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BUDAPEST

Assessing the genetic diversity of basil (*Ocimum* genus) cultivars with Chloroplast markers

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USED ABREVIATIONS

AFLP: Amplified Fragment Length Polymorphism

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

Indel: Insertion or deletion mutations

ISSR: Inter-Simple Sequence Repeat

LB: Lysogeny broth media

PCR: Polymerase Chain Reaction

QTL: Quantitative Trait Locus

RAPD: Random Amplified Polymorphic DNA

SCoT: Start Codon Targeted

SNP: Single Nucleotide Polymorphism

TBE: Tris base, borate and EDTA

µL: Microliter

DEDICATION

To God for being so great and sweet, and for blessing me throughout every step that led me to the obtention of this diploma.

To my parents, Magus and Jorge, for giving me their support and infinite care, for teaching me to appreciate nature, to be curious and eager to learn, for teaching me that effort always has its reward, for being my strength and the best example to follow.

To my siblings, niece, and nephews, for every laugh, for their pure love, for being supportive in every step of my studies.

To Rami, for holding my hand and making me feel home when I need it the most.

To my friends who made this journey fun and full of unforgettable memories in beautiful Budapest.

1. INTRODUCTION

With more than 150 species at present, the genus *Ocimum* is one of the most well-known genera in the Lamiaceae family. Basil is believed to have originated in India. The distribution is primarily in tropical and template climates. It has been grown all over the world as culinary herbs and for the extraction of essential oils (Tangpao *et al.*, 2022). *Ocimum* species poss a high number of pharmacological activities like antimicrobial, immunomodulatory, anti-inflammatory, hepatoprotective, chemoprotective, antihyperlipidemic, cardioprotective, antioxidant, antihypertensive, and anticoagulant activities (Mahajan *et al.*, 2013; Uritu *et al.*, 2018).

Morphological markers including leaf and color, flower, and seed morphology, have been used to distinguish between varieties and within a genus for taxonomical purposes. Unfortunately, extended cultivation and inter- and intra-specific cross hybridization produce numerous polymorphisms that lead to a wide variety of subspecies, each with its own chemistry and biological activity but very similar morphology leading to confusion when identifying cultivars and even species (Kumar *et al.*, 2016a).

Molecular markers are a very popular technique for a wide range of applications, including genome mapping, gene tagging, genetic, phylogenetic analysis, and forensic investigations. This is because of their stability, cost-effectiveness, and convenience of use. Numerous molecular marker approaches have been created and used in numerous systems throughout the world during the past three decades (Grover and Sharma, 2014).

The chloroplast is a type of plastid that is inherited maternally in angiosperm plants. It is an essential component of several metabolic processes, including the metabolism of starch, fatty acids, nitrogen, amino acids, and internal redox signals, because it is where photosynthesis takes place (Fu *et al.*, 2022). The molecule best suited to be used in resolving phylogenetic relationships, especially at higher levels of evolution, is chloroplast DNA (cpDNA), which is the smallest in comparison to the mitochondrian and nuclear genome and is assumed to have undergone very little rearrangement during evolution (Zurawski and Clegg, 2003).

1.1. Aims of the study

The goal of this study on basil cultivars was to assess genetic diversity by using four chloroplast non coding regions, and its potential use for species identification and determining the phylogenetic relationship among these cultivars.

2. LITERATURE REVIEW

2.1. Ocimum basilicum

2.1.1 Taxonomy and identification issues

Basil is frequently referred to as the "king of the herbs" due to its widespread use. It is an herbaceous plant with erect growth habit and branching stems. The stem is aromatic, 4-sided and hairless or slightly hairy. The flowers are white and small (1 cm long), composed of an upper and lower lip, the lower lip being slightly larger (See Figure 1.).

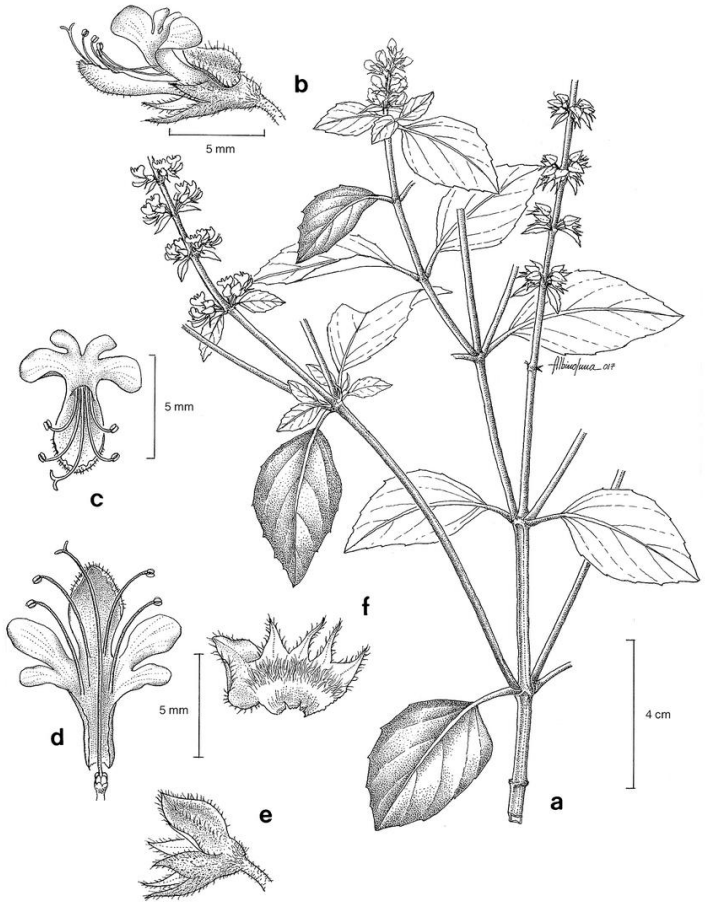

Figure 1. *Ocimum basilicum* -a. Branch with leaves and inflorescences. -b. Flower. -c. Corolla and stamens detail. -d. Detail of corolla, stamens, and gynoecium. -and. Calyx. -F. Detail of the tube and calyx lobes (Martínez Gordillo *et al.*, 2019).

Although basil has many other names and associations, the popular term is most likely derived from the Greek words basileus, which mean "king," or basilikon, which mean "royal." Basilisk, a fabled fire-breathing monster that is so disgusting that a single glance might kill, is described by the Latin term basiliscus. Basil was the remedy to the poison of the basilisk, according to a Roman tale (Makri and Kintzios, 2008a). *Ocimum basilicum* belongs to the Lamiaceae family (See Table 1).

Table 1. Classification for Kingdom Plantae Down to Species *Ocimum basilicum* (US Department of Agriculture, 2023)

Rank	Scientific Name and Common Name
Kingdom	Plantae - Plants
Subkingdom	Tracheobionta - Vascular plants
Superdivision	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Magnoliopsida - Dicotyledons
Subclass	Asteridae
Order	Lamiales
Family	Lamiaceae Martinov - Mint family
Genus	*Ocimum* L. - basil
Species	*Ocimum basilicum* L. - sweet basil

The medicinal and cosmetic qualities of the Lamiaceae family have helped it become more well-known. More than 6900 species belonging to this family are thought to exist in 200 genera around the globe (Mohammadhosseini, 2017).

Ocimum is a very challenging genus because of its extensive genetic diversity and wide range of phenotypic variations (Yaldiz and Camlica, 2021). Despite not being varieties of *Ocimum basilicum*, certain species of the same genus that are similar to each other are referred to as "basil": camphor basil, african basil (*O. kilimandscharicum*) (Ayoubi, Wali and Singh, 2022); clove basil, also African basil (*Ocimum gratissimum*); holy basil (*Ocimum tenuiflorum*, formerly known as *O. sanctum*).

The various chemotypes and cultivars of basil can result from the ease of genetic modification and cross-breeding. This allows for the development of new and different types of plants that have different essential oil compositions (Yaldiz *et al.*, 2023).

