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# CHARACTERIZATION OF MATING SYSTEMS IN TEA

## (*Camellia sinensis* (L.) O. Kuntze)

By

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A thesis submitted in partial fulfillment for the requirements of the degree of Master of Science in Plant Breeding of Egerton University.

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# DECLARATION AND RECOMMENDATION

## DECLARATION

I declare that this thesis is my original work and it has not been previously presented for the award of a degree in this or any other University.

Sign:..... Date: 18.04.07.....

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## RECOMMENDATION

This thesis has been submitted for examination with our approval as the official University supervisors.

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# DEDICATION

...dedicated to Trevor, Dreyson and my parents, Kituku and Ndumi Muoki.

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## ABSTRACT

Knowledge of the predominant mating patterns in plant species is important for the development of efficient breeding strategies. In this respect, an estimation of the mating system parameters for understanding of the genetic structure in *Camellia sinensis* was conducted. Three experiments were set-up among eight *C. sinensis* genotypes in four biclonal isolated seed orchards located in two sites, namely Kericho and Kerugoya. An *in-vitro* germination method was used to study variation in pollen viability (pollen germination) over three flowering seasons during the year 2005. Significant variation in pollen viability among the eight genotypes indicated male gametophyte competition. A significant genotype by environment (location) interaction and a positive correlation between the pollen viability and temperature was observed. Four pollination methods, namely: selfing through bagging; controlled cross-pollination; open-pollination; and controlled self-pollination were compared. The evaluation of crossing success was based on abortion rates, fruit/seed set, seed weight/number per fruit and seed germination percentage. Open pollination had the highest fruit/seed set (44.8%) followed by controlled cross-pollination (32.5%), selfing through bagging (23.7) and lowest in controlled self-pollination (20.6%). Based on molecular markers (RAPD and ISSR), mating parameters of 30 open pollinated breeding progeny arrays of the 6 genotypes were estimated. The mixed mating system estimates obtained ($t_m$ = 0.99) indicated that *C. sinensis* is predominantly outcrossed suggesting that expected levels of genetic variation for open-pollinated families are likely to be maintained. The low $t_m$-$t_s$ (0.024 ± 0.094) indicated negligible biparental inbreeding. Likewise, low estimate of correlation of paternity ($r_p$ = 0.070) indicated that many of the offsprings do not share same paternal genitor despite the isolation of the two putative parents which is indicative of high pollen contamination in the isolated orchards. These results suggest that a review of the current isolation distance (6m between breeding genotypes) is necessary so as to increase pollination efficiency between the two isolated clones and minimize extraneous pollen contamination

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Tea (*Camellia sinensis* (L.) O. Kuntze) belongs to the family Theaceae and is an evergreen perennial beverage crop. It is thought to have originated in the mid-west mainland China (Yunnan) from where it spread to other areas of the world. Tea is now cultivated in a wide range of climates that range from as far North as Turkey and Georgia ($42^0$ N) to as far South as Argentina ($27^0$ S) and altitudes that range from sea level in Bangladesh to 2700m a.m.s.l. in Kenya (Anon, 2000). This wide dispersal resulted in a varietal speciation process and the evolution of three distinct cultivated taxa namely; var. *sinensis* ('China tea'), var. *assamica* (Masters) Kitamura ('Assam tea') and var. *assamica* spp *lasiocalyx* (Planchon ex Watt) ('Cambod tea'). The three main taxa can be differentiated by foliar, floral and growth features and by biochemical and molecular affinities (Owuor *et al.*, 1987c; Wachira *et al.*, 1995; Magoma *et al.*, 2003). Other recently described varieties include *waldenae, dehungensis, publimba* and subspp *buisanensis* whose contribution to the gene pool of cultivated tea is yet unknown (Wachira *et al.*, 1997).

Cultivation of tea dates back to 793 A.D. when the small leafed *sinensis* type was established as a commercial crop (Sealy, 1958). The large leafed type *assamica* was initially grown for use as vegetable rather than as a beverage in Burma, Thailand, Laos and Assam in North East India (Sealy, 1958). Tea cultivation outside its native range was reported as early as 801 A.D. in Yeinsan, Japan (Weatherstone, 1992). Assam tea and hybrids between it and variety *sinensis* became the basis of the tea industries in south, south-east, and west-Asia as well as for those in Africa and South America in the later years of the 1800s. These have grown and flourished since.

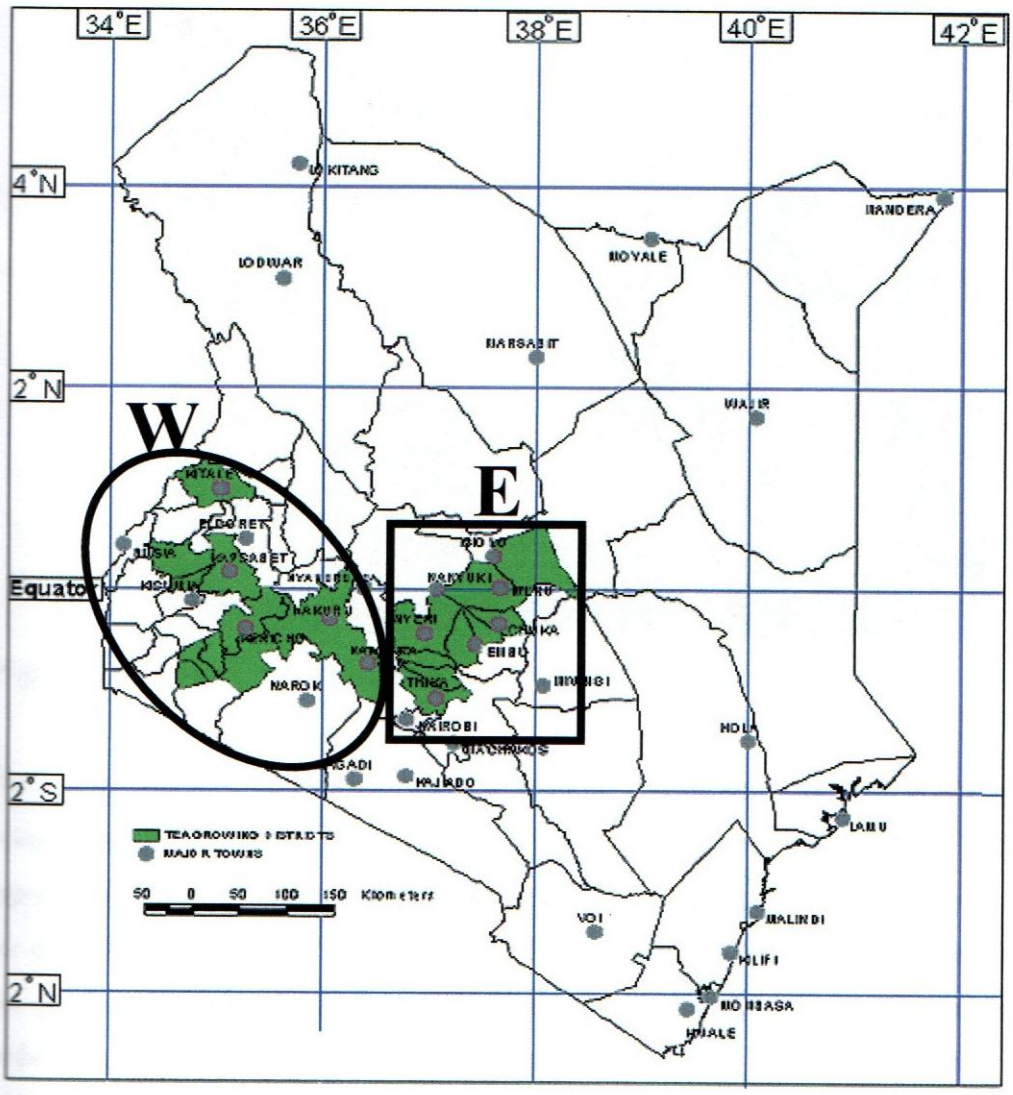
Tea is a major foreign exchange earner and a source of livelihood for millions of people in the world (Reeves *et al.*, 1987). In Kenya, which is the largest exporter of black tea in Africa and the third in the world after India and China, the crop is a source of livelihood for 3 million people and it contributes over 26% of total foreign exchange earnings and 4% of the gross domestic product (Economic survey, 2005). Tea production recorded the highest growth of 10.5% from 293.7 thousand tones in 2003 to 324.6

thousand tones in 2004 earning the country over Kshs 36 billion in foreign exchange earnings, second after horticulture (Economic survey, 2005). Because of the low level of mechanization in its field operations, the tea industry offers direct employment to millions in tea estates and factories as well as in its other subsidiary businesses.

In Kenya, tea was first introduced and planted at Limuru in the early 1900s. Its successful establishment led to the spread of cultivation to other parts of the country. The tea introduced for commercial cultivation in Kenya only comprised seedling plants which were low yielding, and its cultivation then was hampered by lack of adequate planting material (Wilson, 1962). By then, the cheap and effective methods of vegetative propagation from cuttings were not fully developed. In Kenya, tea is grown near the equator with economic production taking place at altitudes ranging from 1400-2700m a.m.s.l. in the highlands west of the Great Rift Valley and the Mount Kenya region (east of the Great Rift Valley ) (Fig. 1). The annual rainfall in these regions is > 1400 mm and is well distributed, and the minimum and maximum mean air temperature of 13°C and 30°C, respectively. The soil is highly leached and acidic (pH 5.0 to 5.6) (Anon, 2002).

Tea has been described as one of the most widely consumed soft beverages in the world. It has many chemical constituents, the unique combination of which are responsible for its popularity. The nutritional significance associated with tea's popularity is linked to its important phytochemicals which include the catechins, their oxidation products and caffeine. These phytochemicals contribute towards tea quality and are important pharmacologically due to their anti-cancer, anti-inflammatory, anti-vascular disorders, anti-hypertensive, anti-fungal, anti-viral and anti-bacterial properties (Adrian and Bolwell, 2000). The three main categories of processed tea include black (fermented or oxidized), green (unfermented) and oolong (semi-fermented or partially oxidized) which differ in the method of manufacture.

The tea species can grow to attain the stature of an undertree or tree depending on the taxon. However, in commercial production, it is maintained as a bush by regular pruning (Fig. 2). The tea crop is thought to be a predominant outbreeder. Owing to this and the apparent lack of crossing barriers between the three main cultivated taxa, a cline of specificness extending from extreme 'China type' plants to distinct Assam type plants




**Map Source: Kenya Tea Development Agency (KTDA)**

Figure 1: Two tea growing regions in Kenya. W and E are West and East of the Great Rift Valley, respectively

has resulted in the species. The outcrossing within the main taxa has been so extensive that indeed it is debatable whether archetypal var. *sinensis,* var. *assamica* or var. *assamica* ssp. *lasiocalyx* still exist (Visser, 1969). Similarly, there are over 200 species within the genus *Camellia* to which tea belongs most of which easily hybridize with tea (Takeda, 1990). Because the centres of origin of these species overlap, extensive interspecific hybridization between tea and these non-tea related species has taken place.



Figure 2: Commercial tea plantation at Timbilil, Kericho

Resulting interspecific hybrids resemble tea and it is thought that numerous such hybrids may exist undetected in commercial plantations (Wachira *et al.*, 1997). Within the tea species, it has been determined that some degree of selfing/inbreeding may occur (Tomo *et al.*, 1956; Rogers, 1975; Wachira and Kamunya, 2005). Generally, selfing (inbreeding) in predominantly allogamous plants reduces genetic diversity and heterosis through inbreeding depression (Muona, 1990; Adams and Birkes, 1991). This is often manifested in poorly endowed progeny lacking in vigour, prone to low fertility and reduced tolerance to environmental stresses which may contribute to reduced sustainability in production systems. However, selfing may facilitate fixation of useful recombinants, resulting in the production of superior clones (Wachira and Kamunya, 2005). The consequences of inbreeding in tea-improvement programs will therefore be important in determining the choice of progenitors, breeding population size and seed orchard design.

The mating system of a plant species determines how the genetic information is transferred from one generation to the next and it produces fundamental information for designing appropriate genetic conservation and breeding programmes (Alves *et al.*, 2003). Understanding the predominant mating system in a crop such as tea is thus of vital importance because it determines the genetic composition of a population and helps

design appropriate breeding strategies to conserve genetic variability and maximize potential for continued evolution, particularly in response to environmental changes (Ritland and Jain, 1981).

## 1.2 Statement of the Problem

Hybrid development in open-pollinated crops is considered an important method of varietal improvement. In tea, hybrids between some species in natural conditions frequently result in superior progenies possibly due to either true heterotic effect and/or a recombination of desirable alleles. However, due to the difficulty in carrying out large numbers of controlled pollinations in tea, the main population of such a programme is typically advanced through open-pollination. This is a simple and low-cost option, allowing rapid turnover of hybrid progenies. However, such hybrids have no known pedigree. The male parentage is assumed to be random. It is not known whether the progeny selected were produced by self-pollination, or are in fact true half-sibs. Open-pollinated families might result in a severe bias when estimating genetic parameters and parental breeding values. Information on the predominant system of mating in such a breeding population is therefore essential as it determines gene and genotypic frequencies between and within families and determines the proportion of co-ancestry.

## 1.3 Significance and Justification of the Project

Long-term breeding is aimed at achieving a balance between continuous genetic gains and maintenance of adequate levels of genetic variation. The success of such programmes is dependent on an understanding of the amount and distribution of the genetic variation present in the genepool. Critical factors that influence the development of promising clones include the choice of mating design and selection method, the intensity of selection applied and proper management of inbreeding. Therefore, to maximize efficiency in tea breeding and selection programmes, detailed knowledge of the reproductive system of the species is necessary. Knowledge of the predominant mating system will be important for the development of the most appropriate breeding strategies. For example, knowledge on how genes will be recombined in the progenies will facilitate selection of superior genotypes while maintaining genetic variability and increasing the

frequency of favorable alleles at loci that control economically important traits. Tea breeding strategies are dictated by the highly outbred nature of the species, its long generation time from seed to flower and its amenability to vegetative propagation. Because of the long breeding cycle, any breeding technique that helps to shorten the cycle could be quite valuable.

## 1.4 Objectives

### 1.4.1 General Objective

The main objective of this study is to enhance tea production through better understanding of mating systems.

### 1.4.2 Specific Objectives

i) To assess the differences in pollen viability within and between three tea taxa i.e. China, Assam and Cambod varieties.

ii) To assess the efficiency of selfing and cross-pollinating techniques.

iii) To test the utility of molecular markers, Randomly Amplified Polymorphic DNAs (RAPD) and Intersimple Sequence Repeats (ISSR), in estimating outcrossing rates in tea.

iv) To determine whether current isolation distance within seed orchards is sufficient in maximizing cross-fertilization between the two clones (progenitors).

## 1.5 Hypothesis

To meet the objectives of this study, the following null hypothesis ($H_O$) were postulated:

i) There is no significant variation in pollen viability among and between the different tea taxa.

ii) There is no significant variation in seed set between the self- and cross-pollination techniques.

iii) The tea species exhibits low outcrossing rate.

iv) The probability of mating between two progenitors is positively correlated with their isolation distance.

## 1.6 Expected Output

i) Information on efficiency of pollination techniques.

ii) Knowledge on molecular marker techniques in estimating outcrossing rates in selected tea clones of Kenya.

iii) A Masters degree thesis.

iv) Publication of research findings.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Breeding and Genetics of Tea

Plant breeding is a dynamic area of applied science. It relies on genetic variation and uses selection to improve plants for characteristics that are of interest for the grower and the consumer. In *Camellia sinensis*, the breeding strategies are dictated by its highly outbreeding nature, the long generation time from seed to flower and its amenability to vegetative propagation (Rogers, 1975). Improving leaf yield and quality have been the prime objectives of tea breeding programmes. Three prerequisites that principally determine progress in breeding are; the existence of an adequate level of genetic variation among breeding stocks and populations; development of recombinant genotypes through intra and interspecific gene introgression, and the availability and use of reliable markers for selection of recombinant genotypes with new sets of attributes from large populations grown in variable environments (Wachira, 2000).

Early tea breeding work concentrated on production of sufficient planting material rather than the enhancement of high yield and quality, by mass seed multiplication or mass selection from random crosses (Wight, 1956). Selection within the populations, coupled with clonal propagation, has resulted in reduced genetic variability within the cultivated gene pool. This maybe particularly so in African tea producing countries including Kenya, since the bulk of their initial populations are thought to have been obtained from a few random open pollinated crosses undertaken in Assam (India) and which possibly account for a small portion of the available gene pool. Because of tea's allogamous nature and its apparent heterogeneity, mass selection failed to produce tea with uniform morphological attributes essential for high yields and high tea quality. Subsequent breeding efforts focused on artificial pollination between plants of different morphophysiological traits for producing superior and heterotic progenies. Selection within the above populations coupled with clonal propagation (multiplication of selected elite mother plants) has however not necessarily enhanced the desired levels of genetic variability (Banerjee, 1992). Elite mother bushes selected in such schemes are either released after field-testing to farmers as clonal varieties or used in hybridization programmes for the production of improved seed varieties.

Unlike most other woody perennials, the harvestable yield of tea is purely vegetative and is confined to the two leaves and a terminal bud, which constitutes only 8.9-26% of the total biomass and dry matter produced by the plant (Wachira and Ngetich, 1999). Leaf characteristics therefore form the basis of morphological selection in tea for yield and quality. Though these characters can be highly variable, some of them have been used successfully as selection criteria in tea breeding. These include bush area and number of flushing points (Visser, 1969; Nyirenda, 1991), rate of shoot extension (Stephen and Carr, 1990), shoot and leaf size (Banerjee, 1991), and leaf area index (Barua and Sharma, 1982).

The main cultivated taxa of tea are diploids (2n=2x=30) (Kondo, 1977). However, the occurrence of naturally evolved autopolyploids (triploid, tetraploid and pentaploid) is common (Wachira and Kiplangat, 1991; Wachira *et al.*, 1999; Magoma *et al.*, 2003). The existence of these polyploids with superior vegetative vigour, stability, enhanced levels of biochemicals and potential resistance to environmental stresses (Kulasegaram, 1980; Ngetich and Wachira, 2003) has facilitated polyploidy breeding. Growth studies have, however, indicated that despite their superior vegetative vigour, polyploids do not always contribute towards high yield and quality (Banerjee, 1992; Wachira, 1994) and a selective approach in polyploidy breeding is therefore recommended (Banerjee, 1992).

