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BUDAPEST

EVALUATION OF BASIL SPECIFIC SSR MARKERS

Vu Phan Thien Nga

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Supervisor(s): Dr. Zsuzsanna György

Reviewers: ……..……..

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Dr. Attila Hegedűs	Dr. Zsuzsanna György
Head of Department	Supervisor

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USED ABBREVATIONS

AFLP: Amplified Fragment Length Polymorphism

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

EST: Expressed sequence tag

ISSR: Inter-Simple Sequence Repeat

NCBI: National Centre for Biotechnology Information

PCR: Polymerase Chain Reaction

RAPD: Random Amplified Polymorphic DNA

RFLP: Restriction Fragment Length Polymorphism

SNP: Single Nucleotide Polymorphism

SSR: Simple Sequence Repeat

STR: Sequence Tagged Sites

TBE: Tris-Borate- EDTA

UTRs: Untranslated Regions

VNTP: Variable Number Tandem repeats

1. INTRODUCTION

Basil (*Ocimum sp.*) belonging the Laminaceae family is considered as the "king of the herbs" due to their wide distribution throughout the world. The aromatic leaves are used fresh or dried as a flavoring agent for foods, confectionery products, and beverages. Traditionally, the plant has been employed in folk medicine for its carminative, stimulant, and antispasmodic properties. The essential oil, mainly used in food industries and perfumery, also possesses antimicrobial activity (Makri and Kintzios, 2008). Most basil cultivars are cultivated in most of tropical and subtropical regions nowadays are members of *O. basilicum* (Simon et al., 1999). According to Nation et al. (1992), although readily self-pollinated, the outcrossing in *Ocimum* species is probably dominant because of the presence of pollinators. By exploitation of this flexibility in basil reproductive system, both natural and artificial selection are likely to make a large contribution in the morphological and volatile diversification of *Ocimum* genus (Pyne et al., 2018). Another factor complicating the taxonomy of basil varieties is the variation in the genome size and chromosome numbers in *Ocimum* genus. The basic chromosome number is x = 12 in *O. basilicum* is considered to be tetraploid (2n = 4× = 48) and hexaploid is *O. americanum*. However, in the diploid *O. tenuiflorum*, the basic chromosome number is x=8 (Carović-Stanko et al., 2010; Rastogi et al., 2015). According to Pyne et al. (2018), it is obvious that there is taxonomical incongruences of different basil cultivars throughout the literature and among seed distributors. The inconsistency in the taxonomical study, unknown geographic origin and the phenotypic abundance of different are served a catalyst as the complication in classification among *O.basilicum* as well as the *Ocimum* genus.

The classification of *Ocimum* species based on morphological characters was firstly implemented by Paton et al. (1999). The construction of phylogenetic map based on the morphological features are used to demonstrate the diversity among *Ocimum* genus (Chutia et al., 2016; Chowdhury et al., 2017). In addition, the volatile composition is another important parameter for the classification of different basil chemotypes into different geographical origin (Varga et al., 2017). However, the reliance in two mentioned parameters above in plant breeding probably leading the false decision with regard to selection and crossing among different germplasm.

The molecular phylogenetic approaches in *Ocimum sp.* have been employed with different non-specific markers such as RAPD (Singh et al., 2004; De Masi et al., 2006)

and ISSR (Aghaei et al., 2012; Kumar et al., 2016). Although each of them is demonstrated to be an efficient tool for genetic diversity, there are some limitations in non-specific molecular markers. Therefore, SSRs are desirable markers for the evaluation genetic diversity due to their multi-allelic feature, polymorphism and wide distribution across the genome (Kalia et al., 2011). Expressed sequence tag (EST) derived SSRs (EST-SSRs) are particularly promising for the assessment of genetic variation owing to their location in coding regions and transferability across related germplasm (Ramchiary et al., 2011).

The objective of this study is to identify the suitable EST-SSRs that were already designed for *Ocimum sp.* and Laminaceae family in previous studies for the evaluation the genetic diversity of different basil cultivars.

2.LITERATURE REVIEW

2.1. *Ocimum sp.*

2.1.1. Origin and distribution of *Ocimum sp.*

Basil (scientific name: *Ocimum sp.*), one of the most wildly distributed herb throughout the world, is indigenous to Middle East and Southeast Asia (India, Pakistan, Iran, Thailand, and other countries) and can be observed growing wild in tropical and sub-tropical regions (Makri and Kintzios, 2008). Basil was probably brought to Europe and America in the sixteenth and in the seventeenth centuries from India (Sullivan, 2009).

According to Makri and Kintzios (2008), owing to its wide distribution, basil is often referred to as the "king of the herbs". Several name derivations and beliefs are associated with basil, but the common name basil is most likely came from the Greek words basileus meaning "king" or basilikon meaning "royal". The botanical name *Ocimum* is derived from the Greek meaning "to be fragrant". According to Khosla (1995), the three diversity centers of basil could be determined based on their geographic data including tropical and subtropical regions of Africa, tropical Asia and tropical part of America (Brazil). Among that, the tropical Africa is more likely to be the original centre since nearly two-thirds of known species was reported in this region.

2.1.2. Traditional use of *Ocimum sp.*

Basil is the herb commonly used in both Western and Eastern culinary arts (Italian, Thailand and Vietnam). *Ocimum sp.*is used as a folk medicine in many regions around the world due to their pharmacological metabolites present in it. In traditional medicine, *Ocimum* species has been used to get rid of headaches, coughs, diarrhea and kidney malfunctions as well as externally for insect stings, snake bites and skin infection (Dharsono et al., 2022). In terms of Eastern traditional medicines, *Ocimum basilicum* is used to prevent and treat diabetics and cardiovascular disorders by Uyghurs (a Turkic ethnic group) while for treating pimples on face by Indians. Regarding folk medicine in Western countries, basil is used as a therapy for aches and pains and as a sedative in Bulgaria and Spain, respectively. In addition, basil could be applied to aromatic and flavor industries, and for extending the food products shelf life (Purushothaman et al., 2018).

In addition, almost all accessions belonging to *Ocimum basilicum* are grown for culinary use while *Ocimum kilimandscharicum* and *Ocimum micranthemum* are mainly

used for medicinal purpose due to their strong scent. *Ocimum sanctum* and *Ocimum thrysiflora* are used in some religious ceremonies and as ornamental plants, respectively (Rindels, 1997).

2.1.3. Cultivation of basil

Basil may be planted directly from seed or transplanted, which grows as a perennial in tropical climates and as an annual in temperate regions. While other members of the Lamiaceace family grow well under competitive circumstances, basil prefers little competition for sun and water (Sullivan, 2009). It is essential for basil to be planted in full sun due to their cold intolerance. In addition, heavy nitrogen fertilization should be circumvented for basil since excessive nitrogen affects oil content and flavor. In terms of harvest, removing terminal growth could promote the four sets of true leaves to be retained on the plant. This encourages bushier growth and increased yield. For best foliage flavor, basil should be cut before flowering because leaf flavor changes after flowers open (Rindels, 1997).

2.1.4. *Ocimum sp.* in Vietnam

The basil cultivars in Vietnam mainly belong to *Ocimum gratissimum* L. and *Ocimum basilicum* L., which is widely grown as a kitchen herb, culinary herb and ornamental herb throughout the country. The researches on *Ocimum* species are limited, mainly focusing on the essential oil content in these species and the effect of purification methods on oil quality (Huong et al., 2020; Hai et al., 2022).

2.1.5. Taxonomy

Ocimum genus is a member of the Lamiaceace family, which comprises 30–160 annual and perennial herbs and shrubs native to the tropical and subtropical regions of Asia, Africa, and Central and South America (Kalita and Khan, 2013; Carovic-Stanko et al., 2010).

According to Kumar et al. (2018), the variability in morphological as well as chemical characteristics within the *Ocimum* genus, its taxonomy and phylogenetic relationships is still controversial. From the previous studies, seven species of *Ocimum* was identified including *O. basilicum* L., *O. americanum* L., *O. tenuiflorum* L., *O. gratissimum* L., *O. kilimandscharicum* Gurke, *O. filamentosum* Forssk. and *O. africanum* Lour (Suddee et al., 2005; Carovic-Stanko et al., 2010).

According to Simon et al. (1999), most of basil cultivars widely distributed in the market belong to the species *O. basilicum*. From the studies of Darrah (1980 and 1984), the *O. basilicum* cultivars was classified into seven types:

(1) tall slender types, which include the sweet basil group;

(2) large-leafed, robust types, including 'Lettuce Leaf' also called 'Italian' basil;

(3) dwarf types, which are short and small leafed, such as 'Bush' basil;

(4) compact types, also described, *O. basilicum var. thyrsiflora*, commonly called 'Thai' basil;

(5) purpurascens, the purple-colored basil types with traditional sweet basil flavor;

(6) purple types such as 'Dark Opal', a possible hybrid between *O. basilicum* and *O. forskolei*, which has lobed-leaves, with a sweet basil plus clove-like aroma;

(7) citriodorum types, which includes lemon-flavored basils.

Holy basil (scientific name: *Ocimum sanctum* L. Syn *Ocimum tenuiflorum* L.) is frequently known as Tulsi in the majority of languages in India, is a herb indigenous to the tropical and subtropical Asia and is nowadays brought and grown in other tropical areas (Baliga et al, 2013). 'Sanskrit' is considered as the origin of Tulsi, which means "matchless one" (Mondal et al., 2009). Thulasi, Vishnupriya are other names of Tulsi in some Indian languages. In traditional medicine, holy basil is known as "The Queen of Herbs," "The Incomparable One," and "The Mother Medicine of Nature" (Rastogi et al., 2015). According to Jamshidi and Cohen (2017), three common types of tulsi are properly identified and classified. Rama or Sri tulsi (green leaves) and Krishna or Shyama tulsi (purplish leaves) belonging to *Ocimum tenuiflorum* (or *Ocimum sanctum* L.) could be straightforwardly distinguished due to their botanical and phytochemical distinctions, while the third type of tulsi is Vana or wild/forest tulsi (scientific name: *Ocimum gratissimum*) possessing dark green leaves. There is a wide range of morphological and phytochemical characteristics even in secondary metabolites in different tulsi cultivars Compared to other *Ocimum* species, holy basil can be differentiated by the color of their yellow pollen, high levels of eugenol, and smaller chromosome number. Traditionally, *Ocimum tenuiflorum* and *Ocimum gratissimum* is used as the same medicinal cure of diseases despite of the existence of six times less DNA in *Ocimum tenuiflorum*.

