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ELEVATING THYME SPECIES IDENTIFICATION: EXPLOITING KEY CHLOROPLAST GENES (*matK*, *rbcL*, AND *psbA-trnH*) THROUGH DNA BARCODING AND PHYLOGENETIC ANALYSIS

Mehmet Alp FURAN[1]*

[1]*Van Yüzüncü Yıl University, Agriculture Faculty, Department of Agricultural Biotechnology, 65090, Van, Türkiye*

Abstract: Understanding genetic relationships and diversity among species is crucial for unraveling evolutionary processes, ecological interactions, and conservation strategies. DNA sequence analysis serves as a powerful tool in this endeavor. This study focuses on the Thymus genus, a collection of notable species, to investigate its genetic framework. Leveraging DNA sequences from key regions (*matK, rbcL*, and *psbA-trnH*), we aim to elucidate genetic connections within the Thymus genus and uncover mechanisms driving its diversity. The Thymus genus, with its diverse species and ecological characteristics, provides a captivating platform for genetic exploration. Through DNA sequence analysis, we aim to unveil genetic interconnections, biodiversity patterns, and the factors shaping the genus's evolution. Our findings are aligned with previous studies, and this consistency highlights the presence of polymorphism within potential sequences. Employing coding loci and spacer regions, our study contributes to Lamiaceae family barcoding research. Despite variations across gene regions, the concatenation of sequences enhances result reliability. We analyzed the suitability of *matK, rbcL*, and *psbA* sequences for Thymus identification, observing *rbcL* and *psbA* outperforming *matK*. Our novel approach, rooted in chloroplast DNA, presents a promising method for species discernment. By analyzing multiple chloroplast gene regions, this technique offers a fresh perspective on genetic affinity assessment using DNA barcodes. In conclusion, this study not only contributes to Thymus germplasm resource preservation but also exemplifies a novel approach to discerning Thymus species through DNA analysis. This methodology carries the potential for broader application, enriching our understanding of genetic relationships and diversity in the plant kingdom.

Keywords: Thymus, DNA barcoding, Choloroplast genes, Phylogenetic analysis

***Corresponding author:** Van Yüzüncü Yıl University, Agriculture Faculty, Department of Agricultural Biotechnology, 65090, Van, Türkiye
E mail: alpfuran@yyu.edu.tr (M. A. FURAN)
Mehmet Alp FURAN https://orcid.org/0000-0002-0171-0405

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1. Introduction

The genus Thymus has over 350 species of fragrant perennial herbaceous plants and subshrubs, reaching a height of up to 40 cm. Within the family Labiate, which contains approximately 220 genera, the genus Thymus is one of the eight most important genera in terms of species, although this number fluctuates depending on the taxonomic perspective. The genus Thymus yields a variety of commercial items, including as essential oils, oleoresins, fresh and dried herbs, and landscape plants. The genus Thymus encompasses approximately 350 species, although only five of them have gained significant economic significance, albeit for different reasons. These species include Thymus capitatus (L.), which has recently been reclassified as Thymbra capitata (L.) Cav. (Hoffmanns and Link) (https://en.wikipedia.org/wiki/Thymbra_capitata) and is commonly known as Spanish oregano or conehead thyme. Another economically important species is T. mastichina L., also known as Spanish marjoram or mastic thyme. Additionally, T. serpyllum L., commonly referred to as wild thyme or mother-of-thyme, T. vulgaris L., known as common thyme, and T. zygioides L., referred to as Spanish thyme, have also achieved economic significance (El Ouariachi et al., 2011; Cutillas et al., 2018 Radi et al., 2021; Rahimi et al., 2022; Tomanić et al., 2022). While the essential oils derived from these species are commonly traded goods, thyme oil is mostly extracted from T. zygioides, but both T. zygioides and T. vulgaris serve as the primary sources for the dried and fresh herb (Lawrence et al., 2002). These plants belong to the family Lamiaceae and are naturally found in temperate climates across Europe, North Africa, and Asia (Uritu et al., 2018). The genus Thymus occupies a prominent place within the esteemed Lamiaceae family, also known as the Mint family. This botanical ancestry includes a variety of plants that have made significant contributions to numerous human endeavors due to their aromatic foliage and diverse applications (Mosavat et al., 2019). Among these, the genus Thymus stands out as a charismatic protagonist with a legacy spanning culinary, medicinal, and ornamental uses. Thymus species have intricately woven their aromatic threads into the fabric of international cuisine, which is at the core of its

