Molecular Evolutionary Genetics Analysis X (MEGA X) software version 10.05 [71] with the MUSCLE algorithm was used for alignment generation. A Maximum Likelihood (ML) fitness assessment for 24 substitution models was carried out for each of the alignments using MEGA X (Table S3). The model with the lowest Bayesian Information Criterion (BIC) value for each alignment was selected to subsequently generate the corresponding ML trees with 1000 bootstrap replicates using MEGA X. The Tamura-Nei (T92) model [72] with discrete gamma distributions (+G = 0.693) was used for trnL, the Kimura 2-parameter (K2-P) model [73] with discrete gamma distributions (+G = 2.119) was used for ITS2, and the Jukes–Cantor model (JC) model [74] with discrete gamma distributions (+G = 0.050) was used for rpoC1 tree reconstructions. The Pisum and Trifolium species in the dendrograms were used as rooting tree outgroups.
5. Conclusions

Legumes, such as faba, peas, and lupins, have the potential to replace animal-based protein sources, such as meat, bone, or fish, as well as provide other important seed fractions, namely, starch and fibre, as raw materials for making a variety of formulated products in the food, feed, and cosmetics industries. Given the importance of several Vicia species in the Mediterranean diet and the value of such ingredients in local PDO and PGI products, we implemented DNA barcoding analysis using the \textit{trnL}, ITS2, and \textit{rpoC1} regions to discriminate Eurasian \textit{Vicia} species. Our analysis indicates that both the \textit{trnL} and ITS2 can effectively discriminate \textit{Vicia} species, whilst the use of the \textit{rpoC1} region was not capable of identifying most of the analysed species. The use of multi-loci analyses may be more powerful to provide higher discrimination accuracies, especially for the closely related species that single DNA barcodes fail to resolve. Broad dendrographic representations for the analysed taxa were also more congruent when considering topologies reconstructed from the \textit{trnL} and ITS2 data, in contrast to the topologies obtained from \textit{rpoC1}. We expect the work presented herein to enrich the available barcoding data for species discrimination in the \textit{Vicia} genus, which will contribute significantly to the resolution of the still inconclusive phylogeny of the genus, as well as for agricultural and culinarian purposes, especially for the local Eurasian communities.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants11070947/s1. Table S1: Multiple sequence alignment statistics, Table S2: Primers used in this study, Table S3: Best Maximum Likelihood model fit for the reconstruction of the \textit{trnL}, ITS2, and \textit{rpoC1} trees.

Author Contributions: Conceptualization, P.M., N.H., C.B. and M.O.; methodology, I.B.; validation, I.B., G.L. and P.M.; formal analysis, I.B. and G.L.; investigation, I.B.; resources, N.H., M.O. and P.M.; data curation, I.B. and G.L.; writing—original draft preparation, I.B. and G.L.; writing—review and editing, I.B., G.L., N.H., C.B. and P.M.; visualization, I.B. and G.L.; supervision, P.M.; project administration, P.M.; funding acquisition, N.H., M.O. and P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Regional Development Fund of the European Union and Greek national funds through the operational program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK-04448). Also, this research was funded by Chiang Mai University.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The generated DNA barcoding data are available through GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with accession numbers MZ334891-MZ334961 for \textit{trnL}, accession numbers MZ338297-MZ338323 for ITS2, and accession numbers MZ285764-MZ285790 for \textit{rpoC1}.

Acknowledgments: We would like to thank the University of Chiang Mai for the financial support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bryant, J.A.; Hughes, S.G. Wild Crop Relatives: Genomic and Breeding Resources; Kole, C., Ed.; Springer: Berlin/Heidelberg, Germany, 2011; ISBN 978-3-642-14386-1.
2. Grusak, M.A. Phytochemicals in plants: Genomics-assisted plant improvement for nutritional and health benefits. \textit{Curr. Opin. Biotechnol.} 2002, \textit{13}, 508–511. [CrossRef]
3. Grusak, M.A. Enhancing Mineral Content in Plant Food Products. \textit{J. Am. Coll. Nutr.} 2013, \textit{21}, 178S–183S. [CrossRef] [PubMed]
4. Velazquez, E.; Silva, L.R.; Peix, A. Legumes: A Healthy and Ecological Source of Flavonoids. \textit{Curr. Nutr. Food Sci.} 2010, \textit{6}, 109–144. [CrossRef]
5. Bromfield, E.S.; Butler, G.; Barran, L.R. Temporal effects on the composition of a population of Sinorhizobium meliloti associated with Medicago sativa and Melilotus alba. \textit{Can. J. Microbiol.} 2011, \textit{47}, 567–573. [CrossRef] [PubMed]
6. Sprent, J.I. \textit{Nodulation in Legumes}; Royal Botanic Gardens, Kew: Richmond, VA, USA, 2001.
7. Arianoutsou, M.; Thanos, C.A. Legumes in the fire-prone Mediterranean regions: An example from Greece. *Int. J. Wildl. Fire* 1996, 6, 77–82. [CrossRef]

