Journal of Molecular Biomarkers & Diagnosis
Bilal, J Mol Biomark Diagn 2020, 11:2
Research Article Open Access

Molecular Characterization of *Ocimum* Species Using Random Amplified Polymorphic DNA Method and Gene Identification Using Sanger Sequencing Method

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Abstract

Ocimum species known for its traditional value,which is used as culinary herb and assigned as medicine and spice plant. We used molecular techniques to assess the genetic variability and relatedness of 20 accessions of three germplasm of *Ocimum* spp. (*Ocimum gratissimum*, *Ocimum sanctum* and *Ocimum basilicum*) collected from different places. DNA was isolated by fixing a sample in alcohol without using liquid nitrogen. Molecular markers were used to assess genetic diversity in basil (*Ocimum L.* spp., Lamiaceae). Varieties were analyzed through RAPD markers with five Inter-simple sequence repeats (ISSR) primers to determine the extent of molecular characterization. PCR amplification of these regions using a single primer yields multiple amplification products that can be used as a dominant multi locus marker system for the study of genetic variation in various organisms. and the result showed various gene expression with the ISSR primer than RAPD markers and we found a maximum number of unique alleles were observed in *O. basillicum* with ISSR primer (1, 5, 8, 9, 11, 12, 13, 14) which mentioned on primer synthesized table. Among the 15 ISSR primers tested, we have got bands in the size range of 300 bp to 850 bp. Our observations suggested that morphological variability and ISSR analysis could help in identifying genetic variations among different varieties of basil, help in plant improvement and develop a well-organized way to conserve the genetic wealth of basil varieties. DNA based markers like ISSR are more useful than the traditional morphological and biochemical markers. Genetic relationships were examined among thirty germplasm accessions belonging to five *Ocimum* species using RAPD and ISSR markers.

Keywords: *Ocimum*; ISSR; RAPD; DNA finger printing (Bands)

Introduction

Ocimum species

Ocimum is a genus of aromatic annual and perennial herbs and shrubs in the family Lamiaceae native to the tropical and warm temperate regions of all 6 inhabited continents, with the greatest number of species in Africa. Its best known species are the cooking herb Cooking basil, *O. basilicum* and the medicinal herb Tulsi (Figure 1).

Scientific classification

Kingdom	:	Plantae
(Unranked)	:	Angiosperms
(Unranked)	:	Eudicots
(Unranked)	:	Asterids
Order	:	Lamiales
Family	:	Lamiaceae
Subfamily	:	Nepetoideae
Tribe	:	Ocimeae
Genus	:	*Ocimum*

Types of species: There are around 60 plus species of *Ocimum* been identified among which we have chosen 5 species of *Ocimum* been taken for our studies (Table 1).

***Ocimum sanctum* (Ramar tulsi):** *Ocimum sanctum*, commonly known as holy basil, tulasi or tulsi, is an aromatic plant in the family Lamiaceae which is native to the Indian subcontinent and widespread as a cultivated plant throughout the Southeast Asian tropics.

***Ocimum sanctum* (Krishna tulsi):** *Ocimum sanctum*, commonly known as holy basil, tulasi or tulsi, is an aromatic plant in the family Lamiaceae which is native to the Indian subcontinent and widespread as a cultivated plant throughout the Southeast Asian tropics. The main difference found in this species compared to above one is it will be

Figure 1: Samples (Leaves of *Ocimum* species).

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Received August 29, 2019; **Accepted** February 25, 2020; **Published** March 03, 2020

Citation: Bilal A (2020) Molecular Characterization of *Ocimum* Species Using Random Amplified Polymorphic DNA Method and Gene Identification Using Sanger Sequencing Method. J Mol Biomark Diagn 9: 423.

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Scientific Name	Commercial Name
Ocimum sanctum	Krishnar Tulsi
Ocimum sanctum	Ramar Tulsi
Ocimum basillicum	Sarkarai Tulsi
Ocimum basillicum Linn	Thiruneetrupachilai
Ocimum teniflorum	Karpoora Tulsi

Table 1: Leaves of *Ocimum* species.

purple in colour in both stem and leaves.

***Ocimum basillicum*:** *Ocimum basilicum* is an aromatic, annual herb, 0.3-0.5 metres tall, but some cultivars can reach up to 1 m. The plant is almost hairless. Some cultivars, such as the 'Dark Opal', have leaves and stems deep purple in colour. The leaves are ovate, often puckered, flowers white or pink, and fruits have four small nutlets.

***Ocimum basillicum Lin*:** *Ocimum basillicum linn* is an aromatic, herb the plant reach about 1m tall, with bright big sized leaves and the stem is hard and fibrous and the plant belongs to Lamiacecae family, widespread as cultivated throughout southeast Asian tropics.

***Ocimum tenuiflorum*:** *Ocimum tenuiflorum* is also commonly known as karpoor tulsi, it is short and it is wide spread throughout South East Asia. The leaves were pale green in colour and leaves were about 5cm.

Nutritional composition: The chemical constituents of this herb include ursolic acid, eugenol, oleanolic acid, rosmarinic acid, linalool and carvacrol. The plant also contains β-caryophyllene, β-elemene and germacrene D. The high eugenol content of this plant helps it act like a pain-killer.

Applications and medicinal uses: Tulsi has known to be used for its medicinal qualities for over 1000 years and ancient Ayurvedic texts refer to tulsi as the 'elixir of life'. The plants leaves, stems and seeds are used for relieving purposes. Tulsi is known to relieve almost all ailments including common colds, digestive problems, breathing problems, stress, blood sugar, heart problems, fever and even ulcers. The oil from the seeds of this herb is now also being used to relieve cancer [1].

Fever: Holy basil helps relieves various kinds of fevers including malaria and dengue. The herbs components induce excessive sweating, thereby, bringing down the body temperature.

Common cold and cough: Raw tulsi leaves are very helpful in providing relief from cold and cough. Being a very effective expectorant, the holy basil is very useful in relieving cough and sore throat.

Kidney stones: Apart from strengthening the functioning of the kidney, regular use of this herb can help one get rid of stones in the kidney.

Heart problems: This herb is very effective in reducing cholesterol levels and it thus helps maintain a healthy heart function.

Insect bites: Extracts from this herb can be used as a preventive as well as reliever for insect bites; they can be used as a mosquito repellent as well as to relieve bites from insects like leeches and scorpions and also snake bites.

Mouth ulcers: Chewing of raw basil leaves helps relieve mouth infections including ulcers. In addition this herb is extremely useful in maintaining dental hygiene; its antibacterial properties help one relieve dental problems like bad breath and pyorrhea.

Stress: Consumption of the holy basil helps combat both physical as well as emotional stress as well as boost energy. The herb helps maintain the balance of the nerves.

Skin problems: Holy basil is used to relieve skin infections like ring worm.

Eye problems: Eye problems like sore eyes and night blindness can effectively be relieved with the regular use of tulsi extracts.

Headaches: External as well as internal consumption of this herb provides relief from headaches.

Breathing problems: Extracts from this plant are often used to relieve respiratory problems like asthma and bronchitis.

Sharpen memory: Extracts from the leaves of this herb act as a tonic for the nerves and help sharpen the memory too.

Blood purifier: This herb is an efficient blood purifier and thus helps attain a clear and glowing skin as well as prevent several other ailments.

PCR

Polymerase chain reaction (PCR) is a primer mediated enzymatic amplification of specifically cloned or genomic DNA sequences. The main purpose of the PCR process is to amplify template DNA using thermostable DNA polymerase enzyme which catalyzes the buffered reaction in which an excess of an oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs) are used to make millions of copies of the target sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40kb in size.

Denaturation the DNA sequence which is to be amplified by PCR is known as the template. The double-stranded DNA template must be denatured into two complementary single strands of DNA before the reaction can commence. Annealing of two oligonucleotide primers to the single-stranded template. After denaturation, the reaction is quickly cooled, preventing immediate reannealing of long DNA strands.

Enzymatic extension of the primers to produce copies that can serve as templates in subsequent cycles. Also known as polymerization, this is the final step of the PCR cycle.

RAPD

RAPDs are DNA fragments amplified by PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1-10 genomic sites simultaneously. Amplified fragments, usually within the 0-5.5 kb size range, are separated by agarose gel electrophoresis, and polymorphisms are detected, after ethidium bromide staining, as the presence or absence of bands of particular sizes. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites. Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

Applications: RAPDs have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers. Variants of the RAPD technique include Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) which uses longer arbitrary primers than RAPDs, and DNA Amplification Fingerprinting (DAF) that uses shorter, 5-8 bp primers to generate a larger number of fragments. Multiple Arbitrary Amplicon Profiling (MAAP) is the collective term for techniques using single arbitrary primers.

DNA sequencing

Sequencing of genomes has been at the center of interest in the biotechnology field over the past several decades and is now leading toward an era of personalized medicine. During this time, DNA sequencing methods have evolved from the labor intensive slab gel electrophoresis, through automated multicapillary electrophoresis systems using fluorophore labeling with multispectral imaging, to the "next generation" technologies of cyclic array, hybridization based, nanopore and single molecule sequencing. Deciphering the genetic blueprint and follow-up confirmatory sequencing of Homo sapiens and other genomes was only possible by the advent of modern sequencing technologies that was a result of step by step advances with a contribution of academics, medical personnel and instrument companies. While next generation sequencing is moving ahead at break-neck speed, the multicapillary electrophoretic systems played an essential role in the sequencing of the Human Genome, the foundation of the field of genomics. In the prospective, researchers wish to overview the role of capillary electrophoresis in DNA sequencing based in part of several research.

Gel electrophoresis

Gel electrophoresis is a technique used to separate DNA fragments (or other macromolecules, such as RNA and proteins) based on their size and charge. Electrophoresis involves running a current through a gel containing the molecules of interest. Based on their size and charge, the molecules will travel through the gel in different directions or at different speeds, allowing them to be separated from one another.

All DNA molecules have the same amount of charge per mass. Because of this, gel electrophoresis of DNA fragments separates them based on size only. Using electrophoresis, we can see how many different DNA fragments are present in a sample and how large they are relative to one another. We can also determine the absolute size of a piece of DNA by examining it next to a standard "yardstick" made up of DNA fragments of known sizes. The end of the gel with the wells is positioned towards the negative electrode. The end without wells (towards which the DNA fragments will migrate) is positioned towards the positive electrode.

Objectives

- To isolate DNA from *Ocimum* species.
- To optimize the similar sequence.
- To check if the RAPD DNA anneals with the sample DNA.

Review of Literature

Polymerase chain reaction involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. It is a reproducible, highly polymorphic marker and is useful in studies of genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology [2,3]. There is a lack of information on the molecular characterization of the *Ocimum* species. PCR conditions are well set, high reproducibility for both RAPD and ISSR markers can be obtained. Almost all PCR applications employes a heat stable DNA polymerase [4].

Detection of genetic variation in *Ocimum* species using RAPD and ISSR markers

Characterisation of 17 genotypes belonging to 5 different species (*O. basilicum*, *O. americanum*, *O. sanctum*, *O. gratissimum* and *O. Polystachyon*) through random amplified polymorphic DNA (RAPD) and the inter simple sequence repeats (ISSR) markers was done by Hardik et al. [5]. Generally, all the studies have reported that ISSR primers produce more reliable and reproducible bands than RAPD primers. In this study, it was observed that once the PCR conditions are well set, high reproducibility for both RAPD and ISSR markers can be obtained. In general, all 22 markers used in this study produced clear consistent and reproducible amplification profiles.

Inter-Simple Sequence Repeat (ISSR) Markers: Are we doing it right?: ISSR markers are easy to use, low-cost, and methodologically less demanding compared to other dominant markers, making it an ideal genetic marker for beginners and for organisms whose genetic information is lacking. Some of the intricacies often overlooked in designing an ISSR experiment, clarify some misconceptions, and provide recommendations on using ISSR markers in genetic variation studies [6].

Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive: RAPDs, AFLPs and SSRs were compared in terms of their informativeness and efficiency in a study of genetic diversity and relationships among 32 olive cultivars cultivated in Italy and Spain. SSRs presented a higher level of polymorphism and greater information content, as assessed by the expected heterozygosity, than AFLPs and RAPDs [7,8].

Inter-species Association of *Ocimum* genus as revealed through random amplified polymorphic DNA fingerprinting: Eight species of *Ocimum* species viz. *Ocimum kilimandscharicum*, *O. canum*, *O. sanctum variety* (var.) black, *O. micranthum*, *O. sanctum* var. green, *O. bassilicum*, *O. gratissimum* and *O. grattisimum* var. clocimum were analyzed through RAPD with ten random primers to determine the extent of genetic variability and phylogenetic relationship within and in between the selected species. This observation suggest that RAPD analysis could help in identifying genetic variations among different species of *Ocimum* and may help in plant improvement and develop a well-organized way to conserve the genetic wealth of *Ocimum* species, and accumulating their effective medicinal use [9].

