DEVELOPMENT OF FUNCTIONAL EST-SSR AND ANALYSIS OF GENETIC DIVERSITY IN *Centella asiatica*

By

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(2013-09-113)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Development of functional EST-SSR and analysis of genetic diversity in *Centella asiatica*"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
Ave_Het	Average hetrozygosity
bp	Base pair
cm	Centimetre
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tags
Exp_Het	Expected heterozygosity
G	Guanine
g	Gram

h	Nei's heterozygosity
HPTLC	High performance thin liquid chromatography
I	Shannon's information index
ISSR	Inter simple sequence repeats
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
MSA	Multiple Sequence Alignment
ng	Nanogram
^{o}C	Degree Celsius
na	Number of alleles per locus
ne	Effective number of alleles
Obs_Het	Observed homozygosity
OD	Optical density

P	Percentage of polymorphic loci
PCR	Polymerase chain reaction
RAPD	Randam amplified polymorphic DNA
RFLPD	Restriction fragment length polymorphism
rpm	Revolution per minute
SD	Standard Deviation
sp.	Species
SNP	Single Nucleotide Polymorphism
SSR	Simple sequence repeat
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Tm	Melting temperature
UPGMA	Unweighted pair group with arithmetic mean
UGT	UDP-glucuronosyltransferase
V	Volt
via	Through

1. INTRODUCTION

Genus *Centella* L. of the family Apiaceae (Umbelliferae), is cosmopolitan in distribution. *Centella* includes about 53 species, most of which are endemic to South Africa, and this region is believed to be its centre-of-origin (Mabberly, 1997). The genus is scarcely represented in the Indian sub-continent, *Centella* with just one species (Hooker, 1879). In peninsular India, there is only a single species of *Centella*- *Centella asiatica* (L.) Urb. The species is a stoloniferous herb, preferring shady moist places. In Indian pharmacopoeia, the plant is listed as useful for the treatment of diarrhoea, dysentery, fever, amenorrhea and female genito-urinary tract diseases, filariasis and tumour (Jain *et al.,* 2007). It is also used as a brain tonic for mentally retarded (Jain *et al.,* 2007).

Centella asiatica contains several bioactive constituents, of which the most important are the triterpenoid saponins such as asiaticoside, madecassoside and centelloside. Pharmacological studies discovered its utility in together with human collagen-1 synthesis (Lee *et al.*, 2006), fast nerve regeneration (Soumyanath *et al.*, 2005), cardio-protection of important neurons (Lee *et al.,* 2000), treatment of blood vessel cardiovascular disease (Incandela *et al.*, 2001) and elevation of inhibitor level (Shukla *et al.,* 1999) etc. The isoprenoid pathway is used by *Centella* to synthesize triterpene saponins by yielding a hydrophobic triterpenoid structure (aglycone) comprising a hydrophilic sugar chain (glycone) and are common secondary plant metabolites (Jacinda *et al.*, 2009).

The high demand of the plant for the manufacture of a variety of herbal products has resulted in its over exploitation from the wild and consequent gene-erosion of the species. The prevailing transformation of habitats of the species – the watersheds – for various purposes is another causative factor for depletion of its populations. Extinction of

potential genotypes of the species is an irreversible set back to the richness of its germplasm, and detrimental to the prospects of developing quality herbal products, its cultivation, and genetic improvement. Therefore, serious efforts to achieve conservation as well as effective utilisation of the species are essential.

On account of the high degree of reticulation of exomorphic traits in the genotypes of *C. asiatica*, study of variation of their morphological characters is needed for authentic identification of its genotypes. Data on intraspecific morphological variation of *C. asiatica* can yield valuable information on the degree and extent of variability by species in morphological characters (qualitative and quantitative). The data is also useful to estimate correlation between characters, heritability and genetic advances of characters and for clustering the genotypes based on genetic and phenetic similarity.

Genetic diversity is the total variability of genetic characteristics within the genetic makeup of a species. With a lot of variation, there is higher probability that some populations possess variations of alleles that are suited to a given environmental condition. The study of genetic diversity and interspecific or intergeneric relationships among a number of species are now being extensively carried out with the help of molecular markers (Bandopadhyay *et al*., 2004).

The advent of phytochemistry in the past few decades has expanded the horizon of researchers. The recent developments in powerful analytical techniques have facilitated screening of a number of taxa in a short time, making available a large quantum of phytochemical characters to be used in plant diversity analysis (Asakawa *et al.,* 1982). The phytochemical study of the six accessions of *C. asiatica* with respect of the content of the triterpenoids is useful on chemotaxonomic point of view and for identifying potential genotypes of the species.

2. REVIEW OF LITERATURE

Centella asiatica comprises of some 53 species, inhabiting tropical and subtropical regions. This genus belongs to the family Apiaceae (previously known as Umbelliferae) and includes the most ubiquitous species *Centella asiatica*. *Centella* is also known as Pennywort, Asiatic Pennywort, Indian Pennywort, Gotu Kola and Brahmi. This plant is a perennial creeper flourishes abundantly in moist areas and is a small herbaceous annual plant. It is present in marshy areas of South East Asia, India, Sri Lanka, China, Madagascar, South Africa, Thailand, Mexico, Malaysia and Eastern South America (Chopra *et al.,* 1956).

Centella is clonally propagated by producing stolons that are characterized by long nodes and internodes. The stems are green to reddish green in colour, long, interconnecting to one another, creeping and rooting at the nodes. Leaves are simple, green, smooth texture with palmately netted veins, fascicled at the node, orbicular, reniform, 1-7 cm long, 1.5-9 cm broad. The flowers are small (less than 1mm) pinkish to red umbel shape. Each flower is partly enclosed in 2-3 green bracts, 5 sepals, 5 petals which are 1-1.5 cm long. Fruits are flattened, 2-3 mm long, 3-4 mm broad. Seeds are very small in size (on average 2.5 mm long and 1.7 broad) and mass (1.3 mg $seed^{-1}$). On germination, the seeds produce seedlings and germinate during the months of September to February (Moharana *et al.,* 1994).

Centella is a wild medicinal plant that has probably been used since prehistoric times and has been reported to have been used for various medicinal and cosmetic purposes, therefore thus becoming an important commercial product. So, this plant is listed as a drug in the Indian Herbal Pharmacopoeia, the German Homeopathic Pharmacopoeia, the European Pharmacopoeia, and the Pharmacopoeia of the People's Republic of China (Schanerberg *et al.*, 2003).

2.1. Plant Description

Figure1: *Centella asiatica*

Indian Pennywort or *Centella asiatica* (L.) Urb. (Fig:1) is a species of the plant family Apiaceae (Umbelliferare). This medicinal herb is known as Brahmi in Unani medicine, Mandookaparni in Ayurveda and Gotu Kola in the Western World. In India, plant was earlier cluttered for *Bacopa monnieri* Wettest., as both were sold in the market by the name Brahmi. However, the controversy has been resolved and it is concluded that Brahmi is *Bacopa monnieri* and Mandookaparni is *Centella. Centella asiatica* is a creeping perennial plant with showel shaped leaves which sprouts clusters at stems nodes. The stems roots at the nodes. Leaves are rounded to reniform, at the tip leaves are round and at the base they are kidney shaped or heart shape sometimes, rounded lobes are overlapping. The petioles are long, erect and green in colour. The peduncles occur in pair of two or three, are less than 1 cm long and usually bear three sessile flowers. The hermaphrodite flowers are dark purple in colour and about 1 mm long, with 5-6 corolla lobes per flower. Each flower bears five stamens and two styles. There are minute fruits which is ovoid, white or green in colour and reticulate also, each with nine sub similar longitudinal ridges. The runners lie along the ground and in the summer greenish to pinkish white flowers are borne in dense

umbel in separate stems. *Centella* matures in three months, and the whole plant, including the roots, is harvested manually.

2.2. Medicinal Importance of the Plant

Centella asiatica is used as a traditional medicinal plant that has probably been used since prehistoric times. The plant is highly valued for various diseases and it is used in Ayurveda, Siddha and Unani System of medicine. Therefore, this plant is listed as a drug in the Indian Herbal Pharmacopoeia, the German Homeopathic Pharmacopoeia, the European Pharmacopoeia, and the pharmacopeia of the People's Republic of China (Jacinda and Ian, 2009).

The major ethnomedicinal use of plant appears to be alleviate gastrointestinal disorders like dysentery, constipation, stomach problems and loss of appetite and to enhance memory or to serve as nerve stimulant.

Around 12 ethnomedicinal reports were from India, 2 from Nepal, six from Bangladesh and two from Africa. Thus, the major ethnomedicinal uses of the plant seemed to be centered in Indian subcontinent (Jahan *et al.*, 2012).

Extract of *Centella asiatica* exhibited cardioprotective activity against myocardial injury in adriamycin-induced rats. Damaged mitochondria were observed from transmission electron microscopy from the control group, while restoration of mitochondria was found from *Centella asiatica* treated rats (Gnanapragasam *et al.,* 2004).

In India *Centella asiatica* is an important herbal medicinal plant used for various applications (Jacinda and Ian, 2009). Since ancient times, *Centella* has been used for wound healing, anti-inflammatory, memory enhancing, immune booster, anti-anxiety, and was used as anti-stress substance (Han *et al.,* 2003). *Centella asiatica* has been clinically used

in mentally retarded children and also in treatment of anxiety neurosis. *Centella* showed positive result in short term memory and learning (Meena *et al.,* 2012).

The saponin containing triterpene acids and their sugar esters of which asiatic acid, medecassic acid and asiaticosides are considered to be most important potent therapeutic substances in *Centella asiatica* (Brinkhaus et al., 2000).

In Europe an extract of *C. asiatica* was used for many years for the treatment of wounds (Maquart *et al.*, 1999). *C. asiatica* is well reputed as a brain tonic for the mentally retarded patients. Significant improvement in general mental ability and mental concentration, overcoming stress and fatigue has been reported by Appa Rao, *et al.*, (1977). The plant extracts are used popularly in memory enhancing tonics and for the treatment of mental and stress-related disorders (Sharan and Khare, 1991; Moharana and Moharana, 1994).

Due to the health benefits such as antioxidant (Ullah *et al.*, 2009), as anti-inflammatory and wound healing memory enhancing property (Subathra *et al.*, 2005) and many others the use of *Centella* in food and beverages has increased over the years. The capability of *Centella* has markedly increased in recent years as an alternative natural antioxidant especially of plant origin and its protection against age-related changes in brain antioxidant defence system (Subathra *et al.*, 2005). Free radicals have been claimed to play an important role in ageing process and capable of damaging many cellular components (Jacinda and Ian, 2009). These changes will affect the brain as it is particularly vulnerable to oxidative damage; as such many studies on its neuroprotection activity have been reported.

Marker is an emblematic feature of a genome and it should be polymorphic. Markers are classified into morphological, biochemical and molecular types. Morphological markers are quantifiable characters

that can be visually determined. Proteins developed by the expression of genes represent biochemical markers and molecular markers can distinguish genetic variations at DNA sequence level. They are solo segments of DNA, found at distinct locations in the genome, and are transmitted from one generation to the next as per the standard laws of inheritance (Semagn *et al.*, 2006). It is not compulsory that molecular markers consistently correlate a phenotypic expression with a genomic trait. But they offer several advantages over conventional, phenotype-based alternatives, as they are more persistent and detectable in all tissues irrespective of growth, differentiation, development, or defence status. Moreover, they are not bewildered by environmental, pleiotropic and epistatic effects (Mondini *et al.*, 2009).

In modern plant breeding studies, molecular markers have become important and efficient tools. Molecular markers linked to agronomic traits are capable of increasing the accuracy of selection, and reducing the field workload, therefore the selection of suitable markers has become one of the key factors in the success of molecular breeding programs.

Molecular markers have been proven to be an impressive tool for many genetic analyses. One of the advantages of molecular markers is that they are more reliable than morphological characteristics since they are not altered by environmental factors (Ali *et al.* 2007). During the last two decades, the invention of polymerase chain reaction (PCR) has accomplished a variety of PCR-based markers including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), sequence-related amplified polymorphisms (SRAP), simple sequence repeats (SSRs) and single nucleotide polymorphism (SNP). Among those markers, SSRs become marker of choice because it requires only a small amount of DNA, easily

detectable by PCR, co-dominantly inherited, multi-allelic, abundant and amenable to high throughput analysis (Kalia *et al.* 2011).

Different molecular markers like restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), inter simple sequence repeats (ISSR) and microsatellites or simple sequence repeats (SSRs), have been developed in different crop plants. Because of their multi-allelic nature, reproducibility, codominant inheritance, high abundance and extensive genome coverage, SSRs become one of the most useful molecular marker systems in plant breeding; hence they are widely used in cultivar fingerprinting, genetic diversity assessment, molecular mapping, and marker assisted breeding (Saha *et al.*, 2004). SSRs also known as the 'marker of choice' are tandem repeats of short (1–6 bp) DNA sequences and are present in both coding and non-coding regions throughout the genome of an organism (Liewlaksaneeyanawin *et al.,* 2004). SSR length variation occurs due to slipped-strand mispairing during DNA replication and this type of mutations occur at a much higher frequency than point mutations and insertions or deletions and so SSRs show higher levels of polymorphism than other marker systems (Pashley *et al.*, 2006).

SSRs, also known as microsatellites, are tandem repeats of 2–6 bp DNA core sequences that are widely distributed in both non-coding and transcribed sequences, generally known as genomic-SSRs and EST-SSRs, respectively (Shirasawa *et al.* 2011). Genomic SSRs are highly polymorphic and widely distributed throughout the genome (Wang *et al.* 2011). However, development of genomic SSR markers is costly, laborious and time-consuming because it requires a small-insert genomic library and performs hybridization with SSR oligonucleotides and sequence candidate clones (Yi *et al.* 2006). Fortunately, the availability of EST sequences in public databases overcomes the

difficulty in developing genomic SSR markers through conventional method. ESTs are particularly attractive for marker development since they represent coding regions of the genome and are also being developed at an extremely fast pace for many genomes (Kumpatla and Mukhopadhyay 2005).

RAPD markers rely on large, intact DNA template sequences, which may not be available for RAPD development if the DNA is of lower quality or has been degraded. Other disadvantages of RAPD include lower resolving power for complex amplified products, difficulty in reproducing results, and the subjective nature of determining the precise characteristics of bands on a gel (Lee *et al.*, 2002). Similarly, AFLP markers also have several disadvantages, such as complexity, low time efficiency, high cost, scoring of the presence or absence of an AFLP band yielding dominant markers, and inaccurate quantitation of band intensities (Tang *et al.*,2009). Moreover, both RAPD and AFLP are dominant markers which are not able to differentiate homozygous from heterozygous genotypes (Zane *et al.*,2002). All these disadvantages significantly limit the wide application of these markers.

Compared with AFLP and RAPD, simple sequence repeat (SSR) markers have the advantages of lack of adornment, potency, ampleness, hypervariability, reproducibility, co-dominant inheritance, extensive genomic coverage, and ease of detection by polymerase chain reaction (PCR) and are treated to be the most robust molecular markers for deciphering genetic diversity and phylogenetic relationships in modern plant breeding systems (Powell *et al.*, 1996). Initially, SSRs were mainly developed by one of two methods, i.e. SSR-enriched libraries or genomic sequence databases, therefore the isolation of SSRs and establishment of the specific primers require large amounts of both funding and time (Varshney *et al.*, 2005).

Recently, the rapid progress in expressed sequence tag (EST) studies has generated a new source for the development of SSRs. Compared with conventional genomic SSR markers, SSRs from EST sequences have several intrinsic advantages: (1) they are embedded in functional gene sequences and directly associated with transcribed genes; (2) they have a lower cost of apperception; and (3) they have a high transferability to related species (Varshney *et al.*, 2005). In recent years, an increasing number of EST-SSRs have been identified in many crops and used extensively for comparative mapping, DNA fingerprinting, genetic diversity and transferability. The brisk and economic development of SSRs from EST databases has become a beneficial way to access high-quality nuclear markers (Gutiereea *et al.*, 2005).

2.3. Molecular marker studies in plants

Genetic diversity is the basis for development of elite varieties with desirable characteristics. Genetic diversity analysis can be performed using morphological, biochemical and molecular markers (Govindaraj *et al.*, 2015). The biological diversity information which is important for efficacious conservation programmes has become refined by the usage of molecular techniques (Oliveira-Miranda *et al.*, 2013). Molecular markers offer consistent results despite the prevailing environmental circumstances. Genetic variation was checked among thirty-eight populations of *Camellia* belonging to three species using RAPD markers. Significant variation was noted among *C. assamica, C. sinensis* and *C. assamica ssp. lasiocalyx* (Wachira *et al.*, 1995). Vierling and Nguyen (1992) studied genetic diversity of two wheat species *Triticum monococcum* and *T. urartu* showing diplod nature using RAPD markers and found that polymorphism was greater for *T. urartu.* RAPD primers were employed for checking genetic diversity among seven populations of *Saxifraga cernua* collected from different regions of Alps and significant variation was not detected within the accessions (Bauert *et al.*, 1998). 12 species of *Jatropha* were studied for genetic diversity

using 26 RAPD primers. 18 primers produced polymorphic bands and the accessions were grouped into three clusters. First cluster included all the populations of *J. curcas* and second cluster contained *J. ramanadensis, J. gosypiifolia, J. podagrica, J. tanjorensis, J. villosa and J. integerrima.* It was noted that *J. glandulifera* showed a greater extent of variation and placed in the third group (Ganesh *et al.,* 2007). 24 RAPD primers were used for testing genetic variation in 58 accessions of sesame. Of the 58 accessions of sesame collected, 36 were from India and 22 accessions were from different parts of the world. Genetic diversity was found more in case of sesame populations from India. Sesame populations from Rajasthan and North Eastern States exhibited higher genetic diversity (Venkataramana *et al.*, 1999).

AFLP markers were used to detect polymorphism in 58 accessions of *J. curcas* from Botanical Garden of South China which were originally collected from different countries. Seven primers were used and 14.3% polymorphism was detected. *J. curcas* in Guizhou was highly diverse among the populations studied (Sun *et al.*, 2008). 37 neem accessions from different parts of India and 4 from Thailand were subjected to genetic diversity study using AFLP markers. Polymorphism Information content (PIC) values ranged from 58 to 83.8% with an average of 69.8%. Indian and Thailand populations were clustered into two different groups based on dendrogram analysis (Singh *et al.*, 1999). Polymorphism among 49 accessions of tea plants from southern parts of India were analysed using AFLP markers. Cluster analysis of these accessions revealed three groups, viz. Assam, China and Cambodia. Highest diversity was shown by Chinese varieties and lowest diversity was exhibited by Assam varieties. Genetic distance was greater between Assam and Cambod cultivars. The study revealed that genetic diversity is relatively lower among the tea cultivars collected from South India and suitable strategies should be adopted conserve the tea germplasm (Balasaravanan *et al.*, 2003). Baydar *et al.* (2004) conducted a study to

check whether the variations shown by different *Rosa damascena* Mill populations from Turkey in the morphological level reflect in the gene level also. 9 SSR and 23 AFLP markers were used to analyse genetic diversity, however polymorphism was not detected among different accessions. 90 accessions of barley including wild, cultivar and elite varieties were examined using three different types of markers- EST-SSR, EST-SNP and AFLP. Highest polymorphism was obtained with SSR markers and highest effective multiplex ratio was obtained with AFLP markers. The wild, cultivar and elite varieties were clustered into three different groups based on the results obtained with all the three different markers (Varshney *et al.*, 2007).