Ocimum kilimandscharicum is an aromatic herb native to Central and East African countries. It can grow both on the plains and on the hills (See Figure 2.). Due to its distribution and abundance in African countries, *O. kilimandscharicum* is often called African blue basil. The plant has attracted attention as an exotic plant with its high camphor content (Charles and Simon, 2011).

Figure 2. *O. kilimandscharicum* (Saxena et al., 2019).

Ocimum gratissimum, an aromatic plant that originated in Africa and spontaneously spread to Brazil, mostly in the Northeast, has been widely introduced in tropical and subtropical areas. Eugenol is the plant's principal chemical component (See Figure 3). This lipophilic, aromatic, and phenolic molecule demonstrated significant biological functions, including antibacterial properties (Silva *et al.*, 2022).

Figure 3. *O. gratissimum* (Tito Mbuvi *et al.*, 2019)

Asia is the place of origin of holy basil (See Figure 4), also known as tulsi in India (*Ocimum tenuiflorum* L. syn. *O. sanctum* L.). Its therapeutic benefits have been discussed in the Ayurveda for several thousand years. In fact, this plant is referred to as a "elixir of life" by Ayurvedic medicine and is used to treat a variety of illnesses, including malaria, asthma, conjunctivitis, heart disease, migraines, stomach problems, and inflammation (Piras *et al.*, 2018).

Figure 4. *Ocimum tenuiflorum* (Ravi et al., 2012).

2.1.2. Distribution

One of the most widely used herbs in the world, basil is indigenous to Asia (including India, Pakistan, Iran, Thailand, and other coutries), where it may be seen growing in the wild in tropical and subtropical areas (Makri and Kintzios, 2008a). As seen on Figure 5. the distribution of basil covers the whole Earth except for the arctic regions.

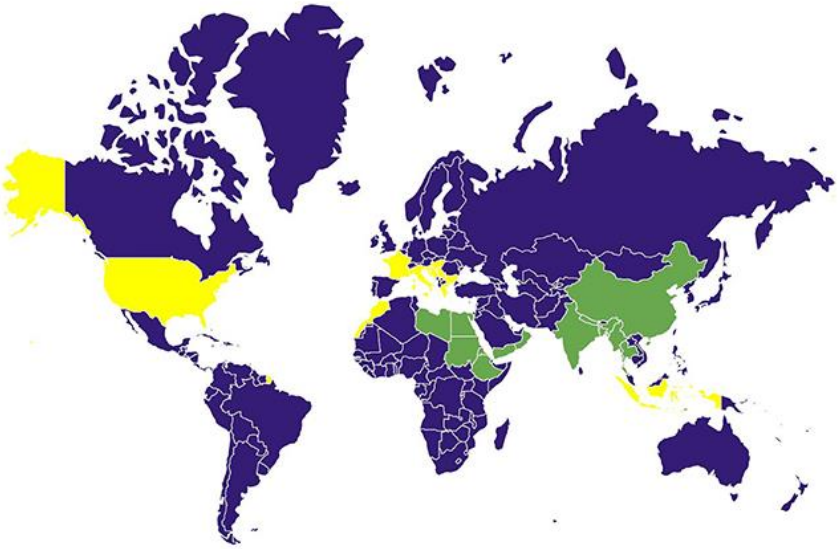

Figure 5. Map of commercial cultivation of basil (Stillroom, 2017).

Basil is probably indigenous to India, as well as several regions of Africa and Asia. In the map above (Figure 5.), the modern countries where it first appeared are highlighted in green. Basil is now grown in several of the countries shown in yellow, in addition to its original regions. It is also grown in gardens worldwide (Stillroom, 2017). For its numerous therapeutic benefits, basil, has been utilized in Indian traditional medicine (Ayurveda) and its related herbalism disciplines for thousands of years. Because the plant is revered and treated as sacred in traditional Hindu temples, sacred groves, and homes all throughout the subcontinent, *O. sanctum* L. is more frequently popular in Indian scientific literature (Bast, Rani and Meena, 2014). Many warm and temperate nations, including France, Hungary, Greece, and other southern European nations, as well as North and South America, commercially cultivate O. basilicum. Basil is a key spice in Italian tomato paste products and is sometimes combined with or used in place of oregano in meatballs, spaghetti sauces, and macaroni and cheese bakes. Basil leaves are widely used for flavoring purposes in soups, meat pies, fish meals, certain cheeses, tomato salads, cooked cucumber dishes, cooked peas, squash, and string beans, as well as vinegars and oils (Pushpangadan and George, 2012). The essential oils that are derived from the leaves and blossoms are utilized in the production of beverages, liqueurs, vinegars, drinks, teas, and cheese, among other things. The seeds are frequently added whole or milled to drinks and ice cream, as well as to bakery products as a source of dietary fiber (Calderón Bravo *et al.*, 2021).

2.1.4. Morphology that helps with identification

The taxonomic characteristics of a plant can help determine its classification. These include its leaf shape, flower, seed morphology and color. Nonetheless, it is difficult to identify species based only on morphology (Fig 6.)

Figure 6. Plant and leaf morphology of five *Ocimum* species (Upadhyay *et al.*, 2015).

The UPOV system of plant variety protection, which is based on individual test recommendations, is an accepted and unified method for evaluating novel cultivars of a species of interest (Table 2.) (UPOV, 2003).

Table 2. International Association for the Protection of New Plant Varieties (UPOV) information of basil genotypes characteristic

No.	Qualitative/quantitative or pseudo-qualitative characteristic	State
1	Plant: habit (PH)	Rounded/Intermediate/Erect
2	Plant: density (PD)	Loose/Medium/Dense
3	Stem: anthocyanin coloration (SAC)	Absent/Present
4	Stem: intensity of anthocyanin coloration (SIAC)	Weak/Medium/Strong
5	Stem: hairiness (SH)	Absent/Present
6	Stem: no. of flowering shoots (at full flowering) (SNFS)	1/ 3/ >3
7	Leaf blade: shape (LBS)	Broad ovate/Ovate/Elliptic
8	Leaf blade: length (LBL)	Short/Medium/Long
9	Leaf blade: width (LBW)	Narrow/Medium/Broad
10	Leaf blade: intensity of anthocyanin coloration of upper side (LBIAC)	Weak/Medium/Strong
11	Leaf blade: distribution of anthocyanin (LBDA)	Few mottles/Many mottles/Total surface
12	(Varieties without anthocyanin only) Leaf blade: green color (LBGC)	Light/Medium/Dark
13	Leaf blade: glossiness (LBG)	Absent or very weak/Weak/Medium/Strong/Very strong
14	Leaf blade: blistering (LBB)	Absent or very weak/Weak/Medium/Strong
15	Leaf blade: profile in cross-section (LBPCS)	Convex/Flat/Concave/V-shaped
16	Leaf blade: serration of margin (LBSM)	Absent/Present
17	Leaf blade: depth of serration (LBDS)	Shallow/Medium/Deep
18	Petiole: length (PL)	Short/Medium/Long
19	Flowering stem: avg. length of internodes (at end of flowering) (FSLI)	Short/Medium/Long
20	Flowering stem: total length (at end of flowering) (FSTL)	Short/Medium/Long
21	Flowering stem: hairiness of bracts (FSHB)	Absent/Present
22	Flower: color of corolla (FC)	White/Pink/Dark violet
23	Flower: color of style (FCS)	White/Light violet

2.1.5. Aims of cultivation

Basil is widely used in folk medicine and is recognized as such in a number of countries. Its leaves are used in pharmacy for their diuretic and stimulant effects, also included in perfume formulations (Chalchat and Özcan, 2008).

Basil has significant amounts of methyl chavicol (estragole), linalool, methyl eugenol, and methyl cinnamate in its essential oils. All morphological features and the chemical makeup of the essential oil differ greatly between basil genotypes collected from various parts of the world (Yaldiz *et al.*, 2023). However, it also contains common sesquiterpenes including caryophyllene, cubebene, and murolene as well as monoterpenes like geranial, neral, and ocimene (Tangpao *et al.*, 2022). Depending on genotypes, cultivation, growth conditions, and post-harvest treatment, basil plants can contain up to 1% essential oil (Zheljazkov, Callahan and Cantrell, 2007). In a study on Turkish basil, it was discovered that the essential oils were more concentrated in the leaves and blossoms and much less in the stems (Chalchat and Özcan, 2008).