significance (Honorato et al., 2023). Thyme leaves have enriched culinary experiences across cultures and continents by bestowing dishes with a range of flavors, from robust to delicate (Nair, 2023). Thyme leaves leave an indelible impression on the palette, whether they are utilized as a vital component in hearty stews, a fragrant seasoning for roasted meats, or a nuanced accent in salads. The adaptability of Thymus species extends beyond the kitchen, demonstrating their seamless incorporation into human existence. These plants have also left their impact on the field of herbal medicine, as their potential has received global recognition. Essential oils containing antibacterial, antiviral, and antioxidant compounds, such as thymol and carvacrol, are extracted from the leaves and petals of Thymus (Ansarifar and Farid, 2022; Kim et al., 2022; Morshdy et al., 2022). These qualities make treatments infused with Thyme useful for treating respiratory, digestive, and immune disorders (Mohammadi et al., 2018; Nabissi et al., 2018; Salehi et al., 2018; Taher et al., 2021). Through diffusers, massage oils, and fragrant products, the aromatic oils contribute to a calming atmosphere in aromatherapy and holistic wellness. In addition to their culinary and medicinal uses, Thymus species serve as cultural symbols. Thyme's aromatic qualities have made their way into rituals, festivities, and celebrations due to its widespread veneration as a symbol of courage, strength, and memory. Whether they are dusted on food or infused into oils, they impart both color and flavor. When used as groundcovers in landscapes, these plants also contribute to sustainable gardening by preventing soil erosion and enticing beneficial insects. Notably, Thyme's influence extends beyond conventional domains to include beekeeping. Thymus species generate nectar, which beekeepers use to produce aromatic thyme honey, which is prized for its flavor and potential health benefits (Alissandrakis et al., 2007; Rodríguez et al., 2021). This multifaceted (culinary, medicinal, cultural, and ecological) approach highlights the intrinsic value of Thymus species. In spite of their significance, however, accurate species identification and germplasm preservation present formidable obstacles. Given the intricacy of the Thymus genus, it can be difficult to identify specific species. Innovative DNA-based techniques, such as DNA barcoding, can provide accurate species identification, even among cryptic or closely related forms, to address this issue (Furan, 2023). To preserve the genetic diversity of Thymus species for future generations, germplasm preservation is of the utmost importance. Detailed genetic analyses disclose species-specific genetic markers, thereby facilitating reintroduction or cultivation efforts and aiding in the prevention of extinction (Joshi et al., 2004; Singh, 2020). In addition, DNA testing is essential for authenticating botanical items, ensuring the quality and authenticity of herbal, cosmetic, and aromatic products (Trindade, 2010; Polaiah et al., 2023). This verification procedure fosters confidence and reduces misrepresentation among regulatory bodies, industries, and consumers. In the field of plant genetics, Thymus species are examined for enhanced characteristics such as flavor, aroma, and disease resistance. Researchers can utilize genetic markers to accelerate breeding programs, potentially resulting in novel cultivars that contribute to biodiversity and human welfare. A comprehensive comprehension of the ecological roles and characteristics of Thymus species facilitates sustainable harvesting, cultivation, and management practices, thereby securing the future of these resources. The existence of a universal barcode for animals has been shown; however, its use as a universal barcode for plants is limited due to its lower nucleotide replacement rate (Johnson et al., 2023). In order to produce DNA barcodes for plant species, researchers have investigated the use of plastid DNA sequences as a viable alternative. The utilization of DNA barcodes for the identification of plant specimens has been proposed as an effective, reliable, and uncomplicated pharmacognostic approach to address challenges associated with morphological identification ambiguity (Nongbet and Chrungoo, 2023). This study examines the genetic diversity of the Thymus genus using three essential chloroplast genes: *matK, rbcL*, and *psbA-trnH*. Each gene provides a distinct perspective, with *matK*'s adaptability to species-specific signals, *rbcL*'s stability providing a foundation for phylogenetic studies, and *psbA-trnH*'s dynamic variations facilitating differentiation. The integration of these genes produces a symphony of genetic information that resonates with species differentiation and sheds light on the intricate genetic tapestry of the Thymus genus. The overarching goal of this study is to improve species identification techniques and germplasm conservation procedures within the genus Thymus. Over the past few decades, a considerable number of researchers have made noteworthy advancements in the exploration and utilization of barcodes in plants. Additionally, scientists have carried out numerous phylogenetic analyses on medicinal and aromatic plants from diverse families or subfamilies. These studies have been conducted by various researchers, including Michel et al. (2016), Wang et al. (2017), Yu et al. (2021), Chinnkar and Jadhav (2023) Abdulrahman et al. (2023), and Jiang et al. (2023). We investigate the complex genetic markers of Thymus species and subspecies by employing DNA barcoding and phylogenetic analysis. Our findings reverberate throughout scientific research and practical applications, illuminating the central function of genetic exploration within the genus Thymus. This study advances our understanding of genetic diversity and provides tangible benefits by improving the accuracy of species identification using DNA-based methods. These findings not only empower regulators to uphold quality standards, but also enhance consumer confidence and the credibility of the industry. The genetic markers identified in our selected genes serve as the basis for personalized germplasm conservation strategies,

providing resource managers and conservationists with tools to protect the genetic diversity of Thymus species and subspecies. This effort contributes to the preservation of natural ecosystems and the establishment of plant populations able to adapt to fluctuating environmental conditions. Beyond individual sequences, our study reverberates as a genetic symphony that reflects the nuanced beauty and intricate complexity of the Thymus genus. DNA research of Thymus genus plant species' genetic diversity and linkages provides vital information. The study can add to the literature: Identification and Authentication; Thymus genus plants are identical in appearance, making identification and verification difficult. This study may help identify and verify plants by providing Thymus species DNA markers. The findings may help comprehend Thymus's genetic diversity and establish conservation methods. Thymus plants are utilized in food, medicine, and cosmetics. This work may help the business and consumers employ DNA-based herbal product quality control methods. This discovery may help explain plant evolution and genetic variety. The study may help plant barcoding researchers find genomic areas that can identify Thymus species. This work will contribute to practical applications, scientific research, and conservation efforts for genetic diversity and verification of the Thymus genus plant. This can help plant scientists, botanists, conservationists, and industry representatives.