Computational approach was used for locating SNP from EST database of wheat and exploited for genetic mapping and genetic diversity purposes. Numerous SNP markers were developed using this strategy and the incidence of one SNP per 540 bp of wheat genotype was observed. PIC was found to be in the range of 0.04 to 0.50 (Somers *et al.*, 2003). DNA probes from *Helianthus annuus* were created from two genomic libraries and four cDNA libraries. Restriction length polymorphism was checked in cultivar forms of sunflower using one hundred and eighty one DNA probes. DNA was isolated from seventeen different genotypes comprising both restorer and maintainer lines of the classical cytoplasmic male sterility. Restriction digestion was performed using four different enzymes and 73 probes were discovered to be polymorphic. UPGMA method was adopted to calculate the genetic distances, through which restorer and maintainer lines of cytoplasmic sterility were evidently discriminated (Gentzbittel *et al.*, 1994).

2.4. Simple sequence repeats (SSR) marker development

Two hundred and ten SSR markers were checked against 48 chickpea accessions and 100 markers which produced 480 polymorphic alleles were used for analysis. Average value of PIC, gene diversity and heterozygosity were greater for wild populations than the cultivated forms (Choudhary *et al.*, 2012). Polymorphism obtained was very high for 65 accessions of *Daucus carota* using SSR markers (Cavagnaro *et al.*, 2011). Cosson *et al.,* (2014) developed 96 polymorphic SSR markers for *Arabidopsis thaliana.* Genetic variation was analysed in orchid species *Masdevallia solomonii* using microsatellite marker and high polymorphism was noted and was suggested for conservation programmes (Lopez-Roberts *et al.*, 2012). 258 microsatellite loci were identified from rubber tree and 126 primer pairs were successfully designed. The transferability of these primers was checked in 12 accessions of cultivated forms of rubber and 7 other species from Euphorbiaceae family. 36 primers were found to produce polymorphic bands (Yu *et al.*, 2011). Genetic diversity was examined in nine accessions of *Sorghum bicolor* grown in Egypt, which were originally collected from different areas using nine microsatellite markers. 58% of the microsatellite markers were observed to be polymorphic (El-Awady *et al.*, 2008). Ten different kinds of tobacco seeds were propagated in tissue culture medium which were Gujarat Tobacco-9, Mosaic Resistance Gujarat Tobacco Hybrid-1, Gujarat Tobacco-7, Gujarat Tobacco-4, Anand-2, Anand BT-10, Anand BD-101, Anand BD-118 and Anand-119. The plants were later studied for the genetic variation using SSR markers. SSR1, SSR2, SSR3, SSR5, SSR6, SSR7, SSR9, and SSR10 showed 100% polymorphism and a small level of polymorphism was shown by SSR4 (Thakur *et al.*, 2013). 41 chloroplast SSR markers were developed from an orchid genus Chiloglottis and concluded that all the markers used were polymorphic across the same genus (Ebert *et al.*, 2009). Genetic diversity study of 31 accessions of barley collected from Algeria, Tunisia and Egypt was done using 11 microsatellite markers. The reason for high level of polymorphism observed among the accessions was hypothesized to be the

different conditions in which the barley plants were grown (Ben Naceur *et al.*, 2012). Kapadia *et al.*, (2016) checked variation among 18 cultivars of Pearl Millet using SSR markers. From the study, 18 genotypes were grouped into three clusters and an average level of genetic diversity was observed. 17 microsatellite markers were utilized for assessing genetic variation in 11 groundnut varieties. Only 24% of the primers produced polymorphic bands and one variety named TMV 2 which is sensitive to leaf infection was independently placed in the dendrogram (Shoba *et al.*, 2010). 34 SSR markers were exploited to distinguish among 21 rice varieties. PIC values of these rice varieties varied from 0.157 to 0.838 with a mean value of 0.488 which indicated that these varieties possess high diversity (Rahman *et al.*, 2012). Genetic variation was checked in 103 Sorghum genotypes from South Africa using 30 microsatellite markers and came up with the conclusion that significant variation exists among different accessions under study (Mofokeng *et al.*, 2014). Genetic diversity was assessed for quality of the protein among maize lines using 34 SSR markers. PIC value for 18 germplasm lines ranged from 0.50 to 0.95 with the average value of 0.83 (Krishna *et al.*, 2012). 19 microsatellite markers were used to analyse variation among 12 wild accessions, 4 landraces and 13 cultivars of apple species from China. Significant diversity was present in the wild populations of apple species as evident from recognition of 10 unique alleles (Zhang *et al.*, 2011). Navel and Valencia, two types of sweet orange varieties were studied for their genetic diversity. 84 navel and 36 valencia genotypes were screened using 26 SSR primers. 15 primers produced bands, of which polymorphic bands were obtained with 7 SSR primers. Navel and Valencia sweet orange varieties were clustered into two different groups based on the results obtained (Polat, 2015). Date palms in Morocco were facing threat because of Bayoud disease. Genetic variation of 128 date palm accessions were checked using eighteen SSR markers and 59% variability was found (Bodian *et al.*, 2012). Genetic diversity of red clover accessions collected from different parts of the world was analysed using 36 SSR markers. High

level of polymorphism was found among the populations with an average PIC value of 0.605 (Gupta *et al.*, 2016).

2.5. EST-SSR (Expressed Sequence Tag- Simple sequence repeats) marker

The availability of expressed sequence tags (ESTs) resulting from large sequencing projects is suitable for the rapid and inexpensive development of SSR (Gupta *et al.*, 2003). The presence of SSRs in transcripts of known genes indicate that they may be having some role in gene expression (Kantety *et al.*, 2002). These EST-derived SSR markers show good transferability across taxonomic boundaries and can be used as anchor markers for comparative mapping and evolutionary studies (Chagne *et al.*, 2004; Liang *et al.*, 2009). EST-SSRs located in coding regions have more transferability than in untranslated regions (Pashley *et al.,* 2006). Due to the vast number of ESTs deposited in public databases, a large number of EST-SSRs have been developed, and the polymorphism and transferability of EST-SSRs were checked in many plant species (Poncet *et al.*, 2006; Aggarwal *et al.,* 2007; Luro *et al.*, 2008). Transcriptome of sesame was sequenced using next generation sequencing technology. Microsatellite markers were developed from this sequence and fifty markers were used to study the genetic diversity. Forty SSR markers produced polymorphic bands in 24 sesame populations (Wei *et al.*, 2011). 82 SSR primers were designed from ESTs of *Hordeum vulgare* and checked the transferability of these primers in *H. chilense*. 21% of the primers exhibited polymorphism in *H. chilense* accessions (Castillo *et al.*, 2008). 24,238 ESTs in peanut were screened for SSR motifs and found 881 SSRs. Tri-nucleotide repeats were greater in number proceeded by di, tetra, hexa and penta nucleotide motifs. 290 primer pairs were designed and analyzed the genetic variation among 16 wild forms and 22 cultivated forms of peanuts. The diversity shown by cultivated and wild populations were

further validated using cloning and sequencing of amplified PCR products (Liang *et al.*, 2009). EST-SSR markers were developed from cassava and tested in *Jatropha curcas L.* 36 microsatellite markers revealed polymorphism in 45 *J. curcas* accessions and these were grouped into six categories based on geographic origins (Wen *et al.*, 2010). EST-SSR markers obtained from apricot and grape were used to check cross species transferability in other members of their families. It was observed that markers from grape produced bands in wide collections of Vitaceae family but only some closely related members from Rosaceae family showed results with markers developed from apricot (Decroocq *et al.*, 2003). Gutierrez et al., (2005) reported that EST-SSR markers developed from the model plant Medicago showed good transferability in other species like faba bean, chickpea and pea. SSRs developed from ESTs of *Helianthus annuus* were employed for assessing genetic variation in *H. verticillatus* and *H. angustifolius*. Instead of 21% transferability shown by anonymous SSRs, EST-SSRs displayed 71% cross species transferability (Pashley *et al.*, 2006).

2.6. *Centella* - asiaticoside

Centella asiatica produces centelloids which contains large quantities of pentacyclic triterpenoid saponins. These triterpenoids include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, sceffoleoside, centellose, asiatic-, brahmic-, centellic- and madecassic acids. The triterpene saponins are common secondary plant metabolites and are constructed via the isoprenoid pathway to yield a hydrophobic triterpenoid structure (aglycone) which comprises a hydrophilic sugar chain (glycone) (Jacinda *et al.*, 2009).

Asiaticoside, an active constituent of *Centella asiatica*, is known for advantageous effects on keloid and hypertrophic scar. The effects of asiaticoside on normal human skin cell behaviours related to healing using *in vitro* systems, were observed. In a wound closure seeding

model, asiaticoside enhanced migration rates of skin cells. In cell proliferation assays, asiaticoside made an upsurge in the number of normal human dermal fibroblasts. Asiaticoside endorses skin cell behaviours involved in wound healing; and as a bioactive component of an artificial skin, may have therapeutic value (Jeong-Hyun Lee *et al.*, 2012).

2.7. Phytochemistry of *Centella asiatica*

Because of medicinal value of *C. asiatica*, many phytochemical studies have been conducted on the species (Karting *et al.*, 1988 and Turton *et al.*,1993). A triterpenoid glucoside, asiaticoside was isolated from the plant which is found to be effective in the treatment of many skin diseases, including leprosy. Initial chemical characterisation of the triterpenoid fraction was thought to comprise the primary biologically active compound (Bhatacharyya *et al.*, 1949; Boiteau *et al.*, 1949). The total terpenoid content is quoted to range from 1.1% to 8% of the leaf dry weight (Rao and Seshadri, 1969). However, these figures represent total N-butanol extracts of undefined plant samples, and most probably overestimate the actual level. Using more data, Das and Mallick (1991) have assumed that asiaticoside comprise 40% of the total triterpenoid fraction, the mean fraction equalling 0.1765% of the leaf dry weight. The terpenoid fraction comprises largely of the sugar esters of two pentacyclic terpenoids, asiaticoside and madecassoside and their aglycons asiatic and madecassic acids. Other triterpenoids that may or may not be present, depending on the plant source, include centelloside, brahmoside, thankuniside and iso-thankuniside. The content and composition of triterpene fraction varies among ecotypes from the different regions of the world.

Das and Mallick (1991) have reported that asiaticoside content varied with genomic diversity. Higher levels of asiaticoside were isolated from

plants selected from sub temperate areas in the Himalayas and those containing two B-chromosomes. In Madagascar samples, asiaticoside registered 40% of the total triterpene content (Turton, 1993). In addition to terpenoid fraction a broad spectrum of phytochemicals have been isolated from *C. asiatica*. This include sugars, fatty oils, amino acids (Malhothra *et al.*, 1961), vallarins tannins, flavonoids and polyphenols (Castillo et al., 1980).

Aziz *et al.* (2007) have determined triterpenoids and asiaticoside in glass house-grown plants of *C. asiatica* and found triterpenoid content highest in leaves. Zhang and Quine (2007) have reviewed the advances in the chemistry and bioactivities of *C. asiatica* particularly monitoring the principal active mass of triterpenoids. Manimaren *et al.* (2006) who standardized Ayurvedic formulation of *C. asiatica* in the laboratory, has dealt with fingerprint method to validate the *Centella* tablets by HPTLC technique.

A comparative quantitative analysis of chemical constituents in Majjaposhak and Shubodak varieties of *Centella asiatica* collected from CIMAP, Hyderabad was carried out by HPLC to estimate the variability in the asiaticoside content. Asiaticoside content was highest in leaves of Subhodak variety (1.42 ±1.60% dw) than the leaves of Majjaposhak variety (0.78 ±0.02 % dw). However, the difference was statistically significant for asiaticoside ($p < 0.05$). Comparative study of two varieties of *C. asiatica* showed a correlation between genomic diversity and asiaticoside content. The leaves of Shubodak variety of *C. asiatica* found to contain around two time's higher concentration of asiaticoside than those from Majjaposhak variety (Madhusudhan *et al.*, 2017).

3. MATERIALS AND METHODS

The present study involved the genetic diversity analysis among thirty different accessions of *Centella asiatica* collected from different parts of Kerala and Tamil Nadu and to develop and use molecular markers in relation to the content of triterpenoids. For this study the following procedures were used.

3.1. Sample Collection

Leaf samples were collected from the JNTBGRI *Centella* conservatory which were originally collected from different regions of Kerala and Tamil Nadu. These collected samples were used for genetic diversity analysis.

Table 1. List of *C. asiatica* accessions used for the study

SL. NO.	SAMPLE (Accession no.)	Location	District
1	302	KAPL Valley near Palode	Thiruvananthapuram
2	315	Nilamel	Kollam
3	317	Pallickal	Kollam
4	341	Thottiode	Thiruvananthapuram
5	342	Thottiode	Thiruvananthapuram
6	359	Kodangavila	Thiruvananthapuram
7	374	Odayaratti(Ooty)	Nilgiri
8	378	Ponmudi	Thiruvananthapuram
9	383	Charalvelai	Kanyakumari
10	386	Vembayam	Thiruvananthapuram
11	392	Thannimoodu vayal	Thiruvananthapuram
12	394	Kochuvila	Thiruvananthapuram
13	395	Banana farm	Thiruvananthapuram
14	396	Garder Station	Thiruvananthapuram
15	398	Alampara, Nanniyod	Thiruvananthapuram
16	400	Thottumukku	Thiruvananthapuram

17	401	Paluvalli, Peraiyam	Thiruvananthapuram
18	402	Patrakali temple, peraiyam	Thiruvananthapuram
19	407	Kadamankode, Kulathupuzha	Kollam
20	414	Kodaikanal	Dindigul
21	415	Kodaikanal	Dindigul
22	417	Kodaikanal	Dindigul
23	423	Varadharajapuram, Srivaikundam	Tuticorin
24	438	Mallakkarappra dam site	Thiruvananthapuram
25	449	Munnar	Idukki
26	451	Munnar	Idukki
27	452	Munnar	Idukki
28	454	Estuary island resort, Poovar	Thiruvananthapuram
29	455	Sirumalai, Dindugal	Dindigul
30	459	JNTBGRI-2	Thiruvananthapuram

3.2. Primer designing of EST-SSR

The raw Fastq read (NCBI PDA/bioProject PRJNA193660, bioSample accession number: SAMN01985855) were analysed and filtered, respectively, with FastQC and Fastx Toolkit to obtain high quality de novo transcriptome sequence data. Each sequence set was filtered with these criteria: first, the read containing the sequencing adaptor was removed, cutadapt was employed to discard adapter sequences; second, the reads with unknown nucleotides comprising more than 5% were removed; and third, low-quality reads with ambiguous sequence "N" were trimmed and discarded. Subsequently, without a reference genome a de novo assembly of the clean reads into transcripts was performed using Trinity, a novel method for the efficient and robust de novo reconstruction of transcriptomes from RNA-Seq

data. SSR regions were detected and suitable primers were designed from this non-redundant dataset using the software WEBSAT (wsmartins.net/websat). A total of 129 primers were designed collectively for the contigs and singletons output obtained from CAP3. The quality of the designed primers was checked using NETPRIMER (premierbiosoft.com/netprimer/index.html). The primers having a score of exactly hundred percent as evidenced by the absence of self-dimer or primer dimer or cross dimers were selected for synthesis. A selected set of 15 primers were synthesized.

3.3. DNA Isolation

DNA isolation was done by using Origin Plant DNA purification kit. Before beginning the isolation, the buffer PW and GD were prepared by adding 60 ml of ethanol (100%) as indicated on the bottle and shaken thoroughly.

- 100 mg of fresh leaf sample was collected and pulverized into fine powder using liquid nitrogen.
- 700µl 65°C preheated GP1along with β-mercaptoethanol, was added to GP1 buffer before use. It was then added to the ground tissue. The final concentration of β-ME was 0.1%. Then it was vortexed for 10-20 seconds to disperse all clumps and then incubated for 20 min at 65°C, mix by inverting the tubes for several times.
- 700µl chloroform was added, mixed by inverting the tube for several times and centrifuged for 5min at 12,000rpm (~13,400×g).
- The supernatant was pipetted out to a new tube and 700µl buffer GP2 was added, followed by inverting the tube for several times.

- This mixture was then transferred into spin column CB3, which was placed in the collection tube. CB3 lid was closed and centrifuged for 30sec at 12,000rpm. The filtrate was discarded and the spin column CB3 was placed into the collection tube.

  If the sample volume exceeded 700 µl, centrifuge successive aliquots in the same column.

- The column was opened carefully and 500 µl Buffer GD was added (Ensure that ethanol (100%) was added to Buffer GD before use), centrifuged at 12000 rpm (~13,400 × g) for 30 s, discarded the filtrate and placed the Spin Column CB3 back into the collection tube.

- 600 µl PW was added (Ethanol (100%) was added to Buffer PW before use) to the Spin Column CB3, and centrifuged for 30 s at 12,000 rpm (~13,400 × g), discarded the flow-through, place the Spin Column CB3 back into the collection tube.
- The above step was repeated for one time.

- Replaced the Spin Column CB3 in the collection tube, centrifuged for 2 min at 12,000 rpm (~13,400 × g), discarded the flow-through. Opened lid of CB3 and kept at room temperature for a while to dry the membrane completely.

- Spin Column CB3 was transferred to a new microcentrifuge tube and 50- 200 µl buffer of TE was directly added to the CB3 membrane, incubated for 2-5 min at room temperature (15-25°C), and then centrifuged for 2 min at 12,000 rpm (~13,400 × g) to elute.

3.4. Quantitative and qualitative analysis of DNA

The quantity and quality of isolated DNA samples were determined using biophotometer (Eppendorf India ltd). The instrument was calibrated with 50 µl Eluting buffer as blank. Biophotometer required a sample size of 1µl DNA diluted with 49 µl eluting buffer for measuring the optical density and to identify the specified parameters. The instrument provided analytical data on,

- The absorbance of abundance of nucleic acid at 260 and 280 nm wavelength
- Concentration of DNA in sample (µg/µl)
- Purity of DNA sample (260/280).

3.5. Agarose gel electrophoresis

Electrophoresis was used to separate nucleic acid or proteins by size and/or charge. Basically, in gel electrophoresis samples were added to the well which was placed in anode side. The DNA which was negatively charged due to their phosphate backbone repelled from anode moves towards cathode. Larger nucleic acids have a harder time moving through the gel matrix, and then the nucleic acid can be separated by size.

The running buffer (1X TBE) which containing Tris base, boric acid and EDTA was prepared in distilled water with pH 8. The gel loading dye consisted of 40% sucrose, 0.25% bromophenol blue and 0.25% xylene cyanol. 3µl DNA along with 2µl gel loading dye was loaded to the agarose gel and the gel was run at 70V for 30 min to check the quality of isolate DNA.