Ocimum's seed-gum is a complex polymer that is mostly derived from holy basil (*Ocimum sanctum*). Gum is a plant-based material that turns into a thick, sticky solution or gel when mixed with water. Its enormous availability, biodegradability, nontoxicity, and low cost are some of its qualities that make it more practical than synthetic polymers in the commercial food and pharmaceutical sectors (Chand Bharati *et al.*, 2022).

2.1.6. Cultivation

2.1.6.1. Growing

Basil is grown in environments where the temperature ranges from 7 to 27 °C. This delicate herbaceous annual is prone to damage from frost and low temperatures. Long, sunny days in full sunlight with well-drained soil are ideal for its development. Once all threat of frost has passed in late spring, basil can either be directly seeded or transferred to the field. Plants are spaced every six inches between rows that are 63 to 88 cm apart. Raised beds can also be used to grow basil in three-line rows. The equipment a grower uses determines the space between rows, which typically ranges from 25 to 40 cm (WIFFSS and USFDA, 2016).

To promote germination and enhance plant establishment, soil is kept moist. After planting seeds, germination happens eight to fourteen days later. Though growth is initially sluggish, it quickly accelerates once the first few sets of leaves appear (Nam, Kang and Kim, 2020).

Basil is grown as an annual crop for the fresh basil market by some small farmers using transplants. Typically, transplants need 28 to 42 days to fully develop. After the plants are five inches tall, you can top them to promote lateral branching and growth (Gill and Randhawa, 2008). When there are three to five sets of genuine leaves, topping encourages branching and aids in maximizing plant growth. As a plant germinates, the second set of leaves—true leaves—appear(Doty & McDonald, 2020).

Basil requires frequent watering because it cannot withstand being under water stress (Khakdan *et al.*, 2021). Since it reduces harm to foliage from moisture contact with the leaves, drip irrigation is favored (Çetin and Akalp, 2019).

The kind of soil, prior fertilizer applications, and previous crop type all influence whether basil has to be fertilized. A fertilizer that contains nitrogen, phosphate, and potassium is often advised (Teliban *et al.*, 2022). A fertilizer can be applied by "side dressing," which is the practice of applying fertilizer in between the rows of a crop that is in growth, or it can be distributed over a field and plowed in (Sharafzadeh and Alizadeh, 2011).

After plant emergence, herbicides are frequently avoided while cultivating this crop economically. Growers use hand weeding, mulch application, mechanized cultivation, dense plant populations, and dense plant populations to manage weeds. Many insect, fungal, viral, and nematode pests can affect basil plants, but only a few insecticides are approved for use on growing basil plants. Biological control utilizing beneficial bacteria or insects, insecticidal soaps, plant extracts, pest traps, and organic pesticides are just a few of the novel or organic methods used. Sometimes, chemical controls are applied before planting or emergence (WIFFSS and USFDA, 2016).

2.1.6.2. Harvesting

The herb's planned usage determines what portion of the plant is harvested and when it is harvested. The plant is removed immediately before the blossoms emerge if it is to be used for dried basil leaves. The plant is collected when the blossoms are fully open to make basil oil (Luiz *et al.*, 2006).

Three to five cuttings can be made each year in warmer climates. Only two cuttings may be possible annually in cooler climates due to the shorter growth season; the first cutting is often taken in the early summer and the second right before blooming (Barroso and Jerez, 2002; Makri and Kintzios, 2008a).

For the fresh and dry markets, basil leaves are plucked above the lowest two to four sets of true leaves. Basil plants are typically clipped four to five inches above the ground in bigger commercial operations to allow for regrowth. The dates for planting and harvesting are spaced apart to ensure a constant supply of fresh basil leaves (WIFFSS and USFDA, 2016).

2.1.7. Breeding

Intense production in climate-controlled greenhouses has lately been established in response to the requirement for a year-round supply. This has been accompanied with a rise in disease infection, necessitating the creation of preventative technologies. The degree of pesticide residues that can be present in the finished product is restricted by today's sophisticated market (Succop and Newman, 2004). All of this led to a thorough breeding process that produced some new cultivars with a focus on appealing look, resistance to diseases, particularly Fusarium, and an extended shelf life (Dudai *et al.*, 2008).

While growing, storing, and transporting, sweet basil is vulnerable to chilling injuries brought on by exposure to temperatures below 12 °C. Brown dots on the leaves that turn necrotic are the first signs of chilling injury. The leaves may then abscise, lose their shine, and become more susceptible to deterioration, which can lead to soft rot from bacteria and fungus like *Botrytis cinerea* (Dudai *et al.*, 2018).

The reason basil breeding initiatives lag behind those of other crops like wheat, rice, or tomato could be due to a lack of advanced genetic material as well as a lack of a well-established and contiguous reference genome. This

delay makes it more difficult to create powerful genome editing tools for basil since it is difficult to get rid of redundancy and off-targeting in the absence of a reference genome. Gonda et al. (2020) continued their earlier work (Dudai *et al.*, 2018), to fill the scientific and breeding deficiencies mentioned above. Using Illumina platforms, the genome of the cultivar "Perrie" was sequenced and assembled using NRGENE's DeNovoMagicTM assembler. The resulting haploid genome was estimated to be 2.13 Gbp in size and contains 12212 scaffolds at this time.

With relation to agronomically significant plants, one of the most generally beneficial uses of biotechnology is probably based on genomic analysis, which includes genetic linkage mapping, QTL analysis of significant phenotypic features, and marker-assisted selection. These methods are presently being applied to the main crops to hasten the creation of new varieties with the appropriate phenotypic characteristics. As aroma is one of the main characteristics for which basil is cultivated, this has been a trait to be used in breeding purposes (Dudai and Belanger, 2016).

Basil genotypes were the subject of an experiment to characterize them based on their morphological and phenological characteristics, herbs, and essential oil output. Days to first cutting (56.92-101.6 days), plant height (13.67-71.90 cm), branch number (3.28-19.43 plants per plant), fresh herb yields (12.94-274.11 g plants per plant), and essential oil yield (0.04-1.71%) are just a few of the traits that showed a wide range of variations and can be helpful for breeding (Yaldiz and Camlica, 2021).

2.2. Molecular markers

DNA sequences with established physical positions on chromosomes are referred to be genetic markers. These are points of variation that may be used to distinguish between different persons or species. They can also be used to relate a gene to an inherited condition by forming genetic relationships with neighboring, but perhaps unrecognized, genes (Nature portfolio, 2022). Molecular markers result from a variety of DNA alterations, including point mutations, insertions and deletions, and errors in the replication of tandemly repeated DNA. Because these markers are often found in non-coding sections of the genomes, they are selectively neutral. Contrary to other markers, DNA markers are unlimited and not influenced by external conditions or by the stage of development of the plant species(Govindaraj, Vetriventhan and Srinivasan, 2015).

2.2.1. Barcodes

The market for medicinal plants is increasingly subject to adulteration and replacement due to growing commercial interest in herbal goods. Herbal products are authenticated using DNA barcoding to guarantee their purity, safety, and effectiveness (Saroja *et al.*, 2022).

For precise species identification, DNA barcoding technique was presented out in 2003. For quick, precise, and automated species identification, this technique uses a DNA barcode, which is a standard, short DNA sequence (Hebert *et al.*, 2003).

Since 2008, the molecular identification and classification technique known as DNA barcoding has been used to identify and classify therapeutic plants. The use of this approach has significantly increased the safety and efficacy

of pharmaceutical products. DNA barcodes are a growing source of genetic data, contributing to everything from single locus-based DNA barcodes to integrated markers and genome-scale levels (Yu *et al.*, 2021).