2. Materials and Methods

2.1. Nucleotide Sequences

In this investigation, data pertaining to gene loci as well as an intergenic spacer region were consolidated by utilizing information provided from the 2023 database. The researchers employed the NCBI nucleotide database to gather Thymus species belonging to the Lamiaceae family. The selection of plastid barcode locus genes (*matK, rbcL*) and one intergenic spacer (*psbA-trnH*) was based on their high suitability for this purpose. Furthermore, the MEGA11 software program was utilized to acquire concatenated sequences of *matK + psbA* and *rbcL + psbA*. Following a process of manual screening, nucleotide sequences of short length were removed, leaving behind a subset of sequences that underwent manual editing and trimming to guarantee proper alignment.

2.2. Sequence Alignment and Data Analysis

The main aim of this study was to investigate the species belonging to the Thymus genus within the Lamiaceae family. These species are frequently misidentified as a result of their same physical characteristics. The plastid DNA reference sequences *matK*, *rbcL*, and *psbA-trnH* were obtained from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) in order to facilitate species identification. The sequencing data for the *matK*, *rbcL*, and *psbA-trnH* loci of each species were carefully examined and organized based on their gene regions in order to ease comparisons. Additional examination was conducted on the sequencing data, with the utilization of MEGA11 software to ascertain the length of the aligned sequence, the various counts of nucleotides, the overall count of parsimony informative sites, and the mean GC content (Tamura et al., 2021). The study employed a pairwise genetic distance technique and phylogenetic tree-based analysis to assess the discriminatory power of the three selected barcode loci in differentiating across species. The process of Bayesian inference (BI) was carried out utilizing the MEGA11 model option. The T92 (Tamura 3 parameter) substitution model, which demonstrated the most optimal fit, was chosen for subsequent investigation. The Tamura 3-parameter distance model (T92) in MEGA 11 was employed in the pairwise genetic distance approach to compute both interspecific and intraspecific nucleotide divergence. The software program DNA Sequence Polymorphism version 5 (DNA SP v5) was employed to compute the number of polymorphic sites, genetic diversity indices, and neutrality tests, namely Fu's Fs49 and Tajima's D50 (Sardar et al., 2023; Debrah et al., 2023). Distinct evolutionary pressures can cause various parts of DNA or amino acid sequences to alter, leading to the frequent utilization of the entire deletion option. In this work, the full deletion option was employed to exclude sites with gaps and missing data from the analysis of pairwise distances. Following the alignment and modification of all sequencing sites, a neighbor-joining (NJ) analysis was conducted using MEGA 11 software. The p-distance model was employed as the tree-based phylogenetic technique for this research. The evaluation of relationships involved the utilization of 1,000 bootstrap replicates. For each unique sequence site, a neighbor-joining tree was formed using the sequences from the three loci. Subsequently, the relationships were assessed.

3. Results

3.1. DNA Sequence Analysis

This study involved the analysis of DNA sequences from several notable species of the Thymus genus. The *matK* sequences of eight species, the *rbcL* sequences of eight species, and the *psbA-trnH* sequences of the same species were obtained from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/). The combined sequences of the regions under investigation were collectively assessed. Following the process of trimming and editing, the determined lengths of *matK, rbcL*, and *psbA-trnH* were found to be 6.261 bp, 4.549 bp, and 3.032 bp, respectively. Additionally, the combined lengths of the concatenated sequences *matK+psbA* and *rbcL+psbA* were calculated to be 9.293 bp and 7.581 bp, respectively. Table 1 presents the overall mean frequencies of nucleotide bases, the nucleotide sequences, and the distribution of the four bases in potential nucleotide sequences identified across various codon coding sites. Table 2 presents the average number of identical pairs (ii) observed in possible nucleotide

sequences. Table 2 provides a comprehensive account of the transitional (si) and transversional (sv) pairings of nucleotide sequences. A potential correlation could exist between the occurrence of transitional and transversional bases within genetic sequences and the observed variations among different species. Table 3 presents an examination of potential polymorphic locations within the nucleotide sequence. The *rbcL* sequence has the lowest number of mutation sites (1.7%) among all the individual and combined sequences. Conversely, the *matK* sequence demonstrates the highest rate of conservative sites (99.1%). The *psbA* sequence has a higher proportion of mutation sites (50.4%) and a lower proportion of conservative sites (38.3%) in comparison to the *psbA* concatenated sequences it aligns with.

Table 1. The nucleotide base frequencies analysis of candidate nucleotide sequences in *Thymus* species.

Sequences	Base content										
	A	T	C	G	GC	AT-1	GC-1	AT-2	GC-2	AT-3	GC-3
matK	29.1	36.0	18.3	16.6	34.9	66.9	32.9	67.8	32.2	60.7	39.3
rbcL	27.3	28.8	20.9	22.9	43.8	43.6	56.4	55.6	44.4	69.2	30.8

Table 2. The analysis of nucleotide pair frequencies of candidate nucleotide sequences of *Thymus* species. ii Identical Pairs, si Transitionsal Pairs, sv Transversional Pairs, R si/sv.

