STUDIES ON AMPLIFIED FRAGMENT LENGTH POLYMORPHISM IN TEA (*CAMELLIA* SPECIES) CLONES

ABSTRACT

By

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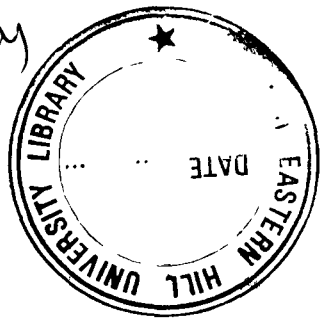

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ABSTRACT

Recent advances in molecular biology led to the development of new techniques, such as DNA based molecular markers. This creates opportunities for plant genetic researchers to assess and characterize genetic variation, which has been the foundation for the selection of desirable qualitative and quantitative traits of agronomical importance. Among the techniques, AFLP is one of the most powerful technologies that could identify a large number of polymorphic loci without prior knowledge of DNA sequences of the genome and has been widely used for genetic fingerprinting, genome mapping and genetic variability studies.

Cultivated tea belongs to the genus *Camellia* and it is a diploid with basic chromosome number 15. It is an important non-alcoholic beverage and a 'health drink' which is widely consumed all over the world. Secondary metabolites, particularly flavonols and polyphenols (chetechins), are of great abundance and contribute to their flavour and health properties. For major tea-producing countries, tea is a major source of foreign currency revenue. So, to meet the high demands of this plantation crop, there is a continuous need for its genetic improvement by application of genetic markers. Tea has long gestation period and is an outcrossing species. Therefore, the conventional methods of breeding take a long time to develop genetically improved plant with desirable traits. Clonal selection is an important and widely adopted method of tea improvement as they are genetically uniform and give uniform yield and quality. In recent years, efforts have been given worldwide, integrating the DNA marker based technologies on genetic improvement of tea.

The development of genetic linkage maps using DNA based markers is a new plant breeding tool that functions as a valuable reference for locating important genes on the maps that allow marker assisted selection of agronomically important traits. Till date, six DNA marker based linkage maps have been developed using different markers such as RAPD (Randomly Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeats), CAPS (Cleaved Amplified Polymorphic Sequences), SSRs (Simple Sequence Repeats), STS (Sequence Tag Sites) markers in different genetic backgrounds (Tanaka 1996, Hackett *et al.* 2000, Huang *et al.* 2005, Huang *et al.* 2006, Kamunya *et al.* 2010 and Taniguchi *et al.* 2012). A molecular linkage map is not only a prerequisite for linking a QTL, it is essential to map them for efficient selection and subsequent transfer of the trait. Therefore, to achieve the ultimate goal of genetic improvement of tea, further efforts are required to construct a high density map and to locate the quantitative trait loci (QTL) and other important agronomic traits. Although the density of integrated genetic maps can be extremely high, it may still not be sufficient to tag a specific gene and more maps would help in filling the existing gaps in the present maps.

Tea genome size is estimated to be 4.0×10^9 bp (Tanaka *et al.* 2006), so AFLP (Vos *et al.* 1995). Therefore conventional AFLP based marker analyses would be cumbersome due to generation of large number of bands. So, a modification of conventional AFLP, TE-AFLP (Three Endonuclease-AFLP) method (van der Wurff *et al.* 2000) that provides high discriminatory power and reduction in the number of

bands would be suitable for tea. Negi *et al.* (2005) also concluded that TE-AFLP was the best technique for fingerprinting of tea.

For the visualization of AFLP products in polyacrylamide gels, silver staining revealed similar sensitivity and resolution with the autoradiography, reduced the time, cost and eliminated hazard of working with radioisotopes (Vantoai *et al.* 1996, Chalhoub *et al.* 1997). In addition, recovery of fragments from the dried gels made silver staining a more useful and versatile detection method.

Therefore, there is a strong case for cheap, reliable, informative silver stained TE-AFLP marker studies on a mapping population of tea for obtaining a possible genetic linkage map and if possible to locate some traits that are related to tea production.

With this background, the present study has the following objectives:

1. Identification of TE-AFLP markers using bi-clonal seed stocks which are maintained at Tea Research Association (TRA), Tocklai, Jorhat, India.
2. Construction of a genetic linkage map based on the above TE-AFLP markers.
3. Nucleotide sequencing and analysis of selected TE-AFLP based amplicons.

In order to achieve these objectives the following approach was followed:

1. Mapping population consisting of 117 genotypes of the F_1 population of the bi-clonal seed stock TS 463, through a cross between two TV clones i.e. TV1, an Assam-China hybrid with another Cambod clone, TV19 were

collected from Tea Research Association (TRA), Tocklai, Jorhat, Assam, India.

2. Genomic DNA extraction was done using modified CTAB method, especially designed for plants containing high polysaccharide and polyphenolic components (Porebski *et al.* 1997).
3. TE-AFLP analysis was performed with minor modifications according to the protocol described by van der Wurff *et al.* (2000).
4. Following TE-AFLP amplification reactions, 6% denaturing polyacrylamide gel electrophoresis were done.
5. After the electrophoresis, silver staining was carried out to visualize the DNA fragments in the sequencing gel. The procedure for silver staining was based on the procedure of Caetano-Anolles and Gresshoff (1994) with few modifications.
6. Elution of AFLP fragments was done according to the protocol described by Ausubel *et al.* (1997) and Chen and Ruffner (1996) with minor modifications.
7. PCR products with only the specific target band were also purified using ethanol precipitation as described by Huang and Cloutier (2007).
8. Purified PCR products were sequenced based on the dideoxy chain termination method of Sanger *et al.* (1977).
9. Binary data obtained for the TE-AFLP profiles were analysed using four statistical parameters i.e. PIC (Polymorphic Information Content), Marker index (MI), Effective multiplex ratio (EMR) and Resolving power (Rp) for

evaluating the discriminating power and the information content of the selected 5 primer combinations.

10. Linkage analysis was performed using all polymorphic markers by observing parental segregation patterns separately using JoinMap 4.0 software (van Ooijen 2006).
11. The correlation between the number of AFLP markers and the length (size) of the linkage groups was analysed using the Pearson correlation coefficient.
12. Two methods were used to estimate genome length: Fishman *et al.* (2001) and method 4 as described by Chakravarti *et al.* (1991).
13. Nucleotide sequences obtained were queried (BLASTn) individually with three different databases on NCBI GenBank (National Center for Biotechnology Information).
14. Open Reading Frame (ORF) for some selected sequences were determined using ORF finder (NCBI).

The results obtained in the present study can be summarised as follows:

1. A 'narrow-down' strategy for the primer combination was beneficial in choosing the optimised TE-AFLP primer combinations. At the final step, 5 out of 36 primer combinations which were highly polymorphic in the tea sample collected were chosen for further studies.
2. There was a lack of correlation among the statistical parameters taken for our study i.e. PIC, MI and Rp indicating that a single parameter was not sufficient to assess the informativeness of a primer combination.

3. Genetic load and the scoring error in AFLP were likely the contributing factors to the segregation distortion observed in our study. However, the exact factor is not known.
4. The map of the TV1 parent consisted of 36 markers in ten linkage groups while TV19 parent consisted of 30 markers in eleven linkage groups. The number of linkage groups obtained in this study is less than the haploid chromosome number (n=15). This discrepancy in the number is due to the low density of markers.
5. Observed genome lengths were calculated as 388.9 cM and 410.7 cM for TV1 and TV19 maps respectively.
6. The observed genome coverage was determined as 50.76% and 45.91% for TV1 and TV19 respectively.
7. The existence of minor linkage groups and unlinked markers (25 markers in TV1 parent and 9 markers in TV19 parent) indicates that there are many large gaps with few markers.
8. The large intervals (> 20cM), observed in both the maps is due to the low-density of the maps obtained.
9. In our study, the bridging marker are clustered among them and not linked with any markers specific to either parents (1:1 markers) which was uninformative for merging linkage groups and thus no integrated map could be developed.
10. The Pearson correlation coefficient (r = 0.91 and r = 0.83) indicated that there was a highly significant positive correlation between the number of markers and linkage group size. This supports the contention that the

distribution of AFLP markers was relatively even in linkage groups of TV1 and TV19 map.

11. BLAST analyses showed that the AFLP marker sequences for functional genes appeared to be relatively less compared to the non coding regions.
12. However, some sequences showed homology with unigenes which were related to nitrogen assimilation, drought, pathogen resistance, heavy-metal stress tolerance, and resistance to biotic and abiotic stresses. The identification of such markers is an important resource for further research aimed at understanding physiological processes important for tea cultivation and quality.

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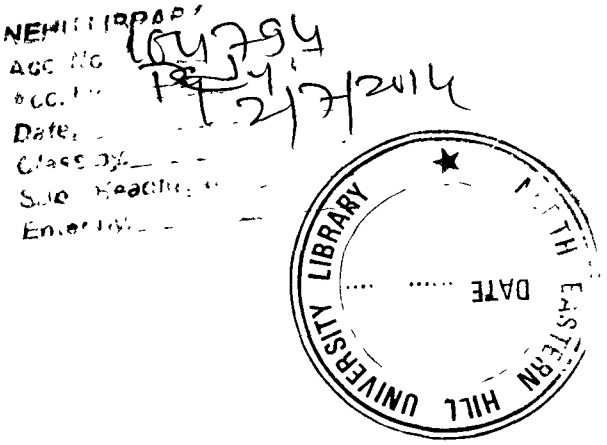

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Dedicated to my Parents

The North-Eastern Hill University

October, 2013

DECLARATION

I, Asem Mipeshwaree Devi, hereby declare that the subject matter of this thesis entitled "**Studies on Amplified Fragment Length Polymorphism in tea (*Camellia* species) clones**" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the North-Eastern Hill University for the award of the degree of Doctor of Philosophy in Botany.

Asem Mipeshwaree Devi

(Asem Mipeshwaree Devi)

31/10/13

(Head of Department) **(Supervisor)** **(Joint Supervisor)**

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Asem Mipeshwaree Devi

(Asem Mipeshwaree Devi)

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
$AgNO_3$	Silver Nitrate
ALPs	*Amplicon Length Polymorphisms*
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
BLASTn	BLAST nucleotide database
BLASTp	BLAST protein databases
bp	base pairs
BSA	Bovine Serum Albumin
χ^2	Chi-square
^oC	degree Celsius
CAPS	Cleaved Amplified Polymorphic Sequences
cDNA	complementary DNA
CHD	Chromodomain Helicase DNA binding protein
cM	centimorgan
cm	centimetre
CP	cross-pollinated
cpDNA	chloroplast DNA
CRE	cytokinin receptor
CRK	Cysteine-rich receptor-like protein kinase
CTAB	cetyltrimethyl ammonium bromide

DCP	Decapping
df	degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EC	epicatechin
ECG	epicatechin gallate
EDTA	Ethylene diamine tetraacetic acid
EGC	epigallocatechin
EGCG	epigallocatechin gallate
EMR	Effective Multiplex Ratio
EST	Expressed Sequence Tag
EST-SSR	EST-based Simple Sequence Repeats
et al.	and others
e-value	expectation value
f	frequency of the marker
F_1 (or F1)	first familial generation (progeny)
G_e	average estimated genome length
G_o	total length of map observed
GTDS	green tea dietary supplements
HIV	Human Immunodeficiency Virus
H_o	Null Hypothesis
I_b	band informativeness
IPC	integral plate chamber
ISSR	Inter Simple Sequence Repeats

Kbp	kilobasepair
L	Litre
LG	Linkage Group
LOD	Log of the odds
µg	microgram
µL	microlitre
µM	Micromolar
M	Molar
MAS	marker-assisted selection
Mbp	megabasepair
mg	milligram
$MgCl_2$	Magnesium Chloride
MI	Marker Index
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger Ribonucleic acid
n (or *x*)	basic chromosome number
Na_2CO_3	Sodium bicarbonate
NaCl	Sodium chloride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADPH-DO	NADPH-dependent oxidoreductase
NCBI	National Center for Biotechnology Information

ng	nanogram
nr/nt	non redundant nucleotide
ORF	Open Reading Frame
%	percentage
P	Probability value
PAL	Phenylalanine ammonia lyase
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR-based Restriction Fragment Length Polymorphism
PIC	Polymorphic Information Content
PIMA	PCR-based isolation of microsatellite arrays
pg	picogram
pH	concentration of hydrogen ions in a solution
pmol	picomole
PVP	Polyvinyl pyrrolidone
QTL	Quantitative trait loci
r	Pearson correlation coefficient
RAPD	Randomly Amplified Polymorphic DNA
REC (Θ)	Recombination frequency
RFLP	Restriction Fragment Length Polymorphism
RLK	Receptor-like protein kinase
Rp	Resolving power
rpm	revolutions per minute
$\sum$	Summation
s	Second

SA	Salicylic Acid
SDL	Segregation Distortion Loci
SDS	Sodium dodecylsulphate
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
STS	Sequence Tag Sites
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA buffer
TE	Tris-EDTA buffer
TE-AFLP	Three Endonuclease-Amplified Fragment Length Polymorphism
TEMED	N,N,N`,N`-tetramethyl ethylenediamine
Tm	melting temperature
TRA	Tea Research Association
Tris-HCl	Tris-Hydrochloride
TS	Tocklai Seed Stock
TV	Tocklai Variety
U	enzyme Unit
UGMS	Unigene derived microsatellite
UPASI	The United Planters` Association of Southern India
UPGMA	Unweighted Pair Group Method Analysis
VNTR	Variable Numbers of Tandem Repeats
WAK	Wall-associated receptor kinase
WGS	Whole Genome Shotgun Sequence
w/v	weight per volume

Chapter 1

Introduction

CHAPTER 1

INTRODUCTION

Over the years, advances in molecular biology have led to the introduction of several types of molecular markers that reveal variation at the DNA sequence level. Molecular markers are considered as the constant landmarks in the genome which are transmitted by the standard laws of inheritance from one generation to the next. The analysis of genomic variation is an essential part of plant genetics and crop improvement programmes. The thrust areas for plant improvement programmes include improvement in sustainable yield, quality; resistance to diseases, pests and nematodes; and tolerance to abiotic stresses such as drought, heavy metals, salt and temperature.

Cultivated tea belongs to the genus *Camellia* of family Theaceae. It is a woody, cross-pollinated, perennial plantation crop with long gestation periods, high inbreeding depression and wide heterogeneity present in the existing seedling population. Therefore, conventional breeding takes several years to develop important agronomic traits. Vegetative propagation of superior clones has been a method of choice in tea industry to overcome the costs and problems related with conventional breeding. Consequently well defined clones and bi-clonal tea population with specific agronomic traits have been developed that have higher productivity and better quality.

World's most important non-alcoholic caffeine containing beverage is in fact the young processed leaves and leaf buds of tea plant. Tea leaves contain more than 700 chemicals, among which the compounds closely related to human health are flavanoides, amino acids, vitamins (C, E and K), caffeine and polysaccharides. In addition to its use as a beverage, it has medicinal properties and is a potential raw material source for pharmaceutical industry (Chen *et al.* 1999). Tea is gaining popularity as an important health drink, and the production of high quality tea with regional characteristics has remained a highly profitable business. It is an important crop for the world socio-economy, providing livelihood to millions of people worldwide and a source of revenue for the producing countries. In this context, DNA markers/technologies that provide high-genetic resolution become important and thus need to be integrated in research on genetics and improvement of tea.

Progress in crop improvement has been reached by manipulating the genetic variation within this plantation crop. Currently, DNA markers have proved valuable in the studies of genetic diversity and variation, molecular identification, molecular phylogenetic, genetic stability and integrity of tea germplasm and the genetic linkage map for breeding of tea. Several molecular markers such as Restriction Fragment Length Polymorphism (RFLP, Matsumoto *et al.* 2004), Randomly Amplified Polymorphic DNA (RAPD, Roy and Chakraborty 2009), Cleaved Amplified Polymorphic Sequences (CAPS, Elangbam 2012), Amplified Fragment Length Polymorphism (AFLP, Sharma *et al.* 2010), Amplicon Length Polymorphisms (ALPs, Kaundun and Matsumoto 2004), Simple Sequence Repeats (SSR, Yuan *et al.* 2010), and Inter Simple Sequence Repeats (ISSR, Liu BY *et al.* 2012)

al. 2012b) have been developed and widely applied in tea plant research. Recent developments in DNA marker technology together with the concept of marker-assisted selection provide new solutions for selecting and maintaining desirable genotypes.

The construction of detailed genetic maps with high levels of genome coverage is a first step for some of the applications of molecular markers in plant breeding (Tanksley *et al.* 1989). It is critical that sufficient polymorphism exists between parents in order to construct a linkage map. Naturally outcrossing species tend to have high levels of DNA polymorphism and virtually any cross that does not involve related individuals will provide sufficient polymorphism for mapping (Helentjaris 1987). Two-way pseudo-testcross strategy has been widely used to construct linkage maps for populations having two heterozygous parents. Since tea is an out-crossing species, this strategy is a suitable approach for linkage map construction. Many linkage maps of tea based on pseudo-testcross strategy have also been developed (Tanaka 1996; Hackett *et al.* 2000; Kamunya *et al.* 2010; Taniguchi *et al* 2012).

Genetic mapping is based on the principle that genes (markers or loci) assort via chromosome recombination during meiosis (i.e. sexual reproduction), thus allowing their analysis in the progeny (Paterson 1996). During meiosis, chromosomes assort randomly into gametes, such that the segregation of alleles of one gene is independent of alleles of another gene. This is stated in Mendel's second law and is known as the law of independent assortment. The chance of a crossover producing recombination between genes is directly related to the distance between two genes - the lower the frequency of recombination between two markers, the closer they are

situated on a chromosome. Therefore, the genetic markers for the linkage map must have a Mendelian segregation, be polymorphic, and be easy to follow in each individual. Genetic maps have been constructed in many crop plants using various molecular markers. Polymorphism in the nucleotide sequence usually is sufficient for it to function as a molecular marker in mapping. Linkage analyses were performed manually for a few markers but it is not feasible to manually analyse and determine linkages between large numbers of markers that are used to construct maps. So, this task requires the analytical power of a computer and software packages are available to carry out this task. Commonly used software programmes include Mapmaker/ EXP (Lander *et al.* 1987; Lincoln *et al.* 1993), CRI-MAP (Donis-Keller *et al.* 1987; Green *et al.* 1989), MapManager QTX (Manly *et al.* 2001) and JoinMap (Stam 1993, van Ooijen 2006).

To establish a genetic map with a marker technique that could identify many polymorphic loci that are dispersed throughout the genome will be an effective approach to analyse the agronomically important traits. Once markers closely linked to desirable traits for marker assisted selection are identified, it would be a great help in early screening and other crop improvement programmes of this plantation crop. Molecular markers such as RFLP, RAPD, AFLP and VNTR are used to map entire genome as they reveal polymorphism.

Among the DNA markers, AFLP is an ideal approach as it is relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers. This technique could identify high polymorphisms with high reproducibility, rapid generation and they generally provide good genome coverage without prior sequence

information. Therefore, AFLP markers play significant role in the programme of plant improvement as the vast amount of genetic variation present in the germplasm can be exploited to the best through this marker system.

However, species with a large genome size generate more AFLP amplification products than species with smaller genomes (Vos *et al.* 1995) and the analysis of a large number of bands thus generated becomes difficult. Modifications of conventional AFLP for characterization related to large genome size were reported to solve this problem. In TE-AFLP (Three Endonuclease-AFLP) method (van der Wurff *et al.* 2000), incorporation of a third endonuclease in the usual AFLP protocol provides a high discriminatory power and reduction in the number of bands. The latter makes it especially suitable for the analysis of complex genomes making the three endonucleases (TE-AFLP) more efficacious for DNA fingerprinting.

Tea has a genome size of $4x10^9$ bp (Tanaka *et al.* 2006) with a basic chromosome number of 15. TE-AFLP method was, therefore, expected to be more suitable for the study of genetic variation in this plant. Due to the reduction in the number of amplified fragments, TE-AFLP profiles would be clearer and easier to score while retaining the ability to generate highly reliable multilocus information with high level of polymorphism and reproducibility and without the need for prior sequence information of the genome. This marker technique which can provide the information of an entire genome will also provide benefits to select improved traits in tea.

For the visualization of AFLP products in polyacrylamide gels, silver staining has similar sensitivity and resolution as with the autoradiography. It also reduces the

time, cost and eliminates hazards of working with radioisotopes (Vantoai *et al.* 1996, Chalhoub *et al.* 1997). In addition, the high resolution and recovery of fragments from the dried gels makes silver staining a more useful and versatile detection method for AFLP amplification products.

Recently some linkage maps have been developed in tea plant using different DNA marker techniques such as RAPD, AFLP, ISSR, CAPS, STS, and SSR. However there is a need to focus on the correlation with functional genomics and proteomics. These constructed maps were still limited to locate QTLs linked with some important traits and other agronomically important traits. As a result, no practical genetic map is available. Further efforts are required to construct a high density map using different molecular markers and to integrate economically important traits onto the map for genetic improvement of tea. Although a practical genetic map is not available for the tea plant right now, the potential of markers assisted selection is clear and strong (Chen *et al.* 2006).

With the emergence of molecular markers, genotyping technologies and related statistical methodologies, identification of markers associated with agronomically important traits becomes possible and thereby offers an opportunity for plant breeders to apply it in marker assisted selection (MAS). Some major challenges in tea production facing today include improvement of the quality, yield and abiotic and biotic stress tolerance. The two most important agronomic traits in tea are yield and quality. It is therefore, necessary to focus on caffeine, flavanols and catechins related genes as quality markers and leaf parameters and shoot density controlling genes as yield markers in tea. Drought and cold susceptibility in tea plant are some

of the abiotic stresses that limits tea yield drastically while biotic stresses which causes damage to the tea plantations include pests (e.g. *Helopeltis species, Andraca species)* and diseases (e.g. blister blight, black rot). The publicly available EST sequence data in the NCBI database (Sharma and Kumar 2005; Park *et al.* 2004; Chen *et al.* 2006), the identification and validation of new Unigene derived microsatellite (UGMS) markers (Sharma *et al.* 2009) and large transcriptome dataset analyses in tea that revealed a large number of candidate genes for major metabolic pathways of tea-specific compounds (Shi *et al.* 2011) are the major breakthroughs in the sequence analyses studies of tea. The success on identifying important genes in turn can serve as important information on gene expression, genomics and functional genomics studies in tea. These would also serve as efficient screening tools for quantitative traits and selection of desirable segregating progenies in a breeding programme, thus leading to consumption of less time and lesser physical and financial resources.

The genetic map is an important tool to study the genome structure and to detect and localise markers which then help to identify genes. Eventually, distances between DNA markers in linkage map need to be described not only by recombination frequency, but by actual physical distance. As mapping techniques advanced, AFLP markers were also sequenced for purposes of physical mapping to some extent and at least in some cases, to generate codominant marker systems. This approach could identify markers closely linked to desirable traits which are a prerequisite for the application of MAS breeding programmes in tea. Development of important traits took several years so selection based on molecular markers linked to a quantitative

trait locus (QTL) could potentially help in early screening. Therefore, there is a strong case of a cheap, reliable, informative silver stained TE-AFLP marker studies on a mapping population of tea for obtaining a possible genetic linkage map and if possible to locate some traits that are related to tea production.

With this background, the present study was designed with the following objectives:

1. Identification of TE-AFLP markers using bi-clonal seed stocks which are maintained at Tea Research Association (TRA), Tocklai, Jorhat.
2. Construction of a genetic linkage map based on the above TE-AFLP markers.
3. Nucleotide sequencing and analysis of selected TE-AFLP based amplicons.

Chapter 2

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2.1. TEA

2.1.1. Tea taxonomy

Tea is a woody evergreen plant of the genus *Camellia* in the Theaceae family. Cultivated tea consists of three species each with specific plant type viz. *Camellia sinensis* (China type), *Camellia assamica* (Assam type) and *Camellia assamica* sub sp. *lasiocalyx* (Cambod type) (Wight 1962). The classifications of these cultivars was initially proposed by Sealy (1958) based on leaf characteristics. This classification was revised by Wight (1962) on the basis of morphological characters such as leaf size, leaf shape, length of pistil and flower sizes (Takeda and Toyao 1980). Based on this, a bush with small leaves, resistant to cold are characterized as China type, while Assam type are tall with large leaves (Sealy 1958) and Cambod type are intermediate between the China and Assam type (Kingdom-Ward 1950; Robert *et al.* 1958). However, differences in caffeine, flavanols and amino acids contents in leaves of cultivated tea were also used for the classification (Nagata and Sakai 1984).

2.1.2. Tea origin

Southeast Asia is the original home for tea. According to Wight (1959), the primary centre of origin of tea is considered to be around the point of intersection of latitude 29°N and longitude 98°E near the source of the river of Irrawaddy, the point of

confluence where lands of Assam, North Myanmar, southwest China and Tibet meet. Secondary centres of origin are considered to be located in southeast China, Mizoram and Meghalaya (Kingdon-Ward 1950). The above areas are, therefore, considered to be the zone of origin and dispersion of the genus *Camellia* as a whole (Sealy 1958). However, presently tea cultivation is spread within the latitudinal range of 45°N–34°S.

2.1.3. Genome Size and Diversity

The genome size in terms of 4C DNA amount for *Camellia sinensis* is 15.61±1.06 pg where 1C DNA is equal to 3824 mega base pair (Mbp) and 1 pg = 980 Mbp (Hanson *et al.* 2001). Generally, tea chromosomes are small and tend to clump together due to 'stickiness'. Tea is diploid ($2n = 2x = 30$; where basic chromosome number $x = 15$) (Morinaga *et al.* 1929). Chromosome lengths range between 1.28μm to 3.44μm (Bezbaruah 1971). The *r* value (ratio of long arm to short arm) for all the 15 pairs of chromosomes ranges from 1.00 to 1.91. This consistency in diploid chromosome number suggests a monophyletic origin for all *Camellia* species. However, few higher ploidy levels, such as triploids, for example, TV-29, HS-10 A, UPASI-3, UPASI-20 ($2n = 3x = 45$), tetraploids ($2n = 4x = 60$), pentaploids ($2n = 5x = 75$) and aneuploids ($2n = 2x\pm1$ to 29) have also been identified (Singh 1980; Zhan *et al.* 1987).

Eighty two species of the genus *Camellia* had been described till 1958 (Sealy 1958). Currently more than 325 species have been described (Mondal 2002a). This may indicate genetic instability and high out-breeding nature of the genus. Presently, world-wide over 600 cultivated varieties are available, of which many have unique

traits such as high caffeine content, blister blight disease tolerant, etc. (Mondal 2009).

2.1.4. Tea clones and bi-clonal stocks

The first scientific attempt to select improved tea in North-East India was made by Stiefelhagen brothers in 1860 by establishing standard sources of tea seeds. Indigenous Assam tea was improved by following the technique of mass selection. The yield increased considerably, because of line breeding for desirable morphological features that are genetically linked with the characteristics of Assam tea. However, the seed grown plants were not uniform and unpredictable as their characters were governed by the genotype of their parents. Therefore, it was felt necessary to develop clonal cultivars in tea (Mondal 2009).

Secondly, with the increase of the region specific need of the industry, almost all tea producing countries have developed their clones or seed stocks. In India, Tea Research Association (TRA), Tocklai, Jorhat released the first three clones i.e. TV1, TV2 and TV3 in 1949. Development of bi-clonal seed stocks was also initiated in the late 1970s and early 1980s. Over the years, 32 clones and 14 bi-clonal seed stocks had been developed in TRA. In South India, the breeding work at UPASI (The United Planters' Association of Southern India), Tamil Nadu has resulted in the release of 28 clones and 5 bi-clonal seed stocks.

Clonal selection is an important and widely adopted method of tea plant improvement because of wide heterogeneity in the existing seedling population (Barua 1963; Shanmugarajah 1994). Clones are genetically uniform and give uniform yield and quality. Genetic stability of clones is vital for tea germplasm

preservation, breeding and production. However, clonal degeneration, a gradual loss of vigour and yield with age of a variety is also a well known phenomenon in vegetatively propagated crops (Forbes and Watson 1992). The increase in clonal plantation and continuous crossbreeding with small selection of superior trees can lead to a reduction in the gene pool of tea. So it is necessary to study the worldwide distributed genetic diversities of tea to overcome future problems associated with narrowness of genetic base of the modern tea cultivars.

2.1.5. Tea as beverage

The custom of brewing leaves of the tea plant for a beverage has its origin in China, with numerous records dating back more than 2,000 years (Tanaka and Taniguchi 2007). Based on the fermentation process, tea are divided broadly into three types: deeply fermented tea (black tea), semi-fermented tea (oolong tea) and non-fermented tea (green tea). Approximately 76-78% of the tea produced and consumed worldwide is black tea, 20-22% is green tea, and <2% is oolong tea (Mukhtar *et al.* 1994; Stoner *et al.* 1995; Bushman 1998; Cabrera *et al.* 2003). Green tea is mainly produced and consumed in East Asia and recently has gained attention as a healthy beverage (Higdon and Frei 2003; Crespy and Williamson 2004; Harada *et al.* 2005) in regions such as the United States and European countries, where green tea was not previously popular (Ujihara *et al.* 2011). Planted area totals about 2.3 million hectares, with China, India, Sri Lanka, Kenya, and Indonesia being the major producers. Major importers include the UK, Russia, Pakistan, the USA, Egypt, and Japan. Black tea accounts for about 70% of the world production, and green tea for most of the remainder (Tanaka and Taniguchi 2007).

2.1.6. Importance of Tea and uses

The economic importance of the genus *Camellia* is attributed primarily to tea. Tea was used initially as medicine, later as beverage and has a proven future potential of becoming an important industrial and pharmaceutical raw material. Many studies have authenticated many beneficial claims of tea, majority of which are attributed to its polyphenolic constituents. There are more than 700 constituent chemicals in tea leaves (Chen 1999). Among them, tea polyphenols and flavonoids have been reported to have strong antioxidant activity (Wiseman *et al.* 1977; Allemain 1999) which is responsible for most of the beneficial effects of tea. Among tea the polyphenols, and in particular catechins, have received immense attention (Cabrera *et al.* 2003). The major green tea catechins are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). EGCG makes up about 40% of the total catechin content and is widely accepted as the major antioxidant ingredient in green tea and commercial green tea dietary supplements (GTDS) (Stoner *et al.* 1995; Cabrera *et al.* 2003).

Tea polyphenols inhibit the absorption of dietary fats and cholesterols (Chen *et al.* 2000). Several experimental evidences point to the potential of tea to protect against cancer. Especially green tea reduces the incidence of cancers of the stomach, small intestine, pancreas, lung, breast, skin, urinary bladder, prostrate, oesophagus and mouth (Vasisht *et al.* 2003). Green tea drinking also has been shown to possess anti-diabetic activity (Gomes *et al.* 1995), anti-arthritic activity (Tapiero *et al.* 2002), anti-plaque activity (Yu *et al.* 1995), antiviral activity (Okubo and Juneja 1997), anti-HIV (Human Immunodeficiency Virus) activity (Hashimoto *et al.* 1996), anorectic

effect (Kwanashie *et al.* 1989) and anti-microbial activity (Hamilton-Miller 1995). It has also been reported that green tea polyphenols exhibit neuromuscular, anti-angiogenic, anti-hepatotoxic, anti-proliferative/apoptotic and immunomodulatory effects (Sueoka *et al.* 2001). Recent studies also showed consumption of green tea had inhibitory effects on cancerous cells of breast (Wu and Butler 2011), oesophagus and lungs (Yuan 2011) and ovaries (Lee *et al.* 2012).

Besides these properties, it is a dietary source of various important constituents among which flavanoids, amino acids, Vitamins (C, E and K), folic acid, Manganese, Potassium, Fluorides and polysaccharides which are important to human health (Chen 1999). Tea is gaining popularity as an important health drink in view of the above properties. The production of high quality tea with regional characteristics has nevertheless remained a highly profitable business. Tea plays a pivotal role in the national economy, a source of revenue and the job opportunities for almost all the producing countries.

2.2. DNA MARKERS AND THEIR APPLICATIONS IN TEA

In the recent years, with the remarkable advances in DNA based molecular markers techniques, several molecular markers, such as RFLP (Restriction Fragment of Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), CAPS (Cleaved Amplified Polymorphic Sequence), ISSR (Inter Simple Sequence Repeat), SSR (Simple Sequence Repeat), EST-SSR (Expressed Sequence Tags based SSR), and ALPs (Amplicon Length Polymorphisms), etc. have been developed and widely applied in the tea plant.

Tea is highly heterogenous, highly cross-pollinated as well as freely out breeding with wild relatives in nature. Consequently a broad genetic variation exists in the cultivated tea gene pool. Some of which have valuable potential for tea industry in future. Therefore, many studies have been focused on estimation of genetic relationship and determination of genetic diversity of the tea germplasms using different molecular markers.

2.2.1. Restriction Fragment Length Polymorphism (RFLP)

Matsumoto *et al.* (1994) employed an RFLP analysis that used Phenylalanine ammonia-lyase sequence (PAL) cDNA as a DNA probe to determine the existence of PAL as a single gene and to study difference between Chinese and Japanese tea. Later, Matsumoto *et al.* (2002) used PAL marker as a tool for the classification and evaluation of tea resource. Matsumoto *et al.* (2004) were the first to assess genetic diversity with RFLP using phenylalanine ammonia-lyase sequence.

2.2.2. Random Amplified Polymorphic DNA (RAPD)

Since the first report on RAPD markers in Kenyan tea germplasm (Wachira *et al* 1995), they have been used to analyze phylogenetic relationships amongst tea genotypes in different regions, including India (Mondal 2000), Japan (Tanaka and Yamaguchi 1996; Chen and Yamaguchi 2002, 2005), China (Chen 1998, Chen *et al.* 1998, 1999, 2002a, b), South Africa (Wright *et al.* 1996), Taiwan (Lai *et al.* 2001), South Korea (Kaundun *et al.* 2000; Kaundun and Park 2002), Portugal (Jorge *et al.* 2003) and Pakistan (Gul *et al.* 2007). Roy and Chakravorty (2010) revealed genetic diversity and relationships among tea (*Camellia sinensis)* cultivar by using RAPD

and ISSR based markers. Recently, Afridi *et al.* (2011) estimated genetic diversity in tea genotypes cultivated in Pakistan using RAPD markers.

2.2.3. Cleaved Amplified Polymorphic Sequence (CAPS)

Based on the sequence information of previously characterized tea genes, such as phenylalanine ammonia-lyase (PAL), chalcone synthase and dihydroflavonol 4-reductase, Kaundun and Matsumoto (2003) reported CAPS markers for the analysis of 52 tea samples of diverse origin. CAPS markers based on the above three genes were also studied for comparative analysis of Korean and Japanese green tea trees (Cho *et al* 2010). Expressed Sequence Tag (EST)-based Cleaved Amplified Polymorphic Sequence (CAPS) markers were also developed in tea plant for cultivar identification (Ujihara *et al.* 2011). Recently, genetic diversity among 30 tea cultivars in Sichuan province of China was investigated by PCR-RFLP analysis of cpDNA using 7 sets of chloroplast primers (Chen *et al.* 2012). CAPS marker for the two genes, phenylalanine ammonia-lyase (PAL) and chalcone synthase 2 (CHS2) were also developed which were related to the catechin content in tea (Elangbam 2012).

2.2.4. Inter Simple Sequence Repeats (ISSR)

Twenty-five diverse Indian tea were analyzed using ISSR markers. A dendogram was constructed using the unweighted pair group method analysis (UPGMA) method and revealed three distinct clusters of Cambod, Assam and China type, which concur with the known taxonomical classification of tea (Mondal 2002b). ISSR markers have also been used to analyze the genetic diversity of 27 Taiwanese tea cultivars (Lai *et al.* 2001), tea plants from Yunnan province of China (Liu *et al.* 2010) and

molecular identification of tea cultivars (Yao *et al.* 2005). The discrimination of tea germplasm at the inter-specific level was analyzed using ISSR markers (Liu B *et al.* 2012).

2.2.5 Simple Sequence Repeats (SSR)

Freeman *et al.* (2004) developed 15 SSR primers which revealed a great variability across a wide range of tea clones. Isolation and characterisation of 11 microsatellite loci from *Camellia sinensis* in Taiwan had been done using PCR- based isolation of microsatellite arrays (PIMA) (Hung *et al.* 2008). Recently, genetic diversity and relationship of clonal tea (*Camellia sinensis)* cultivars in China was revealed using SSR markers (Fang *et al.* 2012).

2.2.6. Expressed Sequence Tags based SSR (EST-SSR)

Zhao *et al.* (2007) generated 24 novel EST derived microsatellites from tea plant (*Camellia sinensis*). Microsatellite markers developed from genomic libraries can belong to the transcribed region or the non transcribed region of the genome. Over the past few years, various EST projects and studies (Sharma and Kumar 2005; Park *et al.* 2004; Chen *et al.* 2006) have generated publicly available EST sequence data in tea. Sharma *et al.* (2009) predicted 1,223 unigenes from 2,181 expressed sequence tags (ESTs) of tea (*Camellia sinensis* L.) which were available in publicly available sequence database. Ninety six primer pairs could be designed from 83.5% of SSR containing unigenes. Of these, 61 (63.5%) primer pairs were experimentally validated and used to investigate the genetic diversity among the 34 accessions of different *Camellia* spp. Seventy four novel polymorphic EST-SSR markers in tea plant were also identified and characterised by Ma *et al.* (2010). Recently, genetic

distribution of China tea germplasm was revealed by EST-SSR markers (Yao *et al.* 2012).

2.2.7. Amplified Fragment Length Polymorphism (AFLP)

Paul and co-workers (1997) were the first to employ AFLP markers to detect genetic diversity and differentiation of different Indian and Kenyan tea clones. Later, AFLP markers were also employed to study genetic variation amongst 49 different south Indian (Balasaravanan *et al.* 2003) and 27 Darjeeling tea cultivars (Mishra and Sen-Mandi 2001), and the phylogenetic relationship among tea cultivars from South Korea (Lee *et al.* 2003) and Japan (Wachira *et al.* 2001). Polymorphism and genetic relations among tea genotypes from turkey were also revealed by AFLP markers (Kafkas *et al.* 2009). Recently, Sharma *et al.* (2010) studied genetic diversity of commercially important tea germplasm in India using seven AFLP primer combinations.

2.3. GENETIC LINKAGE MAP

A mapping project is often started with the objective to detect linkage between one or several markers and a trait of interest. To achieve the ultimate goal of genetic improvement of tea, further efforts are required to construct a high density map using informative DNA markers and to locate the quantitative trait loci (QTL) of important agronomic traits, quality and resistance, showing bright prospects in tea breeding through marker assisted selection to integrate economically important traits onto the linkage map. Although the density of integrated genetic maps can be extremely high, it may still not be sufficient to tag a specific gene.

Many software packages were developed for linkage maps e.g. MAPMAKER (Lander *et al.* 1987; Lincoln *et al.* 1993), JOINMAP (Stam 1993; van Ooijen 2006), CRIMAP MAP (Donis-Keller *et al* 1987; Green *et al.* 1989), MapManager QTX (Manly *et al* 2001), GMENDEL (Liu and Knapp 1990), ANTMAP (Iwata and Ninomiya 2006), etc. Genetic maps have been constructed for several species using various markers like RFLP, RAPD, STS, microsatellites, proteins and recently AFLP markers. Over the years, the number of linkage maps based on AFLP has strongly increased. AFLPs are widely used owing to the large amount of detected polymorphism and the fact that sequence information is not required which is further facilitated by low cost per marker (Schlotterer 2004).

Advantages of AFLP

Since AFLP can generate many polymorphic bands without prior sequence knowledge, it is powerful technique for generating linkage maps. The technique has the following advantages:

- The markers produced are reliable and reproducible within and between the laboratories.
- The AFLP technique can be used for DNA samples of any origin or complexity. Small sequence variations can be detected using only small quantities of genomic DNA (0.05–0.5 mg).
- The capacity to reveal many polymorphic bands in an experiment (high multiplex ratio) is a major advantage of AFLP markers.
- They segregate in a Mendelian fashion and can be used for population genetic and QTL analyses.

- The error level is very low as AFLP amplifications are performed under conditions of high selectivity (at high stringency) (Vos *et al.* 1995; Mueller and Wolfenbarger 1999).