Another type of Tulsi called 'Temperate Tulsi' (scientific name: *Ocimum africanum* Lour.) is a lemon scented basil, which is probably a hybrid of *O. basilicum*

L. and *O. americanum* L. (Paton and Putievsky, 1996). According to Uttar Pradesh State Biodiversity Board (UP), *O. americanum* (Syn. *O. canum*) and *Ocimum africanum* Lour. were commonly got confused by Indian scientists. There is difference in chemical compostion despite of similar appearance. That is why almost all experimental work referred *Ocimum africanum* Lour. as camphor rich chemotype *O. americanum* in India. Different Ocimum species cultivated in India are shown in Figure 1.

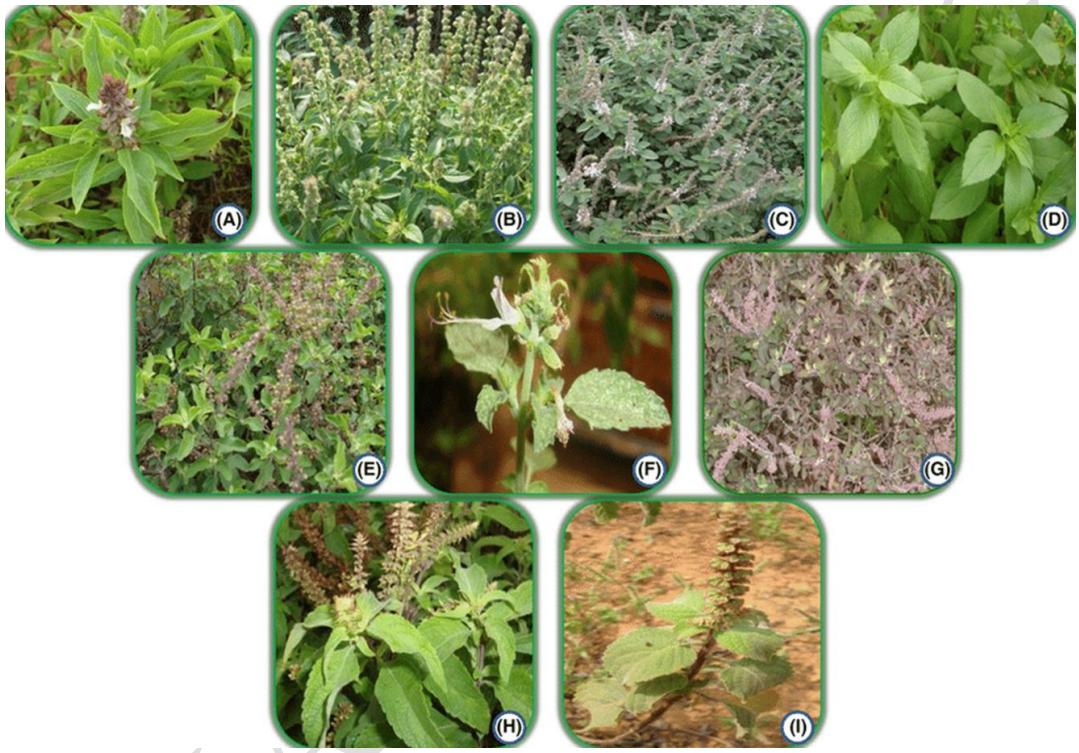

Figure 1. Different *Ocimum* species from India. (A) *Ocimum basilicum*, (B) *Ocimum americanum*, (C) *Ocimum kilimandscharicum*, (D) *Ocimum africanum*, (E) *Ocimum tenuiflorum* var. Ram tulsi, (F) *Ocimum adscendens*, (G) *Ocimum tenuiflorum* var. Krishna tulsi, (H) *Ocimum gratissimum*, (I) *Ocimum viride* (Kumar et al., 2016).

2.1.6. Morphological description

According to Morales et al. (1993), a considerable variability of qualitative traits of *Ocimum* species can be observed through their height of plants, leaf size and color (from green to dark purple), flower color (white, red, lavender or purple), shape and as well as flowering time and aroma.

In terms of the height of plants, the variants belonging to *O. gratissimum* L. are the tallest ranging from 125 to 260 cm while the shortest is *O. americanum* L. (20-60 cm). The variants from *O.* -×- *africanum* Lour. and *O. basilicum* L. are 45-100 cm tall.

The height of accessions from *O. kilimandscharicum* Guerke. and *O. tenuiflorum* L. range from 60 to 120 cm and from 70 to 150 cm respectively (Chowdhury et al., 2017).

The leaf is generally simple, petiolate and the leaf blade is ovate with a rounded base, oblique, and the apex is acute. The size of basil leaf varied significantly from 3.5 cm^2 in *O. citriodorum* to around 45 cm^2 in *O. citriodorum*. The aerials of red and white is a prominent feature for differentiation of *O. sanctum* var. Rama and Shyama. Another noticeable parameter is the divergence in colors of stem and leaf, ranging from red, purple-green, and green among the different varieties of the *O. basilicum* L distributed in Iran. Singh (2012) used the number of leaf veins to show that *O. americanum* was described to have seven distinct veins, and the mid-vein reached the apex, while *O. tenuiflorum* has nine distinct veins, and the mid-vein does not reach the apex. The typical inflorescence of *Ocimum sp.* is a thyrse composed of opposite 1–3-flowered cymes. The calyx is generally a short tube or funnel-shaped; it is straight or slightly curved. The corolla is formed forward (sometimes bent downwards), larger upper lip and a smaller lower one and declinate stamens. The posterior lip of the corolla consists of four lobes. There are always four stamens, an anterior pair that attaches near the corolla mouth and a posterior pair that connects close to the corolla base. The variability in size of basil seeds distinguished on the basis of the phenotype, cultivating location, and moisture content. The differences between varieties sometimes determined by their leaf color. *O. citriodorum* and *O. basilicum var. Thyrsiflorum* are generally discriminated through their complex polysaccharide structure since the unique mucilaginous characteristic of their seed after soaking in water (Tangpao et al., 2022). The morphological features of leaves from different *Ocimum* species are shown in Figure 2.

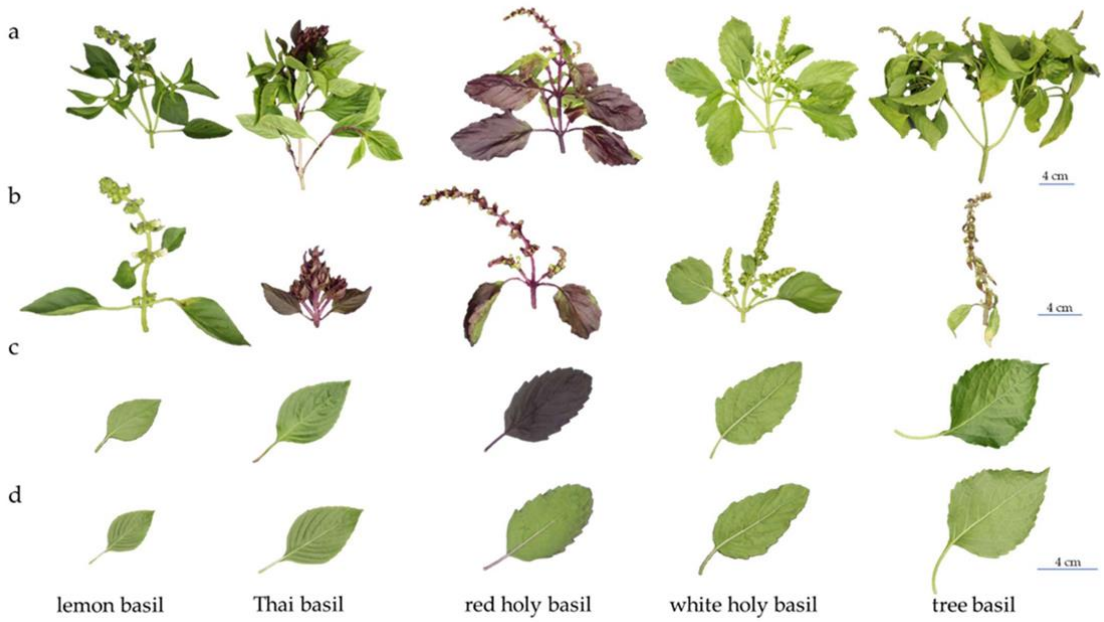

Figure 2. Morphological characteristics of some *Ocimum* species; aerial part (a) inflorescence (b) upper (c) and lower (d) leaf surface of lemon basil (*O. citriodorum*), Thai basil (*O. basilicum* var. *thyrsiflorum*), red holy basil (*O. sanctum* var. Rama), white holy basil (*O. sanctum* var. Shyama) and tree basil (*O. gratissimum*) (Tangpao et al., 2022).

2.1.7. Chemical composition

O. basilicum is mainly composed of about 20 compounds such as linalool, estragole, methyl eugenol, 1, 8-cineole, etc., which has been identified by GC-MS. Linalool (52.42%), methyl eugenol (18.74%), 1,8-cineol (5.61%) are the major compounds in *O. basilicum* which are isolated by the HPLC method. Myrcene, borneol and neral are the minor compounds present at 5%, 9%, 8% w/w respectively (Radulovic et al., 2013). Camphor, limonene, thymol, citral, α-linalool, β-linalool, estragole, are the monoterpene's of *O. basilicum*. Methyl eugenol makes up the largest percentage among different active compounds found in *Ocimum basilicum*. The existence of chichoric acid was identified in the fresh basil leaves (Lee et al., 2009). Crude extract of different botanical parts of basil are a rich source of polyphenol compounds. According to Phippen et al. (1998), the intensely purple pigment of the flowers is determined by the presence of anthocyanins. Fourteen different anthocyanins have been isolated among that 11 has cyanidin based pigments and three has peonidin based pigments.