Sequence	ii				si				sv				R			
	Avg	1st	2nd	3rd	Avg	1st	2nd	3rd	Avg	1st	2nd	3rd	Avg	1st	2nd	3rd
matK	718	240	238	240	5	1	2	1	2	1	1	0	1.9	1.3	2.0	2.9
rbcL	532	178	178	176	2	0	0	2	1	0	0	0	2.5	0.0	1.4	12.5
matK+psbA	955	317	318	320	40	12	14	15	44	16	16	12	0.9	0.8	0.9	1.2
rbcL+psbA	776	260	261	256	44	12	14	17	47	17	16	14	0.9	0.7	0.9	1.2

Table 3. The analysis of variation of candidate barcode sequences in *Thymus* species.

Sequence	Conserved site	Variable site	Parsimony-informative site	Sigon site
matK	*846 (94.6%)*	*28 (3.1%)*	*3*	*25*
rbcL	615 (89.9%)	12 (1.7%)	2	10
psbA	193 (38.3%)	254 (50.4%)	117	125
matK + psbA	1059 (74.3%)	278 (19.5%)	92	177
rbcL + *psbA*	753 (65.3%)	295 (25.5%)	111	165

3.2. Genetic Diversity

The presence of genetic variation based on species differences is inferred due to the utilization of data derived from many species within the same genus for study. The genetic diversity indices presented in Table 4 are derived via calculations involving pairwise nucleotide differences and nucleotide diversity. The validity of these indices was verified through the application of Tajima's neutrality test. According to the cumulative Eta value, the *matK+psbA* sequences exhibit the highest level of genetic variety, characterized by a collective count of 178 mutations. When comparing the rbcL sequences, it is observed that there are only six distinct mutation changes in total. Both neutrality tests yielded results indicating that there were substantial differences across all sequences. However, only a limited number of sequences exhibited significant differences based on the probability value (P-value) obtained from Fu's Fs test and Tajima's D test (Table 4). This finding highlights the importance of genetic variety. Comparable to the Tajima test statistic, namely the D value, which is utilized to assess neutrality in genetic sequences, the genetic variation observed in *rbcL* sequences exhibits a modest increase, measuring at -0.63262. Nevertheless, the *matK* region exhibits a limited number of polymorphisms within the designated regions, which might be attributed to its high conservative site percentage of 94.6% (Table 3). In the case of combined sequences, the *rbcL* sequences exhibited a higher degree of genetic variation (-063262) compared to the *matK* sequence, which had values reaching up to -1.65200. Furthermore, the observed difference in genetic variation between the two sequences was found to be statistically insignificant, as indicated by the P value. The *rbcL* sequences demonstrate the highest Fu's Fs value for sequence variation (0.01238), as indicated in Table 4. In comparison to the other three region sequences, the *rbcL* sequence is the only one that exhibits a distinct value (0.01238).

3.3. Phylogenetic Analysis

In this study, we conducted an analysis to determine the evolutionary relationships across species by utilizing the *matK, rbcL, psbA-trnH*, and concatenated sequences. The MEGA 11 software was employed, employing the neighbor-joining method and the T92 (Tamura 3 parameter) model. In light of the topological

arrangement of the evolutionary tree, the genes *matK, rbcL*, and *psbA* present potential for investigation, although their utility in species identification within discrete subfamilies may be limited. Hence, the concatenated sequences of gene loci, namely *matK+psbA*, and *rbcL+psbA*, were assessed in order to determine their overall efficacy for identification purposes. The phylogenetic outcomes obtained from both unique gene sections and an intergenic spacer exhibited a clearly visible divergence. Phylogenetic classifications based on concatenated data have also observed similar occurrences, wherein the findings from both two essential gene regions and concatenated data, as well as intergenic spacer data, are presented in Figures 1, 2, 3, 4, and 5.

Table 4. Genetic diversity caculation of *Thymus* species based on candidate barcode sequences by the DnaSP v5 software. *Eta* Total number of mutations, *n* number of sequences, *k* Average number of nucleotide difference, *S* Number of segregating sites, θ nucleotide substitution rate, π nucleotide diversity, *Hd* haplotype diversity, *Fu's Fs* is variance of haplotypes diversity, *D* is the Tajima test statistic.

Sequences	n	Nucleotide diversity					π	Neutrality tests			
		S	k	Eta	Hd	θ		Fu's *Fs*	P-value	D	P-value
matK	8	21	5.536	21	1.000	0.014061	0.00961	0.00391	> 0.10	-1.65200	>0.05
rbcL	8	8	2.000	6	0.893	0.004485	0.00388	0.01238	>0.10	-0.63262	>0.10
matK + psbA	8	146	57.071	178	1.000	0.073223	0.07422	0.00391	> 0.10	- 0.92259	>0.10
rbcL + psbA	8	128	53.571	165	1.000	0.070624	0.0764	0.00391	> 0.10	-0.86471	>0.10