Table 2. Composition of 20x TBE Buffer Reagent

Compounds	250 ml	500 ml
Tris base	54 gm	108 gm
Boric acid	27.5 gm	55 gm
0.5M EDTA	20 ml	40 ml

The compounds were weighed and dissolved completely in double distilled water. The pH was adjusted to 8 and stored at room temperature for use.

3.6. Polymerase chain reaction

The newly synthesised primer pair was optimized for PCR parameters such as the annealing temperature and concentration of reagents. The annealing temperature was standardized using gradient PCR. DNA samples were then subjected to PCR amplification with the designed non-redundant primers. The reaction mixture consisted of the following items:

Table 3. The reaction mixture

Reagents	Volume
Sterile water	21.04 µl
1x buffer	2.50 µl
dNTPs (200 µM each)	0.06 µl
Forward primer (15 pmol)	0.15 µl
Reverse primer (15 pmol)	0.15 µl
Template DNA (40 ng μl^{-1})	1.00 µl
Taq Polymerase (0.5 U)	0.10 µl
Total volume	**25.0 µl**

Amplification was carried out on Agilent Technologies thermal cycler (Agilent technologies, United States) using the following temperature profile: Reactions began with a 1 min initial denaturation at 94 °C, followed by 35 cycles with 35 sec at 94 °C, then 35 min at specific annealing temperature and 45 min extension at 72 °C, a final extension step covering 5 min at 72 °C marked the end of the reaction. The amplified products were resolved in 3 % agarose gel with ethidium bromide and were analysed using gel documentation system (UVP, UK). A 100 bp DNA ladder was loaded along with the samples to compare the size of resultant bands. The resultant bands were scored and analysed using population analysis software POPGENE 1.32 version and dendrogram was visualized using MEGA7 software.

3.7. PHENOTYPIC ANALYSIS

Thirty accessions of *C. asiatica* collected from different regions (Tamil Nadu and Kerala) were used to conduct phenotypic analysis in this study. Selected plant accessions along with replica were used to measure two quantitative phenotypic data *viz* leaf area (cm^2), number of leaves per node. The data was collected using leaf tracing method. The obtained data mean and SD values were statistically obtained using the SPSS software.

3.8. ESTIMATION OF TRITERPENOIDS

3.8.1. QUANTIFICATION OF ASIATICOSIDE BY HPTLC

3.8.1.1. CHEMICALS

Standard asiaticoside was procured from Sigma-Aldrich (Steinheim), methanol, n-hexane, n-butanol and ethyl acetate were from Merck (India).

3.8.1.2. EXTRACTION PROCEDURE

Leaf sample from whole plant of *C. asiatica* accessions grown under the same environmental condition for 90 days along with its replica were thoroughly washed with water to remove all debris, dried and powdered. Firstly, the powdered material was defatted with *n*-hexane (20 mL); the hexane insoluble material was extracted with methanol (3 × 20 mL; 72 h) by macerating it in G10 GYROTORY shaker at rpm of 80-120 and temperature 30°C, filtered by Whatman's filter paper No.1 separately. The supernatant was decanted and concentrated by rotary evaporation at 45°C under vacuum and dissolved and made up to 5ml in methanol and stored at 5°C in refrigerator.

3.8.1.3. CHROMATOGRAPHIC CONDITIONS

Chromatography was performed on Merck TLC precoated silica gel 60F254 (20X10 cm) plates. The crude samples were diluted using methanol in the ratio 1:1, 2:3, 3:7, 1:4 (sample:methanol). Methanolic solution of diluted samples and standard compound asiaticoside (1mg/ml) of known concentrations were applied to the layers as 6 mm-wide bands positioned 10 mm from the bottom and 10 mm from side of the plate, using Camag Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150ml/s from application syringe. These conditions were kept constant throughout the analysis of the samples. Following sample application, layers were developed in a Camag twin trough glass chamber which was pre-saturated with mobile phase of n-butanol: ethyl acetate: water (4:1:5) for asiaticoside till proper separation of bands up to 8 cm height. The plate is then derivatised using Anisaldehyde sulphuric acid reagent, heated at 110° C for 5 min. Asiaticoside were simultaneously quantified using Camag TLC scanner model 3 equipped with Camag Wincats software version

1.4.4. Following scan conditions were applied: slit width, 6 x 0.45 mm; wavelength 287 nm; and absorption mode. In order to prepare calibration curves, stock solution of asiaticoside (1 mg/ml) was prepared and various volumes of the solution were analyzed through HPTLC, calibration curves of peak area vs. concentration were also prepared.

4. RESULTS

The chapter includes the results of the project work entitled "Development of functional EST-SSR and analysis of genetic diversity in *Centella asiatica*" carried out at the Biotechnology and Bioinformatics Division of JNTBGRI, Palode during the academic year 2017- 2018.

The main objective of the study was to develop molecular markers such as functional EST-SSR for analysis of genetic diversity in *Centella asiatica* in relation to the content of triterpenoid asiaticoside. The steps involved in the development of functional EST -SSR markers were the following:

4.1. Development of functional EST- SSR

4.1.1. Access reads from SRA (Sequence Read Archive)

The raw transcriptomic sequence was retrieved from SRA with accession number SAMN01985855 for leaf transcriptome of *Centella asiatica.* The quality of the downloaded sequence was checked using FASTQC, the tool which runs on JAVA environment.

4.1.2. Quality checking – FASTQC

FASTQC aims to provide a simple way to do quality control checks on raw sequence data coming from high throughput sequencing pipelines. It provides a molecular set of analyses which you can use to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis.

4.1.3. Output:

FASTQC will process and present the reports in a visual manner. Based on the results, the quality of the sequence data can be assessed. FASTQC generates results in the form of a zipped dictionary and an html summary page for each input file (Figure 2).

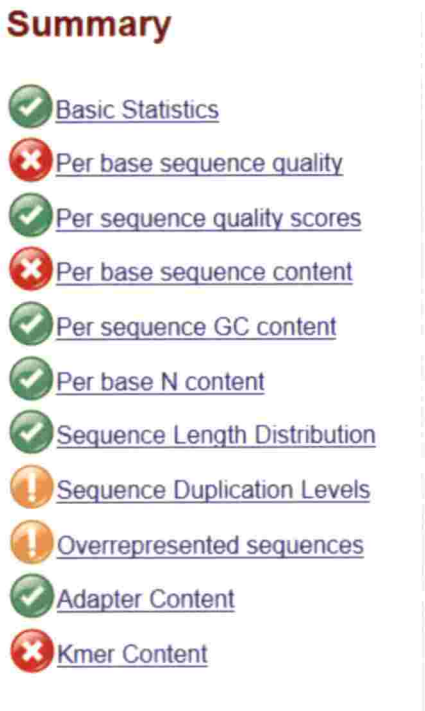

Figure 2: FASTQC summary page

4.1.4. Basic statistics

Figure shows details of transcriptomics sequence. The total number of sequences are 3611520, sequence length was 72 and percentage of GC content was 45% (Figure 3).

Basic Statistics

Measure	Value
Filename	sra_data.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	3611520
Sequences flagged as poor quality	0
Sequence length	72
%GC	45

Figure 3: Basic statistics

4.1.5. Per Base Sequence Quality

Central red line is the median value, blue line represents the mean quality. Yellow box represents the inter-quartile range (25-75%) upper and lower whiskers represents the 10% and 90% points. The y-axis on the graph shows the quality scores. Higher the score, better the base call. The background of the graph shows the quality scores. The higher the quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). The quality of calls on most platforms will degrade as the run progresses, so it is common to see base calls falling into the orange area towards the end of a read.

In this study the base sequence quality score was not normal for further analyses. So, it has to be assembled using DDBJ assembly (Figure 4).

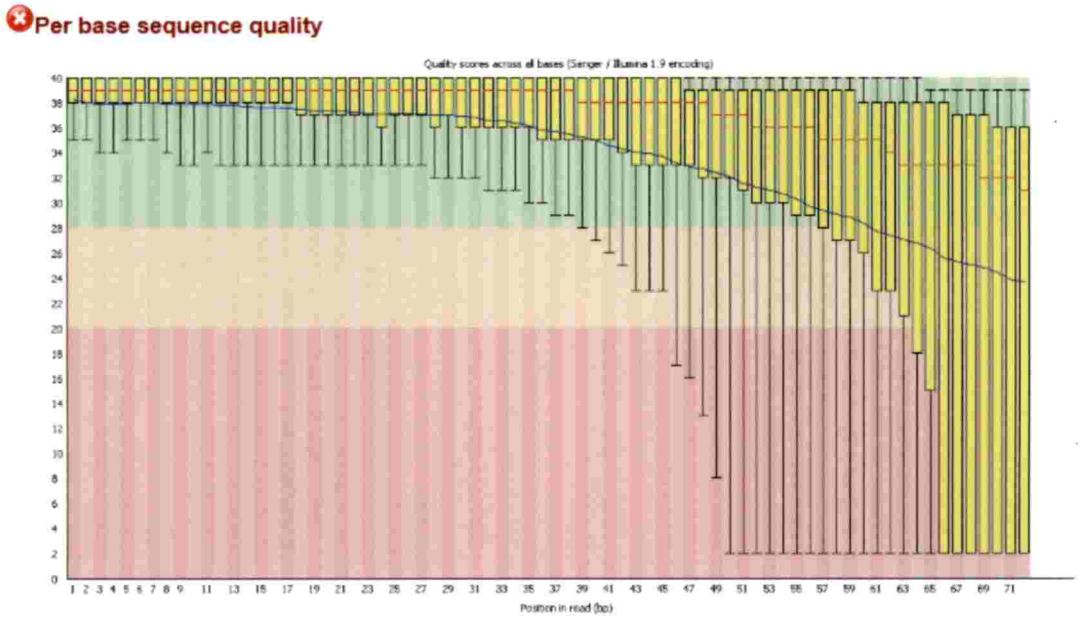

Figure 4: Per Base Sequences Quality

4.1.6. Per Sequences Quality Score

The per sequence quality score report allows to see if a subset of sequences have universally low quality values. It is often the case that a subset of sequences will have universally poor quality, often because they are poorly image, however these should represent only a small percentage of the total sequences. Result shows that sequence quality was normal to our further analysis (Figure 5).

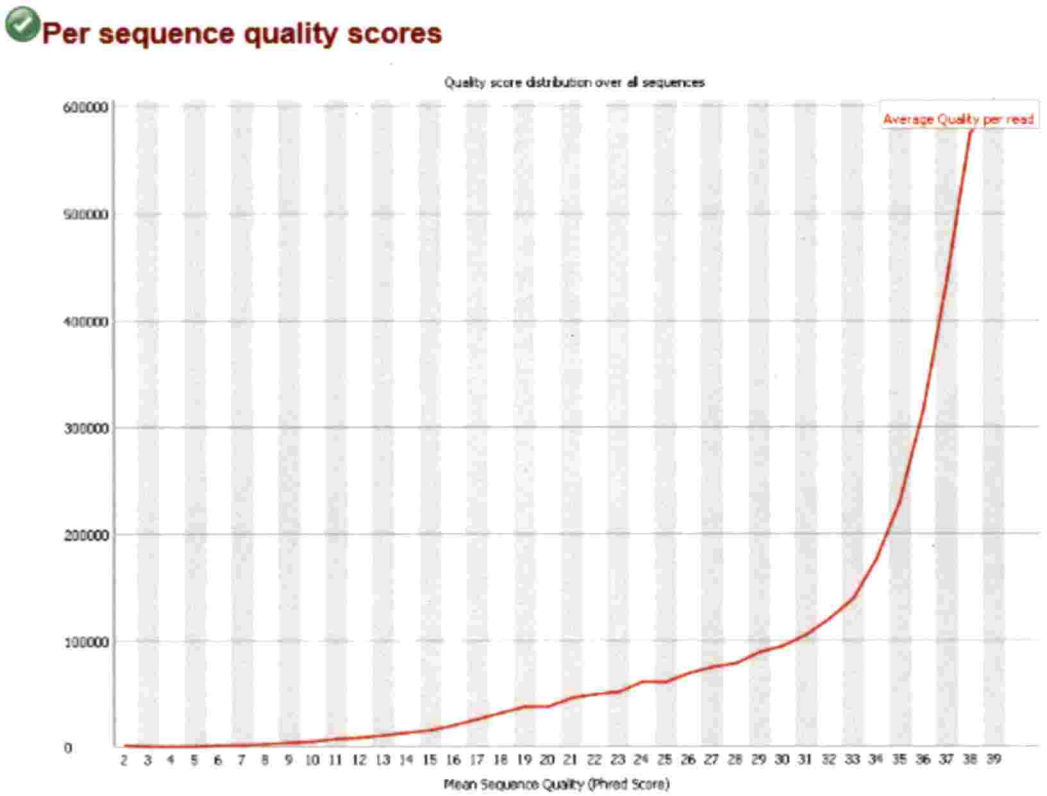

Figure 5: Per sequence quality scores

4.1.7. Adaptor content

No overrepresented sequence was identified in transcriptomic sequence. As well as no adapter content found, so conclude that our transcriptomic sequence was good quality for further analyses (Figure 6).

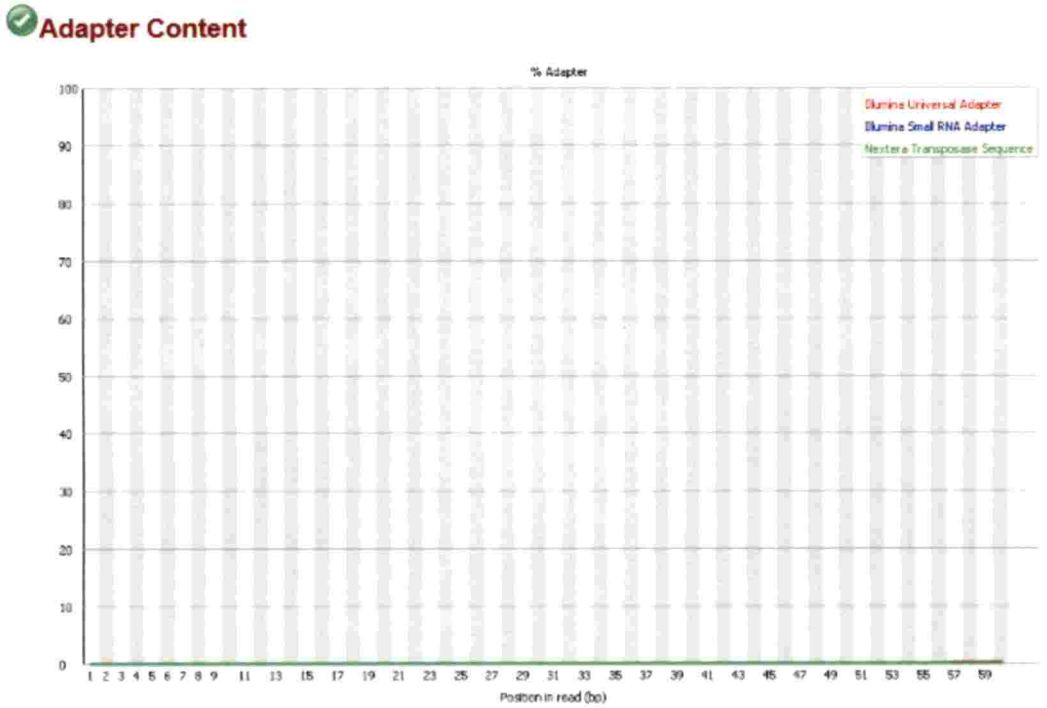

Figure 6: Adaptor Content

4.1.8. De-novo assembly

Using DDBJ server the sequence was assembled and the tool used for de novo assembly was Trinity. Before assembling the sequence, size was 370 MB. After assembling the sequence, the size was reduced to 0.98 MB.

4.1.9. Primer designing

4.1.9.1 Websat

The traditional method for developing microsatellite (SSR) markers can be expensive and time consuming. With the increasing availability of sequence data, cheaper and faster in silico methods have been used. These are based on computational tools that easily screen sequence data and produce complete list of SSRs. Some of these tools concentrate on flanking the SSRs, thus facilitating the marker development process. Websat makes use of Ajax techniques, providing a rich, responsive user interface, allowing submission of sequences, visualization of microsatellites, and design of primers suitable for their amplification, and exportation of the resulting data.

- Yellow color indicates SSR
- Green color indicates primer
- Light blue color indicates selected primer
- Orange colour indicates overlapped SSR

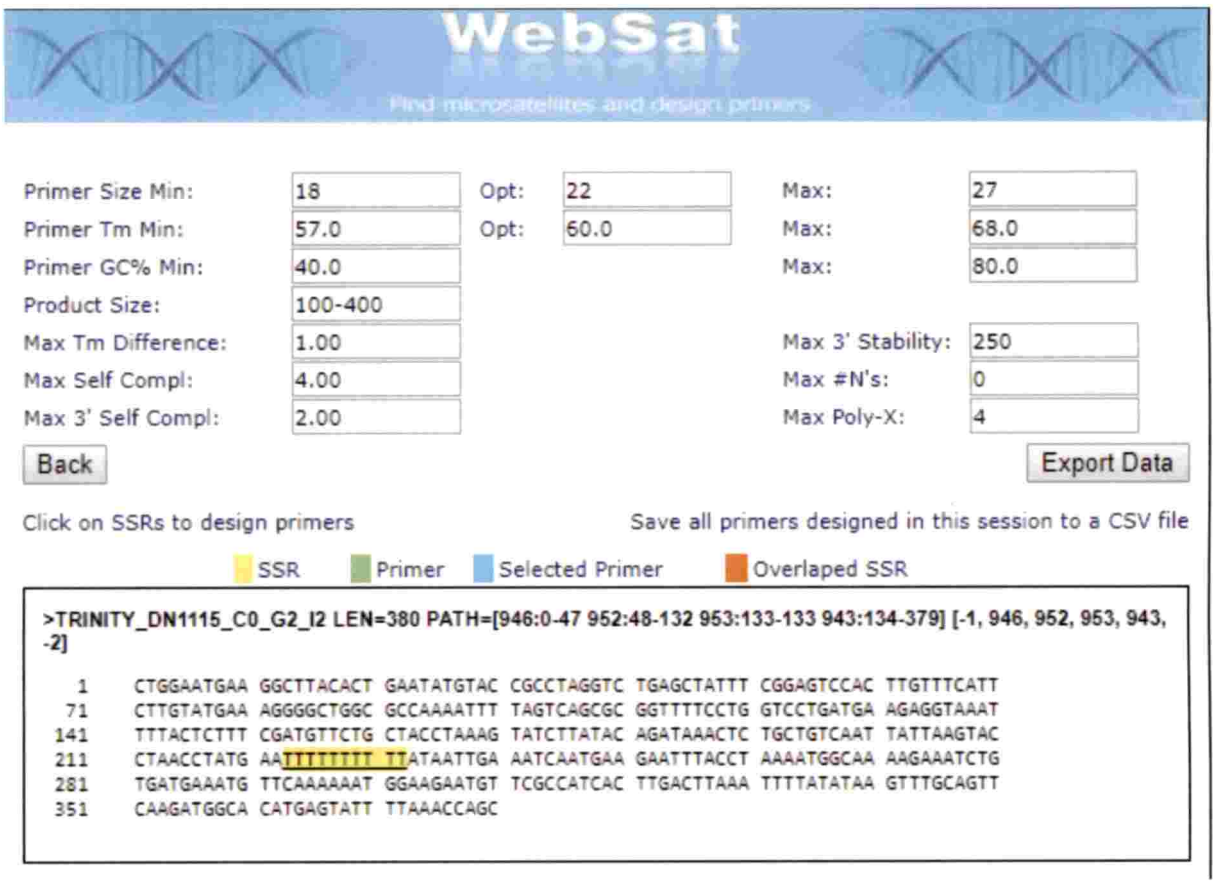

Figure 7: SSR identification

In figure 7 (selected primer), yellow color showed that the selected sequence is an SSR region. By clicking the SSR, it gives corresponding primers. Green color indicates the forward and reverse primer and the light blue indicate the selected primers for the SSR (Figure 8).