Genetic variability among three sweet basil (*Ocimum basilicum L.*) Varieties as revealed by morphological traits and RAPD markers: The genetic diversity among the basil varieties was investigated using 5 RAPD primers, generates discernible, reproducible, banding profiles. The different primers exhibit different levels of polymorphism. Molecular markers are useful complementary tool to morphological and physiological characterization of plants because they have many advantages for example they are plentiful, independent of environmental effects and cultivar identification early in plant development. In addition DNA markers are not influenced by environmental impacts and are useful in describing the levels of genetic variability among plant

populations to discriminate the duplicated accessions within a specific collection of germplasm [10,11].

Genetic diversity of Basil (*Ocimum* spp) based on RAPD markers: RAPD analysis associated with the volatile oils and flavonoids has been reported for *Ocimum gratissimum*. In this study, the phenogram was constructed based on the polymorphism expressed by different markers [12].

RAPD and ISSR Polymorphism in the medicinal plants: *Ocimum sanctum*, *O. basilicum* and *O. gratissimum*: A Both RAPD and ISSR technique is sensitive, precise and efficient tool for genomic analysis in the species' of *Ocimum*. Such kinds of studies are very useful to put *Ocimum* species in appropriate taxonomic groups. DNA based markers like ISSR are more useful than the traditional morphological and biochemical markers. The species specific markers amplified in this study can be converted into simple PCR-based sequence characterized amplified region (SCAR) markers which will allow the screening of large number of samples and populations from different region [13].

Phylogenetic relationships as in *Ocimum* revealed by RAPD markers: Genetic relationships were examined among thirty germplasm accessions belonging to five *Ocimum* species using RAPD markers. A very high degree of polymorphism (98.20%) was observed. UPGMA cluster analysis of genetic similarityindicesindicesgroupedalltheaccessionsintotwomajorclusterscorresponding to previously reported botanical sections. Intra-clustering within the two clusters precisely grouped the accessions belonging to one species in one sub- cluster as expected from their genetic background [14].

Materials and Methods

Materials

Tris Acetate EDTA (TAE) buffer: Stock (50x) in 100 ml: 242 g of Tris Base, 5. 75 ml of Glacial acetic acid and 10 ml of 0.5 M EDTA (pH-8) make up to 100 ml using distilled water and autoclave the buffer. From this 1X TAE buffer is prepared by mixing 1 ml of 50x TAE with 49 ml of distilled water (i.e., for 50 ml 1x TAE buffer.).

Lysis buffer in 100 ml: 35 ml of sodium bromide was added with 35 ml of trichloroacetic acid and 20 ml of 20% sarcosyl detergent was added with 2 ml of Tween 80 and make up to 100 ml using distilled water.

Binding Buffer in 100 ml: 20 ml of potassium acetate was added with 80 ml of absolute ethanol.

Biologicals: The samples were been collected, various species of *Ocimum* from the nursery garden in Uthandi.

Chemicals used: EDTA anhydrous, Sodium Bromide, Trichloroacetic acid, Sarcocyl detergent, Tween 80, Potassium Acetate, Ethanol, Isopropanol, Ethylene-diamine-tetraacetic acid (EDTA), Bromophenol blue, Agarose.

Equipments used: Tissue Lyser, Mini Centrifuge, Cooling Centrifuge, Cyclomixer, Nanodrop Spectrophotometer, PCR, UV Transiluminator, Gel Electrophoresis, Weigh Balance, Concentrator, Deep Freezer, Microwave oven, Primer, Synthesizer, Genetic Analyser.

Glass wares: 50 ml tubes, 15 ml tubes, conical flask, Measuring cylinder, 1 litre reagent bottle, 250 ml reagent bottle.

Method

Sample collection: *Ocimum* samples used for the current study were collected from AET nursery, Uthandi, Chennai.

Sample preparartion: 10 g of samples (leaves and flowers) were homogenised and stored in deep freezer at -20°C and used for further study.

DNA extraction: Lysis Buffer (100 ml): Sodium bromide: 35 ml, Trichloroacetic acid: 35 ml, 20% sarcosyl detergent: 20 ml, Tween 80:2.0 ml and make up to 100 ml using distilled water. Binding Buffer (100 ml): Potassium acetate: 20 ml, 100% ethanol: 80 ml. Elution buffer: Nuclease free water.

Procedure

- 1 gram of sample (Tulsi leaves and flowers) into a 1.5 ml of micro centrifuge.
- 600 µl of lysis buffer have been added and homogenized properly using plastic pestle and bead mill.
- The tubes were centrifuged at the rate of 13000 rpm for 3 minutes.
- 35 µl of the supernatant were transferred to another 1.5 ml tube and 350 µl of binding were added and invert few times to mix properly.
- 700 µl of lysate were loaded into SMS column and centrifuged the tubes at 10000 rpm for 15 seconds and discard the flow through.
- 600 µl of 75% ethanol was added to the SMS column and centrifuged at 10000 rpm for 15 seconds and the flow through discarded.
- This wash step was repeated for one more time to improve the quality of nucleic acid isolation [15].
- The flow through discarded and the SMS column have been placed into new collection tube and centrifuged for 2 minutes at the rate of 13000 rpm to remove residual ethanol.
- This column been transferred into a new 1.5 ml tube and 50 µl of elution buffer been added to column and wait for 1 minute.
- The tubes are centrifuged at 13000 rpm for 1 minute for elution and DNA was collected in 1.5 ml tube.

Quantification of DNA

DNA was quantified using nano drop UV spectrophotometer. 1 µl of isolated DNA was placed in nano drop Spectrophotometer. Concentration of the DNA was read on the detector [16].

Agarose gel electrophoresis

- 2% agarose was prepared. (2 g agarose in 100 ml of 1X TAE buffer and melted using micro oven).
- When the agarose gel temperature was around 60°C, 5 µl of ethidium bromide of was added.
- Warm agarose solution was slowly poured into the gel platform.
- Gel set was kept undisturbed till the agarose solidifies.
- 1x TAE buffer was poured into submarine gel tank.
- Gel platform was carefully placed into the tank. The tank buffer level was maintained 0.5cm above than the gel.
- 5 µl of DNA samples were loaded into the wells after mixing with gel loading dye.

- Electrophoresis was run at 50V till the dye reaches three fourth distance of the gel.
- Gel was viewed in UV Tran illuminator and observed the bands pattern.

Primer synthesis

Random primers were collected from different journal related to our study and it was synthesized in shrimpex biotech services, Uthandi, Chennai under the guidance of our external guide using primer synthesizer (Table 2).

Primer synthesized: The below mentioned primer has been taken with reference with Hardik et al. [5].

RAPD method

PCR program: RAPD-PCR was performed at an initial denaturation at 94°C for 5 min, 38 cycles of 94°C for 1 min, 38°C for 1 min, 72°C for 1. 2 min and final extension at 72°C for 5 min. The optimal annealing temperature for ISSR primers was found to vary according to the base composition of the primers. Therefore, ISSR-PCR was performed at an initial denaturation temperature of 94°C for 5 min, 38 cycles of 94°C for 50s, 35-58°C (depending on primer sequence) for 60 s and 72°C for 1-2 min and a final extension of 72°C for 10 min [5].

PCR purification: 10 µl of PCR product was mixed with 1 µl of 3M sodium acetate (pH 4. 5) and 20 µl absolute ethanol and the mixture is been vortex and stored at -20°C for 30 min, centrifuge at 14,000 rpm for 15 min, the supernantant was discarded and ethanol (75%) wash with 10 µl and resuspend the pellet with 30 µl nuclease free water.

Extraction of DNA from agarose gel

- Visualize the low melting point agarose gel with DNA bands under a UV transilluminator and locate the desired DNA band to cut.
- Carefully cut around the desired DNA band using a scalpel blade.
- Transfer the gel piece into a microfuge tube.
- Add elution buffer into the microfuge tube until the level of buffer is just above the level of gel slice.
- Heat the gel slice at 65°C until it melts.

S. No	Primers
1	CTCTCTCTCTCTCTCTT
2	CACACACACACACACAG
3	ATAATAATAATAAT
4	GAATTATTATTATT
5	AAAAATAATAATAAT
6	TTTATTATTATTATT
7	GAGAGAGAGAGAGAGAC
8	TGTGTGTGTGTGTGTGTGT
9	GAGAGAGAGAGAGAGARGY
10	GAGAGAGAGAGAGAGAC
11	ATATATATATATATATT
12	AGAGAGAGAGAGAGAGYT
13	GGAGAGGAGAGGAGA
14	CATGGTGTTGGTCATTGTTCCA
15	ACTTCCCCACAGGTTAACACA

Table 2: Primer synthesized.

- Freeze the melted gel with DNA by placing in a -70°C freezer for 10 min.
- After freezing, centrifuge for 10minutes and transfer the supernatant into a new microfuge tube.
- Again add half amount of elution buffer that you added in the previous step into the pellet.
- Heat at 65°C until the agarose melts.
- Freeze the melted gel with DNA by placing in a -70°C freezer for 10 min.
- Centrifuge the tube again for 10 minutes and transfer (pool) the supernatant into the previous tube with supernatant.
- Discard the tube with pellet.
- Add an equal volume of n-Butanol to the supernatant and mix the contents well.
- Vortex the tube for 15 minutes in order to remove the Ethidium bromide.
- Discard the upper phase of butanol and repeat the process by adding n-butanol again for one or more times.
- Add 2 times volume of 95% ethanol and mix thoroughly.
- Keep for precipitation in -70°C freezer for 30 minutes to overnight.
- After precipitation, centrifuge for 15 minutes.
- Discard the supernatant into a waste beaker and add 200 µl of 70% ethanol to the pellet.
- Centrifuge for 5 minutes and discard the supernatant again.
- Allow the pellets to dry well.
- Suspend the pellets in 20 µl.

Gel preparation

1% Gel in 1X TAE buffer. Dissolve the Agarose in Microwave Oven. After cooling add 2 µl of Ethidium Bromide & cast the Gel in the boat.

Gel loading dye: Ethidium Bromide - 0. 5 µg/100 ml.

Cycle sequencing

DNA amplification mixture was prepared as follows: RR mix 0.75 µl, Sequencing buffer 1.55 µl, water 5.7 µl mixed well. Then the tube was placed in thermo cycler for amplification.

The program followed is given below:

Step 1: Initial denaturation at 96°C for 1 minute.

Step 2: Melting of DNA stand 96°C for 10 sec.

Step 3: Annealing at 50°C for 5 sec.

Step 4: Primer extension at 60C° for 4 minutes.

Step 5: Go to step 2 (25 cycle)

Step 6: Termination at 4°C for 1 minutes.

Step 7: End.

Cycle sequencing purification

Mix 1: 10 µl of nucleus free water, 2 µl EDTA

Mix 2: 2 µl of sodium acetate (3M, pH-4.1), 50 µl absolute ethanol

Procedure:

Step 1: 12 µl of mix 1 was mixed with 52 µl of mix 2.

Step 2: The mix was centrifuged at 12,500 rpm for 20 minutes.

Step 3: The supernatant was discarded and 250 µl of ethanol was added and vortexed

Step 4: Centrifuge at 12,500 rpm for 10 minutes.

Step 5: Repeat step 3 and step 4.

Step 6: Place the above sample at concentrator for 3 minutes.

Step 7: 10 µl of Hi-Di (highly deionised) formamide is used to re-suspend samples in capillary electrophoresis system.

DNA sequencing

Quality and quantity of purified PCR product was verified and sent to DNA Sequencing. The DNA sequencing was carried out using Big Dye Terminator sequencing kits. Complete sequencing process was carried out on an Applied Biosystems 3500 Genetic Analyzer. The electropherogram data of the sequenced DNA was obtained in the sequence scanner.

Result and Discussion

DNA gel

DNA was isolated from the different *Ocimum* species and they were electorphorisd in 2% agarose gel and viewed under UV transilluminator. The banding pattern was given in the Figure 2.

PCR amplification

Five samples were mixed with 15 sets of primers and amplified using PCR. Thus we got 75 PCR products, ran under 2% agarose gel. The results are shown in the five images given below (Figures 3-7).

From the above study 15 primers were used for amplification of five *ocimum* species leaf samples. Out of 15 only eight primer showed good result for amplification of DNA (primer number - 1, 5, 8, 9, 11, 12, 13, 14). out of five leaf samples only three leaves showed good amplification with above primer mentioned and these were further taken for sequencing to identify the gene since flower's DNA sample did not showed good amplification result with the primers we synthesised. and thus were not considered for further sequencing [17,18].

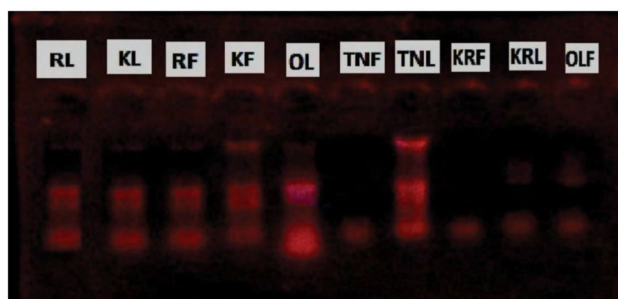

Figure 2: DNA gel image. RL: DNA isolated from *Ocimum sanctum*; KL: DNA isolated from *Ocimum tenuiflorum*; RF: DNA isolated from *Ocimum sanctum*; KF: DNA isolated from *Ocimum tenuiflorum*; OL: DNA isolated from *Ocimum basillicum*; TNF: DNA isolated from *Ocimum basillicum* Linn; TNL: DNA isolated from *Ocimum basillicum* linn; KRF: DNA isolated from *Ocimum tenuiflorum;* KRL: DNA isolated from *Ocimum tenuiflorum*; OLF: DNA isolated from *Ocimum basillicum*.