Modified AFLP methods have also been developed for other species in recent years (Suazo and Hall 1999; Lindstedt *et al.* 2000; Ranamukhaarachchi *et al.* 2000; James *et al.* 2003; Gaafar *et al.* 2003; Kazachkova *et al.* 2004; Masumu *et al.* 2006; Giammanco *et al.* 2007; Esteve-Zarzoso *et al.* 2010; Sharma *et al.* 2011). Seonha *et al.* (2003) used a modified AFLP technique to study the phylogenetic relationship among 37 accessions of the genus *Camellia* consisting of Japanese tea, Korean tea and some *Camellia* species closely related to tea.

Although, AFLP is an important marker that detects high polymorphism but in large genomes (10^8-10^{10}bp) (Blears *et al.* 1998) due to generation of large number of bands the analyses were more cumbersome. A modification of conventional AFLP, TE-AFLP (Three Endonuclease-AFLP) method (van der Wurff *et al.* 2000) provides high discriminatory power and reduction in the number of bands. So, it would be suitable for tea genome which was estimated to be 4.0×10^9 bp.

Negi *et al.* (2005) did a comparative study of three different AFLP based methods and they concluded that TE-AFLP was the best technique for fingerprinting of tea. Recently, TE-AFLP has been successfully used for assessment of genetic diversity of biodiesel species *Pongamia pinnata* accessions (Sharma *et al.* 2011). It had also concluded that the easy scorability of TE-AFLP profiles is desired in studies requiring genotyping of large number of individuals across many gels.

2.3.1. Linkage maps in tea

A first linkage map for tea plant was constructed with RAPD markers by Tanaka (1996) and the markers related with theanine content, date of bud sprouting, resistance to anthracnose and tolerance to cold were detected (Tanaka 1996).

Another linkage map from the female parent, SFS150, was established with RAPD and AFLP markers (Hackett *et al.* 2000). There were 126 markers, covering 1349.7 cM, with an average distance of 11.7 cM between loci on the map.

An AFLP linkage map for tea plant was also constructed in China. The map of a female parent included 17 linkage groups and contained 208 markers, covering a total length of 2457.7 cM. The average distance between markers was 11.9 cM. A map from male parent included 16 linkage groups and located 200 markers, covering a total length of 2545.3 cM, and the average distance between markers was 12.8 cM (Huang *et al.* 2005).

A partial genetic map of backcross F_1 generation between 'Zhenong 129 (selected from the open pollination of 'Fuding Dabaicha'x'Yunnna Dayecha') and 'Fuding Dabaicha' was also generated using RAPD and ISSR markers (Huang *et al.* 2006). However, in previous studies, the number of individuals for mapping was limited and the density is not high enough to meet the demand of precise mapping.

Kamunya *et al.* (2010) used forty seven primers (21 RAPD primers, 20 AFLP and six SSR primer pairs) for complete genotyping of the *Camellia sinensis*. 260 informative markers were generated, out of which 100 markers that showed 1:1 segregation were used to construct a linkage map. The map contained 30 (19

maternal and 11 paternal) linkage groups that spanned 1,411.5 cM with mean interval of 14.1 cM between loci.

Recently, a high reference combined map was developed in a population of 54 F_1 clones derived from reciprocal crosses between 'Sayamakaori' and 'Kana-Ck17'. The parental maps contain 441 SSRs, 7 CAPS, 2 STS and 674 RAPDs. The core map contains 15 linkage groups that covered a total length of 1218 cM (Taniguchi *et al.* 2012).

These constructed maps were still limited to locate QTLs linked with some important traits due to their low distribution of molecular markers.

2.4. SEQUENCING IN TEA

In plants, markers were sequenced for purposes of physical mapping. So, BLAST searches were performed to identify homologous sequences from the public databases. Identification of important markers would facilitate the gene mapping and marker aided selection in tea. The identification and validation of 61 new Unigene derived microsatellite (UGMS) markers from publicly available sequence database and 1,223 unigenes were predicted from 2,181expressed sequence tags (ESTs) of tea. Out of 61 UGMS markers identified and validated, 36 of these UGMS markers correspond to the *Arabidopsis* protein sequence data with known functions (Sharma *et al.* 2009). This will have a major impact on genetic analysis, gene mapping and marker assisted breeding.

Recent advances in large-scale RNA sequencing (2.59 gigabase pairs of the transcriptome from poly(A)$^+$ RNA of *C. sinensis*) was analysed using high

throughput Illumina RNA-seq approach by Shi *et al.* (2011) to generate large expression datasets for functional genomics. This analysis obtained 127,094 unigenes, which consisted of 788 contig clusters and 126,306 singletons. The category of secondary metabolism related genes covered 2.7% (427 of the functional genes) of the total genes identified, out of which thirteen unigenes related to theanine and flavonoid synthesis and were validated. Whole genome sequencing of tea had not been done but four *Camellia* cDNA libraries were available in *Camellia* ESTs in GenBank, including the EST sequences from the young root cDNA library of the tea plant (Shi and Wan 2009) (GenBank accession: GE652554.1-FE861258.1), two reported *C. sinensis* cDNA library respectively named subtractive cDNA library special for young leaves of the tea plant (Park *et al.* 2004) (GenBank accession: CV699876.1-CV699527.1) and the young leaf cDNA library of the tea plant (Chen *et al.* 2005) (GenBank accession: CV067174.1-CV013548.1), and another drought-stressed root SSH cDNA library of *C. sinensis* var. *assamica* (GenBank accession: GW316945.1-GT969202.1).

Therefore, BLAST searches were needed to perform alignments with whole genome shotgun contigs or non reductant nucleotides sequences of *Arabidopsis* and other dicots or alignment with the four *Camellia* cDNA libraries from the public databases. If markers, which are closely linked to desirable traits, are identified then it will facilitate the marker assisted early selection and shorten breeding procedures in tea. Thus, it will speed up the cultivar improvement programs in tea, a perennial crop which has a long juvenile period and is highly heterozygous.

Tea plantation is often affected by many factors ranging from abiotic (drought, cold, etc.) to biotic factors (pest, fungal diseases, bacterial diseases). Understanding and identifying of not only a particular gene but those responsive genes that affect a particular trait are also needed to enhance breeding in tea. For example, drought tolerance is a polygenic trait which affects morphological, physiological, biochemical and molecular processes of plants (Zhu 2002). A set of drought responsive genes and their pattern of expression were identified under controlled condition in tea (Mishra and Sen-Mandi 2001).

So far, one transgenic tea plant obtained by the *Agrobacterium*-mediated transformation of somatic embryos was reported (Mondal *et al.* 2001). Meanwhile, several research projects using both particle bombardment and *Agrobacterium*-mediated or combined transformation methods were also reported (Luo and Liang 2000; Zhao *et al.* 2001; Wu *et al.* 2003, 2005). With advances in technology, if novel genes responsible for drought tolerance or disease resistance are identified, it could be introduced to other drought stress or disease susceptible plants. By this way, the chances of survival of important plant species against biotic and abiotic stresses would be increased and thus the yield of this important cash crop would be increased.

Thus, to achieve the ultimate goal of genetic improvement of tea, further efforts are required to construct a high density map and to locate the quantitative trait loci (QTL) and other important agronomic traits. Although the density of integrated genetic maps can be extremely high, it may still not be sufficient to tag a specific gene. Maps keep improving but to obtain a "complete map" that includes the

sequences and location of all genes of an organism, the work is quite vast and there is still a long way to go.

Chapter 3

Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

3.1 PLANT MATERIAL

The bi-clonal seed stock TS 463 has been produced by crossing TV 1, an Assam-China hybrid with Cambod clone TV19. TV1 is a quality clone which has good quality but average yield with yield potential of about 2,500-2,800 kg made tea/ha. TV19 is a yield clone which has average quality but high yield with yield potential of 4,000 kg made tea/ha and above. Progenies of the bi-clonal seedstock TS 463 are fairly uniform in growth habit with above average yield and quality and tolerance to drought. The stock is suitable for both CTC and orthodox manufacture. The bi-clonal stock was released in 1984.

3.1.1. Sample collection

The mapping population consisted of 117 genotypes of the F_1 population of the bi-clonal seed stock TS 463. Fresh tea leaf samples were collected from Tea Research Station of the Tea Research Association (TRA), at Tocklai, Jorhat, Assam, India. The samples were stored at -80°C for further use.

3.1.2. Genomic DNA extraction

Genomic DNA extraction was done using modified CTAB method, especially designed for plants containing high polysaccharide and polyphenolic components (Porebski *et al.* 1997).

Genomic DNA isolation:

1. 100 mg of leaf tissue was ground in a pre-chilled mortar with liquid nitrogen to obtain fine powder.
2. The powder was scrapped and transferred into a 1.5 mL tube.
3. 650 µL of pre-heated extraction buffer [Tris Base (0.1 M), EDTA (0.1 M), NaCl (1.4 M), CTAB (2% w/v), PVP (1% w/v)] (Appendix A) was added.
4. 20 µL of 10% SDS was added and the mixture was vortexed.
5. It was then incubated for 60 minutes at 65°C in a water bath.
6. The mixture was removed from water bath and brought to room temperature.
7. 650 µL of chloroform-octanol (24:1) was added and gently mixed by inversion till emulsion was formed.
8. After mixing thoroughly, it was centrifuged at 10,000 rpm at room temperature for 10 min.
9. The top aqueous phase was transferred to a new 1.5 mL centrifuge tube using a wide bore pipette tip.
10. Chloroform-octanol extraction was repeated until the cloudiness in the aqueous phase was removed.
11. ½ volume of 5 M NaCl was added and mixed well with the final aqueous solution. Two volumes of chilled absolute alcohol were added and mixed well. It was then kept in -20°C freezer for 15 min.
12. The genomic DNA was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C.

13. The supernatant was discarded and the pellet was washed with 70% alcohol for 2-3 times.
14. The pellet was vacuum dried for 10-15 min and dissolved in 30 µL of ultra pure water.

Purification step of the genomic DNA

1. 3 µL RNase A (1 mg/mL) was added and incubated at 37°C for approximately 60 min.
2. Equal volume of phenol was added and vortexed briefly.
3. Centrifuged at 13,000 rpm for 15 min and solution of the upper layer was collected.
4. Equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed well. Chloroform-isoamyl alcohol extraction was repeated until a clear aqueous phase was obtained.
5. The clear upper phase was collected and transferred to a new 1.5 mL centrifuged tube. 1/10 volume of 2 M sodium acetate and 2 volumes of absolute ethanol were added and gently mixed.
6. It was kept in -80°C freezer for 15 min and the pellet was centrifuged down at 13,000 rpm for 15 min.
7. The pellet was washed with 70% alcohol for 2-3 times and vacuum dried for 10-15 min.
8. The DNA was then dissolved in 30 µL of ultra pure water and stored at -20°C till further use.

3.1.3. Agarose Gel Electrophoresis

For detection of genomic DNA and amplified DNA, 0.8% and 3% agarose gels respectively were used.

1. For preparation of agarose gel, requisite amount of agarose powder was weighed and dissolved in 1X TBE (Tris borate EDTA) buffer.
2. The solution was boiled in a microwave oven (IFB) till agarose dissolved completely and a consistent transparent solution obtained.
3. It was allowed to cool and then poured in the casting tray sealed at the two open ends with cello tape and left for about 15 min to harden after ensuring that the comb was aligned properly.
4. After taking out the cello tape and the comb, the prepared agarose gels were immersed in an electrophoresis tank which also contained 1X TBE buffer.
5. 2 μL of genomic DNA sample or 10 μL PCR amplicons were mixed with 3 μL of the loading buffer separately (Sambrook *et al.* 1989) loaded into the gel wells and electrophoresed at 60 V for 20-30 min. One of the wells was loaded with 3 μL of λ DNA / *Hind* III + *Eco*RI double digest marker.

3.1.4. Visualisation of DNA

DNA was visualized under a U.V. Transilluminator after staining the gel with ethidium bromide. Photographs of agarose gels were captured using KODAK GEL LOGIC 1500 IMAGING SYSTEM. Sizes of the bands of interest were estimated against the standard molecular weight markers using tools which were available in the software.

3.2. TE-AFLP REACTIONS

TE-AFLP analysis was performed with minor modifications according to the protocol described by van der Wurff *et al.* (2000).

Restriction enzymes

Restriction digestion was done using two rare cutter enzymes *Eco*RI and *Pst*I, and one frequent cutter enzyme *Mse*I (Table 3.2.1).

Table 3.2.1. Restriction enzymes used for template preparation

Enzyme	Target sequence	Incubation temperature
***Mse* I**	**T↓TAA**	**37°C**
***Eco*R I**	**G↓AATTC**	**37°C**
***Pst* I**	**CTGCA↓G**	**37°C**

Adapter preparation

Adapters consist of a core sequence and a restriction enzyme – specific sequence (Appendix B). Adapters were prepared by adding equimolar amounts of both strands. The solution was heated at 95°C for 5 min and brought down to 25°C for 10 min. The sequence allowed the ligation of the adapters to the resulting restriction fragments without restoring the original restriction sites. Ligated adapters created a target site for the AFLP primers in the subsequent amplification reactions.

Primer design and preparation

AFLP primers consist of three parts, a core sequence, a restriction enzyme-specific sequence of the adapter and a selective extension. In the pre-amplification, primers having only one additional base at the 3'-end extending into the restriction fragments were used. While for the selective amplification, primers with three additional bases at the 3'-end extending into the restriction fragments were used.

Nomenclature for the primer combination and the loci

Nomenclature for the primer combinations used in the experiments consists of two lower case letters: the first one indicates the *Eco*RI-selective primer and the second letter indicates *Pst*I-selective primer. Nomenclature of the marker/loci obtained, consists of three lower case letter and a numerical: the first two letters indicated the primer combination used, the third letter 'a' or 'b' depend on the marker type (i.e. 'a' for 1:1 marker type and 'b' for 3:1 marker type) and the numerical is based on the numbering of the markers for a particular primer combination.

3.2.1. Digestion and ligation of DNA

Genomic DNA (250 ng) was added to a digestion–ligation mix in a final volume of 20 µL containing digestion-ligation buffer [50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 20 mM DTT, 50 µg/mL nuclease free BSA and 1 mM ATP], 0.5 M NaCl, BSA (10X), 4 µM of both *Eco*RI and *Pst*I adapters, 4U T4 DNA Ligase and 7.5 U each of *Pst*I and *Eco*RI, and 3.5 U *Mse*I. The mixture was incubated for 3 h at 37°C and the reaction was deactivated by bringing the temperature to 72°C for 10 min. The digested-ligated sample was then diluted 20 times and kept at -20°C for further use as template in the pre-amplification step.

3.2.2. Pre-selective amplification

PCR amplification was performed using a pair of primers containing one selective base. The 20 µL PCR reaction mixture contained 5 µL of the diluted digested-ligated sample as DNA template, 0.133 µM each of E-A and P-A primers (Appendix B), PCR buffer [100 mM Tris (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin], 2 mM of each dNTP and 5 U *Taq* Polymerase. The PCR was performed with the following conditions: Initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min and subsequent final extension step of 72°C for 10 min.

3.2.3. Selective amplification

The pre-amplified product was diluted ten times prior to selective amplification. 2.5 µL of the diluted pre-amplified product was used as a template in a 15 µL PCR reaction mix using a pair of primers containing three selective bases (Appendix B), 0.133 µM each of *Eco*RI and *Pst*I selective primers, PCR buffer. 2 mM of each dNTP and 5 U *Taq* Polymerase. A touch down PCR was performed with subsequent decrease of 0.7°C per cycle.

Touchdown PCR

In touchdown PCR the annealing temperature is gradually decreased during the cycling process. At the beginning of the cycling stage, the annealing temperature is set 10°C higher (i.e. 65°C) than the Tm of the primers. The higher temperature favours only the most specific base pairing between the primer and template and therefore only specific products get amplified. In subsequent cycles the temperature is decreased by 0.7°C per cycle. Since the specific products have already been

amplified and are present in excess, these will be preferentially amplified at the lower, more permissive annealing temperatures.

The thermal profile in selective PCR was more stringent. Selective amplifications were optimized as follows: After initial denaturation of 5 min at 94°C, 12 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s (with subsequent decrease of 0.7°C per cycle) and elongation at 72°C for 1 min were carried out. This was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 1 min. A final extension step of 10 min at 72°C was added.

3.2.4. Polyacrylamide Gel electrophoresis

Sample preparation

Following amplification reactions, the PCR product was mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA pH 8.0, bromophenol blue and xylene cyanol) (Appendix A). The amplicons were denatured by heating at 95°C for 4 min and then immediately cooled by keeping on ice. Each sample was then loaded on a 6% denaturing polyacrylamide gel (Sequi-Gen GT electrophoresis system, Biorad).

Glass plate preparation (Bio-Rad Sequi-Gen GT Sequencing Cell)

1. Both the glass plates were cleaned using lab wash detergent, rinsed well with water and dried with paper towels. The two glasses i.e. IPC and the outer glass were wiped with absolute alcohol using lint free kim-wipe tissue paper. While wiping with ethanol, pressure was applied to make sure the glass surface was cleaned enough so that no bubble should be formed.

2. To know the surface of the outer glass that will come into contact with the gel, the surface was marked.
3. IPC glass plate: 1 mL of the repel silane solution was dispensed across the glass surface with a pipette and spread evenly over the whole surface using a kim wipe paper. It was left to dry for 5 min.
4. The outer glass plate: A kim wipe paper was saturated with Bind silane (Appendix A) and wiped both vertically and horizontally on the glass surface. It was allowed to dry. It was then wiped with 95% ethanol (without applying pressure) vertically and horizontally to take out excess bind silane.
5. Spacers were placed on the IPC which was placed horizontally, and then the outer glass plate was placed on the top in such a way that the edges were aligned properly.

Acrylamide gel preparation

1. 10% APS solution: In 1.5 mL centrifuge tube, 0.1 g of Ammonium Persulfate was dissolved in 1000 µL of ultra pure water.
2. 25 µL TEMED and 500 µL 10% fresh APS were added to 50 mL of 6% Acrylamide (Appendix A). The solution was mixed properly.
3. The solution was drawn into the syringe and care was taken to avoid the presence of any bubble inside the solution.
4. A tube was placed on the syringe and air was push out until there was no bubble in either the syringe or the tube. The other end of the tube was inserted into the caster base and the gel solution was injected with moderate pressure.

5. A shark toothed comb was placed with the straight edge in contact with the gel during polymerisation. The comb was inverted to form wells while loading samples and running the gel. The gel was allowed to polymerise for 2-3 h.
6. Bottom tray (universal base) was filled with 1X TBE buffer so that about 1 inch of the bottom of the unit was submerged. The IPC buffer chamber was filled with 1X TBE buffer until one inch above the IPC.
7. The comb was removed and the well/comb region was flushed out well with 1X TBE buffer using a needle syringe to remove any loose acrylamide.
8. The shark toothed comb was inserted in such a way that the comb teeth-side was down. It was placed so that the tip of the comb just touched the gel.
9. 10 µL of the denatured sample was loaded carefully and the electrophoresis was done at 800 volts until the desired fragment size separation was achieved. The gel electrophoresis time was monitored by observing the dye front mobility of the loading dye (approx. 7-8 h).

3.3. SILVER STAINING

After the electrophoresis, silver staining was carried out to visualize the DNA fragments in the sequencing gel. The procedure for silver staining was based on the procedure of Caetano-Anolles and Gresshoff (1994) with few modifications. All the procedures were performed at ambient temperature. Constant gentle agitation of the gel was done during the staining steps.

1. Separation of the plates: After electrophoresis, the plates were carefully separated, ensuring that the gel remained attached strongly to the outer glass plate.
2. Fixing: The gel bound to the long glass plate was fixed in a fixing solution (10% acetic acid) for 45 min and agitated gently.
3. Washing: The gel was rinsed 3 times in deionised water for 10 min. The water was drained from the gel.
4. Staining: The gel was transferred to a tray containing staining solution. The impregnation was performed for 30 min in silver staining solution [0.1% $AgNO_3$, 0.056% formaldehyde] shaking gently. The plate was then removed, vertically drained.
5. Rinsing: The gel was submerged and agitated for 5 s in deionised water. The gel was removed from the tray and vertically drained.
6. Developing: The gel was placed into a tray containing 1 L of cold developing solution [3% Na_2CO_3, 0.056% formaldehyde, 2 µg/mL sodium thiosulphate]. The gel was agitated by gently shaking until the bands appeared. After the bands started appearing, the developing solution was discarded and developing was again continued in a new solution of 1 L chilled developing solution.
7. Fixing/Stopping: The reaction was stopped with the stop solution (10% acetic acid).
8. The gel was rinsed in ultrapure water and left to dry vertically overnight and photographed.

3.4. ELUTION AND RE-AMPLIFICATION OF THE PCR PRODUCT

Elution of AFLP fragments was done according to the protocol described by Ausubel *et al.* (1997) and Chen and Ruffner (1996) with minor modifications:

1. The specific band of interest was excised, directly from the denatured polyacrylamide gels using a razor blade.
2. It was transferred to sterile 1.5 mL microcentrifuge tubes, soaked in 100 µL of sterile water at room temperature and frozen at -80°C for overnight.
3. Finally, it was centrifuged for 20 min at 13,000 rpm at room temperature.
4. 3 µL of the elutant was re-amplified with the same primer combination and conditions used for selective amplification.
5. The amplified product was subjected to two more cycles of elution and amplification as described above to ensure that the product was free of contamination from bands of similar size.

3.5. PURIFICATION OF PCR PRODUCTS

PCR products with only the specific target band were also purified using ethanol precipitation as described by Huang and Cloutier (2007).

1. To a 50 µL PCR reaction mixture 5 µL of 3 M sodium acetate (pH 4.6) and 100 µL of 95% ethanol were added.
2. The tube was vortexed briefly and placed at -20°C for 40 min to precipitate the PCR products.
3. It was then centrifuged at 13000 rpm for 20 min to pellet the DNA.
4. The supernatant was discarded by gently inverting the tube.
5. The pellet was washed with 300 µL of chilled 70% alcohol.

6. It was centrifuged at 13,000 rpm for 5 min and the ethanol was discarded by gently inverting the tube and vacuum dried.
7. The pellet was re-dissolved in 25 µL of ultra pure water.

3.6. SEQUENCING OF PCR PRODUCTS

Purified PCR products were sequenced with the appropriate primers using BigDye Terminator Ready Reaction Mix (Applied Biosystems) (version 3.1) and an Applied Biosystems 3130 Genetic Analyzer located at the Life Sciences Central Instrumentation Facility, North Eastern Hill University (India). Sequencing reactions contained 40–50 ng PCR product as template, 1.9 µL of 5X sequencing buffer, 0.3 µL of 20 µM primer (primers as used in PCR reactions), and 0.4 µL of BigDye reaction mix. The total volume was made up to 10 µL with ultra pure water. The sequencing reaction was carried out using an Applied Biosystems GeneAmp® PCR System 9700 Gold thermalcycler. Following 5 min of denaturation at 96°C, 25 cycles were performed with 30 s at 96°C, 1 min at the appropriate annealing temperature of the primer used (i.e. 56°C) and a final extension step of 4 min at 60°C.

Purification of cycle sequencing products

The cycle sequenced products were purified using ethanol precipitation.

1. To a 10 µL of product 1 µL of 125 mM EDTA was added and mixed well.
2. Then 1 µL of 3 M sodium acetate (pH 4.6) was added and mixed well.
3. The entire solution was transferred to a 0.5 mL tube containing 50 µL of 95% ethanol. It was mixed thoroughly and incubated in the dark for 15 min at room temperature.

4. It was centrifuged at 12,000 rpm for 20 min at room temperature.
5. The supernatant was discarded carefully and 250 µL of chilled 70% alcohol was added, mixed gently and incubated in the dark for 10 min at room temperature.
6. It was then centrifuged at 13000 rpm for 10 min at room temperature.
7. Step 5 and 6 were repeated once more.
8. The pellet was vacuum dried and stored at -20°C for further use.
9. 10 µL of Hi-Di formamide (Applied Biosystems) was added to each tube and centrifuged briefly.
10. It was then denatured in an Applied Biosystems GeneAmp® PCR System 9700 Gold thermalcycler at 95°C for 5 min and chilled on ice.
11. The PCR products were sequenced on a 3130 Genetic Analyser (Applied Biosystems).

3.7. STATISTICAL ANALYSES FOR PRIMER COMBINATION

TE-AFLP band profiles for the polymorphic bands were scored manually for the presence (as '1') and absence (as '0') of an allele at a particular locus. The profiles of each gel across all the 17 accessions for each primer combination were scored. The binary data obtained were used for evaluating the discriminating power and the information content of the 5 primer combinations by assessing four parameters.

3.7.1. Polymorphic information content (PIC)

Polymorphic Information Content (PIC) for dominant markers was calculated according to Roldan-Ruiz *et al.* (2000): $PIC = 1-[f^2 + (1-f)^2]$, where 'f' is the

frequency of the marker in the data set. PIC for dominant markers is a maximum of 0.5 for 'f'=0.5.

3.7.2. Marker index (MI)

Marker index (MI) was calculated according to Powell *et al.* (1996) as amended by Milbourne *et al.* (1997). It is a product of information content as measured by PIC and Effective multiplex ratio (EMR).

3.7.3. Effective multiplex ratio (EMR)

Effective multiplex ratio (EMR) (Powell *et al.* 1996): it is the product of number of polymorphic loci in the germplasm analysed and the fraction of markers that were polymorphic.

3.7.4. Resolving power (Rp)

Resolving power (Rp) of each primer combination was calculated according to Prevost and Wilkinson (1999): $Rp = \sum I_b$ where I_b (band informativeness) can be represented by the formula: $I_b=1-(2x|0.5-p|)$, where p is the proportion of the genotypes containing the band.

3.8. STATISTICAL ANALYSES FOR LINKAGE MAPPING

3.8.1. Linkage mapping

A linkage map is a representation, in the form of a table or a graphic, of the position of genes (or markers) within a linkage group. The map positions are inferred from estimates of recombination frequencies between genes. The co-segregation of markers in families are analysed by gene mapping algorithms, which assemble markers into their respective linkage groups, followed by the calculation of the most

probable order of markers within the linkage groups (Hartl and Jones 2001). The basic principles in map construction are basically the same for the different statistical programmes, and the major steps in linkage analyses by JoinMap are:

1. Verification of the Mendelian segregation for all the markers.
2. Pairwise linkage analysis between all possible two-locus combinations.
3. Allocating the markers to different linkage groups.
4. Ordering of markers in the same linkage group.
5. Estimation of the multipoint recombination fractions between neighbouring loci.

3.8.1.1. *Computer software*

In this study, a pseudo test cross (Grattapaglia and Sederoff 1994) approach was adopted to generate map for tea as it is outcrossing species. Polymorphic markers were visually scored on presence and absence of bands. Linkage analysis was performed using all polymorphic markers by observing paternal and maternal segregation patterns separately using JoinMap 4.0 software (van Ooijen 2006).

The regression mapping function was employed using the following default parameters: recombination frequency (REC) threshold of 0.4, a jump threshold value of 5.0 and ripple 1 function. Jump in the goodness-of-fit represented the difference in goodness-of-fit chi-square values before and after adding a locus to the map (van Ooijen 2006). A large jump (disparity) indicates a poor fit for the added marker and may warrant its removal from the map. Map distances were calculated using the Kosambi mapping function (Kosambi 1944). The position of markers was developed using a sequential map build-up (Stam 1993) and the most informative pair of

markers was selected, followed by sequential addition of other markers. The best fitting position of an added marker was examined on the basis of the goodness-of-fit test (chi-square) for the resulting map. The marker was removed when a marker generated a negative map distance, or a large shift (jump) in goodness-of-fit and map construction was continued as a first-round map. After the first-round marker ordering, the previously removed markers were added back and again subjected to the goodness-of-fit testing. The marker ordering was continued to a third round until an optimum order of markers was found (van Ooijen 2006).

3.8.2. Principles and Statistics used in linkage mapping

3.8.2.1. *Pseudo test cross strategy*

In this strategy, the testcross mating configuration of the markers is not known priori as in a conventional test cross where the tester is homozygous recessive for the locus of interest. Rather the configuration is inferred a posterior after analysing the parental origin of a cross between highly heterozygous parents with no prior genetic information (Grattapaglia and Sederoff 1994). Linkage analysis was done using the pseudo-testcross method and maps were constructed using the 4.0 software package, which permits linkage analysis in out-breeding progenies involving markers with different segregation types performed by the JoinMap 4.0 software (Gratapaglia and Sederoff 1994; Maliepaard *et al.* 1997; Wu *et al.* 2002; van Ooijen 2006).

3.8.2.2. *Mendelian genetics*

Genes, which are units of heredity, are passed along from generation to generation in a sexually reproducing species, following Mendelian inheritance (Liu 1998). In this

study the segregation of markers (the segregation of the parent alleles through the offspring) will be analysed according to expected Mendelian segregation ratios.

3.8.2.3. *Chi square test*

Chi-square analysis is widely used in genomics and measures goodness of fit of the null hypothesis and evaluates observed deviations. The χ^2 value is used to determine how much of the observed deviation can be attributed to chance only. In this study, χ^2 test was done to check whether the segregation ratios for a single marker fit the expected ratios.

The formula for chi-square analysis is:

$$\sum \frac{(O-E)^2}{E}$$

where, O = the observed amounts for a particular category

E = the expected amounts for the specified category

$\sum$ = the sum of the calculated amounts in the ratio

The degrees of freedom are calculated using the number of different categories in the dataset being analysed. Degrees of freedom equals n-1, where n is the number of categories. Once the degrees of freedom have been calculated, the χ^2 value can be interpreted in terms of a probability value (P), which is generally determined using a graph or table (Klug and Cummings 2003).

In our study, the segregation analysis of marker genotypes was performed by JoinMap 4.0 (van Ooijen 2006) with the expected Mendelian ratios of each locus elucidated by the programme. The genotype frequencies for each locus were determined and used to identify any segregation distortion with the use of a chi-

square probability test. Deviation from the expected ratios in any locus was provided by JoinMap 4.0, with seven default significance thresholds (0.1- 0.0001).

3.8.2.4. *Recombination fraction*

The farther the distance between two genes, the more frequent is their crossover leading to higher frequency of recombinations. Therefore, the recombination fraction is calculated from the proportion of recombinants in the gametes produced.

Recombination fraction = number of recombinants / total

The distance between two genes is determined by their recombination fraction.

3.8.2.5. *LOD (logarithm of the odds ratio or likelihood ratio) score.*

It measures the decimal logarithm of the likelihood ratio between the linkage and the independence hypothesis among markers (Morton 1955).

$$\text{LOD score} = \log_{10} \frac{Likelihood\ of\ linkage\ (\Theta)}{Likelihood\ of\ no\ linkage\ (\Theta=0.5)}$$

where, Θ is the recombination fraction

In this study, the threshold was set at an initial LOD score of two with an increasing stringency of one increment to a final threshold of 10 with a default set recombination threshold of 0.4. Linkage groups within a LOD threshold of three were chosen for further analysis (framework markers). The regression mapping algorithm was used to calculate map order. Markers presenting a recombination frequency less than the set threshold and a LOD score greater than the set threshold were considered as linked (van Ooijen 2006).

3.8.2.6. *Map distance*

Map distance is defined as the expected number of crossovers between two loci or one-half the expected number of chiasma (recombination nodules). Map distance is measured in Morgans: 1 Morgan = 1 crossover per chomatid. One crossover between two loci is 1/2 crossover per chromatid or 50% recombination or 0.5 Morgans = 50 cM.

3.8.2.7. *Map function*

Mapping functions convert recombination fractions (Θ) to map distances (d) that are additive. A mapping function translates recombination frequencies between two loci into a map distance in cM. A mapping function gives the relationship between the distance between two chromosomal locations on the genetic map (in centiMorgans, cM) and their recombination frequency. In general, a mapping function depends on the interference assumed. Interference is the effect in which the occurrence of a crossover in a certain region reduces the probability of a crossover in the adjacent region.

Considering the criteria of interference there are two main types of map function: i) Haldane mapping function (with no interference) (Haldane 1919) and ii) Kosambi's mapping function (some interference) (Kosambi 1944).

In our study, Kosambi's function was used as the mapping function:

$$d = \tfrac{1}{4} \ln[(1+2\Theta)/(1-2\Theta)]$$

where, d= kosambi distance

Θ= recombination fraction

3.8.3. Marker distribution

The correlation between the number of AFLP markers and the length (size) of the linkage groups was analysed using the Pearson correlation coefficient 'r' (Yu and Guo 2003; Wang *et al.* 2004; Baranski *et al.* 2006). Thereafter a *t*-test was applied to test the significance of correlation coefficient at the $P = 0.01$ level, using the null hypothesis (H_0) of no correlation. A correlation of +1 indicates a perfect positive relationship (correlation) between the two datasets or variables (the two variables increase together), while a correlation of –1 means there is a negative relationship between the two variables. An *r* value of 0 indicates that there is no correlation between the two variables.

t value for testing the significance of the correlation coefficient was calculated by the equation:

$$t = r\sqrt{\frac{n-2}{1-r^2}}$$

where, n-2 is the degrees of freedom for entering the t-distribution

r is the correlation coefficient

In addition, the AFLP mapped markers were classified according to the 12 primer combinations from which they were derived (Wang *et al.* 2004).

3.8.4. Map length and genome coverage

In this study two methods were used to estimate genome length: the method described by Fishman *et al.* (2001) and method 4 as described by Chakravarti *et al.* (1991).

The average marker spacing/intervals (s) of the two framework maps (maternal and paternal) were calculated by dividing the summed length of the map (all the linkage groups) by the number of intervals (the number of markers minus the number of linkage groups). Similarly, the average marker spacing of each linkage group was calculated by dividing the length of each linkage group by the number of intervals (the number of markers minus 1) occurring in that linkage group (Bratteler *et al.* 2006; Liu *et al.* 2006; Lallias *et al.* 2007).

First, the estimated genome length (G_{e1}) was determined by adding $2s$ (calculated above) to the length of each linkage group to account for chromosome ends (Fishman *et al.* 2001). Secondly, an estimated genome length (G_{e2}) was determined by multiplying the length of each linkage group by $(m + 1)/(m - 1)$, where m is the number of all the markers in each group (Chakravarti *et al.* 1991). The average of the two estimates (G_{e1} and G_{e2}) was used as the estimated genome length (G_e). The observed genome length (G_o) was calculated as the total length considering all markers (framework, accessory and distorted markers). Total genome coverage was determined as G_o/G_e.

3.9. NUCLEOTIDE SEQUENCE ANALYSES

3.9.1. Nucleotide sequence BLAST search

Nucleotide sequences obtained were queried (BLASTn) individually with three different databases on NCBI GenBank (National Center for Biotechnology Information) and the blast hits were filtered on the basis of e-value. The selected threshold e-value was 1e-04 was taken for all database and the hits with e-value more than that were considered as non-homologous. The hits below the threshold limit

were analyzed to determine the homology of the query sequence with already known genes in the GenBank database. Sequences with high quality were selected and analyzed.

e-value

Expectation value (e) threshold is a statistical measure of the number of expected matches by chance in a random data. The lower the e-value or closer to zero, the more likely the match is to be significant.

The three different databases on NCBI GenBank (National Center for Biotechnology Information) which were undertaken for BLASTn studies were:

a) non redundant (nr/nt) nucleotide database of dicot plants (taxid 71240) .
b) Whole genome shot gun sequences databases of *Arabidopsis* (taxid 3702) and other dicot plants (taxid 71240).
c) The identical results from keyword searches and BLAST searches can be used to predict that these genes could be expressed in *C. sinensis.* To discover new genes, all searched unigenes were analyzed by BLAST alignments against the existing tea uniEST database of *Camellia sinensis* (taxid 4442), *Camellia sinensis* var. *sinensis* (taxid 542762), *Camellia assamica* / *Camellia sinensis* var. *assamica* (taxid 261999).

3.9.2. ORF finder (NCBI)

Open Reading Frames (ORF) were found using ORF finder (NCBI) for some selected sequences which showed alignment with EST of tea database. The sequences were translated in all possible six reading frames (three forward and three reverse) and a similarity search against the protein databanks was done.

Chapter 4

Results

CHAPTER 4

RESULTS

4.1. PRIMER COMBINATION SELECTION

4.1.1. Primer selection on different samples

TE-AFLP bands visualised on the silver stained polyacrylamide gel were analysed for selecting primer combinations. Bands between 100bp and 1000 bp were used as reference points and banding patterns of tea were analysed by scoring the prominent bands. A 'narrow-down' strategy of primer combinations (Yang *et al.* 2005) to screen primer combinations showing high polymorphism was carried out in 3 steps using 1, 5, and 17 accessions of tea as described below:

4.1.1.1. *Primer selection with one accession*

In the first step, 36 primer combinations were assayed in one tea sample to determine the number of TE-AFLP bands produced. Primer combinations were represented by two letters where the first letter was used to code the *Eco*RI- selective primer and the second letter was used to code the *Pst*I- selective primer. The number of legible bands ranged from 15 to 38 bands (Table 4.1.1). Of the 36 primer combinations, 18 primer combinations produced more than 25 clear bands and thus were suitable for further selection of polymorphic bands in 5 accessions of tea (Fig. 4.1.1).

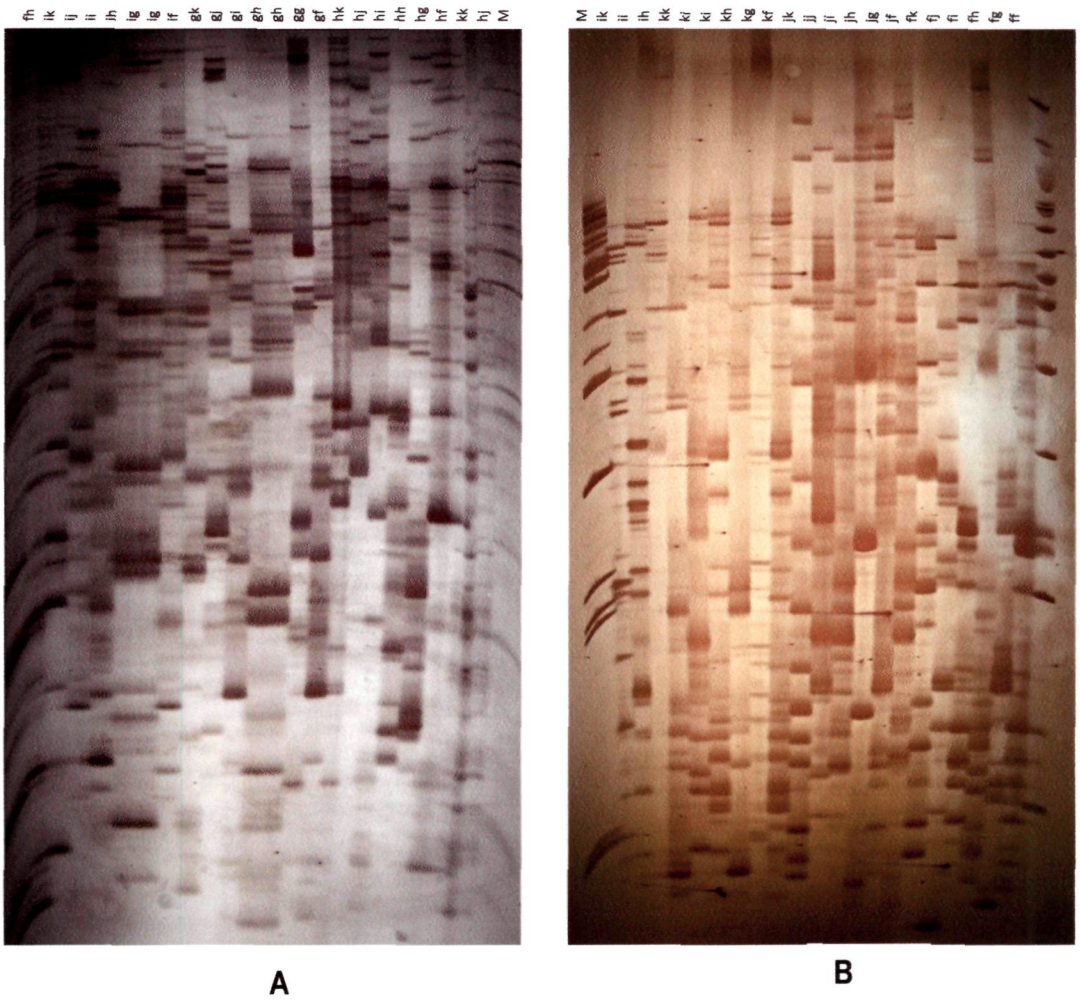

Fig. 4.1.1. TE-AFLP profile for testing 36 primer combinations in one sample. M-100bp ladder marker

A: (From Left to Right) Primer combinations fh, ik, ij, ii, ih, ig, ig, if, gk, gj, gi, gh, gh, gg, gf, hk, hj, hi, hh, hg, hf, kk, hj, M-100bp ladder marker

B: (From Left to Right) M-100bp ladder marker, Primer combinations ik, ii, ih, kk, ki, ki, kh, kg, kf, jk, jj, ji, jh, jg, jf, fk, fj, fi, fh, fg, ff

Table 4.1.1. Total number of TE-AFLP bands generated by 36 primer combinations in a tea sample (Letters in the bracket represent the *Eco*RI- and *Pst*I- selective primers).