About 129 primers were designed using Websat. Then these primers were used for checking primer efficiency using the tool NetPrimer.

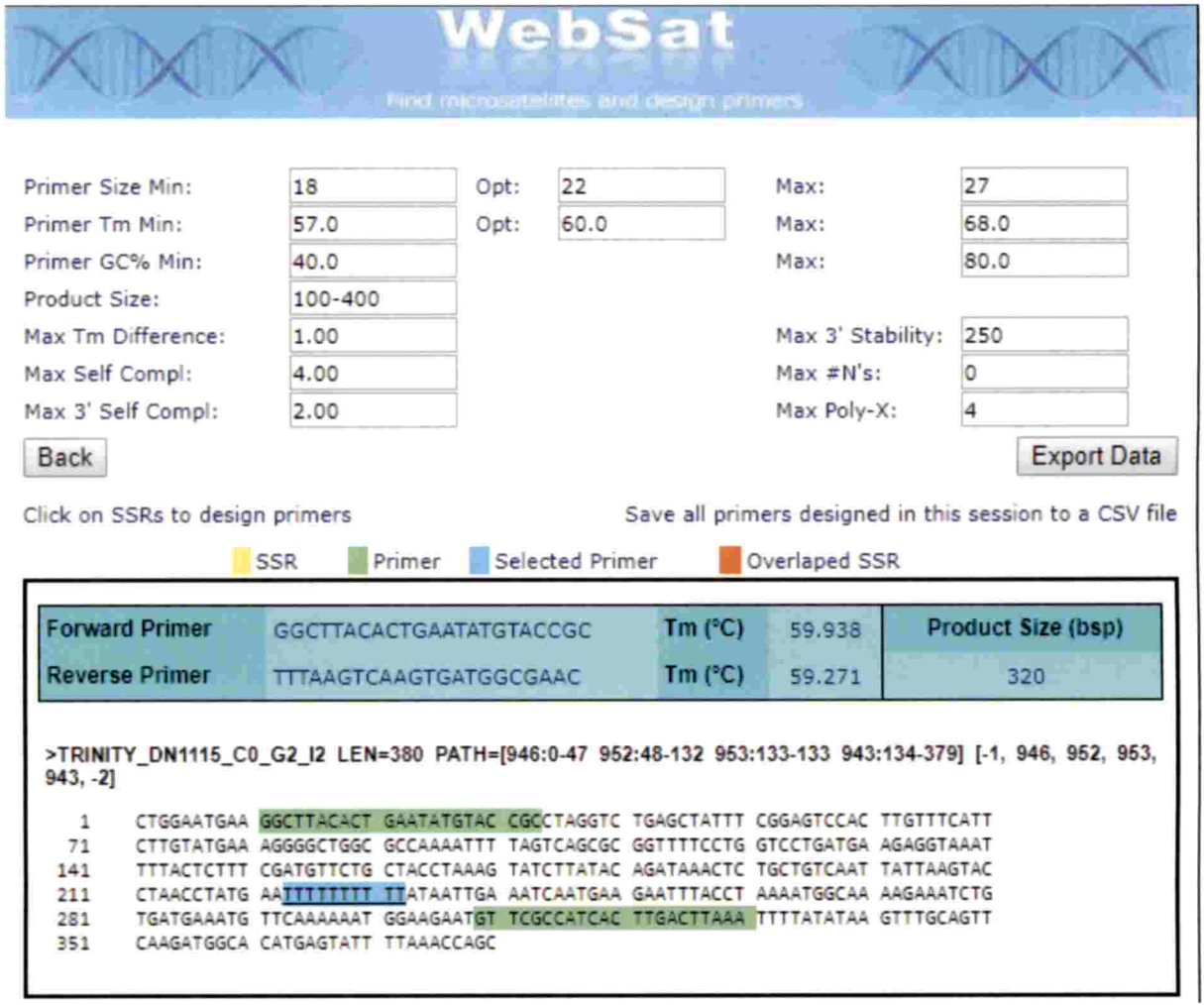

Figure 8: Selected primer

4.1.9.2. NetPrimer

Netprimer is a free web based tool used for analysing primers used in PCR to amplify a DNA sequence. Netprimer also analyses the thermodynamically important secondary structures such as hairpins, self and cross dimers, runs and repeats. These structures significantly affect the primer efficiency and therefore the success of a PCR reaction. Netprimer can be used to determine the best primer pairs for a given set of experimental conditions. The program assigns a rating to each primer analysed (Figure 9). The rating is based on the proximity of the thermodynamic parameters to their scores. Primers are analysed for their GC % (guanine-cytosine content). This important parameter determines their annealing strength.

Analysis Results #1: GGCTTACACTGAATATGTACCGC

Rating	:	91.0		3' end stability	:	-11.16	kcal/mol
Molecular Wt	:	7023.67		ΔH	:	-174.1	kcal/mol
Tm	:	58.74	°C	ΔS	:	-0.46	kcal/°K/mol
GC%	:	47.83		5' end ΔG	:	-9.75	kcal/mol
GC Clamp	:	4		Self Dimer (ΔG)	:	-4.65	kcal/mol
nmol/A_{260}	:	4.54		Hairpin (ΔG)	:	-0.16	kcal/mol
ug/A_{260}	:	31.9		Repeats (# of pairs)	:		kcal/mol
ΔG	:	-37.25	kcal/mol	Run (# of bases)	:		kcal/mol

Analysis Results #2: TTTAAGTCAAGTGATGGCGAAC

Rating	:	91.0		3' end stability	:	-8.47	kcal/mol
Molecular Wt	:	6798.52		ΔH	:	-163.4	kcal/mol
Tm	:	57.45	°C	ΔS	:	-0.43	kcal/°K/mol
GC%	:	40.91		5' end ΔG	:	-6.79	kcal/mol
GC Clamp	:	2		Self Dimer (ΔG)	:	-4.85	kcal/mol
nmol/A_{260}	:	4.52		Hairpin (ΔG)	:		kcal/mol
ug/A_{260}	:	30.75		Repeats (# of pairs)	:		kcal/mol
ΔG	:	-35.28	kcal/mol	Run (# of bases)	:	3	kcal/mol

Cross Dimer (ΔG)	:	-7.75	kcal/mol

Print

Figure 9: Primer analysis results

With the help of Net primer 15 primers were selected that shows 100% efficiency after primer designing. From these 11 primers were selected for analysis of genetic diversity in *Centella asiatica* after screening initial and 4 primers from the previous studied work (Sabu *et al*., Unpublished) also included for analysis are listed below (Figure 10).

	SEQ-ID	SSR	PRODUCT	FW-PRIMER	FW-LEN	TM	RV-PRIMER	RV-LEN	TM	SSR-INDEX	FW-INDEX	RV-INDEX
2	>TRINITY_	(A)10	338	TCTTCACTCTTGTCCTTTTCGG	22	60.763	CCATAGATAGATTCACCGCCTC	22	59.951		74	390
3	>TRINITY_	(AT)6	262	GAAGGATTTGGTATTGGGATTG	22	59.55	AAGTTTGGGATGGATGAAGAGA	22	59.94		47	287
4	>TRINITY_	(TCT)6	189	ATCCAGAACTCATCCTGTCCAT	22	59.824	TAGTAGCAGCAAACCCAAGTCA	22	59.941		376	543
5	>TRINITY_	(GAT)6	371	AAACTGTCCCATCAGCAAATCT	22	60.004	ACTAAAGCCTGAAACCACCAAA	22	60.035		415	764
6	>TRINITY_	(T)10	217	GCTTGGCTGGATTTTGACAT	20	60.081	AAGGCATTTACAAGACCGAAGA	22	60.129		1	196
7	>TRINITY_	(TC)6	181	CACTACACTTTTCTTCGCTCCC	22	60.295	AGATGAGATTCCGATGGATTTG	22	60.286		4	163
8	>TRINITY_	(GT)7	124	ATTCACCATACCCATTGTCCTC	22	59.947	TATTTCCCACTCTATCCTCCCA	22	59.793		1089	1191
9	>TRINITY_	(A)18	329	TTTGCTTCCTACAACAAAGGGT	22	60.035	CACTTCCTACAAATAAACCCAACAG	25	60.193		1449	1753
10	>TRINITY_	(A)10	354	ATAGAAAGAGGGCACAAAAGGG	22	60.807	GAAGCCTCCCAAGTTCCTAAAT	22	59.971		37	369
11	>TRINITY_	(GA)8	241	CACCAGCCCTGAAATAACAAAT	22	60.237	TCTTCTTCTTCTTCCTCCTCCTC	23	59.608		391	609
12	>TRINITY_	(T)23	212	CTGCGGTTGGTCCTTTTC	18	59.215	GTTCGTTCGGCTCTAAACACTT	22	59.825		3	193
13	>TRINITY_	(CAG)6	211	AACAACATCAGCAACAGCAAGT	22	59.848	CATAGCAGCATCATCCAGAGAG	22	60		99	288
14	>TRINITY_	(TGG)6	293	CATTGAAGCAAGGGTCATAGTG	22	59.632	CTCCACCATAAGAACCAAATCC	22	59.704		68	339
15	>TRINITY_	(TGT)7	379	TCGCTTTTGGGGTCATAATAGT	22	59.864	ACAAAGATGCCTATGTTGCTGA	22	59.775		193	550
16	>TRINITY_	(GA)6	317	CGAGGAGAGAGAGGACAGAGAA	22	60.266	AAAGTGGTAGTGGTGGTGGAGT	22	60.168		27	322

Figure 10: primers selected for analysis of genetic diversity

4.2. Genomic DNA isolation of *C. asiatica*

DNA samples were isolated from the thirty accessions for the study using Plant Genomic DNA Kit (Origin, Kerala). Isolated Genomic DNA was resolved in 0.8% agarose gel with ethidium bromide to check the integrity and quality. The quality and quantity of the isolated samples were estimated with biophotometer (Eppendrof Ltd) is represented with table below (Table 4).

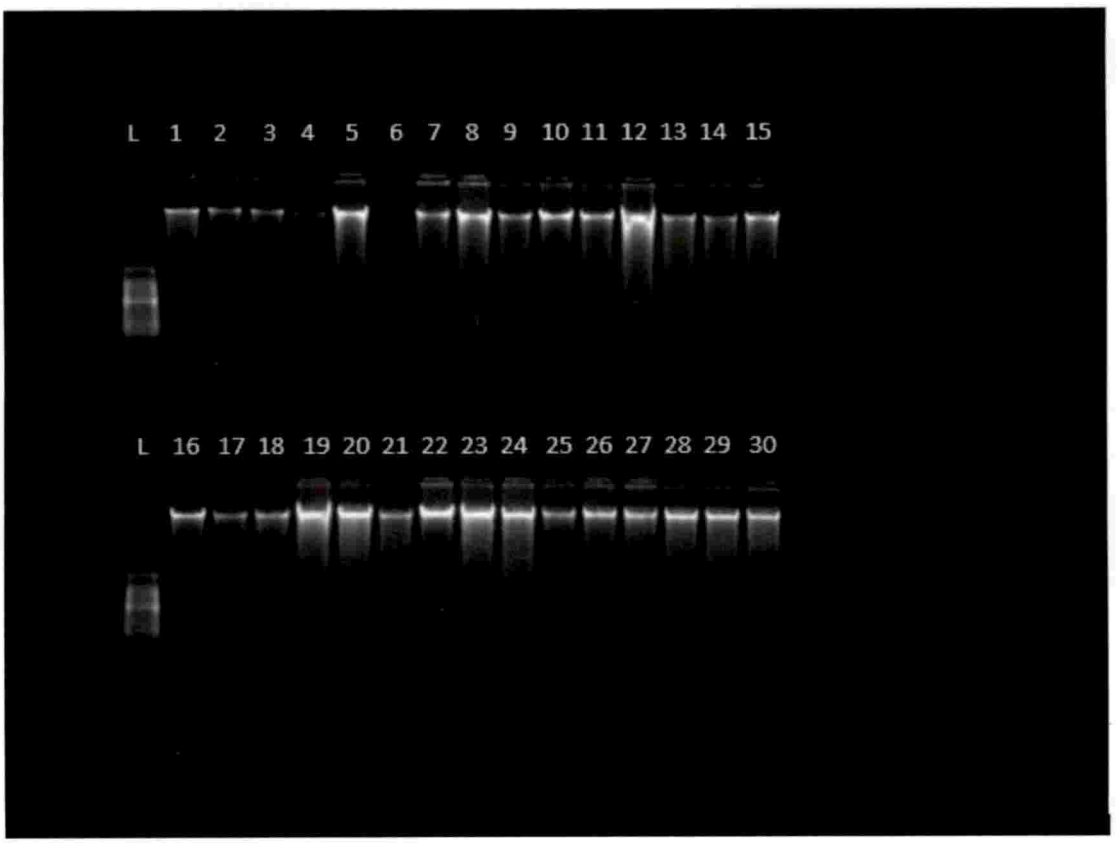

Plate 1: Genomic DNA isolated from 30 *Centella* accessions

Table 4. The quality and quantity of the isolated samples using bio photometer

SL. NO.	SAMPLE (Accession no.)	CONCENTRATION (µg/µl)	PURITY (260/280)	CONCENTRATION (ng/µl)
1	302	0.11	1.9	110
2	315	0.077	1.8	77

3	317	0.116	2.1	116
4	341	0.054	1.8	54
5	342	0.127	1.8	127
6	359	0.041	1.8	41
7	374	0.17	1.8	170
8	378	0.162	1.6	162
9	383	0.139	1.8	139
10	386	0.115	1.9	115
11	392	0.227	1.7	227
12	394	0.237	1.8	237
13	395	0.157	1.7	157
14	396	0.145	1.9	145
15	398	0.145	1.7	145
16	400	0.134	1.9	134
17	401	0.120	1.7	120
18	402	0.154	1.9	154
19	407	0.267	1.9	267
20	414	0.131	2.1	131
21	415	0.089	1.9	89
22	417	0.114	1.8	114
23	423	0.208	1.9	208
24	438	0.155	1.8	155
25	449	0.054	1.9	54
26	451	0.111	1.9	111
27	452	0.242	1.9	242
28	454	0.113	1.9	113
29	455	0.210	1.8	210
30	459	0.135	1.9	135

4.3. Optimization of Primers used for diversity analysis

Gradient PCR was performed at varying temperatures to optimize the annealing temperature of the synthesized 15 primers to perform the diversity

analysis based on the banding resolution. The selected SSR primers along with the optimized annealing temperature used for the study is represented by table 5.

Table 5. Primer details

Sl. No.	PRIMER NAME	FW-PRIMER (5'-3') RV-PRIMER (5'-3')	Ta (°C)	SSR Motifs	PRODUCT SIZE (bp)
1	Centa_DN307	TCTTCACTCTTGTCCTTTTCGG CCATAGATAGATTCACCGCCTC	48	(A)10	338
2	Centa_DN581	GAAGGATTTGGTATTGGGATTG AAGTTTGGGATGGATGAAGAGA	48	(AT)6	262
3	Centa_DN1350	ATCCAGAACTCATCCTGTCCAT TAGTAGCAGCAAACCCAAGTCA	54	(TCT)6	189
4	Centa_DN2679	AAACTGTCCCATCAGCAAATCT ACTAAAGCCTGAAACCACCAAA	48	(GAT)6	371
5	Centa_DN2710	GCTTGGCTGGATTTTGACAT AAGGCATTTACAAGACCGAAGA	54	(T)10	217
6	Centa_DN2983	CACTACACTTTTCTTCGCTCCC AGATGAGATTCCGATGGATTTG	56	(TC)6	181
7	Centa_DN3162	ATTCACCATACCCATTGTCCTC TATTTCCCACTCTATCCTCCCA	56	(GT)7	124
8	Centa_DN3104	TTTGCTTCCTACAACAAAGGGT CACTTCCTACAAATAAACCCAACAG	58	(A)18	329
9	Centa_DN3493	ATAGAAAGAGGGCACAAAAGGG GAAGCCTCCCAAGTTCCTAAAT	56	(A)10	354
10	Centa_DN3440	CACCAGCCCTGAAATAACAAAT TCTTCTTCTTCTTCCTCCTCCTC	58	(GA)8	241
11	Centa_DN3463	CTGCGGTTGGTCCTTTTC GTTCGTTCGGCTCTAAACACTT	52	(T)23	212
12	TBG_CENTA-108	AACAACATCAGCAACAGCAAGT CATAGCAGCATCATCCAGAGAG	58	(CAG)6	211
13	TBG_CENTA-111	CATTGAAGCAAGGGTCATAGTG CTCCACCATAAGAACCAAATCC	60	(TGG)6	293
14	TBG_CENTA-115	TCGCTTTTGGGGTCATAATAGT ACAAAGATGCCTATGTTGCTGA	58	(TGT)7	379
15	TBG_CENTA-117	CGAGGAGAGAGAGGACAGAGAA AAAGTGGTAGTGGTGGTGGAGT	62	(GA)6	317

4.4. Genetic Diversity analysis of *C. asiatica* accessions:

The collected 30 *Centella* accessions diversity were analysed by using the selected 15 SSR primers. The amplified DNA sequences were observed by 3% agarose gel. The banding patterns of collected accessions were found to be varied between some of the accessions studied (Plate 2 to 15).