The effectiveness of seven top potential plastid DNA regions have provided the scientific community a proposal on a common plant barcode (atpF-atpH spacer, matK gene, rbcL gene, rpoB gene, rpoC1 gene, psbK-psbI spacer, and trnH-psbA spacer). CBOL Plant Working Group (2009) suggested the 2-locus combination of rbcL+matK as the plant barcode based on evaluations of recoverability, sequence quality, and degrees of species discrimination. However, unlike in the animal or fungi kingdom, no single universal DNA barcoding fragment has been found for plants mostly due to the low level of mutation of plant organelle genomes (See figure 7.) (Setsuko *et al.*, 2023).

The whole DNA sequence of a plastid is included in the chloroplast genome, which provides more genetic data for species identification than any widely used single-locus marker (Yu *et al.*, 2021).

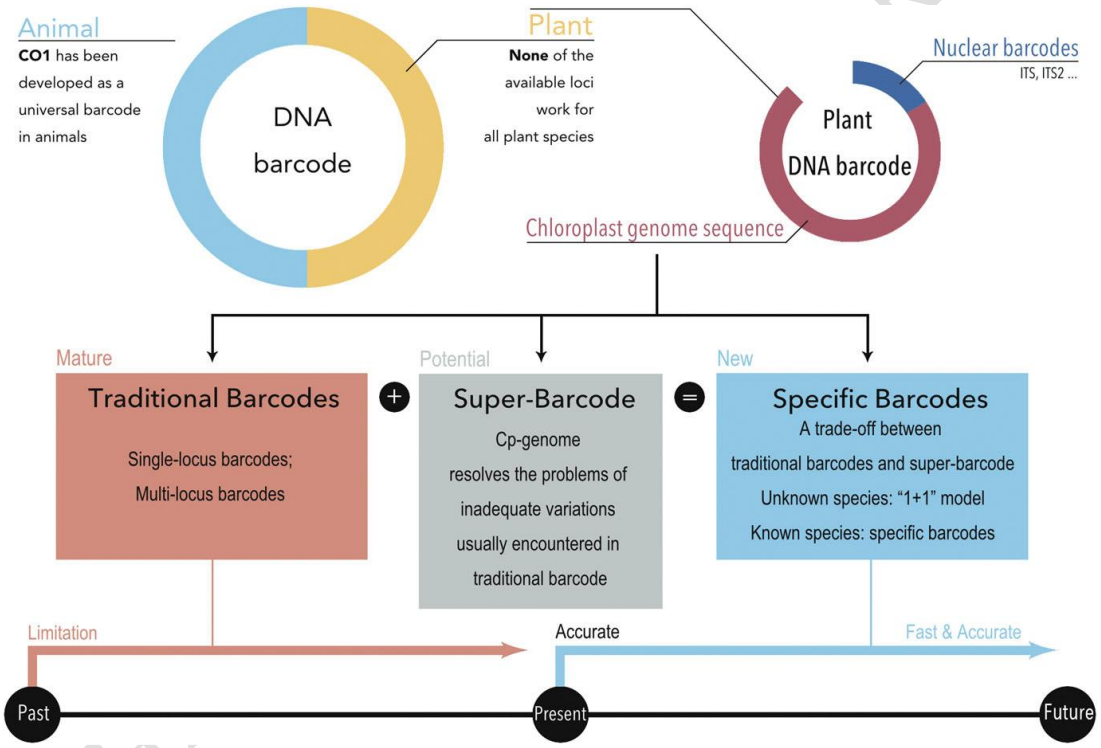

Figure 7. Plant bar coding history (Li *et al.*, 2015).

2.2.2. Molecular markers used in case of basil

According to genome sequencing, basil is a tetraploid organism with a genome size of 2.13 Gbp that was assembled in 12212 scaffolds, with 107 scaffolds making up more than 90% of the assembly. The genome is made up of repeated sequences to a degree of around 76%, the bulk of which are long-terminal repeats (Gonda *et al.*, 2020b). Except for the predominantly autogamous propagation of *O. basilicum*, interspecific hybridization and polyploidy are frequent occurrences within this genus and have caused taxonomic confusion, making any understanding of the genetic link between the several basil species challenging. The occurrence of several botanical variations, cultivator names,

and chemotypes within the species that may not notably differ in appearance further complicates the classification of basil (Makri and Kintzios, 2008b). As a result, relying solely on morphology to address these issues is unsuccessful (Vieira, Goldsbrough and Simon, 2003).

In their study of the relationship between morphological traits, essential oil composition, and ALFP markers in *O. basilicum*, Labra et al. (2004) emphasized that taxonomic classifications should only be based on genetic information and that genomic similarities do not always reflect external characteristics, such as essential oil components.

To determine the degree of variation present in the genus *Ocimum*, Genetic fingerprinting (AFLP) and chemical studies of essential oils were used. 22 *Ocimum* accessions from seven different species were studied. The findings showed some degree of agreement between phenotypic and molecular data among the examined *Ocimum* accessions, showing that phenotypic trait analysis can be a reliable basis for future molecular marker differentiation. Phylogenetically, *O. gratissimum, O. tenuiflorum, O. campechianum,* and *O. kilimandscharicum* are more closely related than *O. africanum, O. americanum*, and *O. basilicum* are (Carović-Stanko et al., 2011).

The genetic link between 14 genotypes of Turkish basil has been determined using RAPD markers. The genotypes of basil were divided into two major clusters based on the RAPD data, with genetic closeness ranging from 46 to 87%. The genotypes under investigation were divided into two major categories. In both dendrograms, the genotypes belonging to the same chemotype were often grouped together (Giachino *et al.*, 2014).

In order to determine the diversity of the genus *Ocimum* by morpho-molecular (RAPD) and chemical (GC-MS) research, a study was conducted in India. The morphological data cluster revealed two distinct groups: the basilicum group and the sanctum group. *O. africanum* and *O. basilicum,* two visually similar species, did not exhibit substantial difference in chemical composition. Nonetheless, RAPD analysis unmistakably established the distinct species boundaries between *O. africanum* and *O. basilicum*. In order to validate taxonomic demarcation, integrated assessments of morphological features, chemical, and genetic markers are therefore the most effective method. Also, they reported *O. africanum* from both this area and West Bengal, India, for the first time (Chowdhury *et al.*, 2017).

The most species-specific loci were found in *O. sanctum* when utilizing RAPD and ISSR markers to detect genetic variation in *Ocimum* species. The findings of this study confirm the effectiveness of both marker types in determining the species/inter-species status of *Ocimum* and determining the distinctiveness of various genera within a family. Additionally, species-specific alleles can be converted into co-dominate SCAR markers for further characterization of the *Ocimum* species from various geographic regions (Patel *et al.*, 2015).

Gupta et al. (2021) used the Start Codon-Targeted Polymorphism (SCoT) and inter-simple sequences repeat (ISSR) markers to examine the diversity among 36 *Ocimum* accessions representing species from various parts of the world. The findings demonstrated that *Ocimum* accessions contain a considerable degree of variety. There were no regionally based clusters, as demonstrated by the cluster analysis using both marker systems. PCA, which demonstrates the population's greatest variance, provided additional evidence for this. When genotypes from the same

locales joined more than one cluster from the same areas, there was no correlation between genetic divergence and geographic origin. This suggests cross-pollination or population-to-population gene flow.

Using 14 ISSR primers, 147 amplified fragments overall, of which 86%, on average, were polymorphic, Alves et al. (2019) sought to analyze the genetic diversity of 27 basil genotypes. Individuals' genetic similarity was determined, resulting in the formation of four different groups. The majority of individuals (40.7%) were assigned to cluster I. The polymorphic information content (PIC) (0.89) showed that genotypes had a high degree of genetic variety. The ISSR markers were effective in identifying polymorphisms between accessions in this way, indicating the genetic diversity of the collection.

2.3. Molecular Genetics results

Patel et al. (2019) located the unique and conserved *O. basilicum* miRNA in the NCBI Expressed Sequence Tags database. These *O. basilicum* discovered miRNA potentially regulate by their cleavage and translational inhibition. They arbitrate the cross kingdom miRNA mediated human gene target networks. As a result, this offers proof of cross-kingdom gene regulation with related biological activities, molecular processes, and pathways. Furthermore, it was discovered that the majority of the possible mRNAs identified in that study were linked to a number of processes, including metabolic processes, cell proliferation, and apoptosis.