**2.2 Seed Orchard**

The first step in any cultivar development programme is the identification or creation of genetically variable populations that may contain desirable genotypes for the character of interest (Siles *et al.*, 2001). Genetically variable breeding populations can be produced through controlled matings between diverse parents, mutagenesis, or through collection of populations that have undergone random mating or natural mutation. Among these means of obtaining genetic variability, the cyclic process of hybridization and selection is the primary method of cultivar development in most crop species. In tea, hybrid seeds among elite plants are obtained by growing the parents in polyclonal or biclonal seed orchards by natural cross-pollination (Bezbaruah, 1981) though cross-pollination can also be effected artificially but with a lower seed set (Fig. 3a and b). As seed orchards are the predominant seed source, the reproductive output of orchard parents



Figure 3a: Polyclonal tea seed orchard at Timbilil, Kericho



Figure 3b: Biclonal tea seed orchard at Timbilil, Kericho

and its role on genetic diversity merit increased scientific and managerial attention. A seed orchard is a plantation of selected genetically superior clones, designed and managed to produce frequent, abundant, easily harvested and genetically improved seed crops. Controlled cross-pollination requires seed orchards to be isolated from surrounding trees, that there be similar fertilities among all orchard trees and that there be minimum inbreeding (Stewart, 1994). Without reproductive isolation, genetic gain is compromised to the extent that non-selected trees contribute gametes to the orchard seed crops. Such an orchard is established by setting out clones or the seedling progeny of trees selected for desired characteristics (Zobel *et al.*, 1958).

The design, establishment and monitoring of seed orchards is determined by factors involved in the reproductive biology of the species. Pollination biology, outcrossing rate, distance that pollen is dispersed, neighbourhood size of tea population and level of diversity between the lines will influence the quality of a seed orchard. If seed orchards are to reach their maximum genetic efficiency, they should perform as a close, perfect population. This efficiency is also described as the degree to which the pollen clones transfer their genetic superiority and diversity to their seed crops. To reach this goal, it was assumed that orchard parents' gametic contribution should reach near equality. However, in most cases, orchards do not fulfill this condition (Kang and El-Kassaby, 2002).

In order to ensure the production of genetically diverse and physiologically high-quality seed crops, seed orchards therefore have to fulfill certain requirements with respect to flowering and pollination. The functioning of tea and generally other tree seed orchards is often far from ideal. There are large differences in flowering abundance between clones and from year-to-year in several varieties (Anon, 2002). Also, variation in the reproductive phenology has an effect on the genetic composition of seed produced in such orchards (Nikkanen *et al.*, 2000). Owing to the abundance of the different genotypes and effective pollen dispersal (Lindgren *et al.*, 1995), high pollen contamination has proven to be a serious problem in the functioning of seed orchards (Paule *et al.*, 1993; Pakkanen *et al.*, 2000). In addition, the genetic composition of the seed produced in seed orchards may be affected by competition among pollen grains produced by different plants in the orchard.

Tea seed orchards are dependant on insect pollination (entomophilous) for transfer of pollen between the selected parents. Inbreeding depression is thought to result if there is a high incidence of selfing (Wachira and Kamunya, 2005). The degree of inbreeding will be determined by orchard design and synchrony of flowering dates (Cottrell and White, 1995). There must therefore be extensive cross-fertilization among clones if the superior alleles of parents are to be fixed in to the hybrid. Precise information on the inheritance of characters of economic importance is, however, still lacking. Due to the practical difficulties associated with tea breeding, few such studies have been undertaken.

Alternatively, several techniques have been developed for hybridization of tea. Some of these techniques have been successfully used in tea but each has its limitations (Wachira and Kamunya, 2005). The difficulty in making crosses and the lack of an efficient crossing technique has resulted in a limited number of detailed genetic studies and, consequently, limited methodical genetic improvement of tea. Therefore, development of an efficient crossing technique is essential in achieving genetic and breeding objectives in tea.

**2.3 Pollination**

Pollination is an important step in plant reproduction. The fitness of male gametophytes depends both on paternal traits, which include phenology of male flowering and the amount of pollen produced, and on pollen grain traits, such as the germination percentage, germination time, pollen tube growth rate and selective fertilization (Nikkanen *et al.,* 2000). Male fertility is a function of pollen production and viability, and can be strongly influenced by the environment (Khatun and Flowers, 1995). Problems with male fertility can result in reduced seed set and yield potential in seed production fields. It is a requirement that there be similar fertilities among clones within an orchard as unequal pollen fertility may generally lower the expected genetic gain of the breeding programme (Stewart, 1994). This is because orchard trees with desirable characteristics, but low pollen fertility, fail to contribute gametes coding for their traits to the orchard seed crop.

Pollen competition among male gametophytes including different rates of germination and tube growth has been suggested as one of the factors affecting the genetic composition of the seed produced in seed orchards (Nikkanen *et al.*, 2000). In most plants, research has indicated that variations in temperature, humidity, and cloud cover can influence pollen production and viability (Brooking, 1979; McLaren and Wehner, 1992). Cold temperature stresses prior to flowering in sorghum reduces pollen viability during anthesis by disrupting meiosis during early stages of microsporogenesis (Brooking, 1979). The physiological effects of such environmental factors as low or high temperature stresses, humidity, and cloud cover on male fertility of tea are not as well characterized.

Pollen viability can be quantified by different procedures. Under field conditions, pollen viability can be tested *in-vivo* by evaluating seed set on self- or cross-pollinated florets. This particular measurement may indicate the presence or absence of fertile pollen. Under laboratory conditions, viability can be quantified more precisely by simple assays: evaluation of pollen germination *in vivo* (Sun *et al.*, 1991) or *in vitro* (Lansac *et al.*, 1994), use of vital stains including aniline blue to detect callose in pollen tubes or iodine to determine starch content (Brooking, 1979; Heslop-Harrison *et al.*, 1984; Wachira and Kamunya, 2005). *In vitro* pollen germination is generally believed to provide the best estimate of pollen viability *in vivo* (Stone *et al.*, 1995).

Recent studies have however, demonstrated that most plant species suffer, at least at times, from pollen or pollinator limitation (either no or not sufficient pollen deposit on the stigma), or as Wilcock and Neiland (2002) stated: "too little, too much, too late, too mixed in composition or too poor in quality". To increase pollination success, plant-pollinator-interactions have evolved in two different ways. On one side, tight specialized pollinator-flower interrelations have evolved (Nilsson, 1992) while on the other hand, broad pollination syndromes with mass flowering, attracting a wide variety of flower visitors have evolved (Bawa and Opler, 1975). The longevity of trees, combined with high pollinator diversity and an oversupply of flowers increases the chances for successful pollination. Additionally, pollination success often depends on attractiveness of the individual tree (amount of pollen and nectar provided), distance to neighboring conspecifics, pollinator diversity and simultaneously flowering species. In the long-term,

pollen limitation can result in a reduced fruit set, leading to a reduced regeneration potential of the species (Farwig *et al.*, 2004). In tea, Kutubidze (1958) reported that supplementary pollination (i.e. extra-pollen) produced more, larger, and heavier capsules, better viability, and higher grade of seed.

The amount and source of pollen required for adequate pollination of any tea genotype is not known. Like in most plants, low seed yields in tea remain common because of inadequate pollen transfer (Free, 1993) and because pollination from the same or closely related varieties sometimes results in low fruit set, sterile seeds and small fruits (Wachira and Kamunya, 2005). Pollen source is an important consideration for obtaining maximum tea seed production. Pollen transferred between varieties can increase yields of seed compared to varietal selfing by producing more seeds and greater fruit set. Generally, pollen from distantly related tea varieties produce more seeds than does pollen from more closely related types (Wachira and Kamunya, 2005). Thus the source of pollen is an important variable to consider in improving fruit set.

In animal-pollinated plants like tea whose main pollinators include fumble bees, ants, coveid bugs, wasps and hymenopteran species, spatial isolation and small population size generally reduce the frequency of pollinator visitation and hence the amount of pollen deposited on the stigma (Hodgins and Barrett, 2006). However, pollinator foraging distances are generally negatively correlated with density, such that at high-density pollen dispersal distances are shorter than at low density (Kameyama *et al.*, 2002). Habitat fragmentation may also reduce the foraging behavior, of flower-visiting insects, disrupt plant-pollinator interactions, and reduce seed set and gene flow of isolated plant populations (Steffan-Dewenter and Tscharntke, 1999)

Similarly, sufficient stigmatic pollen deposition is considered important to maximize yield of fruits. Generally, higher but not excessive numbers of pollen grains are beneficial for optimal seed and fruit production since pollen competition, ovule and seed abortion, pollen tube attrition and physical blockage of pollen grains limit the number of successful fertilization and seed set (Dogterome *et al.*, 2000). Wachira and Kamunya (2005) found that the cross and self pollen tubes of tea grow at different rates and compete to fertilize the ovule. This pollen competition results in ovule fertilization by the

faster pollen tubes. In *Zucchini squash*, this results in better quality seeds and fruits, since faster pollen tubes are more successful at fertilizing ovules (Davis *et al.*, 1987).

The reproductive potential or capacity of a plant is also dependant on the number of inflorescences, the number of pollinated flowers, the number of fertilized ovules, the extent of fruit or seed predation, weather conditions and the ability of the maternal parent to provide resources for development (Gaol and Fox, 2002). Factors that may reduce reproduction include a short growing season, low or high temperature, low nutrient and moisture availability, herbivory, humidity, wind at the time of pollination, resource competition, disease and scarcity of pollinators. These factors may reduce the possibility of flowering and seed set.

**2.4 Self-Incompatibility Mechanisms**

Tea has hermaphrodite flowers (Wachira and Kamunya, 2005). This structure may have evolved to facilitate the deposition of foreign pollen on the stigma and also pollen removal from anthers in a single visit to the flower by insect vectors, but it also potentially enhances the possibilities of self-pollination due to the close proximity of anthers to stigmas. Females often have little control over the genetic relatedness of pollen deposited on stigma. Thus, post-pollination processes are of primary importance for mate discrimination. The ability to screen and select among male gametes during pollen tube growth within the female tissues of the stigma, style and ovary is critical to the process of mate choice and reproductive isolation. It is likely that self-incompatibility mechanisms (*SI*) evolved in flowering plant species to constrain such selfing (Gibbs and Bianchi, 1999). These are genetically controlled physiological mechanisms that either prevent the germination of self-pollen on the stigma, or prevent the successful growth of self pollen tubes to the ovary. Tea had initially been described as virtually self-incompatible and almost entirely cross-pollinated (Wight and Barua, 1939). Initial investigations into the mechanism of *SI* in the species by Simura and Oosone (1956), Tomo *et al.* (1956) and Rogers (1975) indicated that a gametophytic incompatibility system (*GSI*) predominated, although very few partly self-fertile bushes existed.

Three major genetically controlled physiological mechanisms of self-incompatibility (*SI*) have been characterized in flowering plants; homomorphic

gametophytic (*GSI*), homomorphic sporophytic (*SSI*), and heteromorphic *SI* systems (Gibbs and Bianchi, 1999). In the homomorphic *SI* system, compatible individuals are not distinguishable by their morphology while in heteromorphic systems, each compatible group is associated with a distinct morphology. These mechanisms either prevent germination of self pollen tubes on stigmatic lobes or prevent the successful growth of self pollen tubes to the ovary. More recently, another mechanism of control of *SI* called late-acting self-incompatibility (*LSI*) or ovarian sterility (*OS*) was described in the families *Bignoniaceae* (Gibbs and Bianchi, 1999), *Heuchera micrantha* (Rabe Andrea and Soltis, 1999) and *Camellia sinensis* (Wachira and Kamunya, 2005). In this mechanism, selfed flowers fail to produce fruits despite the fact that the self pollen tube grows successfully in the style and successfully penetrates the ovule. Wachira and Kamunya (2005) demonstrated that the *LSI* system in tea contributes upto 20% selfing in some varieties resulting in smaller seeds with reduced germinability or no seed at all. The proportion of ovules that develop into a mature seed is an important factor determining the reproductive potential of most plant species. Surveys have indicated that in outcrossing perennial plant species like tea, approximately 50% of ovules do not form a seed (Marshall *et al.*, 1999).

## 2.5 Plant Mating Systems

Plant mating-system evolution is a dynamic process, as evidenced by the wide variation seen in outcrossing rates among closely related species and among populations within species (Barrett and Eckert, 1990; Takebayashi and Morrell, 2001). An understanding of the forces governing plant mating-system evolution is fundamental to the understanding of evolution in plant populations, because mating system influences evolutionary factors as diverse as the amount and pattern of genetic variation within and among populations, reproductive assurance, ability to colonize new environment, effective recombination rate, and long-term evolvability (Takebayashi *et al.*, 2006).

Detailed information on the mating system is, therefore, important in both natural and artificial populations. For natural populations, information on mating system, fertility variation, and migration is needed for planning gene conservation programmes and for managing breeding and production populations (Muona, 1990). In artificial populations

such as seed collection stands or seed orchards, it is of interest to estimate the mating system to assess accurately the genetic improvement achieved and levels of contamination from external pollen sources (Adams and Birkes, 1991). Such studies help elucidate the dynamics of micro-evolutionary processes, conservation strategies, and the genetic management of a species (Muona, 1990). Estimation of mating system parameters has breeding implications when using open-pollinated seeds for progeny trials, the estimation of genetic parameters and estimation of spatial and temporal variations in the system (Adams and Birkes, 1991).

Population geneticists have classified plant species mating systems into five categories. They are predominantly self-fertilizing (with outcrossing rate $t<0.05$), predominantly outcrossing (self-fertilization rate $s<0.05$), mixed selfing and outcrossing, facultative or obligate apomixis, and intragametophytic or haploid selfing (Brown, 1990). Species showing high outcrossing rates, generally maintain high genetic diversity, with relatively small differences among populations but high variability within populations (Muona, 1990; Adams and Birkes, 1991; Wachira, *et al.*, 1995; 2001). The mating system of plants are affected by a range of factors both external to the plant, such as floral phenology and resource allocation within fruits (Millar *et al.*, 2000; Takebayashi *et al.*, 2006). Floral phenology and pollination vectors can affect the correlation of paternity within fruits. Significant correlation of paternity has been found in several insect-pollinated species and may be a general feature of insect-pollinated plants (Morgan and Barrett, 1990). In *Eucalyptus rameliana* fruits, a significant level of correlated paternity was found which was attributed to the floral structure and phenology of this bird-pollinated species (Sampson, 1998). Animal pollinators are expected to show variation in abundance and behaviour whereas the wind pollinated would be expected to spread pollen more predictably (Barrett and Eckert, 1990).

It has been shown that mating system parameters are often influenced by several non-genetic factors, such as stand density (Morgante *et al.*, 1991) and altitude (Neale and Adams, 1985). Moreover, mating among relatives in neighbourhoods within populations may occur and determine an increment in the proportion of seeds originating from selfing (Garcia *et al.*, 2005). It had been hypothesized that outcrossing rates decreases as stand density decreases, because pollen dispersal decreases exponentially with distance (Farris

and Mitton, 1984). In low-density stands, the pollen cloud of any individual tree contains a relatively higher proportion of its own pollen than in high-density stands and that there is an increase in the local mating competition among sib pollen (Morgante *et al.*, 1991). However, contrasting results have been obtained concerning this hypothesis. Positive correlations between outcrossing rate and plant density have been found in *Pinus pondorosa* (Farris and Mitton, 1984) but Morgante *et al.* (1991) did not find stand density in two Norway spruce (*Picea abies*) populations to have any impact on outcrossing rates. Measurements of outcrossing rates have been further complicated by the fact that single generation estimates of selfing rates made over multiple years demonstrate temporal variation.

## 2.6 Methods for Estimating Mating Systems

Traditional methods used for the measurement of mating systems have been based on the analysis of floral morphology, greenhouse crossing experiments, and the observation of pollinator behavior (Clegg, 1980). In outcrossing plants, the effectiveness of pollen and gene transfer between cultivars depend on complex morphological and behavioral adaptations of the insect vectors that could interact with plant morphology and physiology. The floral structure, the degree of self-incompatibility and the competition between self and foreign pollen determine the outcrossing rate induced by pollinators (Takebayashi *et al.*, 2006). However, the practical use of phenotypic markers in trees is limited by a number of factors such as long period required for progeny to reach maturity for the markers to be scored and lack of consistency between phenotypic markers and outcrossing (Gjuric and Smith, 1996). Such conventional markers are thus less suitable in many applications in plant breeding.

Based on the above shortcomings, various methods for estimating mating system parameters have been proposed for single as well as for multilocus data (Ritland and Jain, 1981; Xie *et al.*, 1991; Ritland, 2002). Most of them use a maximum–likelihood estimation procedure to determine the best simultaneous estimates of allele frequency in the outcrossed pollen pool and the outcrossing rate (Burczyk *et al.*, 2002; Ritland, 2002). While a large number of loci is needed for estimating levels of heterozygosity, genic diversity, and the extent of differentiation, only a few polymorphic loci may be scored to

obtain valid estimates of mating system parameters (Nei, 1975; Ritland and Jain, 1981; El-Kassaby *et al.*, 1994; O'Connell *et al.*, 2004). Such genetic estimation techniques are often the most accurate, precise, and appropriate for tree breeding and orchard management decisions (Stewart, 1994). The use of electrophoretic marker loci in lieu of morphological markers and the development of suitable statistical genetic models have facilitated the detailed quantitative estimation of plant mating systems.

### 2.6.1 Isozyme Polymorphism

An enzyme with multiple molecular forms is called isozyme (Markert 1975). Different structural forms (isomeric) of an enzyme occur within a single organism or even within a cell. These isomeric forms have the same molecular weight and specificity for the substrate, catalyzing the same biochemical reaction, but differing in structural configuration and properties (Bera *et al.*, 1995). Isozymes have been widely used in the determination of genetic diversity and identification of plant varieties (Ilarslan *et al.*, 2001). They have been used in various aspects of breeding such as marker-assisted selection (Ghesquiere *et al.*, 2000), confirming hybridity (Isshiki and Taura, 2003) and in estimation of mating systems (Brown and Allard, 1970; Adams, 1983; Parraguirre *et al.*, 2004). Isozyme variation even at a single locus has been shown to have a major impact upon physiological variation and productivity of qualitative traits (Williamson *et al.*, 1995) making this marker a useful tool for Marker Assisted Selection (MAS). However, in most plants, isozyme variation is limited by both the numbers of polymorphic enzyme systems and loci that can be assayed. Electrophoretic analysis of enzymes can also be difficult particularly for plants with tissue containing high levels of phenolics and tannins, like tea (Magoma *et al.*, 2002).