Primer combinations	P-ACG (P-f)	P-AAT (P-g)	P-CGA (P-h)	P-ATG (P-i)	P-ATT (P-j)	P-AAA (P-k)
E-AAT (E-f)	36	22	32	19	21	21
E-ACT (E-g)	21	19	27	15	29	32
E-AAC (E-h)	29	27	25	34	16	29
E-AAG (E-i)	28	21	34	25	24	38
E-AGT (E-j)	37	27	22	17	23	18
E-ACG (E-k)	30	27	16	27	22	37

(E and P indicate the *Eco*RI and *Pst*I core primers respectively)

4.1.1.2. *Primer selection with five accessions*

The 18 primer combinations selected in the previous step were assessed in five samples to study the TE-AFLP patterns. Polymorphic bands were seen in 7 primer combinations while the rest had identical or less polymorphic TE-AFLP patterns (Fig.4.1.2). The primers revealing polymorphic bands were E-AAC/P-ATG (hi), E-AAT/P-CGA (fh), E-ACG/P-AAA (kk), E-AAC/P-AAT (hg), E-ACT/P-AAA (gk), E-AAG/P-CGA (ih) and E-AAT/P-ACG (ff).

4.1.1.3. *Primer selection with seventeen accessions*

In the final screening, the 7 primer combinations selected in the preceding step were further investigated in 17 samples to examine the TE-AFLP patterns. Five primer combinations that produced polymorphic bands ranging from 10-27 in 17 tea samples were selected (Fig. 4.1.3).

4.1.2. Marker Polymorphism Studies

A total of 181 bands were detected using the five primer combinations, of which 81 fragments showed polymorphism (43.75%). Number of polymorphic bands ranged from 10 for the primer combination E-ACT/P-AAA (gk) (28.57%) to 27 for the primer combination E-ACG/P-AAA (kk) (58.70%) (Fig. 4.1.4).The frequencies of the polymorphic fragments for a given primer combination across the 17 accessions ranged from 0.35 to 0.91 with an average of 0.74 (Appendix C). A large proportion (71.60%) of AFLP fragments had frequencies in the range of 0.70-0.79 and 0.8-0.89 (Fig. 4.1.5). TE-AFLP generated lesser number of fragments which were sparsely distributed as compared to normal AFLP (Sharma *et al.* 2011).

Details for Fig. 4.1.2. (From left to right):

A: Gel profile for testing 4 primer combinations (gk, gi, ff, fh) in five samples.

B: Gel profile for testing 4 primer combinations (hk, hg, hi, gh) in five samples.

C: Gel profile for testing 4 primer combinations (ik, ih, if, jf) in five samples.

D: Gel profile for testing 4 primer combinations (jg, kk, kg, kf) in five samples.

E: Gel profile for testing 4 primer combinations (gh, ki) in five samples.

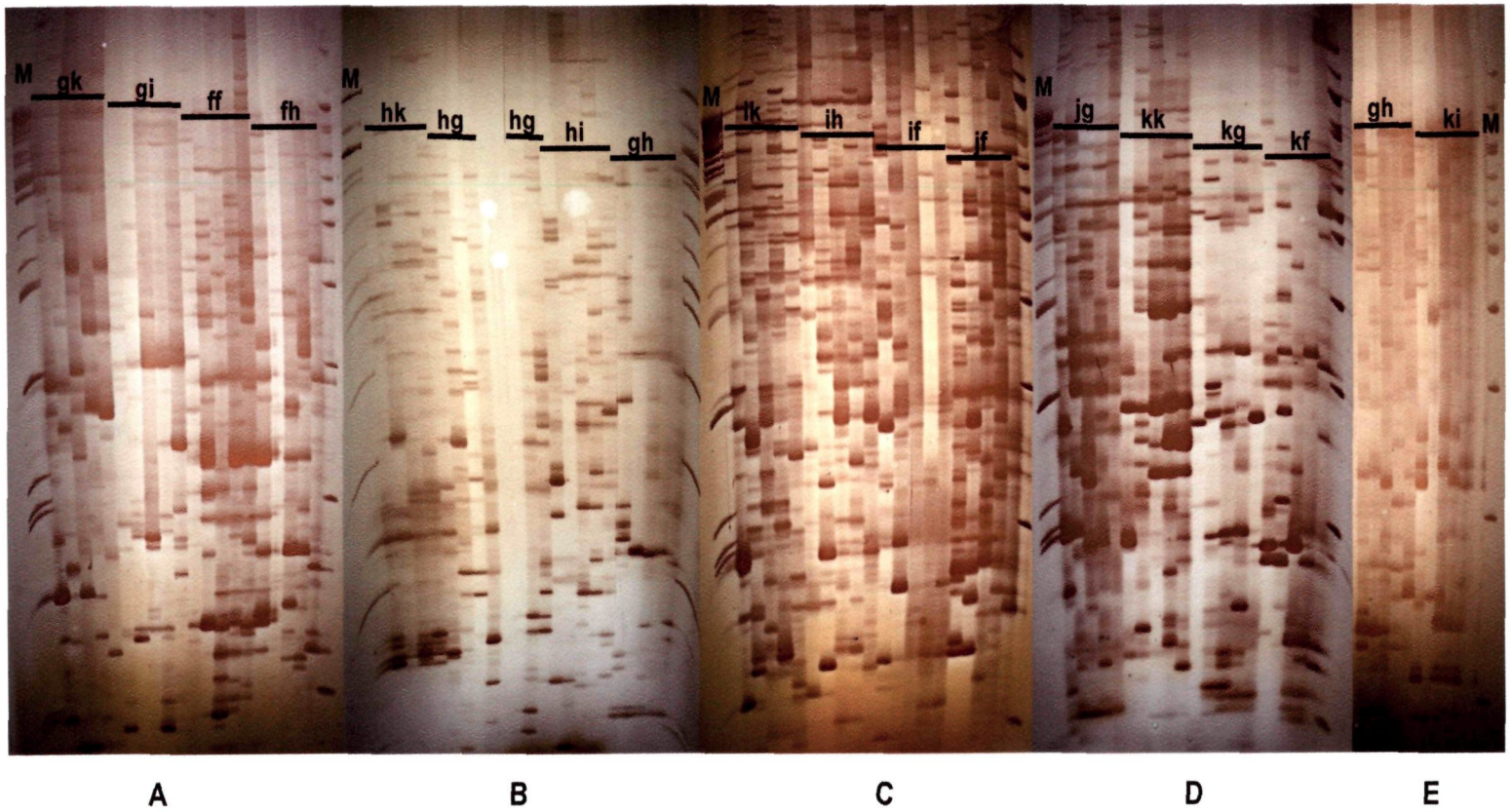

Fig. 4.1.2 TE-AFLP profile for testing 18 primer combinations in five samples. M-100bp ladder marker.

(Letters marked here represent the primer combination of corresponding *Eco*RI/*Pst*I primers; the first letter is *Eco*RI- selective primer and the second letter is *Pst*I- selective primer)

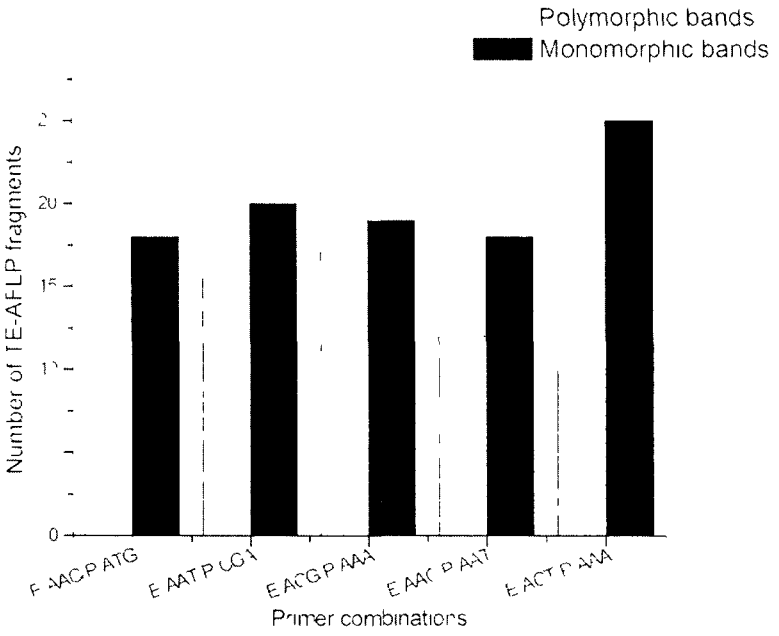

Fig. 4.1.4. Number of fragments scored per primer combination for the seventeen tea samples which was used in the final screening.

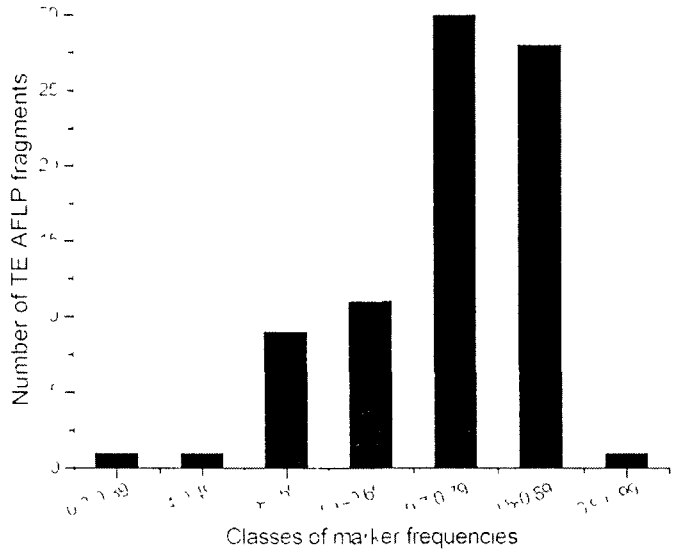

Fig. 4.1.5. Distribution of marker frequencies for polymorphic TE-AFLP fragments generated by the five primer combinations in seventeen tea samples.

The binary data obtained for the five primer combinations was used for evaluating its discriminating power and the information content by using four statistical parameters namely PIC, EMR, MI and Rp.

4.1.2.1. *Polymorphic information content (PIC)*

The PIC values for 81 polymorphic fragments generated by the 5 primer combinations varied from 0.16 to 0.50 with an average of 0.36 per fragment (Appendix C). Majority of the fragments (31 fragments) showed PIC value between 0.31-0.35 while 18 fragments showed PIC value ranging between 0.46-0.50 (Fig. 4.1.6). In terms of the PIC value of the primer combinations, it ranged from 0.32 (E-AAC/P-ATG) (hi) to 0.40 (E-AAT/P-CGA) (fh) with an average of 0.36. Moderate levels of PIC values for TE-AFLP primer combinations in the study can be attributed to highly informative TE-AFLP primer combinations used in this study. Among the different primer combinations, PIC for the primer combination E-AAT/P-CGA (fh) was found highest (overall PIC value 0.40) (Table 4.1.2).

4.1.2.2. *Effective multiple ratio (EMR) and Marker index (MI)*

The higher number of fragments per primer combination is the feature of AFLP that provides higher EMR to AFLP. The higher MI values were detected for combinations presenting the higher number of polymorphic bands. MI for E-ACG/P-AAA (kk) primer combination was the highest (5.73) where the polymorphic bands were highest (27 bands), while MI for E-ACT/P-AAA (gk) was the lowest (1.04) where the polymorphic bands were lowest (10 bands) (Table 4.1.2).

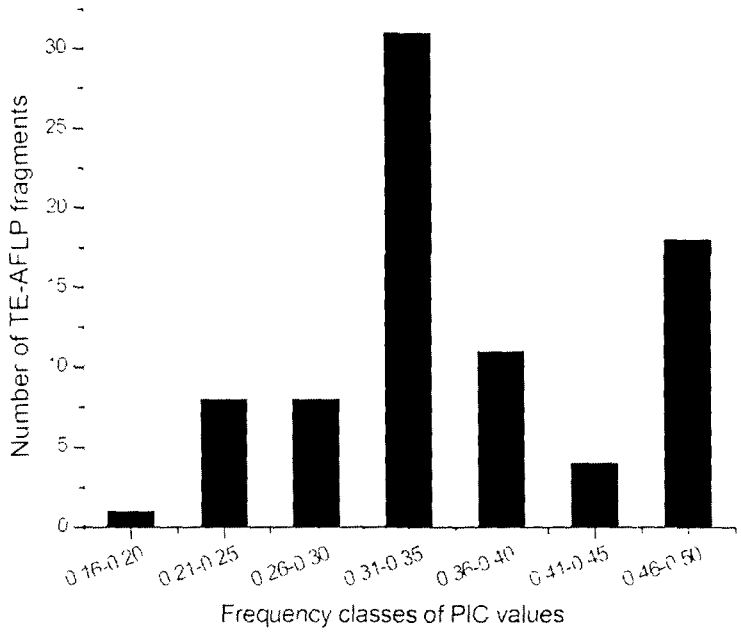

Fig.4.1.6. PIC distribution for all the polymorphic fragments generated by the five TE-AFLP primer combinations.

4.1.2.3. *Resolving power (Rp)*

Resolving power (Rp) predicts the discriminatory potential of different primer combinations. In our study, the resolving power of the primer combinations varied from 6.72 to 19.90 with an average of 11.80 (Appendix C). The resolving power of the primer combination E-ACG/P-AAA (kk) was highest with 19.90. Since Rp is the total sum of all the informativeness of each band, higher the number of polymorphic bands of a primer combination higher is the Rp. Hence the primer combination E-ACG/P-AAA (kk) with the highest Rp value (19.90) was the most informative primer combination for distinguishing the accessions.

Table 4.1.2. Levels of polymorphism and the discriminating capacity of five primer combinations in seventeen tea accessions.

Primer combinations	*Total fragments*	*Monomorphic bands*	*Polymorphic bands*	*% polymorphism*	*PIC**	*EMR**	*MI**	*Rp**
E-AAC/P-ATG	34	18	16	47.06	0.32	7.52	2.48	11.26
E-AAT/P-CGA	36	20	16	44.44	0.40	7.04	2.82	12.18
E-ACG/P-AAA	46	19	27	58.70	0.36	15.93	5.73	19.90
C-AAC/P-AAT	30	18	12	40.00	0.35	4.80	1.68	8.92
E-ACT/P-AAA	35	25	10	28.57	0.36	2.90	1.04	6.72
Total	**181**	**100**	**81**	-	-	-	-	-
Average	-	-	-	**43.75**	**0.36**	**7.64**	**2.75**	**11.80**
Standard deviation	-	-	-	-	**0.03**	**4.99**	**1.80**	**5.00**

*PIC , polymorphism information content; EMR, effective multiplex ratio; MI, marker index; Rp, resolving power

4.2. SEGREGATION ANALYSIS

Primer combinations were screened for level of polymorphism and marker quality. A primer combination's usefulness for linkage mapping was based on the total number of bands produced, the number of polymorphic bands present (between the parents) and the amount of background noise/clarity and band quality (intensity). Of the 36 primer combinations tested, 5 superior primer combinations that were selected in the "narrow down strategy" were used for mapping analysis.

Polymorphic markers were visually scored and recorded as 1 (present) and 0 (absent). Markers present in the two parents but segregating in the progeny were similarly scored. Ambiguous markers in a few genotypes were considered as missing data for map construction purposes.

In this study, a pseudo test cross (Grattapaglia and Sederoff 1994) approach was adopted to generate map for tea as it is an outcrossing species. Markers were subdivided into two groups considering their segregation patterns in the progenies. The first group comprised markers in the testcross configuration between the parents (heterozygous in one parent and homozygous null in the other), which presented a 1:1 segregation ratio in the progeny. The second group concerned markers heterozygous in both parents, and therefore segregating in a 3:1 ratio in the progeny. The cross-pollinated (CP) population type function was used and the marker data were scored according to JOINMAP 4.0 (van Ooijen 2006). Chi-square analysis was performed for goodness of fit to the expected Mendelian segregation ratio for each marker and skewed markers were identified using a threshold of $P < 0.1$. Highly distorted 1:1 and 3:1 markers ($P < 0.05$) were discarded from further analysis.

Table 4.2.1. Comparative table of the different types of segregating markers in a pseudo testcross population.

	1:1 segregating markers	3:1 segregating markers
Marker type	AFLP	AFLP
Parental genotypes	Aa x aa	Aa x Aa
F_1 genotypes	Aa, aa	AA, Aa, aa
Application	Construct parental maps	Identifying potential homologies between parental maps

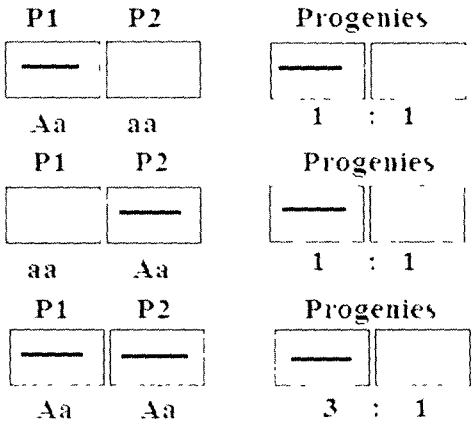

Fig. 4.2.1. Genotypic configuration observed in pseudo testcross population. (P1 and P2 represent the parents).

4.2.1. Segregation analysis in 117 genotypes of the F1 population of bi-clonal stock 463.

The five primer combinations that were selected in the final step of "narrow down strategy" were used for mapping analysis in a F_1 population of bi-clonal stock 463 that consisted of 117 genotypes. Gel profiles for each primer combination are given in the Figures 4.2.2 to 4.2.6. In every gel, lanes for parents and a known molecular marker (i.e. 100 bp ladder) were kept for fine alignment during scoring.

Details for Fig. 4.2.2. (From left to right), M-100bp ladder marker:

A: M; progeny no. 17,16,15,14,13,12,11,10; parents-TV19,TV19,TV1,TV1; progeny no.9,8, 7,6,5,4,3,2,1; M

B: M; progeny no.34,33,32,31,30,29,28,27,26; parents-TV19,TV19,TV1,TV1; progeny no. 25,24,23,22,21,20,19,18; M

C: M; progeny no. 51,50,49,48,47,46,45,44,43,42,41,40,39,38,37,36,35; parents- TV1, TV1,TV19,TV19; M

D: M; progeny no. 69,68,67,66,65,64,63,62,61; parents-TV19,TV19,TV1,TV1; progeny no. 60,59,58,57,56,55,54,53; M

E: M; parents- TV19,TV19,TV1,TV1; Progeny no. 88,87,86,84,82,81,80,79,78,77,76,75,74, 73,72,71,70; M

F: M; Progeny no. 107,106,104,103,102,101,100,99,98; parents-TV19,TV19,TV1,TV1; progeny no. 97,96,95,94,93,92,91,90; M

G: M; Progeny no. 120,119,118,117,116,114,113; parents-TV19,TV19,TV1,TV1,TV1; progeny no. 112,111,110,109,108,107,89,88; M

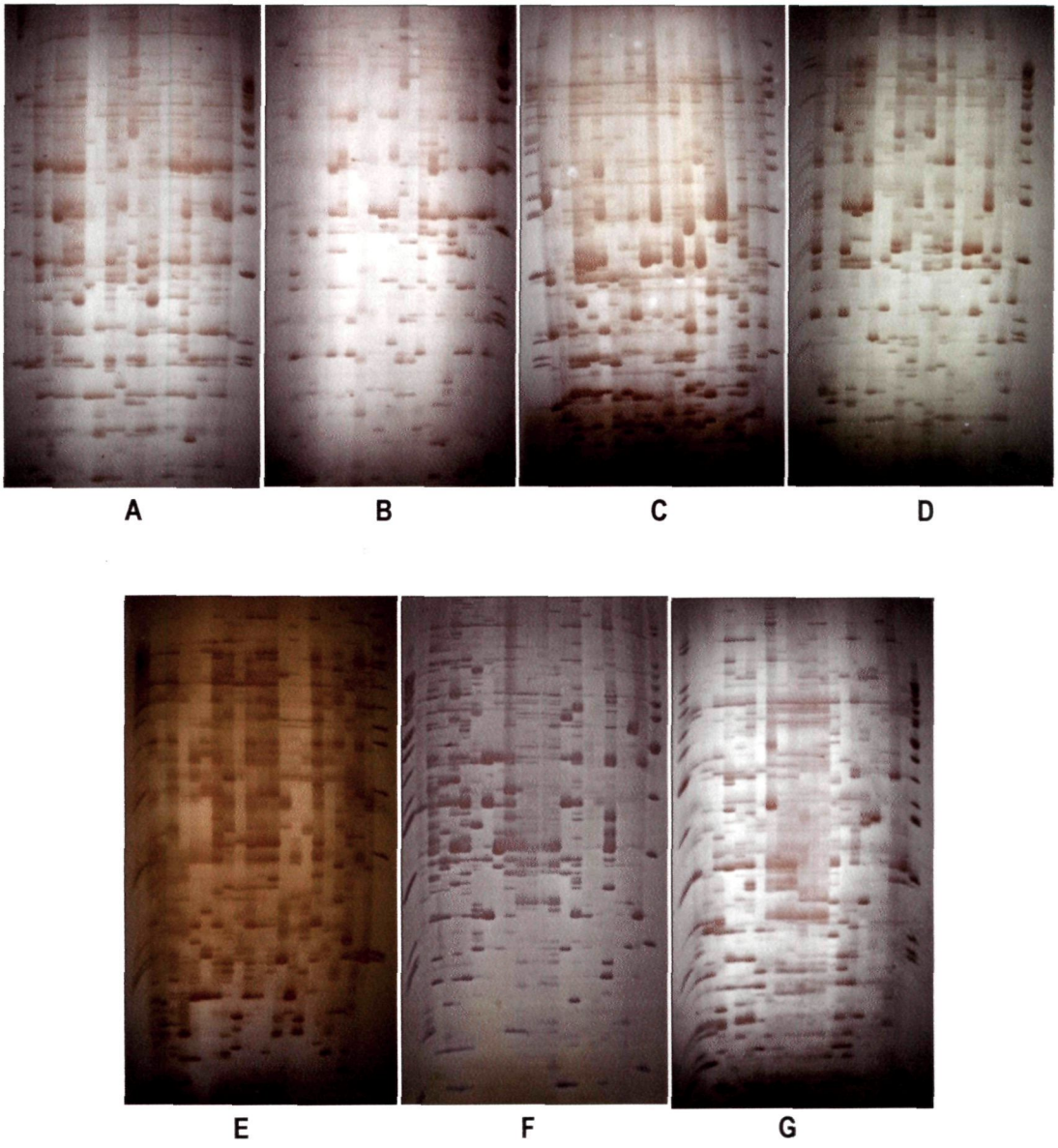

Fig. 4.2.2. Silver stained TE-AFLP profiles generated using E-AAT/P-CGA (fh) primer combination in 117 F1 progenies, TV1 and TV19.

Details for Fig. 4.2.3. (From left to right); M-100bp ladder marker:

A: M; progeny no. 17,16,15,14,13,12,11,--,10,9,8,7,6,5,4,3,2,1, parents-TV1,TV1,TV19,TV19; M

B: Progeny no. 37,36,35,34,33,32,31,30,29; parents- TV19,TV19,TV1,TV1; progeny no. 28, 27,26,25,24,23,22,21,20; M

C: M; parents-TV1,TV1,TV19,TV19; progeny no. 54,53,52,51,50,49,48,47,46,45,44,43,42,41, 40,39,38; M

D: M; parents- TV19,TV19,TV1,TV1; progeny no. 68,67,66,65,64,63,62,61,60,59,58,57,56, 55,54,53,52; M

E: M; parents- TV19,TV19,TV1,TV1; progeny no. 69,70,71,72,73,74,75,76,77,78,79,80,81, 82,84,86,87; M

F: Progeny no. 105,104,102,101,100,99,98,97; parents- TV19,TV19,--,TV1,TV1; progeny no. 96,95,94,93,92,91,90,89,88; M

G: M; parents- TV19,TV19,TV1,TV1; progeny no. 120,119,118,117,116,114,113,112,111, 110,109,108,107,106,105,104,103; M

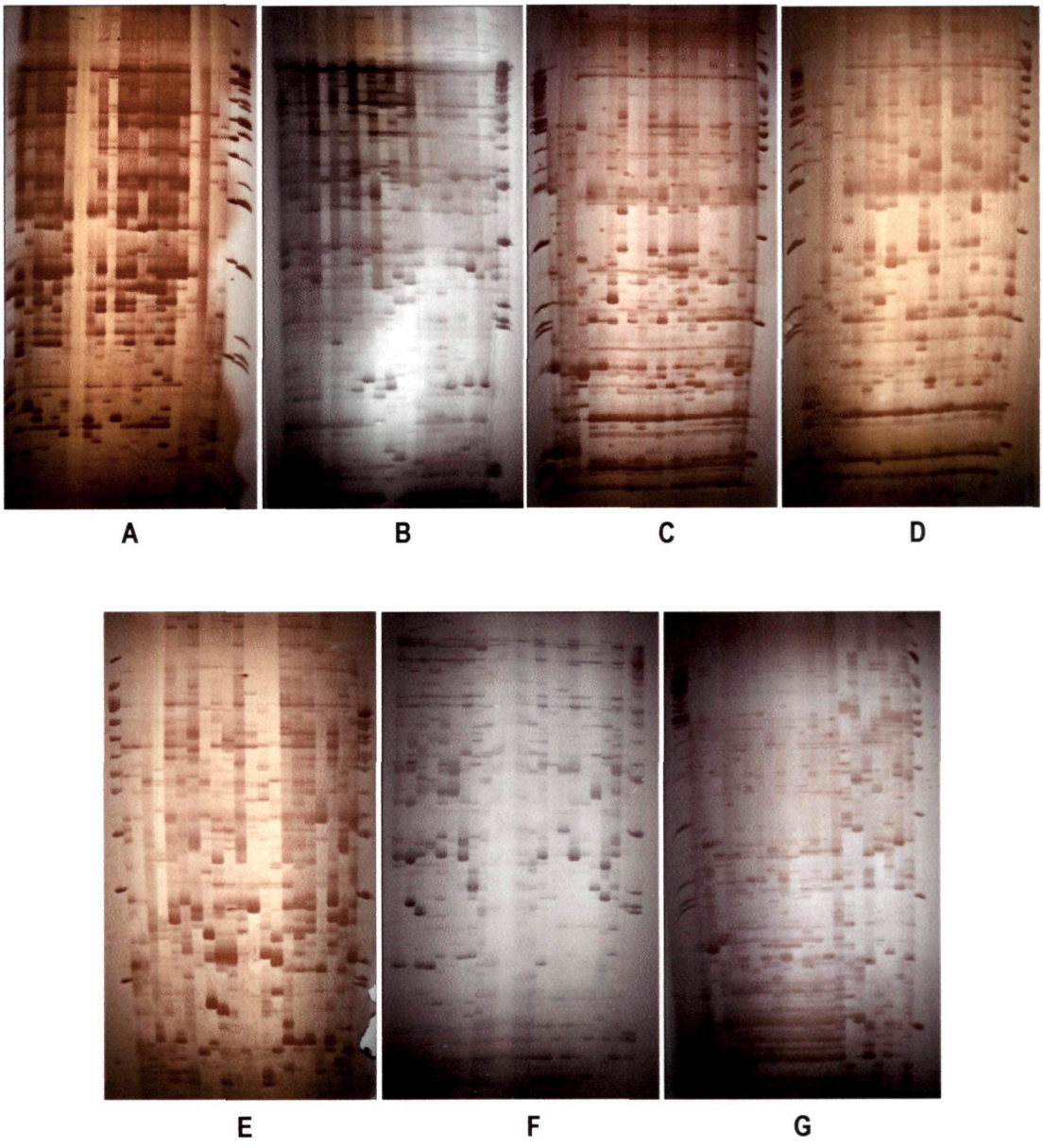

Fig. 4.2.3. Silver stained TE-AFLP profiles generated using E-ACT/P-AAA (gk) primer combination in 117 F1 progenies, TV1 and TV19.

Details for Fig. 4.2.4. (From left to right); M-100bp ladder marker:

A: M; progeny no. 17,16,15,14,13,12,11,10; parents- TV19,TV19,TV1,TV1; progeny no. 9,8,7, 6,5,4,3,2,1; M,M

B: progeny no. 38,34,33,3,30,29,28,27,26; parents- TV19,TV19,TV1,TV1, progeny no. 25,24, 23,22,21,20,19,18; M,--,M

C: M, progeny no. 55,54,53,52,51,50,49,48,47; parents- TV19,TV19,TV1,TV1, progeny no. 46, 45,44,43,42,41,40,39; M

D: Progeny no. 72,71,70,69,68,67,66,65,64; parents- TV19,TV19,TV1,TV1; progeny no. 63, 62,61,60,59,58,57,56; M,M

E: Progeny no. 90,89,88,87,86,--,84,82,81; parents- TV19,TV19,TV1,TV1; progeny no. 80,79, 78,77,76,75,74,73; M, M.

F: Progeny no. 106,105,104,103,102,101,100; parents- TV19,TV19,TV1,TV1; progeny no. 99, 98,97,96,95,94,93,92,91,90; M,M

G: M,--, progeny no. 120,119,118,117,116,114,113,112,111,110,109,108,107,106,105,104; parents- TV19,TV19,TV1,TV1; M

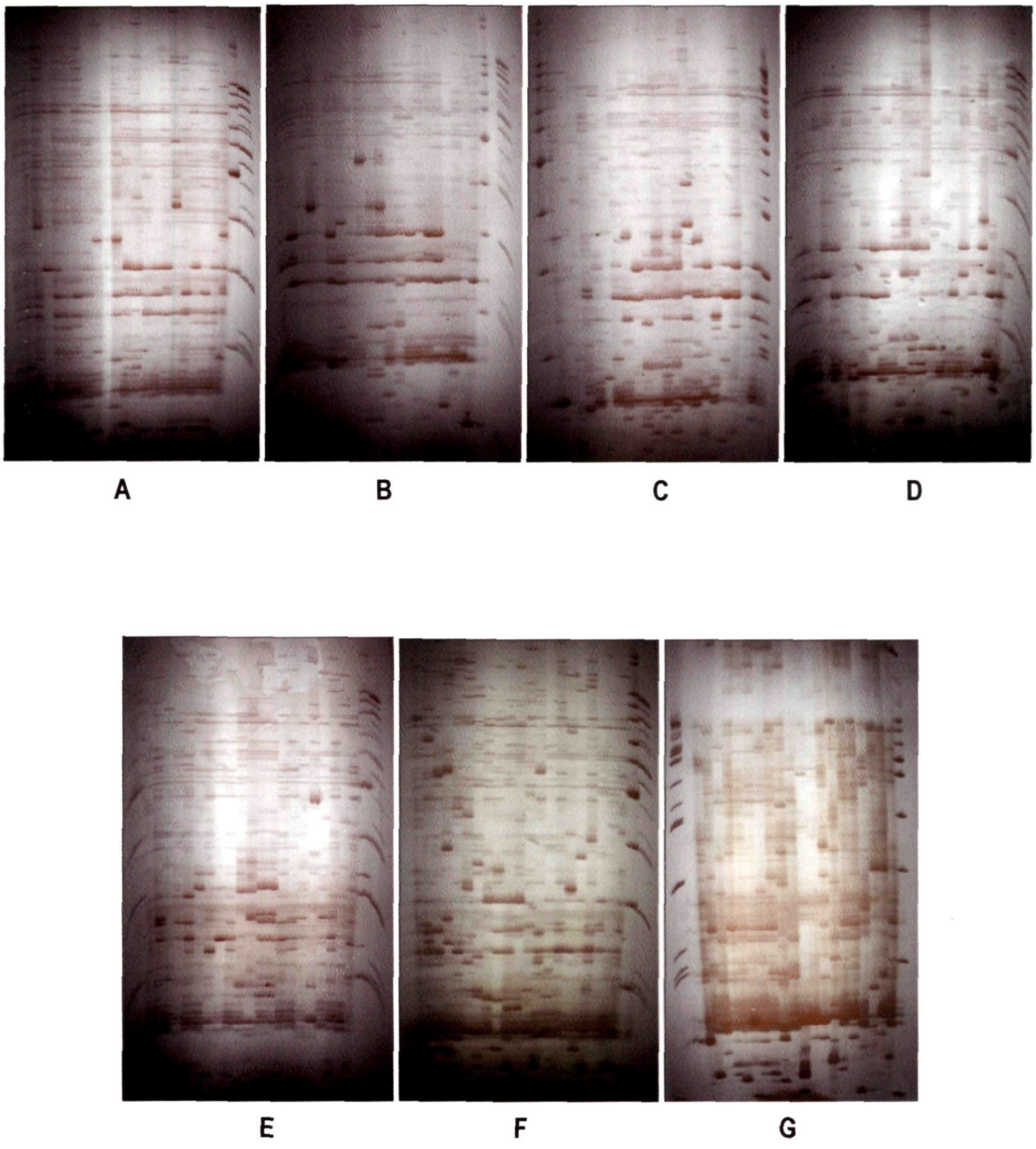

Fig. 4.2.4. Silver stained TE-AFLP profiles generated using E-AAC/P-AAT (hg) primer combination in 117 F1 progenies, TV1 and TV19.

Details for Fig. 4.2.5. (From left to right); M-100bp ladder marker:

A: M; progeny no. 17,16,15,14,13,12,11,10,9,8,7,6,5,4,3,2,1; parents- TV1,TV1,TV19,TV19; M

B: M; progeny no. 34,33,32,31,30,29,28,27,26,25,24,23,22,21,20,19,18; parents- TV1,TV1, TV19,TV19; M

C: M; parents- TV19,TV19,TV1,TV1; progeny no. 51,50,49,48,47,46,45,44,43,42,41,40,39, 38,37,36,35; M

D: M; progeny no. 68,67,66,65,64,63,62,61,60,59,58,57,56,55,54,53,52; parents- TV1,TV1, TV19,TV19; M

E: M, progeny no. 86,--,84,82,81,80,79,78,77,76,75,74,73,72,71,70,69, parents-TV1,TV1, TV19,TV19; M

F: --, Progeny no. 102,101,100,99,98,97,96; parents- TV19,TV19,TV1,TV1; progeny no. 95, 94,93,92,91,90,89,88,87,86; M

G: M; progeny no. 103,104,105,106,107,108,109,110; parents- TV1,TV1,TV19,TV19; progeny no. 111,112,--,113,114,116,117,118,119,120

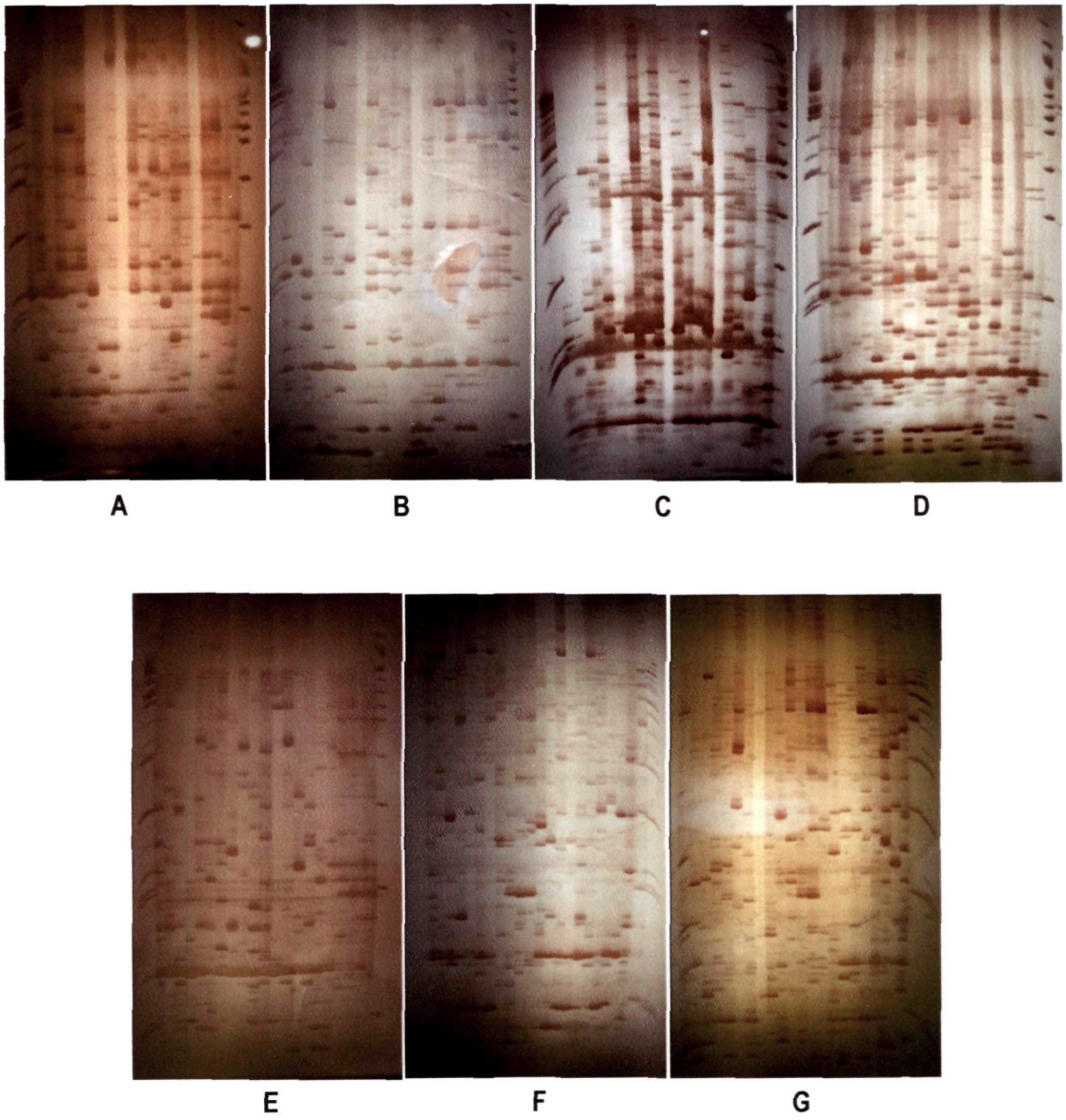

Fig. 4.2.5. Silver stained TE-AFLP profiles generated using E-AAC/P-ATG (hi) primer combination in 117 F1 progenies, TV1 and TV19.

