Study of inheritance and identification of molecular markers for fertility restorers in pigeonpea [*Cajanus cajan* (L.) Millspaugh]

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Osmania University, Hyderabad, Andhra Pradesh, India

By

Rachit Kumar Saxena

International Crops Research Institute for the Semi-Arid Tropics

Patancheru, 502324, Andhra Pradesh, India

icrisat@cgiar.org

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Name: Rachit Kumar Saxena

Institute: Osmania University, Hyderabad, India

Supervisor: Dr. Rajeev K. Varshney

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Abstract

An effort has been made to understand the genetics of fertility restoration (*Fr*) and linking molecular markers with this trait in pigeonpea. A set of 159 A-, B- and R- lines were screened for polymorphism with 148 simple sequence repeat (SSR) markers to choose the most diverse crossing parents. In total, 41 SSR markers showed polymorphism with 2 to 6 alleles and 0.01 to 0.81 polymorphism information content value across the lines surveyed. Two parental combinations for each early (ICPA 2039 × ICPR 2438 and ICPA 2039 × ICPR 2447) and late (ICPA 2043 × ICPR 2671 and ICPA 2043 × ICPR 3467) maturing groups were identified. Segregation data for male fertility/sterility of BC_1F_1 and F_2 populations showed involvement of two duplicate dominant genes in governing male-fertility. Two genetic maps based on ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671 mapping populations with 82 and 117 SSR loci, respectively were developed. QTL analysis of the F_2 mapping populations based on ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671 detected six (four major and two minor) and four (one major and three minor) QTLs involved in the fertility restoration, respectively.

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I. INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is a short-lived perennial shrub that is traditionally cultivated primarily as an annual crop for its grains in Asia, Africa, Caribbean region and Latin America. Considering the vast natural genetic variability available in pigeonpea and presence of its wild relatives in the region, it has been postulated that pigeonpea originated in India and it is concluded as primary center of origin (van der Maesen 1980). It is a hardy, widely adapted and drought tolerant crop. There is a large variation in its maturity that helps in its wide adaptation including diverse locations and cropping systems. Generally, the short-duration (100-140 days) cultivars of pigeonpea are grown as a sole crop, while the medium (160-180 days) and long-duration (> 200 days) types are invariably grown as intercrop or mixed crop with other short-duration cereals and legumes. Besides its main use as dehulled split peas (locally called as *dhal*), its immature seeds and pods are also consumed as fresh vegetable. The broken and damaged seeds are fed to animals, while green leaves are used as quality fodder. The dry stems of pigeonpea are used as fuel wood. Apart from these uses, perennial type pigeonpea is also grown on sloping mountains for reducing soil erosion (Saxena 2006a; 2008).

Globally, pigeonpea is cultivated on 4.68 m ha of land with an annual production of 3.35 m t and productivity of 780 kg ha^{-1}(www.fao.org). India is the major pigeonpea growing country in the world and it accounts for 3.5 m ha area and 2.4 m t of production. The pigeonpea area, production and productivity trends in India in the last five decades showed about 2% annual increase in its area, but the yield levels have remained low and unchanged at around 700 kg ha^{-1} (Saxena et al. 2005; Saxena 2008). In India, Maharashtra stands first with an area of 1.04

m ha followed by Karnataka (0.53 m ha) and Andhra Pradesh (0.52 m ha). The state of Maharashtra also stands first (0.69 m t) in its total production followed by Uttar Pradesh (0.40 m t) and Andhra Pradesh (0.21 m t) (www.fao.org).

Efforts have been made in past to increase the average productivity by developing a number of high yielding pure varieties and in spite of releasing more than 100 varieties, the yield levels remained unchanged (Saxena 2006b). In this scenario, like many cereal crops the use of hybrid technology in enhancing productivity has a potential. A stable male-sterility system in conjunction with existing natural out-crossing can make it possible. Since in pigeonpea no male-sterility system was available at the commencement of hybrid programme, a deliberate search for male-sterile genotypes was made at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in the germplasm that led to the identification of male-sterile plants in ICP 1596. In this accession the genetic male-sterility (GMS) was associated with translucent anthers and it was controlled by a single recessive gene ms_1 (Reddy et al. 1978). Later, another source of male-sterility, characterized by brown anthers and controlled by non-allelic single recessive gene ms_2, was also reported (Saxena et al. 1983). These GMS sources were used for developing hybrids and the world's first pigeonpea hybrid ICPH 8 was released by ICRISAT for cultivation in 1991 (Saxena 1992). Since in any pulse crop no commercial hybrid is available, the release of ICPH 8 is considered a milestone in the history of legume breeding. In spite of high yield ICPH 8 could not become popular with seed producers due to large-scale seed production problems. To overcome such constraints, it was found necessary to have a cytoplasmic nuclear male sterility (CMS) system. ICRISAT took an initiative using wide-hybridization technology. So far seven different cytoplasmic sources have been identified for the utilization in practical pigeonpea hybrid breeding programme (KB Saxena et al. 2010a). These are (i) *Cajanus sericeus* from which CMS with A_1 cytoplasm was