Figure 3: PCR gel image 1. 1KB ladder : Standard Marker; T12: DNA isolated from *Ocimum basillicum* amplified with the primer no. 12; KRL12: DNA isolated from *Ocimum sanctum* amplified with the primer no. 12; T1: DNA isolated from *Ocimum basillicum* amplified with the primer no. 1; KRL7: DNA isolated from *Ocimum sanctum* amplified with the primer no. 7; K5: DNA isolated from *Ocimum tenuiflorum* amplified with the primer no. 5; O4: DNA isolated from *Ocimum basillicum* amplified with the primer no. 4; R4: DNA isolated from *Ocimum sanctum* amplified with the primer no. 4; R13: DNA isolated from *Ocimum sanctum* amplified with the primer no. 13; R15: DNA isolated from *Ocimum sanctum* amplified with the primer no. 15; KRL2: DNA isolated from *Ocimum sanctum* amplified with the primer no. 2; KRL3: DNA isolated from *Ocimum sanctum* amplified with the primer no. 3; R14: DNA isolated from *Ocimum sanctum* amplified with the primer no. 14; KRL13: DNA isolated from *Ocimum sanctum* amplified with the primer no. 13; T2: DNA isolated from *Ocimum basillicum* amplified with the primer no. 2; T3: DNA isolated from *Ocimum basillicum* amplified with the primer no. 3; T4: DNA isolated from *Ocimum basilicum* amplified with the primer no. 4; T6: DNA isolated from *Ocimum basillicum* amplified with the primer no. 6.

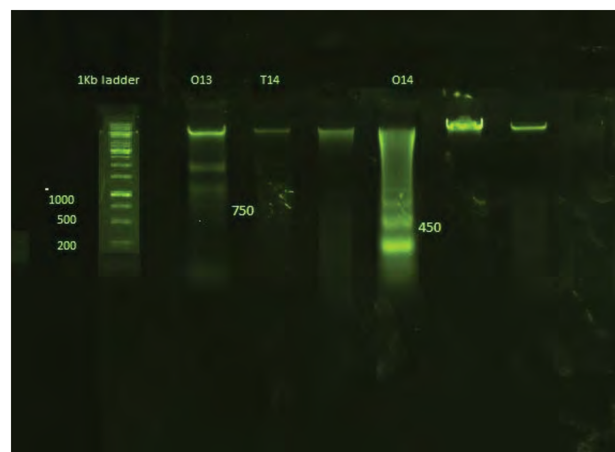

Figure 4: PCR gel image 3. 1KB ladder: Standard Marker. O13: DNA isolated from *Ocimum basillicum* amplified with the primer no. 13; T14: DNA isolated from *Ocimum basillicum* amplified with the primer no. 14; O14: DNA isolated from *Ocimum basillicum* amplified with the primer no. 14

Fasta sequence

Sequence of *Ocimum sanctum* with primer 9:

ACTTCGCTTTGTCGTGTCGCTTCTGAGTCYGCGGTC-GACCGTYGGKAGTTGGTCTMGGTGGGMTCTGATAGAC-GTATSGGGAGCCCGAGAGAGGAGCCCRAAAGATRTG-CAGTAAGCGGGGGACTCGYGCTGTATGGCTCAAAGCTA-AGCTTCGAGGTTCCTGCAGAGATTTGTGGGTTTTTGTAM-WAAGYATGCSGGYAGCAGATTTAGMTWAAAAGAAGT-

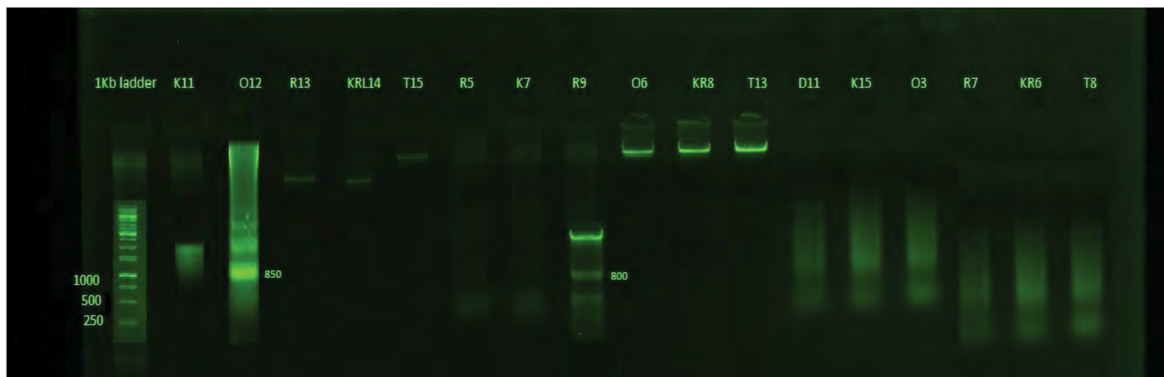

Figure 5: PCR gel image 4. 1KB ladder: Standard Marker. K11: DNA isolated from *Ocimum teniflorum* amplified with the primer no. 11; O12: DNA isolated from *Ocimum basillicum* amplified with the primer no. 12; R13: DNA isolated from *Ocimum sanctum* amplified with the primer no. 13; KRL14: DNA isolated from *Ocimum sanctum* amplified with the primer no. 14; T15: DNA isolated from *Ocimum basillicum* amplified with the primer no. 15; R5: DNA isolated from *Ocimum sanctum* amplified with the primer no. 5; K7: DNA isolated from *Ocimum teniflorum* amplified with the primer no. 7; R9: DNA isolated from *Ocimum sanctum* amplified with the primer no. 5; O6: DNA isolated from *Ocimum basillicum* amplified with the primer no. 6; KRL8: DNA isolated from *Ocimum sanctum* amplified with the primer no. 8; T13: DNA isolated from *Ocimum basillicum* amplified with the primer no. 13; K15: DNA isolated from *Ocimum* teniflorum amplified with the primer no. 15; O13: DNA isolated from *Ocimum basillicum* amplified with the primer no. 13; R7: DNA isolated from *Ocimum sanctum* amplified with the primer no. 5; KRL6: DNA isolated from*Ocimum sanctum* amplified with the primer no. 6; T8: DNA isolated from *Ocimum basillicum* amplified with the primer no. 8.

Figure 6: PCR gel image 5. 1KB ladder: Standard Marker. K1: DNA isolated from *Ocimum* teniflorum amplified with the primer no. 1; O2: DNA isolated from *Ocimum basillicum* amplified with the primer no. 2; R3: DNA isolated from *Ocimum sanctum* amplified with the primer no. 3; O1: DNA isolated from *Ocimum basillicum* amplified with the primer no. 1; KRL4: DNA isolated from *Ocimum sanctum* amplified with the primer no. 4; T5: DNA isolated from *Ocimum basillicum* amplified with the primer no. 5; R1: DNA isolated from *Ocimum sanctum* amplified with the primer no. 1; K6: DNA isolated from *Ocimum* teniflorum amplified with the primer no. 6; O7: DNA isolated from *Ocimum basillicum* amplified with the primer no. 7; KRL1: DNA isolated from *Ocimum sanctum* amplified with the primer no. 1; R8: DNA isolated from *Ocimum sanctum* amplified with the primer no. 8; R12: DNA isolated from *Ocimum sanctum* amplified with the primer no. 12; KRL9: DNA isolated from *Ocimum sanctum* amplified with the primer no. 9; K12: DNA isolated from *Ocimum* teniflorum amplified with the primer no. 12; T10: DNA isolated from *Ocimum basillicum* amplified with the primer no. 10; T9: DNA isolated from *Ocimum basillicum* amplified with the primer no. 9; O9: DNA isolated from *Ocimum basillicum* amplified with the primer no. 9.

TAACTTGAGTTAAGGGAAAACCTAGGAGAGAATATAW-GCACGTTACAATTGTGCCGCTGTAAATAAATGAAT-GTTTACGGGCACCTTGATTGATTGAGGKGKAAGT-CAAAWACATGGAGATSGACCATACCTAGWAGTGRGT-TAGTTTCATGGCAACGAGAGTGGACCCCTCTTCTTGAACK-ACYAACTCYAGCTTCKCTCTCTCTCTCTCACTCTCAMW-CTCTCTCTCTCTCGCACAMGYCTCTCTCTCTCTCTCTCT-CACTCKCTCTCTCTCTCTCTCTCTCACTCTCTCTCTCTCT CTCGCA

Sequence of *Ocimum sanctum* with primer 1:

ACGGTTTMATWGGGTGTCGATTTTAATAATY-AAGAAATATKKGATTGTCTGCTKTTTGTACATGACWAASAA-AAACTTAAAGACCCAKGTTGTCCATTCATTTGATTTCTATCT-GTGCGTACTCTGCCCTYATATCTCGTCGAWTATGTCTA-AGCATGTGGGTCGCCTCCCCCCCTCCCCCCCAMTGAYSAKS-GTYTCTCTCTCTCTCTCA

Sequence of *Ocimum basilicum* with primer 12:

AYYMTATCTCTCTCTCTCTMTMTCTCTCTCTCTC-TATMTCTCTCTCTMTCTCTATMTCTMTCTMTCTMWC-WCWCTCYCTMACAAAAAYYMTATCTCTCTCTCTCTMT-MTCTCTCTCTCTCTATMTCTCTCTCTMTCTCTATMTCT-MTCTMTCTMWCWCWCTCYCTMACAAAA

Sequence of *Ocimum sanctum* with primer 5:

TTCCACCCGACTTTCCCKCACAGATTGTCTGCTKTTT-GTACATGACWAASAAAAACTTAAAGACCCAKGTTGTC-CATTCATTTGATTTCTATCTGTGCGTACTCTGCCCTY-ATATCTCGTCGAWTATGTCTAAGCATGTGGGTCGCCTCC-

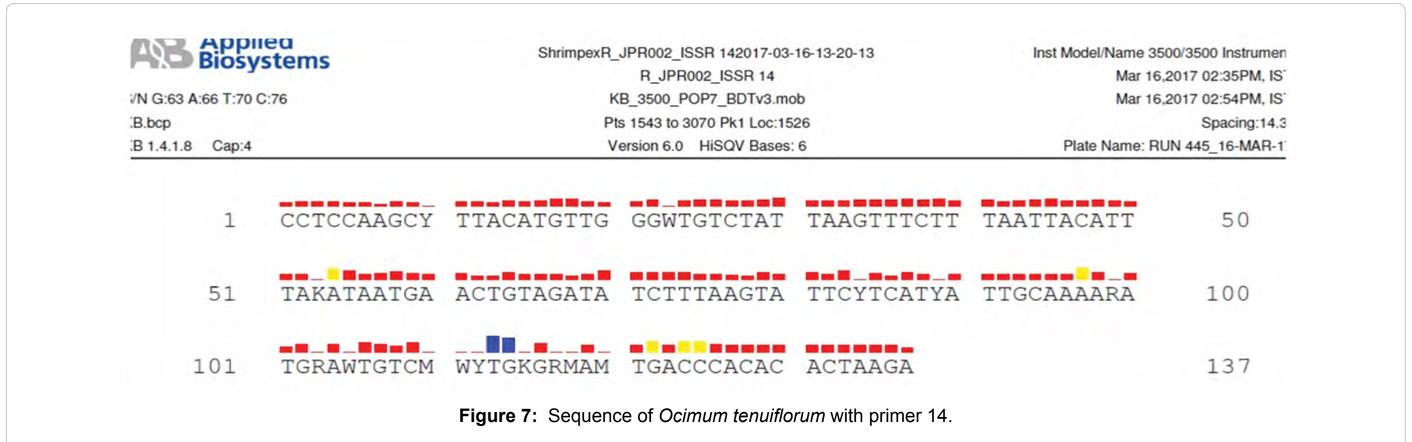

Figure 7: Sequence of *Ocimum tenuiflorum* with primer 14.