Details for Fig. 4.2.6. (From left to right); M-100bp ladder marker:

A: --,progeny no. 17,16,15,14,13,12,11; parents- TV19,TV19,TV1,TV1; progeny no. 10,9,8, 7,6,5,4,3,2,1; M,M

B: M, progeny no. 34,33,32,31,30,29,28,27,26,25,24,23,22,21,20,19,18; parents- TV1,TV1, TV19,TV19; M

C: --,progeny no. 51,50,49,48,47,46,45; parents- TV19,TV19,TV1,TV1; progeny no. 44,43, 42,41,40,39,38,37,36,35; M,M

D: --,progeny no. 68,67,66,65,64,63,62,61; parents- TV19,TV19,TV1,TV1; progeny no. 60, 59,58,57,56,55,54,53,52; M,M

E: progeny no. 87,86,84,--,82,81,80,79,78; parents- TV19,TV19,TV1,TV1; progeny no. 77, 76,75,74,73,72,71,70,69; M,M

F: M; parents- TV1,TV1,TV19,TV19; progeny no. 100,99,98,97,96,95,94,93,92,91,90,89, 88,87,85,84; M

G: M; parents- TV1,TV19,--,TV1,TV19; progeny no. 120,119,118,117,116,114,113,112,111, 110, 109,108,107,106,105,104; M,--

H: --,--, progeny no. 103,102,101,--; M

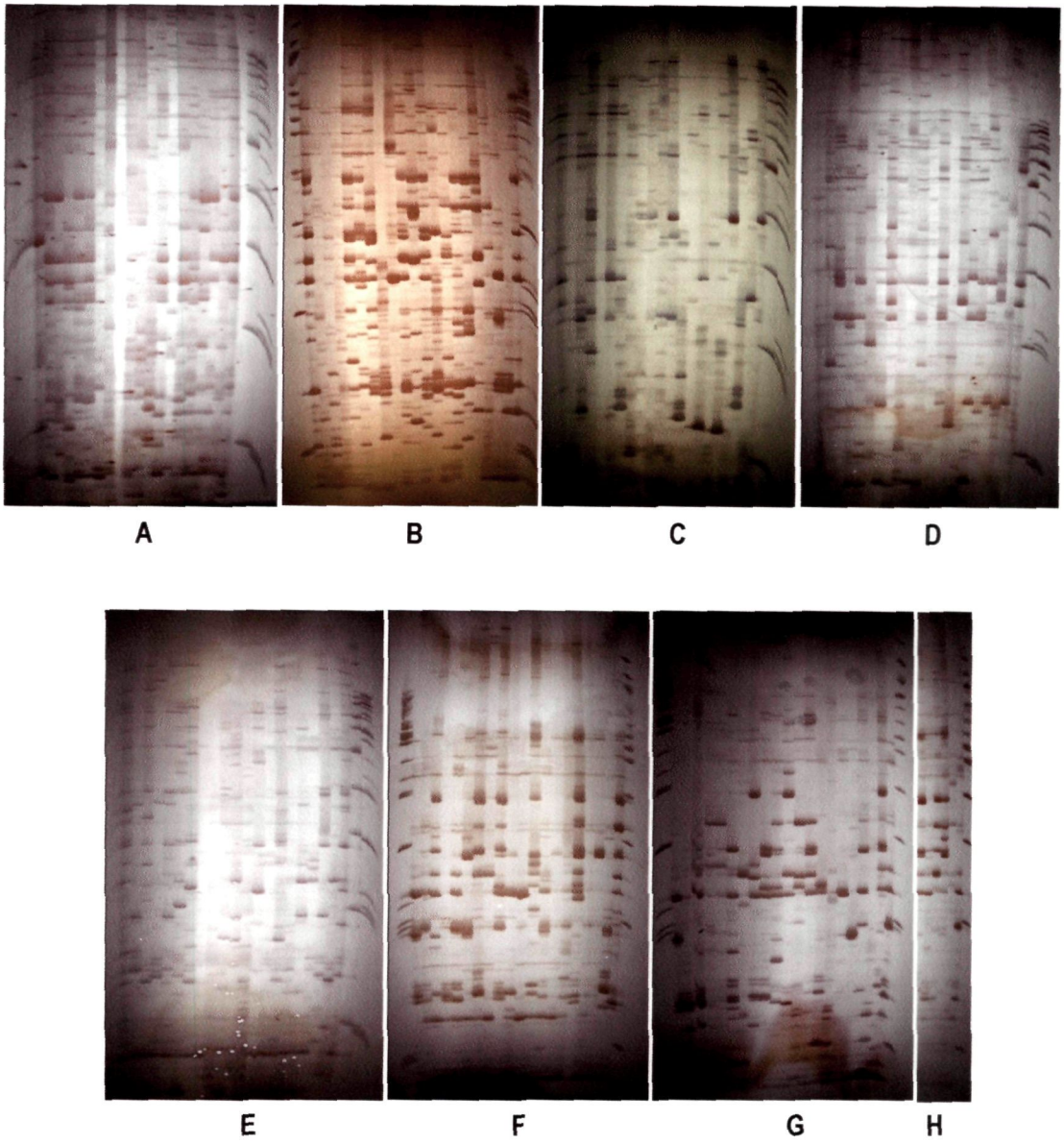

Fig. 4.2.6. Silver stained TE-AFLP profiles generated using E-ACG/P-AAA (kk) primer combination in 117 F1 progenies, TV1 and TV19.

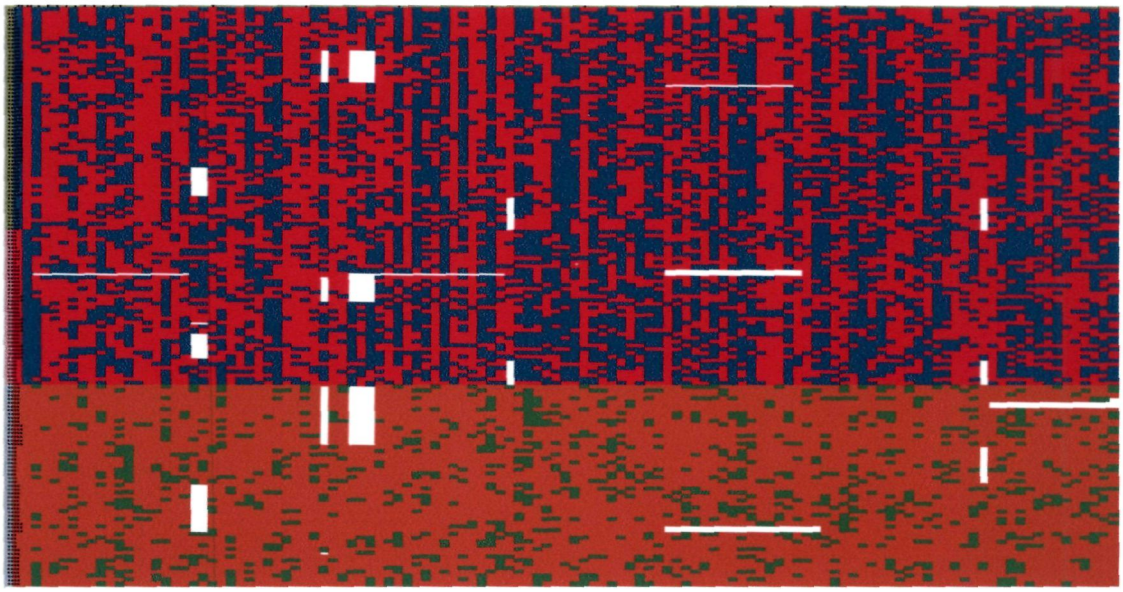

Fig. 4.2.7. Graphical representation of genotyping of all the genotypes used in this study.
Y-axis = 1:1 and 3:1 markers generated by five primer combinations used for linkage study.
X-axis = 117 genotypes and the parents taken for study.
Red and orange colours represent presence of bands for 1:1 and 3:1 markers respectively.
Blue and green colours represent absence of bands for 1:1 and 3:1 markers respectively.
White colour represents the missing data.

Table 4.2.2. AFLP primer combinations employed and polymorphic markers segregation according to 1:1 and 3:1 Mendelian ratios. The letters representing the *Eco*RI and *Pst*I primers respectively are presented in parentheses (columns 1 and 2)

Primer combination		No. of markers generated	TV1	TV19	TV1 and TV19	Distorted markers (TV1+TV19+both parents)
***Eco*RI primer**	***Pst*I primer**		**1:1 marker (a)**	**1:1 marker (a)**	**3:1 marker (b)**	
E-AAC (E-h)	P-ATG (P-i)	27	11	12	4	1+1+1
E-AAT (E-f)	P-CGA (P-h)	26	9	7	10	2+0+5
E-ACG (E-k)	P-AAA (P-k)	40	22	8	10	4+3+3
E-AAC (E-h)	P-AAT (P-g)	32	9	7	16	3+2+9
E-ACT (E-g)	P-AAA (P-k)	28	8	7	13	1+0+6
TOTAL		**153**	**59**	**41**	**53**	**11+6+24=41**
Percentage (%)			**38.56**	**26.80**	**34.64**	**26.80**

Table 4.2.3. Number of 1:1 and 3:1 segregating markers generated by AFLP amplification from the primer pairs tested and scored for linkage analysis.

	Total markers	Type 1:1 Markers	Type 3:1 markers	Distorted markers
Markers scored for linkage analysis	153	100	53	41
Percentage (%)		**65.36**	**34.64**	**26.80**

4.2.1.1. *Markers segregating in a 1:1 Mendelian ratio*

The 5 primer pairs produced a total of 153 bands ranging from 26 (fh) to 40 (kk) per primer pair. On average, each primer combination produced 30 bands. Among the 153 bands, 100 bands were polymorphic (band present in only one parent and absent in the other parent) which accounted for 65.36% (Table 4.2.3). Variation between the primer combinations was evident in the amount of polymorphic fragments generated. The number of polymorphic bands present per primer pair ranged from 15 (gk) to 30 (kk) with an average of 20 bands (Appendix D). Polymorphic loci segregating in a 1:1 ratio were taken for further study, but with exceptionally low band intensity were not included. The number of markers that segregated in the TV1 parent was 59 (38.56%) while 41 (26.80%) markers segregated in the TV19 parent (Table 4.2.2). Segregation distortion remained statistically significant ($P < 0.1$) for 11 (18.6%) markers in the TV1 parent and 6 (14.6%) markers in the TV19 parent.

4.2.1.2. *Markers segregating in a 3:1 Mendelian ratio*

Type 3:1 markers were observed where a band was present in both parents and segregated in the progeny in a 3:1 ratio, which indicated that both parents were heterozygous for the marker. If the loci segregated according to Mendelian expectations ($P = 0.1$), the AFLP marker was considered polymorphic. The number of 3:1 polymorphic markers scored for mapping analysis was 53 (Table 4.2.2) which accounted for 34.64% (Table 4.2.3), segregating through both the parents. On average each primer combination produced 10 markers showing 3:1 ratio. The number of polymorphic markers (3:1 markers) produced per primer pair ranged from 4 (hi) to 16 (hg) with an average of 10 (Appendix D). Initially, of the 53 markers analysed for 3:1 segregation, only 29 segregated according to 3:1 Mendelian expectations at $P = 0.1$. The 3:1 segregating markers were subsequently used to detect any potential homologies between the two parental linkage maps.

Table 4.2.4. The list of loci showing segregating distortions in the mapping population.

Markers	LG[a]	Marker classes[b]					χ^2 value	d.f.	Significance Level[c]	Expected ratio
		ll	lm	nn	np	_				
hia18	TV1-LG8	50	69	0	0	0	3.0	1	*	1:1
hga4	--	47	68	0	0	4	3.8	1	*	1:1
hga15	TV1-LG2	47	68	0	0	4	3.8	1	*	1:1
hga16	--	34	81	0	0	4	19.2	1	*******	1:1
kka3	--	42	63	0	0	14	4.2	1	**	1:1
kka13	--	38	81	0	0	0	15.5	1	*******	1:1
kka15	TV1-LG3	69	50	0	0	0	3.0	1	*	1:1
kka30	--	46	73	0	0	0	6.1	1	**	1:1
gka11	TV1-LG5	49	68	0	0	2	3.1	1	*	1:1
fha13	--	45	72	0	0	2	6.2	1	**	1:1
fha14	--	35	82	0	0	2	18.9	1	*******	1:1
hia19	--	0	0	44	75	0	8.1	1	****	1:1
hga3	--	0	0	38	77	4	13.2	1	******	1:1
hga17	--	0	0	31	84	4	24.4	1	*******	1:1
kka2	--	0	0	46	73	0	6.1	1	**	1:1
kka11	TV19-LG9	0	0	69	50	0	3.0	1	*	1:1
kka27	--	0	0	47	72	0	5.3	1	**	1:1

a LG: Linkage group, --: unlinked.

b The segregation types were according to the manual of JoinMap 4.0 (van Ooijen 2006).

c Significance levels: *0.1; **0.05; ***0.01; **** 0.005; ***** 0.001; ****** 0.0005; ******* 0.0001.

Table 4.2.5. Markers not conforming to the 1:1 and 3:1 Mendelian segregation ratio.

	Total markers	Total distorted markers	Distorted markers (0.05<P<0.1)	Distorted markers (0.01<P<0.05)	Highly distorted markers (P<0.01)	Homozygous excess (+)	Homozygous deficiency (-)
Parent 1 (TV1)	59	11	5	3	3	1	10
Parent 2 (TV19)	41	6	1	2	3	1	5
Both parents (3:1 markers)	53	24	6	2	16	0	24
Total	**153**	**41**	**12**	**7**	**22**	**2**	**39**

4.2.1.3. *Segregation distortion*

Forty eight and 35 heterozygous markers segregated in 1:1 ratio (at the $P > 0.1$ level) in TV1 and TV19 parents respectively, while the rest (i.e. 11 and 6 respectively) were distorted from 1:1 ratio. Out of 53 markers, 24 were found to deviate from 3:1 Mendelian ratio in both the parents. In TV1 parent, out of 11 distorted markers 3 markers were highly distorted ($P < 0.01$) while in TV19 parent, out of 6 distorted markers 3 markers were highly distorted ($P < 0.01$) (Table 4.2.5). While for those markers that deviate from 3:1 ratio, out of 24 distorted markers 16 markers were highly distorted ($P < 0.01$). Loci that showed statistically significant segregation distortion from Mendelian ratio, consisted primarily of homozygote deficiency of 39 markers compared to homozygote excess (no bands) of 2 bands (Table 4.2.5).

4.3. LINKAGE MAPPING

Since tea is outcrossing crop, a pseudo test cross approach was adopted to generate linkage map. Linkage map construction was performed with JoinMap 4.0 which can auto-detect the linkage phase from the marker data. The cross-pollinated (CP) population type function and the marker data were scored according to JoinMap 4.0. For AFLP, lm x ll, nn x np, and hk x hk were used to score the segregation types of locus heterozygous in female parent, locus heterozygous in male parent, and locus heterozygous in both parents (Table 4.3). Mendelian segregation of the markers was tested by chi-square tests. Highly distorted 1:1 and 3:1 markers ($P < 0.01$) were discarded from further analysis. The maps generated are the arrangement of markers corresponding to the best log-likelihood scores.

Table 4.3. Segregation types code for population type CP and marker genotype codes depending on the locus segregation type (according to JOINMAP 4.0).

Segregation type code	*Description*	*Possible genotypes*
<lm x ll>	Locus heterozygous in the first parent	lm, ll, --
<nn x np>	Locus heterozygous in the second parent	nn, np, --
<hk x hk>	Locus heterozygous in both parents, two alleles	hh, hk, kk, h-, k-, --

h- and k- are dominant genotypes:
h- means either hh or hk
k- means either kk or hk
"-" means unknown allele (e.g. no information on that particular genotype)

For both maps, the LOD threshold was set at an initial LOD score of two with an increasing stringency of one increment to a final threshold of 10, to group the markers into linkage groups. To determine marker order within a linkage group, the following JoinMap parameter settings were used: Rec = 0.40, LOD = 1.0 and Jump = 5. Map distance in centimorgans was calculated with Kosambi's mapping function (Kosambi 1944). The linkage map was drawn using the Map Chart 2.1 software (Voorrips 2002) which is inbuilt in JoinMap 4.0.

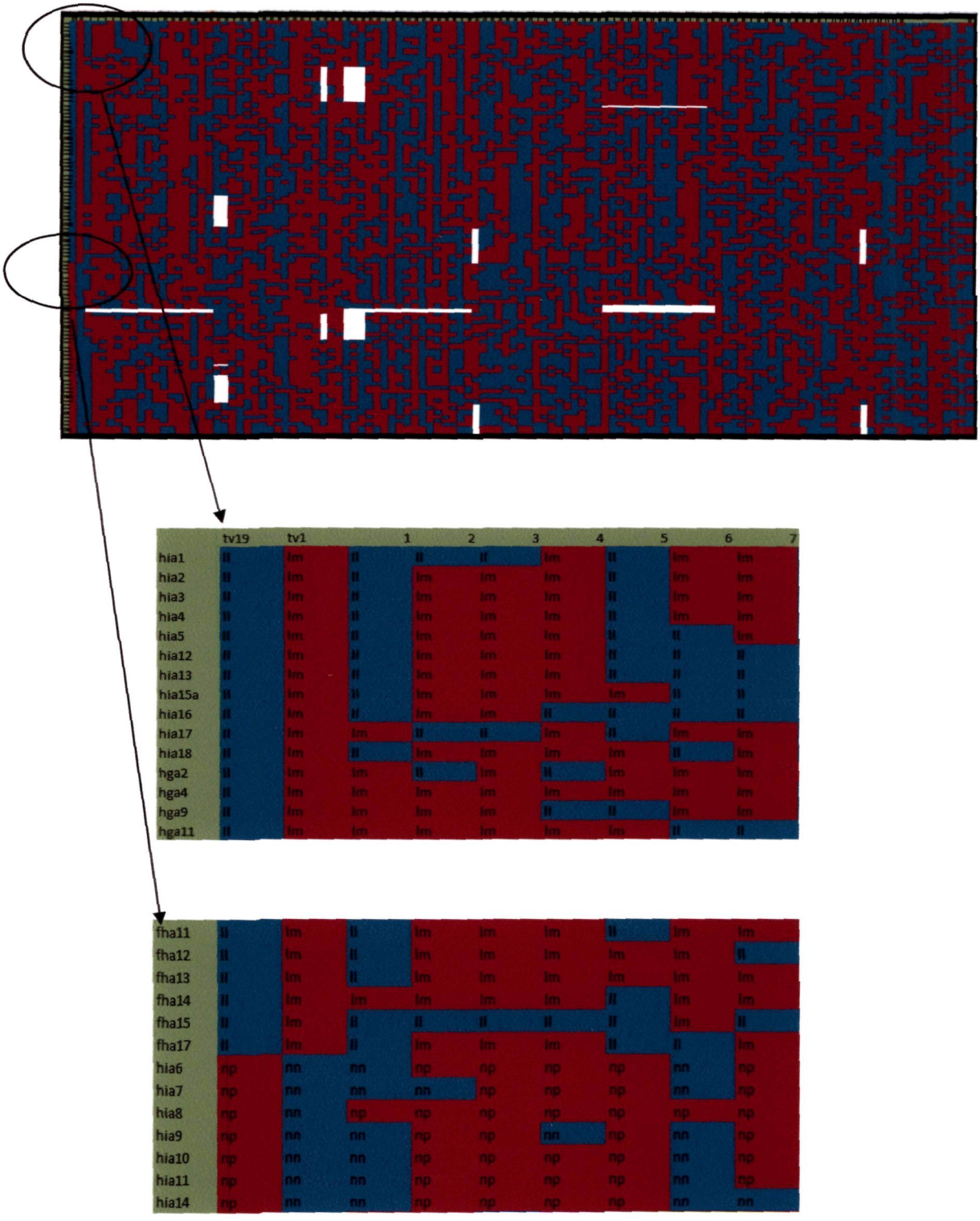

Fig. 4.3. Graphical representation of genotyping of all the genotypes used in this study. Y-axis = 1:1 markers generated by five primer combinations used for linkage study. X-axis = 117 genotypes and the parents taken for study. Red represents presence of bands. Blue represents absence of bands. White represents the missing data.

4.3.1. Parent TV11:1segregating markers linkage map

Out of 59 markers (type 1:1 marker) scored, 3 markers which were highly distorted ($P < 0.01$) from Mendelian ratio were excluded from linkage analysis. Therefore, 56 markers including 8 marginally distorted markers (deviating significantly from the 1:1 Mendelian ratio at $P = 0.1$ level; i.e. $P < 0.1$ and $P > 0.05$) were included in linkage mapping analysis.

Table 4.3.1. Length, number of markers and average spacing of linkage groups of TV1 parent map established with JOINMAP 4.0

Linkage group	Length (cM)	No. of markers	Average marker spacing (cM)	Largest interval (cM)	Smallest interval (cM)
1	87.7	14	6.8	15.4	3.6
2	38.2	3	19.1	38.2	20.0
3	28.3	2	28.3	28.3	--
4	21.8	2	21.8	21.8	--
5	25.5	2	25.5	25.5	--
6	28.9	2	28.9	28.9	--
7	62.8	4	20.9	21.3	20.2
8	41.3	3	20.7	20.8	20.6
9	26.1	2	26.1	26.1	--
10	28.3	2	28.3	28.3	--
Total	**388.9**	**36**	**226.4**	**254.6**	**64.4**
Average	**38.89**	**3.6**	**22.64**	**25.46**	**16.1**

The map of the TV1 parent consisted of 36 framework markers (LOD 3) and no accessory markers (LOD 2) in ten linkage groups, including six doublets (Fig.4.3.1 and pairwise recombination frequency and LOD score table in Appendix E1). There were 20 unlinked markers. The linkage groups ranged in length from 21.8 cM (LG-4) to 87.7 cM (LG-1), with an average length of 38.89 cM. The average distance

between the markers (framework and accessory markers) was 22.64 cM (Table 4.3.1). The 10 linkage groups including the six doublets covered a total map length of 388.9 cM. The largest interval between two markers was 38.2 cM of the LG-2 while the smallest interval was 3.6 cM of the LG-1 (Appendix F1). The number of markers varied from 2 to 14 per linkage group, with an average of 3.6 markers per linkage group (Table 4.3.1). Out of the 36 mapped markers in TV19 parent, 4 were distorted markers.

4.3.2. Parent TV19 1:1 segregating markers linkage map

Out of 41 markers (type 1:1 marker) scored, 3 markers which were highly distorted ($P < 0.01$) from Mendelian ratio were excluded from linkage analysis. Therefore, 38 markers including 3 marginally distorted markers (deviating significantly from the 1:1 Mendelian ratio at $P = 0.1$ level; i.e. $P < 0.1$ and $P > 0.05$) were included in linkage mapping analysis.

The linkage map of TV19 parent consisted of 27 framework markers (LOD 3) and 3 accessory markers (LOD 2) in 11 linkage groups, of which five were doublets (Figure 4.3.2 and pairwise recombination frequency and LOD score table in Appendix E2). There were 8 unlinked markers. The linkage groups ranged in length from 2.6cM (LG-5) to 62 cM (LG-1), with an average length of 37.34 cM. The linkage groups including the five doublets covered a total map length of 410.7 cM. The largest interval between two markers was 30.1cM of LG-9 while the smallest interval was 2.1 of LG-11 (Appendix F2). The number of markers per linkage group ranged from 2 to 5, with an average of 2.73 markers per linkage group. Out of the 30 mapped markers in the TV19 map, only one marker was distorted.

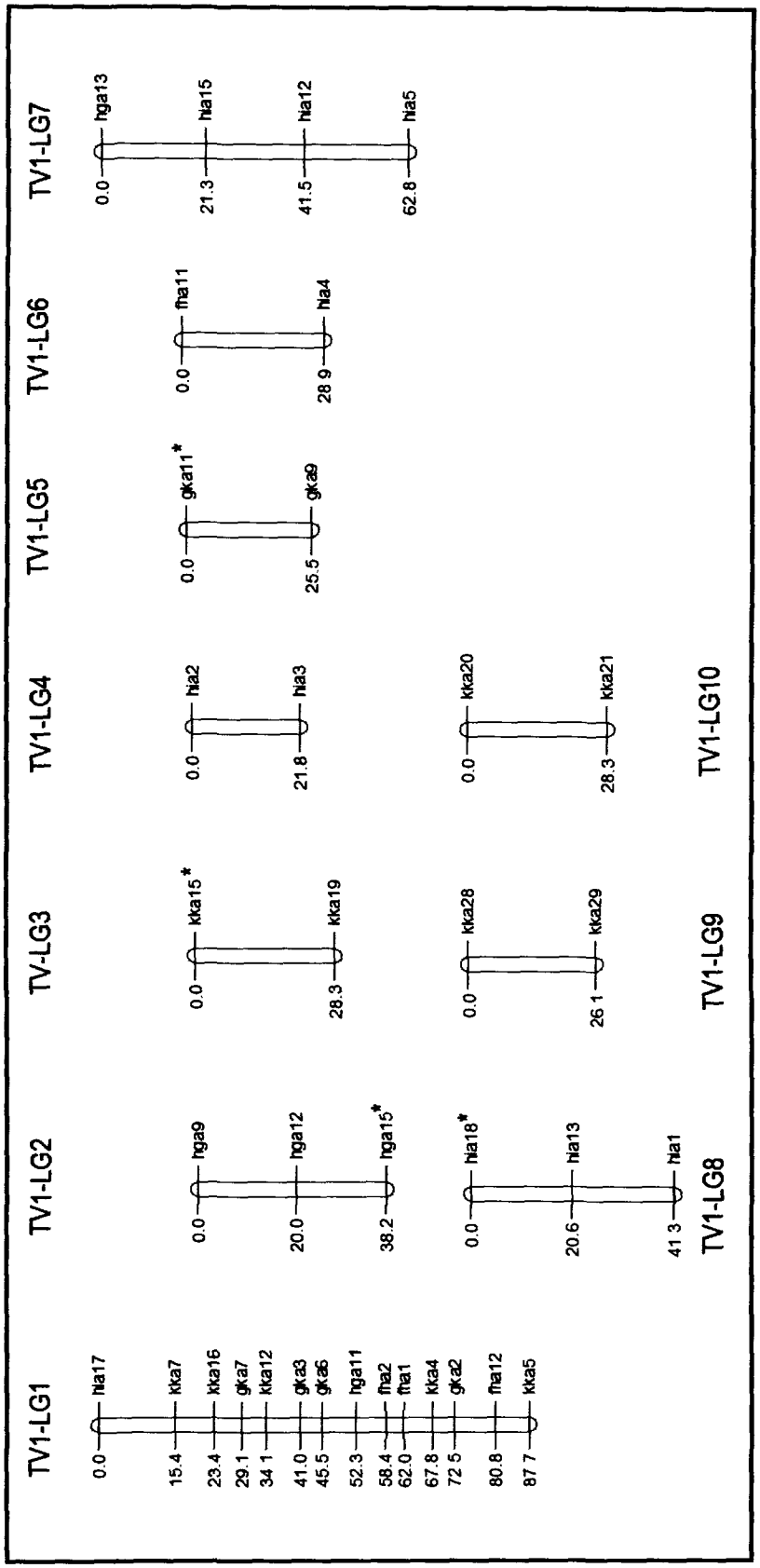

Fig. 4.3.1. Genetic linkage map of TV1 parent with markers indicated on the right and genetic distances (in Kosambi cM) on the left. Distorted markers are marked with asterisks (*). * ($P < 0.1$)

Table 4.3.2. Length, number of markers and average spacing of linkage groups of TV19 parent map established with JOINMAP 4.0.

Linkage group	Length (cM)	No. of markers	Average marker spacing (cM)	Largest interval (cM)	Smallest interval (cM)
1	62.0	5	15.5	20.2	10.7
2	45.7	3	22.9	23.6	22.2
3	47.0	3	23.5	24.3	22.7
4	46.8	3	23.4	26.8	20.0
5	2.6	2	2.6	2.6	--
6	26.6	2	26.6	26.6	--
7	27.2	2	27.2	27.2	--
8	26.1	2	26.1	26.1	--
9	30.1	2	30.1	30.1	--
10	45.2	3	22.6	25.3	20.0
11	51.4	3*	25.7	27.3	2.1
Total	**410.7**	**30**	**246.2**	**260.1**	**97.7**
Average	**37.34**	**2.73**	**22.4**	**23.3**	**16.3**

* Accessory markers (LOD 2)

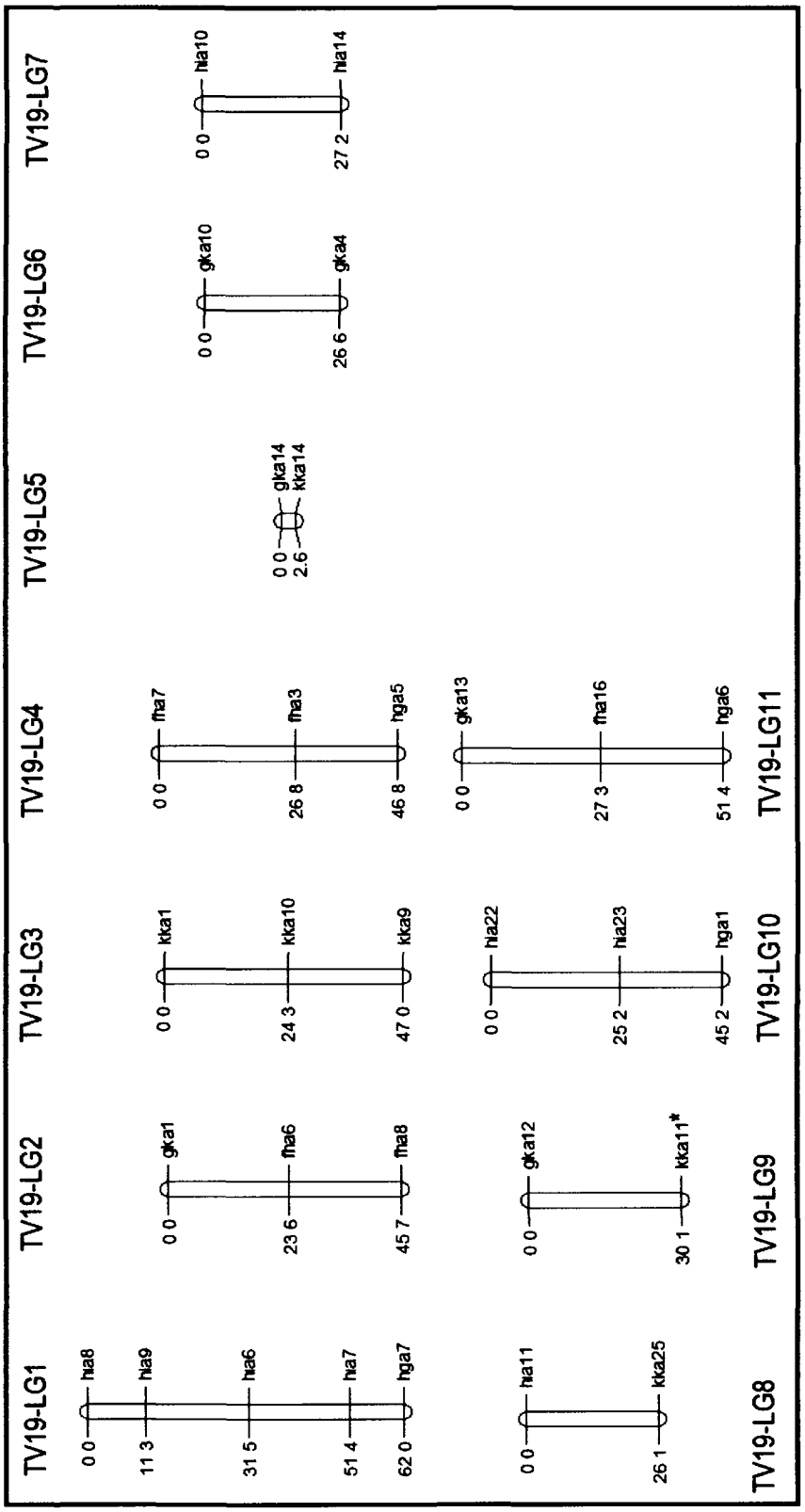

Fig. 4.3.2. Genetic linkage map of TV19 parent with markers indicated on the right and genetic distances (in Kosambi cM) on the left. Distorted markers are marked with asterisk (*). * ($P < 0.1$)

4.3.3. Segregation distortion

A χ^2 test (d.f.=1) was performed to test the null hypothesis of a 1:1 segregation ratio of the marker alleles. At 10% significance level (P = 0.1), 11 (26.83%) and 6 (14.63%) of the markers analyzed were distorted for the TV1 and TV19, respectively.

Three markers belonging to either parent, deviating at 1% significance level ($p<0.01$) were excluded from the linkage analysis because of a major risk of technical artifacts. Therefore, for the TV1 parent data, 8 distorted markers ($0.05 < P < 0.1$) were included in the mapping data set and 4 of them were mapped, whereas 3 distorted markers ($0.05 < P < 0.1$) were included in the TV19 mapping data set and only one of them was mapped. The four distorted markers in TV1 parent were distributed in 4 linkage groups (LG-2, LG-3, LG-5, LG-8) while in TV19 parent one distorted marker was present in the linkage group LG-9 (Fig. 4.3.1. and Fig. 4.3.2.)

4.3.4. Map comparison

The data for the 3:1 segregating markers were later integrated into the analysis to compare the parental linkage maps. Two parental maps based on 1:1 and 3:1 markers were built using JOINMAP 4.0. Out of 53 markers (type 3:1 marker) scored, 16 markers which were highly distorted ($P<0.01$) from Mendelian ratio were excluded from linkage analysis. Therefore, 37 markers including 8 marginally distorted markers (deviating significantly from the 3:1 mendelian ratio at P = 0.1 level; i.e. $P < 0.1$ and $P > 0.05$) were included in linkage mapping analysis.

The intercross markers (3:1 markers) were mapped in 12 linkage groups of the parental maps i.e. 6 and 6 linkage groups in TV1 and TV19 parents respectively. Out

of these 6 linkage groups, linkage groups LG7 and LG12 from TV1 while LG17 from TV19 were comprised of both 1:1 and 3:1 markers, while the remaining linkage groups comprised of 3:1 markers only. The bridging marker were clustered among them and not linked with any markers specific to either parents (1:1 markers) which was uninformative for merging linkage groups (Fig. 4.3.3.3.). Since there is no common marker to merge any linkage groups, the maps could not be combined into a single integrated linkage map.

4.3.5. Marker distribution

The correlation between the number of markers and the length (size) of the linkage groups was analysed using the Pearson correlation coefficient (Yu and Guo, 2003; Wang *et al.*, 2004; Baranski *et al.*, 2006). Thereafter a *t*-test was applied to test the significance of correlation coefficient at the $P = 0.1$ level, using the null hypothesis (H_0) of no correlation.

For TV1 parent, Pearson correlation coefficient was calculated as 0.9098 and the calculated $t = 6.24 > t\ 0.1$ which reject the null hypothesis (H_0) of no correlation. For the TV19 map also, the correlation was significant ($r = 0.8266$, $t = 4.47 > t\ 0.1$). The Pearson correlation coefficient ($r = 0.91$ and $r = 0.83$) indicated the presence of a highly significant positive correlation between the number of markers and linkage group size.

The distribution of the mapped AFLP markers was also classified according to the markers generated by 5 primer combinations used for analysis by visual examination of the linkage groups (Fig. 4.3.4.1 and Fig. 4.3.4.2.).

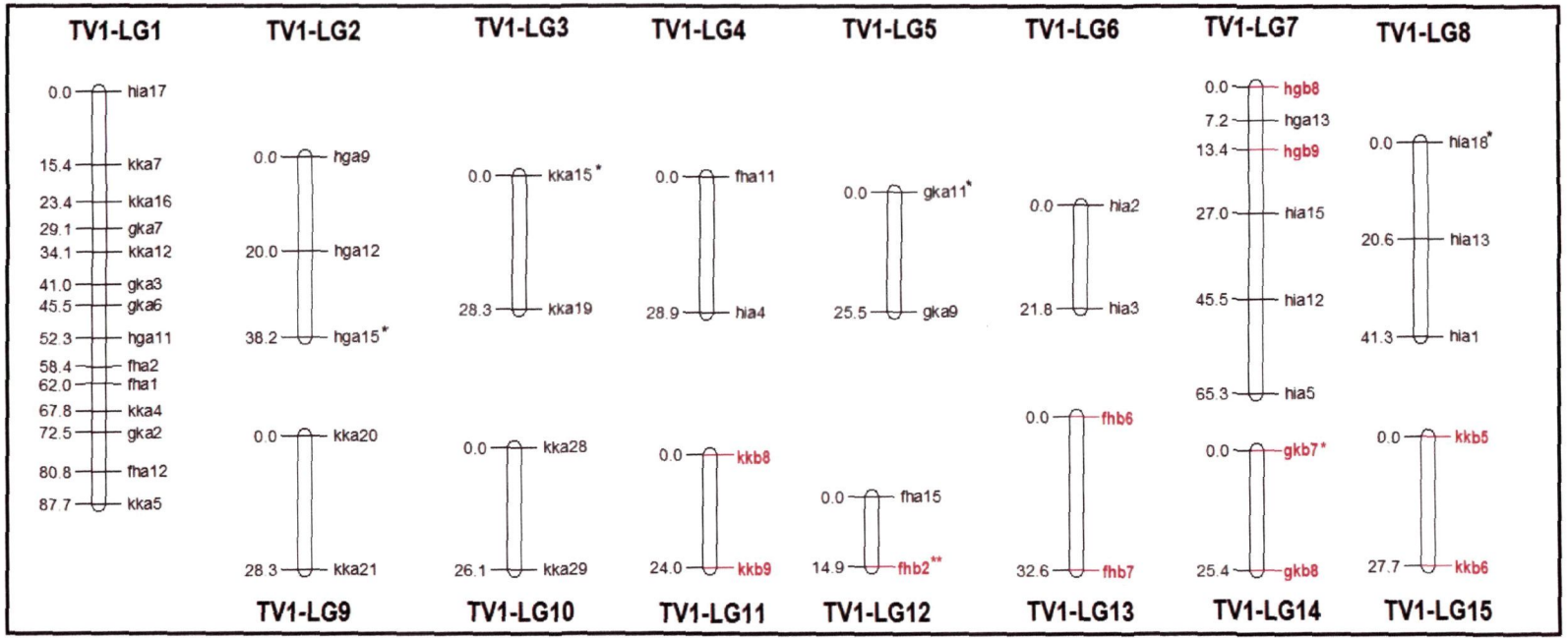

Fig. 4.3.3.1. Genetic linkage map of TV1 parent with markers indicated on the right and genetic distances (in Kosambi cM) on the left. Distorted markers are marked with asterisks (*). * ($P < 0.1$); **($P<0.05$)

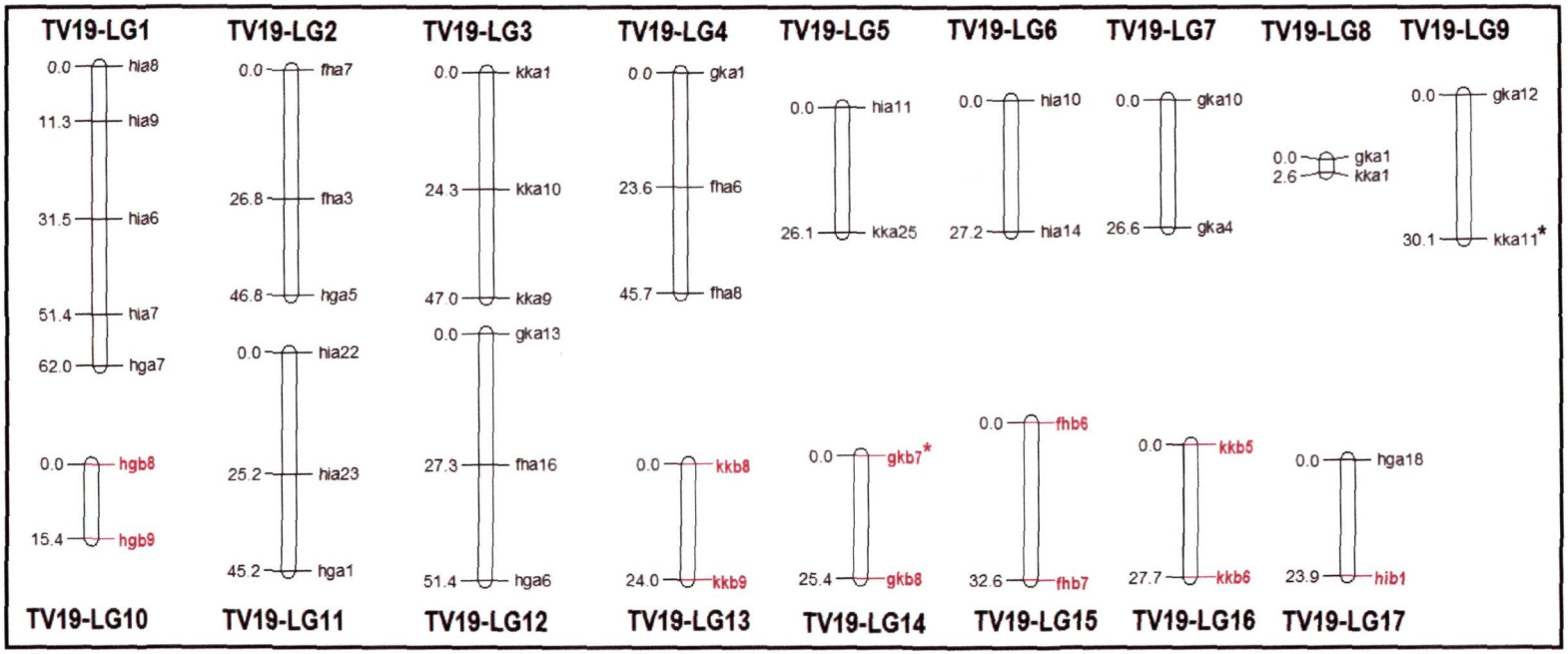

Fig. 4.3.3.2. Genetic linkage map of TV19 parent with markers indicated on the right and genetic distances (in Kosambi cM) on the left. Distorted markers are marked with asterisks (*). * ($P < 0.1$); **($P<0.05$)

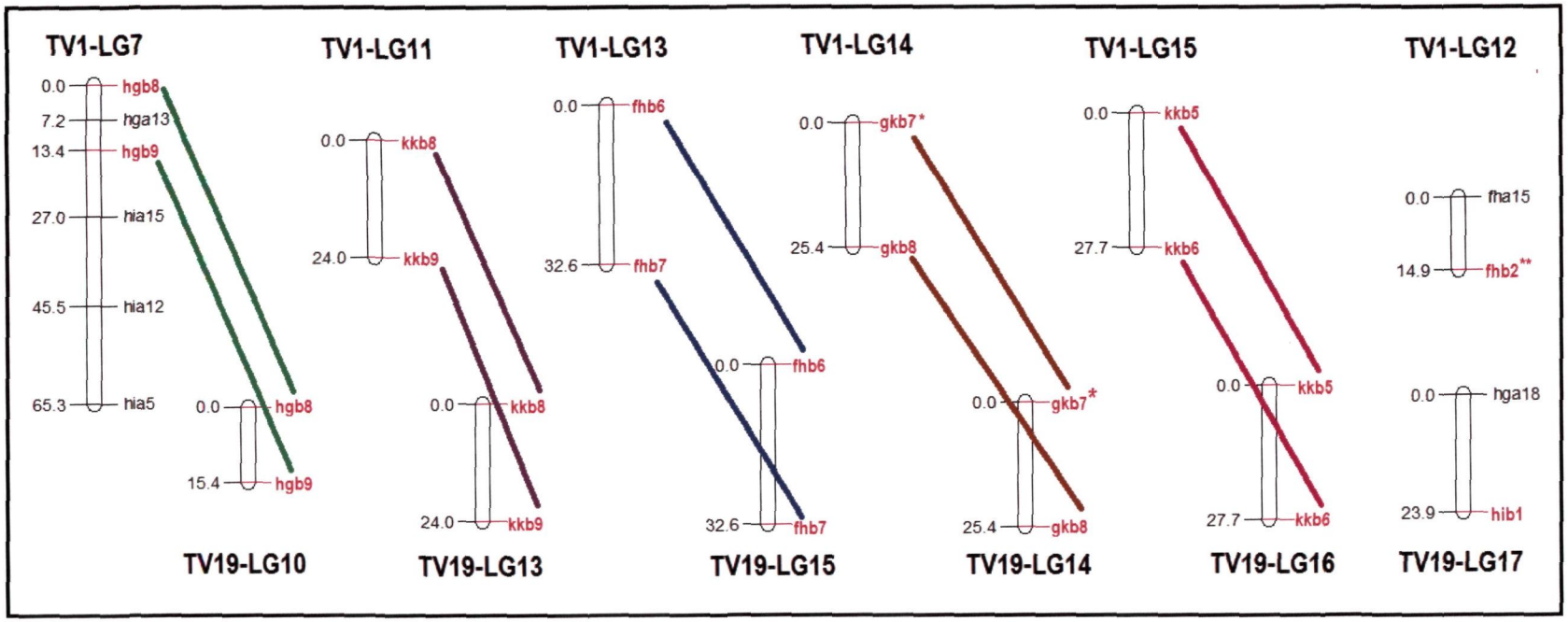

Fig. 4.3.3.3. Comparison of different linkage groups from TV1parent and TV19 parent which have the same 3:1markers from two parental maps. Distorted markers are marked with asterisks (*). * ($P < 0.1$); **($P<0.05$) The colour lines showed the common 3:1 markers between linkage groups.