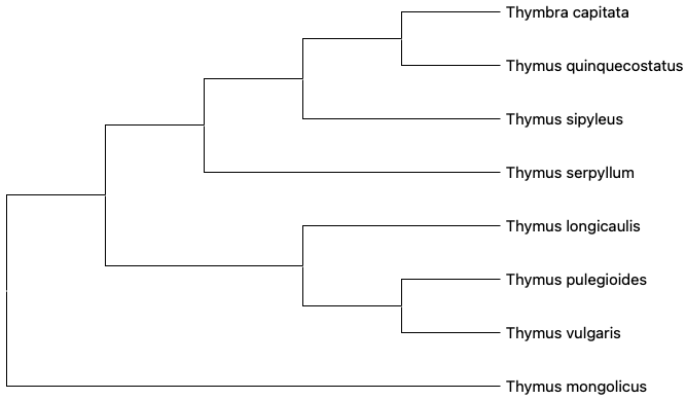

Figure 1. Thymus species NJ tree is derived from a study of the cpDNA matK sequence using the Tamura 3 model. The branches represent the 1000 bootstrap replications test.

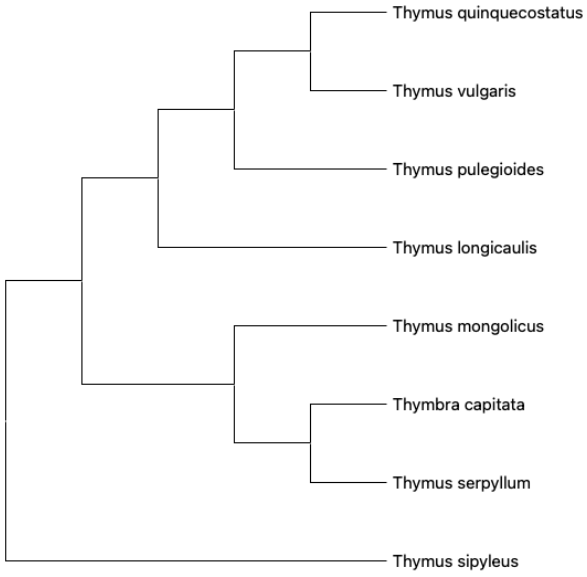

Figure 2. Thymus species NJ tree is derived from a study of the cpDNA rbcL sequence using the Tamura 3 model. The branches represent the 1000 bootstrap replications test.

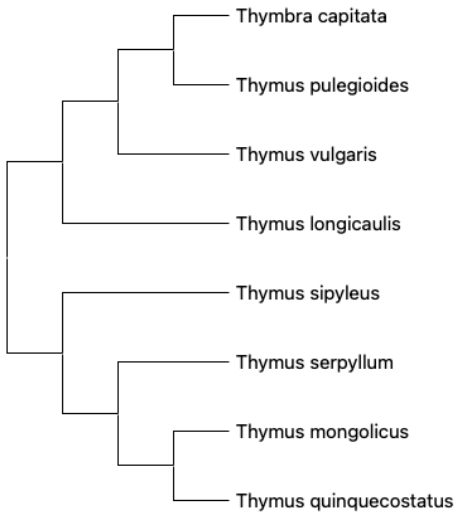

Figure 3. Thymus species NJ tree is derived from a study of the cpDNA psbA sequence using the Tamura 3 model. The branches represent the 1000 bootstrap replications test.

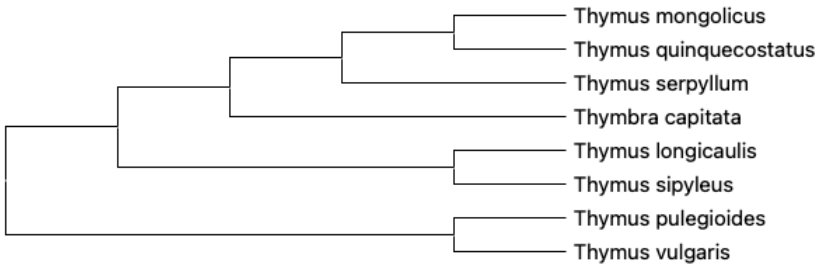

Figure 4. Thymus species NJ tree is derived from a study of the concatenated cpDNA matK+psbA sequence using the Tamura 3 model. The branches represent the 1000 bootstrap replications test.

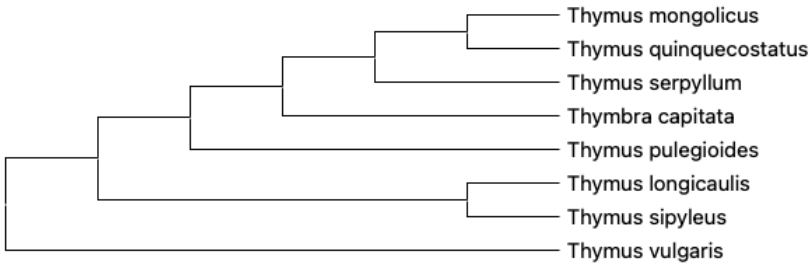

Figure 5. Thymus species NJ tree is derived from a study of the concatenated cpDNA rbcL+psbA sequence using the Tamura 3 model. The branches represent the 1000 bootstrap replications test.