CCCCCCTCCCCCCCAMTGAYSAKSGTYTCTCTCTCTCTCTCA-CAMAMA

Sequence of *Ocimum basilicum* with primer 9:

CTCCCCCGAACACCTSRCACATATTRGTCTGGCATAT-GTGATTAAGTGACTTAAAATTTTCAAACATGTGTTT-TATTGAMATAGTTTCTATCAGTAKGTAMYGAGCGAKAAT-CACCGCAYCTGATAGCTAATCATCTGGGACGCCTCTCC-CCCCCCCCCCCCAAYGAYMAKYSTYTCTCTCTCTCTCT-CAAAMAC

Sequence of *Ocimum sanctum* with primer 11:

TCGCGKGCCCRCTRCACTKCGCRCGC-TACCTATRCCGGCATGTATATCCCCCTATATATGGGG-TATATATATACCTATTTATGTGGGGATATATATATATATATA-GATATATATATATATATATATATATAG-ATATATATATATATATATATATATACATATATACACATA-CATATATATATAGATGTATATCTATATATCTAGATATA-GATATATATATATATAKATATATATATATATATATATA TATGTATATGTATATATACAGGTATGGGGGGSGGGTCG-GCAGMGATATAGATASGGGGGGCCCCCCGGCGTATCGC-CATACAGACGGGCCGCGCCGGCGGGCCTATATAGCCG-GCCSCCGGCCTCCCGCCCCGCCGACGCCCCCCCCCCC-GCGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCKCCCC CCCCCCGCCCCCCCCCCCCCCCCCCCCCCCASGTATATATC-GGGATGTAGASACACCCGTAGGGGGGGCGGGGGCG-GATCTCTYTYTATATATATATCSCTSTATYTKTCT-STATATACCTACCCCCCCCCCCCCCCCCGGCCGGGGGGC-GGGCC CGCCCGCCCCCTCCCTACCG

Sequence of *Ocimum basilicum* with primer 13:

GTCCCGCTACTGTATTCTTTGCTTGTTTGTCTATG-GAGGGCTAGAGCACGAATGTKCGGTKATGCAGKAGWA-GRAAAGGAGGGAGGTGGCTCTCCTCTCCCCTCCACAGGGGC-CRWAGGGGASTGGCTTGTTTCTCCGACAGCACGCTCTGT-TATCCGTGTTTTTATGTTTTAAGAAATAGGGGGGYC-CAATTTTTTACCCCTCACTAAA TTATT

Sequence of *Ocimum basilicum* with primer 14:

ACCGTTGTMTWGTTGGTTATTCTCTAAAK-WAAAACAAAKWWTTTTGGGWTTTTTTTTW-WTTTTTTTTTTTTTTTTTTTTTTTTTGAAAAGCCTCAGGGT-GTTTTGGC

Sequence of *Ocimum basilicum* with primer 8:

GCTCTGCCAAGCTRMTTTCCTCTCTCTTCCTCYCT-GTARGTTCCACACTTWTTAGGCCCYGGCTTTGYTTT-GACCCATTAAAGACGACAAATWCGCTCAKCATGTCTC-GTCACCTTGCTAGTTGTTCACACGCTTGTGTCTGGCTT-TATCAYCCATTTATCCATCCGAAAATCGGCTGGAGTCTG-GTCGGGTGCTTAATTTCTGKTCGAATGAAGATTTTTTTCCW-GATTGATTGAGAAAGGACTTCTTTGGTGTCTCTAAGCAAGT-CAAATACTATACTTGGCTCATAATTTGATGAGTCACACRAG-GATTTGGAATTTCGTCAGCTCATGCATAACGACCTG-MATTCCTTGMASCTCTCTCCCTTCTCT CTCTCTCTCTMT

Sequence of *Ocimum tenuiflorum* with primer 9:

TCATCGCTATGTCGTGCTGCATCTGAGTCTGTCG-GTTGACTCGTCGCMGTGTGGGGCTCGGTGGTGTCT-GACTAGCCGTACGTGGGAGGCCGWGAYAGGAGCCY-WKAGTATRTGCATTAATCGGGGGTCTCTYGCTGTATG-GCTCAGAGCTAGGCTGCGAGGTTCCTGCAGAAATTT-GTGGGTTTTTGTAMTYAGTATTCGGGMAGAAGATTTAGM-TWAAAAGCAGTTGTCTTGAGTTCAGGGGAAACCTAGGM-KGGACTGAGGCACGTTGCAATTGTGCCCCTGTAAATASAT-GAATGTTTACGCGCSCCTTTATTGATTGAGGTGKAAGT-CATAAACATGCTGATSGCCCATACCTKTTAGTGRGT-TAGTTTCATGGCAACGAGAGTGGACCCCTCTTCTCGAW-CKACYAACGCYAGCTTCKCTCTCTCTCTCTCACTCTCACW-CTCTCTCTCTCTCRCACKCTCTCTCTCTCTCTCTCTCTCT-CACTCTCTCTCTCTCTCTCTCTCTCACTCTCTCTCTCTCT CTCTCWCTC

Sequence of *Ocimum tenuiflorum* with primer 12:

CCCCGCGCKATGCGTSATTTCCTACTCCCTTCCGCCCC-GTAGGACTCCWTACTTCTTAGGCCTTGGTTTTATATCT-GACCTGTTTAAGACKACAGATGAGCTSACTCATGWCATCGT-CACGTTGTATGGGCAGTCACTCACTCGGATATGGCTTTAT-CAATTATTTATCCAGCCGATTTTCCGCTGTATATATAGTC-GACTGGCCTGTTCYGATCCAACGACTACCTTTTCTCCK-GACGATGACGAGAGAKCATTTCTTTGWTGTCTCTAAR-GRAGACAAATATAATCCTTGGCCCATAATTWWAGGAGT-CACASCAGGAGTTACAAATTAGWGAACTCWTGCATA-ACGACCTGCATTCCTGCAGCTCKCTCTCTCTCTCTCTCT-CACTATRTCTCTCTCGGACGATTGGGWMTGACTGTCG-GTCAATTATACMGATAGATACTATGSGWTGCTGMKCTCT-GTCTTGWGCAGTCMTGTTGAGGAGSATGACCGACAAC-TARAATGACCATAGTGAAGTSWTTGMTSYTCTCTCY-CTCTCTCTCTCTTCACTMTATCTMTATAGCKMTCTCKCW-CTCTCT ATAYATAYACWATATA

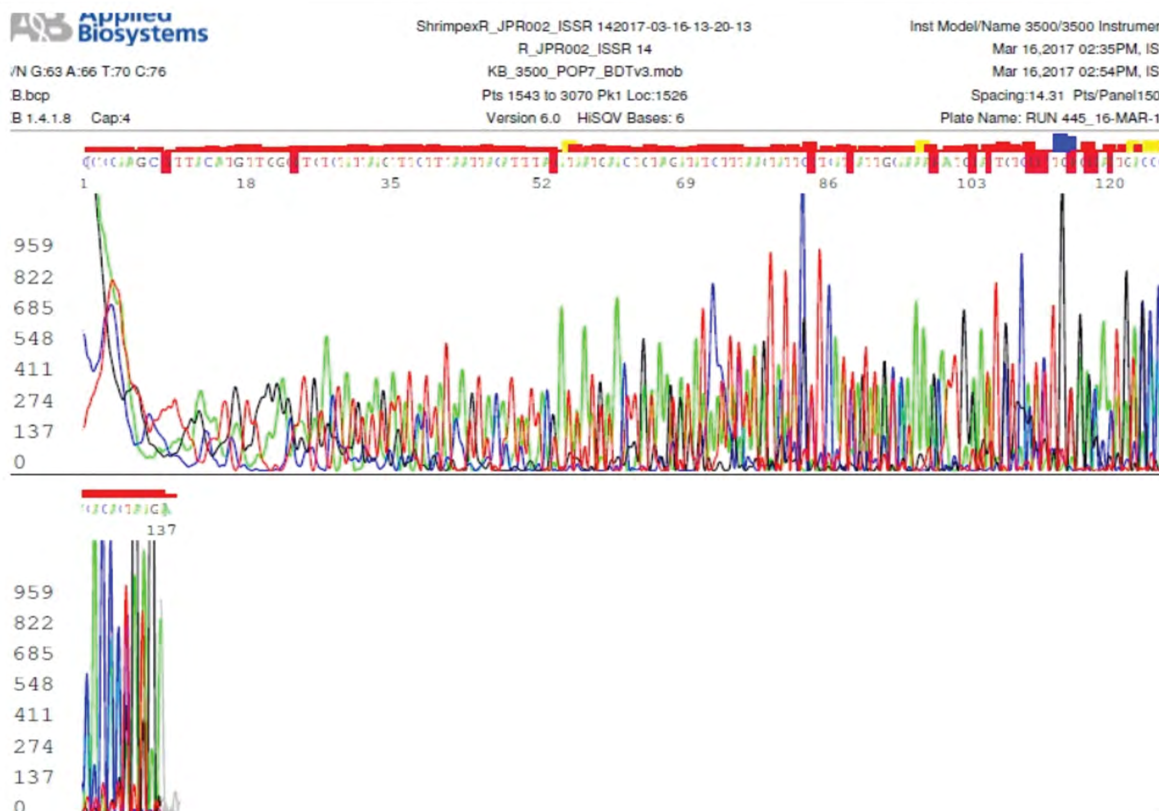

Figure 8: Electrograph of *Ocimum tenuiflorum* with primer 14.

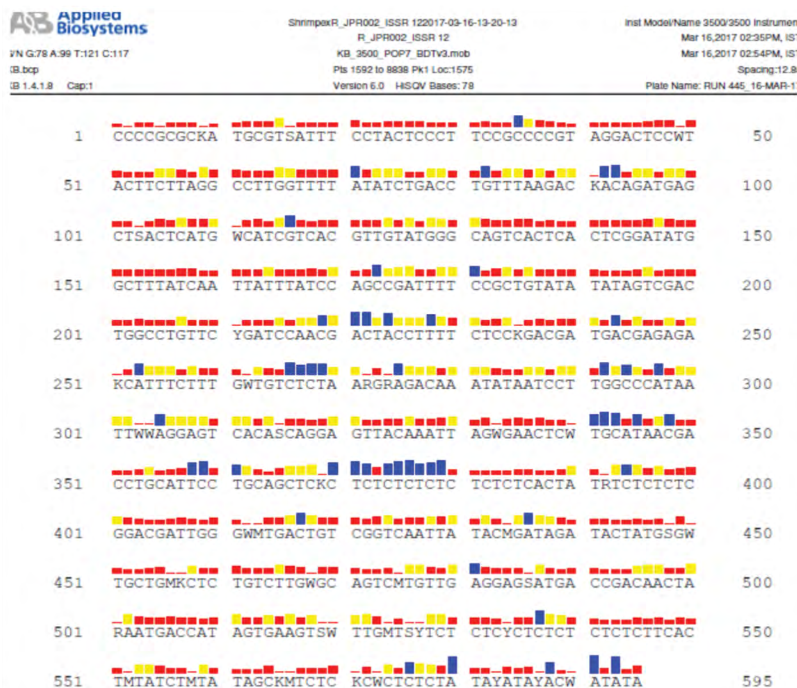

Figure 9: Sequence of *Ocimum tenuiflorum* with primer 12.

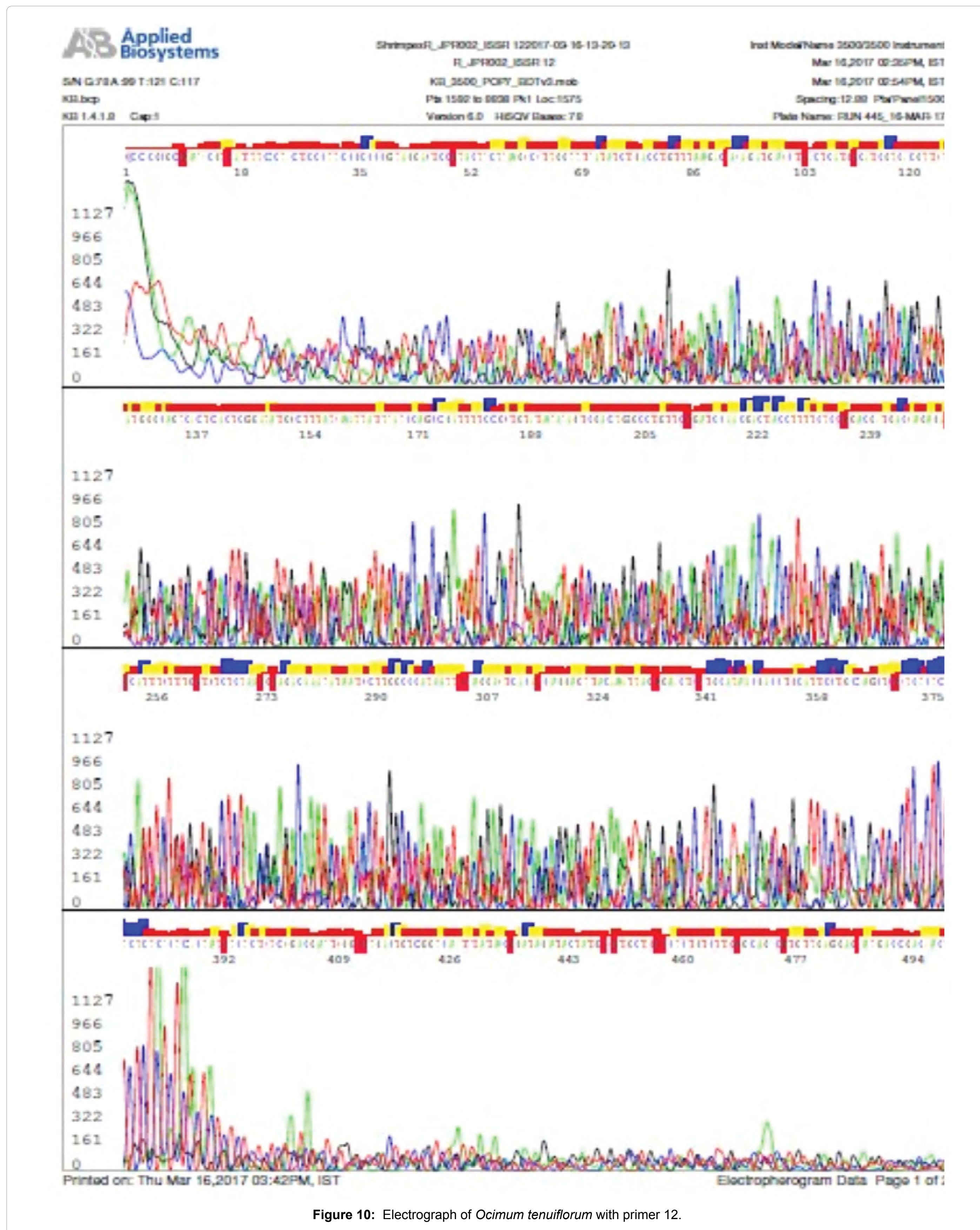

Figure 10: Electrograph of *Ocimum tenuiflorum* with primer 12.