Table 4.3.3. AFLP markers distribution on linkage groups according to their primer combination.

Primer combination		TV1 parent	TV19 parent
*Eco*RI primer	*Pst* I primer		
E-AAC (E-h)	P-ATG (P-i)	10	9
E-AAT (E-f)	P-CGA (P-h)	4	5
E-ACG (E-k)	P-AAA (P-k)	11	6
E-AAC (E-h)	P-AAT (P-g)	5	4
E-ACT (E-g)	P-AAA (P-k)	6	6

In TV1 parent, markers generated by primer combinations E-ACG/P-AAA (kk) and E-AAC/P-ATG (hi) resulted in the higher number of mapped markers in the linkage groups than the other primer combinations (Table 4.3.3). Primer combination E-AAT/P-CGA (fh) was the lowest with only four markers mapped on the linkage groups. All linkage groups except LG1, LG6 and LG7 of TV1map were composed of markers derived from same primer combination. For TV19 parent, primer combination AAC/P-ATG (hi) was the most informative as it contributed the largest number of markers. However the rest of the primer combinations contributed more or less the same amount of markers to linkage groups for TV19 map (Table 4.3.3). Linkage groups LG3, LG6, LG7 were composed of only markers derived from primer combinations E-ACG/P-AAA (kk), E-ACT/P-AAA (gk), AAC/P-ATG (hi) respectively. While the other linkage groups were composed of markers derived from more than one primer combination.

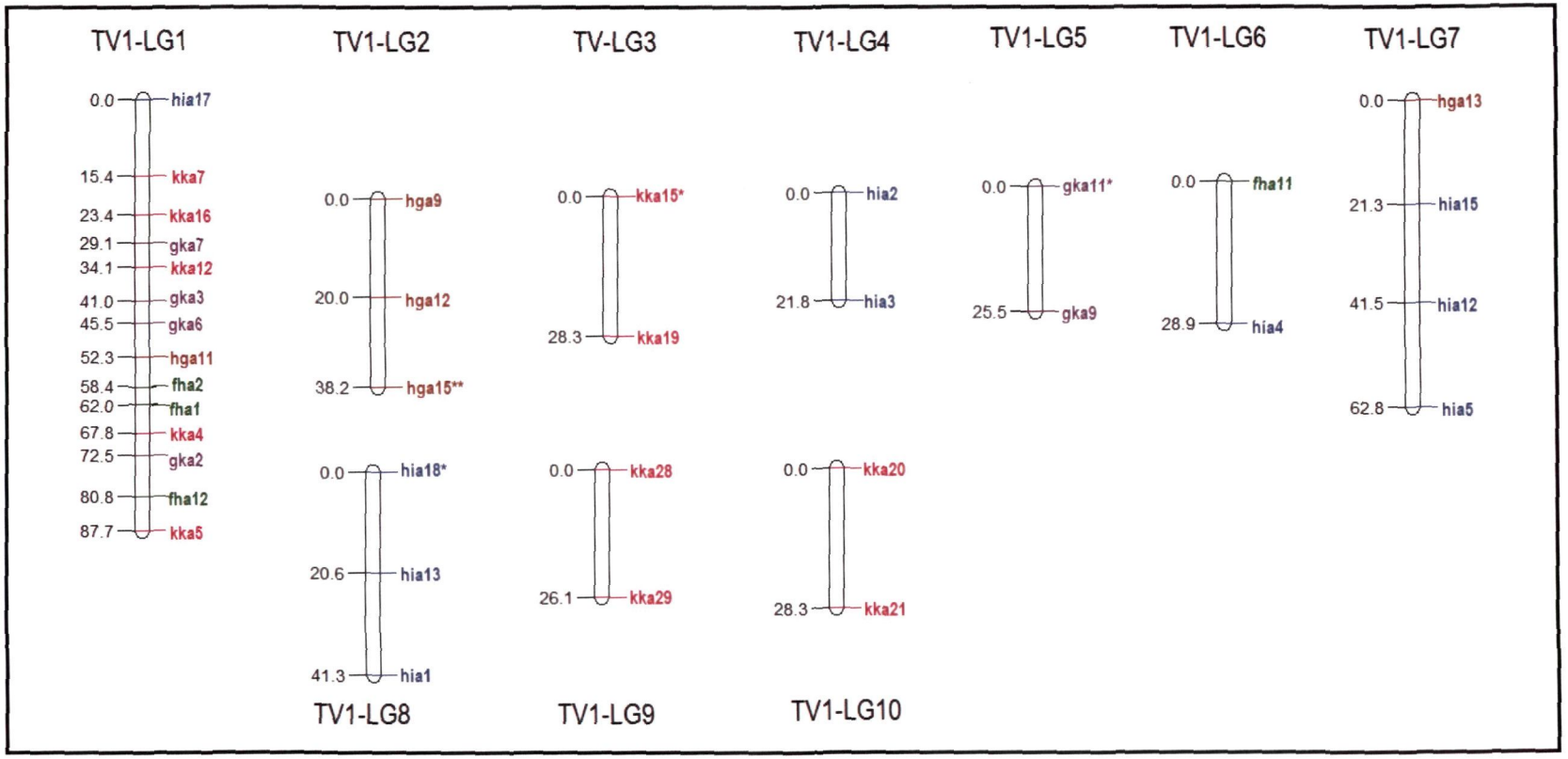

Figure 4.3.4.1. Marker distribution in the TV1 map. Colour scheme used to indicate AFLP markers generated by the same primer combination.

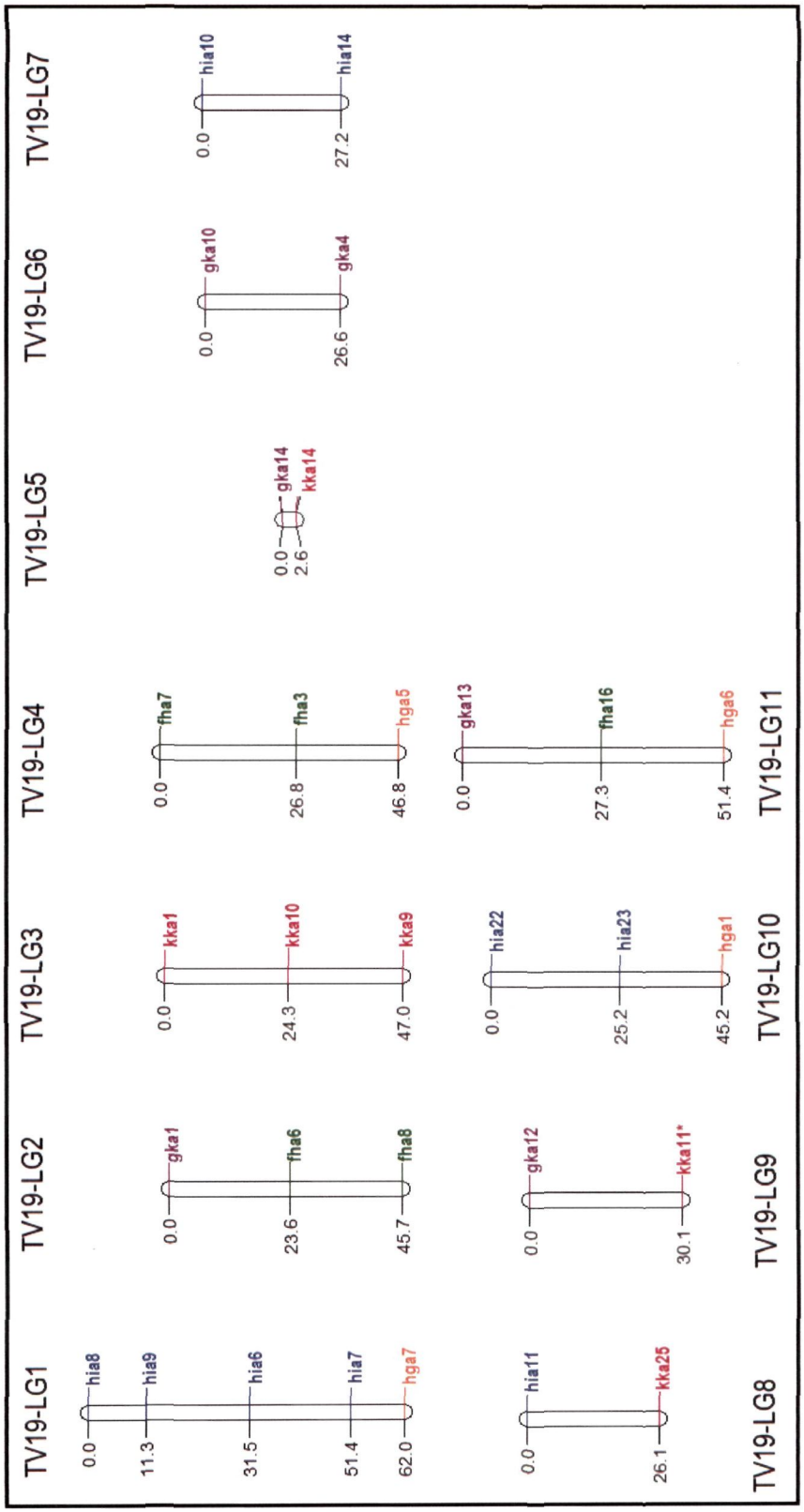

Figure 4.3.4.2. Marker distribution in the TV19 map. Colour scheme used to indicate AFLP markers generated by the same primer combination.

4.3.6. Genome coverage

Observed genome lengths were calculated as the total length of all the linkage groups considering all markers i.e. 388.9 cM and 410.7 cM for TV1 and TV19 maps respectively. Expected genome lengths (G_e) was estimated by the two methods described by Fishman *et al.* (2001) and method 4 as described by Chakravarti *et al.* (1991) (i.e. G_{e1} and G_{e2}) using all the mapped 1:1 segregating markers (Table 4.3.4).

Table 4.3.4. Map length and genome coverage of both the parental maps.

Map length	***1:1 markers***	
	TV1 map	**Tv19 map**
Observed length		
G_o (cM)	388.9	410.7
Expected length		
G_{e1} (cM)	688.9	885.9
G_{e2} (cM)	843.3	903
G_e (average)	766.1	894.5
Genome coverage (G_o/G_e) (%)	**50.76**	**45.91**

For the G_{e1} estimate (Fishman *et al.*, 2001), the average marker spacing of the maps (s) was determined for all the markers: 15 cM for the TV1 parent and 21.6 cM for the TV19 parent. The estimated genome lengths, G_{e1} and G_{e2}, using all the markers were 688.9 cM and 843.3 cM respectively for the TV1 parent and 885.9 cM and 903 cM respectively for the TV19 parent. The genome lengths (G_e) estimated by the two

methods (G_{e1} and G_{e2}) were dissimilar when determined using all the mapped 1:1 segregating markers (Tables 4.3.1 and 4.3.2). So, the average of these two estimates (G_{e1} and G_{e2}) was used as the expected genome length (G_e): 766.1 cM for the TV1 parent and 894.5 cM for the TV19 parent (Table 4.3.4.). The observed genome coverage was determined by G_o/G_e i.e. 50.76% and 45.91% for TV1 and TV19 respectively.

4.4. NUCLEOTIDE SEQUENCE ANALYSES

4.4.1. Nucleotide sequence BLAST search

Nucleotide sequences obtained were BLAST searched individually with three different databases on NCBI GenBank (National Center for Biotechnology Information) and the hits were filtered on the basis of e-value. The selected threshold e-value of 1e-04 was taken for all database and the hits with e-value more than that were considered as non-homologous. The hits below the threshold limit were analyzed to determine the homology of the query sequence with already known genes in the GenBank database. Sequences with high quality were selected and analyzed.

Nucleotide sequences obtained were queried (BLASTn) individually with

a) non redundant (nr/nt) nucleotide database of dicot plants (taxid 71240) on NCBI GenBank (National Center for Biotechnology Information).
b) Whole genome shot gun sequences databases of *Arabidopsis* (taxid 3702) and other dicot plants (taxid 71240) from the public databases NCBI GenBank (National Center for Biotechnology Information).
c) The identical results from keyword searches and BLAST searches can be used to predict that these genes could be expressed in *C. sinensis*. To discover new

genes, all searched unigenes were analyzed by BLAST alignments against the existing tea uniEST database of *Camellia sinensis* (taxid 4442), *Camellia sinensis* var. *sinensis* (taxid 542762), *Camellia assamica* / *Camellia sinensis* var. *assamica* (taxid 261999).

In TV1 parent, a total of 56 markers were used for map construction, of which 36 were mapped and 20 were unlinked markers. Out of 36 mapped TV1 markers, 16 had very low sequence quality, so they were excluded from further sequence analysis. Therefore, 22 mapped TV1 markers were subjected to NCBI BLAST analysis to find out any homology with already known sequences in NCBI GenBank. Out of 22 mapped TV1 markers, 10 markers showed no homology with any known sequence while 12 markers showed homology with the known sequences, of which three were unigenes (Table 4.4.1). Out of 20 unlinked markers, 7 sequences were of bad quality, so 13 sequences were subjected to NCBI BLAST analysis. The result was that 4 sequences showed homology with the known sequences of which two were unigenes. 9 other unlinked markers showed no homology with any sequence.

In TV19 parent, a total of 38 markers were used for map construction, of which 30 were mapped and 8 were unlinked markers. Out of 30 mapped markers, 10 had very low sequence quality. Therefore, 20 mapped TV19 markers were subjected to NCBI BLAST analysis to find out any homology with already known sequences in the three selected data bases of NCBI GenBank.

Out of 20 mapped markers, 7 markers showed no homology with any known sequence while 12 markers showed homology with the known sequences, of which 7 were unigenes (Table 4.4.2). Out of 8 unlinked markers, 4 sequences were of bad

quality, so 4 sequences were subjected to NCBI BLAST analysis. The result was that only one sequence showed homology with the known sequences and 3 sequences showed no homology with any sequence.

4.4.2. Finding ORF using ORF finder (NCBI) for some selected sequences which showed alignment with EST of tea database.

As complete genome has not been sequenced for any of the tea species yet, the trimmed nucleotide sequences were also queried against EST database of tea to find the homologous ESTs. Hits showing e-value above threshold of 1e-04 were considered as novel EST's of tea.

Using the above searches and analyzing the homology, 4 mapped sequences were selected that showed homology with the ESTs of tea. One aspect of the analysis of an unknown DNA sequence is the identification of protein coding regions, also known as open reading frames (ORFs). Therefore, nucleotide sequences that aligned to the tea EST database were subjected to ORF finder tool (NCBI). The sequences were translated in all possible six reading frames (three forward and three reverse) and a similarity search against the protein databanks was done. This approach was found to be successful in predicting gene specific products.

Table 4.4.1.1. Showing the homology of the TV1 sequences with known sequences in three selected databases of NCBI GenBank.

Sl. No.	*TV1 markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
1.	fha1 (TV1-LG1)	Nr/nt, WGS, EST	PREDICTED: *Solanum lycopersicum* uncharacterized LOC101266009 (LOC101266009), mRNA (XM_004235462.1) (7e-20, 85%)	*Camellia sinensis* var. *sinensis* fd_20030 mRNA sequence (GAAC01013247.1) (2e-51, 97%)	FS947410 tea plant mature leaves cDNA library *Camellia sinensis* cDNA clone ML25E06, mRNA sequence (FS947410.1) (1e-54, 97%)
2.	fha2 (TV1-LG1)	Nr/nt, WGS	PREDICTED: *Vitis vinifera* probable peptide/nitrate transporter At5g62680-like (LOC100258695), mRNA (XM_002270776.1) (2e-22, 74%)	*Rauvolfia serpentine* rsa2071 iso1 mRNA sequence (GACE01012116.1) (3e-30, 70%)	
3	fha12 (TV1-LG1)	Nr/nt, WGS	PREDICTED: *Vitis vinifera* uncharacterized LOC100263256 (LOC100263256), mRNA (XM_002272919.2) (4e-15, 79%)	*Camptotheca acuminate* caa8362_iso3 mRNA sequence (GACF01038525.1) (1e-19, 82%)	
4.	gka6 (TV1-LG1)	Nr/nt, WGS	*Vitis vinifera* contig VV78X102999.17, whole genome shotgun sequence (AM465966.2) (1e-39, 83%)	*Camellia sinensis* var. *sinensis* fd_73398 mRNA sequence (GAAC01050786.1) (9e-51, 99%)	

Sl. No.	*TV1 markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
				Arabidopsis lyrata subsp. *lyrata* (ADBK01000432.1) (2e-20,79%)	
5.	gka6 (TV1-LG1)	WGS		*Cajanus cajan* (AGCT01005847.1) (2e-06, 77%)	
6.	hia17 (TV1-LG1)	WGS		*Morus notabilis* contig10186, whole genome shotgun sequence (ATGF01010186.1) (3e-04, 94%)	
7.	hia2 (TV1-LG4)	Nr/nt, WGS	*Ricinus communis* chromodomain helicase DNA binding protein, putative, mRNA (XM_002515399.1) (2e-11, 91%)	*Camellia sinensis* var. *sinensis* fd_21258 mRNA sequence (GAAC01014077.1) (1e-12, 84%)	
8.	hia3 (TV1-LG4)	WGS		*Camellia sinensis* var. *sinensis* fd_70439 mRNA sequence (GAAC01047827.1) (5e-04, 74%)	

Sl. No.	*TV1 markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
9.	gka11 (TV1-LG6)	WGS, EST		*Jatropha curcas* DNA, contig: Jcr4U38027_3, strain: Palawan (BABX02060073.1) (2e-106, 96%)	23E3_A456_TRA_2565 *Camellia assamica* W23E3E4 cDNA library *Camellia sinensis* var *assamica* cDNA 5', mRNA sequence (HS394644.1) (0.0, 96%)
10.	hia5 (TV1-LG7)	EGS		*Camellia sinensis* var. *sinensis* fd_70439 mRNA sequence (GAAC01047827.1) (2e-04, 75%)	
11.	hia12 (TV1-LG7)	Nr/nt, WGS	PREDICTED: *Vitis vinifera* wall-associated receptor kinase 2-like (LOC100257383), mRNA (XM_002284652.2) (1e-15, 81%)	*Vitis vinifera*, whole genome shotgun sequence of line PN40024, contig_118 (CAAP03010908.1) (8e-15, 81%)	
12.	kka21 (TV1-LG10)	WGS		*Camellia sinensis* var. *sinensis* fd_68277 mRNA sequence (GAAC01045665.1) (5e-06, 76%)	

Table 4.4.1.2. Showing the homology of the TV19 sequences with known sequences in three selected databases of NCBI GenBank.

Sl. No.	*TV19 markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
1.	hia6 (TV19-LG1)	WGS		*Camellia sinensis* var. *sinensis* fd_50745 mRNA sequence (GAAC01028133.1) (2e-75, 98%)	
2.	hia7 (TV19-LG1)	Nr/nt, WGS	PREDICTED: *Cicer arietinum* CHD3-type chromatin-remodeling factor PICKLE-like), transcript variant X2 (XM_004501314.1) (3e-18, 85%)	*Camellia sinensis* var. *sinensis* fd_75096 mRNA sequence (GAAC01052484.1) (3e-29, 98%)	
3.	hia8 (TV19-LG1)	Nr/nt, WGS	*Nicotiana benthamiana* phytocalpain (DEK) mRNA, complete cds (AY450851.1) (2e-17, 93%)	*Camellia sinensis* var. *sinensis* fd_8738 mRNA sequence (GAAC01004986.1) (2e-17, 95%) *Arabidopsis thaliana* (AFNC01005203.1) (4e-11, 85%)	
4.	hga7 (TV19-LG1)	Nr/nt, WGS	PREDICTED: *Fragaria vesca* subsp. *vesca* protein decapping 5-like (LOC101294680), mRNA (XM_004304098.1) (5e-05, 67%)	*Camellia sinensis* var. *sinensis* fd_8978 mRNA sequence (GAAC01005128.1) (2e-103, 97%)	

Sl. No.	*TV19 markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
5.	fha6 (TV19-LG2)	WGS		*Camellia sinensis* var. *sinensis* fd_19794 mRNA sequence (GAAC01013079.1) (1e-40, 91%)	
6.	fha8 (TV19-LG2)	Nr/nt, WGS	PREDICTED: *Fragaria vesca* subsp. *vesca* histidine kinase 4-like (LOC101311470), mRNA (XM_004297360.1) (9e-11, 80%)	*Camellia sinensis* var. *sinensis* fd_72915 mRNA sequence (GAAC01050303.1) (1e-38, 95%)	
7.	kka1 (TV19-LG3)	Nr/nt, WGS	PREDICTED: *Fragaria vesca* subsp. *vesca* DNA/RNA-binding protein KIN17-like (LOC101313845), mRNA (XM_004307472.1) (5e-33, 93%)	*Camellia sinensis* var. *sinensis* fd_72739 mRNA sequence (GAAC01050127.1) (3e-41, 92%)	
8.	fha3 (TV19-LG4)	Nr/nt, WGS	*Betula pendula* cytokinin receptor 1 (CRE1) mRNA, partial cds (EU583455.1) (8e-39, 88%)	*Camellia sinensis* var. *sinensis* fd_72915 mRNA sequence; (GAAC01050303.1) (8e-56, 91%) *Arabidopsis thaliana* (AFNC01007619.1) (1e-26, 86%)	

Sl. No.	*TV19 markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
9.	fha7 (TV19-LG4)	Nr/nt, WGS, EST	PREDICTED: *Solanum lycopersicum* uncharacterized LOC101266009 (LOC101266009), mRNA (XM_004235462.1) (5e-16, 82%)	*Camellia sinensis* var. *sinensis* fd_20030 mRNA sequence (GAAC01013247.1) (1e-47, 95%)	FS947410 tea plant mature leaves cDNA library *Camellia sinensis* cDNA clone ML25E06, mRNA sequence (FS947410.1) (9e-51, 95%)
10.	hia14 (TV19-LG7)	WGS		*Camptotheca acuminate* cca169_iso10 mRNA sequence (GACF01000886.1) (3e-05, 80%)	
11.	hia11 (TV19-LG8)	Nr/nt, WGS	PREDICTED: *Solanum lycopersicum* glutamate-rich WD repeat-containing protein 1-like, transcript variant 2 (XM_004243714.1) (5e-08, 84%)	*Camptotheca acuminate* cca11394_iso3 mRNA sequence (GACF01050377.1) (5e-12, 90%)	
12.	hga1 (TV19-LG10)	Nr/nt, WGS, EST	*Vitis vinifera* clone SS0AAG1YJ11 (FQ384421.1) (2e-29, 81%)	*Morus notabilis* contig3307, whole genome shotgun sequence (ATGF01003307.1) (1e-28, 81%) *Arabidopsis lyrata* subsp. *Lyrata* (ABDK01000266.1) (2e-14, 81%)	CsSSHF16 *Camellia sinensis* winter dormancy suppression subtractive hybridization library *Camellia sinensis* cDNA similar to putative NADPH-dependent oxidoreductase, mRNA sequence (FF682712.1) (3e-07, 72%)

Table 4.4.1.3. Showing the homology of the unlinked markers with known sequences in three selected databases of NCBI GenBank.

Sl. No.	*Unlinked markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
1.	fha9 (TV1 marker)	Nr/nt, WGS, EST	PREDICTED: *Fragaria vesca* subsp. *vesca* probable peptide/nitrate transported At5g62680-like (LOC1013606428), misc_RNA (XR_184661.1) (2e-31,87%)	*Lonicera japonica* contig06572 mRNA sequence (GAAY01004592.1) (2e-36, 90%) *Arabidopsis lyrata* subsp. *lyrata* (ADBK01001991.1) (2e-09, 78%)	CsSSHFD563 *Camellia sinensis* drought suppression subtractive hybridization library *Camellia sinensis* cDNA similar to nitrate transporter, mRNA sequence (JK341774.1) (2e-06, 77%)
2.	fha10 (TV19 narker)	WGS		*Camellia sinensis* var. *sinensis* fd_14858 mRNA sequence (GAAC01009478.1) (1e-14, 87%) *Arabidopsis lyrata* subsp. *lyrata* (ADBK01002024.1) (9e-09, 79%)	
3.	hga2 (TV1 marker)	Nr/nt, WGS	PREDICTED: *Vitis vinifera* cysteine-rich receptor-like protein kinase 29 (LOC100265137), mRNA (XM_002280420.1) (1e-12, 71%)	*Camptotheca acuminate* cca6601_iso1 mRNA sequence (GACF01031189.1) (2e-20, 72%)	

Sl. No.	*Unlinked markers*	*Hit*	*Nucleotide (Nr/nt) (e-value, max Idenity)*	*Whole Genome Shot gun contigs (WGS) (e-value, max Identity)*	*Expressed Sequence Tags (ESTs) (e-value, max Identity)*
4.	kka18 (TV1 marker)	WGS		*Camellia sinensis* var. *sinensis* fd_63959 mRNA sequence (GAAC0104134.1) (7e-44, 93%)	
5.	kka23 (TV1 marker)	WGS		*Cannabis sativa* (AGQN01045558.1) (2e-05, 82%)	

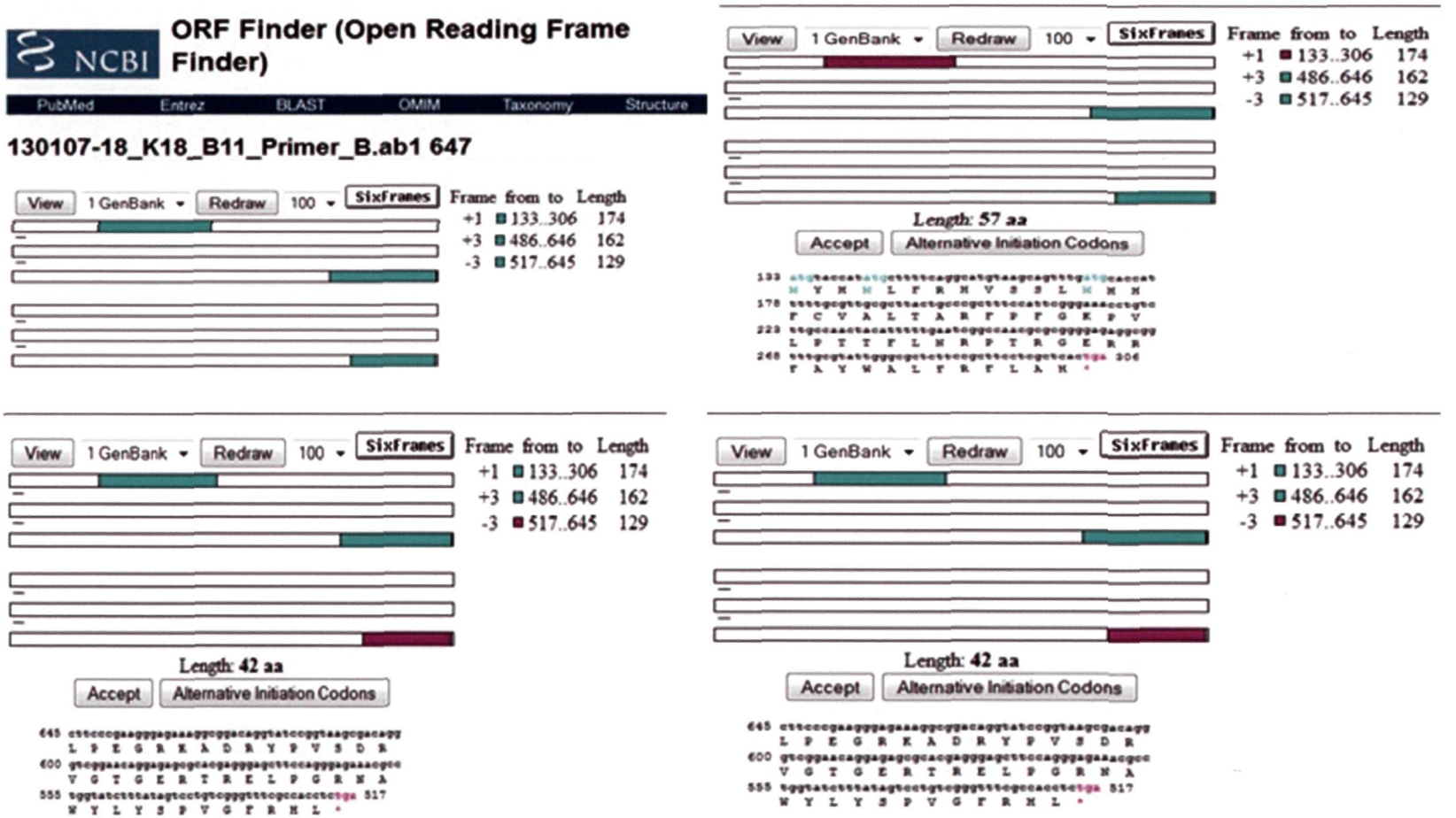

Fig.4.4.1. Finding ORF using ORF finder (NCBI) for gka11 sequence which showed alignment with EST of tea database

Table 4.4.2.1. Protein sequences code by different ORF regions for gka11 marker

Marker	*Frame*	*bp*	*Length*	*Protein sequence*
gka11	+1	133-306	174	133 **atg**taccat**atg**cttttcaggcatgtaagcagttt**g**atg**c**accat **M** Y H **M** L F R H V S S L **M** H H 178 ttttgcgttgcgcttactgcccgctttccattcgggaaacctgtc F C V A L T A R F P F G K P V 223 ttgccaactacatttttgaatcggccaacgcgcggggagaggcgg L P T T F L N R P T R G E R R 268 tttgcgtattgggcgctcttccgcttcctcgctcac**tga** 306 F A Y W A L F R F L A H *
	+3	486-646	162	486 ctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaa L T S I T K I D A Q V R G G E 531 acccgacaggactataaagataccaggcgtttctccctggaagct T R Q D Y K D T R R F S L E A 576 ccctcgtgcgctctcctgttccgaccctgtcgcttaccggatacc P S C A L L F R P C R L P D T 621 tgtccgcctttctcccttcgggaagcg 647 C P P F S L R E A
	-3	517-645	129	645 cttcccgaagggagaaaggcggacaggtatccggtaagcgacagg L P E G R K A D R Y P V S D R 600 gtcggaacaggagagcgcacgagggagcttccagggagaaacgcc V G T G E R T R F L P G R N A 555 tggtatctttatagtcctgtcgggtttcgccacctc**tga** 517 W Y L Y S P V G F R H L *

Table 4.4.2.2. Unigenes which showed homology with the protein sequences code by different ORF regions for gka11 marker.

Description	*ORF frame*	*Max. score*	*Total score*	*Query coverage*	*e-value*	*Max identity*	*Accession*
putative reverse transcriptase [*Zingiber officinale*]	1 ORF in +1 frame	82.4	82.4	85%	2e-19	78%	ABK60177.1
predicted protein [*Populus trichocarpa*]	1 ORF in +3 frame	71.2	71.2	79%	2e-14	81%	EEE75450.1

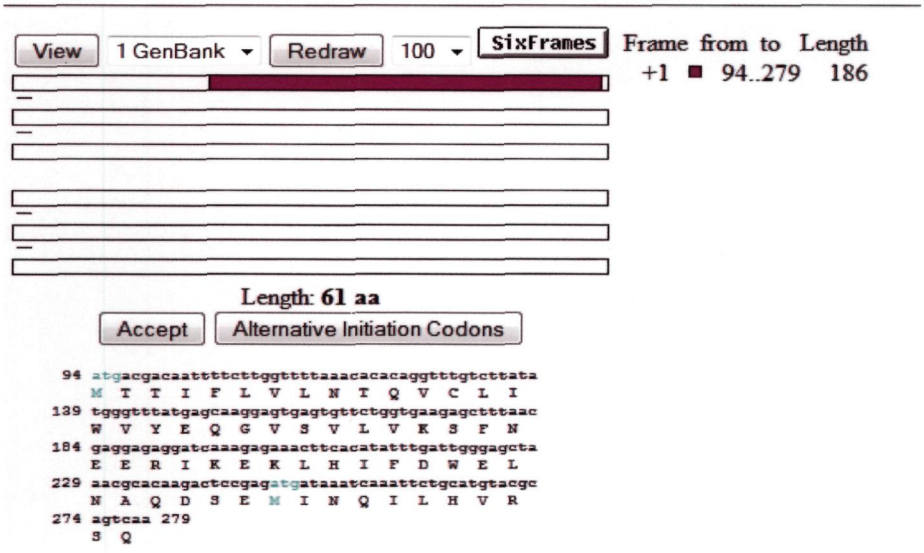

Fig.4.4.2. Finding ORF using ORF finder (NCBI) for hga1 sequence which showed alignment with EST of tea database.

Table 4.4.2.3. Protein sequences code by different ORF regions for hga1 marker

Marker	*Frame*	*bp*	*Length*	*Protein sequence*
hga1	+1	94-279	186	94 **atg**acgacaattttcttggttttaaacacacaggtttgtcttata **M** T T I F L V L N T Q V C L I 139 tgggtttatgagcaaggagtgagtgttctggtgaagagctttaac W V Y E Q G V S V L V K S F N 184 gaggagaggatcaaagagaaacttcacatatttgattgggagcta E E R I K E K L H I F D W E L 229 aacgcacaagactccgag**atg**ataaatcaaattctgcatgtacgc N A Q D S E **M** I N Q I L H V R 274 agtcaa 279 S Q

Table 4.4.2.4. Unigenes which showed homology with the protein sequences code by different ORF region for hga11 marker

Description	*ORF Frane*	*Max score*	*Total Score*	*Query coverage*	*e-value*	*Max. Identity*	*Acession*
PREDICTED: probable NAD(P)H-dependent oxidoreductase 1 (*Vitis vinifera*)	1 ORF in +1 frame	75.1	75.1	74%	4e-15	72%	XP_002285211.1

Out of 4 mapped sequences that aligned with the EST of tea, ORFs of two markers i.e.gka11, hga1 could be obtained using ORF finder (NCBI). Then the protein sequences codes by the corresponding ORFs were subjected to BLASTp to find out any homology with any protein sequence in NCBI database.

gka11 marker showed homology to 23E3_A456_TRA_2565 *Camellia assamica* W23E3E4 cDNA library *Camellia sinensis* var. *assamica* cDNA 5', mRNA sequence (HS394644.1) with the e-value of 0.0, max query coverage of 72% and max identity of 96%.

The ORF finder tool identified 3 putative ORFs in the gka11 marker. Two ORFs were found in the region 133-306 bases (length 174 bases) and 486-646 bases (length 162 bases) in the (+) strand (i.e. +1 and +3 frames respectively). One putative ORF was found in the region 517-645 bases (length 129 bases) in the (-) strand i.e. -3 frame (fig.4.4.1).

The predicted amino acid sequences for each of the 3 putative ORFs (Table no.4.4.1) were subjected to BLASTp (NCBI). The hits with the expected value more than 1e-05, were excluded and considered as no hits found. Out of the three protein sequences code by the three different ORF regions, two showed homology with unigenes. Protein sequence of the ORF region in the +1 frame showed homology with the putative reverse transcriptase of *Zingiber officinale* (accession no.ABK60177.1) with a query coverage of 85%, e-value 2e-19 and maximum identity 78%. While the protein sequence of the ORF region in the +3 frame showed homology with a predicted protein of *Populus trichocarpa* (accession no.EEE75450.1) with query coverage of 79%, e-value 2e-14 and maximum identity

81%. However no hit was found for the protein sequence of the ORF region in the -3 frame.

hga1 marker showed homology to CsSSHF16 *Camellia sinensis* winter dormancy suppression subtractive hybridization library *Camellia sinensis* cDNA similar to Putative NADPH-dependent oxidoreductase, mRNA sequence, (FF682712.1) with the e-value of 4e-07, max query coverage of 36% and max identity of 72%.

The ORF finder tool identified only one putative ORF viz. 94-279 (length 186 bases), in the (+) strand i.e. +1. The predicted amino acid sequences of the putative ORFs were subjected to BLASTp (NCBI). The hits with the expected value more than 1e-05, were excluded and considered as no hits found. The protein sequence of the only one ORF region in the +1 frame showed homology with Predicted probable NAD(P)H-dependentoxidoreductase1 of *Vitis vinifera* (accession no. XP_002285211.1) with a query coverage of 74%, e-value 4e-15 and maximum identity 72%. The result of protein sequence alignment was same as the nucleotide BLAST result i.e. predicted probable NAD (P) H-dependent oxidoreductase.