The essential oil of *Ocimum sp.* have been demonstrated antibacterial activities (Kaya et al., 2008; Moghaddam et al., 2014; Yamani et al., 2016). The methanol extract from *Ocimum basilicum* L. leaves showed antibacterial activities against six strains; namely *P. aeruginosa*, *Shigella* sp., *L. monocytogenes*, *S. aureus* and two different strains of *E. coli* (Kaya et al., 2008). According to Moghaddam et al. (2014), the essential oil had the highest antibacterial activity against *Brenneria nigrifluens*. Moreover, a moderate inhibitory effect was observed on *Pseudomonas syringae* pv. *Lachrymans*, *Pantoea stewartii* subsp. *indologenes*, *Ralstonia solanacearum*, *Rhodococcus fascians* and *Xanthomonas citri*. The oils of *Ocimum tenuiflorum*, at concentrations of 4.5 and 2.25% completely inhibited the growth of *Staphylococcus aureus* (including MRSA) and *Escherichia coli*, while the same concentrations only partly inhibited the growth of *Pseudomonas aeruginosa* (Yamani et al., 2016).

The antifungal properties of basil has been the subject of many studies. Balakumar et al. (2011) showed that the leaf extracts of *Ocimum sanctum* has inhibitory effect on a wide range of dermatophytic fungi. According to Abou El-Soud et al. (2015), the oil concentration of *Ocimum basilicum* L. at 1000 ppm showed complete inhibitory effect on *Aspergillus flavus*, whereas all of the concentrations tested (500, 750 and 1000 ppm) significantly inhibited the aflatoxin B1 production.

In addition, extracts of *Ocimum sp.* was demonstrated to markedly inhibit HIV-1 reverse transcriptase and platelet aggregation (Yamasaki et al., 1998).

2.2. Genetic diversity assessment

Genetic diversity is a part of biological diversity, which is referred to as any variation of genetic materials (nucleotides, genes, chromosomes, or genomes) in all organisms in the ecosystem that they occur at a given time (Ramanatha and Hodgkin, 2002; Fu, 2015).

A variety of laboratory-based techniques such as allozyme or DNA analysis is used to assess the genetic diversity within and between populations, which measure molecular levels of variation directly. Genetic diversity may be assessed through morphological, and biochemical characterization and evaluation. Morphological characterization does not require any expensive technology but their limitations are the dependence on the environmental condition and developmental stages of the plant. While the biochemical analysis gives their result through specific banding patterns by the separation of proteins. However, the resolution of diversity is restricted due to the availability of limited number

of enzymes. In stark contrast, molecular asssessments could be analyzed the variation by an assortment of DNA molecular. A molecular marker can be defined as a genomic locus, detected through probe or specific primers which, in virtue of its presence, distinguishes unequivocally the chromosomic trait which it represents as well as the flanking regions at the 3' and 5' extremity. It is not necessary a molecular marker to associate with the manifestation of a genetic trait into a phenotype. They are superior to traditional, phenotype-based assessements as they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell. Another important feature is that molecular markers are not disturbed by environmental, pleiotropic and epistatic factors (Mondini et al., 2009). These features of DNA-based markers mentioned above make them more superior and popular in studies of genetic diversity compared to other types of evaluation.

2.2.1. Morphological characterization

The first phylogenetic map of different accessions belonging to *Ocimum* genus (Chutia et al., 2016; Chowdhury et al., 2017), *O.* × *africanum* and *O. basilicum* (Makmur et al. 2020) is based on morphological features (leaf, stem and flower). The variability in quantitative morphological parameters may or may not correlate with their geographical distances (Chutia et al., 2016).

2.2.2. Biochemical analysis

Another parameter for *Ocimum* species classification is their aroma compound. Therefore, the determination of genetic diversity based on chemical composition of the essential oils in basil has been the subject of many studies since the early of 2000s (Carovic-Stanko et al., 2011a; De Masi et al., 2006; Kwee and Niemeyer, 2011; Labra et al., 2004; Varga et al., 2017). The dendrograms in these studies showed the relationships among different cultivars of *O. basilicum* based on essential oil content. Nowadays, over 140 compounds have been successfully identified in basil essential oil as a result of the development of analytical techniques throughout the years. The predominant groups in the essential oil of *O. basilicum* is monoterpenes and phenylpropanoids. The physical properties of the oil is determined largely by its proportions of different constituent substances. Different chemotypes have also been classified predominantly on their geographical origin, each with a specific chemical constitution. The European chemotype (Italy, France, Bulgaria) has high concentrations of linalool and methyl chavicol (estragole) while the eugenol-rich is a typical

chemotype from Russia and North Africa. The Tropical chemotype is defined by trans-methyl cinnamate, which originate from India, Guatemala and Pakistan. The Reunion chemotype derived from Thailand, Madagascar and Vietnam is typified by methyl chavicol (Varga et al., 2017).

2.2.3. Molecular markers analysis

DNA markers are the foremost broadly utilized markers basically due to their plenitude. DNA marker techniques are used to reveal sequence differences among species or individuals within a species. Genetic differences among individuals in a species or population are usually as a consequence of abnormal pairing of sister chromosomes or recombination that rearranges the chromosomes, for example, insertions, deletions, inversions, translocation events, reduplication or any mistakes in DNA replication then being tandemly repeated (Yang et al., 2015). Evolution of molecular markers has been chiefly driven by the throughput and cost of detection method and the level of reproducibility. The hybridization-based method like RFLP used to be the most popular molecular marker in plant molecular genetics but the detection of RFLP requires big-budget, proficient technician and time to perform which made these markers ultimately obsolete (Mammadov et al, 2012). Invention of PCR technology and the application of this method for the rapid detection of polymorphisms overthrew low-throughput RFLP markers leading to the emergence of new generation of PCR-based markers in the beginning of nineties. RAPD, AFLP, and SSR markers are prevalent PCR-based markers that have been used in scientific researches on plant systems. RAPDs are able to simultaneously detect polymorphic loci in various regions of a genome. However, the non-specific binding of short, random primers is a main culprit of the indistinctive polymorphism and low reproducibility. Compared to RAPDs, AFLPs are non-specific too, in spite of that the level of their reproducibility and sensitivity is improved extensively due to the longer +1 and +3 selective primers and the presence of discriminatory nucleotides at 3′ end of each primer. However, AFLP markers cannot be applied widely in molecular breeding due to their labor and time-consuming detection method, which was not amenable to automation either. In stark contrast, all limitations of the above-mentioned DNA marker technologies was eliminated in the application of SSR markers in plant genome. SSRs were no longer unspecified; they were highly reproducible, highly polymorphic, and amenable to automation (Amiteye, 2021).

The first researches on *Ocimum* taxonomy and phylogenetic relationship were implemented with Random Amplified Polymorphic DNA (RAPD). By using 20 different random primers, Singh and his colleagues (2004) characterized 32 accessions belonging to five Ocimum species including *O. basilicum* L. (14 accessions), *O.teniuflorum* L. (14 accessions), *O.gratissimum* L. (2 accessions), *O.kilimandscharicum* Guerke (1 accession) and *O. americanum* L. (1 accession). According to De Masi et al. (2006), the seven RAPD markers used showed the degree of polymorphism to 57.5% (23 polymorphic bands out 40 bands scored) on 12 accessions belonging nine cultivars of *Ocimum basilicum* L. in Italy.

The Amplified Fragment Length Polymorphism (AFLP) technique was used to analyze the genetic distances of nine different accession of *O. basilicum* L. (Labra et al., 2004), 120 *Ocimum* accessions belonging to five species and varieties named: *Ocimum ciliatum*, *Ocimum minimum*, *Ocimum basilicum* var. *purpurascens*, *O. basilicum* var. *dianatnejadii* and *O. basilicum* var. *alba* (Moghaddam et al., 2011).

The Inter Simple Sequence Repeat (ISSR) technique was used to determine genetic diversity of 50 Iranian basil (*Ocimum basilicum* L.) accessions (Aghaei et al., 2012) and 14 cultivars belonging seven different species of *Ocimum* (Kumar et al., 2016).

Various random, non-specific marker techniques were applied to identify the taxonomy and phylogenetic relationship of *Ocimum* genus. Although each of them is demonstrated to be an efficient tool for genetic diversity between a wide range of accessions belonging to *O. basilicum* L. and between various species of *Ocimum* genus. The limitations of non-specific markers are low reproducibility and low repeatability. It means that the gel electrophoresis banding pattern depends on the random amplified products and may be different in the repeated experiments. All of them is dominant marker which cannot distinguish between homologous and heterologous individuals.

2.2.4. SSR marker

Simple-sequence repeats (SSRs), also known as microsatellites, are short tandem repeated motifs that may vary in the number of repeats at a given locus (Tautz, 1989). SSR markers have many advantages over other molecular markers, such as genetic co-dominance. They are multi-allelic, relatively abundant, widely dispersed across the genome, and easily and automatically scored (Powell et al., 1996). The number of

repetition units varies greatly among plant species. The repetition sequence is usually simple, consisting of two, three or four nucleotides, also known as di-, tri- and tetranucleotide, respectively, in which, the most abundant repeats found in most of the eukaryotes is dinucleotide repeats (e.g. $(CA)_n$ and $(GA)_n$) (Fan and Chu, 2007). The flanking regions tend to be conserved within species, although sometimes they can also be preserved at a higher taxonomic level. The principle of SSR marker is shown in Figure 3.