2.4. Genetic variability

For effective germplasm management and long-term breeding programs, the preservation of genetic diversity for the future and the effective use of available accessions are crucial. Consequently, the capacity of populations and species to endure across an evolutionary period of time despite changing surroundings depends on genetic variety (Nass and Paterniani, 2000).

Plant breeders have the chance to create new and improved cultivars with desirable traits, including traits that farmers prefer (large seeds, potential for yield, etc.) as well as traits that breeders prefer (pest and disease resistance, photosensitivity, etc.). This is made possible by the diversity of plant genetic resources (PGR). Natural genetic diversity within plant species has been employed since the dawn of agriculture to provide enough food for subsistence, and it is currently being concentrated on producing surplus food for expanding populations (Govindaraj, Vetriventhan and Srinivasan, 2015).

3. MATERIALS AND METHODS

3.1. Plant Materials

Leaves of 17 cultivars, genotypes and breeding materials of basil were used in course of the study. Leaves were used directly for DNA isolation (Table 3).

Table 3. Basil accessions used in this study

Code	Name	Species
S6	Dwarf (törpe)	*Ocimum basilicum*
S7	*O. sanctum* Ethiopian	*O. sanctum*
S8	Vietnamese	*Ocimum basilicum*
S12	Adi F1	*Ocimum basilicum*
S13	*O. americanum*	*O. americanum*
S14	Thai basil	*Ocimum basilicum*
S18	*O. sanctum* Danish	*O. sanctum*
S19	*O. basilicum* mrihani	*Ocimum basilicum*
S24	*O. citronum* lemon basil	*O. americanum*
S26	Iranian lemon	*Ocimum basilicum*
V1	Purple Ruffles	*Ocimum basilicum*
V6	Mrihani	*Ocimum basilicum*
V17	Rama Tulsi	*O. sanctum/tenuiflorum*
V18	Tulsi Vana	*O. gratissimum*
V19	African	*O. gratissimum/africanum*
V20	Penang Lemon (Pinang)	*O. africanum*
V21	African nunum	*Ocimum gratisimum*

3.2. Chloroplast primers

Four primer pairs were selected for assessing genetic diversity in basil, namely matk5/matk6 psbA5'R/matk8F psbB/psbH trn^{SUGA}/$trnfM^{CAU}$. The primers' sequences expected size and annealing temperature during PCR amplification are indicated in Table 4.

Table 4. Chloroplast markers used for sequencing (Shaw *et al.*, 2005)

Code	Name	Sequence (5'-3')	Expected Size (Based on *Nicotiana*)	Annealing temp.
P1	matk5	TGTCATAACCTGCATTTTCC	713 bp	50°C
	matk6	TGGGTTGCTAACTAATGG		
P2	psbA5'R	AACCATCCAATGTAAAGACGGTTT	500 bp	50°C
	matk8F	TCGACTTTCTTGTGCTAGAACTTT		
P4	psbB	TCCAAAAAANKKGGAGATCCAAC	387 bp	57°C
	psbH	TCAAYRGTYTGTGTAGCCAT		
P10	$trnS^{UGA}$	GAGAGAGAGGGATTCGAACC	867 bp	57°C
	$trnfM^{CAU}$	CATAACCTTGAGGTCACGGG		

3.3. DNA extraction and evaluation

The total DNA was extracted by using the CTAB Plant genomic DNA extraction technique. Nano Drop ND-1000 spectrophotometer was used to evaluate DNA concentration and quality parameters (Bioscience, Budapest, Hungary). The Department of Plant Biotechnology at the Hungarian University of Agriculture and Life Sciences kept the isolated DNA in a -20°C freezer.

3.4. PCR

The polymerase chain reactions were performed in a total volume of 15 µl in a Swift MaxPro thermocycler (Esco Healthcare Pte, Singapore). The mix contained 10x PCR buffer, 0.2 mM (0.3 µL) deoxyribonucleotide triphosphates (dNTP) mix, 0.15 unit of DreamTaq polymerase, 0,4 µM (0.5 µL) of primer forward and reverse, 1% BSA, 2% DMSO and sterile distilled water. Each of the reactions contained 20-80 ng of template DNA (1 µl). A standard PCR cycle was used: an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C/57°C for 1 min depending on the primers used (Table 4.), and 72°C for 2 min; the final extension at 72°C was held for 5 min. The amplified products were afterwards stored at 4°C.

3.5. Agarose Gel Electrophoresis

All the PCR products from amplification were run b on 1% TBE agarose gel stained with 1% ethidium bromide (EtBr) for 15-20 min at 120 Volts. DNA bands were visualised under UV light by gel documentation system (Bio-Rad). The approximate fragment size was compared with the GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific).

3.6. DNA Sequencing

Based on the results of the chloroplast markers, 17 genotypes were used for sequencing. Prior to sequencing, in order to remove excess primers and dNTPs, the PCR products were purified using ExoSAP- IT Express reagent (Thermo Fisher Scientific). Seven µl post-PCR reaction product was combined with two µl ExoSAP- IT Express Reagent. The mixture was incubated at 37°C for 4 minutes and incubated at 80°C for 1 minute to inactivate the ExoSAP-IT Express Reagent. The sample was kept at 4°C and then transferred to ice and stored at -20°C. The PCR product is ready for DNA sequencing. The PCR fragments were then sequenced in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

3.7. Data Analysis

Analising of the sequences was done using BioEdit (Hall, 1999). The aligning of reverse and forward sequences was performed in MEGA11, as well as the detection of SNPs, Indels and phylogenetic tree with Tamura 3-parameter as the best model for the constructed phylogenetic tree (Tamura, Stecher and Kumar, 2021).

4. RESULTS AND DISCUSSION

4.1. Amplification and Sequence analysis of basil cultivars using chloroplast markers

From the 17 samples, not all of them showed proper amplification and sequencing with 4 primer pairs. Thirteen cultivars showed good amplification and sequencing with Primer 1 as well as Primer 4 and Primer 10. Meanwhile, fourteen cultivars showed good amplification with Primer 2, showing the highest number of amplifications. Data is summarized in Table 5. Some gel photos from the study can be observed in Figure 8.

Table 5. Chloroplast markers results of sequencing of 17 basil cultivars

Sample	P1	P2	P4	P10
V6	x	x	x	x
V19	x	x	-	x
S19	x	-	-	-
V20	x	x	x	x
V18	x	x	x	x
V17	x	x	x	x
V1	x	x	-	x
S14	x	x	x	x
S13	x	x	x	-
S12	x	x	x	x
S18	x		x	
S24	x	x	x	x
S26	x	x	x	x
V21	-	x	x	x
S8	-	x	x	x
S7	-	x	-	x
S6	-	-	x	-

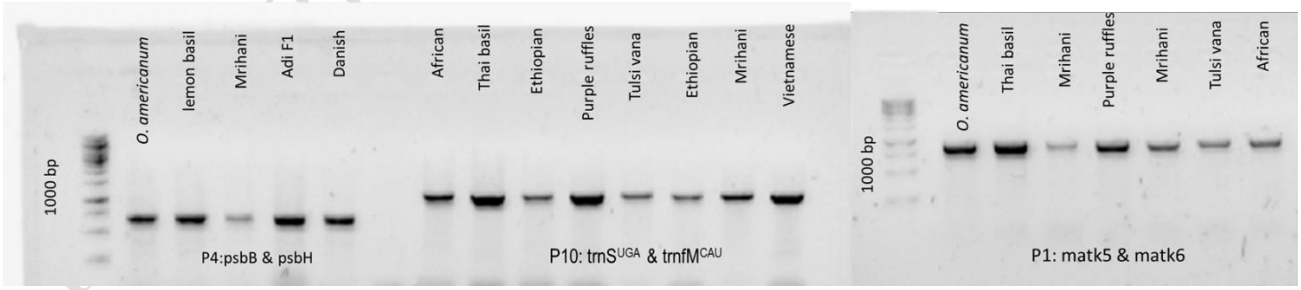

Figure 8. Amplification profile using cultivars of basil

Four chloroplast primer pairs were used for sequencing, and the actual size of each region in the chloroplast genome of basil was also compared with the chloroplast genome of *Nicotiana* (Table 6). Some regions of basil cultivars were larger compared to Nicotiana, namely matk5/matk6 and psbA5'R/matk8F. The remaining regions were smaller

than Nicotiana, namely psbB/psbH and trnS^{UGA}/trnfM^{CAU}. A linear map describing each region in *Nicotiana* chloroplast genome is shown in Figure 9.