Two markers fha1 and fha7 that showed homology with the EST sequences were also subjected to ORF Finder and then to BLASTp (NCBI) but no homology was determined.

Chapter 5

Discussion

CHAPTER 5

DISCUSSION

5.1. PRIMER COMBINATION SELECTION

For revealing polymorphism for an organism with small genome size (10^6-10^7 bp), AFLP primers with one or two selective nucleotides are necessary. According to Vos *et al.* (1995) the number of amplification products generated by the AFLP is related to the size of the genome and the number of selective nucleotides added to the 3' end of the primers. Species with a large genome size are expected to generate a large number of AFLP bands making analyses more cumbersome, therefore, additional selective nucleotides are required for those organisms with larger genomes (10^8-10^{10} bp) (Blears *et al.* 1998). Introduction of one more restriction enzyme limits the number of bands. Theoretically, using the TE-AFLP technique the number of fragments which are potentially amplified can be easily reduced 20 fold compared to traditional AFLP (van der Wurff *et al.* 2000). Considering all the nucleotides are randomly distributed in the genome the expected frequency of the restriction sites of two rare cutters and one frequent cutter in tea genome (4.0×10^9 bp) is estimated to be approximately 1.758×10^7. Due to the addition of a second rare cutter in TE-AFLP, extra sites have been added to increase the discriminatory power of the technique.

Like RAPD, the standard TE-AFLP with only one step also has high probability of generating artifactual bands associated with misannealing of primers and resulting in poor reproducibility. In the present study, to avoid this problem, a pre-amplification

step was added. Addition of the pre-amplification step also gave more of the amplified products for subsequent selective amplification.

Five primer combinations were selected in the final screening. An average of 36 prominent fragments within 1Kbp were obtained per reaction, indicating that silver stained polyacrylamide gel retained the resolution power of the radio-labelling technique. High resolution silver stained polyacrylamide gel without radioactive isotopes proved to be a more efficient technique to visualize TE-AFLP markers in tea. Silver-staining reduced the time and cost as well as eliminated hazard of working with radioisotopes (Vantoai *et al.* 1996, Chalhoub *et al.* 1997). In addition, the high resolution and recovery of fragments from the dried gels made silver staining a more useful and versatile detection method for AFLP amplification products.

In the biclonal population taken for the present study, the selected primer combinations could resolve differences at the level of intra-species. Mueller and Wolfenbarger (1999) also reported that AFLP markers have the potential to resolve genetic differences at the level of 'DNA fingerprints' for individual identification and parentage analysis. Several studies had identified inter- and intraspecific hybrid individuals with AFLP (Liu *et al.* 1998; Congiu *et al.* 2001; Bensch *et al.* 2002; Chauhan *et al.* 2004). However, the usefulness of AFLP markers for systematics rests more on the rapid grouping of closely related lineages. Phylogenetic inferences based on similarities of AFLP profiles become problematic for higher taxonomic levels, because the high variability of AFLP markers reduces similarities between distant taxa to the level of chance (Mueller and Wolfenbarger 1999).

A 'narrow-down' strategy for the primer combination and increasing number of samples from 1 to 5, and finally to 17 samples in three different steps was followed. This strategy was beneficial in choosing the optimised TE-AFLP primer combinations. Four marker indices were used to examine the overall efficiency of the primer combinations selected. PIC has been used extensively in diversity and marker studies (Vos *et al.*1995; 245 Milbourne *et al.* 1997; Rosales *et al.* 2005; Tatikonda *et al.* 2009). PIC values for the TE-AFLP primer combinations in the study showed that it is an efficient parameter to reveal the diverse nature of the genotypes but it is independent to the number of fragments per primer combination. In the study, the PIC value ranged from 0.33 (E-AAC / P-ATG) to 0.40 (E-AAT / P-CGA) with an average of 0.36 which is higher than the TE-AFLP primer combinations used in *Pongamia pinnata* diversity study (Sharma *et al.* 2011).

The AFLP generates a large number of fragments per primer combination. This leads to a higher EMR because the number of polymorphic bands is directly proportional to EMR. The multilocus nature of AFLP markers is well suited for detecting polymorphism and distinguishing genotypes using few primer combinations. In this study, EMR for E-ACG/P-AAA (kk) primer combination was the highest (15.93) where the polymorphic bands were highest. Since MI is a product of two parameters: EMR and PIC, it is used to calculate the overall utility of a maker system. MI together with PIC value has been used to assess the discriminatory power of AFLP primer combinations used in several cross pollinated plants including Radish (PIC = 0.24, MI = 5.14) (Muminovic *et al.* 2005); *Jatropha curcus* L. (PIC = 0.26, MI =

25.13) (Tatikonda *et al.* 2009) and *Pongamia pinnata* L. (PIC = 0.31, MI = 30.75) (Kesari *et al.* 2010).

The discriminatory power of the TE-AFLP primer combinations was also evaluated using the two parameters i.e. MI and PIC in *Pongamia pinnata* accessions (MI =7.95, PIC = 0.30) (Sharma *et al.* 2011). In the present investigation, MI values were reported in the range of 1.04 to 5.73 (average 2.75) and PIC in the range of 0.33 to 0.40 (average 0.36). Discriminating as many accessions as possible would be the most important feature of a given primer combination. Various studies reported a high MI in AFLP markers compared with other marker system (Bohn *et al.* 1999; Hongtrakul *et al.* 1997; Powell *et al.* 1996; Russell *et al.* 1997). There is a reduction in the number of bands generated in TE-AFLP. Consequently the MI value was much lower than the standard AFLP.

Rp is well suited for comparing primers, primer-enzyme combinations or probe-enzyme combinations generated by RFLP, RAPD, ISSR-PCR or AFLP analyses (Prevost and Wilkinson 1999). Since Rp is the sum of all the informativeness of each band, higher the number of polymorphic bands of a primer combination, higher is the Rp. In the present work, Rp value was highest for E-ACG/P-AAA (kk) primer combination and MI value was also the highest for the same. The combined use of all these parameters clearly showed the ability of the primer combinations to distinguish the different genotypes. The primers with higher Rp values have a greater capacity to separate genotypes (Prevost and Wilkinson 1999) hence they are useful for bigger applications.

Laurentin and Karlovsky (2007) reported that there was a lack of correlation among PIC, MI and Rp indicating that a single parameter was not sufficient to assess the informativeness of a primer combination. We too found no specific relation among the parameters used by us. In this study, the PIC was highest in E-AAT/P-CGA (fh) whereas EMR, MI, Rp were highest in E-ACG/P-AAA (kk). However, the values of the latter three parameters are directly proportionate to the number of polymorphic fragments generated while PIC is independent of it. There is not much difference of PIC values (an average of 0.36) in the primer combinations undertaken which showed that all the five primer combinations could be used for variation studies on a wider scale. However, EMR, MI and Rp were markedly higher in E-ACG/P-AAA (kk) showing that it was the most informative among the five primer combinations. Thus, TE-AFLP is better suited for analysis of larger genomes. The addition of the pre-amplification step helped improve the accuracy and reproducibility of amplification, and produced much cleaner bands. The number and sequence of the selective bases of the primers in the selective amplification further enhanced the efficacy of this protocol. Silver staining was found to be more advantageous compared to radio-labelling and was used for visualizing the DNA fragments of the selective PCR amplification in the sequencing gel. Therefore, we recommend use of silver stained TE-AFLP for analysing a larger number of tea samples.

5.2. SEGREGATION STUDIES

5.2.1. Segregation analysis

Five TE-AFLP primer combinations which were selected in the final screening were used in mapping analysis of 117 genotypes of the bi-clonal population of TS 463 i.e.

F_1 generation of a cross between two tea clones TV1 (assam-china) and TV19 (combod). Two kinds of segregating AFLP markers could be identified. Type 1:1 markers, where one parent was heterozygous (presence of band, *A/a*) and the other was homozygous (absence of bands, *a/a*) and the F_1 offspring were expected to segregate in a 1:1 (presence of band, *A/a*: absence of band, *a/a*) ratio. The other type was a 3:1 marker where both parents were heterozygous (presence of bands in both cases; *A/a*) and the offspring were expected to segregate in a 3:1 (presence of bands, *A/a* or *A/A*: absence of bands, *a/a*) ratio. All segregating markers were tested for goodness of fit to the 1:1 and 3:1 Mendelian ratio using chi-square analysis and a significance level of $P = 0.1$. A total of 153 polymorphic markers were generated, out of which 100 markers comprised of the first type (type 1:1 marker) and 53 markers comprised of the second type (type 3:1 marker). The number of 1:1 markers that segregated in the TV1 parent was more than TV19 parent (59 and 41 respectively).

Linkage maps of tea had been generated using different tea plant sources and various types of molecular markers. Hackett *et al.* (2000) generated 420 (RAPD and AFLP) markers of which 304 markers comprised of 1:1 marker type while 116 markers comprised of 3:1 marker type in tea (*Camellia sinensis*). Kamunya *et al.* (2010) generated 260 markers (RAPD,SSR,AFLP) of which 149 markers showed 1:1 segregation ratio and 118 displayed 3:1 segregation ratio and Taniguchi *et al.* (2012) generated 1124 markers (SSR,RAPD,CAPS,STS). The number of markers generated in our study was much lower when compared due to lower number of primer combinations used in the study. However, primer combinations used in this study

generated on average 20 polymorphic markers between the parents (type 1:1 markers) which was higher than AFLP markers generated by Hackett *et al.* (2000) (an average of 10.5 polymorphic bands per primer combination). The high polymorphic rate observed in our study may be due to the fact that the samples taken for the study were from artificially selected populations (Moore *et al.* 1999; Li *et al.* 2006) since tea is a plantation crop. In addition, the F_1 population was obtained by crossing two highly heterozygous parents belonging to different species both of which were open pollinated.

Using the same primer combinations, 59 and 41 markers segregated through TV1 and TV19 parent respectively. This showed that TV1 parent was heterozygous for a larger number of loci than TV19 parent. But in case of informativeness for linkage analysis, markers segregating through TV19 were more informative as 30 out of 41 markers (73.17%) were mapped in the TV19 map while 36 out of 59 markers (61.02%) were mapped in TV1 map. Three markers which were highly distorted ($P < 0.01$) from Mendelian ratio were excluded from linkage analysis from each parent. Therefore, eight TV19 markers and twenty TV1 markers remained unassigned to linkage groups. Among the five primer combinations, primer combination E-ACG/P-AAA (kk) was most informative, generating the highest number (40) of markers. If the markers segregating in either parent (1:1 marker type) were considered, markers generated by E-ACG/P-AAA (kk) and E-AAC/P-ATG (hi) primer combinations were highest (11 and 9) in TV1 parent and TV19 parent respectively.

5.2.2. Segregation distortion

All segregation markers were checked with χ^2 test ($P < 0.1$) to identify deviations from Mendelian 1:1 ratio. Distorted markers were suffixed with a minus (-) for homozygote deficiency and a plus (+) for homozygote excess. The markers from the TV1 parent had a higher proportion of distortions compared to TV19 parent (i.e.11 markers in TV1 parent and 6 markers in TV19). The proportion of distorted segregating markers in this study was 17% for markers heterozygous in either parent. The percentage of significantly distorted markers detected (17%) was similar to that observed in linkage analyses of pine (14–15%; Kubisiak *et al.* 1995), eucalyptus (15%; Marques *et al.* 1998), *Quercus* (18%; Barreneche *et al.* 1998), willow (18%; Hanley *et al.* 2002), and artichoke (14%; Lanteri *et al.* 2006).

The markers with highly distorted segregation ratios at the 1% level ($P<0.01$) were excluded from linkage analysis. However, we included the markers showing distorted segregation ratios ($0.05 < P < 0.1$) in the linkage analysis. In TV1 parent and TV19 parent data, 8 and 3 distorted markers ($0.05 < P < 0.1$) were included respectively in the mapping data sets.

Markers with distorted segregation ratios ($0.05 < P < 0.1$) were included in the linkage analysis to see if these markers could help in connecting between markers to obtain a longer linkage group. In addition to this, it would also help in identifying possible regions of distortion by investigating regions of clustering of markers. However, most of the distorted markers from both the parents were found to be unlinked and could not be placed on the map. No clustering of the distorted markers was also observed thereby regions of segregation distortion could not be identified in

this study. This was due to less distorted markers ($0.05 < P < 0.1$) (to avoid false linkages) were linked to the linkage groups in the study, i.e. only 4 distorted markers were linked to the TV1 map while only one distorted marker was linked to TV19 map.

Several reasons for distortion of segregation ratios in many plants have been reported that range from biological to non biological factors. The biological factors include chromosome loss (Kasha and Kao 1970), genetic isolation mechanisms (Zamir and Tadmor 1986), and the presence of viability genes (*e.g.*, Hendrick and Muona 1990; Beavis and Grant 1991; Liedl and Anderson 1993; Bradshaw and Stettler 1994). Nonbiological factors include scoring errors (Devey *et al.* 1994; Xu *et al.* 1997; Nikaido *et al.* 1999), marker sampling error and low population size (Lu *et al.* 1998) and sampling errors (Plomion *et al.* 1995; Echt and Nelson 1997). The nonbiological factor for the distortion may also be the superimposition on the gel of non-allelic amplified products corresponding to different loci (Virk *et al.* 1998). Transmission distortion between genetically different genomes (Fishman *et al.* 2001); zygotic selection (Rick 1969) or using parents from different populations containing a high genetic load (Fishman *et al.* 2001) can also lead to distortion in segregation ratios. Another significant issue that hampers all aspects of map development is experimental error such as wrongly scored individuals or contamination.

Segregation distortion may also be caused by a shortage in identical-by descent homozygotes due to a high genetic load (Launey and Hedgecock 2001). This is due to harmful recessive alleles being filtered out of the family line in outcross species. In our study, homozygote deficiency accounted for more of the segregation distortion

(76.47%) compared to homozygous excess (3.92%). Genetic load is likely a contributing factor to the segregation distortion observed. Similar results have also been reported in other outbreeding tree species such as populus (Bradshaw and Stettler 1994) and eucalyptus (Gion *et al.* 2000). Another contributing factor for segregation distortion in our study could be scoring error as AFLP has been reported to have and error rate of 0.6-2% per band (Arens *et al.* 1998; Janssen *et al.* 1997).

5.3. LINKAGE MAPPING

5.3.1. Linkage map

Two separate genetic linkage maps for TV1 and TV19 were constructed using the two-way pseudo-testcross strategy (Grattapaglia and Sederoff 1994) based on the two separate data sets obtained for heterozygous segregating genetic markers. The pseudo-testcross approach has been widely used for construction of linkage maps in tea (Tanaka 1996; Hackett *et al.* 2000; Kamunya *et al.* 2010; Taniguchi *et al.* 2012) and in many other outbreeding species.

In our study, TV1 map consisted of 36 loci, spread over 10 linkage groups while in TV19 map, 30 loci were distributed in 11 linkage groups. The number of linkage groups obtained in this study was therefore less than the haploid chromosome number (n=15) for this species. Kamunya *et al.* (2010) also could obtain only 19 maternal and 11 paternal linkage groups in tea.

Estimates of total genome map size have been calculated on tea in various studies including a female map that covered 1349.7 cM with an average distance of 11.7 cM between loci (Hackett *et al.* 2000); a maternal map spanning 1,012 cM, while the paternal map covered total length of 399.5cM, with mean distance between markers

being 14.7 and 12.9 cM, respectively (Kamunya *et al.* 2010). Recently, in a high reference map developed by Tanaguchi *et al.* (2012) the core map of tea covered a total length of 1218 cM . In our study, 10 linkage groups of TV1 map covered a total map length of 388.9 cM at an average distance of 38.89 cM between the markers while in TV19 map, 11 linkage groups covered a total map length of 410.7 cM with an average distance of 37.34 cM between the markers.

Therefore, linkage maps generated in this study had comparatively low genome length due to less number of markers. The linkage maps were also incomplete as indicated by the lower number of linkage groups when compared to the haploid chromosome number. Large intervals (> 20cM) were also observed in both the maps generated including all the markers. The existence of these minor linkage groups and unlinked markers (20markers in TV1 parent and 8 markers in TV19 parent) also indicated that there were many large gaps with few markers (Kesseli *et al.* 1994). All these shortcomings were attributed to the less number of markers used for linkage map generation.

The density and resolution of the linkage map will be increased if more molecular markers were added. The addition of markers to these maps should reduce the larger intervals and thus the gaps (Lallias *et al.* 2007). Furthermore, increasing the marker density may result in the linkage groups containing only two or three markers to coalesce into larger linkage groups (Baranski *et al.* 2006).

Few distorted markers ($0.05 < P < 0.1$, to avoid false linkages) were included in the linkage analysis and only 5 markers were linked in the parental maps (4 marker in TV1 parent and 1 marker in TV19 parent). Since only 5 markers were linked to

linkage groups and no clustering was observed. regions of segregation distortion could not be identified in this study.

Another factor contributing to the low map length could be the presence of genotyping errors which can overestimate the proportion of double crossovers and thus severely inflating map lengths (Hackett and Broadfoot 2003). Therefore, the rigorous data checking and exclusion of numerous scored markers with an apparent excess of double crossovers may have had the effect of keeping map length to a minimum. The experiences gained in this study shall be used in subsequent studies to overcome these limitations.

5.3.2. Map comparison

The heteroduplex markers which were descended from both the parents (3:1 marker type) were essential for the integration of the separate parental linkage maps into one consensus map. However, in our study bridging markers were clustered among themselves and not linked with markers specific to either parent (1:1 markers) (Fig. 4.3.3.3). This uneven distribution throughout the parental maps may be due to the low density of markers generated. So, these bridging markers could not be used to detect homology between linkage groups with markers specific to either parent. Since there is no information on homology of the linkage groups, integration of linkage groups to make an integrated/ consensus map was not possible.

The addition of more markers (both marker types) to the parental maps is required for the establishment of a consensus map. The addition of more informative co-dominant markers such as microsatellites, SNP, CAPS (Omura *et al.* 2000, Yamamoto *et al.* 2002, 2005, 2007), will further increase the accuracy of the

consensus map, as they will serve as anchor loci between the two parental maps (Powell *et al* 1996). Recently a consensus map based on a combination of different markers (AFLP, SSR and CAPS) has been established in tea by Taniguchi *et al.* (2012).

5.3.3. Marker distribution

The AFLP marker distribution was analyzed by calculating the Pearson correlation coefficient between the number of AFLP markers in the linkage groups and the size of the linkage groups (Yu and Guo 2003), *t-test* was applied to test the significance of correlation coefficient at $P = 0.1$ level.

The Pearson correlation coefficient ($r = 0.91$ and $r = 0.83$) indicated that there was a highly significant positive correlation between the number of markers and linkage group size. This supports our contention that the distribution of AFLP markers was relatively even in linkage groups of TV1 and TV19 maps.

The distribution of the mapped AFLP markers was also classified according to the markers generated by 5 primer combinations used for analysis by visual examination (Fig. 4.3.4.1 and fig 4.3.4.2). In this study, we found the accumulation of markers (1:1 markers) on a particular linkage group amplified by the same primer combination and the distribution was relatively even in both the parental maps. The number of AFLP markers in the linkage groups that had high positive correlation with the size of the linkage groups also supports this conclusion.

The AFLP markers segregating at 3:1 ratio were not distributed along with the 1:1 markers, and therefore remained uninformative in finding homology between linkage groups.

5.3.4. Genome coverage

The estimated genome lengths determined for both the parental maps in this study were 766.1 cM and 894.5 cM for TV1 and TV19 parent respectively. The difference seen between the two maps is not an uncommon occurrence as it had also been observed in maps of other tea plants (Kamunya *et al.* 2010; Tanaguchi *et al.* 2012). Possible reasons for the variations between the estimates for both the parents are: (*i*) the distribution of markers along the chromosome differs in both parents, (*ii*) recombination frequencies occurring in both gametes may have differed, and (*iii*) errors within the data, such as statistical and experimental errors (Wu *et al.*, 2004). The map coverage of both the maps was low i.e. 50.76% and 45.91 % for TV1 and TV19 respectively.

The addition of genetic markers, especially different genetic markers, such as additional microsatellites, EST and SNPs (Wang *et al.* 2004) are needed to improve genome coverage. Although low, it has potential to serve as a basis for the development of a denser and more saturated linkage map.

5.4. SEQUENCE ANALYSIS

The current results demonstrate the ability of combining the advantages of PCR and restriction pattern fingerprinting methods in AFLP analysis method that could detect single nucleotide polymorphisms in addition to minor insertions, deletions, transpositions, duplications or inversions. Those sequences that turned up in our

analysis indicated that the particular sequences were subjected to relatively frequent sequence variations. Most of the genes for which map positions are reported in this linkage map were polymorphic regions that reveal putative functions relating to housekeeping genes, functioning in general plant metabolism. Thus it showed that a number of multilocus housekeeping genes accumulate neutral variation/mutation providing more discrimination in the genome. Sometimes housekeeping genes may be of considerable value in determining evolutionary mechanisms and evaluating diversity. Although the similarity of housekeeping genes might be an indication of genome similarity, there are many examples, particularly in large genomes, where dramatic differences in gene content have been found in organisms (e.g. Rocap *et al.*, 2003). Even distantly related plant species have in common these genes with similar functions and a high degree of sequence conservation. In our study, 12 mapped markers each of both TV1 and TV19 showed homology with the sequences from different database of NCBI GenBank. The homologous sequences came from several species; unigenes that showed homology with TV1 markers were from *Solanum lycopersicum, Ricinus communis* and majority were *Vitis venifera*. All the 12 sequences also showed homology with sequences from the whole genome shot gun sequence database, 7 mapped markers and one unlinked marker showed homology with whole genome sequences of *Camellia sinensis* var *sinensis* (Table 4.4.1.1. and 4.4.1.3.). In case of TV19 markers, homologous unigenes were from various plant species e.g. *Populus trichocarpa, Fragaria vesca supsp vesca, Betula pendula, Solanum lycopersicum, Cicer arietenum*. 9 out the 11 TV19 mapped markers and one unlinked marker showed homology with whole genome sequences of *Camellia sinensis* var *sinensis* (Table 4.4.1.2. and 4.4.1.3).

Those sequences that did not identify any similarities of the DNA fragment with any known genes or anonymous sequences after a BLASTn search at NCBI were subjected to find ORF region by ORF finder (NCBI). The sequences were translated for all possible reading frames but did not result in any known protein (BLASTp). The absence of homology could be caused by several factors. A large proportion of sequences were too short to allow statistically meaningful matches. However, for some sequences, the absence of homologous sequences in the public databases may indicate specific roles for them in *C. sinensis* (Shi *et al.* 2011).

Unigenes of various plant species showed homology with some of the sequences in our study. In TV 1 map, out of 12 sequence (mapped) markers, three markers showed homology with unigenes in the database (Table 4.4.1.1.). The details of the unigenes are as follows:

1. Peptide/nitrate transporter

Two of the sequences aligned with the putative mRNA for nitrate transporter of *Vitis vitifera* and *Fragaria vesca* subsp. *vesca.* This unigene is involved in primary assimilation of inorganic nitrogen and amino acid metabolism. So, this would be helpful in understanding physiological processes important for tea cultivation and quality.

2. Chromodomain helicase DNA binding protein

The CHD (Chromodomain Helicase DNA Binding Protein) family of proteins is known to be involved in the regulation of gene expression, recombination and chromatin remodelling via their chromatin specific interactions and activities. e.g. CHD2 is a multifunctional protein that is involved in the regulation of the vital cellular processes of pre-mRNA splicing, DNA repair and DNA damage induced

transcriptional activation of stress response genes at the molecular and cellular levels (Woodage *et al.* 1997)

3. Wall-associated receptor kinase 2-like

So far, WAKs have been identified only in *Arabidopsis* and they play important roles in cell expansion, heavy-metal stress tolerance and pathogenic bacteria resistance in the plants (Lally *et al.* 2001; He *et al.* 1998; Hou *et al.* 2005).

In TV 19 map, 8 mapped markers showed homology with unigenes in the database (Table 4.4.1.2). The details of the unigenes are as follows:

1. Chromatin-remodeling factor PICKLE –like

It is related to the function of inhibition of some late embryogenesis genes after imbibition to promote germination (Perruc *et al.* 2007).

2. Phylocalpain

Calpain, a calcium-dependent cysteine, plays an essential role in basic cellular cells. Phytocalpain plays a key role in the determination of the proliferation and differentiation fates of cells during organ development of dicotyledonous plants (Ahn *et al.* 2004).

3. Protein decapping 5-like

A study in *Arabidopsis thaliana* found that Decapping 5 (DCP5) is required for mRNA decapping, P-body formation and translational repression during postembryonic development (Xu and Chua 2009).

4. PREDICTED: histidine kinase 4-like

In *Arabidopsis thaliana*, His-kinase was recently suggested as a sensor for cytokinins which have important function in cell division and differentiation (Nishimura *et al.* 2004).

5. DNA/RNA-binding protein KIN17-like

Kin17 is believed to be involved in the cellular response to DNA damage, gene expression, and DNA replication.

6. PREDICTED: cytokinin receptor 1 (CRE1) mRNA

Elevated cytokinin levels maintain high cellular redox potentials during drought and resistance to biotic and abiotic stresses in tobacco (*Nicotiana tabacum*) (Rivero *et al.* 2007). However, recent findings have shed light on a distinct role of cytokinins in plant immune responses (Choi *et al.* 2010; Sano *et al.* 1994; Sano *et al.* 1996). For these reasons, cytokinin receptor might be an important application target for generating drought tolerant and disease resistant in tea.

7. PREDICTED: glutamate-rich WD repeat-containing protein 1-like

These proteins form a very large family that is both diverse in function and structure. They play key roles in the formation of protein-protein complexes in nearly all the major cellular pathways.

8. Putative NADPH-dependent oxidoreductase

NADPH-DOs include essential enzymes for a variety of cell functions in plants, such as NADPH oxidases which control defence mechanisms in plants (Mehdy *et al.* 1996) and also play role in the control of plant growth by zinc and auxin (Oguchi 2004).

One unlinked TV1 marker also showed homology with the unigenes in the GenBank.

1. Cysteine-rich receptor-like protein kinase 29

Cysteine-rich repeat RLKs (CRKs) functions during oxidative stress, pathogen attack and the application of salicylic acid (SA) (Du and Chen 2000; Wrzaczek *et al.* 2010).

Using the above searches and analyzing the homology, 4 sequences were selected which showed homology with the existing tea EST database. EST sequences (regulatory sequences + exons) could be considered as a good representation of coding sequences. The sequences were translated in all possible six reading frames (3 forward and three reverse) and a similarity search was done against the protein databanks. This approach was found to be successful to predict gene specific products. ORFs of two sequences i.e. gka11 and hga1 showed homology with unigenes in the NCBI database.

AFLP markers were sequenced for purposes of physical mapping and, at least in some cases, to generate codominant marker systems. However no informative codominant markers (intercross marker) from the sequences could be achieved that could be linked to any of the linkage groups.

Current studies have shown that functional genes appear to be relatively less compared to the non coding regions. There have been reports that AFLPs cluster around the centromeres and telomeres where functional genes are rare. Moreover, the noncoding nature of AFLPs may provide a rational explanation for their high variability.

However, some sequences showed homology with unigenes which were related to nitrogen assimilation, drought, pathogen resistance, heavy-metal stress tolerance, resistance to biotic and abiotic stresses. The identification of such markers is an important resource for research aimed at understanding physiological processes important for tea cultivation and quality. The identification of markers closely linked to desirable traits is a prerequisite for the application of MAS breeding programmes, as the markers will be used as identifiers to locate the presence of desirable traits in breeding families (Wang *et al.* 2004).

These findings will lead to the genetic enhancement of this species, which is the ultimate goal of genetic improvement of tea. Further efforts are required to construct a high density map and to locate the quantitative trait loci (QTL) and other important agronomic traits.

Chapter 6

Conclusion

CHAPTER 6

CONCLUSION

1. A 'narrow-down' strategy for the primer combination was beneficial in choosing the optimised TE- AFLP primer combinations. At the final step, 5 out of 36 primer combinations which were highly polymorphic in the tea sample collected were chosen for further studies.
2. There was a lack of correlation among the statistical parameters taken for our study i.e. PIC, MI and Rp indicating that a single parameter was not sufficient to assess the informativeness of a primer combination.
3. Genetic load and the miss-scored error in AFLP were likely contributing factors to the segregation distortion observed in our study. However, the exact factor is not known.
4. Two separate parental maps based on the 1:1 markers were constructed using the software JoinMap 4.0. The map of the TV1 parent consisted of 36 markers in ten linkage groups while TV19 parent consisted of 30 markers in eleven linkage groups.

   The number of linkage groups obtained in this study is less than the haploid chromosome number (n=15). This discrepancy in the number is due to the low density of markers scored.
5. Observed genome lengths calculated considering all markers were 388.9 cM and 410.7 cM for TV1 and TV19 maps respectively. The observed genome coverage was determined as 50.76% and 45.91% for TV1 and TV19 respectively.

6. The existence of minor linkage groups and unlinked markers (25 markers in TV1 parent and 9 markers in TV19 parent) indicates that there are many large gaps with few markers. The large intervals (> 20cM), observed in both the maps is due to the low-density of the maps obtained.
7. In our study, the bridging marker are clustered among them and not linked with any markers specific to either parents (1:1 markers) which was uninformative for merging linkage groups and thus no integrated map could be developed.
8. The Pearson correlation coefficient (r = 0.91 and r = 0.83) indicated that there was a highly significant positive correlation between the number of markers and linkage group size. This supports our contention that the distribution of AFLP markers was relatively even in linkage groups of TV1 and TV19 map.
9. BLAST analyses of the AFLP marker sequences showed that the functional genes appear to be relatively less compared to the non coding regions.
10. However, some sequences showed homology with unigenes which were related to nitrogen assimilation, drought, pathogen resistance, heavy-metal stress tolerance, resistance to biotic and abiotic stresses, etc. The identification of such markers is an important resource for further research aimed at understanding physiological processes important for tea cultivation and quality. Some of them may be tested further for potential use as markers for selecting clones tolerant to various biotic and abiotic stresses.

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Appendices

APPENDIX A

I. Buffers used for DNA extraction and agarose gel electrophoresis.

DNA EXTRACTION BUFFER (pH 8.0)

Component	*Quantity (per100 mL)*
Tris-HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	20 mM
NaCl	1.4 M
CTAB	2% (w/v)
PVPP	1% (w/v)

EDTA 0.5M pH 8.0

186 1 g of disodium EDTA 2 H_2O

800 mL of distilled water and stir vigorously

Adjust the pH to 8 0 with NaOH pellets (~ 20 g)

Note EDTA will not dissolve until proper pH is attained

GEL ELECTROPHORESIS BUFFERS

10x TBE Buffer (1 L)

Reagents	*Amount*
Trisbase	108 g
Boric acid	55 g
EDTA	40 mL of 0.5M (pH 8.0)

Double distilled water was added to make the total volume upto 1000 mL of 10x TBE buffer

0 5xTBE 50 mL of 10x TBE was added to 950 mL of double distilled water to make 1000 mL of 1x TBE buffer

Type III Loading Buffer (6X)

Reagents	*Quantity (w/v)*
Bromophenol blue	0.25%
Xylene Cyanol FF	0.25%
Glycerol	30% in water

Formamide Loading Buffer (50mL)

Reagents	*Quantity (50 mL)*	*Final Concentration*
Formamide	Add up to 49 mL	
0.5 M EDTA pH 8.0	1 mL	10 mM
Xylene Cyanol	50 mg	1 mg/mL
Bromophenol Blue	50 mg	1 mg/ml

All the components are mixed stored in freezer (-20°C)

II. Preparation of chemicals/solutions for Polyacrylamide gel.

40% Acrylamide Solution (100 mL)

38 g acrylamide : 2 g bis-acrylamide was dissolved in approx. 50 mL double distilled water and finally the volume was made upto 100 mL with double distilled water. The stock solution of 40% acrylamide was stored in into a brown glass bottle and kept at 4°C.

6% Acrylamide Solution (50mL)

Reagents	*Quantity (50 mL)*
Urea	21 g
40% acrylamide solution	7.5 mL
10x TBE	5 mL
Double distilled water	made to 50 mL

10% APS:

0.1 g APS (Ammonium Persulfate) was dissolved in 1000 µL ultra-pure water.

Bind silane solution:

Reagents	*Quantity (50 mL)*
Ethanol	950 µL
Water	45 µL
Glacial acetic acid	5 µL
Bind silane	3 µL

Repel silane solution:

2% dichlorodimethylsilane in heptane.

III. Preparation of chemicals/solutions for Silver staining

Fix/Stop Solution (2 L) (10% acetic acid)

200 mL of glacial acetic acid was added to 1,800 mL of double distilled water.

Staining Solution (2 L)

2 g of silver nitrate (AgNO3) was dissolved in 2 L of double distilled water. 3 mL of 37% formaldehyde was added to the solution.

Developing Solution (2 L)

60g of sodium carbonate (Na_2CO_3) was dissolved in 2 L of double distilled water. It was kept in -20°C for about 30 min. 3mL of 37% formaldehyde and 400 µL of sodium thiosulfate as added immediately before staining.

APPENDIX B

Table B1. Sequences of the primers used in the pre-selective amplification and selective amplifications. In parentheses (columns 3) are the letters representing the *Eco*RI and *Pst*I primers.

Primer	*Primer sequence 5'-3'*	
E-A	5'GACTGCGTACCAATTCA3'	
P-A	5'GACTGCGTACATGCAGA3'	
E-AAT	5'GACTGCGTACCAATTCAAT3'	(E-f)
E-ACT	5'GACTGCGTACCAATTCACT3'	(E-g)
E-AAC	5'GACTGCGTACCAATTCAAC3'	(E-h)
E-AAG	5'GACTGCGTACCAATTCAAG3'	(E-i)
E-AGT	5'GACTGCGTACCAATTCAGT3'	(E-j)
E-ACG	5'GACTGCGTACCAATTCACG3'	(E-k)
P-ACG	5'GACTGCGTACATGCAGACG3'	(P-f)
P-AAT	5'GACTGCGTACATGCAGAAT3'	(P-g)
P-CGA	5'GACTGCGTACATGCAGCGA3'	(P-h)
P-ATG	5'GACTGCGTACATGCAGATG3'	(P-i)
P-AAA	5'GACTGCGTACATGCAGAAA3'	(P-k)
P-ATT	5'GACTGCGTACATGCAGATT3'	(P-j)

Table B2. Sequences of the adapter used in the template preparation.

Adapters	**Adapter sequence 5'-3'**
EcoR I adapter 1	CTCGTAGACTGCGTACC
EcoR I adapter 2	AATTGGTACGCAGTC
Pst I adapter 1	CTCGTAGACTGCGTACATGCA
Pst I adapter 2	TGTACGCAGTCTAC

APPENDIX C

Tables showing Allele frequency, PIC value and Ib value of all the bands of 5 primer combinations.