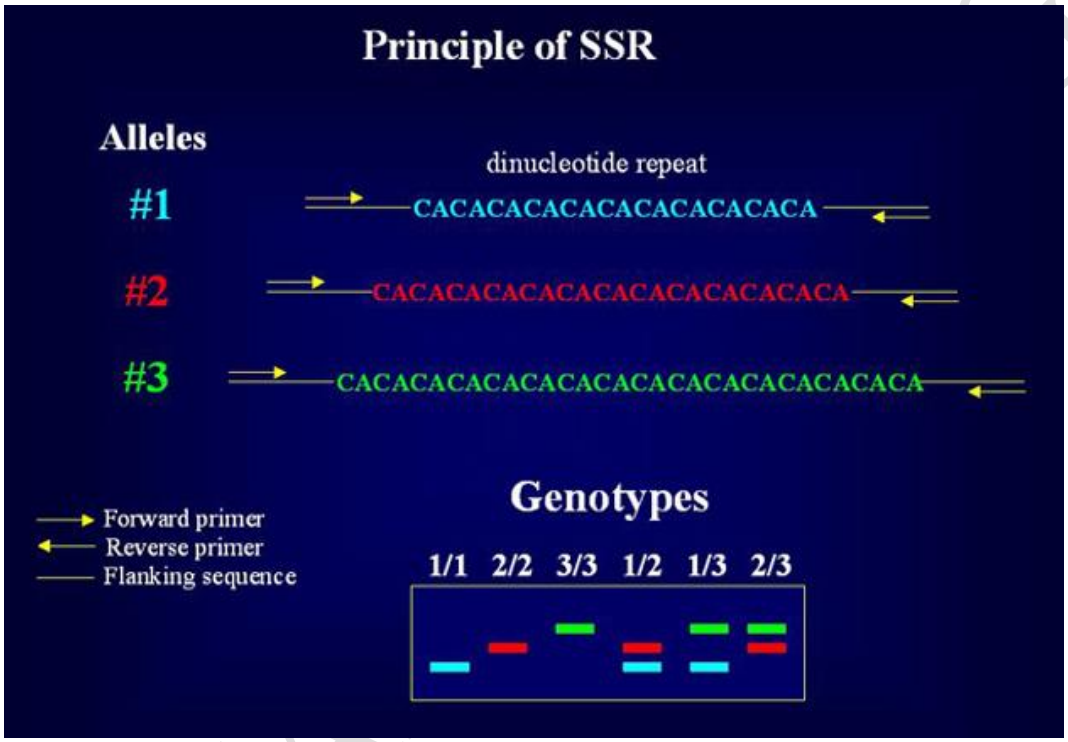

Figure 3. Principle of microsatellite (SSR marker) (http://www.cdfd.org.in/2010/10/12/analysis-of-simple-sequence-repeats-(ssr)).

2.2.5. SSR markers used in genetic diversity analysis of *Ocimum sp.*

Thanks to the advancement in genome sequencing and molecular marker technique, the Simple Sequence Repeat (SSR) markers from expressed sequence tags (ESTs) databases was developed to analyze genetic diversity different accessions belonging to four species of *Ocimum* genus (Mahajan et al., 2015) and 180 *Ocimum* spp. accessions (Pyne et al., 2018). In comparison with other markers, SSR markers have advantageous features including abundant genomic distribution, high levels of allelic polymorphisms, co-dominant mode of inheritance and high reproducibility (Kalia et al., 2010). In addition, SSR markers derived from non-coding regions show polymorphism at cultivar level while genic-SSRs (EST-SSRs), derived

from coding sequences, are polymorphic across species (Scott et al., 2000). Phylogenetic analysis revealed that *O. basilicum* was genetically closer to two accession of *O. canum* (Oc I and Oc II) while *O. gratissimum*, *O. viride* and two accessions from a species belonging to Lamiaceae family - *Hyptis suaveolens* (Hs I and Hs II) clustered together (Mahajan et al., 2015). According to Pyne et al. (2018), multivariate analysis of the genetic similarity data grouped accessions belonging to *Ocimum* spp. into three major clusters. In general, the first cluster was demonstrated lower genetic diversity due to the majority of accessions belonging to *Ocimum basilicum* while the accessions of second cluster derived from *O. basilicum*, *O. citriodorum* or *O. americanum*. The accessions of third cluster mainly belong to *O. americanum, O. tenuiflorum, O. kilidmandsharicum, O. x africanum, O. basilicum*, in which several of them from seed companies and USDA-GRIN are phenotypically indistinguishable sources.

3. MATERIALS AND METHODS

3.1. Plant materials

Leaves of 28 genotypes and cultivars of *Ocimum* originating from different parts of the world were evaluated in the experiment. Fresh plant material derived from the genebanks of MATE Institute of Horticulture, Department of Medicinal and Aromatic Plants and Center for Ecological Research, Lendület Seed Ecology Research Group (Table 1). Leaves were directly used for DNA isolation.

3.2. DNA extraction

The DNA isolation from leaf samples (approx. 1-2 pieces) was done by NucleoSpin Plant II DNA kit (Macherey-Nagel, Germany) using manufacturer's recommendations. Nano Drop ND- 1000 spectrophotometer (Bioscience, Budapest, Hungary) was used to verify DNA concentration and quality parameters. Isolated DNA was stored at -20°C in the Dept. of Genetics and Plant Breeding until used. Some cultivars were duplicated in the experiment as a control.

3.3. SSR primers

A set of 13 SSR primers pairs (four *Salvia spp.* primer pairs) were used for assessing genetic diversity and were selected based on previous study performed. Primer sequences are shown in Table 2. The forward primers were fluorescently labelled with 6-FAM.

3.4. PCR (Polymerase Chain Reaction)

PCR amplifications were performed in a Swift MaxPro thermocycler (Esco Healthcare Pte, Singapore) using the program SSR with the following cycling parameters: initial denaturation at 98°C for 4 min; followed by 35 cycles of 98°C for 30 s, 50°C for 40 s, 72°C for 60 s; and a final synthesis at 72°C for 5 min. Amplifications were performed in final reaction volume of 15 µl with 1 µl DNA sample, 5X PCR buffer, 0.4 µl 1% of DMSO, 0.3 µmol of each 5' and 3' end primers and 0.3 µl Phire Hot Start polymerase (Thermo Scientific, Szeged, Hungary) and dNTP and sterile distilled.

Table 1. Names and codes of the studied *Ocimum* accessions.

Code	Species	Cultivar or accession name
S8	*Ocimum basilicum*	Vietnamise
S9	*Ocimum basilicum*	'Mittelgroßblättriges Grünes'
S10	*Ocimum basilicum*	'Cinnamum'
S15	*Ocimum basilicum*	'Dark Opal'
S16	*Ocimum basilicum*	'Fine Verde'
S17	*Ocimum basilicum*	'Anisse'
S20	*Ocimum basilicum*	Thai long
S21	*Ocimum americanum*	Togo basil
S23	*Ocimum basilicum*	Licorice scent
S24	*Ocimum americanukm*	Lemon basil
V1	*Ocimum basilicum*	'Purple Ruffles'
V2	*Ocimum basilicum*	'Red Rubin'
V3	*Ocimum basilicum*	'Rubin Kaira' (Stavia)
V4	*Ocimum basilicum*	'Fine Verde Compacto'
V5	*Ocimum africanum*	Tulsi Temperate
V6	*Ocimum basilicum*	Mrihani
V7	*Ocimum basilicum*	'Aromatto'
V15	*Ocimum basilicum*	'Barhat'
V17	*O. tenuiflorum*	Rama Tulsi
V18	*Ocimum gratissimum*	Tulsi Vana
V20	*O. africanum*	Penang Lemon (Pinang)
V22	*Ocimum basilicum*	'Raspberry Muscat'
V23	*Ocimum basilicum*	(Fioletovij) 'Karlik'
V24	*Ocimum basilicum var. thyrsiflorum*	'Siam Queen'
V25	*O. sanctum/tenuiflorum?*	'Holy'
V26	*Ob/O. x citriodorum??*	'Spicy Globe'
S26	*Ocimum basilicum*	Iranian lemon
S22	*Ocimum basilicum*	Clove Scent

3.5. Electrophoresis

The verification of PCR products was done by Agarose Gel Electrophoresis (AGE). The amplified products were loaded on 1% ethidium-bromide stained agarose Tris-Borate-EDTA (TBE) gels and run for 30-40 minutes at 80 Volts. To determine size and quality of DNA products a 100bp DNA ladder was used (Fermentas, Szeged, Hungary). UV transilluminator was used to photograph the separated DNA samples under the Gel documentation system.

Table 2. The name and sequence of the applied SSR primer pairs.

Primer codes	Forward and reverse primer sequence	Repeat motif	References
OB158	CAGCTGAGTATTTCAAGGGG TCTCCGGACTGTAGTGTGTG	Not known	Mahajan et al. (2015)
OB174	CCTTTAGACCTCGCAAGAGA GTTTGGTAGAGATGGGCAGA	Not known	
OB181	CACCCTTGTTCACCCTATCA CCCTAAAATTGGGGAACC	Not known	
OB212	GAGAAGCTGGGTCCTATTGA AATGGTAATCGGCTCCCT	Not known	
OBNJR2sg04	ACGATATGAGACATGGGCCT CGCAGGTACAAGCTTCTCAA	$(GA)_{17}$	Pyne et al. (2018)
OBNJR2sg30	GCCAAATAATTCCTATCCGGT CTTGGCTTTGGGAGATTCAC	$(AG)_{22}$	
OBNJR2sg33	GCCTCTCCCTCCTCCATAAC AGGCGACGAGCATGAAGTAG	$(AC)_{16}$	
OBNJR3sg145	GGAAATGTGGTCGTTATTCACA CCAAAGGAAGCGACAATGAT	$(GCT)_{8}$	
OBNJR3cn240	AACACACAAAGATCCAAACCC TTATTTCCCAACCCACTACCA	$(ATA)_{16}$	
LAB008	AGTATTTGTGCCGAGGGTTG ACAGGAAAGGGAGAGGGAGA	$(TC)_{15}$	Karaca et al. (2013)
LAB029	AATTCGGCACGAGGTAACAC TGGGGAAATCGGGTATGAC	$(GCA)_{7}$	
LAB030	TGCCTCGACTACTATTGAGCGTA GTGTCTTATTTCTTCGTGGGTCA	$(CA)_{10}$	
LAB042	TCGGCACGAGGTATCAGTCT CCAGTTCCTCACCGACACTT	$(TC)_{10}$	

3.6. Fragment length analysis

Fragment length analysis was done by capillary electrophoresis where amplified SSR fragments were run on an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary) at Biomi Ltd. (Gödöllő, Hungary). Fragment length determination was done by a Peak Scanner software 1.0 (Applied Biosystems 2006) manually. Results were collected in MS Excel.