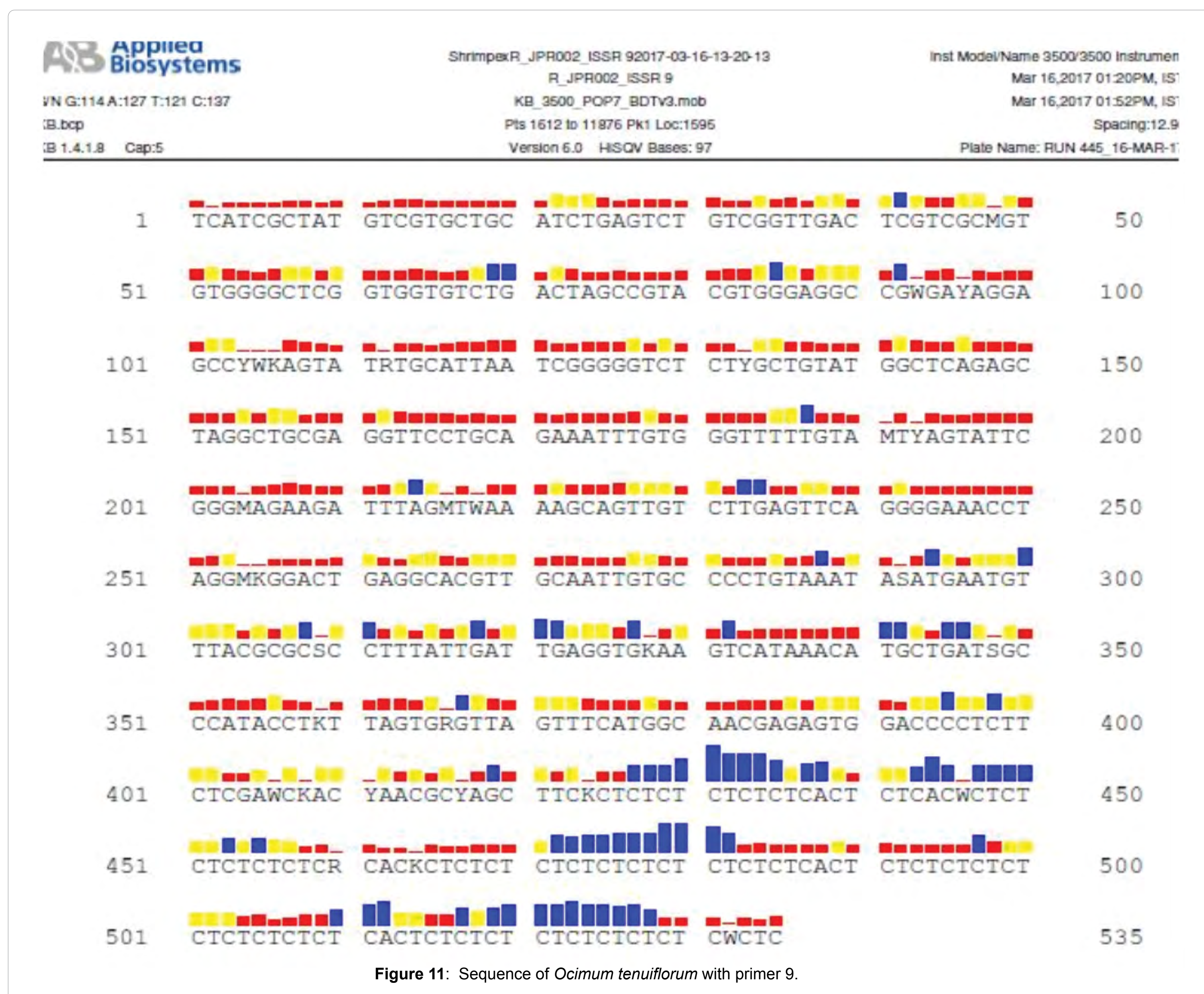

Figure 11: Sequence of *Ocimum tenuiflorum* with primer 9.

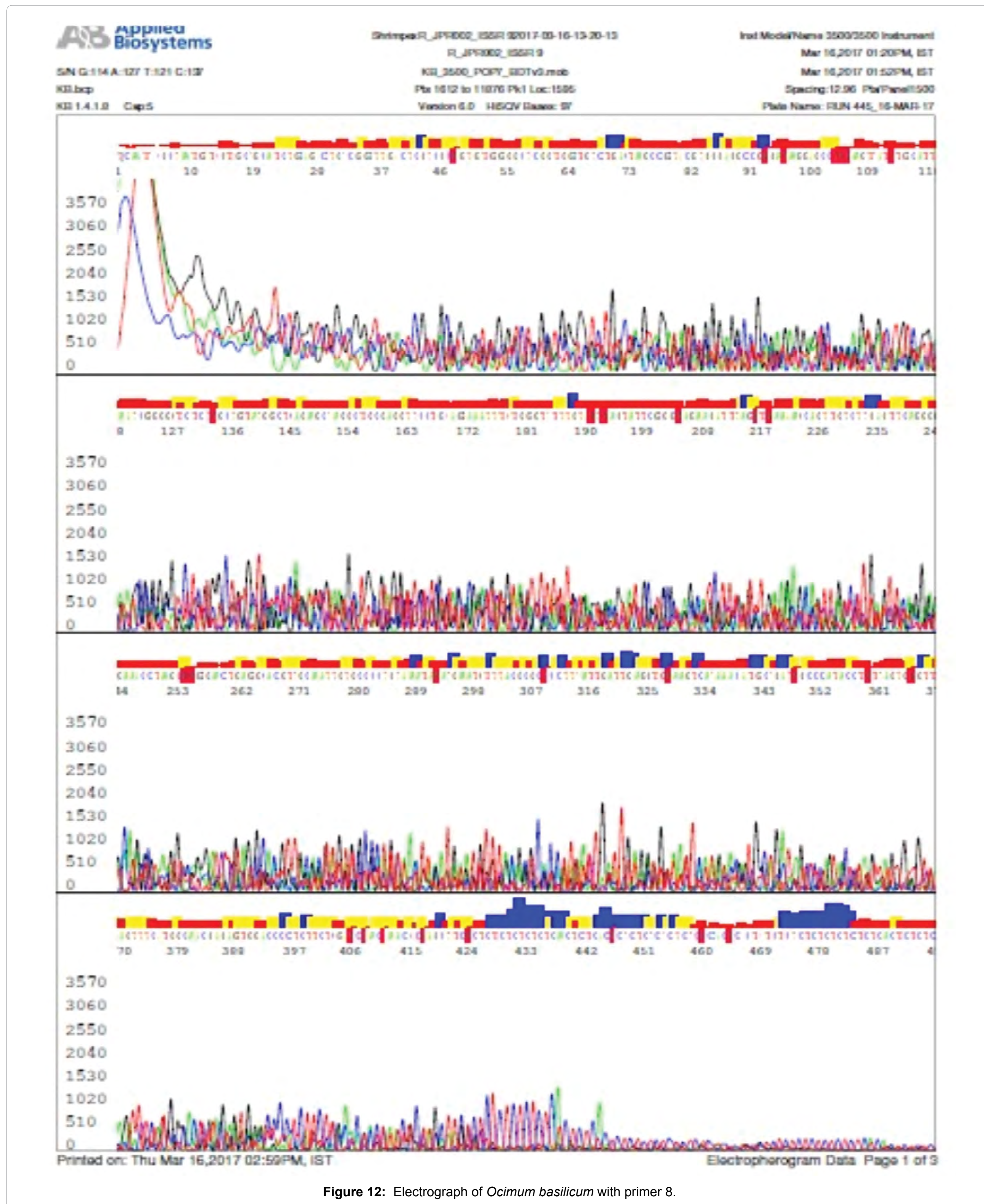

Figure 12: Electrograph of *Ocimum basilicum* with primer 8.

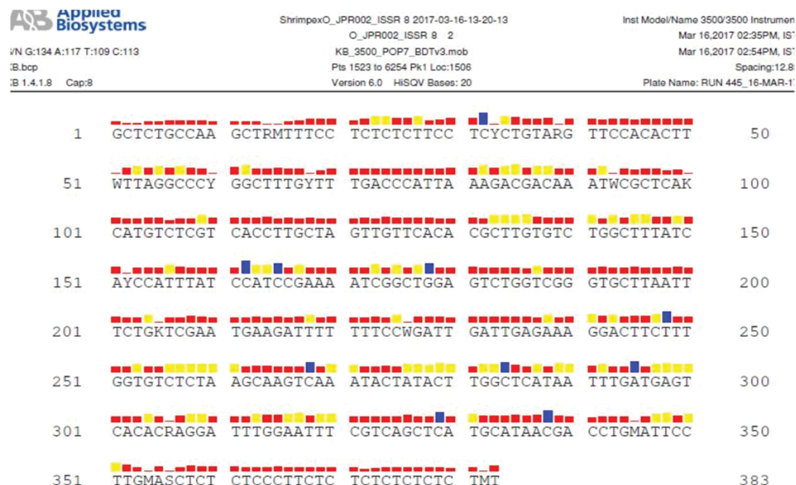

Figure 13: Sequence of *Ocimum basilicum* with primer 8.

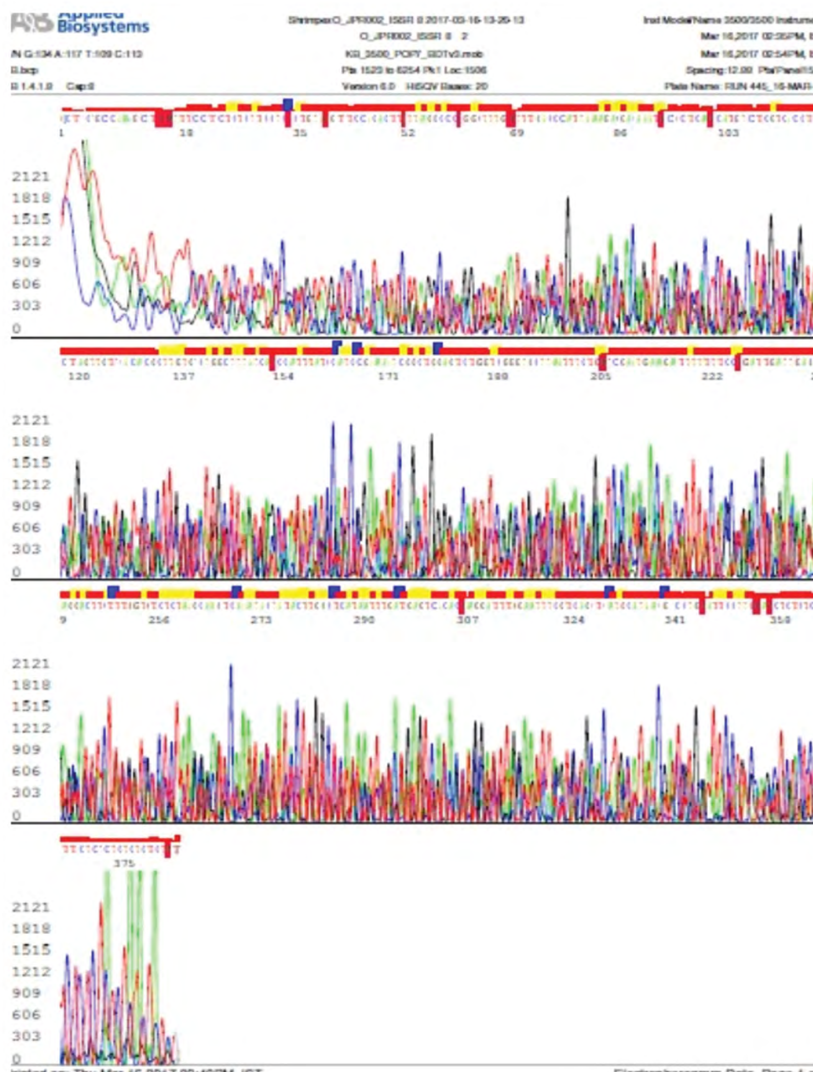

Figure 14: Electograph of *Ocimum basilicum* with primer 8.