8. Graham, P.H.; Vance, C.P. Legumes: Importance and Constraints to Greater Use. *Plant Physiol.* 2003, 131, 872–877. [CrossRef]

9. Keskin, S.O.; Ali, T.M.; Ahmed, J.; Shaikh, M.; Siddiq, M.; Uebersax, M.A. Physico-chemical and functional properties of legume protein, starch, and dietary fiber—A review. *Legum. Sci.* 2021, 4, e117. [CrossRef]

10. Siegel, A.; Fawcett, B. Food legume processing and utilization: With special emphasis on application in developing countries. In *IDRC-TSI*; International Development Research Centre: Ottawa, ON, Canada, 1976; p. 88. ISBN 0-88936-086-3.

11. Schuysuter, M.A.; Pelgrum, P.J.M.; van der Goot, A.J.; Boom, R.M. Dry fractionation for sustainable production of functional legume protein concentrates. *Trends Food Sci. Technol.* 2015, 45, 327–335. [CrossRef]

12. Soyland, J.; Luckow, M.A. The Rest of the Iceberg. *Legume Diversity and Evolution in a Phylogenetic Context. Plant Physiol.* 2003, 131, 900–910. [CrossRef]

13. Lewis, G.P.; Schrire, B.; Mackinder, B.; Lock, M. *Legumes of the World*; Royal Botanic Gardens: London, UK, 2005; ISBN 9781900347808.

14. Cacan, E.; Kokten, K.; Inci, H.; Das, A.; Sengul, A.Y. Fatty Acid Composition of the Seeds of Some Vicia Species. *Chem. Nat. Compd.* 2016, 52, 1084–1086. [CrossRef]

15. Schaefer, H.; Hechenleitner, P.; Santos-Guerra, A.; De Sequeira, M.M.; Pennington, R.T.; Kenicer, G.; Carine, M.A. Systematics, biogeography, and character evolution of the legume tribe Fabaeae with special focus on the middle-Atlantic island lineages. *BMC Evol. Biol.* 2012, 12, 250. [CrossRef] [PubMed]

16. Lupicha, F.K. The infrageneric structure of *Lathyrus*. *Notes Roy Bot. Gard Edinb.* 1983, 41, 209–244.

17. Lupicha, F.K. The infrageneric structure of *Vicia*. *Notes Roy Bot. Gard Edinb.* 1976, 34, 287–326.

18. Hanelt, P.; Mettin, D. Biosystematics of the genus *Vicia* L. (Leguminosae). *Annu. Rev. Ecol. Syst.* 1989, 20, 199–223. [CrossRef]

19. Ruffini Castiglione, M.; Frediani, M.; Gelati, M.T.; Ravalli, C.; Venora, G.; Caputo, P.; Cremonini, R. Cytological and molecular characterization of *Vicia esdraelonensis* Warb. & Eig: A rare taxon. *Protoplasma* 2007, 231, 151–159. [PubMed]

20. Keiša, A.; Maxted, N.; Forde-Lloyd, B. The assessment of biodiversity loss over time: Wild legumes in Syria. *Genet. Resour. Crop Evol.* 2007, 55, 603–612. [CrossRef]

21. Haider, N.; Nabulsi, I.; Mirali, N. Identification of species of *Vicia* subgenus *Vicia* (Fabaceae) using chloroplast DNA data. *Turkish J. Agric. For.* 2012, 36, 297–308. [CrossRef]

22. Chernoff, M.; Plattmann, U.; Klselev, M.E. Seed characters and testa texture in species of the vicieae: Their taxonomic significance. *Isr. J. Bot.* 1992, 41, 167–186.