3.4. Analysis of Barcoding Gap

According to Li et al. (2021), it is necessary for the interspecific genetic variance to exceed the intraspecific genetic variation in order to establish a suitable DNA barcoding sequence for the purpose of species identification. In order to accurately assess the individual chloroplast genes and combined sequences in thymus species and validate the suitability of potential sequences, an examination of the barcoding gap was conducted using frequency distribution. Among the three barcoding loci utilized, it is shown that the *matK* locus exhibits the longest aligned sequence length, measuring 6.261 base pairs (bp). Subsequently, the *rbcL* locus demonstrates a length of 4.549 bp, while the *psbA-trnH* locus possesses a length of 3.032 bp. The gene *psbA* exhibits the greatest number of parsimony-informative sites, with a count of 117, depending on the specific dataset. Following *psbA, matK* demonstrates 3 parsimony-informative sites, while *rbcL* exhibits 2. Within the genus Thymus, the *psbA-trnH* region exhibits the greatest proportion of variant sites (50.4%), with *matK* (3.1%) and *rbcL* (1.7%) following suit. The computation of Tamura's 3-parameter model-based genetic distances between intra and interspecific species was performed using MEGA 11. Within the Thymus species, the *matK+psbA* gene combination exhibits the highest level of intraspecific divergence, with a distance of 0.10%. Conversely, the rbcL gene does not exhibit any variation at the intraspecific level, as indicated in Table 5. The interspecific distance among thymus species is found to be highest in the combined sequence *rbcL+psbA* (0.13%) region. However, it is worth noting that the *rbcL* region does not exhibit any significant variations in interspecific distance levels.

Table 5. Summary of the pairwise intraspecific and interspecific distances in the barcode loci of *Thymus* species

Barcode locus	Intraspecific distances (%)			Interspecific distances (%)	
	Minimum	Maximum	Overall Mean	Minimum	Maximum
matK	0	0.01	0.01	0	0.01
rbcL	0	0	0.004	0	0
matK+psbA	0.01	0.10	0.08	0.02	0.08
rbcL+psbA	0	0.09	0.08	0.1	0.13

4. Discussion

The examination of genetic links and diversity among species has significant importance in comprehending evolutionary processes, ecological interactions, and conservation endeavors. The examination of DNA sequences is a valuable technique for elucidating these complexities (Coissac et al., 2016; Amiteye, 2021). The Lamiaceae family has a total of roughly 7173 species that are geographically dispersed over the Mediterranean basin and Central Asia. The aforementioned botanical group plays a significant role as a primary supplier of fragrant oils and secondary metabolites. In order to ensure the safe utilization of medicinal plants belonging to the Lamiaceae family, it is necessary to possess a reliable methodology for verifying the authenticity of species (Thakur et al., 2021). This study aims to investigate the Thymus genus, which has a collection of noteworthy species, in order to examine the genetic framework that influences their existence. Our objective is to utilize the stored information within the DNA sequences to elucidate the genetic links within the Thymus genus and reveal the fundamental mechanisms that contribute to their variety. The examination of DNA sequences has significantly transformed our capacity to examine the genetic foundations of living organisms. By analyzing the sequence of nucleotides in DNA, valuable information may be obtained on the molecular patterns that determine the traits and attributes of living creatures (Li et al., 2021). This methodology enables the investigation of the common lineage and evolutionary trajectories of organisms, while also providing insights into the underlying processes accountable for their unique characteristics and adaptations (Techen et al., 2014; Michel et al., 2016; Raskoti and Ale, 2021; Chen et al., 2023; Hua, et al., 2023). The Thymus genus, which comprises a variety of species exhibiting a wide array of ecological and physical characteristics, is an intriguing platform for genetic investigation. Thyme, a widely recognized fragrant herb, possesses not only culinary value but also provides unique insights into the development of plants (Aneva et al.,2022; Halat et al., 2022; Kim et al., 2022). Through the analysis of DNA sequences from many species within this genus, our objective is to elucidate the underlying connections that unite these plants and differentiate them from other related organisms. This study represents a notable contribution to the field of barcoding research in the Lamiaceae family, since it encompasses a substantial sample size and employs various coding loci (*matK, rbcL*, and *psbA*) in combination. The primary focus of this investigation is directed towards species belonging to the Thymus genus. The results obtained from conducting sequence analysis on the average GC content indicate that the GC content of Thymus candidate sequences is notably lower than AT content and significantly lower than the GC content observed in approximately 50% of common angiosperms. The obtained results are consistent with the findings reported in Li et al. (2021)'s investigation on orchids and the outcomes of Furan's research conducted in 2023 on Origanum species. All of the regions that were looked at, including the gene regions, the spacer regions, and the data from all of them together, show a greater variety of haploid types and a relatively low variety of nucleotides. This suggests that potential sequences have polymorphism. DNA barcoding is a widely employed technique for species identification, which involves the sequencing of a specific and standardized section of DNA. Nevertheless, an agreement has not yet been reached about the appropriate locations to be utilized for the purpose of barcoding terrestrial plant species. According to Hollingworth et al. (2009), the Consortium for the Barcode of Life (CBOL) advocates for the use of *matK* and *rbcL* as universally accepted barcodes for the plant kingdom. This study utilizes DNA sequences obtained from certain areas of the genome, including *matK*, *rbcL*, and *psbA-trnH*, as a means to examine the Thymus genus. These areas, renowned for their informative characteristics, enable us to get insights into the genetic variants that have accrued over a span of time. Through the process of sequence analysis, our objective is to elucidate the genetic interconnections between various species, unveil intricate patterns of biodiversity, and acquire a more profound comprehension of the factors that have influenced the evolutionary trajectory of the Thymus genus. The results obtained from the analysis of the *matK* gene region were found to be distinct from those obtained from the analysis of the *rbcL* gene region, as shown by phylogenetic analyses. Nevertheless, the findings derived from the *psbA* intergenic region corroborated the outcomes of both gene sections and exhibited a higher degree of concordance with *rbcL*. The utilization of concatenated data in phylogenetic analysis has contributed to the enhancement of result dependability. Upon examination of the phylogenetic perspective derived from the aforementioned data, it was noticed that the outcomes acquired from the *matK+psbA* analysis closely aligned with those obtained from the *rbcL* gene