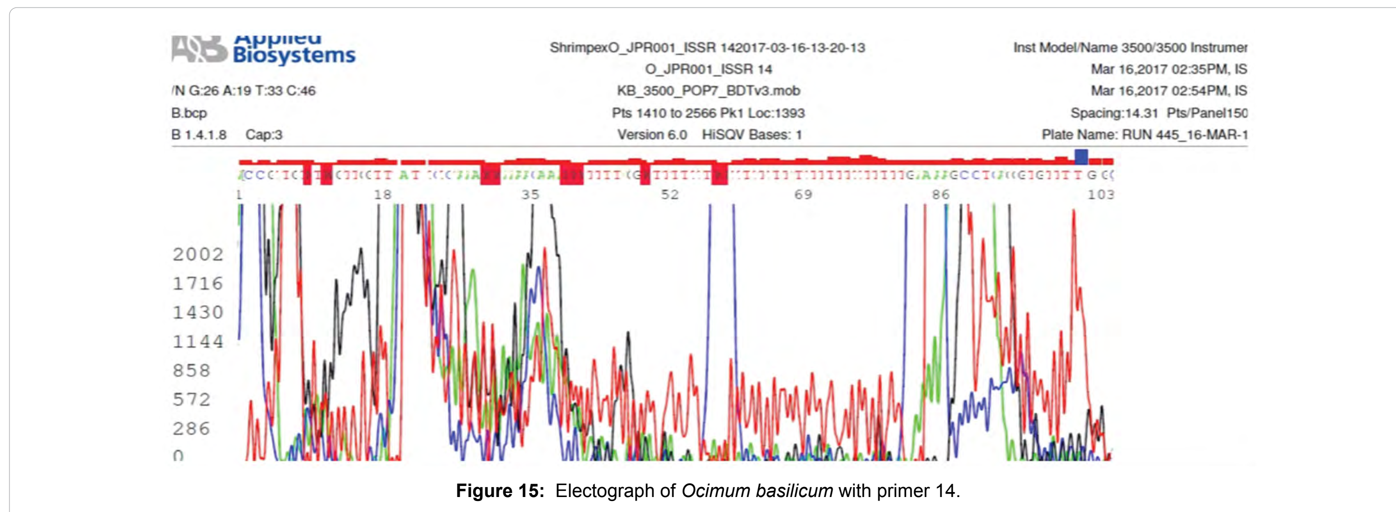

Figure 15: Electograph of *Ocimum basilicum* with primer 14.

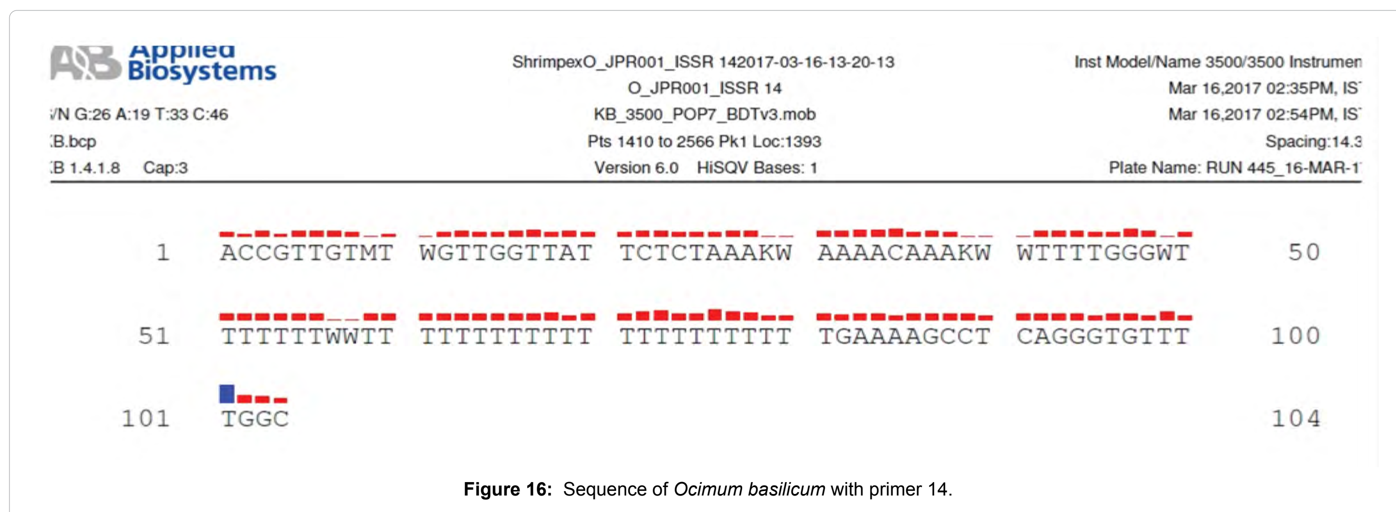

Figure 16: Sequence of *Ocimum basilicum* with primer 14.

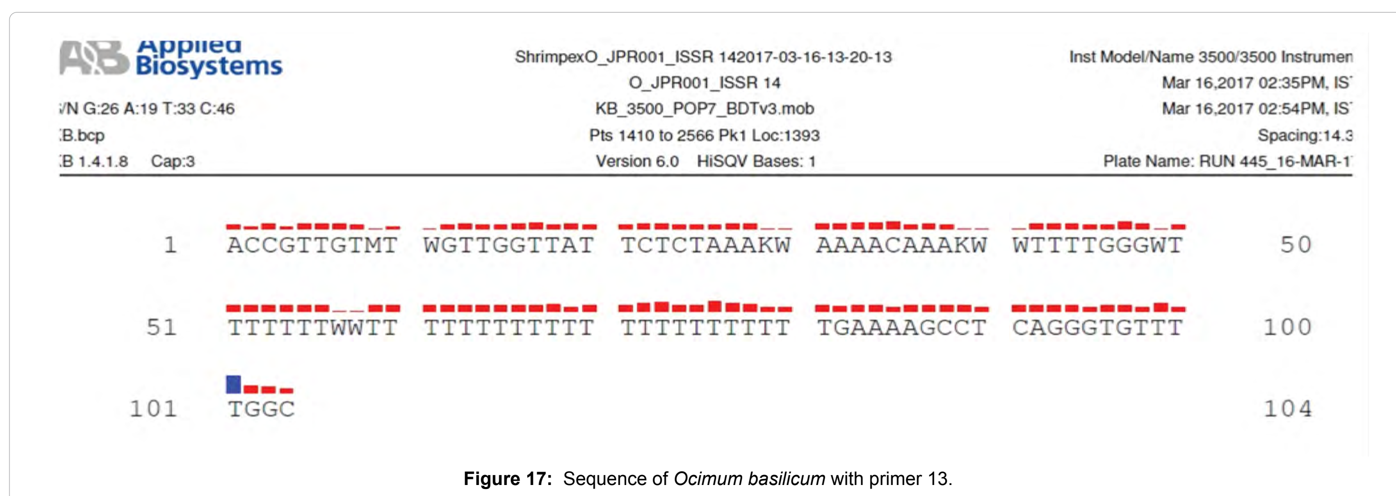

Figure 17: Sequence of *Ocimum basilicum* with primer 13.

Figure 18: Electograph of *Ocimum basilicum* with primer.

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Pts 1625 to 12008 Pk1 Loc:1608
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Inst Model/Name 3500/3500 Instrumen
Mar 16,2017 01:20PM, IS
Mar 16,2017 01:52PM, IS
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Plate Name: RUN 445_16-MAR-1

VN G:4 A:8 T:7 C:4
B.bcp
B 1.4.1.8 Cap:7

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101  ATATAGATAT ATATATATAT ATATATAGAT ATATATATAT ATATATATAT  150
151  ACATATATAC ACATACATAT ATATATAGAT GTATATCTAT ATATCTAGAT  200
201  ATAGATATAT ATATATATAK ATATATATAT ATATATATAT ATGTATATGT  250
251  ATATATACAG GTATGGGGGG SGGGTCGGCA GMGATATAGA TASGGGGGGC  300
301  CCCCCGGCGT ATCGCCATAC AGACGGGCCG CGCCGGCGGG CCTATATAGC  350
351  CGGCCSCCGG CCTCCCGCCC CGCCGACGCC CCCCCCCCCC GCGCCCCCCC  400
401  CCCCCCCCCC CCCCCCCCCC CCCCKCCCCC CCCCCGCCCC CCCCCCCCCC  450
451  CCCCCCCASG TATATATCGG GATGTAGASA CACCCGTAGG GGGGCGGGGG  500
501  CGGATCTCTY TYTATATATA TATCSCTSTA TYTKTCTSTA TATACCTACC  550
551  CCCCCCCCCC CCCCGGCCGG GGGGCGGGCC CGCCCGCCCC CTCCCTACCG  600
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Figure 19: Sequence of *Ocimum basilicum* with primer 11.

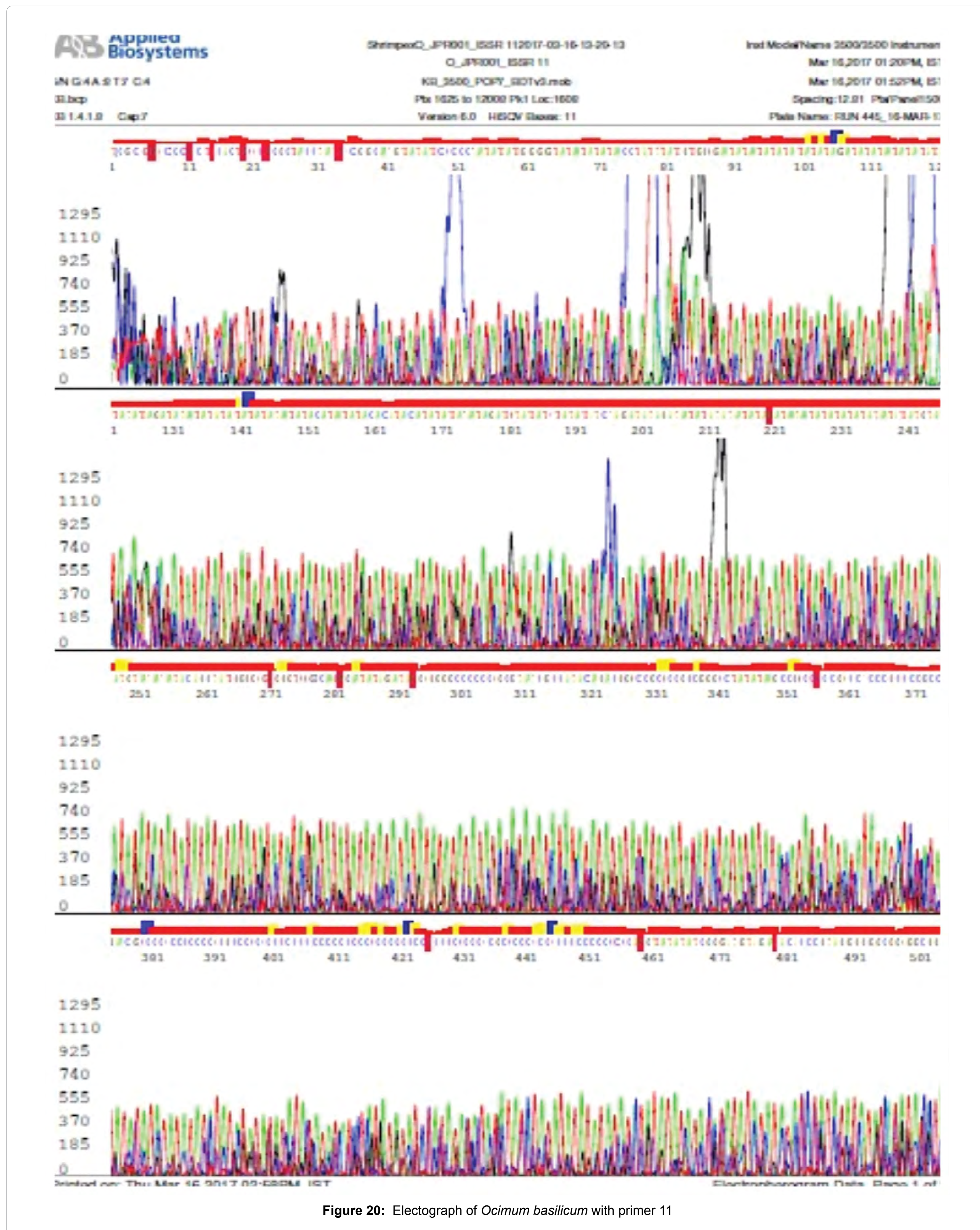

Figure 20: Electograph of *Ocimum basilicum* with primer 11

Figure 21: Electograph of *Ocimum basilicum* with primer11.