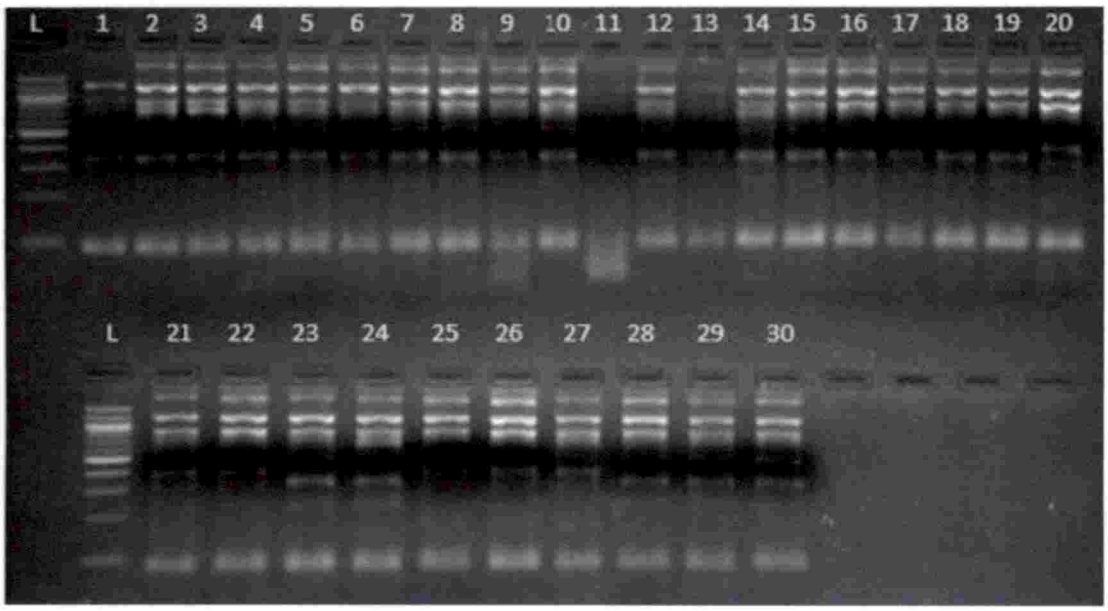

Plate 2: SSR profile obtained for primer Centa_DN307(Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

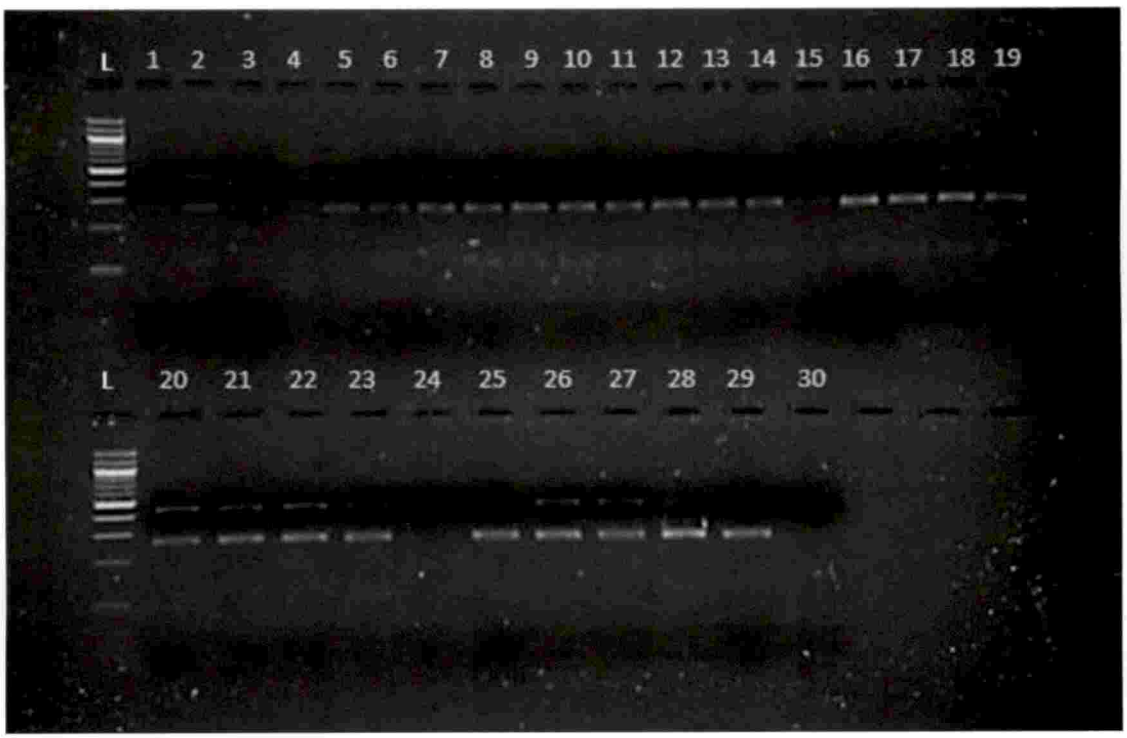

Plate 3: SSR profile obtained for primer Centa_DN581 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

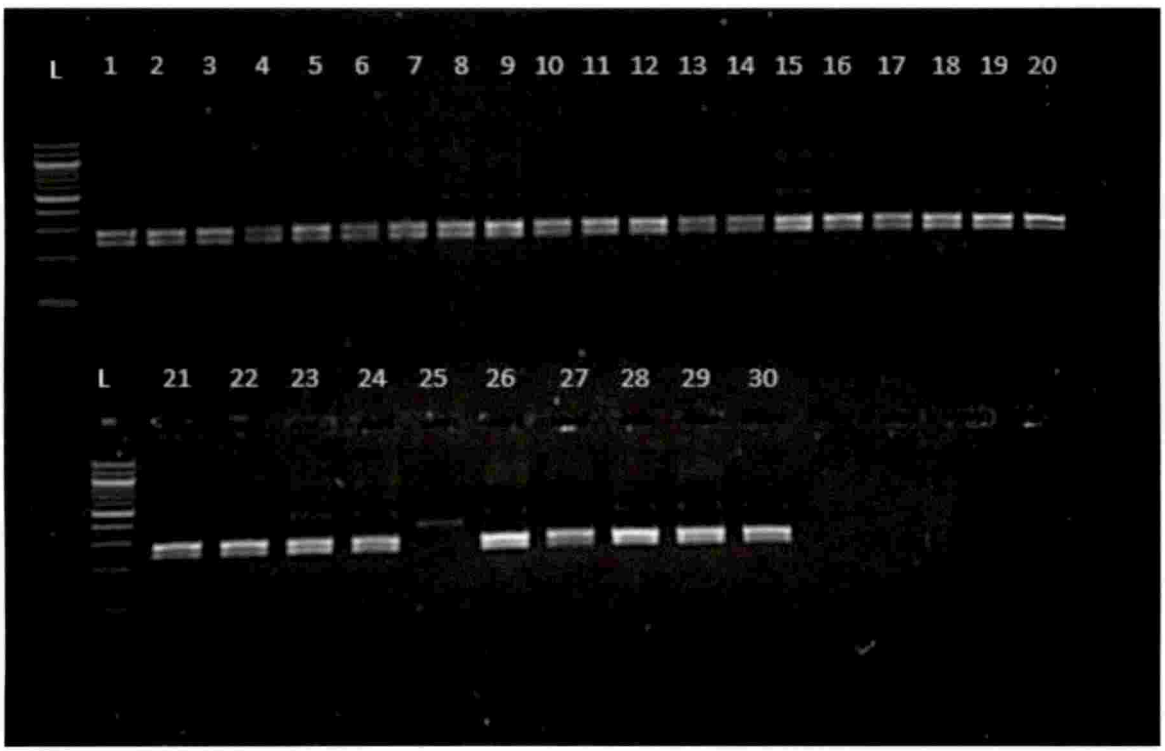

Plate 4: SSR profile obtained for primer Centa_DN1350 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

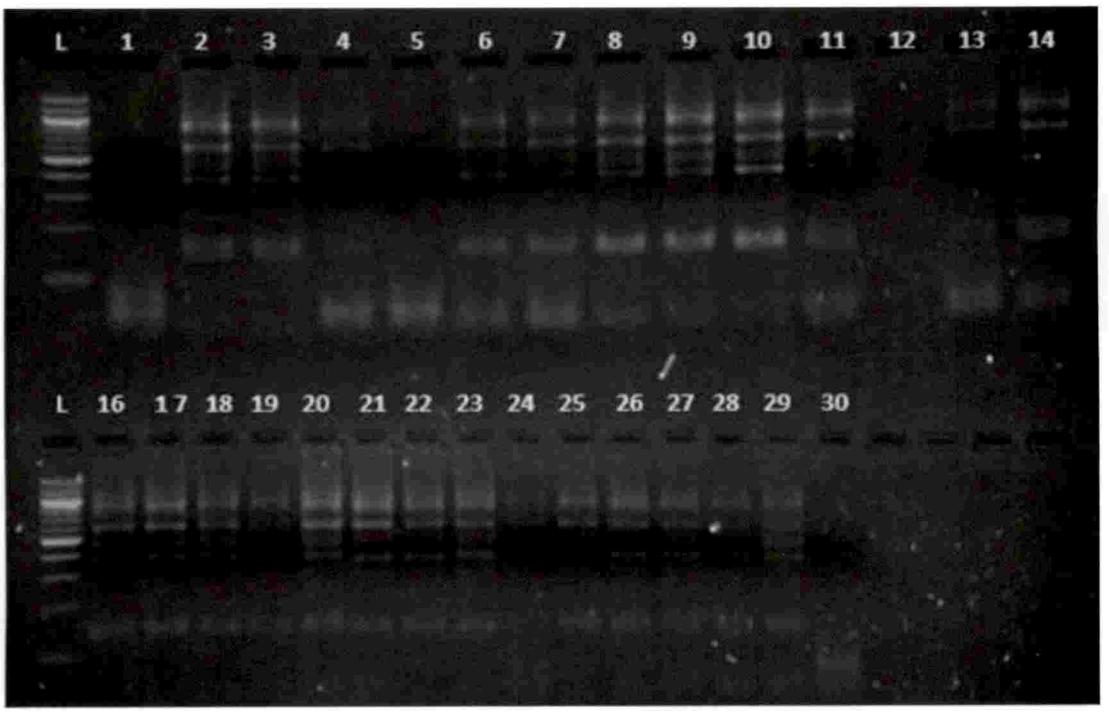

Plate 5: SSR profile obtained for primer Centa_DN2679 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

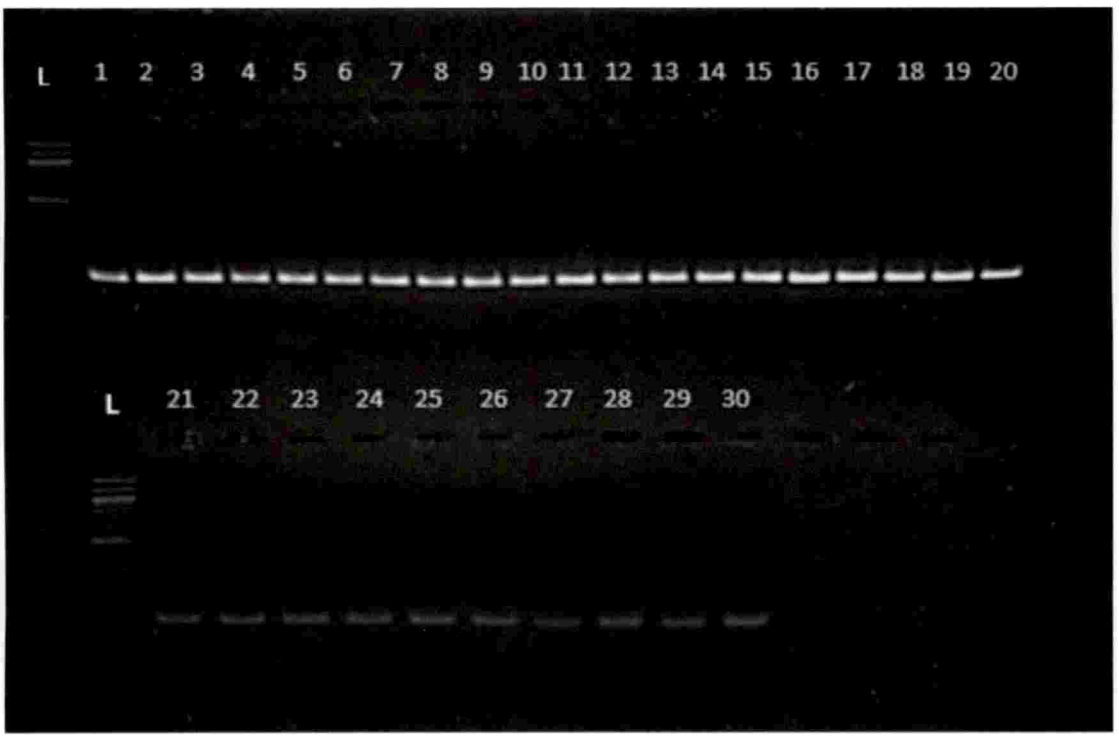

Plate 6: SSR profile obtained for primer Centa_DN2983 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

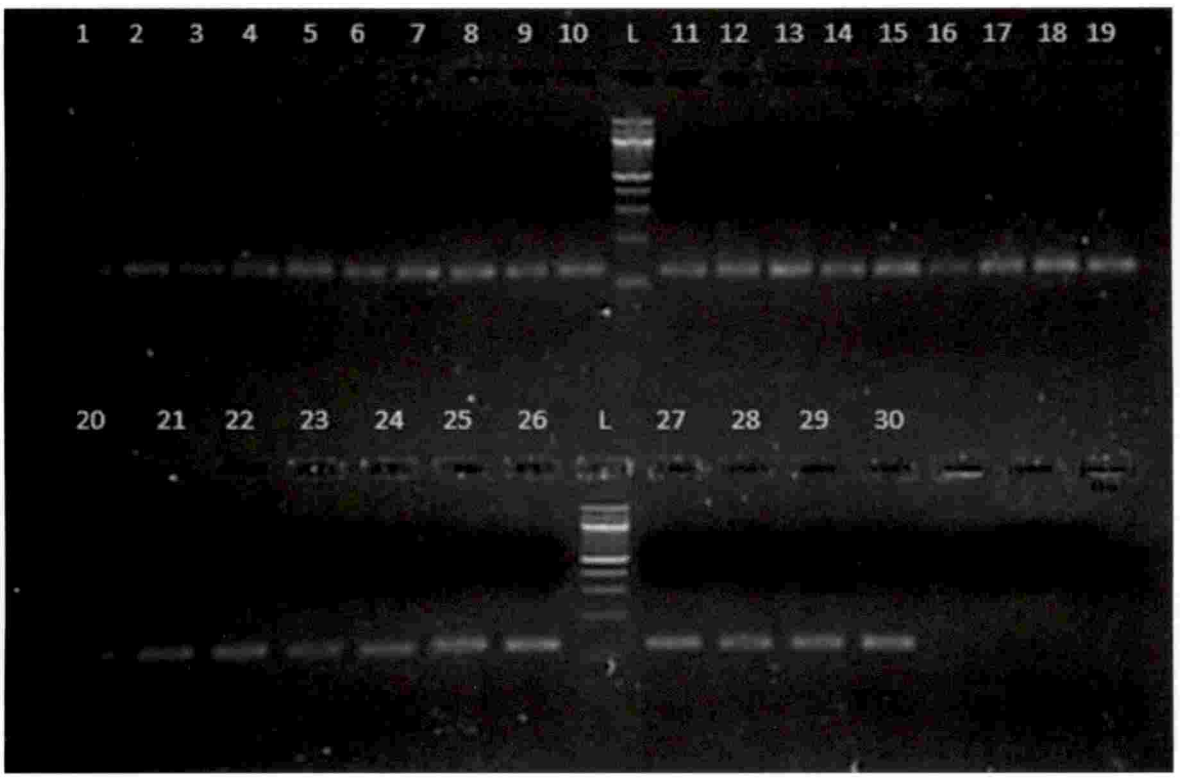

Plate 7: SSR profile obtained for primer Centa_DN3162 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

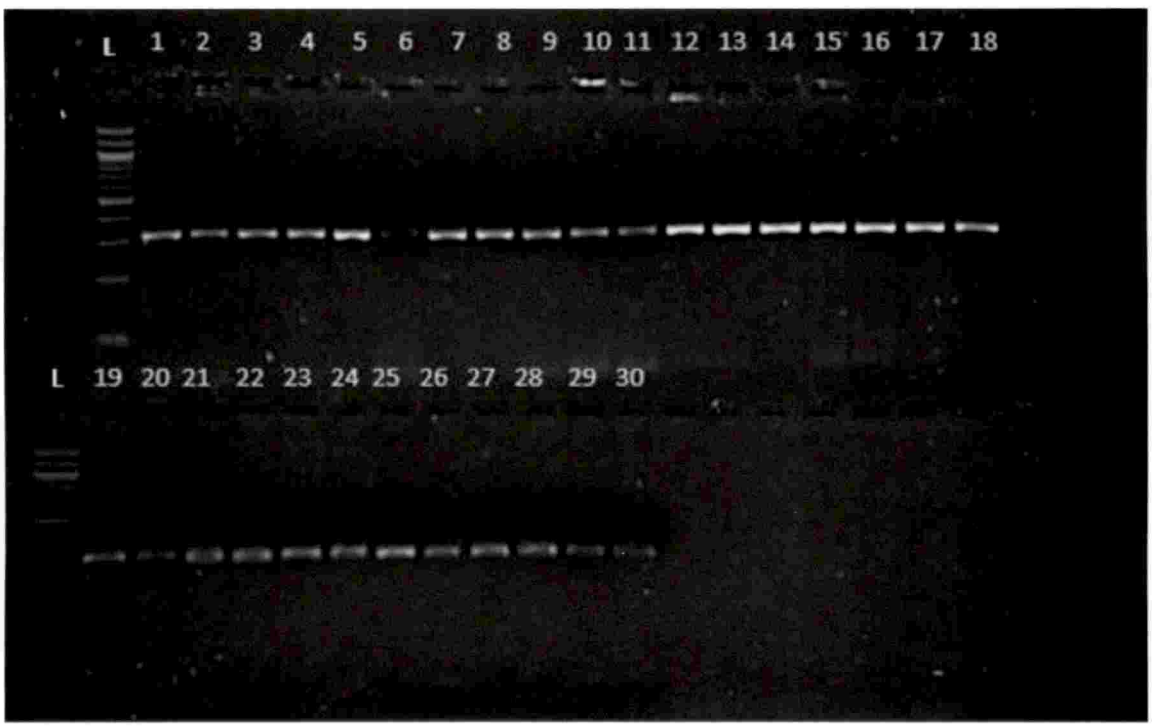

Plate 8: SSR profile obtained for primer Centa_DN3104 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

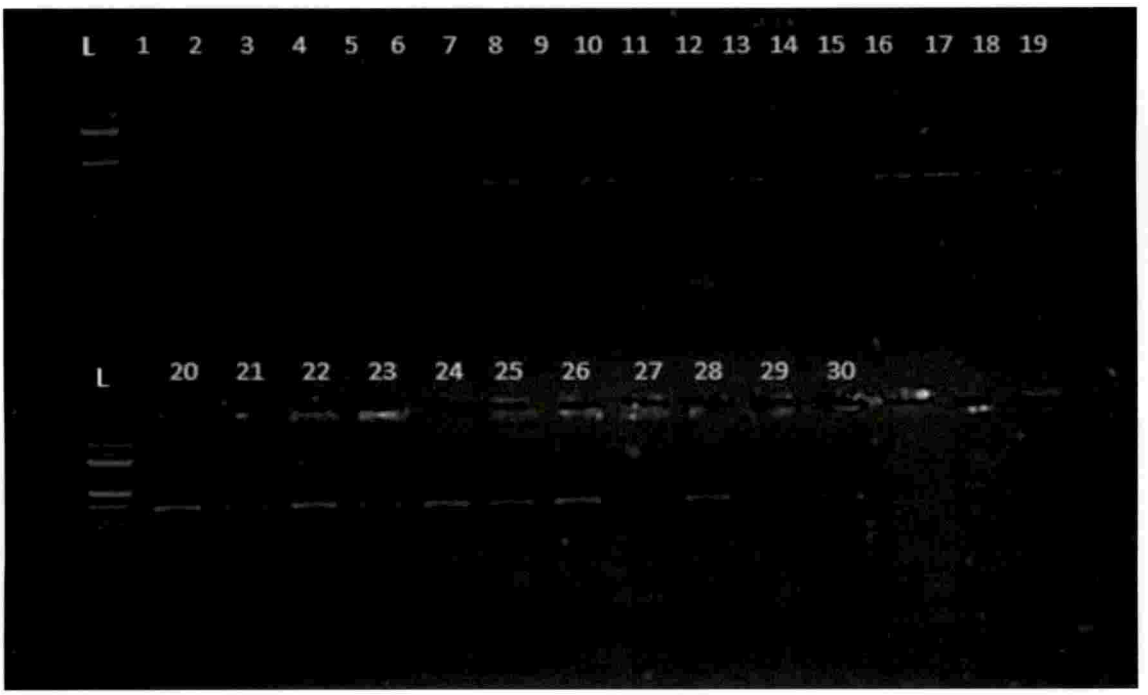

Plate 9: SSR profile obtained for primer Centa_DN2679 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