23. Jalilian, N.; Rahiminejad, M.R.; Maassoumi, A.; Maroofi, H. Taxonomic revision of the genus *Vicia* L. (Fabaceae) in Iran. *Iran. J. Bot.* 2014, 20, 155–164.

24. Endo, Y.; Ohashi, H. The morphology of styles and stigmas in *Vicia* (Leguminosae), and its systematic implications. *J. Plant Res.* 1995, 108, 17–24. [CrossRef]

25. Nam, B.M.; Park, M.S.; Oh, B.U.; Chung, G.Y. A cytotaxonomic study of *Vicia* L. (Fabaceae) in Korea. *Korean J. Plant Taxon.* 2012, 42, 307–315. [CrossRef]

26. Hosseinzadeh, Z.; Pakravan, M.; Tavassoli, A. Micromorphology of Seed in Some *Vicia* Species from Iran. *Rostaniba* 2009, 9, 230.

27. Ali, M.N.; Yeasmim, L.; Gantait, S.; Goswami, R.; Chakraborty, P. Screening of rice landraces for salinity tolerance at seedling stage. *Int. J. Wildl. Fire*, 77–82. [CrossRef]

28. Maxted, N.; Khettab, M.A.; Bisby, F.A. The newly discovered relatives of *Vicia* L. (Fabaceae) in Iran. *Iran. J. Bot.* 2012, 155–164.

29. Chen, S.; Yao, H.; Han, J.; Liu, C.; Song, J.; Shi, L.; Zhu, Y.; Ma, X.; Gao, T.; Pang, X.; et al. Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS ONE* 2010, 5, e8613. [CrossRef]

30. Gregory, T.R. DNA barcoding does not compete with taxonomy. *Nature* 2005, 434, 1067. [CrossRef]

31. Hollingsworth, P.M.; Graham, L.D. Choosing and Using a Plant DNA Barcode. *PLoS ONE* 2011, 6, e19254. [CrossRef]

32. Stavridou, E.; Lagiotis, G.; Karapetsi, L.; Osathanunkul, M.; Madesis, P. DNA Fingerprinting and Species Identification Uncovers the Genetic Diversity of Katsouni Pea in the Greek Islands Amorgos and Schinoussa. *Plants* 2020, 9, 479. [CrossRef]

33. Lagiotis, G.; Stavridou, E.; Bosmali, I.; Osathanunkul, M.; Haider, N.; Madesis, P. Detection and quantification of cashew in commercial tea products using High Resolution Melting (HRM) analysis. *J. Food Sci.* 2020, 85, 1629–1634. [CrossRef]

34. Taberlet, P.; Coissac, E.; Pampanon, F.; Gielly, L.; Miquel, C.; Valentini, A.; Vernat, T.; Corthier, G.; Brochmann, C.; Willerslev, E. Power and limitations of the chloroplast trnL (UA) intron for plant DNA barcoding. *Nucleic Acids Res.* 2007, 35, e14. [CrossRef] [PubMed]

35. Chase, M.W.; Cowan, R.S.; Hollingsworth, P.M.; van den, B.C.; Madriñán, S.; Petersen, G.; Seberg, O.; Jørgensen, T.; Cameron, K.M.; Carine, M.; et al. A proposal for a standardised protocol to barcode all land plants. *Taxon* 2007, 56, 295–299. [CrossRef]

36. Kress, W.J.; Wurdack, K.J.; Zimmer, E.A.; Weigt, L.A.; Janzen, D.H. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA* 2005, 102, 8369–8374. [CrossRef] [PubMed]
38. Hollingsworth, M.L.; Andra Clark, A.; Forrest, L.L.; Richardson, J.; Pennington, R.T.; Long, D.G.; Cowan, R.; Chase, M.W.; Gaudeul, M.; Hollingsworth, P.M. Selecting barcoding loci for plants: Evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. *Mol. Ecol. Resour.* 2009, 9, 439–457. [CrossRef]

39. Madesis, P.; Panopoulou, I.; Ralli, P.; Tsatsarlis, A.; Parthenopio, R.; Tsatsarlis, A. Barcoding the major Mediterranean leguminous crops by combining universal chloroplast and nuclear DNA sequence targets. *Genet. Mol. Res.* 2012, 11, 2548–2558. [CrossRef]

40. Gao, T.; Chen, S. Authentication of the Medicinal Plants in Fabaceae by DNA Barcoding Technique. *Planta Med.* 2009, 75, 13. [CrossRef]