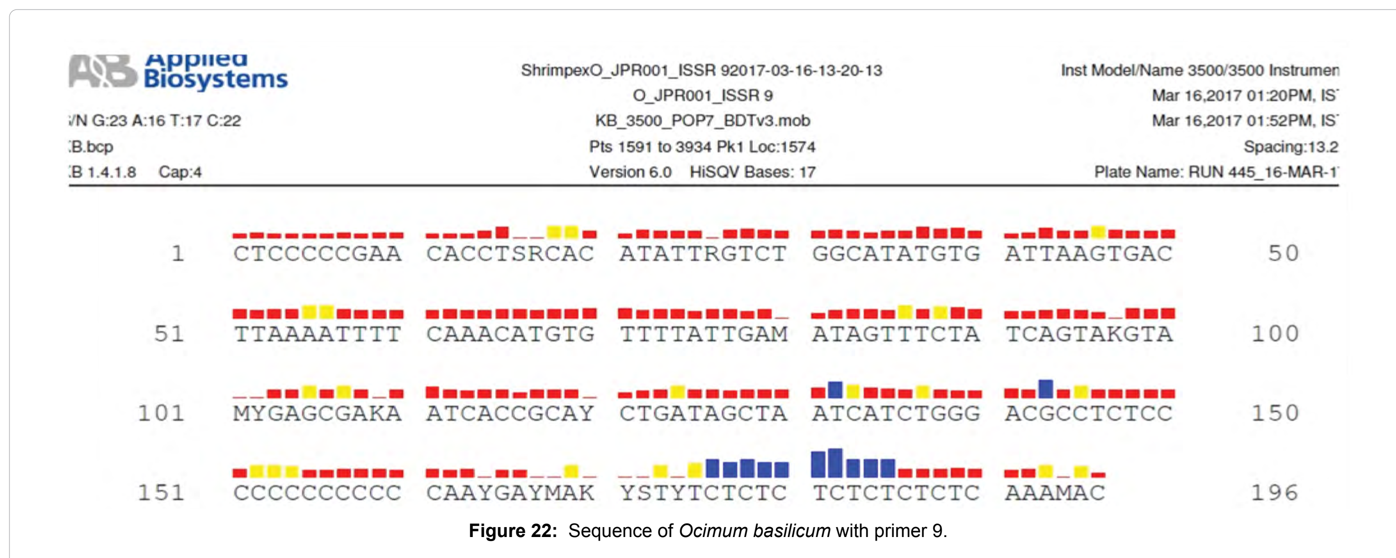

Figure 22: Sequence of *Ocimum basilicum* with primer 9.

Figure 23: Electograph of *Ocimum basilicum* with primer 8.

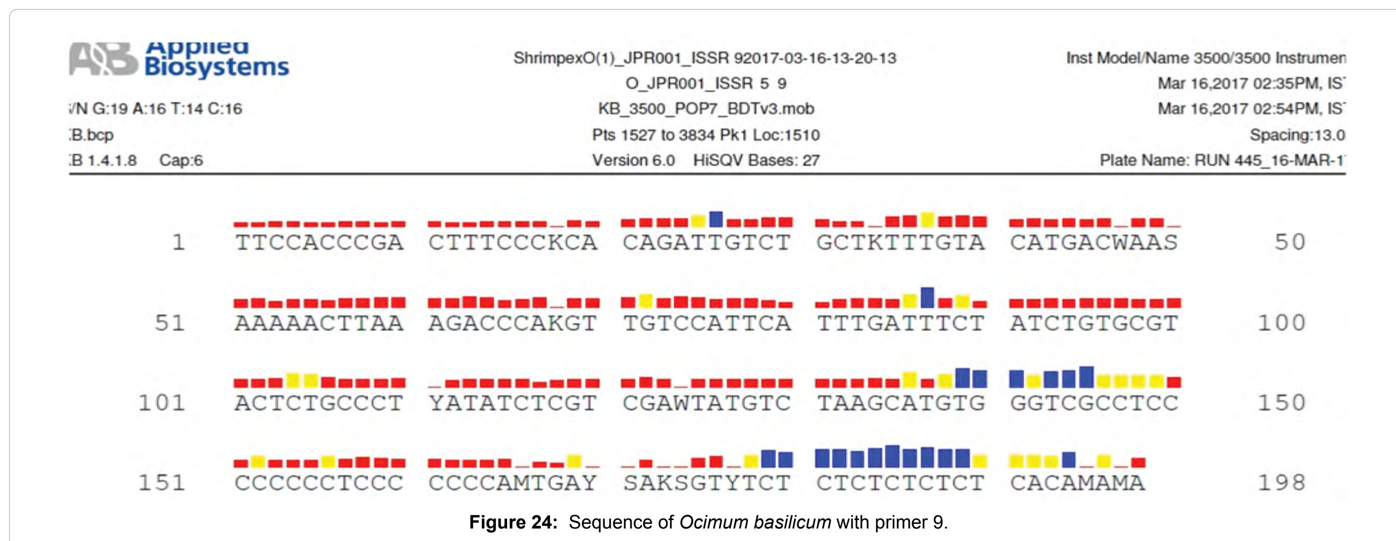

Figure 24: Sequence of *Ocimum basilicum* with primer 9.

Figure 25: Electograph of *Ocimum basilicum* with primer 9.

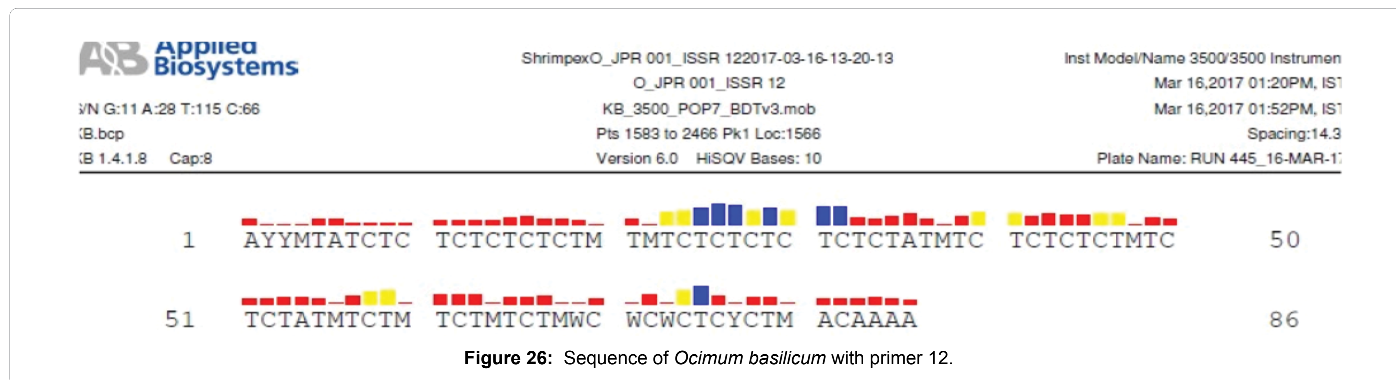

Figure 26: Sequence of *Ocimum basilicum* with primer 12.

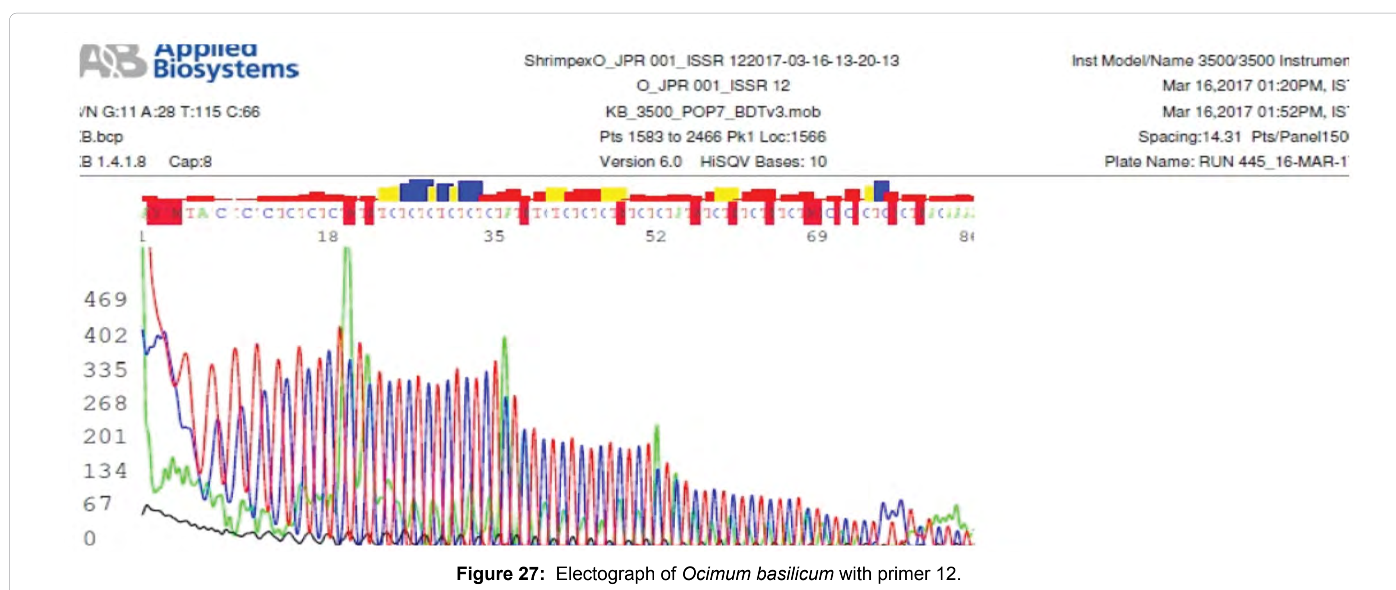

Figure 27: Electograph of *Ocimum basilicum* with primer 12.

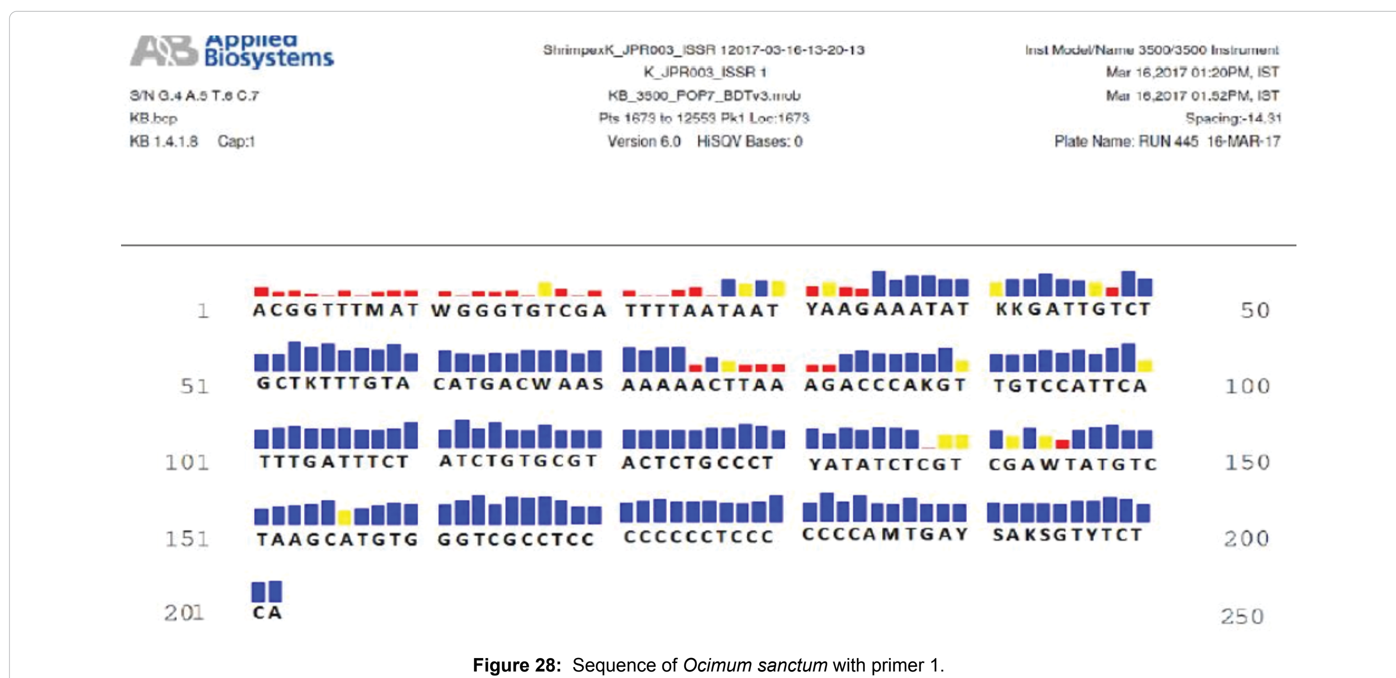

Figure 28: Sequence of *Ocimum sanctum* with primer 1.