### 2.6.2 Molecular Markers

Advances in knowledge of the genetic code have led to robust techniques for genetically typing individuals and for detecting the movement of genetic material from one plant to another (Powell *et al.*, 1996). The use of nuclear DNA markers potentially explains the portion of the genome that can be examined. There are numerous types of

molecular marker technologies which differ in the source and level of revealed polymorphism.

### 2.6.2.1 Restriction Fragment Length Polymorphisms (RFLP)

Genetic variability can be readily assessed using restriction enzymes. RFLPs can elicit considerable polymorphism on the basis of the restriction size variation in the genomic DNA (Paterson *et al.*, 1991). In plant breeding, RFLPs have immediate application in assessing the extent and nature of genetic variation in base populations, strain and varietal identification, seed quality control and characterization of breeding systems (Luebberstedt *et al.*, 2000). However, their use in perennial tree crops has been constrained by high costs, low throughput, lack of adequate technical expertise and the low quantity and quality of DNA extractable from such species. In recent years, several high–throughput PCR technologies have been developed to assay genetic polymorphism at the DNA level. These technologies are based on the Polymerase Chain Reaction (PCR).

### 2.6.2.2 Polymerase Chain Reaction (PCR)

The PCR technique for *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA was first developed by Millis and Faloona (1987). It involves three steps: DNA denaturation, primer binding and DNA synthesis. Target duplex DNA is first denatured, followed by hybridization and annealing to two oligonucleotide primers that flank the specific target DNA segment. The annealed primers are then extended (5'→3') on the template strand by a DNA polymerase (Taylor, 1991). There are numerous variations of the PCR technique.

#### *Randomly Amplified Polymorphic DNA (RAPDs)*

Williams *et al.*, (1990) and Welsh and McClelland (1990) described a procedure for the identification of genetic polymorphism in plants based on the arbitrary amplification of specific DNA segments between small inverted DNA sequences scattered throughout the genome by use of single, short (10mers i.e. bases long) synthetic oligonucleotide primers. Sequences amplified yielded stable genetic markers. While

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classical PCR required nucleotide sequence information for the design of the primers, Williams *et al.*, (1990) demonstrated that priming with random oligonucleotides could give stable genetic (RAPD) markers. This provided an innovative technology for DNA mapping, fingerprinting and related research (Waugh and Powell, 1992; Tingey and del Tufu, 1993). Primer sequences are normally designed so as to at least contain 60% G≡C content to ensure efficient annealing and also so as to reduce internal palindromic pairing which can provide PCR artifacts.

The procedure described by Williams and his colleagues, ensured that fragments (bands) of up to 2.0 Kb in length were obtained which could be easily visualized after electrophoresis by staining with ethidium bromide. The size and number of polymorphic fragments was a function of the sequence variability between the termini of the primers, which resulted in changes in priming site. Such changes in priming sites could also be due to point mutations, or genetic changes that modify the size or block of the amplification of the target DNA, such as insertions, deletions and inversions in the target genome.

The advantages and uses of RAPD markers have been reviewed (Powell, 1992) and include the following attributes;

- The process is technically simple, can be automated, requires small amounts of crude DNA and does not require radio-labeling.
- RAPDs may provide markers in regions of the genome inaccessible to RFLP analysis due to the presence of repetitive DNA sequences.
- A set of primers can be used for detection of polymorphisms in a wide variety of species.
- RAPDs are suitable for numerous types of genetic studies including genetic mapping, linkage studies and phylogenetic studies, DNA fingerprinting and plant identification.

Despite the above advantages, RAPD markers cannot distinguish between heterozygous and homozygous individuals such as those likely to be found in an $F_2$ population because of their dominant nature of inheritance (Powell, 1992). Nevertheless, they have been widely used in the assessment of the degree of polymorphism and diversity, relatedness and cultivar identification for instance in banana (Pillay *et al.*,

2003), *Camellia* (Wachira *et al.*, 1995) and the tropical tree *Terminalia* (Pither *et al.*, 2003), phylogenetic and pedigree studies for example in maize (Parentoni *et al.*, 2001), tea (Wachira *et al.*, 1997); genetic mapping and identification of markers linked to genes controlling important traits in lettuce (Casasoli *et al.*, 2001), tea (Hacket *et al.*, 2000) and estimation of outcrossing rates (Bamberg *et al.*, 2004). RAPD amplicons can be sequenced and primers flanking the amplicon designed. Such primers can be used to amplify a co-dominant marker, the Sequence Characterized Amplified Region (SCAR).

***Amplified Fragment Length Polymorphism (AFLP)***

Amplified Fragment Length Polymorphism (AFLP), is a novel PCR based technique for DNA fingerprinting which selectively amplifies DNA fragments (Zabeau and Vos, 1993; Vos *et al.*, 1995). This marker system combines RFLP and PCR technologies. Prior to PCR amplification, genomic DNA is digested by two restriction endonucleases and site-specific adaptors are then ligated to the resulting DNA fragments to generate template DNA for PCR. Template DNA is amplified using PCR primers complimentary to the adaptor sequences. Addition of extra nucleotides at the 3' end of the PCR primers allows the selective amplification of only those restriction fragments starting with nucleotides homologous to those of the primers. In this way, AFLP can be tailored to produce patterns of desired complexity. Resolution of the resulting DNA fragments on standard sequencing gels allows for the detection of amplified fragment length polymorphisms (AFLPs).

The reproducibility of AFLP profiles is assured by using primer sequences of at least 16 nucleotides at stringent amplification conditions and is demonstrated by the consistency in relative band intensity. AFLP has been used to establish the extent of genetic variation and population differentiation in plants (Loh *et al.*, 2000; Sole *et al.*, 2004; Muluvi *et al.*, 2004). AFLPs are particularly useful since they have a high multiplex ratio. The use of short-lived radioisotopes was initially a major limitation of this technique especially in studies including large numbers of samples. However, other methods of detection that eliminate the use of non-radioisotopes like the silver staining and fluorescence detection have rekindled the interest in the use of this technique (Wachira *et al.*, 2001).

***Inter-Simple Sequence Repeats (ISSR)***

Simultaneous analysis of DNA sequence variation at multiple loci increases the information content of any genome assay. Conserved, highly repetitive sequences in the genome can be used as markers for such a multiplexed assay. A relatively new approach called the Inter-Simple Sequence Repeat amplification (ISSR) (Kantety *et al.*, 1995) has the potential to serve as a powerful tool for genome analysis. The ISSR analysis involves the PCR amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR)-containing primer. The technique can be applied for any species that contains a sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required (Goodwin *et al.*, 1997). The primers used in ISSR can be based on any di-, tri-, tetra-, or pentanucleotide SSR motifs found at microsatellite loci, giving a wide array of possible amplification products (Blair *et al.*, 1999). The primers may also be achored at the 5' or 3' end with a few nucleotides to increase specificity of priming. Because of greater length of ISSR primers (i.e. upto 20 mers), they may show greater repeatability and stability of map position in the genome when comparing genotypes of closely related individual (Zietkiewicz *et al.*, 1994). The technique is more reliable than RAPD and generates larger numbers of polymorphisms per primer because variable regions in the genome are targeted (Hantula *et al.*, 1996; Mondal, 2002). The potential use of ISSR markers depends on the variety and frequency of microsatellites, which changes with the species and with the targeted SSR motifs (Morgante and Olivieri, 1993). In addition, the number of bands produced by an ISSR primer with a given microsatellite repeat should reflect the relative frequency of that motif in the genome and would provide an estimate of the motif's abundance as an alternative for library hybridization (Blair *et al.*, 1999). ISSR-PCR has been widely used to genetically characterize various plant species (Kantety *et al.*, 1995; Blair, *et al.*, 1999; Mondal, 2002).

***Information content of different molecular marker systems***

Powell *et al.*, (1996) showed that the utility of a given marker system is a function of its marker index; (MI)= H x E; where H= Diversity Index and E= effective multiplication ratio (proportion of polymorphic loci x number of loci analyzed per gel). A

comparison of the informativeness of the various marker systems in the tea species revealed the following descending order: AFLP> SSR > RAPD >RFLP (Wachira *et al.*, 2001). The SSRs, AFLP and RAPDs can be classified as ideal genetic markers because of their informativeness, abundance and ease of use.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Plant Materials and Experimental Sites

The experiments were carried out in four isolated *Camellia sinensis* biclonal seed orchards (two genotypes) at the two Tea Research Foundation of Kenya (TRFK) research sites located at Timbilil estate, Kericho ($0^0$ 22'S and $35^0$ 21'E, elevation 2178m a.m.s.l.) and Kangaita, Kirinyaga ($0^0$ 26'S and $37^0$ 15'E, elevation 2020m a.m.s.l.). The genotypes in each of the four seed orchards consisted of six clones namely TRFK 6/8, AHP SC 31/37, TRFCA SFS 150, GW Ejulu-L, TRFK 301/5 and TRFK 301/4 with clones AHP SC 31/37 and GW Ejulu-L being at both sites (Table 1). Each seed orchard had an area of 0.08ha and was established in 1998. The six breeding genotypes were planted in each orchard in the center surrounded by a pollen dilution zone approximately 20m wide (Fig. 4a and b). The zone consisted of the two parental genotypes and was expected to effectively eliminate pollen contamination (gene migration).

Table 1: Description of *Camellia* genotypes studied in four isolated seed orchards

| Orchard site | Genotype/Clone | Variety type* | Important attributes |
|---|---|---|---|
| 1. Timbilil (Kericho) | TRFK 6/8 | China/Assam Hybrid | High quality |
| | AHP SC 31/37 | Assam | High yield, pest resistant |
| 2. Timbilil (Kericho) | TRFCA SFS 150 | Assam | Pest/cold/drought tolerant |
| | GW Ejulu-L | China | High quality |
| 3. Kangaita (Kirinyaga) | TRFK 301/5 | Cambod | High yield, RKN resistant** |
| | GW Ejulu-L | China | High quality |
| 4. Kangaita (Kirinyaga) | TRFK 301/4 | Cambod | High yield |
| | AHP SC31/37 | Assam | High yield, pest resistant |

* *Delineation of variety type based on morphological traits*
***RKN – Root knot nematode*

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Figure 4a: Planting arrangement in seed orchards with two genotypes, A (+) and B (x), each with three breeding plants in the centre separated by 6 meters and surrounded by clones of the same genotypes at the boarder




Figure 4b: Breeding (stock) genotypes of *C. sinensis* in the centre surrounded by pollen dilution zone with two genotypes 6m wide in an isolated biclonal seed orchard at Timbilil, Kericho

## 3.2 Methods

### 3.2.1 Experiment 1: Pollen Viability

***Pollen Collection***

Ten to twenty freshly opened flowers were randomly harvested from each of the three bushes of a genotype within a seed orchard and were used to estimate pollen viability. Each bush of a genotype was considered a replication. The tea species flowered from March to August. All flowers were collected between 0900 and 1000h. The mean daily maximum and minimum temperatures and other weather parameters i.e. rainfall and humidity registered during flower collection were noted. Pollen viability study was carried out over three flowering seasons (i.e. early (March), mid (May) and late (July)) in 2005.

***Pollen Germination and Viability***

Pollen viability was estimated using a modified method of Tuinstra and Wedel (2000) by suspending dry pollen in a petri dish on a paper towel soaked in 1ml of 30% sucrose (W/V sucrose) with 1.5 $mgl^{-1}$ borate as sodium tetraborate for 24 hours. The germination temperature and illumination were controlled by incubating the pollen in a growth cabinet at a constant temperature of 25°C; illumination (15 Lux – Quantum Model LI-185 (LI-COR)). After incubation, counts were made at random in three fields (each with a minimum of 50 pollen grains per replication) under a low power (magnification X6.3) light photomicroscope and rated as germinated or non-germinated. Pollen grains were considered germinated when the pollen tube had gained a length equal to or longer than the diameter of the pollen grain (Fig. 5). The percentage germination was obtained as:

$$\text{Germination (\%)} = \frac{\text{number of germinated pollen per field}}{\text{total number of pollen per field}} \times 100$$

***Statistical Analysis***

The experiment was set up in a completely randomized design with three replications for each genotype. The study was carried out in three seasons: May, July and

September corresponding to early, middle and late flowering seasons, respectively. Observation means for each genotype were used for statistical analysis. The arcsine transformation was applied to the percentages of pollen germinated prior to statistical analysis. Analysis of variance was performed in a completely randomized design using GENSTAT 5 (1987) for each flowering season and Duncan's Multiple Range Test (DMRT) used to compare the means of pollen viability of the parent genotypes of the three varieties.

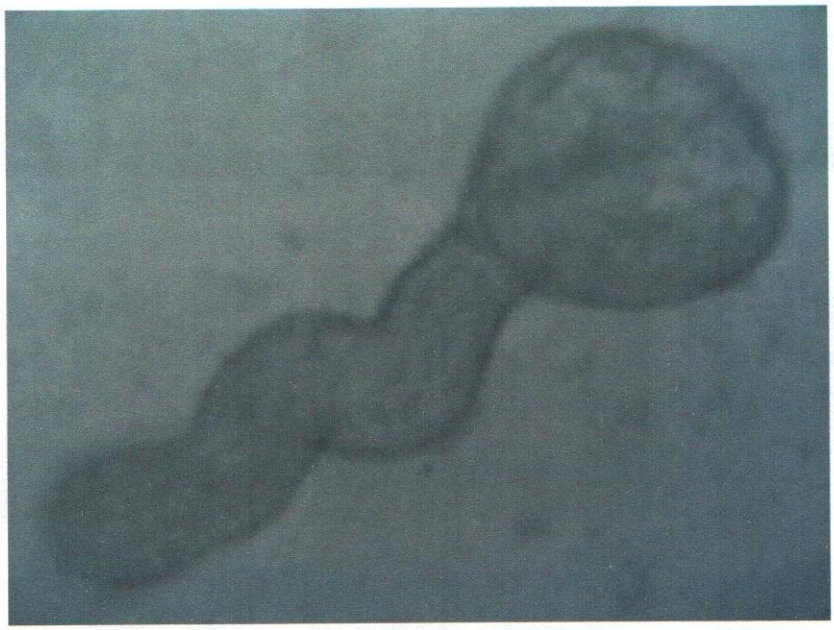


Figure 5: Germinating tea pollen *in-vitro*

### 3.2.2 Experiment 2: Self- and Cross-Pollination

***Pollination Techniques, Seed and Fruit Set Determination.***

Four pollination techniques were compared in the isolated orchards over a single breeding season to assess the reliability of various pollination methods (Fig. 6a-l). The experiment was set up in a completely randomized design with three replications for each genotype:

a) *Self-pollination with bagging*: 200 near mature closed flower buds were bagged (Khaki bags) two days prior to flower opening on each of the 2 clones in each

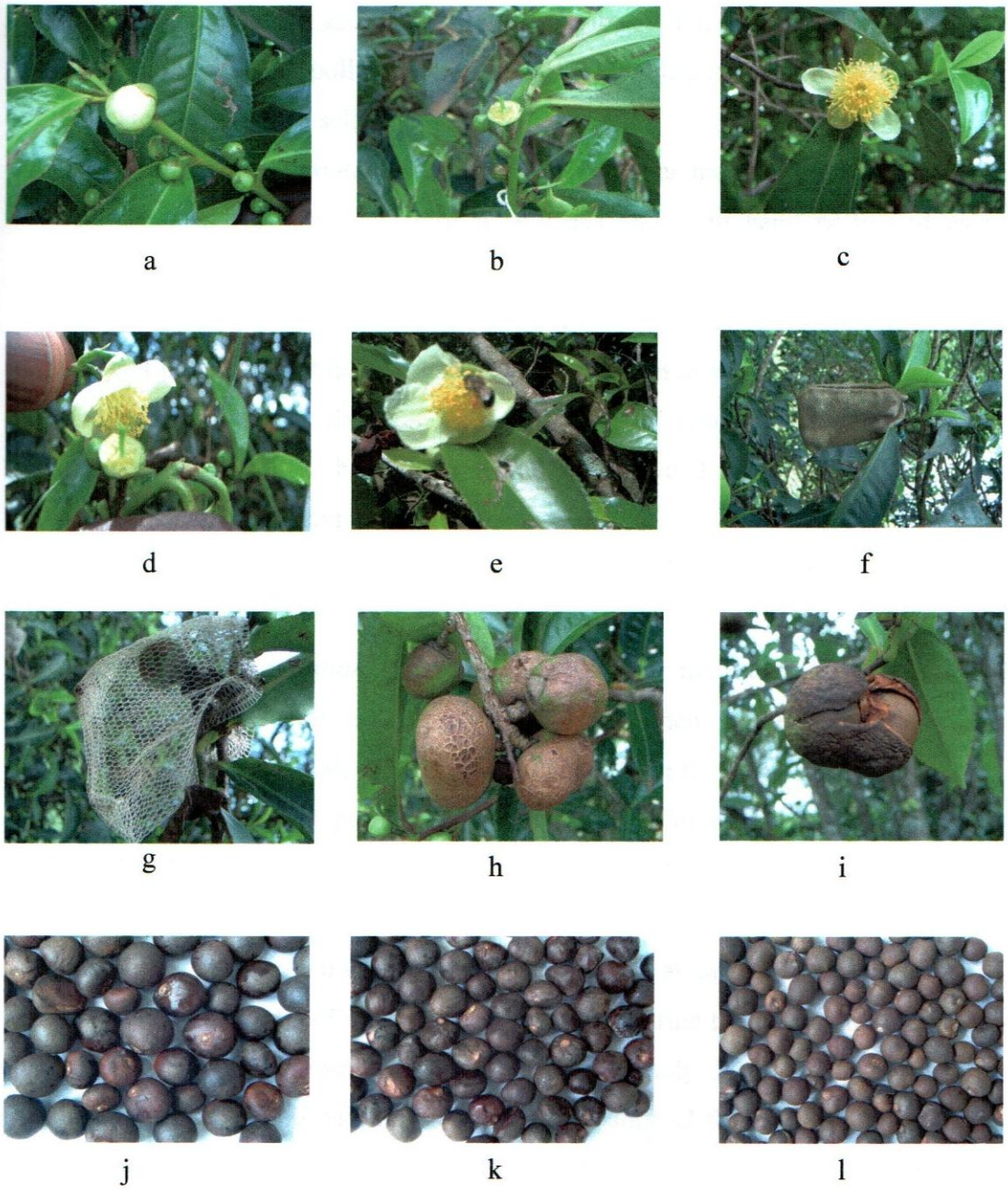



Figure 6: Stages involved in fruit/seed formation; a) unopened flower bud, b) emasculated flower bud, c) opened flower, d) controlled hand pollination, e) pollination by a vector, f) bagging after pollination, g) fruit netting, h) maturing fruits, i) fully mature cracked fruit capsule, j) variety Cambod seeds, k) variety Assam seeds, l) variety China seeds

genotype in the seed orchards. After bagging, the buds were marked individually with a colored thread and tagged around the base of the flower. The bags were removed 7 days after pollination. A total of 600 buds/genotype (i.e. 200 buds x 3 replicates = 600) were selfed.

b) *Controlled-cross pollination with bagging:* 200 near mature closed flower buds were emasculated two days prior to anthesis and were hand pollinated using mature fresh pollen collected from the male genotype in an orchard 48hrs later i.e. reciprocal crosses between genotypes in an orchard. The stigma was covered with wet pollination (Khaki) bags after emasculation as well as immediately after hand pollination until the fruit started forming to avoid pollen contamination. These were replicated, bagged and tagged as above. For all pollinations, fresh pollen was collected from flowers at anthesis.

c) *Open-pollination:* 200 near mature unopened flower buds were tagged and left for open pollination.

d) *Controlled-self pollination with bagging:* 200 near mature closed flower buds were emasculated two days prior to anthesis, then subsequently pollinated manually 48hrs later with fresh pollen collected from the same clone. The stigma was covered with wet pollination (Khaki) bags after emasculation as well as immediately after hand pollination until the fruit started forming to avoid contamination. These were replicated, bagged and tagged as above. For all pollinations, fresh pollen was collected from flowers at anthesis.