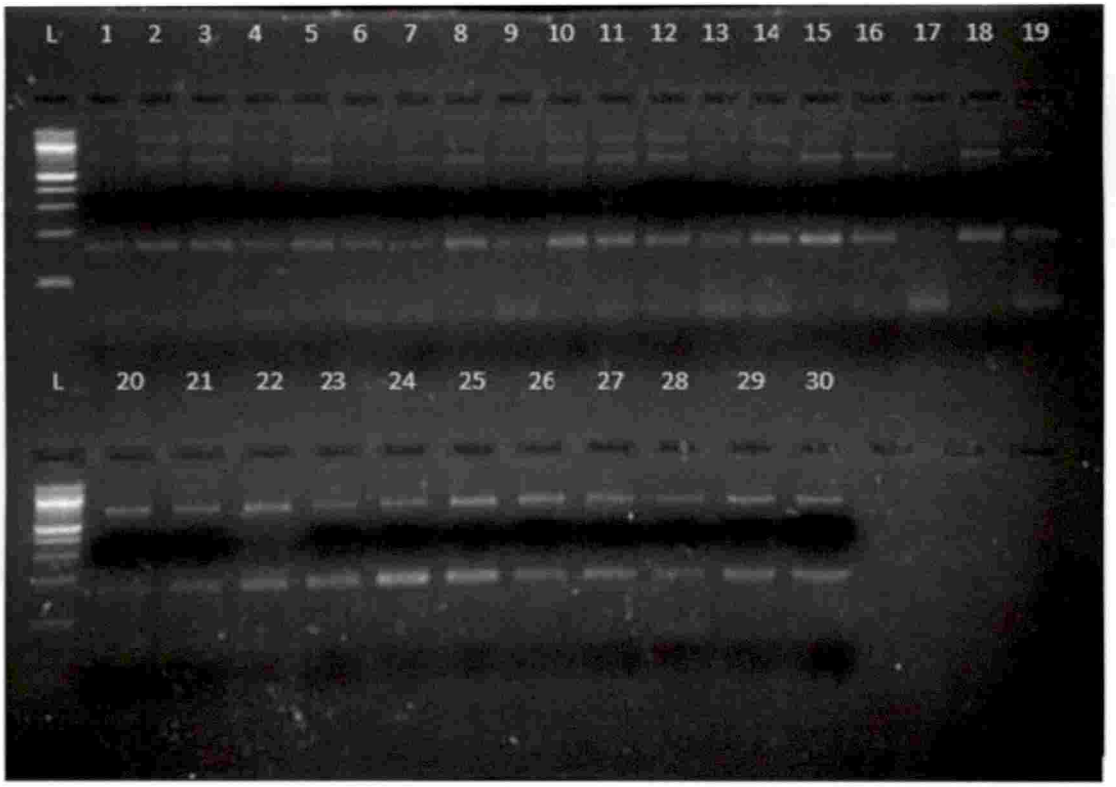

Plate 10: SSR profile obtained for primer Centa_DN3493 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

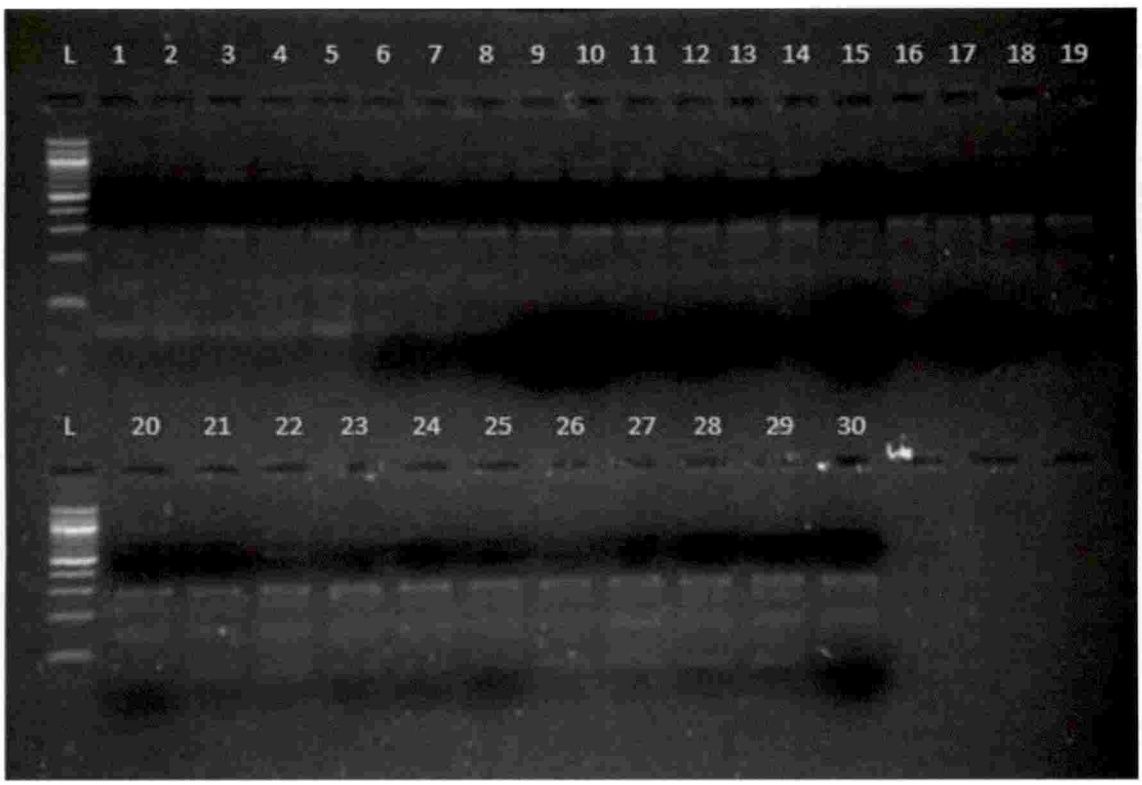

Plate 11: SSR profile obtained for primer Centa_DN3440 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

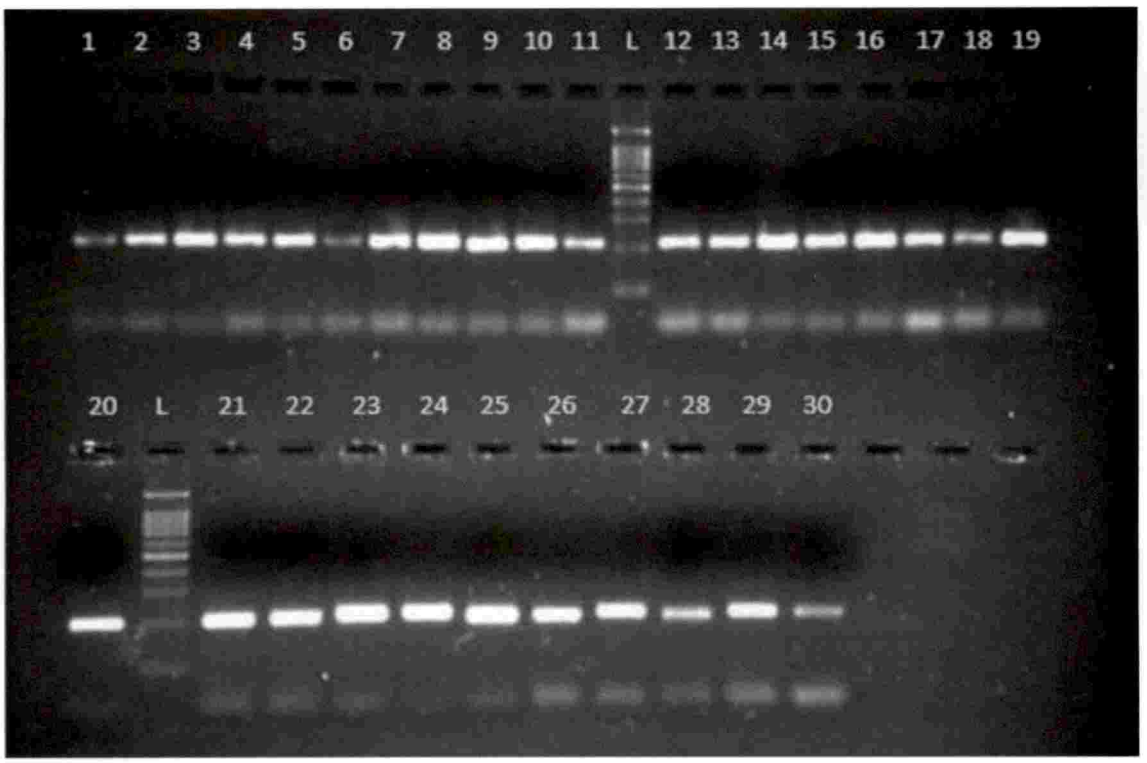

Plate 12: SSR profile obtained for primer TBG_CENTA-108 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

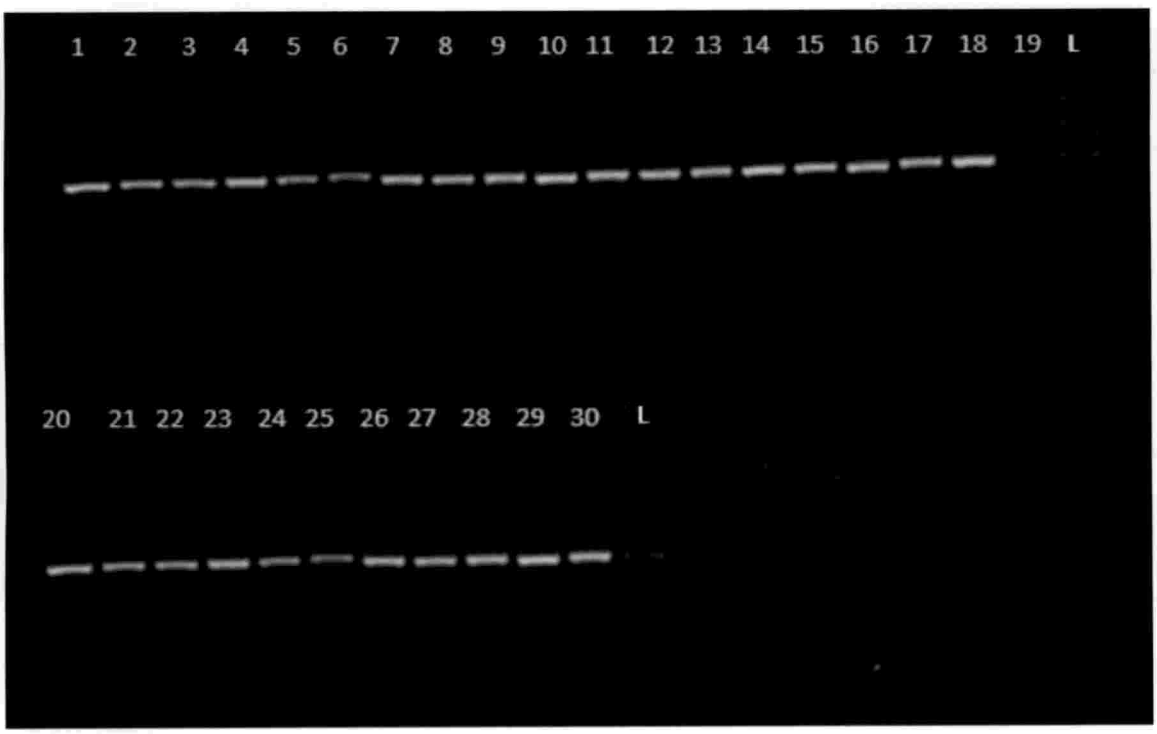

Plate 13: SSR profile obtained for primer TBG_CENTA-111 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

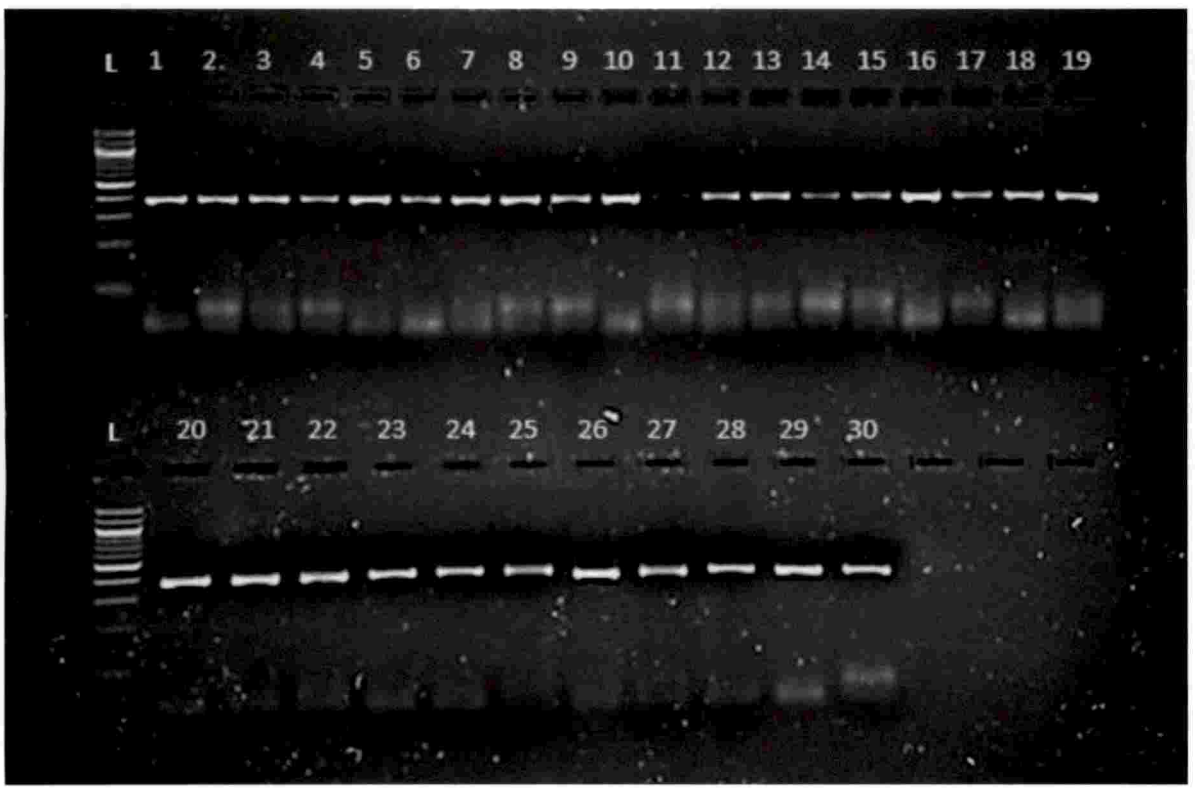

Plate 14: SSR profile obtained for primer TBG_CENTA-115 (Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

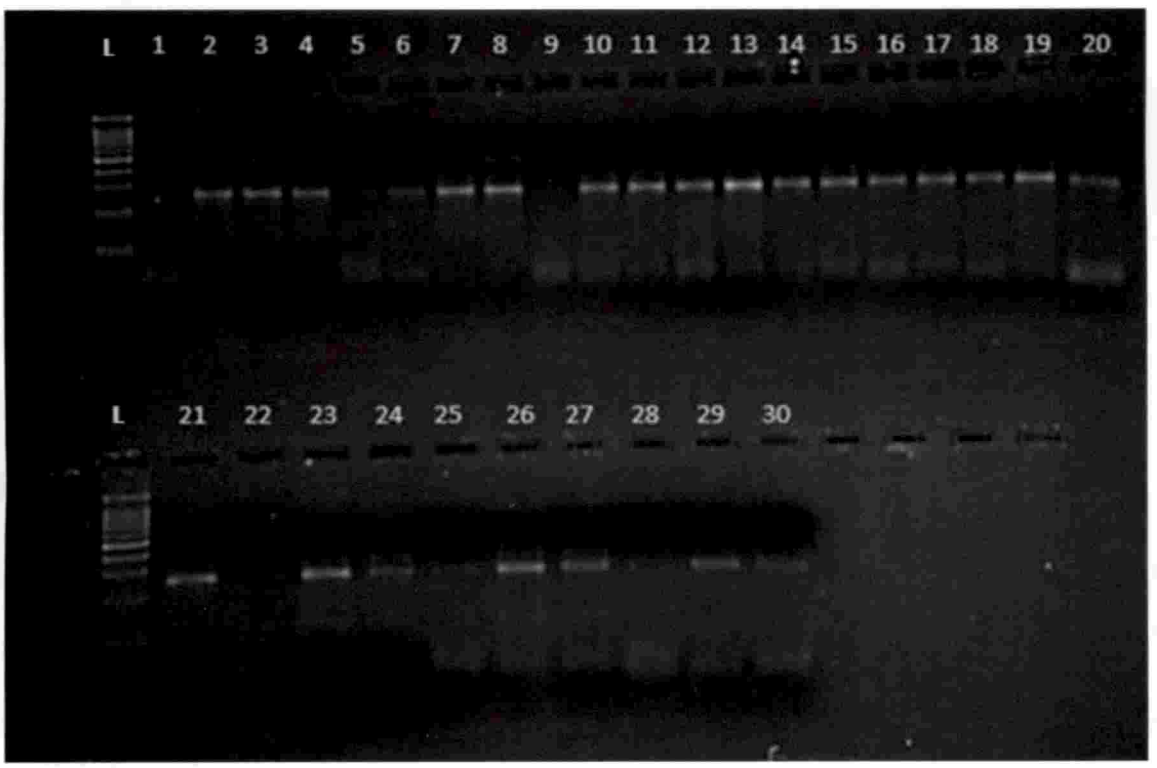

Plate 15: SSR profile obtained for primer TBG_CENTA- 117(Lanes: 1 - 302, 2 – 315, 3 – 317, 4 – 341, 5 – 342, 6 – 359, 7 – 374, 8 – 378, 9 – 383, 10 – 386, 11 – 392, 12 – 394, 13 – 395, 14 – 396, 15 – 398, 16 – 400, 17 – 401, 18 – 402, 19 – 407, 20 – 414, 21 – 415, 22 – 417, 23 – 423, 24 – 438, 25 – 449, 26 – 451, 27 – 452, 28 – 454, 29 – 455, 30 – 459, L – ladder)

4.5. GENETIC DIVERSITY DATA ANALYSIS

Genetic diversity of the 30 accessions was subjected to microsatellite analysis using 15 loci and their summary of genetic variation and distance was statistically generated using POPGENE (Yeh and Boyle, 1997). The number of alleles per locus ranges from 1.00 to 2, maximum Shannon's information index and Percentage of polymorphic loci obtained was 0.6931. Results are represented in Table 8. Dendrogram was constructed using MEGA 7 software based on the genetic distance (Figure 10). The dendrogram shows the cluster formation among the studied accessions. It shows grouping of 30 accessions in to 6 groups based on the genetic relationship. In this, group 6 (Paluvalli, Patrakalitemple, Kadamankode) form genetically distant cluster from other groups (Group 1 – KAPL valley, Thottiode, Group 2 – Kodangavila, Odayaratti, Ponmudi, Thottumukku, Kodaokanal, Munnar, Sirumalai, JNTBGRI, Charalvel, Kodaikanal, Group 3- Nilamel, Pallickal, Thottiode, Group 4- Vembayam, Kochuvila, Bananafarm, Garderstation, Alampara, Thannimoduvayal, group 5- Varadharajapuram, Munnar, Estary Island resort, Mallakkarappara dam site.