41. Endo, Y.; Choi, B.H.; Ohashi, H.; Delgado-Salinas, A. Phylogenetic relationships of New World Vicia (Leguminosae) inferred from nrDNA internal transcribed spacer sequences and floral characters. *Syst. Bot.* 2008, 33, 356–363. [CrossRef]

42. Ruffini Castiglione, M.; Frediani, M.; Gelati, M.T.; Ravalli, C.; Venora, G.; Caputo, P.; Cremonini, R. Cytology of Vicia species. X. Karyotype evolution and phylogenetic implication in Vicia species of the sections Atossa, Microcarinae, Wiggersia and Vicia. *Protoplasma* 2011, 248, 707–716. [CrossRef]

43. Shiran, B.; Kiani, S.; Sehgal, D.; Hafizi, A.; ul-Hassan, T.; Chaudhary, M.; Raina, S.N. Internal transcribed spacer sequences of nuclear ribosomal DNA resolving complex taxonomic history in the genus Vicia L. *Genet. Resour. Crop Evol.* 2014, 61, 909–925. [CrossRef]

44. Cold Spring Harbor DNA Learning Center. Using DNA Barcodes to Identify and Classify Living Things. Available online: http://www.dnabarcoding101.org/ (accessed on 8 September 2021).

45. Raveendar, S.; Lee, J.R.; Shim, D.; Lee, G.A.; Jeon, Y.A.; Cho, G.T.; Ma, K.H.; Lee, S.Y.; Sung, G.H.; Chung, J.W. Comparative efficacy of four candidate DNA barcode regions for identification of Vicia species. *Plant Genet. Resour. Characterisation Util.* 2017, 15, 286–295. [CrossRef]

46. Raveendar, S.; Lee, J.-R.; Park, J.-W.; Lee, G.-A.; Jeon, Y.-A.; Lee, Y.J.; Cho, G.-T.; Ma, K.-H.; Lee, S.-Y.; Chung, J.-W. Potential use of ITS2 and matK as a Two-Locus DNA Barcode for Identification of Vicia Species. *Plant Breed. Biotechnol.* 2015, 3, 58–66. [CrossRef]

47. Wu, F.F.; Gao, Q.; Liu, F.; Wang, Z.; Wang, J.L.; Wang, X.G. DNA barcoding evaluation of Vicia (Fabaceae): Comparative efficacy of six universal barcode loci on abundant species. *J. Syst. Evol.* 2020, 58, 77–88. [CrossRef]

48. Han, S.; Sebastian, R.; Wang, X.H.; Lee, K.J.; Cho, G.T.; Hyun, D.Y.; Chung, J.W. Identification of Vicia Species Native to South Korea Using Molecular and Morphological Characteristics. *Front. Plant Sci.* 2021, 12, 14. [CrossRef] [PubMed]

49. Tiffney, B.H. The Eocene North Atlantic land bridge: its importance in Tertiary and modern phytogeography of the Northern Hemisphere. *J. Arnold Arboretum.* 1985, 66, 243–273. [CrossRef]

50. van de Wouw, M.; Maxted, N.; Chabane, K.; Ford-Lloyd, B.V. Molecular taxonomy of Vicia ser. Vicia based on Amplified Fragment Length Polymorphisms. *Plant Syst. Evol.* 2001, 229, 91–105. [CrossRef]

51. Bennett, S.J.; Maxted, N. An ecogeographic analysis of the Vicia narbonensis complex. *Genet. Resour. Crop Evol.* 1997, 44, 411–428. [CrossRef]

52. van de Wouw, M.; Maxted, N.; Ford-Lloyd, B.V. A multivariate and cladistic study of Vicia L. ser. Vicia (Fabaceae) based on analysis of morphological characters. *Plant Syst. Evol.* 2003, 237, 19–39. [CrossRef]

53. Van Itersum, K.; Candel, M.J.J.M.; Torelli, F. The market for PDO/PGI protected regional products: Consumers’ attitudes and behaviour. In Proceedings of the 67th EAAE Seminar, Le Mans, France, 28–30 October 1999.