For each treatment, a total of 4800 crosses were done during the flowering season. All genotypes within each site flowered together during this study. Reproductive success of each genotype was calculated based on the number of aborted flowers/fruits, number of mature fruits produced per pollination treatment, number of seeds per fruit and number of mature seeds germinated in each treatment. In each case, fruit abortion was evaluated through survival counts done every two months throughout the fruiting season (March 2005– May 2006). Mature fruits had an outer covering that splits when mature, exposing one to six black seeds (Fig. 6i). Fruits were harvested at maturity (about one year after crosses) i.e. with seed falling in muslin bags. The number of seeds per fruit and weight of 100 seeds randomly selected were determined. Seed viability and percentage germination

were determined by floatation test and subsequent germination of sinkers in the nursery, respectively. The percentage fruit set was calculated as:

$$\text{Fruit set (\%)} = \frac{\text{number of harvested/mature fruits}}{\text{number of pollinated flowers}} \times 100$$

***Statistical Analysis***

Analysis of variance was conducted to test whether reproductive characteristics differ between the pollination techniques. The difference in fruit set in open, bagged and artificial pollination for each genotype was tested by an analysis of variance in a completely randomized design using GENSTAT statistical programme. Duncan's Multiple Range Test was used to compare the means of fruit set among the eight varieties. The arcsine transformation was applied to the percentages of fruit set prior to statistical analysis.

### 3.2.3 Experiment 3: Molecular Characterization

***DNA Isolation***

A random sample of open-pollinated (OP) progeny arrays of 30 individuals from six genotypes (Experiment 2) and their six maternal parents; TRFK 6/8, TRFK 301/4, TRFK 301/5, TRFCA SFS 150, GW Ejulu-L and AHP SC31/37 (i.e. a total of 186 individuals), were used for molecular characterization. Young healthy fresh leaves were harvested from the nursery plants and frozen at -20°C. Genomic DNA was extracted from the frozen leaf samples using a modified Cetyltrimethylammoniumbromide (CTAB) procedure and purified as described by Wachira *et al.*, (1995). The frozen leaf material (3 leaves) weighing 0.6g was put in an ethanol rinsed mortar containing 0.3g of polyvinylpolypyrrollidone. The mixture was then quickly ground with the addition of liquid nitrogen to a fine powder using a pestle before adding 8ml of preheated ($65^{O}$C) CTAB extraction buffer (100mM Tris HCL, pH 7.5; 1.4mM EDTA, pH 8.0; 2% CTAB; 0.1% DTT). The suspension obtained were poured into clean centrifuge tubes and incubated at $65^{O}$C for 30minutes with constant shaking. After incubation, 6ml of chloroform: isoamyl alcohol (24:1) was added into each tube and the contents thoroughly

mixed to denature the proteins in the suspension. The contents in the tubes were centrifuged (Medifuge, model Heraus; 3200rpm, 5min). The resulting supernatant (2ml) was removed into clean centrifuge tubes and a one and a half fold volume (3ml) of ice-cold isopropanol added followed by gentle shaking (by inverting) to precipitate the total nucleic acids. The threadlike nucleic acids were then spooled by sterile forked Pasteur pipette tubes into microcentrifuge tubes containing 1ml 70% ethanol. The contents were centrifuged (Microfuge, Hermle, model Z-252M; 10,000rpm, 5mins), resulting pellets rinsed thrice in 70% ethanol and vacuum dried before being suspended in 400µl of sterile distilled water (SDW).

***Purification of Isolated DNA***

The RNA which was co-precipitated with DNA was removed by digestion with Ribonuclease A (RNase A) as follows: A stock solution of RNase A (10mg/ml) was prepared in Tris buffer (Tris-HCl, p.H. 7.5; 1mM NaCl), and an aliquot of 20µl added to each DNA sample. The samples were then incubated at $60^{O}$C for 15min to allow for complete digestion of the RNA. Further purification of the DNA and removal of the insoluble material was carried out by addition of 400µl chloroform: isoamyl alcohol (24:1) and mixing the contents thoroughly before centrifugation (Microfuge, Model Heraus; 12000rpm, 15mins). The aqueous phase was transferred to a new tube and 200µl NaCl-TE (1mM NaCl, 100mM Tris HCl, pH 7.5; 1.4mM EDTA, pH 8.0) added to the old tube. This was thoroughly shaken and centrifuged (12,000rpm, 15mins). The resulting aqueous phase was transferred to the new eppendorf tube with the previously aliquoted supernatant and the two mixed and centrifuged (12,000rpm, 15mins). The resulting supernatant (500µl) was removed into clean eppendorf tube and a one and a half fold volume of ice-cold isopropanol added followed by gentle shaking (by inverting) to precipitate the nucleic acid. The contents were centrifuged (5,000rpm, 5mins) and the supernatant removed. The pellet was washed by adding 1ml 75% ethanol with gentle inverting and centrifuged (5,000rpm, 5mins). The supernatant was poured, the pellet vacuum dried and suspended in 200µl sterile double distilled water with overnight incubation at $55^{\circ}$C.

***Estimation of DNA Concentration and Quality***

The DNA yield and quality (intactness) was visually determined using the agarose gel electrophoresis method as described by Manniatis *et al.*, (1982). Lambda (λ) phage DNA was used as the standard. A working stock of λ DNA (20ng/µl) was prepared and used for quantification. Different concentrations of λ DNA (40-200ng) were electrophoresed with the test DNA samples (5µl), on 1.5% agarose in 1 X TBE running buffer (89mM Tris-HCl, pH 8.3; 89mM Boric Acid; 2.5mM EDTA) at a constant voltage of 150V for 30min. After electrophoresis, the gel was stained in ethidium bromide (0.1% w/v) before viewing under ultraviolet light (312nm) on a transilluminator. The concentrations of the samples were determined by comparing band sizes and staining intensities of the test DNA samples with those of the standard λ DNA. The DNA samples were then standardized to concentrations of between 10-30ng/µl by appropriate dilutions using sterile distilled water.

***Primer Screening for Polymerase Chain Reaction (PCR)***

Five randomly selected DNA samples were used to screen for polymorphism within RAPD and ISSR primers using a thermal cycler (Techne, Model Genius FGEN02TD). Thirty RAPD and 16 ISSR primers (Table 2) were screened for PCR amplification and their reproducibility of discrete fragments. The PCR constituents were used at the following final concentrations per reaction: 40ng template genomic DNA; 200µM of each of the Deoxynucleoside Triphosphates (dNTPs); 2.5mM $MgCl_2$, 0.5U Taq DNA polymerase (Roche) and 200nM arbitrary primer kits obtained from Operon Technologies, USA (OP) and Advanced Biotechnologies, UK (AB).

A stock solution of the PCR mix for the 5 samples per treatment was prepared as follows; 5.0µl primer; 2.5µl dNTPs; 5.0µl $MgCl_2$; 29.5µl sterile double distilled water and 0.5U Taq DNA polymerase. Nine and a half micro-liters (9.5µl) from this stock was drawn and added to 0.5µl of the test DNA samples in 0.2ml PCR tubes. The above reaction mixtures were overlaid with a drop of sterile mineral oil (Sigma) and incubated in the thermal cycler and subjected to a hot start (93$^{O}$C for 5 min) followed by 40 amplification cycles as follows:

Table 2: Randomly Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR) oligonucleotide primer sequences screened in tea mating systems experiment

| | RAPDs | | | ISSRs | |
|---|---|---|---|---|---|
| | 5' | 3' | | 5' | 3' |
| 1. | AB4-04 | AAGTCCGCTC | 1. | 801 | ATATATATATATATATT |
| 2. | AB4-13 | GTCAGAGTCC | 2. | 803 | TATATATATATATATAC |
| 3. | AB4-19 | GGTGCACGTT | 3. | 804 | TATATATATATATATAA |
| 4. | OPB-07 | GGTGACGCAG | 4. | 806 | TATATATATATATATAG |
| 5. | OPC-02 | GTGAGGCGTC | 5. | 807 | AGAGAGAGAGAGAGAGT |
| 6. | OPD-05 | TGAGCGGACA | 6. | 808 | AGAGAGAGAGAGAGAGC |
| 7. | OPD-10 | GGTCTACACC | 7. | 810 | GAGAGAGAGAGAGAGAT |
| 8. | OPD-18 | GAGAGCCAAC | 8. | 813 | CTCTCTCTCTCTCTCTT |
| 9. | OPD-20 | ACCCGGTCAC | 9. | 816 | CACACACACACACACAT |
| 10. | OPE-01 | CCCAAGGTCC | 10. | 817 | CACACACACACACACAA |
| 11. | OPF-01 | ACGGATCCTG | 11. | 821 | GTGTGTGTGTGTGTGTT |
| 12. | OPG-06 | GTGCCTAACC | 12. | 842 | GAGAGAGAGAGAGAGATG |
| 13. | OPI-13 | CTGGGGCTGA | 13. | 849 | GTGTGTGTGTGTGTGTCA |
| 14. | OPM-04 | GGCGGTTGTC | 14. | 850 | GTGTGTGTGTGTGTGTTC |
| 15. | OPM-17 | TCAGTCCGGG | 15. | 851 | GTGTGTGTGTGTGTGTCG |
| 16. | OPU-08 | GGCGAAGGTT | 16. | 857 | ACACACACACACACACTG |
| 17. | OPU-15 | ACGGGCCAGT | | | |
| 18. | OPU-18 | GAGGTCCACA | | | |
| 19. | OPU-19 | GTCAGTGCGG | | | |
| 20. | OPV-02 | AGTCACTCCC | | | |
| 21. | OPV-03 | CTCCCTGCAA | | | |
| 22. | OPV-06 | ACGCCCAGGT | | | |
| 23. | OPV-14 | AGATCCCGCC | | | |
| 24. | OPV-17 | ACCGGCTTGT | | | |
| 25. | OPW-02 | ACCCCGCCAA | | | |
| 26. | OPW-03 | GTCCGGAGTG | | | |
| 27. | OPW-04 | CAGAAGCGGA | | | |
| 28. | OPW-05 | GGCGGATAAG | | | |
| 29. | OPW-07 | CTGGACGTCA | | | |
| 30. | SC10-56 | CCAGCGTCTA | | | |

| RAPD | ISSR | Process |
|---|---|---|
| 93 $^{O}$C for 1min | 94 $^{O}$C for 30sec | Template DNA denaturation step |
| 42 $^{O}$C for 1.5min | 52 $^{O}$C for 45sec | Primer- template DNA annealing step |
| 72 $^{O}$C for 1min | 72 $^{O}$C for 2min | Elongation step |

After completion of the 40 cycles, the samples were maintained at 72$^{O}$C for 5 minutes to extend all the PCR products. The samples were then cooled at 4$^{O}$C until removal and separation by agarose gel electrophoresis. Primers that were most polymorphic were further tested for their ability to give reproducible discrete fragments by replicating the samples into three and running a PCR and checking for consistency in the profile.

***Randomly Amplified Polymorphic DNA (RAPD) Marker Analysis***

Polymerase chain reaction (PCR) mixtures (total volume of 10µl) contained 40ng template genomic DNA; dNTPs (dATP, dCTP, dGTP and dTTP mixture) at 100nM final concentration; 200nM arbitrary sequence oligonucleotide primer (each containing 50-70% GC content); 1 X Taq DNA polymerase buffer, 2.5mM $MgCl_2$; and 0.5 units of Taq DNA polymerase. Reactions were set in 0.2ml strip-ease PCR tubes with a mineral oil overlay. Each PCR run included a negative control (with sterile distilled water instead of template DNA). Samples for enzymatic amplification were subjected to 93$^{O}$C for 5 minutes (Hot start step); 40 cycles of 93$^{O}$C for 1 minute (denaturation step), 42$^{O}$C for 1.5 minutes (primer/template annealing step), 72$^{O}$C for 1 minute (polymerization step); and a final extension phase of 10 minutes at 72$^{O}$C (Williams *et al.*, 1990). Amplification was carried out on a Techne Genius thermal cycler (Model FGEN02TD).

### *Inter-Simple Sequence Repeat Polymorphism (ISSR) Marker Analysis*

The ISSR PCR mixtures (total volume of 10µl) contained the same constituents as those for the RAPD protocol. The primers used for ISSR amplification however contained numerous simple sequence repeats e.g. GT (n) *et cetera*. The primers were also longer than those for RAPD analysis. PCRs were set in 0.2ml PCR tubes with an oil overlay. The thermal cycling profile was different; i.e. a hot start at 94$^{O}$C for 5minutes; 40 cycles of 94$^{O}$C for 30 seconds (denaturation step), 57$^{O}$C for 45 seconds (primer/template annealing step), 72$^{O}$C for 2 minute (polymerization step); and a final extension phase of 10 minutes at 72$^{O}$C (Kantety *et al.*, 1995). The thermal cycling was done on a Techne Genius thermal cycler (Model FGEN02TD). Each PCR run included a negative control (with sterile distilled water instead of template DNA).

### *Fractionation of DNA by Gel Electrophoresis*

PCR samples (both RAPD and ISSR) were mixed with 3µl of gel loading buffer (50% Glycerol, 250mM EDTA (pH 8.0), 0.01% Bromophenol blue) and fractionated in 1.5% agarose gels with 1 X TBE both as gel and running buffer. The running voltage was 150 volts. The agarose gels was then stained in Ethidium Bromide [Et Br] (50µl of 10mg/ml solution in 1litre 1 X TBE buffer) for 45 minutes, and visualized and photographed on a UVP transilluminator at 312nm.

### *Data Analysis*

RAPD and ISSR are typically dominant markers. Scoring of bands was done on the basis of two possible alleles, namely band presence (1) and band absence (2). Scoring was carried out twice to ensure correct typing. The binary matrix data file created was then configured as an input file for data analysis. The maximum-likelihood based programme, MLTR (version 3.0; Ritland, 2002) was used to estimate parental inbreeding coefficients ($F$), ovule and pollen allele frequencies (o and p, respectively), and mating-system parameters. Mating-systems parameters that were estimated include: single- and multi-locus outcrossing rates ($t_m$ and $t_s$, respectively; Ritland and Jain, 1981) and correlation of paternal parentage through self-fertilization and through outcrossing

between sibs ($r_s$ and $r_p$, respectively; Ritland, 1988, 2002), and correlation of selfing rates among loci ($r_{s,\ loci}$; Ritland, 2002). The traditional mixed-mating model (e.g. Ritland and Jain, 1981) assumes that a fraction of progeny are produced through self-fertilization and the remaining progeny are derived from random outcrossing. The correlated-matings model, used in MLTR, incorporates an additional aspect of plant mating system: that siblings may share the same male parent. For instance, if a plant is fertilized only by the closest neighbor, paternal parentage among the outcrossed sibs is highly correlated (i.e. high $r_p$). Under low pollinator availability, a high correlation of selfing ($r_s$) is also likely because seeds derived from a flower that has been visited may be all outcrossed, whereas seeds derived from an unvisited flower will also be selfed. Biparental inbreeding can be suggested by low $r_{s,loci}$ because biparental inbreeding causes variation in $t_s$ and reduces the correlation of $t_s$ among loci (Ritland 2002). Maximum-likelihood estimates of mating-system parameters were calculated for the populations by assuming that both groups experienced the same pollen allele frequencies. Maternal genotypes were inferred from the progeny arrays by the maximum-likelihood method of Brown and Allard (1970). Newton-Raphson iteration was used to find the maximum-likelihood estimates for all parameters. The standard error of each estimate was calculated as the standard deviation of 500 bootstrap values with the maternal progeny array as the unit of resampling.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 Experiment 1: Pollen Viability.

### 4.1.1 Difference in pollen viability among genotypes

There were significant differences in pollen viability among the eight genotypes and seasons (Table 3). After 24 hours *in-vitro* germination, the mean percentage of pollen germination (collected in 2005) varied from 44.1% for clone GW Ejulu-L at Timbilil, Kericho to 51.2% for clone AHP SC 31/37 at Kangaita, Kirinyaga with the mean germination percentage of 48.5% (± 1.35) for both sites. These results confirm the earlier report of considerable variation in pollen viability between tea genotypes (Wachira and Kamunya, 2005). Such differences among the genotypes suggest that there might be pollen competition leading to unequal reproductive success in tea. This has been demonstrated in other plants (Nakamura and Wheeler, 1992; Skrøppa and Lindgren, 1994). Nikkanen *et al.*, (2000) found that pollen germination is similar both *in vitro* and *in vivo*, although it occurs faster in the former.