Table 6. Profile of Chloroplast markers in Nicotiana vs. basil cultivars.

No	Primer's Name	Expected Size (Based on *Nicotiana* cpDNA) (bp)	Actual Size (bp) (Basil cpDNA)
1	matk5-matk6	500	883
2	psbA5'R-matk8F	454	887
4	psbB-psbH	1021	659
10	trnS^{UGA}-trnfM^{CAU}	1282	1051

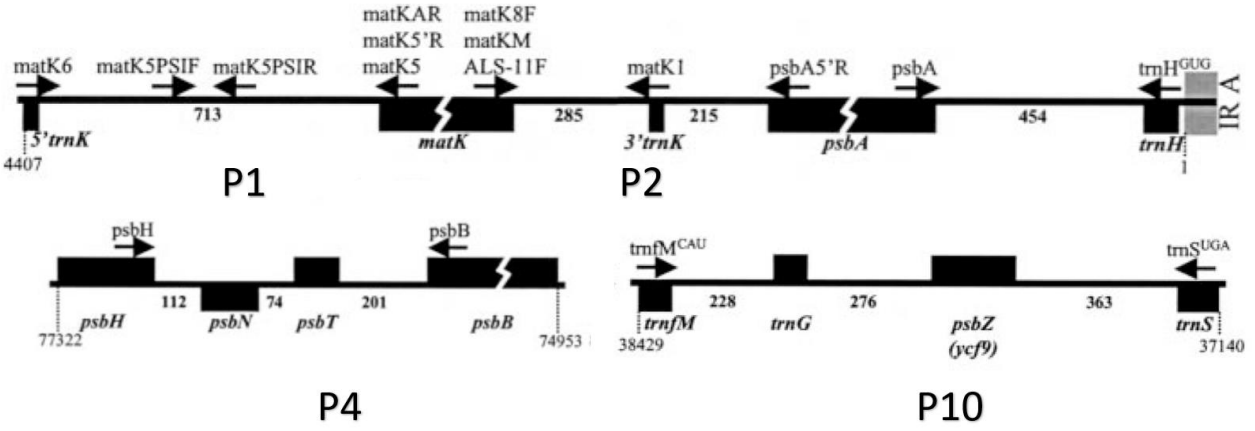

Figure 9. Linear map of the 4 noncoding cpDNA regions used in this study (based on the *Nicotiana* chloroplast genome) (Wakasugi *et al.*, 1998; Shaw *et al.*, 2005).

Table 7. Summarised polymorphism results of 17 basil cultivars with 4 Chloroplast primer pairs.

Code	Name	Number of INDEL mutations	Number of SNPs
P1	matk5 & matk6	3	19
P2	psbA5'R & matk8F	7	21
P4	psbB & psbH	4	76
P10	trnS^{UGA} & trnfM^{CAU}	12	23

4.2.1 Primer 1: matk5/matk6

Fourteen genotypes were sequenced with matk5/matk6, belonging to the cultivars marked with an "x" in Table 5. In general, the size of the PCR product was 883 bp. Unfortunately, out of them the African nunum cultivar (*O. gratisimum*) did not show its full length, lacking 298 bp. In the alignment of the sequences, 19 SNP and 3 Indels were found. The biggest Indel was of 5 bp in *O. basilicum.*

Nine out of the 19 SNP loci were shared between V17 (*O. basilicum*), V18 (*O. gratissimum* vana), and *O. gratissimum/africanum* African). RAPD and ISSR markers have been used in the past to confirm genetic stability in *O. gratisimum*, because of the importance of keeping a stable gene pool after micropropagation and for pharmaceutical uses (Soumen *et al.*, 2011; Saha, Sengupta and Ghosh, 2014); the findings of the present study show that even though there are clear differences between the chloroplast genome of *O. gratissimum*, and most of the cultivars in study. Nevertheless, the cultivar of *O. basilicum* also shares these polymorphisms which could interfere in the correct use of this region as a fingerprint for species identification between *O. basilicum* and *O. gratissimum*. Jiang et al. (2023), compared three cultivated varieties of *Scutellaria baicalensis* by using their chloroplast genomes, and found out that the complete chloroplast, codon use analysis, and repeat sequence analysis all revealed significant conservation. However, reveal important variations in matK-rps16 and petA-psbJ amongst the three cultivated kinds. They discovered that three cultivars may be identified using a barcode created from the matK-rps16 sequence.

4.2.2. Primer 2: psbA5'R/matk8F

Fifteen genotypes were sequenced with psbA5'R/matk8F, belonging to the cultivars marked with an "x" in Table 5. In general, the size of the PCR product was 887 bp. In the beginning of the sequence of Mrihani *O. basilicum* there were 49 bp missing. In the alignment of the sequences, 21 SNP and 7 Indels were found.

There are many similarities between the sequences of Rama tulsi (*O. sanctum*), *O. Gratissimum/ africanum*, African nunum *O. gratissimum*; including an Indel with a lenght of 6 bp. Harini, Balaji and Parani (2021), have elucidated the complete chloroplast genome of the Rama tulsi subtype from *O tenuiflorum* and performed a phylogenetic analysis involving other *Ocimum* species, they confirmed that this subtype belongs in the clade of the genus *Ocimum* under the Lamiaceae family; in the present study and also previous authors (Saran and Kalariya, 2019), Rama Tulsi was considered a cultivar from the species *Ocimum sactum*, instead of *O. tenuiflorum*, nonetheless both of them are considered synonyms (Hackney, 2016).

Many sizable Indel mutations could be observed. One was of six bp in African *O gratissimum/ africanum*. And one of 10 bp in Rama tulsii (*O. sanctum/tenuiflorum*). These regions could be useful for the development of DNA barcodes for species identification.

Maheswari, Kunhikannan and Yasodha (2021) studied the phylogenetic relationships among Lamiaceae members, providing a clear example of the use of chloroplast genomes to better understand taxonomic and evolution relationships in the plant kingdom. In China, a comparative analysis of the chloroplast genomes of patchouli plants (Lamiaceae) also showed portrayed information to be used for phylogenetic reconstruction, species delimitation and identification in the future (Zhang et al., 2020).

4.2.3. Primer 4: psbB/psbH

Thirteen genotypes were sequenced with psbB/psbH, belonging to the cultivars marked with an "x" in Table 5. In general, the size of the PCR product was 659 bp. The sequences had to be trimmed from both sides due to unclear

parts: 50 bp in the beginning of the sequence, 47 bp in the end. In the alignment of the sequences, 76 SNPs and 4 Indels were found. An Indel loci with a length of 8 base pairs was found in a microsatellite region in Tulsi vana *O. gratissimum* which could be useful for species identification, since *O. gratissimum* is in the present known for its many pharmacological uses; in the drug fabrication industry the authentication of the plant material is key for the assurance of quality and effectiveness (Ugbogu *et al.*, 2021).

Fu et al. (2022) sequenced the cp genome sequences of *Dracocephalum heterophyllum* (Lamiaceae) and compared it with closely related species. Their findings offer a solid starting point for future studies on the genetic resources of this plant and may be used to develop more efficient conservation and management strategies for species within the genus.

4.2.4. Primer 10: trnS^{UGA}/trnfM^{CAU}

Thirteen genotypes were sequenced with trn^{SUGA}/trnfM^{CAU}. In general, the size of the PCR product was 1051 bp. A portion of 57 bp was trimmed from the beginning of the Ethiopian cultivar (*O. sanctum*) due to low accuracy in the sequence which was confirmed by reading the chromatogram on the BioEdit software. Additionally, the Tulsi Vana cultivar (*O. gratissimum*) lacked 581 bp in the end of the sequence. After aligning the sequences, 23 SNP and 12 Indels were found.