54. Sakowicz, T.; Cieslikowski, T. Phylogenetic analyses within three sections of the genus Vicia. *Cell. Mol. Biol. Lett.* 2006, 11, 594–615. [CrossRef]

55. Venora, G.; Blangiforti, S.; Frediani, M.; Maggini, F.; Gelati, M.T.; Castiglione, M.R.; Cremonini, R. Nuclear DNA contents, rDNAs, chromatin organization, and karyotype evolution in Vicia sect. faba. *Protoplasma* 2000, 213, 118–125. [CrossRef]

56. Fuchs, J.; Strehl, S.; Brandes, A.; Schweizer, D.; Schubert, I. Molecular-cytogenetic characterization of the Vicia faba genome—Heterochromatin differentiation, replication patterns and sequence localization. *Chromosom. Res.* 1998, 6, 219–230. [CrossRef]

57. Przybylska, J.; Zmięka-Przybylska, Z. Electrophoretic seed albumin patterns and species relationships in Vicia sect. Faba. (Fabaceae). *Plant Syst. Evol.* 1995, 198, 179–194. [CrossRef]

58. van de Ven, W.T.G.; Duncan, N.; Ramsay, G.; Phillips, M.; Powell, W.; Waugh, R. Taxonomic relationships between V. faba and its relatives based on nuclear and mitochondrial RFLPs and PCR analysis. *Theor. Appl. Genet.* 1993, 86, 71–80. [CrossRef] [PubMed]

59. Renning, S.B.; Rudi, K.; Berdal, K.G.; Holst-Jensen, A. Differentiation of important and closely related cereal plant species (Poaceae) in food by hybridization to an oligonucleotide array. *J. Agric. Food Chem.* 2005, 53, 8874–8880. [CrossRef] [PubMed]

60. Ward, J.; Peakall, R.; Gilmore, S.R.; Robertson, J. A molecular identification system for grasses: A novel technology for forensic botany. *Forensic Sci. Int.* 2005, 152, 121–131. [CrossRef] [PubMed]

61. Daniell, H.; Lin, C.S.; Yu, M.; Chang, W.J. Chloroplast genomes: Diversity, evolution, and applications in genetic engineering. *Genome Biol.* 2016, 17, 134.

62. Ahmed, I.; Biggs, P.J.; Matthews, P.J.; Collins, L.J.; Hendy, M.D.; Lockhart, P.J. Mutational Dynamics of Aroid Chloroplast Genomes. *Genome Biol. Evol.* 2012, 4, 1316–1323. [CrossRef]

63. Ahmed, I.; Matthews, P.J.; Biggs, P.J.; Naeem, M.; Mclenachan, P.A.; Lockhart, P.J. Identification of chloroplast genome loci suitable for high-resolution phylogeographic studies of Colocasia esculenta (L.) Schott (Araceae) and closely related taxa. *Mol. Ecol. Resour.* 2013, 13, 929–937. [CrossRef]

64. Xu, J.H.; Liu, Q.; Hu, W.; Wang, T.; Xue, Q.; Messing, J. Dynamics of chloroplast genomes in green plants. *Genomics* 2015, 106, 221–231. [CrossRef]
65. Li, X.; Yang, Y.; Henry, R.J.; Rossetto, M.; Wang, Y.; Chen, S. Plant DNA barcoding: From gene to genome. *Biol. Rev. Camb. Philos. Soc.* **2015**, *90*, 157–166. [CrossRef]

66. Choi, B.H.; Seok, D.I.; Endo, Y.; Ohashi, H. Phylogenetic significance of styal features in genus Vicia (Leguminosae): An analysis with molecular phylogeny. *J. Plant Res.* **2006**, *119*, 513–523. [CrossRef]

67. Maxted, N. *An Ecogeographical Study of Vicia Subgenus Vicia*; IPGRI (International Plant Research Institute): Rome, Italy, 1995; ISBN 978-92-9043-240-1.

68. Fennell, S.R.; Powell, W.; Wright, F.; Ramsay, G.; Waugh, R. Phylogenetic relationships between Vicia faba (Fabaceae) and related species inferred from chloroplast trnL sequences. *Plant Syst. Evol.* **1998**, *212*, 247–259. [CrossRef]

69. Jaaska, V. Isoenzyme diversity and phylogenetic affinities in Vicia subgenus Vicia (Fabaceae). *Genet. Resour. Crop Evol.* **1997**, *44*, 557–574. [CrossRef]

70. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.

71. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]

72. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526.

73. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef]

74. Jukes, T.H.; Cantor, C.R. Evolution of Protein Molecules. *Mamm. Protein Metab.* **1969**, *3*, 21–132.