Table C1. E-AAC/P-ATG (hi) primer combination

Bands	*frequency*	*PIC*	*Ib*
1	0.73	0.4	0.94
2	0.64	0.46	0.82
3	0.77	0.35	0.82
4	0.59	0.48	0.7
5	0.87	0.22	0.5
6	0.87	0.22	0.5
7	0.73	0.4	0.94
8	0.84	0.26	0.58
9	0.77	0.35	0.82
10	0.87	0.22	0.5
11	0.81	0.3	0.7
12	0.81	0.3	0.7
13	0.84	0.26	0.58
14	0.77	0.35	0.82
15	0.77	0.35	0.82
16	0.84	0.26	0.58
Total	**12.52**	**5.18**	**11.32**
Average		0.32375	

Table C2. E-AAT/P-CGA (fh) primer combination

Bands	*frequency*	*PIC*	*Ib*
1	0.77	0.35	0.82
2	0.73	0.4	0.94
3	0.77	0.35	0.82
4	0.54	0.5	0.58
5	0.73	0.4	0.94
6	0.54	0.5	0.58
7	0.73	0.4	0.94
8	0.73	0.4	0.94
9	0.87	0.22	0.5
10	0.54	0.5	0.58
11	0.77	0.35	0.82
12	0.64	0.46	0.82
13	0.73	0.4	0.94
14	0.54	0.5	0.58
15	0.81	0.3	0.7
16	0.81	0.3	0.7
Total	**11.25**	**6.33**	**12.2**
Average		0.395625	

Table C3. E-ACG/P-AAA (kk) primer combination

Bands	*Frequency*	*PIC*	*Ib*
1	0.84	0.26	0.58
2	0.84	0.26	0.58
3	0.49	0.5	0.48
4	0.54	0.5	0.58
5	0.87	0.22	0.5
6	0.77	0.35	0.82
7	0.59	0.48	0.7
8	0.64	0.46	0.82
9	0.77	0.35	0.82
10	0.54	0.5	0.58
11	0.77	0.35	0.82
12	0.77	0.35	0.82
13	0.73	0.4	0.94
14	0.73	0.4	0.94
15	0.81	0.3	0.7
16	0.77	0.35	0.82
17	0.69	0.42	0.94
18	0.84	0.26	0.58
19	0.81	0.3	0.7
20	0.77	0.35	0.82
21	0.77	0.35	0.82
22	0.77	0.35	0.82
23	0.81	0.3	0.7
24	0.77	0.35	0.82
25	0.81	0.3	0.7
26	0.81	0.3	0.7
27	0.77	0.35	0.82
Total	**20.09**	**9.66**	**19.92**
Average		0.357778	

Table C4. E-AAC/P-AAT (hg) primer combination

Bands	*Frequency*	*PIC*	*Ib*
1	0.81	0.3	0.7
2	0.64	0.46	0.82
3	0.84	0.26	0.58
4	0.73	0.4	0.94
5	0.64	0.46	0.82
6	0.87	0.22	0.5
7	0.64	0.46	0.82
8	0.35	0.46	0.24
9	0.77	0.35	0.82
10	0.87	0.22	0.5
Total	**7.16**	**3.59**	**6.74**
Average		0.359	

Table C5. E-ACT/P-AAA (gk) primer combination

Bands	*Frequency*	*PIC*	*Ib*
1	0.91	0.17	0.36
2	0.77	0.35	0.82
3	0.69	0.42	0.94
4	0.69	0.42	0.94
5	0.84	0.26	0.58
6	0.59	0.48	0.7
7	0.81	0.3	0.7
8	0.81	0.3	0.7
9	0.87	0.22	0.5
10	0.69	0.42	0.94
11	0.73	0.4	0.94
12	0.64	0.46	0.82
13	0.9	0.18	0.2
Total	**9.94**	**4.38**	**9.14**
Average		0.350833	

Table D1. Score chart for E-ACG/P-AAA (kk) primer combination in 117 progenies and TV1 and TV19 parents

		TV1	TV 19	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	kka1	np	nn	nn	np	nn	np	np	np	np	np	np	nn	nn	np	np	nn	nn	np	np	nn	nn	np	np	np	np	np	nn	nn	np	np	np	np
2	kka2	np	nn	nn	nn	np	np	nn	np	np	nn	nn	nn	np	np	np	np	nn	np	np	np	np	np	np	np	nn	np	nn	nn	np	np	np	np
3	kka9	np	nn	nn	nn	nn	np	np	np	nn	nn	nn	nn	nn	nn	np	np	nn	np	np	np	nn	np	nn	np	np	np	nn	nn	nn	np	np	np
4	kka10	np	nn	nn	np	np	np	np	np	nn	nn	np	nn	nn	np	np	np	nn	np	np	np	np	np	nn	np	np	np	np	nn	nn	np	np	np
5	kka11	np	nn	nn	np	nn	np	np	np	nn	nn	np	np	nn	nn	nn	nn	np	np	nn	np	np	np	nn	np	np	np	np	nn	nn	nn	np	np
6	kka14	np	nn	nn	np	np	np	nn	nn	nn	np	nn	np	np	np	nn	np	nn	np	nn			nn	np	np	np	nn	np	nn	nn	np	np	np
7	kka25	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	np	np	np	nn	nn	np	nn	np	nn	np	nn	np	nn	np	nn	nn	np	np	np	np
8	kka27	np	nn	nn	np	np	nn	nn	np	np	np	np	np	nn	np	nn	np	np	np	np	nn	np	np	nn	np	nn	np	np	nn	nn	np	np	np
9	kka3	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	ll	ll
10	kka4	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	li	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm
11	kka5	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	ll	lm	ll	lm	lm	ll	ll	lm	lm	lm
12	kka6	ll	lm	ll	ll	lm	lm	ll	ll	lm	ll	ll	lm	ll	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm
13	kka7	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	ll	ll	ll	lm	lm	lm	lm	lm	ll	lm	lm	ll	lm	lm	lm
14	kka8	ll	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	ll	ll	ll	ll	lm	lm
15	kka12	ll	lm	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm
16	kka13	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm
17	kka15	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	lm	ll	ll	ll	ll	lm	ll	ll	lm	ll	ll
18	kka16	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	lm
19	kka17	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm
20	kka18	ll	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm
21	kka19	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	ll	lm	ll	ll	lm	lm	ll	lm	lm	ll
22	kka20	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm	ll
23	kka21	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll
24	kka22	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll	ll	ll	lm	ll	ll	ll	ll
25	kka23	ll	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	ll
26	kka24	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm
27	kka26	ll	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	lm	lm	ll	ll	ll	ll	ll	lm	lm
28	kka28	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll
29	kka29	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm
30	kka30	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	ll
31	kkb1	h	h	kk	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	h	h
32	kkb2	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
33	kkb3	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	kk	kk	kk	h	h	kk	h	h	h	h	h	h	h	h
34	kkb4	h	h	h	h	h	kk	kk	h	h	kk	kk	h	kk	h	h	kk	h	h	h	kk	h	kk	h	h	h	h	h	kk	h	kk	kk	h
35	kkb5	h	h	kk	h	kk	h	h	h	h	kk	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	kk	h	kk	h	h	h	h	h
36	kkb6	h	h	kk	h	kk	kk	h	h	h	kk	kk	h	h	h	h	kk	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h	kk	h
37	kkb7	h	h	kk	h	kk	h	h	h	h	kk	h	kk	kk	h	h	h	h	h	h	h	h	h	h	kk	h	kk	h	h	h	kk	h	h
38	kkb8	h	h	kk	h	h	h	h	h	kk	kk	h	kk	h	h	h	kk	h	h	h	h	kk	h	h	kk	h	h	h	h	kk	kk	h	h
39	kkb9	h	h	kk	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	h	kk	h	h	kk	h	kk	h	h	h	kk	h	h
40	kkb10	h	h	kk	h	h	h	h	h	h	kk	h	kk	h	h	h	h	kk	h	h	kk	h	kk	h	kk	h	h	h	h	h	h	h	h

		TV1	TV 19	30	31	32	33	34	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	kka1	np	nn	np	np	nn	np	np	nn	nn	nn	np	nn	np	nn	nn	np	nn	np	nn	np	nn	nn	np	np	nn	nn	nn	np	nn	nn	nn	np
2	kka2	np	nn	np	np	np	np	np	np	nn	np	nn	nn	np	np	nn	np	nn	np	np	nn	np	np	np	np	np	nn	nn	np	nn	np	nn	np
3	kka9	np	nn	np	nn	np	nn	np	nn	nn	np	np	nn	np	nn	np	np	nn	nn	nn	np	nn	nn	np	np	np	nn	nn	np	np	nn	nn	np
4	kka10	np	nn	np	np	nn	np	np	np	nn	nn	np	nn	np	nn	nn	np	nn	nn	nn	np	nn	nn	np	np	nn	nn	nn	np	nn	nn	nn	np
5	kka11	np	nn	np	nn	nn	nn	np	nn	nn	np	np	nn	np	nn	nn	np	nn	nn	nn	nn	nn	nn	np	np	nn	nn	nn	nn	nn	nn	np	np
6	kka14	np	nn	np	np	nn	np	np	np	nn	nn	np	nn	nn	nn	nn	np	nn	np	np	np	nn	nn	np	np	nn	nn	nn	nn	nn	nn	nn	np
7	kka25	np	nn	np	np	np	np	nn	np	nn	nn	np	nn	np	nn	np	np	nn	np	nn	np	nn	nn	np	np	np	nn	np	nn	nn	np	nn	np
8	kka27	np	nn	np	np	nn	nn	nn	np	nn	np	nn	nn	np	nn	nn	np	nn	np	nn	np	nn	np	np	np	np	np	np	nn	nn	nn	np	np
9	kka3	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	lm	lm	lm
10	kka4	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm
11	kka5	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	ll
12	kka6	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll	ll	lm
13	kka7	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm
14	kka8	ll	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm
15	kka12	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm
16	kka13	ll	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm
17	kka15	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	ll	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll	lm	lm	ll	ll	lm
18	kka16	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm
19	kka17	ll	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll	ll
20	kka18	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll	ll	ll	ll	ll	lm
21	kka19	ll	lm	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm
22	kka20	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm
23	kka21	ll	lm	ll	ll	ll	lm	lm	lm	lm	lm	lm	ll	lm	ll	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm
24	kka22	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm
25	kka23	ll	lm	ll	lm	ll	ll	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm
26	kka24	ll	lm	lm	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll	lm	ll	ll	ll	lm	ll	ll	lm	lm	ll	ll	ll	lm
27	kka26	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	lm	ll	ll	lm	lm
28	kka28	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm
29	kka29	ll	lm	lm	lm	lm	lm	lm	lm	ll	lm	ll	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	ll	ll	ll	lm
30	kka30	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	ll	ll	lm
31	kkb1	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	kk	h	h	h	h	h
32	kkb2	h	h	h	h		h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
33	kkb3	h	h	n	h	h	h	h	h	h	h	h	h	h	kk	kk	kk	h	h	kk	h	h	kk	kk	kk	h	h	h	h	h	h	h	h
34	kkb4	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	h	kk	kk	h	h	h	h	h	kk
35	kkb5	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	kk	h	h	h	kk	h	h
36	kkb6	h	h	h	h	kk	h	h	h	h	h	kk	kk	h	h	h	h	h	h	kk	kk	h	h	kk	kk	h	h	h	h	h	h	kk	h
37	kkb7	h	h	h	h	kk	h	h	kk	h	h	h	kk	h	h	kk	h	kk	h	h	h	h	kk	h	h	kk	h	h	h	h	h	h	h
38	kkb8	h	h	h	h	h	h	kk	h	h	h	h	h	kk	h	kk	h	h	h	h	h	kk	h	h	h	h	h	kk	kk	h	h	h	kk
39	kkb9	h	h	h	h	h	h	kk	h	kk	h	kk	h	kk	h	kk	h	h	h	kk	h	kk	h	h	h	h	h	kk	kk	h	h	h	kk
40	kkb10	h	h	h	h	h	h	h	h	kk	h	kk	h	kk	h	h	h	h	h	h	h	kk	h	h	h	h	h	kk	h	h	h	kk	kk

		TV1	TV 19	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	84	86	87	88	89	90	91	92
1	kka1	np	nn	np	np	nn	nn	nn	np	np	np	nn	np	nn	nn	np	nn	nn	nn	np	nn	np	np	np	nn	np	nn	np	nn	np	np	nn	nn
2	kka2	np	nn	nn	np	nn	np	np	nn	nn	nn	np	nn	np	nn	nn	nn	np	np	np	nn	np	np	np	nn	np	np	np	nn	np	np	np	nn
3	kka9	np	nn	np	np	np	nn	np	nn	nn	nn	np	nn	np	nn	np	nn	nn	nn	nn	np	nn	nn	nn	nn	np	np	np	nn	nn	np	np	nn	nn
4	kka10	np	nn	np	nn	nn	np	np	np	np	nn	np	nn	np	nn	np	nn	nn	nn	nn	nn	nn	nn	nn	nn	np	np	nn	nn	np	np	nn	nn
5	kka11	np	nn	np	nn	nn	nn	np	np	nn	nn	np	nn	np	nn	np	nn	np	nn	nn	nn	np	np	np	nn	np	np	nn	nn	np	nn	nn	nn
6	kka14	np	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn	nn	np	np	nn	np	nn	nn	np	nn	np	np	nn	np	nn	np	nn	np	np	nn	nn
7	kka25	np	nn	nn	np	nn	nn	np	nn	nn	nn	np	nn	np	np	np	nn	np	nn	nn	nn	nn	np	np	np	np	np	nn	nn	np	nn	nn	nn
8	kka27	np	nn	np	nn	np	nn	np	nn	np	np	np	nn	np	nn	np	np	nn	nn	np	nn	nn	np	np	nn	np	np	np	np	np	np	np	np
9	kka3	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll															lm	lm	ll	ll	ll	lm	ll	ll
10	kka4	ll	lm	lm	ll	ll	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	ll	ll	ll	ll	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	ll	ll
11	kka5	ll	lm	ll	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	ll
12	kka6	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm
13	kka7	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	ll
14	kka8	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	ll	ll	ll	lm	ll	ll	ll	lm	ll	ll	ll	lm	ll	ll	lm	ll	ll	ll
15	kka12	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll
16	kka13	ll	lm	ll	ll	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm
17	kka15	ll	lm	ll	ll	ll	ll	lm	lm	ll	ll	ll	ll	ll	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	ll
18	kka16	ll	lm	lm	ll	ll	ll	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm
19	kka17	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	ll	ll	ll	ll	lm	ll	ll	ll	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	ll	ll
20	kka18	ll	lm	ll	ll	lm	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm
21	kka19	ll	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	ll	ll	ll	ll	lm	ll	ll	lm	lm	ll	ll
22	kka20	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	ll	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm
23	kka21	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	ll	lm
24	kka22	ll	lm	ll	ll	ll	ll	lm	lm	lm	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm
25	kka23	ll	lm	ll	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll
26	kka24	ll	lm	ll	lm	ll	lm	ll	ll	ll	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	lm	lm
27	kka26	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm
28	kka28	ll	lm	ll	lm	ll	lm	ll	ll	ll	ll	lm	lm	ll	lm	ll	ll	ll	ll	ll	ll	ll	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll
29	kka29	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	ll	lm	ll	ll	ll	lm	ll	ll	ll	ll	lm	lm	lm	ll	ll	ll	lm	ll	lm	ll	ll
30	kka30	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm
31	kkb1	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	kk	kk	kk	h	h	h	h	h	h	h	h	h	h
32	kkb2	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
33	kkb3	h	h	kk	kk	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h
34	kkb4	h	h	h	h	h	h	h	kk	kk	kk	h	kk	h	h	h	h	h	h	h	h	kk	h	kk	h	kk	kk	h	h	kk	h	h	h
35	kkb5	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h
36	kkb6	h	h	h	h	h	h	kk	h	kk	h	kk	h	kk	h	kk	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h
37	kkb7	h	h	h	h	h	h	h	h	h	h	kk	h	h	kk	h	h	h	h	kk	kk	h	kk	h	h	h	h	h	h	kk	h	h	h
38	kkb8	h	h	kk	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	kk	h
39	kkb9	h	h	kk	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	kk	h	kk	kk	h	h	h	h	h	h	h	kk	h	h	h
40	kkb10	h	h	h	h	kk	h	h	kk	kk	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h

		TV1	TV 19	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	116	117	118	119	120
1	kka1	np	nn	np	nn	nn	np	nn	np	np	nn	nn	np	np	np	np	np	nn	nn	nn	nn	nn	np	nn	np	np	np	nn	np	nn
2	kka2	np	nn	np	nn	np	np	nn	nn	np	nn	nn	np	nn	np	np	np	nn	np	nn	nn	nn	np	nn	np	np	np	nn	nn	nn
3	kka9	np	nn	np	nn	np	np	np	nn	np	nn	nn	np	np	nn	np	np	nn	nn	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn
4	kka10	np	nn	np	nn	np	np	np	nn	np	nn	nn	np	np	np	np	np	nn	nn	np	nn	nn	nn	nn	np	np	np	nn	np	np
5	kka11	np	nn	nn	nn	nn	np	nn	np	nn	nn	nn	np	np	nn	np	nn	nn	nn	np	nn	nn	np	nn	np	np	nn	np	nn	nn
6	kka14	np	nn	nn	nn	np	np	nn	nn	np	nn	np	np	np	np	np	np	np	np	np	nn	nn	nn	nn	np	np	np	np	nn	np
7	kka25	np	nn	np	nn	np	np	np	np	nn	nn	nn	np	np	np	nn	np	np	nn	nn	nn	nn	nn	nn	nn	np	np	nn	nn	np
8	kka27	np	nn	nn	nn	np	np	nn	np	nn	np	np	np	np	np	np	np	np	nn	nn	np	nn	nn	nn	np	np	np	np	nn	nn
9	kka3	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	lm
10	kka4	ll	lm	lm	ll	ll	ll	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm
11	kka5	ll	lm	lm	ll	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll
12	kka6	ll	lm	lm	ll	ll	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm
13	kka7	ll	lm	ll	ll	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll	lm	ll	ll	ll
14	kka8	ll	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm
15	kka12	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	ll	ll	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	ll
16	kka13	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll
17	kka15	ll	lm	ll	ll	ll	ll	ll	lm	ll	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll
18	kka16	ll	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	ll	ll	ll
19	kka17	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	ll	ll	ll	ll	lm	ll	ll	ll	ll	ll
20	kka18	ll	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll
21	kka19	ll	lm	ll	ll	ll	ll	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm
22	kka20	ll	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm	lm	ll	ll	ll	lm	lm	ll	ll	ll	ll
23	kka21	ll	lm	lm	ll	ll	ll	ll	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	ll	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	lm
24	kka22	ll	lm	lm	ll	ll	ll	ll	ll	lm	lm	ll	lm	ll	ll	lm	ll	ll	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll	ll	ll
25	kka23	ll	lm	lm	ll	ll	ll	lm	lm	ll	ll	ll	ll	ll	lm	lm	ll	ll	ll	ll	lm	ll	ll	ll	lm	lm	lm	lm	lm	ll
26	kka24	ll	lm	ll	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm
27	kka26	ll	lm	ll	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	ll	ll	ll	ll	lm	ll	ll	ll	lm	ll	ll	ll	ll	lm
28	kka28	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll
29	kka29	ll	lm	ll	ll	ll	ll	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	ll	ll	ll	ll
30	kka30	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll
31	kkb1	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h
32	kkb2	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
33	kkb3	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
34	kkb4	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	kk	h	h	h
35	kkb5	h	h	kk	h	kk	h	h	kk	kk	h	h	kk	h	h	kk	h	h	kk	kk	h	h	h	h	h	h	kk	h	h	h
36	kkb6	h	h	h	h	kk	h	kk	h	kk	h	h	kk	h	h	kk	h	h	kk	kk	h	h	h	h	h	h	kk	h	h	h
37	kkb7	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h
38	kkb8	h	h	h	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
39	kkb9	h	h	h	h	h	kk	h	h	kk	h	h	h	kk	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h
40	kkb10	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	kk	h	h	h	kk	h	h	h	h	h

Table D2. Score chart for E-AAT/P-CGA (fh) primer combination in 117 progenies and TV1 and TV19 parents

		tv19	tv1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	fha1	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	lm
2	fha2	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	ll	lm	lm	ll	lm	lm	lm
3	fha9	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	ll	ll	ll	ll	ll	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm
4	fha11	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm	lm
5	fha12	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	ll	ll	ll	ll	lm	lm
6	fha13	ll	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	lm	ll	ll	lm	ll	lm
7	fha14	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	lm
8	fha15	ll	lm	ll	ll	ll	ll	ll	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	ll	ll	lm	ll	lm	ll	ll	ll
9	fha17	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	lm	ll	ll	ll	lm	lm
10	fha3	np	nn	nn	np	np	np	np	np	np	nn	nn	nn	nn	np	np	np	nn	np	np	np	nn	np	np	nn	np	nn	np	nn	nn	np	np	np
11	fha5	np	nn	nn	np	nn	np	nn	np	np	nn	nn	nn	nn	np	np	nn	nn	np	np	np	nn	nn	nn	nn	np	nn	np	np	nn	np	nn	nn
12	fha6	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	nn	np	np	np	np	np	nn	nn	np	np	nn	np	np	np	np	nn	nn	np	np	np
13	fha7	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	nn	np	np	np	nn	np	nn	np	nn	np	nn	nn	nn	nn	np	nn	nn	np	np	np
14	fha8	np	nn	nn	np	np	np	nn	np	np	nn	np	np	nn	np	np	np	nn	np	np	nn	np	np	nn	np	np	np	np	nn	nn	np	np	np
15	fha10	np	nn	np	nn	np	nn	nn	np	np	np	np	np	np	np	np	np	nn	np	np	nn	nn	np	nn	np	nn	nn	np	nn	nn	nn	np	nn	np
16	fha16	np	nn	nn	np	np	np	np	nn	np	nn	np	np	nn	nn	np	np	nn	np	np	nn	nn	np	nn	np	nn	nn	nn	np	nn	np	nn	nn	nn
17	fhb1	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-
18	fhb2	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-
19	fhb3	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	kk	kk	kk	h-	h-	h-	h-	kk	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-
20	fhb4	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-
21	fhb5	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-
22	fhb6	h-	h-	kk	h-	h-	kk	kk	h-	h-	h-	h-	h-	kk	h-	h-	kk	h-	h-	kk	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-
23	fhb7	h-	h-	kk	h-	h-	kk	kk	h-	h-	h-	h-	kk	kk	h-	h-	kk	h-	h-	kk	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-
24	fhb8	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	h-	h-	h-
25	fhb9	h-	h-	kk	h-	h-	h-	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	kk	kk	kk	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	kk	h-	h-
26	fhb10	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	kk	h-	kk	h-	kk	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-

		tv19	tv1	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	fha1	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	ll	ll	ll	ll	lm		ll	ll	lm	lm	ll	ll	ll	lm
2	fha2	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	lm		ll	ll	ll	lm	ll	ll	ll	lm
3	fha9	ll	lm	ll	ll	lm	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll	ll		ll	ll	lm	ll	lm	ll	lm	lm
4	fha11	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	ll	lm		ll	ll	lm	lm	lm	ll	ll	lm
5	fha12	ll	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	ll	ll	ll	ll	ll	lm	lm	lm	ll	ll		ll	ll	lm	lm	ll	ll	ll	lm
6	fha13	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	lm		ll	lm	lm	lm	ll	ll	ll	lm
7	fha14	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	ll	lm		lm	lm	lm	lm	ll	ll	lm	ll
8	fha15	ll	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll		lm	lm	lm	lm	ll	ll	lm	lm
9	fha17	ll	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	ll	lm	ll	lm	ll	lm	ll	ll	ll		lm	lm	lm	lm	ll	ll	lm	lm
10	fha3	np	nn	np	nn	np	np	nn	nn	nn	nn	np	nn	np	nn	nn	np	nn	np	np	np	np	nn	np		nn	nn	nn	np	np	nn	nn	np
11	fha5	np	nn	np	np	np	np	np	np	nn	nn	np	nn	np	nn	np	np	nn	nn	np	np	nn	np	np		nn	nn	nn	nn	np	np	np	np
12	fha6	np	nn	nn	nn	np	np	nn	nn	nn	np	np	np	np	nn	np	nn	nn	np	nn	np	np	nn	nn	-	nn	nn	np	nn	np	nn	nn	np
13	fha7	np	nn	nn	nı	np	nn	np	nn	np	nn	np	np	nn	np	nn	np	nn	np	np	nn	np	nn	np		nn	nn	nn	np	nn	nn	nn	np
14	fha8	np	nn	np	nn	np	np	nn	nn	np	np	np	np	nn	nn	nn	np	nn	np	nn	np	np	nn	np		nn	nn	nn	np	np	nn	nn	np
15	fha10	np	nn	np	nn	np	np	nn	np	np	nn	np	np	nn	np	nn	np	nn	nn	np	nn	nn	np	np		nn	nn	nn	nn	np	nn	nn	nn
16	fha16	np	nn	np	np	np	np	nn	np	nn	np	nn	np	nn	np	np	np	np	nn	np	np	np	nn	nn		np	np	np	nn	nn	np	np	nn
17	fhb1	h	h	kk	h	kk	h	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	kk	kk	kk	h	h	h	kk	h	h	h	kk	h
18	fhb2	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	kk	kk	h	h	kk	kk	h	h	h	h	h	h
19	fhb3	h	h	kk	kk	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h
20	fhb4	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	kk	h	h	h	h
21	fhb5	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	kk	h
22	fhb6	h	h	kk	kk	h	h	h	h	h	kk	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	h	kk	h	h	kk	h	h	h
23	fhb7	h	h	kk	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	kk	h	h	h	kk	h	h	h
24	fhb8	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	kk	h	h	h	h	h	kk	h	h	h	h	h	h	h	kk	h	h
25	fhb9	h	h	kk	h	h	h	kk	h	h	h	h	h	h	kk	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	h	kk	h	h
26	fhb10	h	h	kk	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h

		tv19	tv1	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	84	86	87	88	89	90	91	92
1	fha1	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	ll	ll	ll	lm	lm	lm	ll
2	fha2	ll	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm
3	fha9	ll	lm	ll	ll	ll	lm	lm	ll	ll	ll	ll	lm	lm	ll	lm	ll	ll	ll	ll	ll	ll	lm	lm	ll	lm	lm	ll	ll	ll	lm	ll	ll
4	fha11	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	ll	ll
5	fha12	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	ll	ll	lm	lm	ll	ll	ll	ll	ll
6	fha13	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm
7	fha14	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	ll
8	fha15	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm
9	fha17	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	ll	lm
10	fha3	np	nn	nn	nn	nn	np	nn	np	nn	nn	np	nn	np	nn	np	np	np	nn	nn	nn	np	np	np	nn	nn	np	np	nn	np	np	nn	nn
11	fha5	np	nn	np	np	nn	np	nn	nn	nn	nn	np	nn	np	np	nn	nn	nn	nn	np	nn	np	np	np	nn	nn	np	nn	nn	np	nn	nn	nn
12	fha6	np	nn	np	nn	nn	nn	nn	np	nn	nn	np	np	nn	np	nn	nn	np	nn	nn	nn	np	np	np	nn	np	np	nn	nn	nn	np	nn	nn
13	fha7	np	nn	nn	np	nn	np	np	np	nn	nn	np	nn	np	nn	np	np	np	nn	np	nn	np	np	np	nn	np	np	np	np	np	nn	nn	nn
14	fha8	np	nn	np	nn	nn	np	np	np	nn	nn	np	nn	nn	nn	nn	nn	np	nn	np	nn	np	np	np	nn	np	np	np	nn	nn	np	np	nn
15	fha10	np	nn	nn	np	nn	np	np	np	nn	nn	np	nn	np	np	nn	nn	np	nn	nn	nn	np	np	np	nn	np	nn	nn	nn	np	nn	np	nn
16	fha16	np	nn	nn	np	np	nn	np	nn	np	np	np	nn	nn	np	np	nn	nn	nn	np	nn	nn	np	np	nn	np	np	nn	nn	nn	np	np	nn
17	fhb1	h	h	h	kk	kk	h	kk	h	h	kk	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	kk	h	h	h	h
18	fhb2	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	kk
19	fhb3	h	h	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h
20	fhb4	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	kk	h	kk	h	h	h	h	h
21	fhb5	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	h	h	h	h	h
22	fhb6	h	h	h	kk	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h
23	fhb7	h	h	h	h	h	kk	h	h	h	kk	h	h	kk	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h
24	fhb8	h	h	h	h	kk	h	kk	h	kk	h	h	h	h	h	h	h	h	kk	kk	h	h	h	kk	kk	h	h	h	kk	kk	h	h	h
25	fhb9	h	h	h	h	kk	h	kk	h	kk	kk	h	h	h	h	h	kk	h	kk	kk	h	h	h	h	kk	h	h	kk	h	h	h	kk	h
26	fhb10	h	h	kk	kk	h	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	kk

		tv19	tv1	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	116	117	118	119	120
1	fha1	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm		lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	ll	ll	ll
2	fha2	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm	lm	lm		lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll
3	fha9	ll	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm		lm	ll	ll	ll	ll	ll	lm	ll	lm	lm	ll	ll	lm	lm
4	fha11	ll	lm	ll	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm		lm	lm	lm	ll	ll	ll	lm	ll	ll	lm	ll	lm	ll	lm
5	fha12	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	ll	lm	lm		ll	ll	ll	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll
6	fha13	ll	lm	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll		lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	ll	lm
7	fha14	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm	lm	lm		lm	lm	lm	ll	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll
8	fha15	ll	lm	ll	lm	lm	ll	ll	ll	ll	ll	ll	ll	lm	lm		ll	lm	ll	ll	lm	ll	ll	lm	ll	ll	ll	ll	lm	lm
9	fha17	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	lm		lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm
10	fha3	np	nn	np	np	np	np	nn	np	nn	np	np	np	np	np		np	nn	nn	np	np	nn	nn	nn	np	np	np	nn	nn	nn
11	fha5	np	nn	np	nn	nn	nn	nn	np	nn	nn	nn	np	np	nn		np	nn	np	nn	np	np	np	nn	np	np	np	np	np	np
12	fha6	np	nn	np	nn	nn	np	np	np	nn	np	np	np	np	np		np	nn	nn	nn	nn	nn	np	np	np	np	nn	nn	np	nn
13	fha7	np	nn	nn	nn	nn	np	np	np	nn	nn	nn	np	np	np		np	nn	np	nn	nn	nn	nn	nn	np	np	nn	nn	nn	nn
14	fha8	np	nn	nn	nn	np	np	nn	nn	nn	nn	nn	np	np	np		np	nn	nn	nn	nn	nn	nn	nn	np	np	nn	np	np	nn
15	fha10	np	nn	np	np	np	np	nn	nn	nn	np	np	np	np	nn		np	np	np	np	np	nn	nn	np	nn	np	np	np	nn	nn
16	fha16	np	nn	nn	np	np	np	np	nn	nn	nn	nn	np	np	np		nn	np	nn	nn	np	np	nn	nn	np	np	nn	np	np	np
17	fhb1	h	h	h	h	h	h	h	h	h	h	h	h	h	h		h	h	h	h	h	h	h	h	h	h	h	h	kk	h
18	fhb2	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h		h	h	h	h	h	h	h	h	h	h	h	h	h	kk
19	fhb3	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h		h	h	kk	h	h	h	h	h	h	h	h	h	h	h
20	fhb4	h	h	h	h	h	h	h	h	h	h	h	h	h	n		h	kk	h	h	h	h	h	h	h	h	h	h	h	h
21	fhb5	h	h	h	h	kk	h	h	h	h	kk	h	kk	h	h		h	h	h	h	h	h	kk	h	h	h	h	h	h	h
22	fhb6	h	h	h	h	h	h	h	kk	h	kk	kk	h	h	h		h	kk	h	h	h	h	kk	h	h	h	h	kk	h	kk
23	fhb7	h	h	h	h	h	h	h	h	h	h	h	kk	h	kk		kk	kk	h	h	h	h	h	kk	h	h	h	h	kk	kk
24	fhb8	h	h	kk	h	h	kk	h	h	h	h	kk	h	h	h		h	h	h	kk	h	h	h	h	kk	h	kk	h	h	h
25	fhb9	h	h	h	kk	h	h	h	h	h	kk	h	h	h	h		h	h	h	h	h	h	h	h	h	h	h	h	kk	h
26	fhb10	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h		h	h	kk	kk	h	h	h	h	h	kk	h	h	h	h

Table D3. Score chart for E-AAC/P-AAT (hg) primer combination in 117 progenies and TV1 and TV19 parents

		tv19	tv1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	hga1	np	nn	np	nn	nn	nn	nn	nn	nn	np	np	np	np	nn	nn	np	np	nn	np	nn	nn	np	nn	np	np	nn	nn	np	np	np	np	np
2	hga3	np	nn	np	np	np	np	nn	np	np	nn	nn	np	nn	np	np	np	nn	np	np	nn	nn	np	nn	np	np	np	nn	nn	nn	nn	np	np	np
3	hga5	np	nn	np	np	np	np	np	np	np	nn	np	nn	nn	np	np	np	nn	np	nn	np	nn	np	np	np	np	nn	nn	nn	nn	np	np	np
4	hga6	np	nn	np	np	np	np	np	nn	np	nn	np	np	nn	nn	nn	np	nn	np	np	nn	nn	np	nn	np	nn	np	nn	np	np	np	nn	nn
5	hga7	np	nn	nn	np	np	np	np	np	np	nn	nn	nn	np	np	np	np	nn	np	np	nn	nn	nn	nn	np	np	nn	nn	np	nn	np	np	np
6	hga17	np	nn	np	np	np	np	np	np	np	np	np	np	np	np	np	np	nn	np	np	nn	nn	np	nn	nn	np	np	np	np	np	nn	np	np
7	hga18	np	nn	nn	np	np	np	nn	np	nn	np	np	nn	np	nn	nn	np	nn	np	nn	np	np	np	np	nn	nn	nn	np	nn	nn	np	np	nn
8	hga2	ll	lm	lm	ll	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	ll	ll	ll	lm	lm	lm
9	hga4	ll	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll
10	hga9	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	ll
11	hga11	ll	lm	lm	lm	lm	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	ll	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	lm
12	hga12	ll	lm	lm	lm	lm	ll	lm	lm	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll
13	hga13	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	lm
14	hga14	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	ll	lm	ll	ll	ll	ll	lm	ll	lm	ll	lm	ll	ll	lm	lm	lm
15	hga15	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	lm	ll
16	hga16	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	lm	lm
17	hgb1	h	h	kk	h	h	h	h	kk	h	kk	h	h	h	h	h	h	kk	h	h	kk	h	h	kk	h	h	h	h	h	h	h	h	kk
18	hgb2	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	kk	h	h	kk	h	h	h	h	h	kk	kk	h	kk
19	hgb3	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
20	hgb4	h	h	h	h	kk	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	kk	h	h	h	h	h	kk	h	h	kk
21	hgb5	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h
22	hgb6	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h
23	hgb7	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
24	hgb8	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	kk
25	hgb9	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	h	h
26	hgb10	h	h	h	h	h	h	h	h	h	kk	h	kk	h	h	h	h	kk	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h
27	hgb11	h	h	h	h	h	h	kk	kk	h	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	h	kk
28	hgb12	h	h	h	kk	h	h	h	kk	h	h	h	kk	h	h	h	h	kk	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h
29	hgb13	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h
30	hgb14	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	kk	h	h	h	h	h	kk	h	kk	kk	h	h	h	h	h	kk
31	hgb15	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h
32	hgb16	h	h	h	h	h	h	h	kk	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h

		tv19	tv1	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	hga1	np	nn	np		np	np				nn	np	np	np	np	np	np	np	np	nn	np	nn	nn	np	nn	nn	nn	np	nn	nn	np	nn	np
2	hga3	np	nn	np		np	np				np	np	nn	np	np	nn	np	nn	np	np	np	nn	nn	np	np	np	nn	np	nn	nn	np	np	np
3	hga5	np	nn	np		np	np				nn	np	nn	np	nn	np	nn	nn	np	np	np	np	nn	np	nn	nn	nn	nn	np	nn	nn	np	np
4	hga6	np	nn	np		np	np				nn	nn	np	nn	np	nn	np	nn	nn	nn	np	nn	nn	nn	np	np	np	np	nn	nn	np	np	nn
5	hga7	np	nn	nn		nn	np				nn	nn	np	nn	nn	nn	np	nn	np	nn	np	nn	nn	np	np	np	nn	nn	nn	np	nn	np	np
6	hga17	np	nn	np		np	nn				np	np	nn	np	nn	nn	np	nn	np	np	np	np	nn	np	nn	nn	np	nn	np	np	nn	np	np
7	hga18	np	nn	np		np	np				nn	nn	np	nn	nn	np	nn	np	nn	nn	np	nn	nn	nn	np	np	nn	nn	np	nn	nn	nn	nn
8	hga2	ll	lm	lm		ll	lm				lm	lm	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll	lm	lm
9	hga4	ll	lm	lm		lm	lm				lm	lm	lm	lm	ll	ll	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	ll
10	hga9	ll	lm	lm		lm	lm				ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm
11	hga11	ll	lm	lm		lm	lm				ll	lm	ll	ll	ll	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	ll
12	hga12	ll	lm	ll		lm	lm				lm	lm	lm	ll	ll	lm	ll	ll	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll	lm	ll	ll	ll	lm
13	hga13	ll	lm	ll		ll	lm				lm	lm	ll	lm	ll	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	ll	lm	ll	ll	ll	lm
14	hga14	ll	lm	ll		ll	lm				lm	ll	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm
15	hga15	ll	lm	lm		lm	lm				lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	lm	ll	ll	lm
16	hga16	ll	lm	lm		lm	ll				lm	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	lm
17	hgb1	h	h	h		h	kk				h	h	kk	h	h	h	kk	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	kk
18	hgb2	h	h	h		h	kk				h	h	h	h	kk	kk	h	h	h	h	h	kk	h	h	h	h	kk	h	h	h	h	h	h
19	hgb3	h	h	h		h	h				h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	kk	kk	h	h	h	h	h	h
20	hgb4	h	h	h		h	h				h	h	h	h	kk	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	h	h	kk	h
21	hgb5	h	h	h		h	h				h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	h	h	h	h
22	hgb6	h	h	h		h	h				h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	h	kk	h	h	h
23	hgb7	h	h	h		h	h				h	h	h	h	kk	h	kk	h	h	h	h	kk	kk	h	h	kk	kk	h	h	h	h	h	kk
24	hgb8	h	h	h		kk	h				h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	kk	kk	h	h	h
25	hgb9	h	h	h		kk	h				h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	kk	h	h
26	hgb10	h	h	h		kk	kk				kk	h	h	h	h	h	h	kk	h	h	h	h	kk	h	h	h	kk	kk	kk	h	h	h	h
27	hgb11	h	h	h		h	h				h	h	h	h	h	h	h	h	kk	h	h	kk	h	h	h	h	kk	kk	kk	kk	h	h	h
28	hgb12	h	h	h		h	h				h	h	kk	h	h	h	h	h	kk	h	h	h	h	kk	h	h	h	h	h	h	h	h	h
29	hgb13	h	h	h		h	h				h	h	h	h	kk	h	h	h	h	h	h	kk	h	h	h	h	kk	h	h	h	h	h	kk
30	hgb14	h	h	kk		h	h				h	h	h	h	h	h	h	kk	h	h	kk	h	kk	h	h	h	kk	h	h	h	h	kk	kk
31	hgb15	h	h	h		h	h				h	h	h	kk	kk	h	h	h	h	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h
32	hgb16	h	h	h		h	h				h	h	h	kk	kk	h	kk	h	h	h	h	h	kk	h	h	h	kk	kk	h	kk	h	kk	h

		tv19	tv1	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	84	86	87	88	89	90	91	92
1	hga1	np	nn	nn	nn	np	np	np	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	nn	nn	nn	np	np	np	np	nn	nn	np	np	nn	nn
2	hga3	np	nn	np	nn	np	nn	np	np	np	np	np	nn	np	nn	nn	np	nn	np	nn	np	nn	np	np	np	nn	np	np	nn	np	np	np	nn
3	hga5	np	nn	nn	nn	np	nn	nn	np	np	nn	np	nn	np	nn	np	nn	np	nn	nn	nn	np	np	np	nn	np	np	nn	nn	np	np	np	nn
4	hga6	np	nn	nn	np	np	nn	nn	nn	nn	np	np	nn	nn	nn	nn	nn	nn	np	nn	np	np	np	np	nn	nn	np	nn	np	nn	np	np	nn
5	hga7	np	nn	nn	nn	nn	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn	np	nn	np	nn	np	np	np	nn	np	np	nn	nn	np	np	nn	nn
6	hga17	np	nn	np	np	nn	np	nn	nn	nn	nn	np	np	np	np	np	np	np	nn	nn	np	np	np	np	np	np	np	np	np	np	nn	nn	np
7	hga18	np	nn	nn	nn	np	np	np	nn	np	nn	np	nn	np	nn	np	np	np	nn	nn	nn	nn	np	np	np	np	nn	np	np	np	np	np	nn
8	hga2	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	ll	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	ll	ll	lm
9	hga4	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	li	ll
10	hga9	ll	lm	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	lm	ll	ll	ll	lm	ll	ll	ll	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	ll
11	hga11	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	ll	ll	ll
12	hga12	ll	lm	lm	lm	ll	ll	ll	lm	ll	ll	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll
13	hga13	ll	lm	ll	ll	ll	ll	lm	ll	ll	lm	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll
14	hga14	ll	lm	ll	ll	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll
15	hga15	ll	lm	lm	lm	ll	ll	ll	lm	ll	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll
16	hga16	ll	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll
17	hgb1	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	kk	kk	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	h	kk
18	hgb2	h	h	kk	kk	h	h	h	h	kk	kk	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	kk	h	h	kk	h	h
19	hgb3	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h
20	hgb4	h	h	h	h	kk	h	h	h	kk	h	h	h	h	kk	kk	kk	h	kk	h	h	h	h	h	h	h	h	h	kk	kk	kk	kk	h
21	hgb5	h	h	h	kk	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h
22	hgb6	h	h	h	kk	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h
23	hgo7	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
24	hgb8	h	h	h	h	h	kk	h	h	h	h	h	kk	h	kk	h	h	h	kk	kk	h	h	h	h	kk	h	h	h	h	h	h	h	kk
25	hgb9	h	h	h	h	h	h	h	h	h	h	h	kk	h	kk	h	h	h	kk	kk	h	h	h	h	kk	h	h	h	h	h	h	kk	h
26	hgb10	h	h	h	kk	kk	kk	h	h	kk	h	h	h	h	kk	h	kk	h	h	kk	kk	h	h	h	kk	h	h	h	h	h	h	kk	h
27	hgb11	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	kk	h	kk	h	h	h	h	h	h	kk	h	h	h	h	h	h
28	hgb12	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h
29	hgb13	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
30	hgb14	h	h	kk	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	h	kk	kk	h	h	kk	h	h	h	h	kk	h	h	h	h
31	hgb15	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
32	hgb16	h	h	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	kk	kk	h	h	kk	h	h	h	h

		tv19	tv1	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	116	117	118	119	120
1	hga1	np	nn	nn	nn	np	nn	np	nn	np	nn	np	np	nn	np	np	np	np	nn	nn	nn	np	np	np	np	np	np	nn	nn	np
2	hga3	np	nn	nn	np	np	np	np	nn	np	np	np	np	nn	nn	np	np	nn	np	nn	np	np	np	np	np	np	np	nn	np	np
3	hga5	np	nn	np	np	np	np	nn	np	nn	nn	np	np	np	np	nn	np	np	nn	nn	nn	nn	nn	nn	np	np	nn	np	nn
4	hga6	np	nn	nn	np	np	np	np	nn	np	nn	nn	nn	np	np	np	np	np	nn	nn	np	np	np	np	np	np	nn	nn	np
5	hga7	np	nn	np	nn	nn	np	np	np	np	nn	np	np	nn	np	np	np	nn	nn	nn	nn	np	np	nn	np	np	np	np	np
6	hga17	np	nn	np	np	np	nn	np	np	np	nn	nn	np	np	np	np	np	np	np	np	np	nn	np	np	np	np	np	np	nn
7	hga18	np	nn	np	nn	np	nn	nn	nn	np	nn	nn	np	nn	np	nn	np	nn	nn	nn	nn	np	nn	np	np	np	nn	np	np	nn
8	hga2	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	ll	ll	ll	ll	lm	lm	lm	lm	lm	lm	ll	ll
9	hga4	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	ll	ll	lm	ll	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	ll
10	hga9	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	ll	ll	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	ll
11	hga11	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	lm
12	hga12	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm	ll	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll
13	hga13	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	lm	ll
14	hga14	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll
15	hga15	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	ll
16	hga16	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll
17	hgb1	h-	h-	h-	kk	kk	h-	kk	h-	h-	kk	h-	kk	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	kk
18	hgb2	h-	h-	h-	kk	h-	h-	kk	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk
19	hgb3	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-
20	hgb4	h-	h-	h-	kk	kk	h-	h-	kk	kk	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	--
21	hgb5	h-	h-	h-	h-	h-	h-	h	kk	h	h-	h-	h-	h-	h-	h-	--	--	--	--	--	-	-	--	--	--	--	--	--	--
22	hgb6	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	--	--	--	--	--	--	--	--	--	--	--	--	--	--
23	hgb7	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-
24	hgb8	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk
25	hgb9	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk	h-	kk	h-	h-	h-	h-	h-	kk	h-	kk	h-	kk
26	hgb10	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-
27	hgb11	h-	h-	kk	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	kk	h-	h-	kk	kk	h-	h-	h-	h-	h-
28	hgb12	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	kk	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h	h-	kk	h-	h-	kk
29	hgb13	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk	kk	kk	h-	h-	h	h	h-	h-	h-	h-	h-	kk
30	hgb14	h-	h-	h-	h-	kk	h-	h-	kk	h-	h-	kk	h-	kk	h-	h-	h-	h-	kk	kk	h	h-	h-	kk	h-	h-	h-	h-	kk	h-
31	hgb15	h-	h-	h	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h	h-	h	h-	h-	h-	h-	h-	h-	h-
32	hgb16	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk	h-	kk	h-	h-	kk	h	h-	h-	h-	h-	h	kk	h-	h-	kk