The reproductive biology of *C. sinensis* provides an opportunity for male gametophyte competition through differential germination ability. However, a late-acting self-incompatibility system that constrains selfing has been reported in tea (Wachira and Kamunya, 2005). Thus the genotypes homozygous for lethal, sublethal or defective genes are eliminated either by early abortion of zygotes or, later on, through embryo competition. This is a feature that might affect the results of gametophyte competition. There was also significant seasonal variation in percentage pollen grain germinated in 2005. Seasonal mean pollen germination in the year ranged from 44.2% during the late season to 50.7% in the mid season, an indication that pollination is most effective in the early to mid season but germination reduced towards the late season, clonal variability is evident. Clone GW Ejulu-L at Timbilil, Kericho had a significantly low pollen germination (47.4%) in the early season as compared to clone AHP SC 31/37 at Kangaita, Kirinyaga that had the highest germination percentage (55.9%). In the mid season, percentage pollen germination ranged from 44.3% (TRFK 301/5) to 58.2% (TRFCA SFS 150). Late season percent germination were significantly lower than early and mid season and ranged from 38.9% for GW Ejulu-L at Timbilil, Kericho to 51.5%

Table 3: Percentage pollen viability over three flowering seasons (early, mid and late) in eight tea genotypes

| | Mean pollen germination percentage | | | |
|---|---|---|---|---|
| Clone | Early | Mid | Late | Mean |
| TRFK 6/8 | $51.2^{(ab)}$ | $56.1^{(a)}$ | $41.3^{(cd)}$ | $49.4^{ab}$ |
| AHP SC 31/37 | $53.1^{(ab)}$ | $44.4^{(b)}$ | $43.8^{(bcd)}$ | $47.0^{ab}$ |
| GW Ejulu-L | $47.4^{(b)}$ | $46.0^{(b)}$ | $38.9^{(d)}$ | $44.1^{b}$ |
| TRFCA SFS 150 | $48.6^{(ab)}$ | $58.2^{(a)}$ | $41.6^{(cd)}$ | $49.3^{ab}$ |
| TRFK 301/5* | $49.7^{(ab)}$ | $44.3^{(b)}$ | $46.3^{(abc)}$ | $46.7^{ab}$ |
| AHP SC 31/37* | $55.9^{(a)}$ | $54.9^{(a)}$ | $43.0^{(bcd)}$ | $51.2^{a}$ |
| TRFK 301/4* | $50.6^{(ab)}$ | $51.3^{(ab)}$ | $47.4^{(ab)}$ | $49.8^{ab}$ |
| GW Ejulu-L* | $47.9^{(ab)}$ | $51.3^{(ab)}$ | $51.5^{(a)}$ | $50.2^{ab}$ |
| Mean | $50.5^{ab}$ | $50.7^{a}$ | $44.2^{b}$ | 48.5 |

**clones in Kangaita sub-station (Kirinyaga district);the others at Timbilil (Kericho)*
*$^{a,b}$-means followed by the same letter are not significantly different at $P \geq 0.05\%$ level*
*$^{(a,b,c,d)}$Means followed by same letters are not significantly different at $P \geq 0.05\%$ level within the season.*

for GW Ejulu-L at Kangaita, Kirinyaga. Although data on seed set is unavailable, the current study indicates that hybridization work in tea needs to be programmed/staggered such as to synchronize with seasons when optimal success is expected i.e. genotype specific.

The results of the present study show that the fitness of the *C. sinensis* pollen with respect to the germination ability varies. Analysis of variance revealed significant differences among seasons and variety by season interaction effect during the year (Table 3). The varying behaviour of the genotypes in the year 2005 collections may be caused by a number of factors like the duration for which anthers continue shedding pollen and the range of environmental factors to which they are exposed, are critical for cross-pollinated

Table 4: Weather parameters at the experimental sites

| Season/Location | March | | May | | July | |
|---|---|---|---|---|---|---|
| | Timbilil | Kangaita | Timbilil | Kangaita | Timbilil | Kangaita |
| Air Temperature ($^{O}$C) | 17.6 | 17.2 | 16.0 | 16.8 | 15.2 | 13.4 |
| Rainfall (mm) | 133.9 | 84.2 | 362.8 | 528.7 | 194.8 | 82.2 |
| Humidity (%) | 62 | 81 | 81 | 91 | 69 | 94 |

species like tea. The weather conditions over the three seasons during flowering were different as shown in Table 4. This study suggests that pollen germination among genotypes is affected by weather changes i.e. temperature, rainfall and relative humidity, indicating the adaptation of different *C. sinensis* genotypes to produce fast-germinating pollen for different environmental conditions.

### 4.1.2 Effect of environmental conditions on pollen viability

Potential relationships between pollen viability and environmental factors and other characteristics of the donors were also studied. The effect of environmental conditions was studied on two genotypes, i.e. clone AHP SC31/37 (Assam) and GW Ejulu-L (China) that were planted in both sites. The analysis of variance revealed that there were significant seasonal effects on the pollen viability (Table 3). The genotypes were affected differently over the three seasons. Whereas clone AHP SC31/37 pollen germination was not greatly affected by environmental factors (4.2% difference in pollen germination between the genotype in the two sites), the difference in pollen germination in clone GW Ejulu-L between the two sites was greater (6.1%) (Table 3). Genotype AHP SC 31/37 is an Assam while clone GW Ejulu-L is Chinary. The stable performance of a tea cultivar over wide range of environments is regarded desirable (Wachira *et al.*, 2002). The above results suggest that the Assam variety has a low response to different environmental conditions as compared to the China variety. This could be a possible explanation for the wide adaptation of Assam varieties throughout the world than the other varieties (Paul *et al.*, 1997). Indeed, nearly all the popular cultivars in Kenya are of

Assam varieties. Nikkanen *et al.* (2000) pointed out that genotype-environment interactions in pollen performance promote the maintenance of genetic variation within populations even if pollen performance is related to fitness and that some environmental signals during the reproductive process taking place in the female flowers, may cause variation in the phenology traits of the progeny. In seed orchards, these phenomena may contribute to the variable genetic composition of seed produced in different seasons or years.

The effect of specific environmental parameters i.e. rainfall, relative humidity and temperature was studied using linear correlation analysis ($P<0.05$). The mean pollen germination percentage revealed significant positive correlation with temperature in both genotypes ($r= 0.77$) (Fig. 7). During flowering period of tea, the weather conditions in both sites differ considerably from year to year, the daily temperatures values based on ten year mean data varied from +15.3 to +16.9$^{O}$C during 1994-2004, with a mean of +16.3$^{O}$C in Timbilil and from +14.7 to +15.7$^{O}$C with a mean of +15.2 $^{O}$C during the same period in Kangaita (Anon, 2004). The field conditions are thus colder in Kangaita than in Timbilil site.

It was observed that there is some response of flowering to rainfall (personal observation). Flowering commenced with the onset of long rainfall season (March/April) and that flower production varies considerably among the tea genotypes and varieties. Cambod variety was most prolific with flowering occurring almost throughout the year. It appeared that good rainfall is an important requirement for flowering as flower numbers reduced drastically with the break of the long rains in July. However, no correlation was found between the mean germination percentage and relative humidity, nor with rainfall in the year during the study (data not presented).

The significant variation in pollen viability found among the *C. sinensis* genotypes in the present study indicates a potential for male gametophyte competition. Together with the observed genotype-environment interactions in pollen performance, competition may contribute to the variable genetic composition of the seed produced in the orchard. The magnitude of the effects of pollen viability can, however, be assumed to be smaller than the effects of variation in flowering abundance and phenology within the seed orchard and of pollination by non-orchard sources.

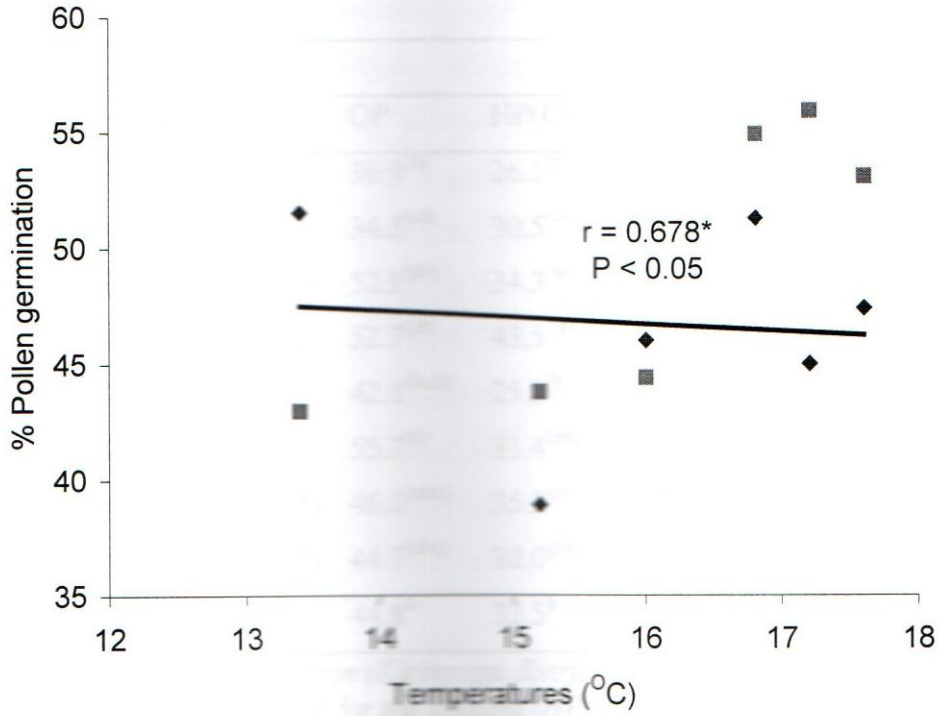



Figure 7: Correlation coefficients between *in-vitro* pollen germination and field temperature conditions during flowering

### 4.2 Experiment 2: Pollination techniques

#### 4.2.1 Fruit and seed set

The reproductive success of each *Camellia* genotype was calculated based on the number of fruits aborted, number of fruits produced, seeds per fruit, mean seed weight and viable seeds. In this experiment, the number of fruits and seeds harvested at maturity was highly dependent on the pollination technique used which principally affected severity of fruit drop. Significant differences ($P \leq 0.05$) were found among genotypes for fruit and seed set in all the four pollination methods studied (Table 5). On the average, the open pollinated treatment had a significantly higher percentage of fruit set (44.8%; Table 5) ranging from 30.9% in genotype TFRK 6/8 to 55.7% in genotype GW Ejulu-L at Kangaita. The least fruit set was found in selfing by emasculation and hand pollination

Table 5: Percent fruit set in eight tea genotypes under different pollination techniques

| Clone | Percent fruit set | | | | |
|---|---|---|---|---|---|
| | OP | HP(C) | SP(B) | HP(S) | Mean |
| TRFK 6/8 (AHP SC 31/37) | 30.9$^{(d)}$ | 26.1$^{(b)}$ | 18.2$^{(b)}$ | 18.0$^{(bc)}$ | 23.7$^{b}$ |
| AHP SC 31/37 (TRFK 6/8) | 34.5$^{(cd)}$ | 30.5$^{(ab)}$ | 19.1$^{(b)}$ | 20.5$^{(ab)}$ | 26.7$^{ab}$ |
| GW Ejulu-L (TRFCA SFS 150) | 52.1$^{(ab)}$ | 34.3$^{(ab)}$ | 25.8$^{(ab)}$ | 22.8$^{(ab)}$ | 34.5$^{ab}$ |
| TRFCA SFS 150 (GW Ejulu-L) | 52.7$^{(ab)}$ | 43.5$^{(a)}$ | 20.0$^{(ab)}$ | 21.7$^{(ab)}$ | 35.7$^{a}$ |
| TRFK 301/5* (GW Ejulu-L*) | 42.1$^{(bcd)}$ | 25.3$^{(b)}$ | 30.5$^{(a)}$ | 18.7$^{(bc)}$ | 29.9$^{ab}$ |
| GW Ejulu-L* (TRFK 301/5*) | 55.7$^{(a)}$ | 31.4$^{(ab)}$ | 21.0$^{(ab)}$ | 10.0$^{(c)}$ | 31.8$^{ab}$ |
| TRFK 301/4* (AHP SC 31/37*) | 46.0$^{(abc)}$ | 35.2$^{(ab)}$ | 29.9$^{(a)}$ | 29.0$^{(a)}$ | 35.2$^{a}$ |
| AHP SC 31/37* (TRFK 301/4*) | 44.7$^{(abc)}$ | 32.0$^{(ab)}$ | 22.2$^{(ab)}$ | 19.8$^{(ab)}$ | 30.5$^{ab}$ |
| Mean | 44.8$^{a}$ | 32.5$^{b}$ | 23.7$^{bc}$ | 20.6$^{c}$ | |

**Genotypes in Kangaita sub-station (Kirinyaga district); the others at Timbilil (Kericho). In parenthesis is the pollen donor for the crossed (HP) treatment.*
*OP=open pollination; HP(C)=cross hand pollination through emasculation; SP(B)=self pollination through bagging; HP(S)=self hand pollination through emasculation.*
*$^{a,b,c,}$ Means followed by same letters are not significantly different at $P \geq 0.05\%$ level.*
*$^{(a,b,c,d),}$ Means followed by same letters are not significantly different at $P \geq 0.05\%$ level within the treatments.*

(20.6%) ranging from 10.0% in genotype GW Ejulu-L (Kangaita) to 29.0% in genotype TRFK 301/4 (Kangaita). Similarly, open-pollination produced a significantly ($P \leq 0.05$) higher number of seeds per plant (48%) (data not presented). The controlled self- and cross-pollination treatments had significantly lower percent fruit set (Table 5). However, controlled cross-pollination (HP) produced a significantly higher mean number of fruits per plant (32.5%) than any of the selfing by emasculation treatment. Percentage fruit set in the treatment ranged from 25.3% in genotype TRFK 301/5 (Cambod) to 43.5% in genotype TRFCA SFS 150 (Assam), both of which had a common pollen donor i.e. genotype GW Ejulu-L (China). This data on fruit set (Table 5) suggests that the Assam variety combines better with the China variety than with the Cambod variety and hence more fruit set.

Open pollen transfer was the most efficient pollination technique with almost 44.8% of the open-pollinated flowers developed fruits. Although there was no significant difference among the selfing techniques, selfing through bagging had more fruit set per plant (23.7%) than controlled selfing following emasculation (HP) (20.6%) (Table 5). Percentage fruit set in the self through bagging ranged from 18.2% in clone TRFK 6/8 (China/Assam hybrid) to 30.5% in genotype TRFK 301/5 (Cambod), while in the selfing through hand pollination (HP), percent fruit set ranged from 10.0% in clone GW Ejulu-L (China, Kangaita) to 29.0% in clone TRFK 301/4 (Cambod). Such variation in fruit set within the same treatment suggests variable expression of the *SI* trait (Wachira and Kamunya, 2005). The variations in fruit set between the two selfing techniques might be due to injuries caused by emasculation and subsequent death of embryos. Low fruit set in selfed flowers suggests that *C. sinensis* relies heavily on cross-pollination for seed production.

Percent fruit set may also be determined by the degree of early abscission of fertilized fruits in *C. sinensis*. A severe drop of fruits after fertilization resulted in extremely low final fruit sets especially in the selfed-treatments (about 20%) (Fig. 8a and Table 5). The number of aborted fruits (abscission) per plant after pollination was significantly higher between the 8-16 weeks after pollination (Fig. 8a and b). However, in the open pollinated treatment, the number of aborted fruits at 24 weeks after pollination, was not significantly different from the number of fruits at harvest (over 52 weeks). By contrast, the number of fruits that had aborted by the 16$^{th}$ week after pollination in the open pollinated treatment was considerably less (46.5%) compared to 68.5% in cross-pollination by emasculation, 80.8% in selfing through bagging and 85.7% in controlled selfing by emasculation (Fig. 8a). Details of fruit abscission in different tea genotypes are presented in Figure 8b. In the open pollinated plants, no further significant abortion occurred after the 16$^{th}$ week, while in the other treatments there were further fertilized fruit loss (about 10%) between the 16$^{th}$ week and harvest time (over 52 weeks). In all genotypes there were fewer abortions at 16$^{th}$ week when the flowers were open pollinated or crossed than when the genotypes were selfed. Total fruit abscission during the early phase from 8$^{th}$ – 16$^{th}$ week post fertilization was highest on the selfing by emasculation and pollination. At the eighth week post-anthesis, for example, the selfed by

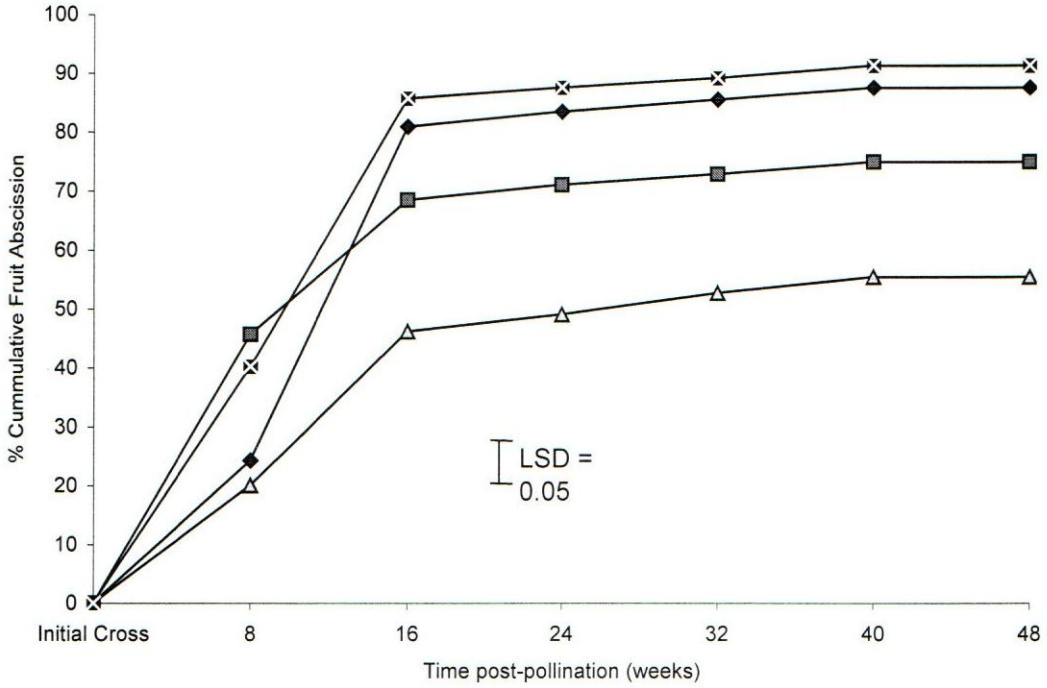



Figure 8a: Percentage mean fruit abscission in different pollination techniques

emasculation treatment in clone TRFCA SFS 150 shed 169 fruits, accounting for an abscission rate of 84%. During the same period, the open-pollinated treatment in the same genotype shed only 62 fruits, representing an abscission rate of 31%.