4. RESULTS AND DISCUSSION

4.1. Markers OB212, OB181, OB174, LAB008, LAB029, LAB030, LAB042

In the beginning all SSR markers were tested on a restricted number of ten basil genotypes, to see if amplification was successful and amplified SSR alleles were polymorphic. The allele sizes are summarized in Table 3. As it can be seen from the table, OB212, LAB008 and LAB030 were monomorphic, resulting one homozygous allele in all samples. OB174 and LAB042 resulted two kinds of alleles, while OB181 resulted three. LAB029 also resulted two allele sizes and an additional out of the expected range. Due to the low polymorphism we decedied to not work further with these markers.

Table 3. The allele sizes of different basil samples with primer OB212, OB181, OB174, LAB008, LAB029, LAB030, LAB042

Expected size / Code	OB212	OB181	OB174	LAB008	LAB029	LAB030	LAB042
	452	373	398	134	232	210	246
S8	151	480	389; 391	346		267	282
S9	151	464; 477	389	346	279	267	282, 292
S10	151	480	391	346	212		282, 292
S15	151	480	391	346	279	267	282, 292
S16	151	464; 477	389	346	279		282, 292
S17	151	464, 480	389	346			
S20		480					
S21	151	480	389	346	279, 348	267	282, 292
S23	151						
S24	151	456, 481	389	346	212, 279	267	282, 292

4.1.1. Markers OB212, OB181, OB174

The basil primer pairs (OB212, OB181, OB174) were designed from the expressed sequence tags (ESTs) databases of *Ocimum basilicum* (Mahajan et al., 2015). According to Scott et al. (2000), SSR markers derived from non-coding regions show polymorphism at cultivar level while genic-SSRs (EST-SSRs), derived from coding sequences, are polymorphic across species. On the other hand, the EST-SSR primers are designed from the flanking regions of genic SSRs, which are conserved even at genus level. This enabling the use of EST-SSRs markers for comparative analysis.

In terms of OB212, the amplified allele size was significantly lower than the expected product size as the original paper mentioned. In addition, the amplification products only showed the homologous allele size in nine basil samples. This result just

demonstrated that tested samples belong to the *Ocimum* genus but could not showed the genetic variation among different basil species.

In case of OB181, the amplified allele sizes were significantly lower than the expected product size as the original paper mentioned. Among nine out of ten samples giving the amplified products, the polymorphism was illustrated in four basil samples (S9, S16, S17 and S24). Interestingly, the fragment sizes identified from amplification product of S24 – Lemon basil are different from other tested basil samples. This could be because this samples belong to *Ocimum americanum* while the majority of tested basil samples are members of *Ocimum basilimum* except for S21 – Togo basil. Figure 4 illustrates the chromatogram of S17 - 'Anisse' with marker OB181

In terms of OB174, the amplified allele sizes were approximately similar to the expected product size as the original paper mentioned. Among eight out of ten samples giving the amplified products, the polymorphism was illustrated in all of them with two fragment sizes at 389 and 391 basepairs (bp).

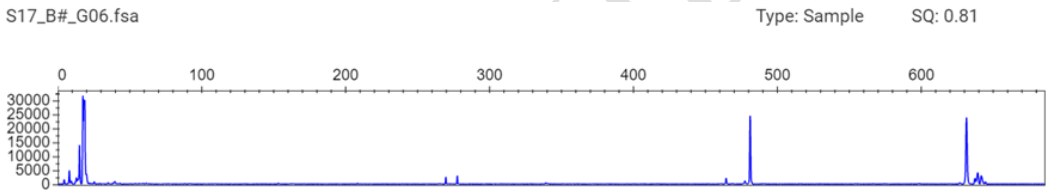

Figure 4. Example of the fragment length analysis of sample S17 - 'Anisse' with marker OB181.

4.1.2. Markers LAB008, LAB029, LAB030, LAB042

According to Karaca et al. (2013), primer pairs (LAB008, LAB029, LAB030, LAB042) were designed from the expressed sequence tags (ESTs) databases of three *Salvia spp*. (*S. fruiticosa*, *S. miltiorrhiza* and *S. sclarea*), which are members of Lamiaceae family. In the original paper, EST-SSR markers also were used for the amplification of different genera of Lamiaceae family including *Ocimum* species. In the experiment of investigation of the cross-genera transferability of microsatellite markers, nine primer pairs could produce amplified products with the *Ocimum basilicum*. That is why primer pairs (LAB008, LAB029, LAB030, LAB042) were decided to used with selected basil samples.

In terms of LAB008, LAB030 and LAB042, the amplified products resulted significantly higher allele sizes than the expected product size as the original paper mentioned. In addition, the amplification products only showed the homologous allele

size in all tested basil samples. This result just demonstrated that tested samples belong to the *Ocimum* genus but could not showed the genetic variation among different basil species. Figure 5 illustrate the chromatogram of S24 - Lemon basil with marker LAB030.

In case of LAB029, the amplified allele sizes were higher than the expected product size as the original paper mentioned. Among six out of ten samples giving the amplified products, the polymorphism was illustrated in three basil samples (S10, S21 and S24). The polymorphic microsatellites are present in two cultivars (S21 and S24) of *O. americanum* while S10- 'Cinnamum' belongs to *O. basilicum*.

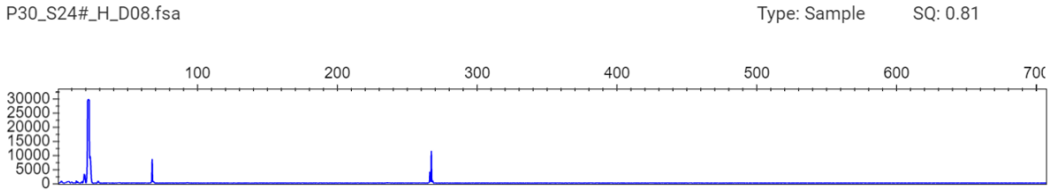

Figure 5. Example of the fragment length analysis of sample S24 - Lemon basil with marker LAB030.

4.2. Marker OB158

OB158 was the first primer pair that we tested with most of the basil samples. In the first trial, the amplification was successfully performed in 25 out of 39 tested samples (Figure 6-A). Those DNA samples which did not give any amplification were repeatedly used in a following PCR. At the second time, most of the remaining samples (S17 and S20) also showed amplification (Figure 6-B).

In each sample more than two fragment sizes were identified. Evaluating all fragment sizes we identified the non-polymorphic sizes, we eliminated them, and hence we attempted identification of the true microsatellite alleles. The filtered allele sizes are summarized in Table 4.

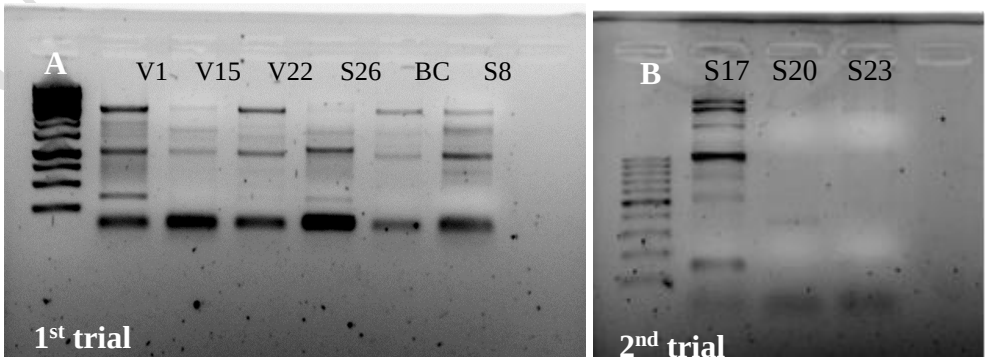

Figure 6. Gel photos of all trials of OB158.

Primer pair OB158 was designed from the EST databases of *O.basilicum* (Mahajan et al., 2015). The amplified products i.e. the allele sizes were in the range lower than the expected product size as the original paper mentioned. The polymorphism was illustrated in 10 basil samples (S20, S21, S24, V2, V5, V6, V18, V20 V23 and V26) with four fragment sizes at 129, 130, 131 and 132. Interestingly, only four of them (S20, V2, V6 and V23) are members of *O. basilicum* while S21 and S24 belong to *O. americanum*. Samples V5 and V20 are *O. africanum* cultivars whereas V18 is included in *O. gratissimum*. V26 is probably one of *Ob/O. x citriodorum* cultivars. However, the differences between four identified fragment sizes only 1-2 bp. The polymorphic microsatellites only showed in a just over one-third among 28 basil samples tested. It is concluded from the length analysis that the polymorphism with specific primer pair OB158 is not likely to be enough to demonstrate the genetic variation among tested basil samples. Figure 7 illustrates the chromatogram of S26 - Iranian lemon with marker OB158.

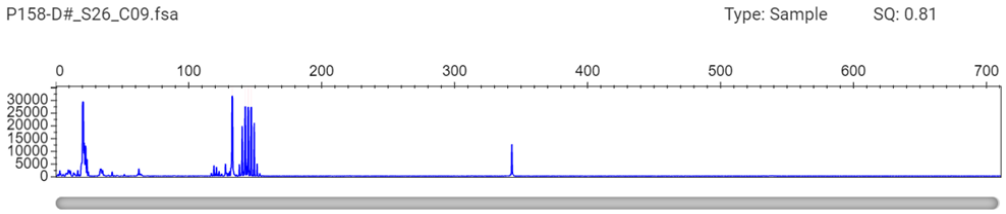

Figure 7. Example of the fragment length analysis of sample S26 - Iranian lemon with marker OB158.

4.3. Marker OBNJR3cn240

Amplification was successfully performed in most of samples for the first trial (Figure 8). Those DNA samples which did not give any amplification were repeatedly used in a following PCR. At the second and third time, most of the remaining samples (S20, V6, V22 and S22) also showed clear bands. Sample V18 showed very faint band in all trials, hence the fragment length analysis was not successful for this sample.

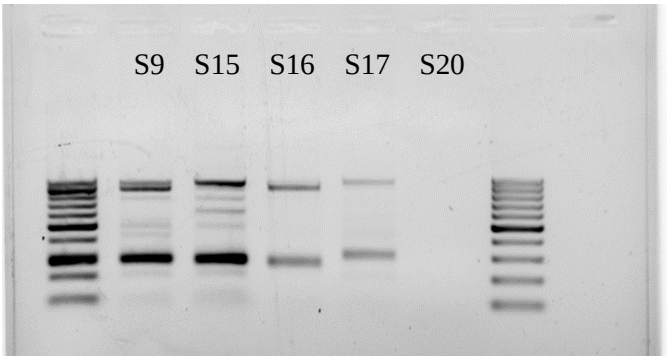

Figure 8. Gel photos of the PCR products with five basil samples of OBNJR3cn240.