Table D4. Score chart for E-AAC/P-ATG (hi) primer combination in 117 progenies and TV1 and TV19 parents

		tv19	tv1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	hia1	ll	lm	ll	ll	ll	lm	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm	lm	ll	lm	lm	lm
2	hia2	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll	ll	lm	lm	lm
3	hia3	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	lm
4	hia4	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm
5	hia5	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	lm	lm	ll	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll
6	hia13	ll	lm	ll	lm	lm	lm	ll	ll	ll	ll	ll	ll	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	lm
7	hia15	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm
8	hia16	ll	lm	ll	lm	lm	ll	ll	ll	ll	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	fm	ll	lm	ll	ll	ll	lm	lm	ll
9	hia17	ll	lm	lm	ll	ll	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	ll	lm	lm	lm
10	hia18	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll
11	hia6	np	nn	nn	np	np	np	np	nn	np	nn	nn	nn	nn	np	np	np	np	np	np	nn	nn	nn	nn	np	np	nn	np	np	nn	np	np	np
12	hia7	np	nn	nn	nn	np	np	np	nn	np	nn	nn	nn	nn	np	nn	np	np	nn	np	nn	nn	nn	nn	np	np	nn	np	np	nn	np	np	np
13	hia8	np	nn	np	np	np	np	np	np	np	np	nn	nn	nn	np	np	np	np	nn	np	nn	nn	nn	np	np	np	np	np	nn	nn	np	np	np
14	hia9	np	nn	nn	np	np	nn	np	nn	np	nn	np	nn	np	nn	nn	np	np	np	np	nn	nn	np	nn	np	np	np	np	np	nn	np	np	np
15	hia10	np	nn	nn	np	np	np	np	nn	np	nn	np	nn	nn	nn	np	np	nn	np	np	np	np	np	nn	np	nn	nn	np	np	np	np	nn	nn
16	hia11	np	nn	nn	np	np	np	np	nn	np	nn	np	np	nn	np	np	np	np	np	nn	nn	nn	nn	nn	np	np	nn	nn	nn	nn	np	np	np
17	hia14	np	nn	nn	np	np	np	np	nn	nn	nn	np	np	nn	np	np	nn	nn	np	nn	nn	nn	np	np	np	np	nn	np	np	nn	np	nn	nn
18	hia19	np	nn	np	np	nn	np	np	np	np	nn	np	np	nn	np	np	np	np	np	np	np	np	np	np	np	nn	nn	np	np	np	np	np	nn
19	hia20	np	nn	np	np	np	nn	np	np	np	nn	np	np	nn	np	np	nn	np	nn	nn	nn	nn	np	np	np	np	nn	nn	nn	nn	np	np	np
20	hia21	np	nn	np	nn	nn	nn	nn	np	nn	nn	np	np	np	nn	np	np	np	nn	nn	np	nn	nn	np	np	nn	nn	np	nn	np	np	np	nn
21	hia22	np	nn	np	np	nn	nn	nn	np	np	nn	nn	np	np	np	nn	np	np	np	nn	nn	nn	np	nn	np	np	nn	nn	nn	np	np	np	np
22	hia23	np	nn	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	nn	nn	nn	np	np	np	nn	nn	nn	np	np	np	np
23	hib1	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	kk	kk	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-
24	hib2	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h	kk	h-	h-	h-	h-	h-	kk	h-	h-
25	hib3	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	kk	h-	h-
26	hib4	h-	h-	h-	h-	h-	h-	h-	kk	h-	h	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-

		tv19	tv1	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	hia1	ll	lm	ll	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	ll	lm
2	hia2	ll	lm	ll	ll	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	ll
3	hia3	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm
4	hia4	ll	lm	ll	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm
5	hia5	ll	lm	lm	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm
6	hia13	ll	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm
7	hia15	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm	ll	lm	lm	ll
8	hia16	ll	lm	lm	lm	lm	lm	lm	lm	ll	lm	ll	ll	lm	ll	ll	ll	ll	lm	ll	lm	ll	ll	lm	ll	lm	lm	ll	ll	ll	lm	lm	lm
9	hia17	ll	lm	ll	lm	lm	lm	ll	lm	ll	lm	lm	ll	ll	ll	ll	ll	ll	ll	ll	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm
10	hia18	ll	lm	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm
11	hia6	np	nn	nn	nn	np	np	np	nn	nn	np	np	nn	np	nn	nn	nn	nn	np	nn	np	np	nn	np	np	nn	nn	nn	nn	nn	nn	np	np
12	hia7	np	nn	nn	nn	np	np	nn	nn	np	nn	np	nn	np	nn	nn	np	nn	np	nn	np	nn	nn	np	np	np	nn	nn	nn	np	nn	nn	np
13	hia8	np	nn	np	nn	np	np	np	nn	nn	np	np	nn	np	nn	np	np	nn	np	nn	np	nn	nn	np	np	nn	nn	nn	np	np	nn	nn	np
14	hia9	np	nn	nn	nn	np	np	np	np	nn	nn	np	nn	np	nn	nn	np	nn	np	np	np	np	nn	np	nn	nn	nn	nn	np	nn	nn	nn	np
15	hia10	np	nn	np	nn	np	nn	np	np	np	nn	np	nn	np	nn	np	np	nn	np	nn	np	nn	nn	np	np	nn	nn	nn	nn	np	np	nn	np
16	hia11	np	nn	np	np	nn	np	np	np	nn	nn	np	nn	np	nn	nn	nn	nn	nn	nn	np	nn	nn	np	np	nn	nn	nn	nn	nn	nn	nn	np
17	hia14	np	nn	np	nn	np	np	np	np	nn	nn	np	nn	np	nn	np	np	nn	np	np	np	nn	nn	np	np	nn	nn	nn	nn	np	nn	np	np
18	hia19	np	nn	np	np	np	np	nn	np	nn	nn	np	nn	np	nn	nn	np	nn	np	np	np	nn	nn	np	np	np	np	np	nn	nn	nn	nn	nn
19	hia20	np	nn	np	nn	np	nn	np	np	nn	nn	np	nn	np	nn	np	np	nn	np	np	np	np	nn	np	np	nn	nn	nn	nn	np	nn	np	np
20	hia21	np	nn	nn	np	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	nn	np	np	np	nn	nn	nn	nn	np	np	np	np	np	nn	np	np
21	hia22	np	nn	np	nn	np	nn	nn	nn	nn	nn	np	nn	nn	np	nn	np	nn	np	nn	np	nn	nn	np	np	nn	nn	np	nn	nn	np	nn	np
22	hia23	np	nn	np	np	np	np	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	np	nn	nn	np	nn	nn	np	nn	np
23	hib1	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk	h-	h-	kk	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-
24	hib2	h-	h-	kk	h-	h-	h-	h-	kk	kk	h-	h	kk	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	kk	h-	h-	h-	h-	h-	kk	kk
25	hib3	h-	h-	h-	h-	h-	h-	kk	h-	kk	h-	kk	kk	h-	h-	h-	h-	kk	h-	h-	h-	kk	kk	h-	h-	kk	kk	h-	h-	h	h-	kk	h-
26	hib4	h-	h-	h-	h	h	h-	kk	h-	kk	h-	h-	kk	h-	h-	h-	h-	kk	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	kk	h-	h-	h	h

		tv19	tv1	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	84	86	87	88	89	90	91	92
1	hia1	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	ll	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	ll	ll
2	hia2	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll
3	hia3	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	lm	lm
4	hia4	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	ll	ll
5	hia5	ll	lm	ll	ll	lm	ll	lm	ll	ll	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	lm	ll
6	hia13	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll
7	hia15	ll	lm	ll	ll	ll	ll	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll
8	hia16	ll	lm	lm	lm	lm	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm
9	hia17	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll
10	hia18	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	ll
11	hia6	np	nn	nn	nn	nn	nn	nn	nn	nn	nn	np	nn	np	np	np	nn	nn	nn	nn	nn	np	np	np	nn	nn	np	nn	nn	np	np	nn	np
12	hia7	np	nn	nn	np	np	nn	np	nn	np	np	np	np	np	np	np	nn	np	nn	nn	nn	np	nn	np	np	nn	np	nn	nn	nn	nn	nn	nn
13	hia8	np	nn	nn	nn	nn	np	nn	nn	nn	nn	np	np	np	nn	np	nn	nn	np	nn	np	np	nn	nn	nn	np	np	nn	nn	nn	np	nn	np
14	hia9	np	nn	nn	np	nn	nn	np	nn	nn	nn	nn	nn	nn	nn	np	nn	nn	nn	np	np	nn	nn	np	nn	nn	np	nn	nn	np	np	nn	nn
15	hia10	np	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn	np	nn	np	nn	np	nn	nn	nn	np	nn	np	nn	np	nn	nn	nn	nn	np	nn	nn
16	hia11	np	nn	np	np	nn	nn	np	nn	nn	nn	np	nn	np	np	np	nn	np	nn	nn	np	np	np	np	nn	np	np	nn	nn	np	np	nn	nn
17	hia14	np	nn	nn	nn	np	nn	nn	nn	np	nn	np	nn	nn	nn	np	nn	np	nn	nn	nn	np	np	np	nn	np	nn	nn	np	np	np	nn	nn
18	hia19	np	nn	nn	np	np	nn	np	nn	np	np	np	np	np	nn	np	nn	np	np	nn	nn	np	np	np	np	nn	np	nn	nn	np	nn	nn	np
19	hia20	np	nn	nn	np	nn	nn	np	nn	np	nn	np	nn	np	np	np	np	np	np	nn	nn	np	np	np	np	np	np	nn	np	nn	nn	nn	nn
20	hia21	np	nn	nn	nn	np	nn	np	nn	np	np	np	np	np	nn	nn	nn	np	nn	nn	nn	nn	np	np	nn	np	np	nn	nn	np	np	nn	np
21	hia22	np	nn	nn	nn	np	nn	np	nn	nn	nn	--	--	--	--	--	--	--	--	--	--		--	--	--	--	np	nn	nn	np	nn	nn	nn
22	hia23	np	nn	nn	nn	np	nn	np	nn	nn	np	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	np	nn	nn	np	np	np	nn
23	hib1	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	h-	h-	h-	kk	kk	h-	kk	kk	h-	h-	h	h-	h-
24	hib2	h-	h-	h-	h-	h-	kk	h-	h-	kk	h-	h-	kk	kk	h-	h-	h-	h-	h-	kk	h	h	h-	h-	kk	h-	h-	kk	kk	h-	h-	h-	h-
25	hib3	h-	h-	h-	h-	h	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-	h-
26	hib4	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h	h-	kk	h-	h-	h-	kk

		tv19	tv1	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	116	117	118	119	120
1	hıa1	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	ll	ll	lm	lm
2	hıa2	ll	lm	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	lm	ll
3	hıa3	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	ll	lm	ll	lm	ll	ll	lm	lm	ll	lm	ll
4	hıa4	ll	lm	ll	ll	ll	ll	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	ll	lm	ll	ll	lm	ll	ll	ll	lm	lm	ll	ll	ll
5	hıa5	ll	lm	ll	ll	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	ll	lm	lm	ll	ll	lm	ll	lm	lm	ll	ll	lm	ll
6	hıa13	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	ll	lm	ll	ll	lm	lm
7	hıa15	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	ll	lm	ll	lm	ll	lm	ll
8	hıa16	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	ll	lm	ll	ll
9	hıa17	ll	lm	ll	ll	lm	lm	ll	lm	ll	ll	ll	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	lm	lm	ll	ll	lm	ll	lm	lm
10	hıa18	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	ll	ll	lm	ll
11	hıa6	np	nn	nn	nn	nn	np	np	nn	np	nn	nn	np	np	nn	np	np	nn	nn	nn	nn	np	nn	nn	np	np	np	nn	nn	nn
12	hıa7	np	nn	nn	nn	np	np	np	np	nn	nn	nn	np	np	nn	np	np	nn	nn	nn	nn	nn	np	nn	np	np	np	nn	nn	nn
13	hıa8	np	nn	np	nn	np	np	np	nn	np	nn	nn	np	np	nn	np	np	nn	nn	nn	nn	nn	np	nn	nn	np	nn	nn	np	np
14	hıa9	np	nn	nn	nn	np	np	np	nn	nn	np	nn	np	np	nn	np	np	nn	nn	nn	nn	np	nn	nn	nn	np	np	nn	np	nn
15	hıa10	np	nn	nn	np	np	np	np	nn	nn	nn	nn	np	np	nn	np	np	nn	nn	nn	nn	np	nn	nn	np	nn	np	nn	np	nn
16	hıa11	np	nn	nn	np	np	np	np	nn	nn	nn	nn	nn	np	np	nn	np	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn	np	nn
17	hıa14	np	nn	nn	nn	np	nn	np	nn	nn	np	nn	np	np	nn	np	np	nn	nn	nn	np	nn	np	nn	nn	nn	np	np	nn	nn
18	hıa19	np	nn	np	np	np	nn	nn	nn	nn	np	np	np	np	nn	np	np	nn	nn	np	np	nn	np	nn	nn	np	nn	np	nn	nn
19	hıa20	np	nn	np	np	np	np	nn	nn	nn	nn	np	np	np	nn	np	np	nn	np	np	np	nn	np	nn	nn	np	nn	nn	nn	nn
20	hıa21	np	nn	np	nn	np	np	np	nn	np	nn	np	nn	np	nn	np	np	nn	nn	np	np	nn	np	nn	nn	np	np	np	np	nn
21	hıa22	np	nn	nn	nn	np	nn	np	np	nn	nn	np	np	np	nn	np	nn	np	nn	np	np	nn	np	nn	nn	nn	nn	nn	nn	nn	nn
22	hıa23	np	nn	np	np	np	nn	np	np	np	nn	np	np	nn	nn	np	nn	np	nn	nn	nn	nn	np	nn	np	nn	np	nn	np	np
23	hıb1	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-	kk	h-	kk	h-	h-	h-	kk	kk	h-
24	hıb2	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	kk	kk	h-	h-	h	kk	h-	kk	kk	h-	h-
25	hıb3	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	kk	h-	h-	kk	h-	h	h-	h	h-	h	kk	h-	h-	kk	h-	h-
26	hıb4	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	kk	kk	kk	h-	h	h-	h	h-	h-	h-	h-

Table D5. Score chart for E-ACT/P-AAA (gk) primer combination in 117 progenies and TV1 and TV19 parents

		tv19	tv1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	gka1	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	nn	np	np	np	np	np	nn	--	--	nn	nn	np	nn	np	np	nn	nn	np	np	np
2	gka4	np	nn	nn	np	np	np	nn	np	np	nn	np	nn	nn	np	np	np	np	np	nn	--	--	np	np	np	np	nn	np	np	nn	np	np	np
3	gka10	np	nn	nn	np	np	np	np	np	np	nn	np	np	nn	np	np	np	nn	nn	nn	--	--	nn	nn	np	np	nn	nn	np	nn	np	np	np
4	gka12	np	nn	nn	np	np	np	np	np	np	nn	np	np	nn	np	np	np	nn	np	nn	--	--	np	nn	np	nn	np	np	nn	nn	nn	np	np
5	gka13	np	nn	nn	np	np	np	nn	nn	nn	nn	nn	np	np	np	np	np	nn	np	nn	--	--	np	nn	np	nn	np	nn	np	np	np	nn	nn
6	gka14	np	nn	nn	np	np	np	nn	nn	nn	np	nn	np	np	nn	nn	np	nn	np	nn	-	--	nn	np	np	np	nn	np	nn	nn	np	np	np
7	gka15	np	nn	nn	np	np	nn	nn	np	np	nn	nn	np	np	np	nn	np	nn	nn	np	--	--	np	np	nn	np	nn	np	nn	nn	np	nn	np
8	gka2	ll	lm	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	ll	--	--	ll	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm
9	gka3	ll	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	ll	lm	lm	lm	ll	lm	ll	--	--	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm
10	gka5	ll	lm	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll	lm	lm	lm	ll	lm	lm	--	--	ll	ll	lm	lm	lm	ll	lm	lm	lm	ll	lm
11	gka6	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	ll	--	--	lm	lm	lm	lm	ll	lm	ll	ll	lm	lm	lm
12	gka7	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm	lm	lm	ll	lm	lm	--	--	lm	ll	ll	lm	lm	lm	ll	ll	lm	lm	lm
13	gka8	ll	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	ll	--	--	lm	lm	lm	lm	ll	ll	ll	ll	ll	lm	ll
14	gka9	ll	lm	lm	lm	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm	lm	lm	ll	--	--	lm	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm
15	gka11	ll	lm	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	lm	ll	lm	ll	--	--	lm	ll	lm	ll	ll	ll	ll	ll	lm	lm	lm
16	gkb1	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	h-	h-	h-	h-	h-	h-	h-	--	--	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-
17	gkb2	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	--	--	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-
18	gkb3	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-	--	--	h-	h-	kk	h-	h-	h-	kk	kk	h-	h-	h-
19	gkb4	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	--	--	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-
20	gkb5	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	kk	h-	h-	--	--	kk	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-
21	gkb6	h-	h-	h	h-	h-	h-	h-	kk	h-	h-	kk	kk	kk	h-	h-	h-	h-	h-	h-	--	--	h-	h-	h-	kk	kk	kk	h-	h-	h-	h-	h-
22	gkb7	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	kk	kk	kk	h-	h-	h-	h-	h-	--	--	kk	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-
23	gkb8	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	kk	kk	h-	h-	h-	--	--	kk	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-
24	gkb9	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	--	--	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-
25	gkb10	h-	h-	kk	h	h-	h-	kk	h-	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	h-	--	--	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-
26	gkb11	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	--	--	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-
27	gkb12	h-	h-	kk	h-	kk	h-	kk	h-	h-	kk	h-	h-	kk	h-	h-	h-	h-	h-	kk	--	--	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-
28	gkb13	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	--	--	kk	h-	h-	h	h-	h-	h-	h-	h-	h-	h-

		tv19	tv1	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	gka1	np	nn	nn	nn	np	np	nn	nn	np	nn	np	nn	np	np	nn	np	nn	np	np	np	nn	nn	nn	np	nn	nn	nn	nn	nn	nn	nn	np
2	gka4	np	nn	np	nn	np	np	nn	np	nn	nn	np	nn	np	np	nn	np	nn	np	np	np	nn	nn	np	nn	nn	nn	nn	nn	nn	np	nn	np
3	gka10	np	nn	np	nn	np	nn	nn	np	nn	nn	nn	nn	np	nn	nn	np	nn	np	np	np	nn	nn	np	np	nn	nn	nn	nn	nn	np	nn	np
4	gka12	np	nn	np	np	np	np	nn	np	nn	np	np	nn	np	np	nn	np	nn	nn	nn	nn	np	np	nn	np	nn	nn	nn	np	nn	nn	nn	np
5	gka13	np	nn	np	np	np	np	nn	np	nn	np	np	np	np	np	np	np	np	np	np	np	nn	np	nn	np	np	np	np	nn	np	np	nn	nn
6	gka14	np	nn	np	nn	np	np	nn	np	nn	nn	np	nn	nn	nn	nn	np	nn	np	np	np	nn	nn	np	np	nn	nn	nn	nn	nn	nn	nn	np
7	gka15	np	nn	np	nn	nn	np	nn	np	nn	nn	np	nn	np	nn	np	np	nn	np	nn	np	nn	nn	nn	nn	np	np	np	np	np	nn	nn	np
8	gka2	ll	lm	lm	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	lm	lm	ll	ll	ll	lm	ll	ll	lm	lm	ll	ll	ll	lm
9	gka3	ll	lm	lm	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm
10	gka5	ll	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm
11	gka6	ll	lm	lm	lm	lm	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	ll	ll	lm
12	gka7	ll	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm	ll	lm	lm	ll	lm	lm	ll	ll	ll	lm	ll	ll	ll	lm
13	gka8	ll	lm	lm	ll	lm	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	ll	lm	ll	ll	lm	lm	ll	lm	lm	lm	ll	ll	ll	lm
14	gka9	ll	lm	lm	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm	lm	ll	ll	ll	ll	lm
15	gka11	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	ll	ll	lm	ll	lm	ll	lm	lm	ll	lm	lm	lm	lm	lm	lm	ll	lm	lm	ll	ll	ll	lm
16	gkb1	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-
17	gkb2	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-	h-	kk	kk	h-
18	gkb3	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	h-	h-	h-	kk	h-	kk	h-
19	gkb4	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	kk	h-	h-	h-	kk	h-
20	gkb5	h-	h-	h-	kk	h-	h-	kk	kk	h-	h-	h-	kk	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	h-	h-	h-	h-	h-	h-
21	gkb6	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-
22	gkb7	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-
23	gkb8	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-
24	gkb9	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-
25	gkb10	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	kk	kk	h-	kk	h-
26	gkb11	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	kk	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-
27	gkb12	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	kk	kk	h-	h-	kk	h-	h-	h-	h-	h-	h-	h-	h-	h-
28	gkb13	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	h-	kk	h-	h-	h-	kk	kk	kk	h-	h-	h-

		tv19	tv1	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	84	86	87	88	89	90	91	92
1	gka1	np	nn	nn	np	nn	np	np	nn	nn	nn	nn	np	np	nn	nn	nn	np	nn	np	nn	nn	nn	nn	nn	np	np	nn	nn	np	np	nn	np
2	gka4	np	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn	np	np	nn	nn	np	np	nn	np	np	np	np	nn	np	np	nn	nn	np	np	nn	nn
3	gka10	np	nn	nn	nn	nn	nn	nn	nn	nn	nn	np	np	nn	nn	nn	np	np	nn	nn	nn	np	np	np	nn	np	np	nn	nn	np	np	nn	nn
4	gka12	np	nn	np	np	nn	nn	np	np	nn	nn	np	nn	np	nn	np	nn	np	nn	nn	nn	np	np	np	np	np	np	nn	nn	np	nn	np	nn
5	gka13	np	nn	nn	np	nn	nn	nn	nn	np	np	np	np	nn	np	nn	nn	nn	np	nn	nn	np	np	np	nn	np	np	nn	np	np	np	nn	nn
6	gka14	np	nn	nn	nn	nn	nn	np	nn	nn	nn	np	nn	nn	np	np	nn	np	nn	nn	np	nn	np	np	nn	np	nn	np	nn	np	np	nn	nn
7	gka15	np	nn	nn	nn	np	nn	nn	np	nn	nn	np	nn	nn	nn	nn	nn	np	np	nn	nn	np	np	np	np	nn	np	nn	nn	nn	np	nn	nn
8	gka2	ll	lm	ll	ll	ll	ll	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	ll	ll	ll	lm	ll	ll	ll
9	gka3	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	ll
10	gka5	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	ll	ll	lm	ll	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	lm	lm	lm	ll	ll	ll
11	gka6	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	lm	ll	ll	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	ll	ll	ll	lm	ll	ll	lm
12	gka7	ll	lm	ll	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	ll	lm	ll	ll	lm	ll	ll
13	gka8	ll	lm	ll	ll	lm	ll	lm	lm	lm	ll	lm	ll	lm	ll	ll	ll	lm	ll	ll	ll	lm	lm	lm	ll	lm	ll	ll	ll	lm	lm	lm	ll
14	gka9	ll	lm	ll	ll	ll	ll	lm	lm	ll	ll	lm	lm	lm	lm	lm	lm	ll	ll	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	lm	lm	ll	lm
15	gka11	ll	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	ll	lm	ll	lm	lm	ll	ll	lm	ll	ll	lm	lm	ll	ll	lm	lm	ll	lm	ll	lm	lm
16	gkb1	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h
17	gkb2	h	h	h	kk	kk	h	kk	h	kk	kk	h	h	kk	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h
18	gkb3	h	h	h	h	h	kk	h	h	h	h	h	kk	h	kk	kk	h	h	kk	kk	h	h	h	kk	kk	h	h	h	h	h	kk	h	h
19	gkb4	h	h	h	h	kk	h	kk	h	h	h	kk	h	kk	h	h	h	h	h	h	kk	h	h	kk	kk	h	h	h	kk	h	h	kk	h
20	gkb5	h	h	h	h	kk	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	h	h	h	h	h	kk	h	h	kk	h	h	kk	h
21	gkb6	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	kk	h	h	h	kk	kk	h	kk	h	h
22	gkb7	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	kk	kk	kk	kk	h	h	h	kk	h	h	h	h	kk	h	kk	h
23	gkb8	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	kk	kk	kk	h	h	h	h	h	h	h	h	h	h	h	kk	kk
24	gkb9	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	h	kk	h	h	kk	h	h	h	kk	kk	h	kk	h	h
25	gkb10	h	h	h	h	kk	h	h	kk	h	h	h	h	kk	kk	h	kk	h	h	h	kk	h	h	kk	h	kk	h	h	h	h	kk	kk	h
26	gkb11	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h	h	kk	kk	h	h	h	h	h	h	kk	kk	h	h
27	gkb12	h	h	h	h	h	h	h	h	h	h																		h	h	kk	h	h
28	gkb13	h	h	h	h	h	h	h	h	h	h																		h	h	kk	kk	h

		tv19	tv1	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	116	117	118	119	120
1	gka1	np	nn	nn	nn	np	np	np	np	nn	np	np	np	np	np	np	np	nn	nn	nn	nn	nn	np	nn	np	np	nn	np	np	np
2	gka4	np	nn	np	nn	np	np	np	nn	nn	np	np	np	nn	np	np	np	np	np	nn	np	nn	nn	nn	np	np	np	nn	np	nn
3	gka10	np	nn	nn	nn	nn	np	np	nn	np	nn	nn	nn	nn	np	np	np	np	np	nn	np	nn	np	nn	np	nn	np	nn	nn	nn
4	gka12	np	nn	nn	nn	np	np	np	nn	nn	np	nn	nn	np	nn	np	nn	np	nn	nn	np	nn	np	np	np	np	np	np	nn	np
5	gka13	np	nn	nn	np	np	np	nn	nn	nn	nn	nn	nn	np	np	np	np	np	np	nn	nn	nn	nn	nn	np	np	nn	nn	np	nn
6	gka14	np	nn	nn	nn	np	np	nn	nn	np	nn	np	np	np	np	np	np	nn	nn	np	np	nn	np	nn	np	np	np	np	nn	np
7	gka15	np	nn	nn	np	nn	nn	np	nn	nn	np	nn	np	np	np	nn	np	nn	np	nn	np	np	np	np	np	np	nn	np	nn	np
8	gka2	ll	lm	ll	ll	lm	lm	lm	lm	lm	ll	ll	ll	lm	lm	lm	lm	lm	ll	ll	lm	ll	lm	ll	lm	lm	lm	ll	ll	ll
9	gka3	ll	lm	lm	ll	ll	lm	lm	lm	ll	ll	lm	ll	lm	lm	lm	lm	lm	ll	ll	lm	lm	lm	lm	ll	lm	lm	ll	ll	ll
10	gka5	ll	lm	lm	lm	ll	ll	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	ll	ll	lm	ll	lm	ll	ll	lm	ll	ll	lm	ll
11	gka6	ll	lm	lm	ll	ll	ll	lm	lm	ll	lm	ll	lm	ll	lm	lm	lm	lm	ll	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll
12	gka7	ll	lm	lm	ll	ll	lm	ll	lm	ll	ll	ll	ll	ll	lm	lm	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm	lm	ll	lm	lm
13	gka8	ll	lm	ll	ll	ll	ll	lm	lm	ll	lm	lm	lm	ll	lm	lm	ll	lm	ll	ll	lm	ll	ll	ll	lm	lm	lm	lm	ll	lm
14	gka9	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	lm	lm	ll	lm	ll	ll	lm	lm	ll	ll	ll
15	gka11	ll	lm	lm	ll	ll	lm	lm	lm	ll	lm	ll	lm	lm	lm	lm	ll	ll	ll	ll	lm	lm	lm	ll	lm	lm	ll	lm	lm	lm
16	gkb1	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	h
17	gkb2	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	kk	h	kk	h	kk	kk	h	h	h	h	h	h	kk	kk	kk
18	gkb3	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	h	kk	h	h	h
19	gkb4	h	h	h	h	kk	h	kk	h	h	kk	h	h	h	h	h	kk	h	h	h	h	h	kk	kk	h	h	h	h	kk	h
20	gkb5	h	h	h	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	kk	h	kk	kk	h	h	h	kk	h
21	gkb6	h	h	h	h	h	kk	h	kk	kk	h	h	h	h	h	kk	h	h	h	h	h	kk	kk	kk	h	h	kk	kk	h	h
22	gkb7	h	h	h	h	kk	h	h	h	h	h	h	h	h	kk	kk	h	h	h	h	h	kk	h	h	h	h	kk	h	h	h
23	gkb8	h	h	h	kk	kk	kk	h	h	h	h	h	h	h	kk	h	h	kk	h	kk	h	h	h	h	h	h	kk	h	h	h
24	gkb9	h	h	h	h	kk	h	kk	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	h	h	h	kk	h	kk	h	kk
25	gkb10	h	h	h	h	kk	h	h	h	kk	h	h	h	h	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	kk	h
26	gkb11	h	h	h	h	h	h	h	h	h	h	h	kk	h	h	h	h	kk	h	kk	h	h	h	h	h	h	h	h	h	kk
27	gkb12	h	h	n	h	kk	ı	h	kk	h	kk	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h
28	gkb13	h	h	h	t	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h

APPENDIX E1

Pairwise recombination frequency value and LOD score generated by JOINMAP 4.0 for each possible pair of markers in each linkage group of TV1 parent.

TV1-LG1

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	51	fha1	46	gka6	0.2124	8.59
2	51	fha1	44	gka3	0.2655	5.57
3	55	fha12	43	gka2	0.2566	6.03
4	55	fha12	44	gka3	0.2743	5.26
5	52	fha2	46	gka6	0.1947	9.78
6	52	fha2	44	gka3	0.2478	6.49
7	43	gka2	55	fha12	0.2566	6.03
8	43	gka2	44	gka3	0.2609	6.08
9	44	gka3	27	kka12	0.2174	8.39
10	44	gka3	25	kka7	0.2261	7.83
11	46	gka6	52	fha2	0.1947	9.78
12	46	gka6	47	gka7	0.2000	9.54
13	47	gka7	46	gka6	0.2000	9.54
14	47	gka7	30	kka16	0.2522	6.17
15	15	hga11	46	gka6	0.2252	7.65
16	15	hga11	27	kka12	0.2478	6.49
17	10	hia17	25	kka7	0.2308	7.52
18	10	hia17	47	gka7	0.2696	5.34
19	27	kka12	46	gka6	0.2174	8.39
20	27	kka12	44	gka3	0.2174	8.39
21	30	kka16	25	kka7	0.1709	11.65
22	30	kka16	44	gka3	0.2261	7.83
23	22	kka4	23	kka5	0.2222	8.22
24	22	kka4	46	gka6	0.2522	6.34

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
25	23	kka5	22	kka4	0.2222	8.22
26	23	kka5	52	fha2	0.2783	4.96
27	25	kka7	30	kka16	0.1709	11.65
28	25	kka7	44	gka3	0.2261	7.83

TV1-LG2

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	16	hga12	19	hga15	0.1858	10.24
2	16	hga12	14	hga9	0.2035	9.18
3	19	hga15	16	hga12	0.1858	10.24
4	19	hga15	14	hga9	0.2832	4.76
5	14	hga9	16	hga12	0.2035	9.18
6	14	hga9	19	hga15	0.2832	4.76

TV1-LG3

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	29	kka15	33	kka19	0.2564	6.77
2	33	kka19	29	kka15	0.2564	6.77

TV1-LG4

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	2	hia2	3	hia3	0.2051	9.20
2	3	hia3	2	hia2	0.2051	9.20

TV1-LG5

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	50	gka11	49	gka9	0.2348	6.98
2	49	gka9	50	gka11	0.2348	6.98

TV1-LG6

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	54	fha11	4	hia4	0.2609	6.12
2	4	hia4	54	fha11	0.2609	6.12

TV1-LG7

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	17	hga13	8	hia15	0.2743	5.07
2	6	hia12	5	hia5	0.2393	7.26
3	8	hia15	17	hga13	0.2743	5.07
4	5	hia5	6	hia12	0.2393	7.26

TV1-LG8

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	1	hia1	7	hia13	0.2564	6.15
2	7	hia13	11	hia18	0.2564	6.08
3	11	hia18	7	hia13	0.2564	6.08

TV1-LG9

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	40	kka28	41	kka29	0.2393	7.26
2	41	kka29	40	kka28	0.2393	7.26

TV1-LG10

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	34	kka20	35	kka21	0.2564	6.16
2	35	kka21	34	kka20	0.2564	6.16

APPENDIX E2

Pairwise recombination frequency value and LOD score generated by JOINMAP 4.0 for each possible pair of markers in each linkage group of TV19 parent.

TV19-LG1

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	17	hga7	1	hia6	0.2478	6.67
2	17	hga7	2	hia7	0.3274	3.03
3	1	hia6	17	hga7	0.2478	6.67
4	1	hia6	4	hia9	0.2479	6.65
5	2	hia7	1	hia6	0.2479	6.65
6	2	hia7	4	hia9	0.3248	3.11
7	3	hia8	1	hia6	0.2564	6.66
8	3	hia8	4	hia9	0.3333	3.03
9	4	hia9	1	hia6	0.2479	6.65
10	4	hia9	2	hia7	0.3248	3.11

TV19-LG2

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	37	fha6	39	fha8	0.2348	7.36
2	37	fha6	28	gka1	0.2566	6.10
3	39	fha8	37	fha6	0.2348	7.36
4	39	fha8	28	gka1	0.3009	4.03
5	28	gka1	37	fha6	0.2566	6.10
6	28	gka1	39	fha8	0.3009	4.03

TV19-LG3

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	20	kka1	23	kka10	0.2393	7.23
2	20	kka1	22	kka9	0.3248	3.31
3	23	*kka10*	22	*kka9*	*0.2222*	*8.60*
4	23	kka10	20	kka1	0.2393	7.23
5	22	kka9	23	kka10	0.2222	8.60
6	22	kka9	20	kka1	0.3248	3.31

TV19-LG4

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	35	fha3	15	hga5	0.1982	9.08
2	35	fha3	38	fha7	0.2609	6.05
3	38	fha7	35	fha3	0.2609	6.05
4	38	fha7	15	hga5	0.3243	3.13
5	15	hga5	35	fha3	0.1982	9.08
6	15	hga5	38	fha7	0.3243	3.13

TV19-LG5

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	33	gka14	25	kka14	0.0261	29.45
2	25	kka14	33	gka14	0.0261	29.45

TV19-LG6

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	30	gka10	29	gka4	0.2435	7.74
2	29	gka4	30	gka10	0.2435	7.74

TV19-LG7

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	5	hia10	7	hia14	0.2479	6.69
2	7	hia14	5	hia10	0.2479	6.69

TV19-LG8

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	6	hia11	26	kka25	0.2393	7.52
2	26	kka25	6	hia11	0.2393	7.52

TV19-LG9

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	31	gka12	24	kka11	0.2696	6.53
2	24	kka11	31	gka12	0.2696	6.53

TV19-LG10

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	13	hga1	12	hia23	0.2239	4.56
2	11	hia22	12	hia23	0.2794	3.67
3	12	hia23	13	hga1	0.2239	4.56

TV19-LG11

S/n	Nr1	Locus1	Nr2	Locus2	Rec. Freq.	LOD
1	41	fha16	16	hga6	0.2432	6.57
2	32	gka13	41	fha16	0.2920	4.16
3	16	hga6	41	fha16	0.2432	6.57

APPENDIX F1

Position of each marker and interval between the loci for each linkage group of TV1 parent as generated by JOINMAP 4.0.

TV1-LG1

S/n	Nr	Locus	Position	interval (adjacent loci)
1	10	hia17	0.000	--
2	25	kka7	15.439	15.439
3	30	kka16	23.395	7.956
4	47	gka7	29.082	5.687
5	27	kka12	34.056	4.974
6	44	gka3	40.980	4.974
7	46	gka6	45.518	4.538
8	15	hga11	52.304	6.786
9	52	fha2	58.411	6.107
10	51	fha1	61.996	3.585
11	22	kka4	67.831	5.835
12	43	gka2	72.529	4.698
13	55	fha12	80.768	8.239
14	23	kka5	87.663	6.895

TV1-LG2

S/n	Nr	Locus	Position	interval (adjacent loci)
1	14	hga9	0.000	--
2	16	hga12	19.971	19.971
3	19	hga15	38.175	38.175

TV19-LG3

S/n	Nr	Locus	Position	interval (adjacent loci)
1	29	kka15	0.000	--
2	33	kka19	28.327	28.327

TV1-LG4

S/n	Nr	Locus	Position	interval (adjacent loci)
1	2	hia2	0.000	--
2	3	hia3	21.796	21.796

TV1-LG5

S/n	Nr	Locus	Position	interval (adjacent loci)
1	50	gka11	0.000	0.000
2	49	gka9	25.476	25.476

TV1-LG6

S/n	Nr	Locus	Position	interval (adjacent loci)
1	54	fha11	0.000	--
2	4	hia4	28.936	28.936

TV1-LG7

S/n	Nr	Locus	Position	interval (adjacent loci)
1	17	hga13	0.000	0.000
2	8	hia15	21.304	21.304
3	6	hia12	41.546	20.242
4	5	hia5	62.815	21.269

TV1-LG8

S/n	Nr	Locus	Position	interval (adjacent loci)
1	11	hia18	0.000	--
2	7	hia13	20.569	20.569
3	1	hia1	41.315	20.746

TV1-LG9

S/n	Nr	Locus	Position	interval (adjacent loci)
1	40	kka28	0.000	--
2	41	kka29	26.060	26.060

TV1-LG10

S/n	Nr	Locus	Position	interval (adjacent loci)
1	34	kka20	0.000	--
2	35	kka21	28.327	28.327

APPENDIX F2

Position of each marker and interval between the loci for each linkage group of TV19 parent as generated by JOINMAP 4.0.

TV19-LG1

S/n	Nr	Locus	Position	interval (adjacent loci)
1	3	hia8	0.000	--
2	4	hia9	11.305	11.305
3	1	hia6	31.471	20.166
4	2	hia7	51.365	19.894
5	17	hga7	62.045	10.68

TV19-LG2

S/n	Nr	Locus	Position	interval (adjacent loci)
1	28	gka1	0.000	--
2	37	fha6	23.565	23.565
3	39	fha8	45.749	22.184

TV19-LG3

S/n	Nr	Locus	Position	interval (adjacent loci)
1	20	kka1	0.000	--
2	23	kka10	24.328	24.328
3	22	kka9	46.992	22.664

TV19-LG4

S/n	Nr	Locus	Position	interval (adjacent loci)
1	38	fha7	0.000	--
2	35	fha3	26.764	26.764
3	15	hga5	46.769	20.005

TV19-LG5

S/n	Nr	Locus	Position	interval (adjacent loci)
1	33	gka14	0.000	--
2	25	kka14	2.611	2.611

TV19-LG6

S/n	Nr	Locus	Position	interval (adjacent loci)
1	30	gka10	0.000	--
2	29	gka4	26.603	26.603

TV19-LG7

S/n	Nr	Locus	Position	interval (adjacent loci)
1	5	hia10	0.000	--
2	7	hia14	27.181	27.181

TV19-LG8

S/n	Nr	Locus	Position	interval (adjacent loci)
1	6	hia11	0.000	--
2	26	kka25	26.060	26.060

TV19-LG9

S/n	Nr	Locus	Position	interval (adjacent loci)
1	31	gka12	0.000	--
2	24	kka11	30.146	30.146

TV19-LG10

S/n	Nr	Locus	Position	interval (adjacent loci)
1	11	hia22	0.000	--
2	12	hia23	25.247	25.247
3	13	hga1	45.249	20.002

TV19-LG11

S/n	Nr	Locus	Position	interval (adjacent loci)
1	32	gka13	0.000	--
2	41	fha16	27.311	27.311
3	16	hga6	51.434	2.123