The factors causing such differences in reciprocal crosses (Table 5) or the severe fruit drop in tea are unclear. Late-acting self-incompatibility (*LSI*)-type which acts post-fertilization maybe a possible factor accounting for the differences in the pattern and level of fruit abscission between the open-pollinated, crossed and selfed plants (Wachira and Kamunya, 2005). The fruit set results (Table 5) further suggests that there are differences in self-incompatibility levels of different genotypes as reflected in the varying number of fruits/seeds among the genotypes after fertilization. These results of the fruit and seed set from self- and cross-pollinated treatments can also be used to evaluate pollen viability among and between the genotypes as the measurement may indicate the presence or absence of fertile pollen.

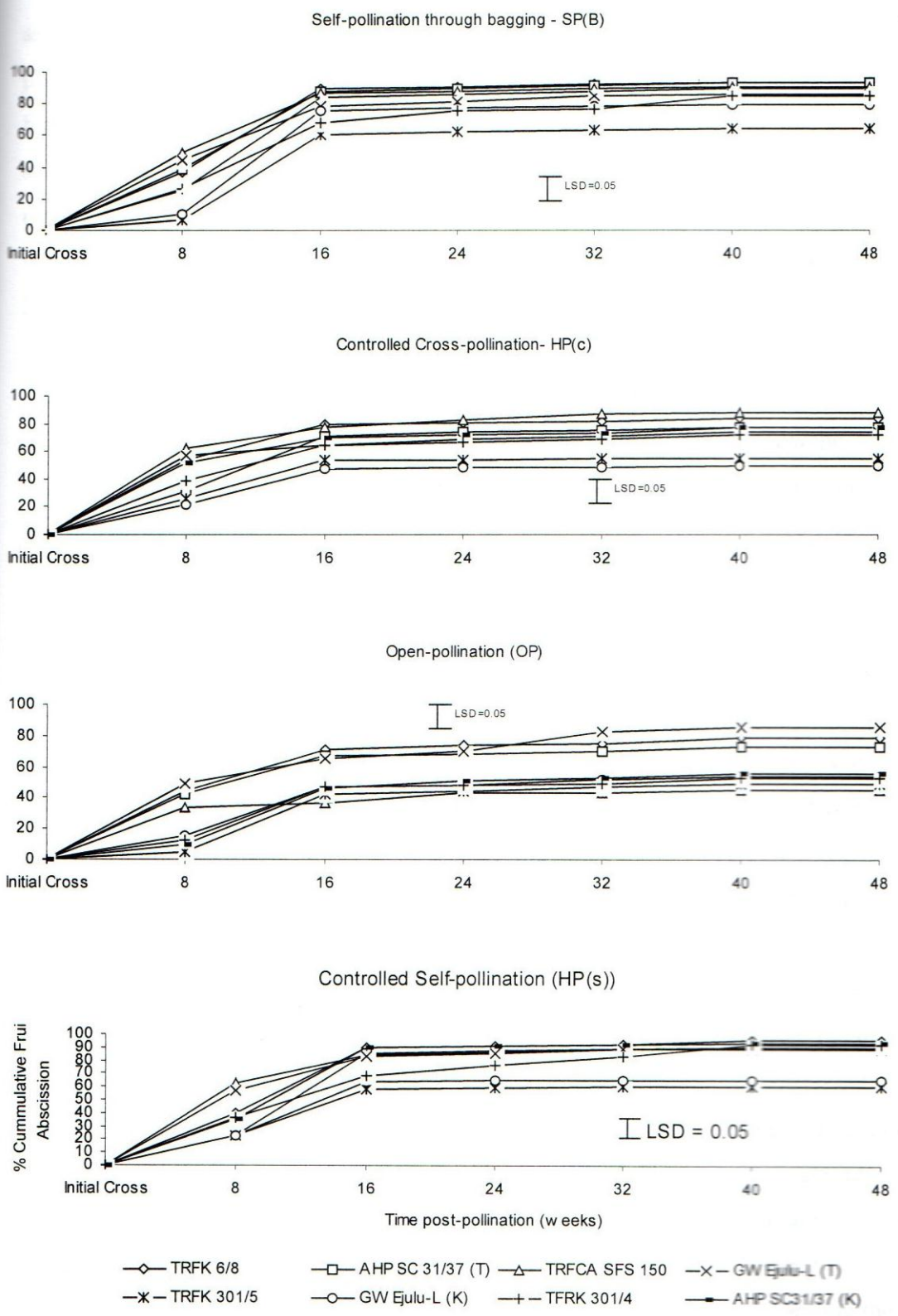



Figure 8b:Percentage mean fruit abscission in different pollination techniques

Abscission between $8^{th}$ – $16^{th}$ weeks post-fertilization was much greater on the selfs- than on cross- or open-pollinated treatments, so that selfs fruit set at the end of this phase were much less than those of cross or open treatments. This indicates that post-fertilization type of self-incompatibility mechanism is involved in the tea genotypes studied. The variations in abscission rates among the treatments suggests that abscission, upto sixteen weeks after pollination, is due primarily to genetic factors (gametophytic) whilst subsequent abscission maybe due to other non-genetic factors. Such an observation has been made in *Trifolium repens* (Marshall *et al.*, 1999). Premature fruit drop was virtually over by 24 weeks post-pollination so that, this period of abscission is completed before fruits are fully mature and while they are still rapidly gaining fresh weight. Several other causes (non-genetic) have been suggested to account for the low percentages of flowers retained as fruits for many plant species, including inadequate pollination, pollen parent effects, resource availability, predation and disease (Trueman and Truebull, 1994; Gaol and Fox, 2002). The reduced fruit set was also as a result of predation experienced during the experiment. Birds were the main predators as they harvested the thread color codes used in labeling the experiment but this was soon realized and changed to tag labels. The damage caused could however not be accurately quantified.

In Acacia species, Gaol and Fox, 2002 (2002) suggested that the resources available to the flower or fruit depend on the total resources of the individual or branches and the number of reproductive structures drawing upon those resources. The plant matched reproductive output to resources available by flower and juvenile fruit abscission. It is possible that like in many plant species, tea regularly aborts flowers and immature fruits producing mature fruits from only a small portion of the flowers. However, it is difficult to know whether the abscission was caused by unfertilized flowers or the drop of fertilized fruits.

Trueman and Wallace, (1999) found that limited pollen supply and inadequate maternal resources may also constrain fruit production during a single reproductive season, particularly if pollen supply and resource availability vary spatially or temporally with reduced carbohydrate supply negatively affecting the number of fruits in Macademia. It is possible that the significant variation in seed numbers among the tea

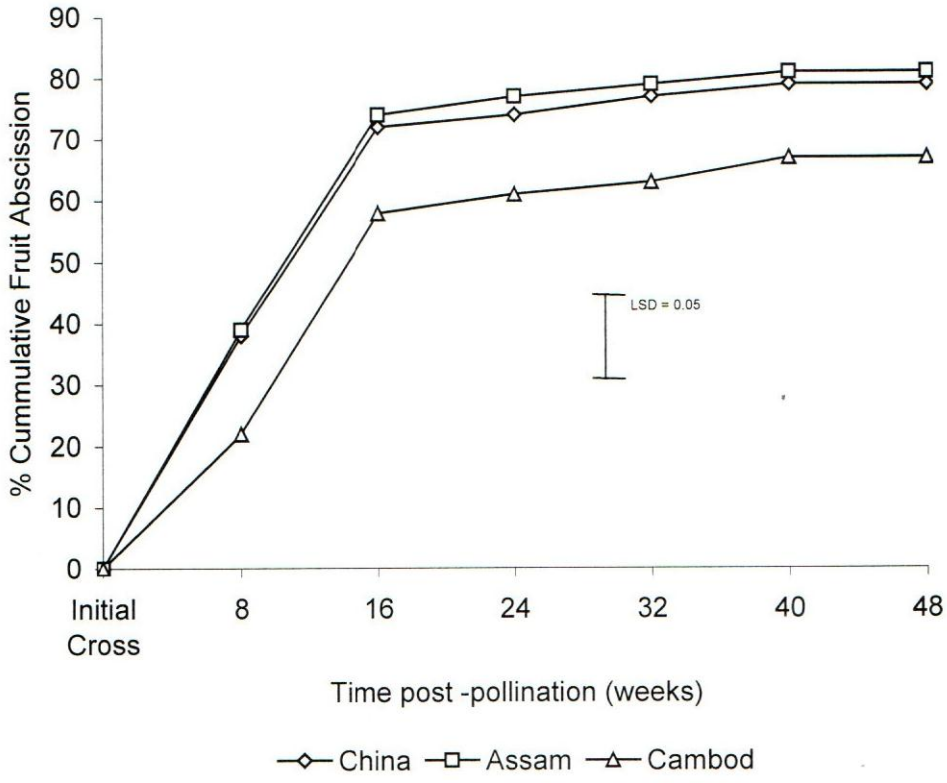



Figure 8c: Percentage mean rates of fruit abscission in three *C. sinensis* varieties

genotypes and varieties might also be attributed to maternal resource variation among the genotypes. Sakai and Nagao (1985) suggested that the onset of abscission is more likely to be associated with developmental changes occurring within the fruits, and the possibility exists that at least the early stages of premature fruit drop amount to an early adjustment of crop load, before peak depletion of storage carbohydrates. In other plant species, accumulating evidence indicates that changes in resource supply changes seed numbers (Trueman and Wallace, 1999). This is an area awaiting further research in tea.

At harvest, a variation in the proportion of fruit abscission in the three tea varieties was observed (Fig. 8c). In the Cambod variety, fruit abscission was approximately 68% compared to 80% in the Assam and 83% in China varieties. Reciprocal crosses within an orchard (Treatment two) indicated that there were significant variations in fruit set depending on the genotype used as the seed bearer (Table 5). For example, in orchard 2 where clones TRFCA SFS 150 (Assam) and GW Ejulu-L (China) were crossed with each other, there was more fruit set when the Assam

variety (43.5%) was used as the seed bearer compared to when the Chinese variety (34.3%) was used as the seed bearer (Table 5). Among the genotypes, clone TRFCA SFS 150 (Assam variety) produced significantly higher mean fruits per plant than the other genotypes. Of the eight genotypes studied, the best seed set was obtained from clone TRFCA SFS 150 (Assam variety) followed by clone TRFK 301/4 (Cambod variety), while the poorest seed set was obtained in clone TRFK 6/8 (Assam/China variety) (Table 5). The significant variation in fruit set among the three tea taxa suggests that Cambod variety has the potential to carry more fruits to harvest than China and Assam varieties, and therefore contributing to more seeds per capsule.

### 4.2.2 Seed weight

When seed number per fruit and seed weight were considered, there were significant variations among the genotypes (Table 6). A total of 6960 seeds (pooled data) were harvested from the eight genotypes and tested for seed viability. Of these 40.0% were non-viable (floaters) with only 33.65% of the viable seeds germinating. Seed weight at harvest was not significantly affected by pollination treatment (cross *vs.* self), but was significantly different among the genotypes ($P \leq 0.05$). Mean seed weight was 1.10 ± 0.06 gm (Table 6). The number of seeds per genotype ranged from 384 (AHP SC 31/37 - Kangaita) to 1201 (GW Ejulu-L - Kangaita) (Fig. 9). Heavy seeds were obtained from genotype TRFK 301/5 (1.51gm/seed) while genotype GW Ejulu-L (0.82gm/seed) produced lighter number of seeds.

The Assam tea variety had 2213 seeds (pooled total) each weighing on average 1.12gm, of these 35.68% were floating (non-viable). Genotype AHP SC 31/37 (Assam) had on average 1.35 (pooled mean) seeds per fruit (1 – 4 seeds/fruit) with a mean seed weight of 1.12gm. Of these 38.31% were floaters, and 28.16% were viable seeds and germinated. Genotype TRFCA SFS 150 (Assam) had on average 1.70 seeds per fruit (ranging 1 – 5 seeds/fruit) with a mean seed weight of 1.11gm; 34.37% were floaters, and 32.43% of the viable seeds successfully germinated. The China variety genotype GW Ejulu-L had on average 1.69 (pooled mean) seeds per fruit (ranging 1 – 4 seeds/fruit) with a mean seed weight of 0.95gm (pooled mean) and some 41.29% floaters, 40.89% of

Table 6: Mean seed weight (gms) and seeds per fruit among *C. sinensis* genotypes

| Clone | Mean seed weight (gms) | Mean seeds per fruit |
|---|---|---|
| TRFK 6/8 | $0.90^{b}$ | $1.29^{cd}$ |
| AHP SC 31/37 | $1.10^{b}$ | $1.38^{bcd}$ |
| GW Ejulu-L | $1.08^{b}$ | $1.54^{abcd}$ |
| TRFCA SFS 150 | $1.11^{b}$ | $1.70^{abc}$ |
| TRFK 301/5* | $1.51^{a}$ | $1.71^{ab}$ |
| GW Ejulu-L* | $0.82^{b}$ | $1.87^{a}$ |
| TRFK 301/4* | $1.13^{b}$ | $1.78^{ab}$ |
| AHP SC 31/37* | $1.14^{b}$ | $1.29^{d}$ |
| Mean | 1.10 | 1.57 |

$^{a,b,c,d}$ *Means followed by same letters are not significantly different at $P \leq 0.05\%$ level*
**clones in Kangaita sub-station (Kirinyaga district); the others at Timbilil (Kericho)*

the viable seeds germinated while genotype TRFK 6/8 (China/Assam hybrid) had on average 1.29 seeds per fruit (ranging 1 – 4 seeds/fruit) with a mean seed weight of 0.90gm and some 19.13% floaters, 25.68% of the viable seeds germinated. Generally, the Chinese variety had 2615 seeds (pooled total), each weighing on average 0.99gm and 37.18% non-viable. The Cambod variety had 2132 seeds (pooled data) each weighing on average 1.14gm, of these 48.9% were non-viable. Genotype TRFK 301/4 had on average of 1.57 seeds per fruit (ranging 1 – 6 seeds/fruit) and a mean seed weight of 1.13gm. Of these seeds, about 52.8% were non-viable while 21.54% germinated. Clone TRFK 301/5 (Cambod) had on average 1.69 seeds per fruit (ranging 1 – 6 seeds/fruit), a mean seed weight of 1.51gm and 48% floating, 29.89% of the seeds germinated.

Higher number of seeds per fruit was associated with larger fruit size, though this association was not significant with single seed weight, but can suggest more resources were devoted to larger fruits. A greater number of seeds per fruit were also associated with more varied seed sizes, indicating that competition occurs for resources within seeds in the fruit. Seed weight may also affect seedling survival as in some species larger

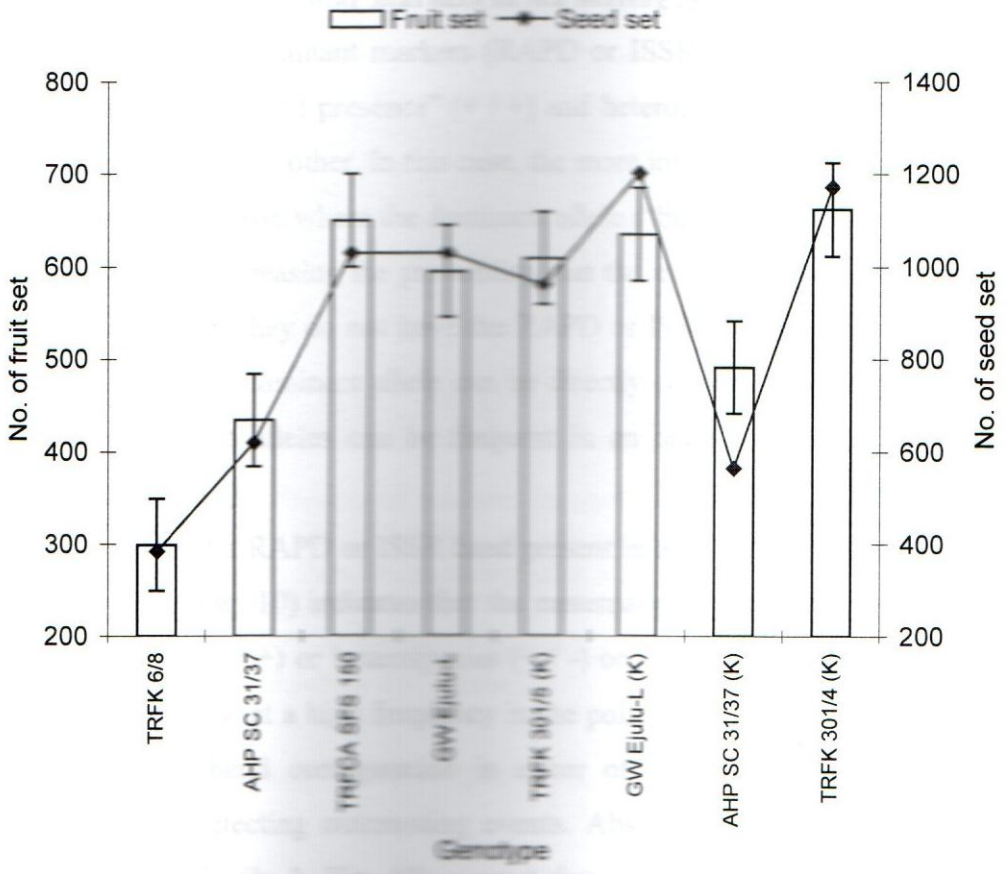



Figure 9: Fruit and seed set among *C. sinensis* genotypes

(heavier) seeds can produce more vigorous seedlings and may be better suited to endure unfavourable climatic conditions to produce a seedling (Wyllie-Echeverria *et al.*, 2003). Many of the floating (non-viable) seeds did not have cotyledon/embryo or had cotyledon that started to develop but died before full maturation. Ultimately, the production of very large numbers of flowers among the three tea genotypes, far more than the number of fruits that reach maturity, may have functions relating to such factors as pollinator attraction, pollen dissemination, and maximization of the genetic variability of initially set fruits. The precise stage of generative development that is most sensitive to environmental stress and the degree of susceptibility among the tea varieties awaits further investigation.