Among the studied accessions the Observed number of alleles per locus ranges from 1.00 to 1.1429, average heterozygosity ranges from 0.0051 to 0.0393. The highest Observed heterozygosity and Expected heterozygosity obtained was 0.1429 (Table 6 and Table 7).

The Nei's heterozygosity maximum 0.0714 was obtained in our study among accessions (Table 7). The highest genetic identity value based on Nei's 1978 Unbiased Measures of Genetic Identity and Genetic distance was found to be 1.01 and the lowest value was 0.71. Genetic distance between each accession ranged from -0.02 to 0.38 (Table 9). The average percentage of diversity among accessions 5.2 % (I*) was obtained.

Table 6. Summary of genetic variation statistics for 30 accessions analyzed using 15 SSR primer pairs

Sl. No	Sample Name	P*	Na*	Ne*	I*
1	302	6.67	1.0769	1.0769	0.0533
2	315	13.33	1.1333	1.1333	0.0924
3	317	13.33	1.1333	1.1333	0.0924
4	341	13.33	1.1333	1.1333	0.0924
5	342	13.33	1.1429	1.1429	0.0990
6	359	6.67	1.0667	1.0667	0.0462
7	374	6.67	1.0667	1.0667	0.0462
8	378	6.67	1.0667	1.0667	0.0462
9	383	6.67	1.0714	1.0714	0.0495
10	386	6.67	1.0667	1.0667	0.0462
11	392	6.67	1.0714	1.0714	0.0495
12	394	6.67	1.0667	1.0667	0.0462
13	395	6.67	1.0667	1.0667	0.0462
14	396	6.67	1.0667	1.0667	0.0462
15	398	6.67	1.0667	1.0667	0.0462
16	400	6.67	1.0667	1.0667	0.0462
17	401	6.67	1.0714	1.0714	0.0495
18	402	6.67	1.0667	1.0667	0.0462
19	407	6.67	1.0667	1.0667	0.0462
20	414	6.67	1.0667	1.0667	0.0462
21	415	6.67	1.0667	1.0667	0.0462
22	417	6.67	1.0714	1.0714	0.0495
23	423	6.67	1.0667	1.0667	0.0462
24	438	0.00	1.0000	1.0000	0.0000
25	449	6.67	1.0667	1.0667	0.0462
26	451	6.67	1.0667	1.0667	0.0462

27	452	6.67	1.0714	1.0714	0.0495
28	454	6.67	1.0667	1.0667	0.0462
29	455	6.67	1.0667	1.0667	0.0462
30	459	6.67	1.0667	1.0667	0.0462

* na = Observed number of alleles; * ne = Effective number of alleles

* I = Shannon's Information index; *P=percentage of polymorphic loci

Table 7. Summary of genetic variation statistics for 30 accessions analyzed using 15 SSR primer pairs

Sl. No.	Sample	Obs_Het	Exp_Het*	Nei**	Ave_Het
1	302	0.0769	0.0769	0.0385	0.0423
2	315	0.1333	0.1333	0.0667	0.0367
3	317	0.1333	0.1333	0.0667	0.0367
4	341	0.1333	0.1333	0.0667	0.0367
5	342	0.1429	0.1429	0.0714	0.0393
6	359	0.0667	0.0667	0.0333	0.0367
7	374	0.0667	0.0667	0.0333	0.0367
8	378	0.0667	0.0667	0.0333	0.0367
9	383	0.1429	0.1429	0.0714	0.0393
10	386	0.0667	0.0667	0.0333	0.0367
11	392	0.1429	0.1429	0.0714	0.0393
12	394	0.0667	0.0667	0.0333	0.0367
13	395	0.0667	0.0667	0.0333	0.0367
14	396	0.0667	0.0667	0.0333	0.0367
15	398	0.0667	0.0667	0.0333	0.0367
16	400	0.0667	0.0667	0.0333	0.0367
17	401	0.1429	0.1429	0.0714	0.0393
18	402	0.0667	0.0667	0.0333	0.0367
19	407	0.0667	0.0667	0.0333	0.0367
20	414	0.0667	0.0667	0.0333	0.0367
21	415	0.0667	0.0667	0.0333	0.0367

22	417	0.1429	0.1429	0.0714	0.0393
23	423	0.0667	0.0667	0.0333	0.0367
24	438	0.0000	0.0000	0.0000	0.0051
25	449	0.0667	0.0667	0.0333	0.0367
26	451	0.0667	0.0667	0.0333	0.0367
27	452	0.1429	0.1429	0.0714	0.0393
28	454	0.0667	0.0667	0.0333	0.0367
29	455	0.0667	0.0667	0.0333	0.0367
30	459	0.0667	0.0667	0.0333	0.0367

Obs_Het - Observed heterozygosity Exp_Het* - Expected heterozygosity

Nei** - Nei's heterozygosity Ave_Het - Average heterozygosity

Table 8. Summary of Genetic Variation Statistics for All Loci

Sl. No	Locus	na*	ne*	I*	Nei*
1	Centa_DN3	1.0000	1.0000	0.0000	0.0000
2	Centa_DN5	1.0000	1.0000	0.0000	0.0000
3	Centa_DN1	2.0000	2.0000	0.6931	0.5000
4	Centa_DN2	2.0000	1.1421	0.2449	0.1244
5	Centa_DN2	1.0000	1.0000	0.0000	0.0000
6	Centa_DN2	1.0000	1.0000	0.0000	0.0000
7	Centa_DN3	1.0000	1.0000	0.0000	0.0000
8	Centa_DN3	1.0000	1.0000	0.0000	0.0000
9	Centa_DN3	2.0000	1.5077	0.5196	0.3367
10	Centa_DN3	1.0000	1.0000	0.0000	0.0000
11	Centa_DN3	1.0000	1.0000	0.0000	0.0000
12	TBG_CENTA	1.0000	1.0000	0.0000	0.0000
13	TBG_CENTA	2.0000	1.4706	0.5004	0.3200
14	TBG_CENTA	2.0000	1.3846	0.4506	0.2778
15	TBG_CENTA	2.0000	1.2366	0.3405	0.1913

* na = Observed number of alleles * ne = Effective number of alleles [Kimura and Crow (1964)]

* I = Shannon's Information index [Lewontin (1972)] Nei* - Nei's heterozygosity

Table 9. Nei's original measures of genetic diversity and genetic distance (Nei, 1972) estimated from the SSR analysis

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	***	0.92	0.92	0.92	0.96	0.94	0.94	0.94	0.90	0.87	0.82	0.86	0.86	0.86	0.86
2	0.08	***	1.02	1.02	0.91	0.93	0.93	0.93	0.89	0.86	0.81	0.86	0.86	0.86	0.86
3	0.08	-0.02	***	1.02	0.91	0.93	0.93	0.93	0.89	0.86	0.81	0.86	0.86	0.86	0.86
4	0.08	-0.02	-0.02	***	0.91	0.93	0.93	0.93	0.89	0.86	0.81	0.86	0.86	0.86	0.86
5	0.04	0.09	0.09	0.09	***	0.96	0.96	0.96	0.92	0.89	0.85	0.89	0.89	0.89	0.89
6	0.06	0.07	0.07	0.07	0.04	***	1.01	1.01	0.98	0.94	0.90	0.94	0.94	0.94	0.94
7	0.06	0.07	0.07	0.07	0.04	-0.01	***	1.01	0.98	0.94	0.90	0.94	0.94	0.94	0.94
8	0.06	0.07	0.07	0.07	0.04	-0.01	-0.01	***	0.98	0.94	0.90	0.94	0.94	0.94	0.94
9	0.10	0.12	0.12	0.12	0.08	0.02	0.02	0.02	***	0.90	0.86	0.90	0.90	0.90	0.90
10	0.15	0.15	0.15	0.15	0.12	0.06	0.06	0.06	0.10	***	0.98	1.01	1.01	1.01	1.01
11	0.20	0.20	0.20	0.20	0.17	0.10	0.10	0.10	0.15	0.02	***	0.98	0.98	0.98	0.98
12	0.15	0.15	0.15	0.15	0.12	0.06	0.06	0.06	0.10	-0.01	0.02	***	1.01	1.01	1.01
13	0.15	0.15	0.15	0.15	0.12	0.06	0.06	0.06	0.10	-0.01	0.02	-0.01	***	1.01	1.01
14	0.15	0.15	0.15	0.15	0.12	0.06	0.06	0.06	0.10	-0.01	0.02	-0.01	-0.01	***	1.01
15	0.15	0.15	0.15	0.15	0.12	0.06	0.06	0.06	0.10	-0.01	0.02	-0.01	-0.01	-0.01	***
16	0.06	0.07	0.07	0.07	0.03	-0.01	-0.01	-0.01	0.02	0.06	0.10	0.06	0.06	0.06	0.06
17	0.20	0.12	0.12	0.12	0.26	0.18	0.18	0.18	0.15	0.28	0.34	0.28	0.28	0.28	0.28
18	0.15	0.07	0.07	0.07	0.20	0.14	0.14	0.14	0.10	0.22	0.28	0.22	0.22	0.22	0.22
19	0.15	0.07	0.07	0.07	0.20	0.14	0.14	0.14	0.10	0.22	0.28	0.22	0.22	0.22	0.22
20	0.06	0.07	0.07	0.07	0.03	-0.01	-0.01	-0.01	0.02	0.06	0.10	0.06	0.06	0.06	0.06
21	0.06	0.07	0.07	0.07	0.03	-0.01	-0.01	-0.01	0.02	0.06	0.10	0.06	0.06	0.06	0.06
22	0.11	0.12	0.12	0.12	0.08	0.02	0.02	0.02	-0.01	0.10	0.15	0.10	0.10	0.10	0.10
23	0.15	0.07	0.07	0.07	0.12	0.06	0.06	0.06	0.10	0.14	0.18	0.14	0.14	0.14	0.14
24	0.24	0.24	0.24	0.24	0.20	0.13	0.13	0.13	0.18	0.22	0.28	0.22	0.22	0.22	0.22
25	0.15	0.07	0.07	0.07	0.12	0.06	0.06	0.06	0.10	0.14	0.18	0.14	0.14	0.14	0.14
26	0.06	0.07	0.07	0.07	0.03	-0.01	-0.01	-0.01	0.02	0.06	0.10	0.06	0.06	0.06	0.06
27	0.11	0.12	0.12	0.12	0.17	0.10	0.10	0.10	0.15	0.18	0.24	0.18	0.18	0.18	0.18
28	0.15	0.07	0.07	0.07	0.12	0.06	0.06	0.06	0.10	0.14	0.18	0.14	0.14	0.14	0.14
29	0.06	0.07	0.07	0.07	0.03	-0.01	-0.01	-0.01	0.02	0.06	0.10	0.06	0.06	0.06	0.06
30	0.06	0.07	0.07	0.07	0.03	-0.01	-0.01	-0.01	0.02	0.06	0.10	0.06	0.06	0.06	0.06

pop ID	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	0.94	0.82	0.86	0.86	0.94	0.94	0.90	0.86	0.79	0.86	0.94	0.90	0.86	0.94	0.94
2	0.93	0.89	0.93	0.93	0.93	0.93	0.89	0.86	0.79	0.86	0.93	0.89	0.86	0.93	0.93
3	0.93	0.89	0.93	0.93	0.93	0.93	0.89	0.86	0.79	0.86	0.93	0.89	0.86	0.93	0.93
4	0.93	0.89	0.93	0.93	0.93	0.93	0.89	0.86	0.79	0.86	0.93	0.89	0.86	0.93	0.93
5	0.96	0.77	0.82	0.82	0.96	0.96	0.92	0.89	0.82	0.89	0.96	0.85	0.89	0.96	0.96
6	1.01	0.83	0.87	0.87	1.01	1.01	0.98	0.94	0.88	0.94	1.01	0.90	0.94	1.01	1.01
7	1.01	0.83	0.87	0.87	1.01	1.01	0.98	0.94	0.88	0.94	1.01	0.90	0.94	1.01	1.01
8	1.01	0.83	0.87	0.87	1.01	1.01	0.98	0.94	0.88	0.94	1.01	0.90	0.94	1.01	1.01
9	0.98	0.83	0.90	0.90	0.98	0.98	1.01	0.90	0.83	0.90	0.98	0.86	0.90	0.98	0.98
10	0.94	0.76	0.80	0.80	0.94	0.94	0.90	0.87	0.81	0.87	0.94	0.83	0.87	0.94	0.94
11	0.90	0.71	0.76	0.76	0.90	0.90	0.86	0.83	0.76	0.83	0.90	0.79	0.83	0.90	0.90
12	0.94	0.76	0.80	0.80	0.94	0.94	0.90	0.87	0.81	0.87	0.94	0.83	0.87	0.94	0.94
13	0.94	0.76	0.80	0.80	0.94	0.94	0.90	0.87	0.81	0.87	0.94	0.83	0.87	0.94	0.94
14	0.94	0.76	0.80	0.80	0.94	0.94	0.90	0.87	0.81	0.87	0.94	0.83	0.87	0.94	0.94
15	0.94	0.76	0.80	0.80	0.94	0.94	0.90	0.87	0.81	0.87	0.94	0.83	0.87	0.94	0.94
16	***	0.83	0.87	0.87	1.01	1.01	0.98	0.94	0.88	0.94	1.01	0.90	0.94	1.01	1.01
17	0.18	***	0.98	0.98	0.83	0.83	0.86	0.76	0.68	0.76	0.83	0.79	0.76	0.83	0.83
18	0.14	0.02	***	0.01	0.87	0.87	0.90	0.80	0.73	0.80	0.87	0.83	0.80	0.87	0.87
19	0.14	0.02	-0.01	***	0.87	0.87	0.90	0.80	0.73	0.80	0.87	0.83	0.80	0.87	0.87
20	-0.01	0.18	0.14	0.14	***	0.01	0.98	0.94	0.88	0.94	1.01	0.90	0.94	1.01	1.01
21	-0.01	0.18	0.14	0.14	-0.01	***	0.98	0.94	0.88	0.94	1.01	0.90	0.94	1.01	1.01
22	0.02	0.15	0.10	0.10	0.02	0.02	***	0.90	0.84	0.90	0.98	0.86	0.90	0.98	0.98
23	0.06	0.28	0.22	0.22	0.06	0.06	0.10	***	0.95	1.01	0.94	0.98	1.01	0.94	0.94
24	0.13	0.38	0.31	0.31	0.13	0.13	0.18	0.05	***	0.95	0.88	0.91	0.95	0.88	0.88
25	0.06	0.28	0.22	0.22	0.06	0.06	0.10	-0.01	0.05	***	0.94	0.98	1.01	0.94	0.94
26	-0.01	0.18	0.14	0.14	-0.01	-0.01	0.02	0.06	0.13	0.06	***	0.90	0.94	1.01	1.01
27	0.10	0.24	0.18	0.18	0.10	0.10	0.15	0.02	0.09	0.02	0.10	***	0.98	0.90	0.90
28	0.06	0.28	0.22	0.22	0.06	0.06	0.10	-0.01	0.05	-0.01	0.06	0.02	***	0.94	0.94
29	-0.01	0.18	0.14	0.14	-0.01	-0.01	0.02	0.06	0.13	0.06	-0.01	0.10	0.06	***	1.01
30	-0.01	0.18	0.14	0.14	-0.01	-0.01	0.02	0.06	0.13	0.06	-0.01	0.10	0.06	-0.01	***

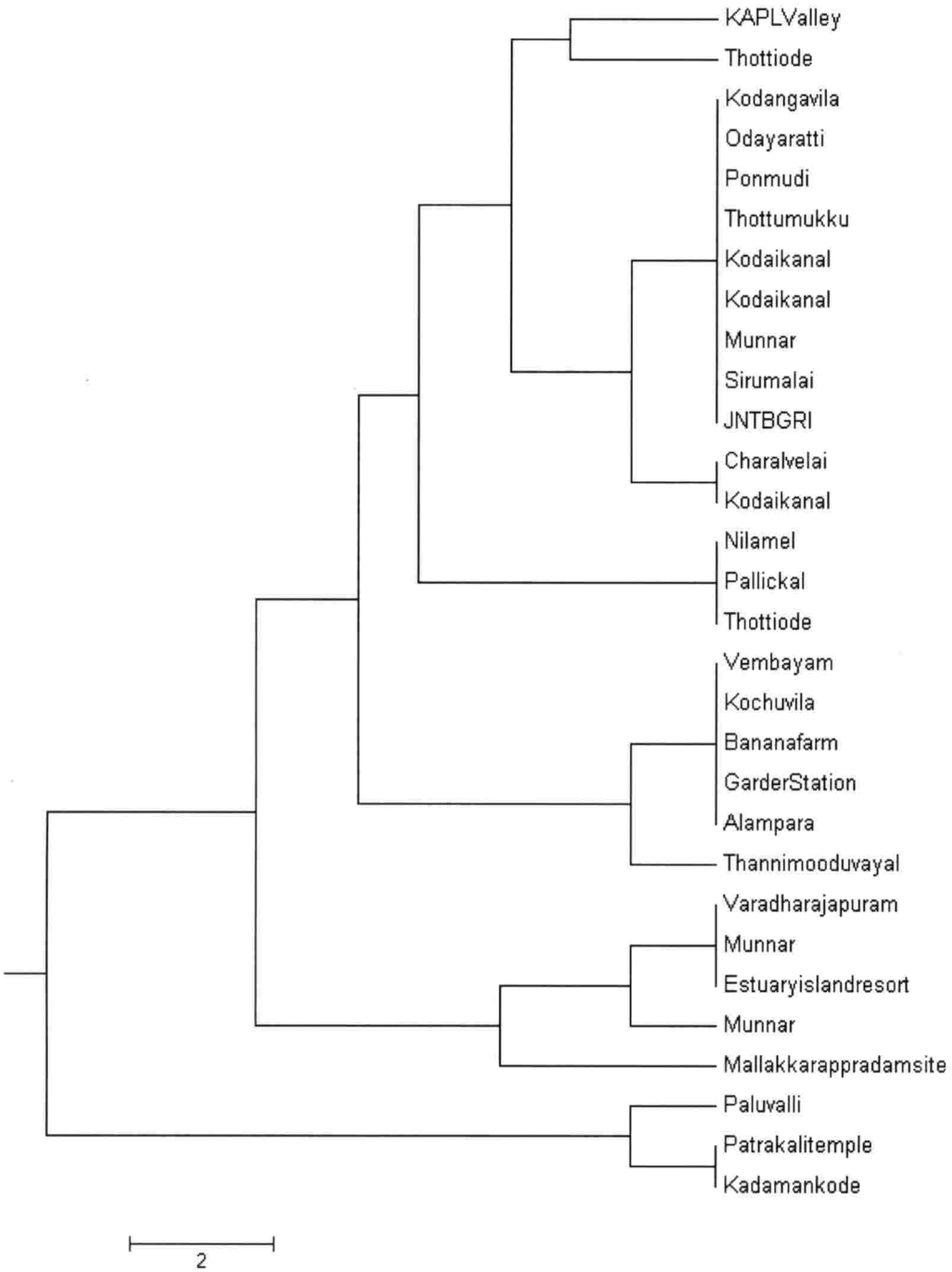

Figure 11: Dendrogram based on Nei's (1978) genetic distance method assessed using 15 SSR primers in 30 *Centella* accession denoted by the location in which they were collected

4.6. Phenotypic character and asiaticoside content analysis:

Leaf area (cm^2) and number of leaves per node of the 30 different accessions were studied. The leaf area (cm^2) obtained was in the range of 4.1 - 9.9 and in the case of number of leaves per node, it was from 2.4 – 7(Table 10). Based on the clusters formed during genetic analysis, an accession was selected from each cluster randomly to conduct the asiaticoside content analysis. Totally six clusters were formed (Figure 11), one accession from each group was taken to asiaticoside content analysis using HPTLC to represent each group. So, from out of the six accessions analysed, the asiaticoside content was 5.6 % and lowest was 1.35 % when compared to other selected studied accessions. Biomass content (% dry wt.) obtained ranges from 17.22 to 21.52 (Table 11) and Peak obtained while doing HPTLC also represented as 3D graph in the figure 12. The value of asiaticoside obtained in the present study was significantly higher than as reported by Thomas *et al.*, 2010. This shed an interesting impact on the present study.