A deletion of 6 bp was found in lemon basil (*O. americanum*) and Pinang (*O. africanum*). Furthermore, in the sequence belonging to lemon basil (*O. americanum*), there is an insertion mutation of 3 bp. This region could be considered for the design of species-specific primer for identification since it was only found for this individual. Kholilatul Izzah et al. (2023) developed three insertion/deletion (Indel) markers that were successfully taken from the regions of trnA-UGC-rrn23, trnK-UUU-rps16, and rps16 intron, based on comparative chloroplast genome analysis of two cacao genotypes.

4.3. Phylogenetic analysis

For the final evaluation of the DNA sequences, only the ones with successful sequencing for all the four primer pairs were taken into consideration (Table 8.). Even though V18 is available in all the primers, it has been omitted from the phylogenetic analysis due to its highly incomplete sequence when analysed with Primer 10: trn^{SUGA}/trnfM^{CAU}.

Table 8. List of sequences used in phylogenetic analysis (highlighted in green).

Sample	P1	P2	P4	P10
V6	x	x	x	x
V19	x	x	-	x
S19	x	-	-	-
V20	x	x	x	x
V18	x	x	x	x
V17	x	x	x	x
V1	x	x	-	x
S14	x	x	x	x
S13	x	x	x	-
S12	x	x	x	x
S18	x		x	
S24	x	x	x	x
S26	x	x	x	x
V21	-	x	x	x
S8	-	x	x	x
S7	-	x	-	x
S6	-	-	x	-

The phylogenetic tree was constructed using chloroplast sequence data from four primers, namely matk5/matk6 psbA5'R/matk8F psbB/psbH trn^{SUGA}/$trnfM^{CAU}$. The result of the phylogenetic tree is shown in Figure 10. The Neighbor-Joining approach was used to infer the evolutionary history (Saitou and Nei, 1987). With branch lengths in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is drawn to scale. The evolutionary distances were calculated using the Maximum Composite Likelihood technique and are expressed in base substitutions per site (Tamura, Nei and Kumar, 2004). There were 7 nucleotide sequences in this investigation. Codon positions 1st+2nd+3rd+Noncoding were included. For each set of sequences, all ambiguous locations were eliminated (pairwise deletion option). There was a total of 3336 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura, Stecher and Kumar, 2021).

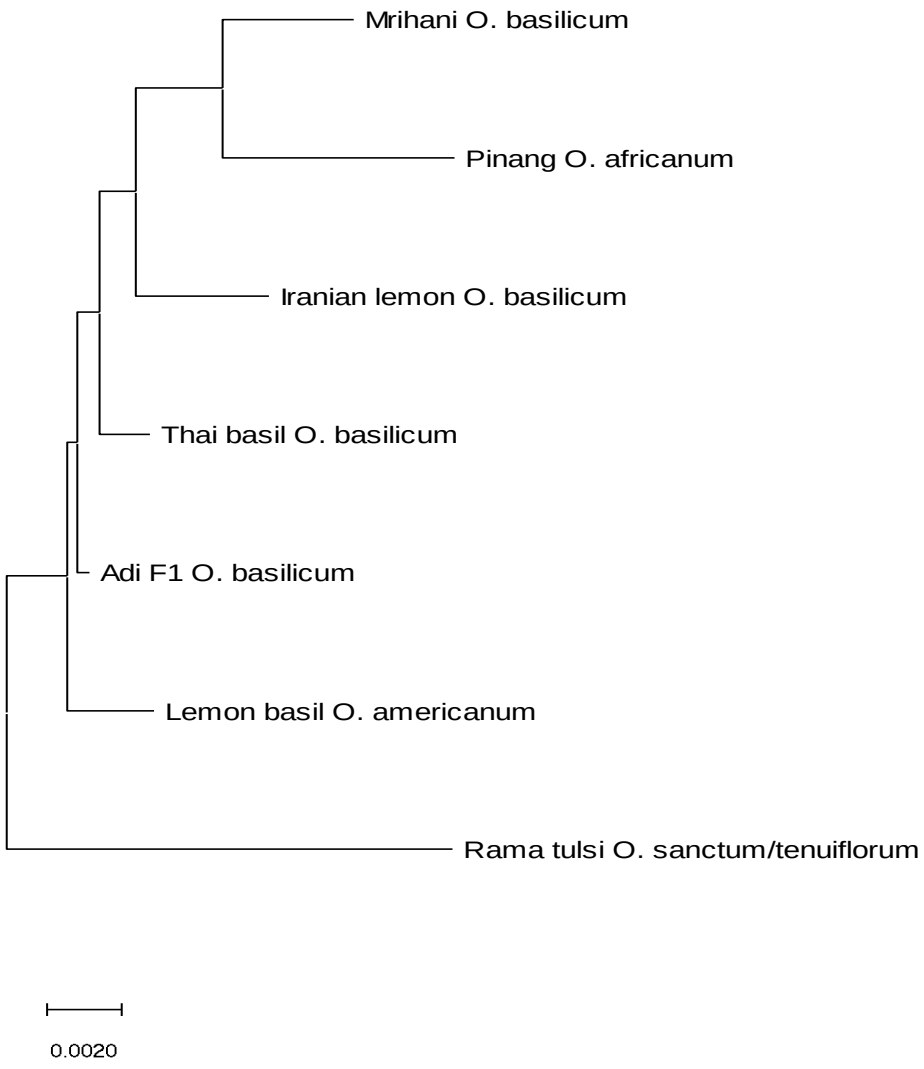

Figure 10. Phylogenetic tree of 7 basil cultivars.

Based on the phylogenetic tree result (Figure 10.), two groups can be identified, separating Rama Tulsi (*O sanctum/tenuiflorum*) from the other analised individuals, four cultivars belonging to *O. basilicum*, one from *O. africanum* and one to *O. americanum*. This implies that the chloroplast regions used in the present study are useful to identify Rama Tulsi from other species from the *Ocimum* genus. In New Dehli, Kumar et al. (2016) used a different chloroplast region to analyse 14 *Ocimum* individuals. According to their phylogenetic tree based in psbA-trnH, *Ocimum basilicum*, *Ocimum americanum* and *Ocimum africanum* species are closely related but they are separated in a different clade from Rama Tulsi (*O. tenuiflorum*) (Figure 11.)