### 4.3 Experiment 3: Molecular markers in tea mating system

When using dominant markers (RAPD or ISSR), the homozygous genotype for the dominant-allele "band presence" (+ / +) and heterozygous genotype (+ / -) cannot be distinguished from each other. In this case, the more informative markers for outcrossing-rate estimation are those where the dominant allele ("band presence") is at low frequency in the population, increasing the probability that the maternal plants are homozygous for "band absence", i.e. they do not have the RAPD or ISSR marker so that outcrossed off-spring that have the dominant allele can be directly counted. Typically, it was observed that some dominant alleles can be frequent in an open-pollinated family and rare in another one (Fig. 10).

Presence of a RAPD or ISSR band present in all offspring (e.g., band A in family 3 - GW Ejulu-L, Fig. 10) indicates that the maternal plant could be homozygous for this marker (genotype + / +) or heterozygous (+ / -) or homozygous null (- / -), and that the marker could also be at a high frequency in the pollen pool, i.e. in the population. In this case the marker band configuration is either of very little use, or else it is non-informative, for detecting outcrossing events. Absence of markers in a few offspring (e.g., band C in family 3, Fig. 10) suggest that the maternal genotype could be either heterozygous (+ / -) or homozygous null (- / -), with the marker band at high frequency. In this case, offspring originating from outcrossing cannot be distinguished based solely on this marker. Finally, the most-informative configuration is when a marker band is present in just a few offspring (e.g., band B in family 3- GW Ejulu-L, Fig. 10) and is absent in the maternal parent. In this case, it can be inferred that the maternal plant has homozygous genotype (- / -) for the marker, and that the outcrossed progeny individuals that show the marker are most surely heterozygous (+ / -). Outcrossing events can therefore be readily identified if the maternal plant has a homozygous null genotype (-/ -). Evidently even in this case, not all of the outcrossing events can be identified based only on this marker, as progeny individuals that were outcrossed but received the "band-absent" allele from the paternal plant are not directly detectable. It is by accumulating data from several markers and estimating allele frequencies (Table 7) in the population for the RAPD or ISSR markers

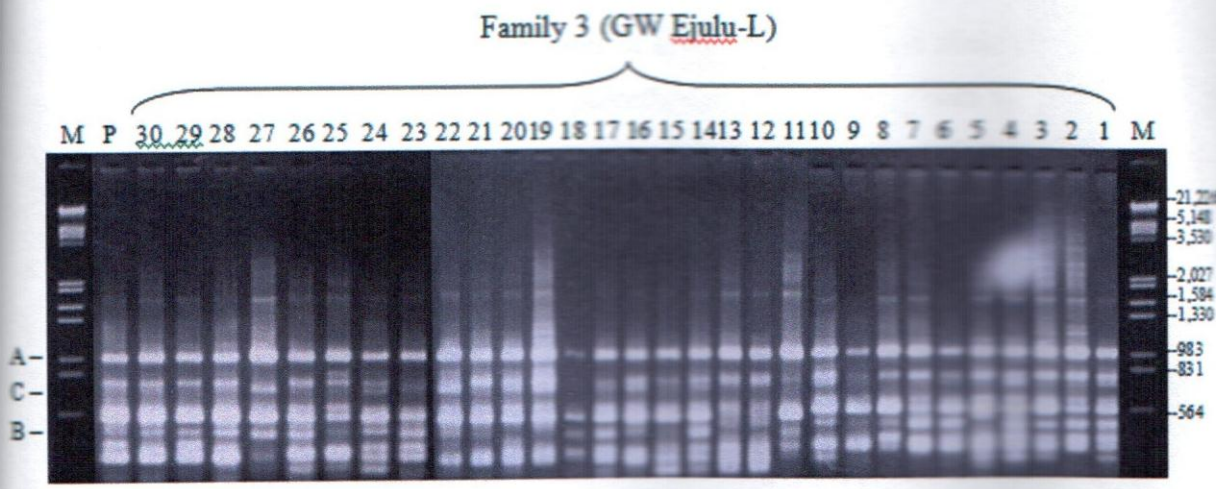



Figure 10: Molecular fingerprint patterns in open pollinated (O.P) families. Randomly Amplified Polymorphic DNA (RAPD) gel was generated with primer OPW-17 in Family 3 (lane 1-30 progenies). Band A: monomorphic in the family; band B: rare in the family; band C: frequent in the family
*M – Molecular marker EcoR I/Hind III digested λ DNA*
*P – Maternal parent: genotype GW Ejulu-L*

used, that the most-likely maternal genotypes can be inferred and all the outcrossing events detected.

To estimate outcrossing among six families of tea, 30 RAPD and 13 ISSR primers were screened. Nine RAPD and 5 ISSR primers were selected for assaying the 6 families since they produced repeatable and scorable amplification products. However, unstable or weak bands were not scored for data analysis. Amplification of DNA from the six tea families with the 9 RAPD and 5 ISSR primers yielded a total of 114 RAPD and 40 ISSR bands, of which 52 RAPD and 19 ISSR markers were polymorphic with only 26 RAPD and 10 ISSR of the polymorphic bands being informative enough for use in the study (Table 8). On average, about 3 RAPD and 2 ISSR polymorphic loci were scored per primer. The size of the polymorphic amplified RAPD and ISSR fragments that were scored ranged from 200bp to 1000bp. Such a large range of variability is perhaps caused by small insertions/deletions in the genomic regions amplified and between the arbitrary/microsatellite-anchored regions. The maximum and minimum numbers of bands observed for the RAPD primers were 20 (OPU-19) and 8 (OPD-18) while for ISSR primers they were 9 (850) and 7 (817), respectively.

Table 7: Dominant marker loci, estimates of the dominant marker allele frequencies and their respective standard deviations (in parentheses) for agreement with the mixed-mating model

| Gene frequencies | | | Gene frequencies | | |
|---|---|---|---|---|---|
| Locus | Pollen | Ovule | Locus | Pollen | Ovule |
| OPV-17-1 | 0.710 (0.072) | 0.667 | OPW-03-2 | 0.292 (0.065) | 0.500 |
| OPV-17-2 | 0.384 (0.125) | 0.417 | OPW-03-3 | 0.442 (0.055) | 0.250 |
| AB4-13-1 | 0.179 (0.056) | 0.167 | OPD-18-1 | 0.283 (0.047) | 0.250 |
| AB4-13-2 | 0.316 (0.107) | 0.250 | OPG-06-1 | 0.341 (0.111) | 0.333 |
| AB4-13-3 | 0.409 (0.120) | 0.500 | OPW-04-1 | 0.058 (0.027) | 0.333 |
| OPU-19-1 | 0.256 (0.072) | 0.250 | OPW-04-2 | 0.046 (0.017) | 0.083 |
| OPU-19-2 | 0.751 (0.067) | 0.750 | OPW-04-3 | 0.744 (0.063) | 0.417 |
| OPU-19-3 | 0.121 (0.037) | 0.083 | OPW-04-4 | 0.035 (0.023) | 0.333 |
| OPW-07-1 | 0.461 (0.127) | 0.333 | 850-1 | 0.262 (0.039) | 0.083 |
| OPW-07-2 | 0.083 (0.025) | 0.167 | 850-2 | 0.570 (0.048) | 0.250 |
| OPW-07-3 | 0.927 (0.043) | 0.833 | 850-3 | 0.306 (0.044) | 0.167 |
| OPW-07-4 | 0.796 (0.066) | 0.667 | 857-1 | 0.403 (0.085) | 0.417 |
| OPW-07-5 | 0.633 (0.057) | 0.250 | 857-2 | 0.662 (0.054) | 0.333 |
| OPW-07-6 | 0.536 (0.130) | 0.500 | 817-1 | 0.077 (0.022) | 0.000 |
| OPB-07-1 | 0.104 (0.030) | 0.083 | 817-2 | 0.478 (0.085) | 0.333 |
| OPB-07-2 | 0.675 (0.043) | 0.250 | 817-3 | 0.032 (0.013) | 0.000 |
| OPB-07-3 | 0.507 (0.092) | 0.500 | 851-1 | 0.441 (0.061) | 0.167 |
| OPW-03-1 | 0.319 (0.060) | 0.250 | 851-2 | 0.374 (0.067) | 0.250 |

Allele frequencies for the dominant allele "band presence" and their standard errors were estimated by the MLTR programme both for RAPD and ISSR assays. The estimates of multilocus outcrossing rates ($t_m$) and single-locus outcrossing rates ($t_s$) obtained from MLTR, both from RAPD and ISSR data, clearly indicate that outcrossing is predominant in the isolated seed orchards of *C. sinensis* open-pollinated breeding population (Tables 9 and 10). The mean multilocus outcrossing and single-locus outcrossing rate estimates based on all 26 RAPD loci and 10 ISSR loci, all varieties pooled, was $t_m = 0.995 \pm 0.094$ and $t_s = 0.971 \pm 0.011$, respectively. These values suggest high levels of outcrossing in tea and that expected levels of genetic variation for open-

Table 8: Number of amplification products generated with 5 ISSR and 9 RAPD primers in tea

| Primer Code | Sequence (5' – 3') | Total no of bands per primer | Total no of polymorphic bands per primer | Percentage of products detecting polymorphis |
|---|---|---|---|---|
| ISSR 817 | CAC ACA CAC ACA CAC AA | 7 | 3 | 42.86 |
| ISSR 842 | GAG AGA GAG AGA GAG ATG | 8 | 3 | 37.50 |
| ISSR 850 | GTG TGT GTG TGT GTG TTC | 9 | 5 | 62.50 |
| ISSR 851 | GTG TGT GTG TGT GTG TCG | 8 | 4 | 50.00 |
| ISSR 857 | ACA CAC ACA CAC ACA CTG | 8 | 4 | 50.00 |
| RAPD AB4 – 13 | GTCAGAGTCC | 18 | 10 | 55.56 |
| RAPD OPB - 07 | GGTGACGCAG | 11 | 4 | 36.36 |
| RAPD OPD – 18 | GAGAGCCAAC | 8 | 7 | 87.50 |
| RAPD OPG – 06 | GTGCCTAACC | 12 | 5 | 41.67 |
| RAPD OPU – 19 | GTCAGTGCGG | 20 | 10 | 50.00 |
| RAPD OPV – 17 | ACCGGCTTGT | 11 | 5 | 45.45 |
| RAPD OPW – 03 | GTCCGGAGTG | 11 | 6 | 54.54 |
| RAPD OPW – 04 | CAGAAGCGGA | 10 | 3 | 30.00 |
| RAPD OPW - 07 | CTGGACGTCA | 13 | 2 | 15.38 |
| | Total | 154 | 71 | |

pollinated families are likely to be maintained. Both multilocus and single-locus outcrossing rates estimated both from RAPD and ISSR data were over 90%. The high multilocus outcrossing rate estimated for the *Camellia sinen*sis is comparable to ones described for other tropical tree species such as *Pinus jeffreyi* ($t_m$ = 0.881 – 0.971) by Furnier and Adams, (1986), *Picea sitchensis* ($t_m$ = 0.981) by Cottrell and White (1995), *Eucalyptus urophylla* ($t_m$ = 0.89 – 0.93) by Gaiotto *et al.*, (1997), *Theobroma grandiflorum* ($t_m$ = 1.000) by Alves *et al.*, (2003), Acacia species ($t_m$ = 0.914 – 1.000) by Casiva *et al.*, (2004), and in *Prunus mahaleb* ($t_m$ = 0.41 – 1.01) by Garcia *et al.*, (2005).

The estimates obtained with RAPD markers were however slightly higher than those obtained with ISSR (Table 10). These might be attributed to the fact that RAPD and ISSR markers have different genetic base and sample different genomic regions, RAPD

Table 9: Mating system parameters estimated from open-pollinated populations of *C. sinensis*

| Parameter | Species | | | |
|---|---|---|---|---|
| | *Camellia sinensis* (Pooled data) | *C. sinensis* var. *assamica* | *C. sinensis* var. *sinensis* | *C. sinensis* var. *assamica* ssp. *lasiocalyx* |
| Families | 6 | 2 | 2 | 2 |
| Offspring | 180 | 60 | 60 | 60 |
| $t_m$ | 0.995 (0.094) | 1.200 (0.000) | 0.959 (0.041) | 1.200 (0.000) |
| $t_s$ | 0.971 (0.011) | 0.976 (0.021) | 0.900 (0.048) | 1.042 (0.013) |
| $t_m$-$t_s$ | 0.024 (0.092) | 0.224 (0.021) | 0.059 (0.029) | 0.158 (0.013) |
| $r_p$ | 0.070 (0.017) | 0.020 (0.024) | 0.033 (0.095) | 0.025 (0.058) |
| $r_s$ | 0.193 (0.312) | -0.200 (0.001) | 0.179 (0.157) | -0.200 (0.001) |
| $r_{s,\ loci}$ | 0.001 (0.280) | 0.033 (0.520) | 0.076 (0.478) | 0.139 (0.409) |
| *F* (Observed) | -0.200 (0.001) | -0.200 (0.001) | -0.200 (0.001) | -0.200 (0.001) |
| *F* (Expected) | 0.003 | -0.091 | 0.021 | -0.091 |

Estimates of standard errors (in parentheses) were obtained from 500 bootstraps of the data. $t_m$ = multi-locus outcrossing rate; $t_s$ = single locus outcrossing rate; $t_m$-$t_s$ – biparental inbreeding; $r_p$ –multi-locus correlation of outcrossed paternity within progeny arrays; $r_s$ = correlation of selfing; $r_{s,\ loci}$ = correlation of selfing among loci; *F* - fixation inde.

markers are based on amplified arbitrary sequences and sample a wider part of the genome compared to ISSR markers which are based on microsatellite anchored regions. Although a significant difference might exist in the estimates of $t_m$, the difference was only approximately 6%. In practical terms such a numerical difference does not change the main conclusion that outcrossing is highly predominant in tea population.

The present study demonstrated the use of dominant RAPD and ISSR markers in tea mating-system study. It demonstrated that both markers, although less informative than the traditional co-dominant markers such as isozyme and SSRs, are adequate for study of mating sytems in tea populations. These high-throughput marker technologies allow the analysis of large individuals population with large number of markers in a

Table 10: Mating system parameters comparing dominant markers, RAPD and ISSR, in *C. sinensis* populations

| Parameter | Marker | |
|---|---|---|
| | RAPD | ISSR |
| Families | 6 | 6 |
| Offspring | 180 | 180 |
| $t_m$ | 0.988 (0.051) | 0.936 (0.022) |
| $t_s$ | 0.986 (0.015) | 0.889 (0.031) |
| $t_m$-$t_s$ | 0.002 (0.050) | 0.047 (0.024) |
| $r_p$ | 0.071 (0.017) | 0.051 (0.027) |
| $r_s$ | -0.072 (0.240) | 0.244 (0.216) |
| $r_{s,\ loci}$ | 0.336 (0.493) | 0.503 (0.285) |
| $F$ (Observed) | -0.200 (0.001) | -0.200 (0.018) |
| $F$ (Expected) | 0.006 | 0.033 |

Estimates of standard errors (in parentheses) were obtained from 500 bootstraps of the data. $t_m$ = multi-locus outcrossing rate; $t_s$ = single locus outcrossing rate; $t_m$-$t_s$ – biparental inbreeding; $r_p$ –multi-locus correlation of outcrossed paternity within progeny arrays; $r_s$ = correlation of selfing; $r_{s,\ loci}$ = correlation of selfing among loci; $F$ - fixation index

relatively short time as only a few RAPD and ISSR primer pairs allow the generation of sufficient markers to obtain a robust estimate of outcrossing rate. This study also points to the fact that when using dominant markers for outcrossing rate estimation it is imperative that an adequate screening of primers be done so as to maximize the probability of amplifying a large number of polymorphic markers in the progeny arrays (Gaiotto *et al.*, 1997; Mondal, 2002). Only by amplifying a large number of markers does one have the flexibility of selecting highly reproducible ones that can be readily scored across families and which display low-to-intermediate frequency for the dominant marker allele in the population.

The outcrossing rate for hermaphroditic species depends on the presence and intensity of self-incompatibility mechanisms; the degree of protandry or protogyny; pollinators foraging behavior between and within the trees, which is affected by flowering density in the population and selective abortion of selfed seeds or fruits (Alves

*et al.*, 2003). Flowering was reasonably synchronous in all clones and therefore the low selfing rate was not attributable to lack of self-pollen when the stigma was receptive. The mixed mating system ($t_m$ = 0.99) described for this species is consistent with previous studies which indicated that tea is an outbreeder species with a complex self-incompatibility system (Rogers, 1975; Wachira and Kamunya, 2005). The self-incompatibility reaction may be at the ovary and pre-zygotic stage, leading to flower abscission before embryo formation. The presence of selfing in *Camellia sinensis* has been studied using either flower bud isolation (spontaneous selfing) or by controlled pollination with pollen from the same tree (Wachira and Kamunya, 2005; Experiment two in this study). However, this was artificial selfing and cannot reflect what happens naturally.

According to Seoane *et al.*, (2001), the occurrence of selfing in predominantly outbreeding species derives from deficiencies in the mechanism of self-incompatibility during the flowering period, favoring selfing during specific phases. In general, tropical tree species have demonstrated that they have a mixed mating system with the predominance of outbreeding rate above 85% (Alves *et al.*, 2003). Plants with long life span, such as tea, tend to accumulate large somatic mutations and selfing could lead to extinction, thus natural selection might have driven such species to a high outcrossing rate. Also a higher outcrossing rate in young trees such as the ones used in this study, i.e. seven years old, is expected because they produce less pollen, and therefore, their pollen constitutes a smaller proportion of the pollen cloud and increases the chance of being fertilized by outcross pollen (O' Connell *et al.*, 2001).