In course of the fragment length analysis, more than two fragment sizes were identified in each sample. Evaluating all fragment sizes we identified the non-polymorphic sizes, we eliminated them, and hence we attempted identification of the true microsatellite alleles. The filtered allele sizes are summarized in Table 4.

By using NCBI-EST database, primer pair OBNJR3cn240 was designed from the Contig2142 (Pyne et al., 2018). Half of the amplification products give 11 allele sizes in the expected product range as the original paper mentioned. The remaining samples gives the polymorphic alleles at 187, 195, 197, 201, 204, 218, 245, 248 basepairs. This could be because *Ocimum* species extraction used in this study are different from those used in Pyne et al. (2018) research. In addition, there are 2-4 allele sizes which were identified in each amplified product except for S21, V5, V7 and S22. Both V7 and S22 are members of *O. basilicum* while S21 and V5 belong to *O. americanum* and *O. africanum*, respectively. Although the polymorphic microsatellites were identified in nearly all of the basil samples tested but the removal of non-polymorphic microsatellites is difficult in several amplified products (S15, S16, S17, V22, V24 and V25) for further statistical analysis. Figure 9 illustrates the chromatogram of V22 - 'Raspberry Muscat' with marker OBNJR3cn240.

Table 4. The allele sizes of different basil samples with marker OB158 and OBNJR3cn240.

Code	Expected allele size / Name	OB158	OBNJR3cn240	
		148	254-370	Filtered allele sizes
S8	Vietnamise	130	197; 269; 272; 275; 278	272; 275
S9	'Mittelgroßblättriges Grünes'	130	269; 272	269; 272
S10	'Cinnamum'	130	197; 269; 272; 275	269; 272
S15	'Dark Opal'	130	195; 260; 263; 266	260; 263; 266
S16	'Fine Verde'	130	195; 245; 248; 260; 263; 266	245; 248; 260; 263
S17	'Anisse'	130	195; 296; 299; 302; 305	296; 299; 302
S20	Thai long	130; 132	195; 251; 254; 257; 290; 293; 296	254; 293
S21	Togo basil	129; (131)	182; 187; 195	195
S23	Licorice scent	130	195; 204	195; 204
S24	Lemon basil	132	187; 218	187; 218
V1	'Purple Ruffles'	130	197; 257; 260; 263; 266	260; 263
V2	'Red Rubin'	130; 132	197; 260; 263; 266	260; 263
V3	'Rubin Kaira' (Stavia)	130	197; 245; 248; 251; 299; 302; 305	245; 248
V4	'Fine Verde Compacto'	130	197; 263; 266; 269; 272	263; 266
V5	Tulsi Temperate	130; 132	185; 190; 199; 201; 204; 207	204
V6	Mrihani	130; 132	195; 269; 272	269; 272
V7	'Aromatto'	130	204	204
V15	'Barhat'	130	195; 278; 281; 284; 287; 290	281; 284
V17	Rama Tulsi	130	269; 272	269; 272
V18	Tulsi Vana	130; 132		
V20	Penang Lemon	130; 132	185; 191; 198; 201; 204	201; 204
V22	'Raspberry Muscat'	130	195; 260; 263; 299; 302	260; 263; 299; 302
V23	'Karlik'	130; (131)	195; 257; 260; 263; 266	260; 263
V24	'Siam Queen'	130	195; 296; 299; 302; 305	296; 299; 302
V25	'Holy'	130	197; 201; 204; 207; 248; 251	204; 248; 251
V26	'Spicy Globe'	130; 132	195; 204	195; 204
S26	Iranian lemon	130	185; 191; 197; 201; 204	197; 204
S22	Clove Scent	130	195	195

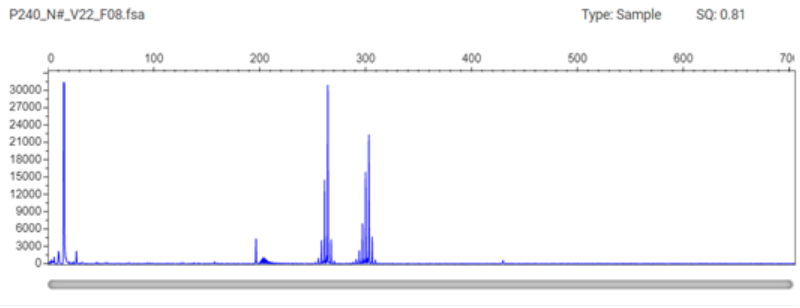

Figure 9. Example of the fragment length analysis of sample V22 - 'Raspberry Muscat' with marker OBNJR3cn240.

4.4. Markers OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145

Amplification was successfully performed in most of samples for the first time trial (Figure 10). Those DNA samples which didn't give any amplification were repeatedly used in the PCR procedure.

In course of the fragment length analysis, more than two fragment sizes were identified in each sample. Evaluating all fragment sizes we identified the non-polymorphic sizes, we eliminated them, and hence we attempted identification of the true microsatellite alleles. The filtered allele sizes are summarized in Table 5. (primer OBNJR2sg04, OBNJR2sg30) and Table 6. (primer OBNJR2sg33, OBNJR3sg145).

In terms of OBNJR2sg04, in several basil samples, allele sizes (231; 241; 247; 255 and 259 bp) were probably microsatellite but we eliminated them because they were not in the expected range compared to the original paper. The appearance of allele sizes was not in range the expected allele size since basil samples used in our study was different from basil cultivars used in original paper.

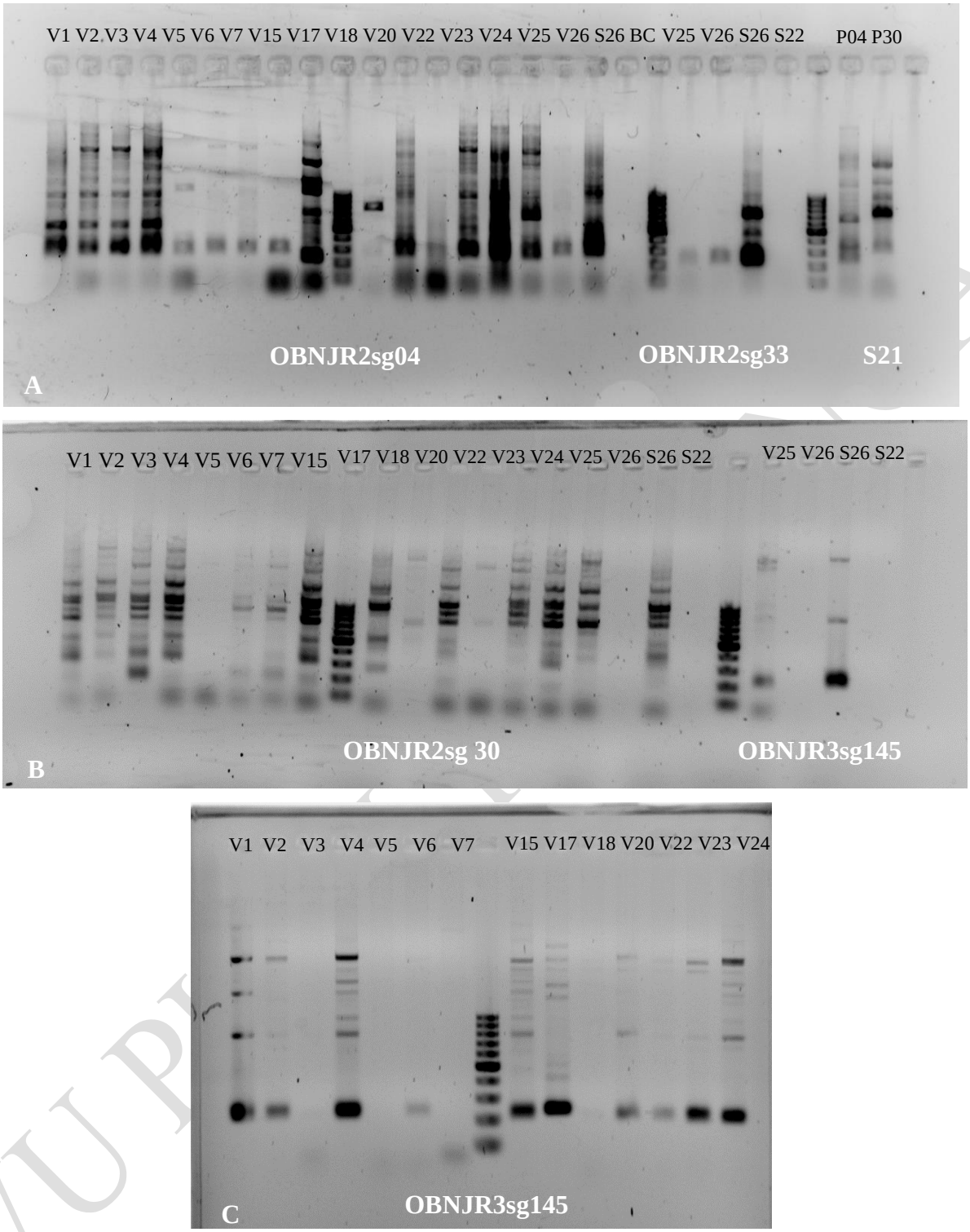

Figure 10. The gel photos of the amplified products of OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145.

By using NCBI-EST database, primer pair OBNJR2sg04 was designed from the genebank item DY343638 (Pyne et al., 2018). The amplification products i.e. eight allele sizes were in the expected product range as the original paper mentioned. The

allele sizes that are not in the expected range include the five microsatellites at 231; 241; 247; 255 and 259 basepairs. This could be because *Ocimum* species extraction used in this study are different from those used in Pyne et al. (2018) research. In addition, there are 2-6 allele sizes which were identified in each amplified product. Moreover, the microsatellite at 267 bp was identified in nearly all of the amplified products except for V17 – Rama tulsi, which belongs to *O. tenuiflorum*. It is concluded from the length analysis that the polymorphism with specific primer pair OBNJR2sg04 probably an appropriate SSR marker for the diversity assessment among basil species. Figure 11. illustrates the chromatogram of S21 - Togo basil with marker OBNJR2sg04.