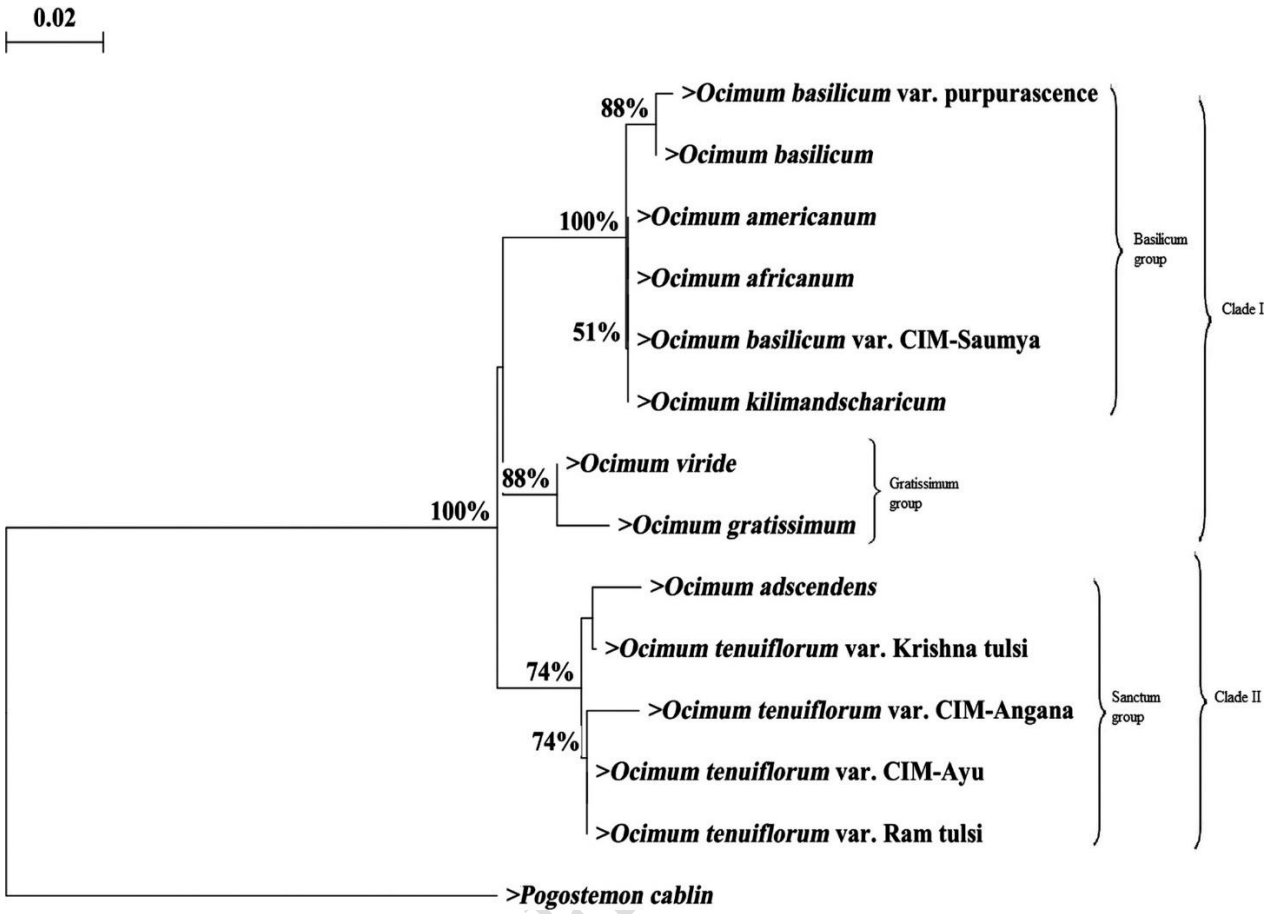

Figure 11. Neighbor-joining bootstrap tree using Kimura 2-parameter algorithm. Phylogram resulting from psbA-trnH *Ocimum* nucleotide sequence data (Kumar *et al*., 2016b).

According to Paton, Harley and Harley (1999), *Ocimum* was separated into the subsections *Ocimum*, *Gratissima*, and *Hiantia Benth*. Later, Pushpangadan and Bradu (1995) developed a separate infrageneric classification based on morphological traits and split the whole taxon into two groups: the *Basilicum* group and the *Sanctum* group. The species in the *Sanctum* group consisted of perennial shrubs with brown, globose, non-mucilaginous, or weakly mucilaginous seeds, whereas the species in the *Basilicum* group were herbaceous annuals/perennials with black, ellipsoid, highly mucilaginous seeds (Carović-Stanko *et al*., 2011b). In Figure 10. the Rama Tulsi cultivar (*O. sanctum/tenuiflorum*) is the oldest ancestor of all the other studied cultivars. Carović-Stanko et al., (2011b) conducted a study on different *Ocimum* species using AFLP markers, they showed that *O. tenuiflorum* accessions, together with the *O. gratissimum* accessions, had distinct positions from the rest of the species on phylogenic trees. *O. tenuiflorum*'s distinct positions in the Carović-Stanko et al., (2011b) study and the present work, might be explained by its membership in the *Sanctum* group, whereas *O. basilicum*, *O. americanum*, and *O. africanum* are members of the *Basilicum* group (Khosla, 1995). Furthermore, it would be very interesting in the future to solve the species interrogant from the Rama Tulsi cultivar that unfortunately was not solved in the course of this work.

5. CONCLUSIONS

Regions of basil's chloroplast genome are highly variable. Such regions are the first to be considered when identifying the best loci for DNA barcoding and phylogenetic analysis to distinguish between closely related species or taxa.

A comparative analysis of 4 regions from the *Ocimum* chloroplast genome in seventeen cultivars was performed, it revealed 139 single nucleotide polymorphisms (SNPs). Twenty-six common indels were detected. The phylogenic analysis based on seven cultivars showed two clades, separating Rama Tulsi (*O. tenuiflorum*) from the other analised individuals, four cultivars belonging to *O. basilicum*, one from *O. africanum* and one *to O. americanum*. This implies that the chloroplast regions used in the present study are useful to identify *O. tenuiflorum* from other species from the *Ocimum* genus.

The chloroplast genome of basil is a source of reliable and valuable molecular markers for revealing the extent of genetic diversity and identifying different species. Future research on the phylogeny, population genetics, and evaluation of the genetic diversity of basil would greatly benefit from the study's findings.

6. SUMMARY

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Assessing the genetic diversity of basil (*Ocimum*) cultivars with Chloroplast markers

MSc in Agricultural Biotechnology (Plant)

The genus *Ocimum* is one of the most well-known genera in the Lamiaceae family. Basil is believed to have originated in India. The distribution is primarily in tropical and template climates. It has been grown all over the world as culinary herbs and for the extraction of essential oils. *Ocimum* species posses a high number of pharmacological, nutritional, cosmetic and industrial.

Morphological markers including leaf and color, flower, and seed morphology, have been used to distinguish between varieties and within a genus for taxonomical purposes. Unfortunately, extended cultivation and inter- and intra-specific cross hybridization produce numerous polymorphisms that lead to a wide variety of subspecies, each with its own chemistry and biological activity but very similar morphology leading to confusion when identifying cultivars and even species.

Molecular markers result from a variety of DNA alterations, including point mutations, insertions and deletions, and errors in the replication of tandemly repeated DNA. Because these markers are often found in non-coding sections of the genomes, they are selectively neutral. Contrary to other markers, DNA markers are unlimited and not influenced by external conditions or by the stage of development of the plant species. Because chloroplast markers are maternally inherited in angiosperms and evolve at a slower pace than nuclear genomes, they can be utilized to investigate genetic diversity and phylogeny. The identification of species, the investigation of their genetic diversity, and phylogenetic analyses may all be accomplished using chloroplast markers.

This study aimed to assess the genetic diversity of 17 basil (*Ocimum* genus) cultivars with Chloroplast markers and its potential use for species identification and determining the phylogenetic relationship among these cultivars.

The total DNA was extracted by using the CTAB Plant genomic DNA extraction technique. Four primer pairs were used (matk5/matk6 psbA5'R/matk8F psbB/psbH trnSUGA/trnfMCAU). The PCR amplification was performed in a Swift MaxPro thermocycler. The PCR products of two markers were run through electrophoresis and visualized under UV light using a gel documentation system (Bio-Rad). The PCR fragments were first purified using ExoSAP- IT Express

reagent (Thermo Fisher Scientific), then sequenced in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Analysis of the sequences was done using BioEdit (Hall, 1999). The aligning of reverse and forward sequences was performed in MEGA11, as well as the detection of SNPs, Indels and phylogenetic tree with Tamura 3-parameter as the best model for the constructed phylogenetic tree.

The study revealed 139 single nucleotide polymorphisms (SNPs). Twenty-six common indels were detected. The phylogenic analysis based on seven cultivars showed two clades, separating Rama Tulsi (*O. tenuiflorum*) from the other analised individuals, four cultivars belonging to *O. basilicum*, one from *O. africanum* and one *to O. americanum*. This implies that the chloroplast regions used in the present study are useful to identify *O. tenuiflorum* from other species from the *Ocimum* genus.

The chloroplast genome of basil is a source of reliable and valuable molecular markers for revealing the extent of genetic diversity and identifying different species. Future research on the phylogeny, population genetics, and evaluation of the genetic diversity of basil would greatly benefit from the study's findings.

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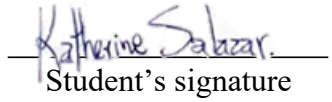

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