On average, estimates suggested that only 1% of the progeny would result from selfing. Multilocus estimation ($t_m$) is statistically more efficient than single-locus estimation ($t_s$) because multilocus data sets contain more information about outcrossing than is available at any one single locus (Furnier and Adams, 1986). Single-locus estimation is more sensitive to related matings other than selfing (Furnier and Adams, 1986). To investigate the possibility of biparental inbreeding (inbreeding arising from mating among related plants), the difference between the multilocus outcrossing rate estimates and the mean of the single locus estimate was calculated (Table 9). This difference was low, suggesting low tendency of mating between relatives. Thus, if

inbreeding other than selfing occurs, $t_s$ will generally underestimate outcrossing to a much greater extent than $t_m$. In this study, the multilocus estimates did not differ significantly from the single-locus estimates, suggesting a negligible biparental inbreeding ($s_b = t_m - t_s$). Biparental inbreeding estimate provides little evidence that selfing naturally occurs in tea.

Additionally, the maternal families showed positive but low correlation of selfing ($r_s$=0.033). Correlation of selfing ($r_s$) can be interpreted as the normalized variance of the selfing rate among families (Ritland, 2002), or as a measure of departure from an assumption in the 'mixed-mating' model, which is commonly used for estimating outcrossing rates (Ritland and Jain, 1981). The mixed-mating model assumes that the probability of a particular mating event (selfing or crossing) is statistically independent of other mating events in the same progeny array. If this assumption is satisfied, $r_s$ estimates are close to zero. Generally, correlated selfing ($r_s$) indicates that under the above assumption some rate of biparental inbreeding can be found (Table 9 and 10) arising when plants vary in floral traits or pollinator rates, hence in outcrossing rates (Takebayashi *et al.*, 2006). A positive value of $r_s$ means that two selfed progeny samples from the same progeny array are not the result of two independent selfing events. For instance, such correlated selfing may occur when movement of a pollinator causes within-flower selfing. Therefore, such correlations are expected to be high among seeds within a single fruit. Although sampling in this study was completely randomized among the open pollinated families, there might have been sampling of seeds from the same fruit resulting in the positive correlated selfing rate. The maternal families also showed low correlation of selfing among loci ($r_{s,loci}$) suggesting that they experienced strong biparental inbreeding depression (Table 9 and 10).

Possible reason for the non-significant differences between the allele frequencies of ovules and incoming pollen (Table 7) could be the evolution of a pre-zygotic gametophytic self-incompatibility system that reduces the incidence of selfing (Rogers, 1975; Wachira and Kamunya, 2005). Tea displays strong inbreeding depression (Wachira and Kamunya, 2005), therefore, it is expected that inbred seed would be at a selection disadvantage and that the amount of viable inbred seed available for the detection of inbreeding would be low. Several lines of evidence indicate that selection operates

against inbred progeny in self-incompatible species (Louati-Namouchi *et al.*, 2000). This view is supported by high levels of seed abortion in hand self-pollination when compared to open pollination. Thus, higher levels of embryo abortion may be responsible for maintaining elevated levels of outcrossing rates in populations. However, theoretical and empirical results suggest that species which are more outcrossed would probably be more susceptible to inbreeding depression than historically inbred species (Premoli, 1996)

The correlation of the paternity within progeny arrays ($r_p$) which is an estimate of the fraction of siblings that share the same father was low (0.070), suggesting frequent and successful multiple matings in this population and therefore a high probability that a randomly chosen pair of progeny from the same array are only half sibs. This indicates that many of the offsprings were derived from non-related crosses, not sharing the same paternal genitor despite the isolation of the two putative parents i.e. each plant is pollinated by an unlimited number of male parents. Few studies with tropical tree species have reported estimation of correlation of paternity. The $r_p$ estimation for *Esenbeckia leiocarpa* populations ranged from 0.749 to 0.986 (Seoane *et al.*, 2001). For *Cariniana legalis* populations, the correlation of paternity estimated ranged from 0.212 to 0.324, while for *Tabebuia cassinoides*, it varied from 0.295 to 0.547 (Sebbenn *et al.*, 2001). In *Theobroma grandiflorum* this value was 0.930 (Alves *et al.*, 2003). The causes for high correlated outcrossing might be derived from pollinator behavior, visiting mostly neighboring trees; polyandry or deposit of multiple pollen grains from one pollinator; small number of close neighboring trees or mass flowering and flower asynchrony among trees (Alves *et al.*, 2003). The low $r_p$ in this study may be a consequence of all flowers within the orchard opening simultaneously, giving the probability that different insects pollinate different flowers with pollen from different paternal clones. In this study, seeds were randomly sampled from different fruits that resulted in high half sibs proportion therefore suggesting that pollinating insects tend to visit fewer flowers in the same orchard before moving to a different plant. A similar observation was made by Casiva *et al.*, (2004). Also, the isolated orchards are bordered by other tea genotypes in plantations that regularly flower even when they are maintained in vegetative state. These could have been a possible source of pollen and the observed high level of outcrossing.

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Families from open-pollinated crosses are mostly considered as random, resulting in half-sibs. High-density genotype populations of trees have more diversified (multiple) origins than less density populations. However, the location of tea seed orchards within plantations that occasionally flower (Fig. 3a) and the limited separation distance between orchards, approximately 200 meters within a site, might not be sufficient for insect isolation. The effect of such background pollen contamination can be a serious problem in isolated seed orchards as such pollen is assumed to be of lower genetic value and can reduce the genetic gain of the orchard (Adams and Birkes, 1991; Stoehr *et al.*, 1994). The results of this study indicate that dilution zone (living wall) around the seed bearers which is expected to eliminate pollen contamination (gene migration) is not effective in excluding extraneous pollen as more than one paternal parent contributed pollen to the maternal breeder i.e. low $r_p$. As a result, it will be important to review the current isolation distance between the orchards as well as the density of breeding plants within the orchard. Alternatively, isolated seed orchards can be established outside tea plantations, e.g. amidst Eucalyptus stand, thus reducing pollen contamination from surrounding populations/genotypes.

Effective pollen contribution from neighbouring trees within an orchard may be enhanced by increasing the density of the fertelizing genotypes, thus reducing the current distance among genotypes within an orchard. Increasing the distance between the orchards will improve the efficiency of isolated tea orchards. Kameyama *et al.*, (2002) reported that pollinator foraging distances are generally negatively correlated with density, such that at high-density pollen dispersal distances are shorter than at low density. These would promote the repetitive movement of insect pollinators between adjacent trees thus leading to an increase in the correlation of paternity (Millar *et al.*, 2000; Garcia *et al.*, 2005). Studies have demonstrated that high levels of outcrossing may remain stable in a fairly wide range of densities within trees and that correlation among paternal gametes is inversely related to distance (Robledo-Arnuncio *et al.*, 2004). Mating systems are also influenced by environmental variations which affect pollinator behavior and/or density, therefore affecting the outcrossing rate of the species in distinct populations. All these facts suggest that restricted pollen dispersal result in a substantial proximity advantage for pollen from the adjacent individual. Further experimental

replication would be needed, however, to generalize these results, because it is based on observations from a small population size.

The half-sib relationship among progeny within open pollinated families often assumed when estimating additive genetic variance and the heritability of traits, is not usually achieved as shown in these results especially when mature fallen seed is harvested from breeding bushes as is the practise in polyclonal seed orchards. With a half-sib relationship, the genetic correlation among progeny is 0.25, indicating that the progeny have a quarter of their genes in common. Any degree of correlated mating, mating among relatives, or self-fertilization will cause an increase in coefficient of relationship (Louati-Namouchi *et al.*, 2000). The violation of the half-sib assumption will result in overestimations of additive genetic variance, heritability and, subsequently, genetic gains. In an entomophilous species, like *C. sinensis*, the combination of pollinator behavior and genetic substructuring may create a hierarchy of correlated matings within inflorescences and individual plants. Therefore, the most effective way to ensure additive genetic variance and the heritability of traits is by maintaining tree seed harvest i.e. before seed drop.

The fixation index, $F$, in the progeny was lower than expected based on the estimate of $t_m$. Taking $t_m = 0.995$, the expected fixation index was $[F = (1 - t)/(1 + t)] = 0.002$. Similarly, the index was lower when data for RAPD and ISSR markers were analyzed independently (Table 10). For RAPD markers taking $t_m = 0.98$, expected $F = 0.006$ while for ISSR marker with $t_m = 0.93$, expected $F = 0.033$. A lower than expected $F$ suggests less inbreeding than expected in progeny population used to carry out the study. Since the mating system in *C. sinensis* involves only limited selfing, a reduction of homozygotes in progenies would be expected if the populations are in mating-system equilibrium (Furnier and Adams, 1986; Muluvi *et al.*, 2004).

The assessment of outcrossing rates was undertaken in an exceptionally good flowering year with the majority of clones producing large number of flowers. However, this does not guarantee that the seed orchard will perform as well every year. Certainly, the extent of flowering varies from year to year. This clonal and annual variation in the intensity of flowering is a major problem in the design and management of seed orchards. To a certain extent, clonal variation in flowering intensity can be taken into account at the

design stage when additional bushes of poor flowering clones can be incorporated to compensate for their low flower production. This approach has been adopted in pine seed orchards when it was realized that flower production varied considerably between the selected clones (Cottrell and White, 1995). The annual variation in flowering cannot be dealt with at the planning stage and management strategies must be adopted to optimize flowering. In trees, it is known that stress, e.g. drought and heat, accompanied by injection of Gibberellic Acid (GA) can greatly promote flowering (Cottrell and White, 1995). The breeder is dependent on the weather and has minimal control over these variables, except through site selection. However, it is relatively simple to inject GA to promote flowering in tea.

The mating system pattern of a plant species is expected to show variation that is due to either genetic or environmental factors (Clegg, 1980). In species that are vector-dependant for their pollination like tea, plant density represents one of the most important environmental factors affecting their mating system pattern, extent of gene flow, and level of genetic differentiation among and within populations (El-Kassaby and Jaquish, 1996). High plant densities are expected to affect pollinator flight distance. Thus, higher inbreeding, caused by mating among near-by relatives, restricted gene flow, and a pronounced level of genetic differentiation, is common. Therefore, in *Camellia sinensis*, plant density is expected to represent an important factor affecting mating pattern. However, plant density should be considered in concert with other factors, such as reproductive phenology and presence of pollen barriers, that might work as mechanisms counteracting the limitation of unrelated pollen in low plant density. Density differences (fewer trees per unit area) between the seed tree populations permits greater vector movement, thus allowing less restricted gene flow among mates. In tea seed orchards, more trees per unit area may result in reduced vector movement allowing more restricted gene flow among the genotypes of interest.

# CHAPTER FIVE

# CONCLUSIONS AND RECOMENDATIONS

The mating system is an important life history characteristic of a plant species. It has a large impact on plant population genetic structure, with selfing species showing on average lower genetic diversity within and higher diversity among populations, compared to outcrossing species. Assessment of the mating system for a crop is particularly important for the design of adequate breeding and genetic conservation programmes. The ability to measure mating patterns is central to a number of important problems in plant population biology. In this thesis, an estimation of the mating system parameters for the understanding of population genetic structure and evolution in *C. sinensis* was studied.

First, the pollen viability was investigated among and between three cultivated tea taxa. A significant variation in pollen viability found among the *C. sinensis* genotypes in the seed study indicates male gametophyte competition. The observed genotype-environment interactions in pollen performance may contribute to the variable genetic composition of the seed produced in the orchard. The magnitude of the effects of pollen viability can, however, be assumed to be smaller than the effect of variation in flowering abundance and phenology within the seed orchard and of pollination by non-orchard sources. This research showed that response to *in-vitro* pollen germination of different tea genotypes varied with temperature i.e. positive correlation. There is no significant variation in pollen germination between the three different tea varieties.

In the second experiment, cross- and self-pollination methods were compared. Like other crops, *C. sinensis* can benefit from hybridization techniques employed by plant breeders. The results of the pollination technique revealed that fruit retention in *C. sinensis* populations is constrained potentially by both internal and external factors. Fertilization failure occurred in all treatments but it was most severe in the self-pollinated treatments. Early abscission was the result of fertilization failure in some flowers, and little abscission occurred later after fruit initiation. Fruit/seed set is thus determined primarily by events occurring within the ovary in the early weeks of pollination. The natural level of fruit set in the study population was very high and could not be increased by hand-pollination. Comparisons of the different field methods confirmed that open-

pollination was the most effective technique in producing seeds within the isolated biclonal orchard.

Although *Camellia sinensis* seems to be predominantly cross-pollinated, as indicated by both the pollination and molecular experiments, about 20% fruit set in controlled selfing (bagging and selfing effected manually by hand pollination) indicates that some self-pollination is still possible. It would be important to investigate if there is any type of temporal separation of gender in tea and the stage at which the stigma becomes most receptive to pollen as even small amounts of pollen that remain viable in nature after flowering period might be responsible for self fruit set.

This study indicates that outcrossing varies both within and between varietal groups. The results suggest that outcrossing can be enhanced where adequate pollinators are present and other environmental conditions are favorable. However, cultivars differ in the extent of cross-pollination, therefore, greater isolation distances may be used than have commonly been used in the past (6 meters between breeding genotypes) to produce genetically pure seed in small plots bordered by other genotypes. From the results, it is evident that some cultivars have higher potential for outcrossing than others.

In the third experiment, the proportion of selfing and outcrossing in *C. sinensis* was estimated using molecular (RAPD and ISSR) markers. Data revealed that both markers are adequate for the study of the mating system in tea populations. Against the hypothesis that tea species exhibits low outcrossing rate, the mixed mating system estimates obtained ($t_m$ = 0.99) indicate that the mode of pollination in tea is predominantly outcrossing, suggesting high levels of genetic variation are likely to be maintained. It can be concluded that pollen gene dispersal is extensive as it is clear that insect pollinators are very effective in promoting cross-fertilization in the *C. sinensis* seed orchard though causing pollen contamination. Pollen contamination, in general, increases the gene diversity of the crop. Most of the effective pollen in natural systems appears to come from pollen outside the orchards, despite the fact that a dilution zone exists around the orchard mother trees. However, contamination is not desired in seed orchards due to its negative impact on the genetic gain and adaptation. A reduction of the current spacing (6 meters) of breeding genotypes within the isolated seed orchards might promote the repetitive movement of insect pollinators between adjacent trees that could lead to an

crease in the correlation of paternity, therefore, increasing the efficiency of cross pollination and minimizing extraneous pollen contamination. Due to the fact that measurements of outcrossing rates based on single generation demonstrate temporal variation, it would be recommendable to estimate selfing rates over multiple years at known distances to confirm these results.

To conclude, the combined approach of field with molecular data sets that complement each other has broadened our understanding of mating system in tea.

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# APPENDICES

Appendix I: Analysis of variance for percent pollen *in-vitro* germination in eight *C. sinensis* genotypes

| Source | Degrees of freedom | Sum of squares | Mean of squares |
|---|---|---|---|
| Replication | 2 | 39.760 | 19.880 |
| Clone | 7 | 348.823 | 49.832* |
| Season | 2 | 691.970 | 345.985* |
| Clone x Season | 14 | 821.956 | 58.711* |
| Error | 46 | 755.371 | 16.421 |
| Total | 71 | 2657.880 | |

*Coefficient of variation = 8.34%*
** significant at $P \leq 0.05\%$;*

Appendix II: Analysis of variance for mean percent pollen viability among the three tea varieties (China, Assam and Cambod) after *in-vitro* germination

| Source | Degrees of freedom | Sum of Squares | Mean Square |
|---|---|---|---|
| Replication | 2 | 13.080 | 6.540 |
| Season | 2 | 276.318 | 138.159* |
| Variety | 2 | 5.606 | $2.803^{NS}$ |
| Season x Variety | 4 | 96.104 | 24.026* |
| Error | 16 | 104.343 | 6.521 |
| Total | 26 | 495.451 | |

*Coefficient of variation = 5.25%*
** significant at $P \leq 0.05\%$;*
*NS = not significant.*

Appendix III: Analysis of variance comparing site effects (Timbilil *vs* Kangaita) on mean percent pollen viability in two (GW Ejulu-L and AHP SC 31/37) *C. sinensis* genotypes

| Source | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Replication | 2 | 35.659 | 17.829 |
| Genotype | 1 | 36.441 | $36.441^{NS}$ |
| Season | 2 | 296.968 | 148.484* |
| Genotype x Season | 2 | 117.527 | 58.764* |
| Site | 1 | 243.880 | 243.880* |
| Genotype x Site | 1 | 9.881 | $9.881^{NS}$ |
| Season x Site | 2 | 61.887 | $30.944^{NS}$ |
| Genotype x Season x Site | 2 | 150.187 | 75.094* |
| Error | 22 | 342.649 | 15.575 |
| Total | 35 | 1295.079 | |

*Coefficient of variation = 8.19%*
**significant at P ≤ 0.05%;*
*NS = not significant*

Appendix IV: Analysis of variance for percentage fruit set

| Source | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Replication | 2 | 320.698 | 160.349 |
| Genotype | 7 | 1663.350 | 237.621* |
| Pollination technique | 3 | 8792.728 | 2930.909* |
| Genotype x Pollination technique | 21 | 2176.685 | 103.652* |
| Error | 62 | 2628.282 | 42.392 |
| Total | 95 | 15581.743 | |

*Coefficient of variation = 21.96%*
** significant at $P \leq 0.05\%$;*

Appendix V: Analysis of variance for seed weight in *C. sinensis*

| Source | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Replication | 2 | 0.334 | 0.167 |
| Genotype | 7 | 3.477 | 0.497* |
| Pollination technique | 3 | 0.287 | $0.096^{NS}$ |
| Genotype x Pollination technique | 21 | 1.144 | $0.054^{NS}$ |
| Error | 62 | 2.571 | 0.041 |
| Total | 95 | 7.812 | |

*Coefficient of variation = 18.51%*
**significant at P ≤ 0.05%*
*NS = not significant*

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Appendix VI: Analysis of variance for seeds per fruit in *C. sinensis*

| Source | Degrees of freedom | Sum of squares | Mean square |
|---|---|---|---|
| Replication | 2 | 0.224 | 0.112 |
| Genotype | 7 | 4.400 | 0.629* |
| Pollination technique | 3 | 0.625 | 0.208* |
| Genotype x Pollination technique | 21 | 1.562 | $0.074^{NS}$ |
| Error | 62 | 3.900 | 0.063 |
| Total | 95 | 10.713 | |

*Coefficient of variation = 15.97%*
**significant at P ≤ 0.05%*
*NS = not significant*