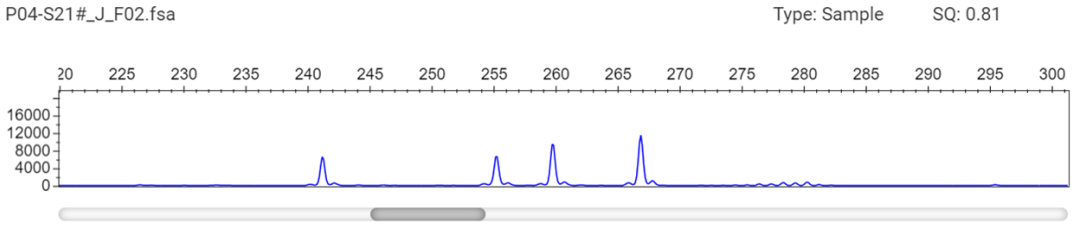

Figure 11. Example of the fragment length analysis of sample S21 - Togo basil with marker OBNJR2sg04.

By using NCBI-EST database, primer pair OBNJR2sg30 was designed from the genebank item DY336727. The amplification products i.e. 19 allele sizes were in the range lower than the expected product size as the original paper mentioned. This could be because *Ocimum* species extraction used in this study are different from those used in Pyne et al. (2018) research. The polymorphism was illustrated in nearly all of amplified products except for V17 – Rama tulsi, which belongs to *O. tenuiflorum*. It is concluded from the length analysis that the polymorphism with specific primer pair OBNJR2sg30 probably an appropriate SSR marker for the diversity assessment among basil species. Figure 12. illustrates the chromatogram of V7 – 'Aromatto' with marker OBNJR2sg30.

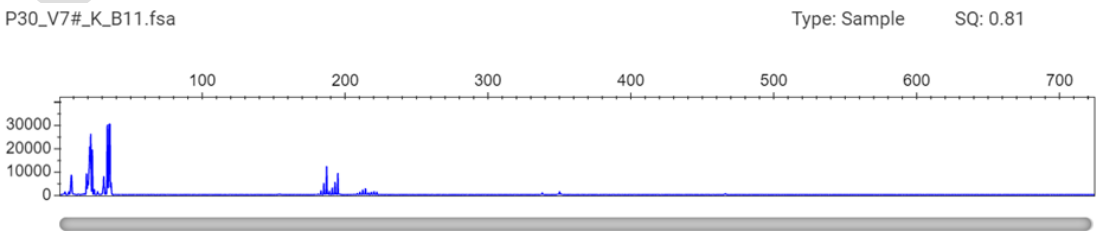

Figure 12. Example of the fragment length analysis of sample V7 – 'Aromatto' with marker OBNJR2sg30.

Table 5. The allele sizes of different basil samples with marker OBNJR2sg04 and OBNJR2sg30.

Code	Expected allele sizes / Name	OBNJR2sg04		OBNJR2sg30	
		268-357	Filtered allele sizes	241-279	Filtered allele sizes
S8	Vietnamise	267; 280	267; 280	186; 190; 194	186; 190
S9	'Mittelgroßblättriges Grünes'	267; 292	267; 292	186; 198; 208	186; 198; 208
S10	'Cinnamum'	267; 290; 292	267; 292	196; 215	196; 215
S15	'Dark Opal'	267; 285	267; 284	206	206
S16	'Fine Verde'	267; 280; 288	267; 280; 288	186; 192; 210; 218	186; 210
S17	'Anisse'	241; 255; 259; 267; 280	267; 280	189; 194	189; 194
S20	Thai long	267; 280; 286; 288	267; 280; 288	186; 190; 215	186; 190
S21	Togo basil	241; 255; 259; 267; 280	267; 280	172; 178	172; 178
S23	Licorice scent	267; 286; 288	267; 288	184; 186; 211; 214	186; 214
S24	Lemon basil	267; 269; 280	267; 280	178; 200	178; 200
V1	'Purple Ruffles'	242; 267; 286; 288	267; 288	153; 184; 215	186; 215
V2	'Red Rubin'	242; 259; 267; 288	267; 288	154; 174; 194; 214	194; 214
V3	'Rubin Kaira' (Stavia)	267; 280; 288	267; 280; 288	186; 215	186; 215
V4	'Fine Verde Compacto'	267; 278; 280	267; 280	153; 194; 215; 219	194; 215
V5	Tulsi Temperate	241; 259; 267; 274	267; 274	153; 196; 198; 200; 214	200; 214
V6	Mrihani	241; 255; 259; 267; 290; 292	267; 292	153; 192; 194; 196; 215	196; 215
V7	'Aromatto'	241; 255; 267	267; 290	153; 186; 194; 213	186; 194
V15	'Barhat'	241; 267; 280; 288	267; 288	153; 192; 210	192; 210
V17	Rama Tulsi	231; 290	290	153	153
V18	Tulsi Vana	241; 255; 259; 267	267	153; 184; 192; 194; 204	184; 192; 204
V20	Penang Lemon	267; 269; 280	267; 280	153; 178; 198; 200	178; 200
V22	'Raspberry Muscat'	241; 255; 259; 267; 282	267; 282	153; 184; 189	184
V23	'Karlik'	241; 255; 259; 267; 285	267; 284	153; 206; 212	206

Code	Name / Expected allele sizes	OBNJR2sg04		OBNJR2sg30	
		268-357	Filtered allele sizes	241-279	Filtered allele sizes
V24	'Siam Queen'	241; 247; 255; 259; 267; 280	267; 280	152; 189; 194	189; 194
V25	'Holy'	247; 259; 267	267	153; 194; 214	194; 214
V26	'Spicy Globe'	241; 255; 259; 267; 278; 280	267; 280	153; 192; 210	192; 210
S26	Iranian lemon	263; 267; 280	267; 280	153; 198; 202; 206	202
S22	Clove Scent	267; 280; 290; 292	267; 280; 292	153; 186; 190; 215	186; 215

By using NCBI-EST database, primer pairs OBNJR2sg33 and OBNJR3sg145 were designed from the genebank items DY333933 and DY328393, respectively. In terms of OBNJR2sg33, the amplification products i.e. 14 allele sizes were in the range lower than the expected product size as the original paper mentioned. The amplification with marker OBNJR3sg145 gave the polymorphism at 9 fragment sizes, which was lower than the expected product size. This could be because *Ocimum* species extraction used in this study is different from those used in Pyne et al. (2018) research. In course of OBNJR2sg33, there are 2-6 allele sizes which were identified in each amplified product except for V18 – Tulsi Vana belonging to *Ocimum gratissimum*. Although the polymorphic microsatellites were identified in nearly all of thebasil samples tested but the removal of non-polymorphic microsatellites is difficult in several amplified products (e.g. V20 and S26 with both primer pairs) for further statistical analysis. It could be concluded that the use of both markers OBNJR2sg33 and OBNJR3sg145 for the diversity evaluation is not appropriate to apply for all types of *Ocimum* species. Figure 13. and 14. illustrate the chromatogram of V1 – 'Purple Ruffles' with marker OBNJR2sg33 and V23 – 'Karlik' with marker OBNJR3sg145, respectively.

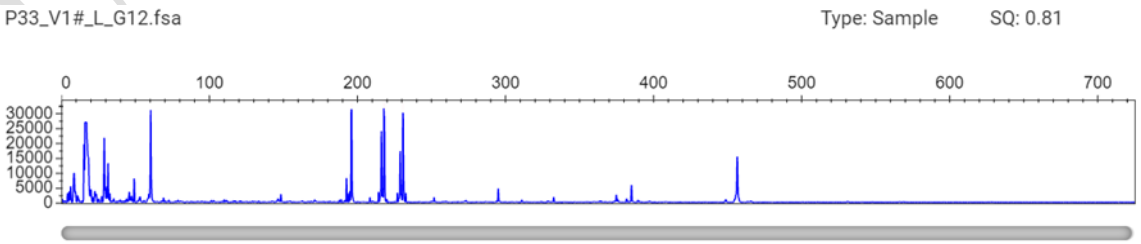

Figure 13. Example of the fragment length analysis of sample V1 – 'Purple Ruffles' with marker OBNJR2sg33.

Table 6. The allele sizes of different basil samples with marker OBNJR2sg33 and OBNJR3sg145.

Code	Expected allele size / Name	OBNJR2sg33		OBNJR3sg145	
		271-299	Filtered allele sizes	271-313	Filtered allele sizes
S8	Vietnamise	195; 217; 221; 230	217; 230	217; 240	217; 240
S9	'Mittelgroßblättriges Grünes'	195; 219; 230	219; 230	217; 240	217; 240
S10	'Cinnamum'	195; 217; 230	217; 230	217; 240	217; 240
S15	'Dark Opal'	195; 217; 230	217; 230	217; 243	217; 243
S16	'Fine Verde'	195; 210; 221; 230	210; 221; 230	217; 240	217; 240
S17	'Anisse'	195; 221; 230	221; 230	217; 237; 240	217; 240
S20	Thai long	195; 219; 228	219; 228	217; 228; 237; 240	217; 240
S21	Togo basil	195; 208; 214; 217; 219	208; 219	215; 225; 234	215; 234
S23	Licorice scent	215; 217; 228; 230	217; 230	217; 237; 240	217; 240
S24	Lemon basil	195; 210; 219; 225; 233	210; 219	217; 221; 228	217; 221; 228
V1	'Purple Ruffles'	195; 217; 230	217; 230	217; 240; 243	217; 243
V2	'Red Rubin'	195; 217; 230; 232	217; 232	217; 240; 243	217; 243
V3	'Rubin Kaira' (Stavia)	195; 210; 219; 221; 228; 230	210; 221; 230	217; 240	217; 240
V4	'Fine Verde Compacto'	195; 219; 221; 228; 230	221; 230	217; 225; 237; 240	217; 240
V5	Tulsi Temperate	189; 195; 208; 219	210; 219	215; 233; 243	215; 233; 243
V6	Mrihani	195; 208; 215; 217; 228; 230	217; 230	215; 217; 221; 228; 237; 240	217; 221; 228; 240
V7	'Aromatto'	215; 217; 228; 230	217; 230	217; 225; 240; 243	225; 240; 243
V15	'Barhat'	195; 217; 228; 230	217; 230	217; 237; 240	217; 240
V17	Rama Tulsi	195; 205; 230; 236	205; 230; 236	215; 235; 240; 246	235; 246
V18	Tulsi Vana	215	215	217; 221; 235; 240; 243	221; 243
V20	Penang Lemon	195; 210; 217; 219; 225	210; 219; 225	217; 221; 225; 228	217; 221; 228
V22	'Raspberry Muscat'	195; 215; 217	217	217; 240	217; 240