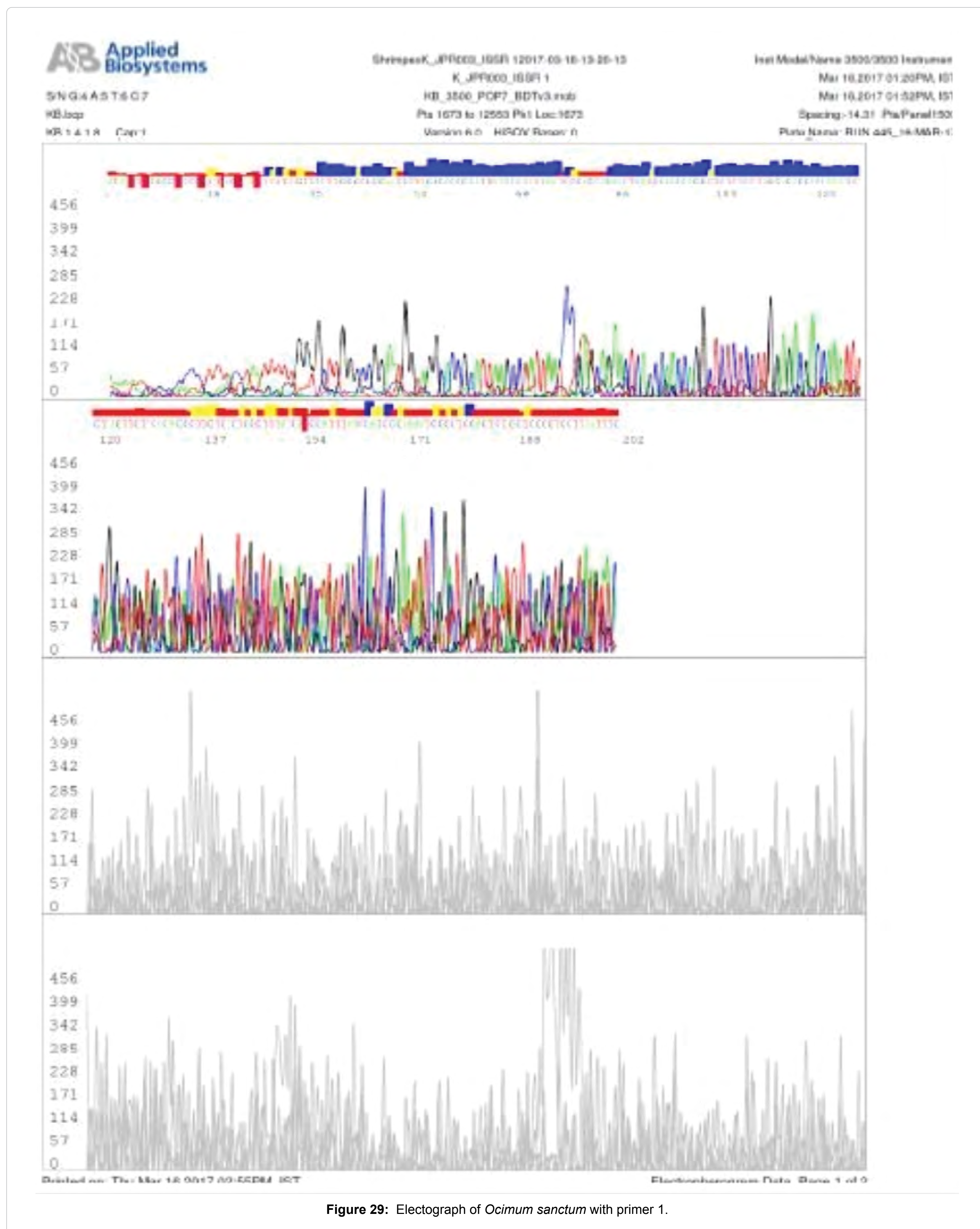

Figure 29: Electograph of *Ocimum sanctum* with primer 1.

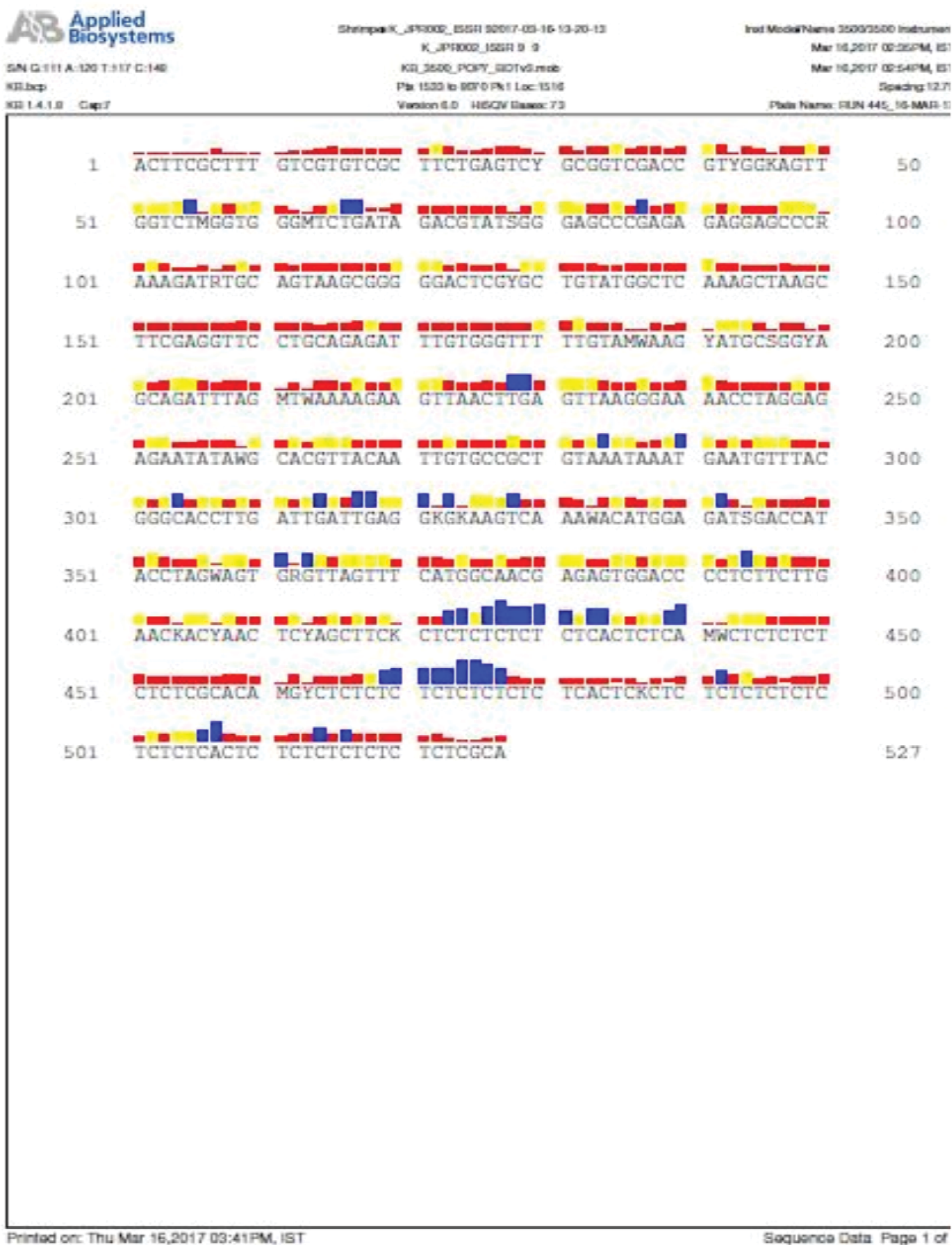

Figure 30: Sequence of *Ocimum sanctum* with primer 9.

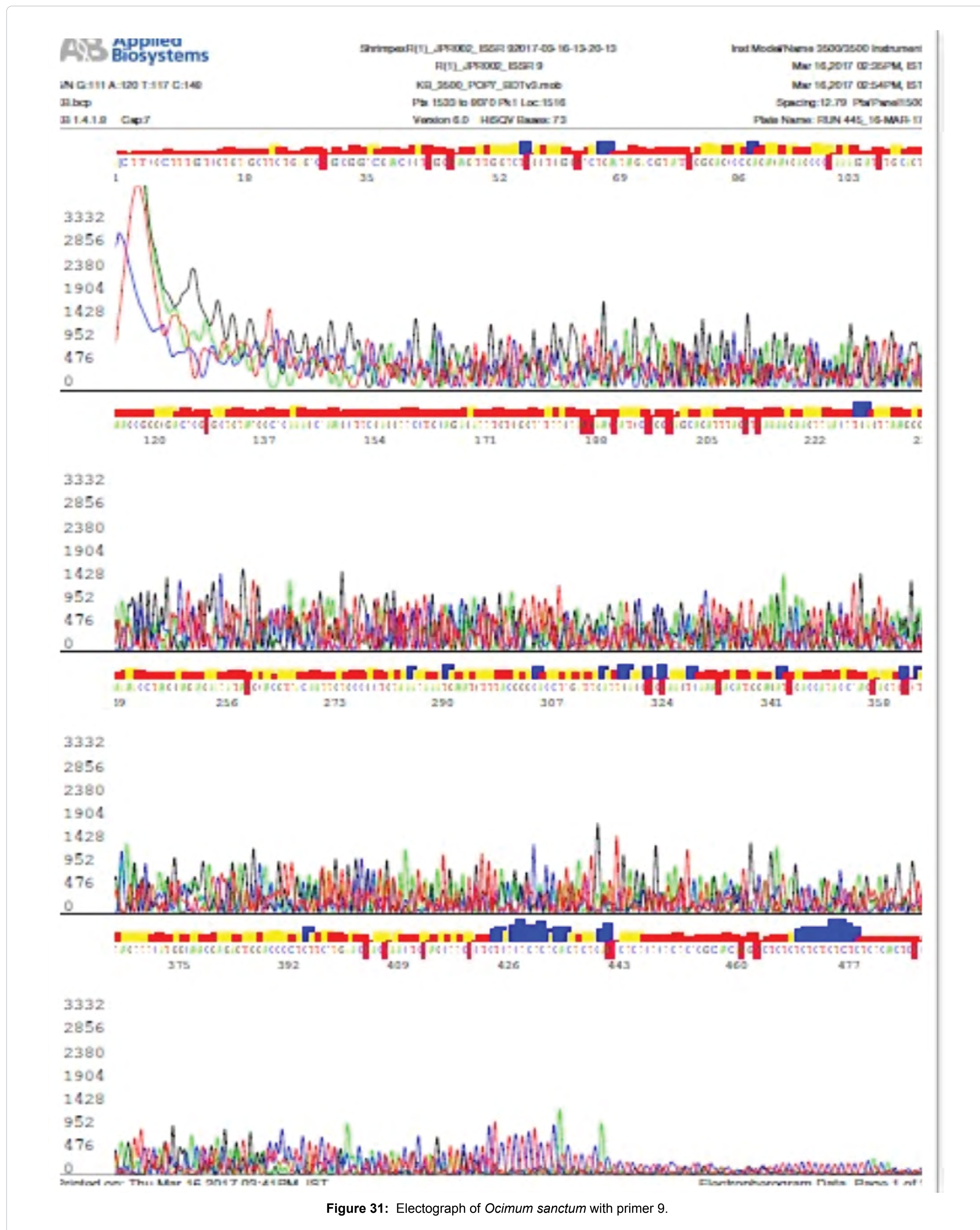

Figure 31: Electograph of *Ocimum sanctum* with primer 9.

Sequence of *Ocimum tenuiflorum* with primer 14:

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Sequencing results

Sanger sequencing was done in genetic analyser (Hitachi 3500) at Shrimpex biotech services,uthandi,Chennai (Figures 8-31).

Conclusion

We started our study using *Ocimum* species to isolate and study of our taditional medicinal plant Tulsi gene to identify the marker for the *Ocimum* species,we started our work by collecting the samples from various nursery garden located in E. C. R, uthandi, Chennai. As a first step we had isolated DNA from the samples both leaves and flowers which was collected and then a specific primers sequence needed for our study was collected from the reference with Hardik and was synthezied in our lab under the supervision of our external guide at Shirmpex Biotech Services,Uthandi,Chennai. These synthesized primers were amplified with our DNA samples using PCR and after the purification of these samples using sodium acetate method, the sample was loaded to a 2% agarose gel electrophorosis, run under 50v for 45 minutes then low melting point agarose gel with DNA bands has been visualized under a UV transilluminator and located the desired Marker band with equal number of base pairs was cut and purified. Since DNA samples from flowers did not amplify well with any of the 15 primers we had chosen, thus were not considered for further sequencing, the leaves samples which has amplified has shown a better result. These samples which is been removed from the gel were subjected to cycle sequencing. After purification it was loaded in the genetic analyzer for Sanger sequencing.

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