Table 10. Phenotypic Data of thirty *Centella asiatica* accession studied

SL. NO.	Accession Number (Sample code)	Leaf area (cm^2) *	Number of leaves per node*
1	302	4.95±1.40	4.00±2.11
2	315	9.40±1.71	7.00±1.49
3	317	7.05±3.05	6.10±1.91
4	341	6.20±2.90	2.40±1.07
5	342	5.25±1.75	6.00±1.25
6	359	4.10±0.84	4.80±1.32
7	374	5.25±0.98	5.00±2.00
8	378	6.30±1.18	4.50±2.59
9	383	5.40±1.50	6.50±1.51
10	386	5.70±1.62	5.80±1.62
11	392	7.45±3.26	4.20±1.13
12	394	6.40±1.07	4.90±1.85
13	395	5.50±1.03	5.10±1.91
14	396	5.80±2.03	5.60±1.84

15	398	6.35±1.37	4.10±1.20
16	400	6.95±1.55	5.30±2.00
17	401	5.90±1.12	5.80±1.99
18	402	6.10±1.54	3.90±1.20
19	407	7.20±1.80	7.10±2.08
20	414	7.20±1.32	5.30±0.95
21	415	6.55±1.74	5.80±1.69
22	417	8.35±1.41	5.70±1.57
23	423	6.20±1.44	4.70±1.64
24	438	8.05±2.01	4.90±1.73
25	449	7.55±1.92	5.90±1.37
26	451	9.90±2.85	5.50±1.78
27	452	9.40±2.69	6.20±1.47
28	454	5.00±1.35	6.60±1.35
29	455	7.60±2.32	4.10±1.85
30	459	4.40±1.17	5.90±2.28

*Mean and SD value

Table 11. Table shows the percentage of asiaticoside Content and dry weight

Sl. No.	Accession No.	Dry Wt. (%)	Asiaticoside Content (%)
1	315	21.52	2.0
2	342	17.97	1.45
3	386	19.49	5.6
4	400	17.22	1.35
5	407	18.48	2.0
6	423	19.37	1.45

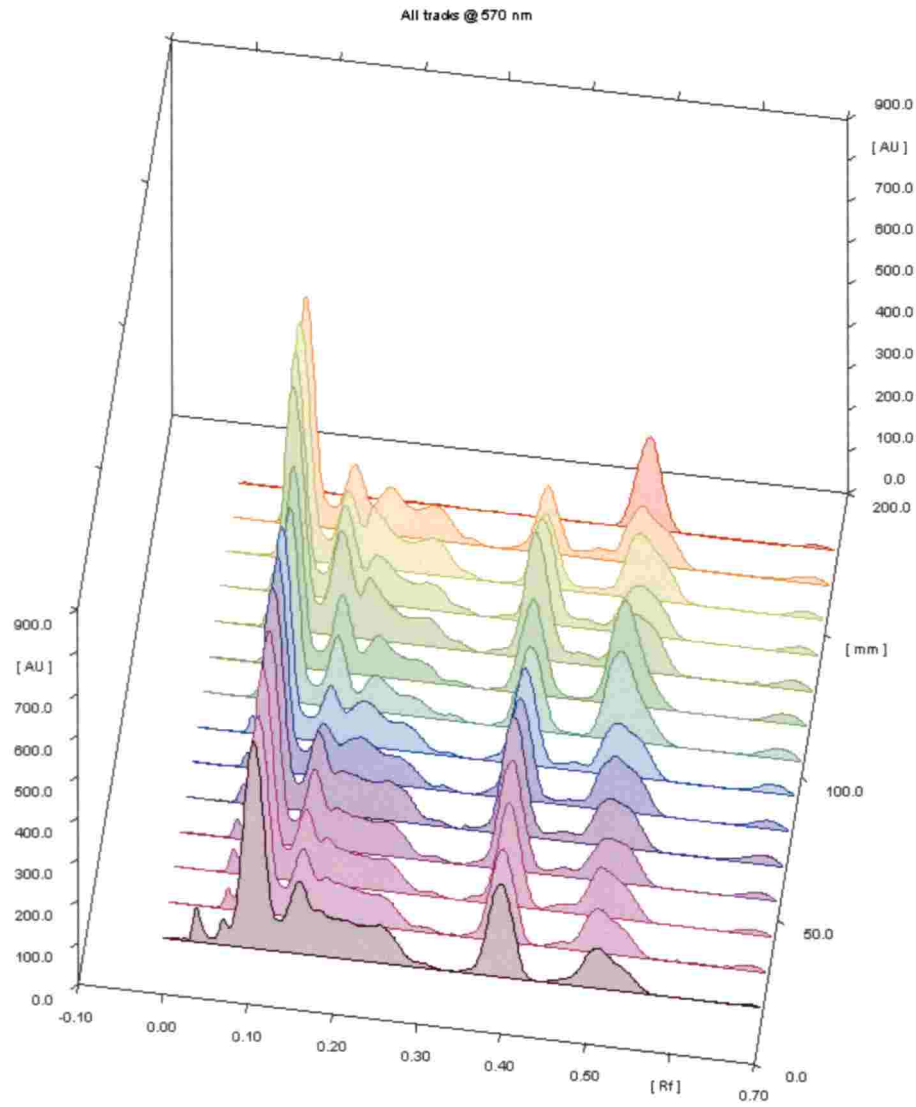

Figure 11: 3D graph showing the peak of all samples

4.7. Correlation study:

The Pearson correlation analysis was conducted to study the relationship between the various collected genetic, phenotypic and chemical parameters analysed in the collected accessions using XLSAT software. There was a strong positive correlation found between the genetic variation variables. The weak positive correlation (0.142) was observed between asiaticoside content and genetic heterozygosity and additionally one phenotype character i.e. number of leaves per

node show more positive correlation (0.291) with triterpenoid content (Table 12). These works conclude that genetic diversity has the positive relationship with the analyzed characters.

Table 12. Correlation Coefficient

Variables	P*	Na*	Ne*	I*	Nei*	Leaf area (cm2)	Number of leaves per node	asiaticoside content
P*	**1**	**0.995**	**0.995**	**0.994**	**0.994**	**0.008**	**0.061**	**0.153**
Na*	**0.995**	**1**	**1.000**	**1.000**	**1.000**	**-0.003**	**0.063**	**0.143**
Ne*	**0.995**	**1.000**	**1**	**1.000**	**1.000**	**-0.003**	**0.063**	**0.143**
I*	**0.994**	**1.000**	**1.000**	**1**	**1.000**	**-0.002**	**0.063**	**0.142**
Nei*	**0.994**	**1.000**	**1.000**	**1.000**	**1**	**-0.002**	**0.063**	**0.142**
Leaf area (cm2)	**0.008**	**-0.003**	**0.003**	**0.002**	**0.002**	**1**	**0.190**	**0.002**
Number of leaves per node	**0.061**	**0.063**	**0.063**	**0.063**	**0.063**	**0.190**	**1**	**0.291**
asiaticoside content	**0.153**	**0.143**	**0.143**	**0.142**	**0.142**	**0.002**	**0.291**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

P* =percentage of polymorphic loci, Na* = Observed number of alleles,

Ne* = Effective number of alleles, I* = Shannon's Information index,

Nei* - Nei's heterozygosity

5. DISCUSSION

Medicinal Plants have been used as treatments for thousands of years; *C. asiatica* is an important medicinal herb that is widely used in the treatment of many diseases. It is perennial herbaceous creeper belonging to the family Umbellifere (Apiceae) is found in most tropical and subtropical countries growing in swampy areas, including parts of India, Pakistan, Sri Lanka, Madagascar, and South Africa and South pacific and Eastern Europe. The whole plant is used for medicinal purposes. Triterpenoid, saponins, the primary constituents in this plant are mainly responsible for its wide therapeutic actions.

Genetic diversity is the total variability of genetic characteristics within the genetic makeup of a species. It is the necessity for populations to adapt to dynamical environments. The importance of plant genetic diversity (PGD) has being recognized as a specific area since exploding population with urbanization and decreasing cultivable lands were the critical factors contributing to food insecurity in developing world.

Agricultural scientists realized that PGD can be captured and stored in the form of plant genetic resources (PGR) such as gene bank, DNA library, and so forth, in the biorepository which preserve genetic material for long period. However, conserved PGR must be utilized for crop improvement in order to meet future global challenges in relation to food and nutritional security (Govindaraj *et al.,* 2015).

Many genetic diversity works were carried out in variety of plant species to find out the PGD. Sakthipriya *et al.,* 2018 have reported that, through molecular screening by 10 microsatellite markers showed low polymorphism (0.019) between the 30 *centella* samples analyzed. And also, it was reported that effect of environmental factors, genetic composition or possibility of inbreeding, were required to analyze the probable reason for the low variability exists in the species.

Using transcriptomic sequence of *Trichosanthes kirilowii* Maxim. Silpa *et al.*, 2016 developed and validated EST-SSR markers and EST sequences of *T. dioica* Roxb. for identification of EST-SNPs in *T. cucumerina* var. *cucumerina* L. A total of 382

primers were developed and out of which 58 were found to be suitable for genetic analysis.

Transcriptomes based SSR markers were developed for the plant genus *Orinus* (Poaceae) by Yu-Ping Liu *et al* in 2017.They have designed 16 polymorphic SSR markers from two cDNA libraries for investigating population structure in *Orinus* and these markers provide positive result by showing considerable polymorphisms for 248 individuals from three populations each of the three species of *Orinus*.

By using 32 nuclear simple sequence repeat (SSR) markers and 7 cytoplasmic gene markers to analyze a total of 357 individuals from 162 accessions of 9 *Lolium* species Xuanli Guan *et al.*, 2017 assess the genetic diversity and population structure of *Lolium* species. This survey revealed a high level of polymorphism, with an average number of alleles per locus of 23.59 and 5.29 and an average PIC-value of 0.83 and 0.54 for nuclear SSR markers and cytoplasmic gene markers, respectively.

Thomas *et al.,* 2010 reported that methanol extracts of *C. asiatica* (whole plant) gave well resolved twin spots of both madecassoside and asiaticoside in the mobile phase (4:1:5, v/v) butanol: ethyl acetate: water, at Rf values 0.34±0.02 and 0.47±0.02, respectively. Both triterpene glycosides were evaluated densitometrically at 570 nm. Of the sixty accessions, highest detected madecassoside and asiaticoside contents were 5.67±0.08% (dry wt. of whole plant) and 1.70±0.02%, respectively.

In this study, SSR regions were identified from the leaf transcriptome sequence of *C. asiatica* and suitable primers were designed from this non-redundant dataset using the software WEBSAT (wsmartins.net/websat). A total of 129 primers were designed collectively for the contigs.

The genetic relationship with triterpenoid content and genetic diversity was analyzed within 30 accessions of *Centella asiatica* from Tamil Nadu and Kerala using designed transcriptome based SSR markers. Low percentage of polymorphism was observed using 15 markers. This indicated that percentage of genetic diversity existed among studied accessions. This could be due to the reasons were reported previously (Sakthipriya *et al.,* 2018)

The dendrogram based on SSR marker analysis showed that most accessions from the adjacent regions clustered together. Totally 6 groups were formed, from each group one accessions was taken for asiaticoside content analysis.

The asiaticoside content found to be have relationship with the genetic heterozygosity as well as analyzed phenotypic characters. Apart from the terpenoid content, two phenotype characters were subjected to study. These characters show the positive correlation with the terpenoid content. However, results based on the contents of asiaticoside and genotype did show low level of correlation but it was a positive, so this was a good initial step towards the development of functional related markers.

6. SUMMARY

The research work entitled "Development of functional EST-SSR and analysis of genetic diversity in *Centella asiatica.*" was carried out at Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during 2017-18. The objective of the study was to develop and use functional molecular markers such as EST-SSR for analysis of genetic diversity in *Centella asiatica* in relation to the content of triterpenoids.

The *Centella asiatica* is a medicinal plant which has been used since centuries. It is also known as Brahmi, Asiatic pennywort or Gotu kola. It is a small trailing perennial herb and is the only species of *Centella* seen in India. Stem is green to greenish red colour and rooting at nodes. These connects plants each other. Petiole is long, smooth, green in colour. Leaves are palmately veined fleshy leaves. Plants are mainly found in marshy land of tropical and subtropical regions.

Centella asiatica contains large quantities of pentacyclic triterpenoid saponins. Major triterpenoids are asiaticoside, centelloside, madecassoside, brahmoside, asiatic-, brahmic-, centellic- and madecassic acids. Common secondary plant metabolites are triterpene saponins and in these triterpenoids asiaticoside is the major one. In this work asiaticoside is estimated in 6 samples which was selected from the cluster analysis using POPGENE. The amount of asiaticoside ranges from 1.35 % to 5.6% of the dry weight.

Thirty accessions of *Centella asiatica* from different geographical locations that are conserved in the *Centella* conservatory of JNTBGRI, were used for the present study. DNA purity estimated using bio photometer ranges from 1.6 – 2.1.

Over all observed number of alleles (na) ranges from 1.00 to 2. Effective number of alleles (ne) ranges from 1.00 to 2. Shannon's Information index (I) ranges from 0.00 to 0.06931. Among the accessions observed heterozygosity ranges from 0.000 to 0.1429. Expected heterozygosity (Exp_Het) ranges from 0.000 to 0.1429. Nei's heterozygosity (Nei) ranges from 0.000 to 0.0714. Average heterozygosity (Ave_Het) ranges from 0.0051 to 0.0393. Phenotypic data was also collected from the all the accessions

during study. Leaf area ranges from 4.10 – 9.9 cm^2 and Leaves per node ranges from 2.4 – 7.10 was observed.

Genetic diversity analysis using dendrogram revealed the thirty accessions taken for the study was divided into 6 groups.

Asiaticoside was extracted using methanol and asiaticoside content was estimated using HPTLC in these groups. The variation in asiaticoside content was low which shows a slight genetic diversity. Asiaticoside content (%) which is the major Triterpenoid in *Centella asiatica* ranges from 1.35 – 5.6. Biomass content (% dry wt.) ranges from 17.22 – 21.52 %.

Correlation analysis showed positive correlation between genetic diversity and asiaticoside content. Additionally, one phenotype character (number of leaves per node) showed higher positive correlation with asiaticoside content. It infers that asiaticoside content depends on both genetic and phenotypic variations, it varies when changes in genetic as well phenotypic level since it shows positive correlation.

7. REFERENCE

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8. APPENDIX I

TBE Buffer (20X) for 100ml solution

Tris base	21.6g
Boric acid	11.0g
0.5M EDTA (Ph 8.0)	8.00ml

APPENDIX II

Tracking dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%
Water	69.5%

APPENDIX III

AE Buffer

Tris HCL	10Mm
EDTA (Ph 9.0)	0.5Mm

APPENDIX IV

Composition of Gel for AGE

Agarose	0.75g
20X TBE buffer	2.5ml
EtBr	2.5µl

DEVELOPMENT OF FUNCTIONAL EST-SSR AND ANALYSIS OF GENETIC DIVERSITY IN *Centella asiatica*

ARYA ARAVIND

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Abstract of Thesis

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B.Sc. - M.Sc. (Integrated) Biotechnology

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2018

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9. ABSTRACT

Centella asiatica is a small trailing perennial herb and is the only species of *Centella* seen in India. Stem is green to greenish red colour and rooting at nodes. These connects plants each other. Petiole is long, smooth, green in colour. Leaves are palmately veined fleshy leaves. Plants are mainly found in marshy land of tropical and subtropical regions.

Genetic diversity is that the total variety of genetic characteristics within the genetic makeup of a species. It plays a very important role within the survival and flexibility of a species and is important for a species to evolve.

We aim to develop and use transcriptome based SSR molecular marker for analysis of genetic diversity in *Centella asiatica* in relation to the content of triterpenoids. To achieve that 129 Primers were designed from the transcriptome sequence obtained from NCBI. To validate the designed primers 15 primers were selected and used for genetic diversity analysis in 30 different *Centella* accessions collected from different regions of Tamil Nadu and Kerala. The genetic diversity analysis result grouped the accessions in to six group based on the genetic distance. Single accessions was randomly selected from each group to chemical analysis the asiaticoside content and additionally two phenotypic characters were also characterised in all 30 collected accessions.

The result of genetic diversity analysis shows the less Nei heterozygosity (0.0433) between the studied accessions and the positive correlation (0.142) with the asiaticoside content. From the analysed phenotypic characters, number of leaves per node character shows the interesting positive correlation (0.291) with asiaticoside content than genetic diversity. This study gives the positive and initiative step to the genetic diversity and the asiaticoside content correlations. This will be helpful to find out the functional marker related to asiaticoside content which will be useful in MAS studies in future.