Code	Expected allele sizes / Name	OBNJR2sg33		OBNJR3sg145	
		271-299	Filtered allele sizes	271-313	Filtered allele sizes
V23	'Karlik'	195; 215; 217; 228; 230	217; 230	217; 240; 243	217; 243
V24	'Siam Queen'	195; 219; 221; 228; 230	221; 230	217; 237; 240	217; 240
V25	'Holy'	208; 219	208; 219	215; 217; 233	215; 233
V26	'Spicy Globe'	219; 221; 230; 232	208; 219	217; 237; 240	217; 240
S26	Iranian lemon	195; 210; 217; 225; 233	210; 217; 225	217; 221; 225; 240; 243	217; 221; 243
S22	Clove Scent	195; 219; 230	219; 230	217; 237; 240	217; 240

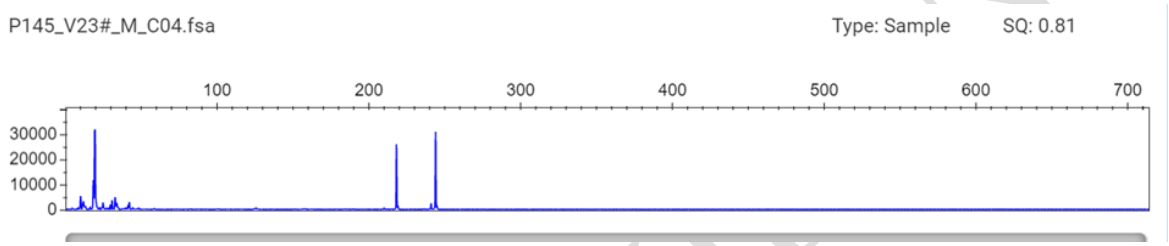

Figure 14. Example of the fragment length analysis of sample V23 – 'Karlik' with marker OBNJR3sg145.

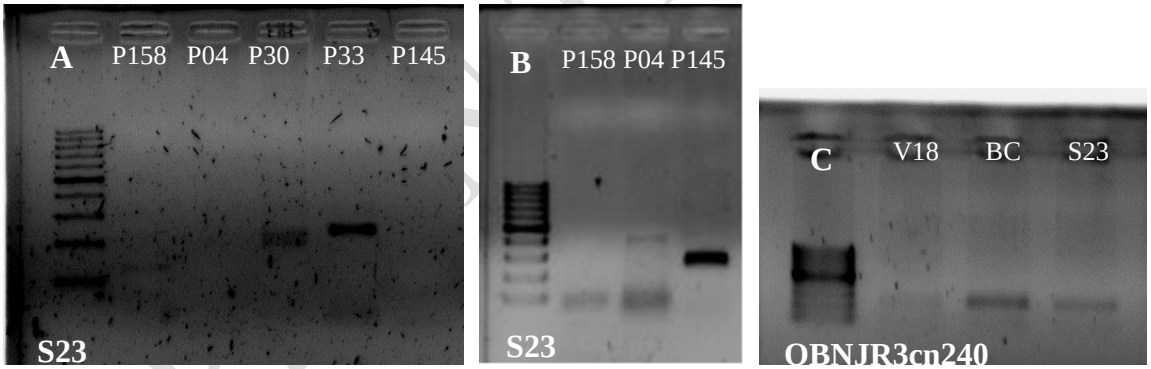

Figure 15. Gel photos of S23 with primer OB158, OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145 and OBNJR3cn240.

Sample S23 showed very faint bands or did not show the bands in the gel photos in the first trial with the first three basil primers tested (OB158, OB174, OB181) and with four *Salvia spp.* primer tested (LAB08, LAB 29, LAB 30, LAB 42). It only showed clear bands with the trial of OB212. Therefore, when the other basil primers (OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145 and OBNJR3cn240) were performed for the first trial, sample S23 was not included. Until when the primer OBNJR3cn240 was performed for the second trials, the S23 showed clear bands

amplification (Figure 15- C). Therefore, we decided to perform the S23 with other remaining primers (OB158, OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145) (Figure 15- A, B).

In conclusion, the primer pairs (OBNJR2sg04, OBNJR2sg30) are probably appropriate SSR markers for the diversity assessment among basil genotypes. In addition, the use of markers OBNJR2sg33, OBNJR3sg145 and OBNJR3cn240 for the diversity evaluation is not appropriate to apply for all types of *Ocimum* species due to the difficulty in removal of non-specific fragments. Markers OB174, OB181 and LAB042 should be tested with more basil genotypes before a conclusion could be made. The polymorphic microsatellites were identified only in one-third of the genotypes tested with OB158 so this marker is not likely to be suitable for the evaluation the genetic diversity among *Ocimum* genotypes and cultivars. OB212, LAB008 and LAB030 just gave the monomorphic alleles, which just demonstrated that tested samples belong to the *Ocimum* genus but could not showed the genetic variation among different basil species. Therefore, markers (OB212, LAB008 and LAB030) could not be used for analysis of genetic diversity for *Ocimum* genus.

SUMMARY

Hungarian University of Agriculture and Life Sciences

Faculty of Horticultural Science

Department of Genetics and Plant Breeding

Vu Phan Thien Nga

EVALUATION OF BASIL SPECIFIC SSR MARKERS

MSc in Agricultural Biotechnology

The *Ocimum* genus is one of the most important herbs all over the world due to their economic value in foods and beverages as well as in aromatic industries. Basil is also a desirable medicinal cure due to their secondary metabolites present in it. The complexity in the genome sizes and chromosome numbers as well as the variety of morphological and chemical composition among different basil cultivars serve a catalyst as the inconsistency of taxonomical analysis for *Ocimum* genus. Therefore, genetic data could be a promising foundation for the more congruency in taxonomical analysis of *Ocimum* genus but very few published studies are done in its genetic diversity. EST-SSRs are a potential marker for genetic variation assessment due to their advantages. Hence, the present study aimed to identify the EST-SSR markers designed from previous researches that give the most efficient amplified products for evaluation the genetic diversity among *Ocimum* variants.

Thirteen SSR primer pairs (OB212, OB181, OB174, OB158, LAB008, LAB029, LAB030, LAB042, OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145 and OBNJR3cn240) were chosen from previous studies designed for *Ocimum sp.* and *Salvia spp.* DNA was isolated from fresh leaves of 28 genotypes and cultivars of *Ocimum sp.* originating from different parts of the world. After successful DNA extraction, PCR amplification was performed in a Swift MaxPro thermocycler (Esco Healthcare Pte, Singapore). Agrose Gel Electrophoresis was used to verify PCR products. Determination of exact fragment length size was done by fragment length analysis, fragment sizes were determined by Peak Scanner software 1.0 (Applied Biosystems 2006) manually and results were collected in MS Excel.

94 alleles were produced in total with an average of 6.57 ranging from 3-13 alleles per locus with size ranged between (130-480 bp) where five markers (OB158, OBNJR2sg04, OBNJR2sg30, OBNJR2sg33, OBNJR3sg145) were successfully amplified in all genotypes. Primer pair OBNJR3cn240 has the amplification percentage as 96%. Seven primer pairs (OB212, OB181, OB174, LAB008, LAB029, LAB030 and LAB042) were tested on a restricted number of ten basil genotypes. The amplification percentage of OB212 and OB181 was 90% while that of OB174 and LAB008 was 80%. The amplification percentage for remaining primers is 70%. OBNJR2sg30 and

OBNJR3cn240 were the most informative as they showed high variability with 19 alleles followed by OBNJR2sg33, OBNJR2sg04 and OBNJR3sg145 with 14, 13 and 9 alleles, respectively.

In terms of number of alleles per sample, OBNJR2sg04 and OBNJR3sg145 resulted from two to six alleles while OBNJR2sg30 and OBNJR2sg33 produced from one to five microsatellite alleles. The amplified products with markers OB212, OB174, LAB008 and LAB030 produced only one allele size per sample while OB158, OB181, LAB029 and LAB042 produced from one to two alleles per sample. The range for OBNJR3cn240 was one to seven alleles per sample. In addition, OB212, LAB008 and LAB030 were monomorphic, resulting one homozygous allele in all samples while OB042 resulted two kinds of homologous alleles in all amplified products. OB174 resulted two kinds of alleles, which showed the polymorphism in all amplified products. In terms of OB181, three polymorphic alleles just appeared in four out of nine amplified products. LAB029 also resulted two allele sizes and an additional out of the expected range. OB158 resulted three polymorphic microsatellite alleles out of four created alleles, which was illustrated in one-third of amplified products. OBNJR2sg30 and OBNJR3cn240 showed the polymorphism in all amplified products with 19 polymorphic alleles. In terms of OBNJR2sg04, only one homozygous allele size was created at 267 bp. OBNJR2sg33 and OBNJR3sg145 resulted 14 and 9 heterozygous allele sizes in all amplified products.

Further statistical analysis cannot be performed due to the difficulty in the removal of non-specific fragment sizes.

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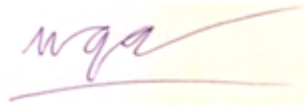

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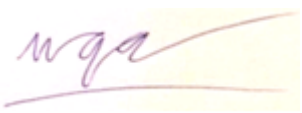

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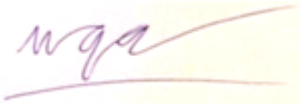

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As a supervisor of Vu Phan Thien Nga (Code-Neptun: XRCLM3), I here declare that the master's thesis has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

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