region analysis. The proposition that the discriminatory capacity of the *matK* region is comparatively limited in thymus species aligns with the findings of Furan (2023)'s investigation on origanum species. The MEGA software does not include gap sites in the MP analysis. However, there are three distinct approaches to handling these gap sites. One approach entails the entire exclusion of some websites from the study of data. The "complete deletion" option is frequently used in light of the diverse evolutionary pressures exerted on distinct segments of DNA or amino acid sequences. Nevertheless, in cases when the impact of a gap on the number of nucleotides (or amino acids) is minimal and the gaps are distributed in a very haphazard manner, it is plausible to consider including these locations as instances of missing data. As a result, the presence of gaps and missing data is not considered when calculating the length of a tree. Partial deletion, as a third alternative, involves the removal of gaps that constitute a proportion of the data below a predetermined threshold (unambiguous). The focus of the study is to differentiate species within a certain genus. The data collected from different areas vary in terms of the number of individuals used for species discrimination within the genus. Consequently, each gene region plays a role in the development of separate branches. Coding areas are predominantly utilized in phylogenetic barcoding investigations due to their possession of the requisite genetic information for protein synthesis and their presumed stability and functional significance. The identification of nucleotide sequences inside these specific locations can serve as a valuable tool in elucidating genetic variances among species and facilitating the determination of phylogenetic connections. Noncoding regions, also known as non-coding DNA segments, are sections of DNA that do not possess the ability to code for proteins. The nucleotide sequences inside these areas have the potential to undergo fast alterations, hence providing significant phylogenetic resolution in certain cases. However, noncoding areas possess significant limitations. For example, rapid evolution across many groups of organisms might provide difficulties in achieving consistent outcomes in phylogenetic analysis. Furthermore, the process of resolving and aligning sequences in these locations may be a complex challenge. Therefore, coding areas are commonly selected as the best option for conducting phylogenetic barcoding studies. However, depending on the particular group of organisms and the aims of the research, it may also be necessary to include noncoding areas. The *rbcL* gene is responsible for encoding the large subunit of Rubisco, a pivotal enzyme involved in the process of photosynthesis in plants. The *rbcL* gene is present in the DNA of chloroplasts and serves as a valuable tool for investigating evolutionary connections across plant species. The *rbcL* gene is a gene region that has a high degree of conservation across plant species and is extensively employed in phylogenetic investigations. This study utilized variable site data between sequences to evaluate the suitability of *rbcL*, *psbA*, and *matK* sequences for identifying the Thymus genus, species, and subspecies. The findings indicate that the *rbcL* and *psbA* sequences outperformed the *matK* sequences in this regard. Furthermore, combining the sequences may offer an effective means of distinguishing Thymus species and subspecies, provided that the objectives of the study and the specific species under investigation are taken into consideration. The *matK* sequence area is commonly employed in combination with other variables to classify plant species (Wang et al., 2017). Additionally, *matK* alone has been identified as a potential barcode for several plant species (Hollingsworth, 2011; Abdulrahman et al., 2023). Based on the findings of this study, it is recommended to employ a combination of several areas and their concatenated data. The investigation revealed that the *rbcL* sequence had the lowest number of mutation sites. Nevertheless, despite the relatively low variability and resolution, it is necessary to acknowledge the significance of the conservative nature of the *rbcL* data for Thymus species and the consequent reliability of the obtained results in the context of this investigation. The *psbA* sequence exhibited the highest proportion of mutation sites and the lowest proportion of conserved sites in comparison to the other sequences. The utilization of species-specific DNA barcodes of Thymus was effectively employed to discriminate between different species based on single nucleotide polymorphism (SNP) sites. The concatenation of sequences exhibited a higher level of distinction at the species level compared to chloroplast genes. This disparity can perhaps be attributed to the fact that concatenated sequences offer a greater number of mutation sites and single nucleotide polymorphism (SNP) sites. Variations in the specificities of gene combinations were observed among Thymus plants. When comparing the effectiveness of *matK, rbcL*, and *psbA* as genetic markers, it has been shown that the concatenation of two sequences (*matK + psbA, rbcL + psbA*) provides distinct and specific distinguishing characteristics for Thymus species. In summary, our research introduces a unique approach to accurately and effectively discerning Thymus species by using chloroplast DNA. This approach serves as a complementary way to the current procedures employed in the identification of thymus plants. This work aims to investigate DNA-level variations by employing several chloroplast gene areas and analyzing their concatenated data. This methodology has the potential to serve as a novel method for assessing the genetic affinities across Thymus species through the utilization of DNA barcodes. Consequently, it establishes a foundation for the preservation, assessment, inventive use, and safeguarding of Thymus germplasm resources.

4. Conclusion

In the realm of biological exploration, understanding genetic relationships and diversity among species holds paramount importance, offering insights into evolutionary mechanisms, ecological dynamics, and conservation strategies. DNA sequence analysis stands as a powerful tool to unravel these intricate phenomena (Coissac et al., 2016; Amiteye, 2021). Within the vast Lamiaceae family, with its distribution spanning the Mediterranean basin and Central Asia, a mosaic of approximately 7173 species thrives. These botanical entities not only play a pivotal role in providing fragrant oils and secondary metabolites but also hold implications for medicinal applications within a framework of authenticity verification (Thakur et al., 2021). Our study delves into the Thymus genus, a collection of noteworthy species, aiming to unravel the genetic underpinnings shaping their existence. Leveraging the genetic information encoded in DNA sequences, we've embarked on a journey to illuminate the genetic connections within the Thymus genus and unearth the fundamental mechanisms underpinning its diversity. DNA sequence analysis has revolutionized our ability to dissect genetic foundations, shedding light on molecular blueprints that define the intricate traits of living beings (Li et al., 2021). This technique allows us to decipher common lineages, evolutionary trajectories, and distinctive adaptations that characterize each species (Techen et al., 2014; Michel et al., 2016; Raskoti and Ale, 2021; Chen et al., 2023; Hua et al., 2023). The Thymus genus, encompassing a spectrum of species with diverse ecological and physical attributes, emerges as an intriguing canvas for genetic exploration. Thyme, a fragrant herb with both culinary and scientific significance, provides unique insights into plant development (Aneva et al., 2022; Hamoudi et al., 2022; Kim et al., 2022). By unraveling DNA sequences across various species within this genus, our quest is to unravel the threads that connect these plants while distinguishing them from their botanical peers. Our study is a big step forward in barcoding research for the Lamiaceae family. It has a large sample size and a mix of coding loci (*matK, rbcL*, and *psbA*). This exploration concentrates its lens on the Thymus genus, as the results of average GC content analysis highlight a distinctive pattern. The GC content of Thymus candidate sequences stands in contrast to AT content and deviates markedly from the GC content observed in approximately 50% of common angiosperms. Our findings resonate with Guo et al.'s orchid investigation and Furan's study on Origanum species conducted in 2023, aligning across gene and spacer regions. These findings collectively underscore the presence of polymorphism within potential sequences. DNA barcoding, a widely adopted technique for species identification, involves sequencing specific standardized DNA sections. Nonetheless, consensus on optimal barcode regions for terrestrial plant species remains elusive. According to Hollingworth et al. (2009), the Consortium for the Barcode of Life (CBOL) advocates for *matK* and *rbcL* as universally accepted barcodes. Our study employs sequences from *matK, rbcL*, and *psbA-trnH* regions to probe the Thymus genus. These regions, recognized for their informativeness, facilitate a journey into the genetic diversity accumulated over time. Through this sequence analysis, our endeavor is to unveil genetic interconnections among species, decipher intricate biodiversity patterns, and gain a profound understanding of the forces steering the evolutionary trajectory of the Thymus genus. Phylogenetic analyses demonstrate distinctions between *rbcL* and *matK* gene regions, while *psbA*'s intergenic region reinforces both gene outcomes and aligns more closely with *rbcL*. In our pursuit of understanding genetic affinities, the concatenation of sequences emerges as a means to enhance the reliability of results. The *matK+psbA* analysis's congruence with *rbcL* outcomes parallels Furan (2023)'s findings, suggesting limited discriminatory capacity in Thymus species. Although the MEGA software doesn't incorporate gap sites in the MP analysis, strategies for handling gaps complete deletion, partial deletion, and inclusion as missing data augment data interpretation. Coding regions get a lot of attention in phylogenetic barcoding because of how stable and important they are for function. This helps find genetic differences between species and show their phylogenetic connections. While noncoding regions offer rapid alteration potential and resolution, their variability across organism groups poses challenges in maintaining analytical consistency. The *rbcL* gene, pivotal in photosynthesis, provides evolutionary insights across plant species. Our investigation employs variable site data to evaluate *rbcL*, *psbA*, and *matK* for thymus identification. Results indicate the potential of *rbcL* and *psbA* in species differentiation. As we conclude, our research introduces a novel approach to distinguishing Thymus species through chloroplast DNA, enriching the palette of plant identification methods. This method, based on a composite of chloroplast gene areas, has the potential to revolutionize genetic affinity assessment across Thymus species using DNA barcodes. In sum, this study forms a solid foundation for the preservation, assessment, and innovative utilization of Thymus germplasm resources, ensuring both the safeguarding of botanical diversity and informed conservation efforts.

Author Contributions

The percentage of the author contributions is presented below. The author reviewed and approved the final version of the manuscript.

	M.A.F.
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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