developed (Saxena et al. 1997), (ii) *C. scarabaeoides* from which CMS with A_2 cytoplasm was developed (Tikka et al. 1997; Saxena and Kumar 2003), (iii) *C. volubilis* from which CMS with A_3 cytoplasm (Wanjari et al. 2001), (iv) *C. cajanifolius* from which the most stable CMS with *A4* cytoplasm was developed (Saxena et al. 2005), (v) *Cajanus cajan* (*L.*) Millsp. from which A_5 cytoplasm was developed, (vi) *C. lineatus* from which A_6 cytoplasm was developed (Saxena et al. unpublished), and (vii) *Cajanus platycarpus* from which *A7* cytoplasm was developed (Mallikarjuna et al. 2006). *C. cajanifolius* is reported to be genetically closest to the cultivated pigeonpea and differs only by a solitary gene (De 1974). The male-sterile plants in this (developed from *C. cajanifolius*) material showed no morphological deformity and produced plenty of pollen grains in hybrid combinations with the restorers. This male-sterile source has been reported to be stable at different locations and it is capable of producing high-yielding hybrids. Therefore, it has a great potential for use in commercial hybrid pigeonpea programs. It was also observed that the frequency of fertility restorers of this CMS source is higher than that of other sources.

Cytoplasmic nuclear male–sterility is a maternally inherited trait that is characterized by inability of plants to produce viable and/or functional pollen grains. This trait was first discovered in 1921 in two strains of flax (*Linum usitatissimum* L.) (Bateson and Gairdner 1921). Now this CMS has been reported in more than 150 plant species (Kaul 1988). CMS results from interaction of sterile (S) cytoplasm with homozygous recessive alleles (*fr/fr*) of nuclear fertility restorer genes. When a dominant nuclear fertility restorer allele (*Fr*) is present, male fertility is restored in plant irrespective of a sterile (S) or normal (N) cytoplasm. Because of their value in hybrid seed production, CMS systems have been identified and characterized in many crop species, such as maize (Levings and Pring 1976), sorghum (Chen et al. 1995), rice (Tan et al. 1998), rapeseed (Erickson et al. 1986), wheat (Harold et al. 1993), sunflower (Rieseberg et al. 1994), rye (Dohmen et al. 1994), common bean

(Hervieu et al. 1993), tobacco (Nikova et al. 1991), petunia (Edwardson and Warmke 1967), pearl millet (Sujata et al. 1994) and more recently in pigeonpea (Saxena et al. 2005).

Molecular markers are reliable diagnostic tools for various plant breeding applications and it allows to analyze thousands of genotypes during a breeding season rapidly and effectively. Molecular maker techniques provide powerful tools to identify and map the target genes. Molecular markers tightly linked to fertility restoration (*Fr*) loci will have several applications in breeding programmes. Molecular markers that are tightly linked to fertility restorer genes have been identified in several crops like maize (Sisco 1991; Wise and Schnable 1994), sorghum (Wen et al. 2002), rice (Akagi et al. 2004), brassica (Delourme et al. 1998), cotton (Zhang and Stewart 2004), petunia (Bentolila et al. 2002), wheat (Ma and Sorrells 1995; Ahmed et al. 2001), pepper (Wang et al. 2004), coffee (Coulibaly et al. 2003), suger beet (Hjerdin-Panagopoulos et al. 2002) and winter rye (Miedaner et al. 2000). However so far similar linkage analyses have not been reported in pigeonpea because of lower level of polymorphism among the cultivated genotypes and absence of ample amount of genomics tools such as mapping populations, molecular markers and linkage maps etc. In the past only 10 SSR markers were available in pigeonpea (Burns et al. 2001). Therefore, with an objective to enrich genomics resources in pigeonpea, SSR markers were developed from genomic enriched libraries (Odeny et al. 2007; 2009; RK Saxena et al. 2010a) and Bacterial Artificial Chromosome (BAC) libraries (Bohra et al. 2010). As a result > 3000 SSR markers were developed for use in pigeonpea.

The mapping of fertility restoration in various crops have been carried out using different types of mapping populations like F_2 and/or F_2-derived generations, BC and/or BC-derived generations, NILs and RILs developed from either A × R crosses or B × R crosses in the later case, the phenotyping has been done by testcrosses.

Although knowledge of the genetics of male-sterility and fertility restoration behaviour of the CMS systems would have considerable impact on breeding hybrid parents with greater efficiency; the current knowledge regarding the genetics of CMS, the linkage between fertility restorer gene(s), if any is not well understood in pigeonpea. The identification of molecular markers tightly linked to fertility restoration loci would further enhance the breeding efficiency by enabling for the classification of inbred lines or germplasm as either maintainer (B- line) or restorer (R- line) without field evaluation of their test crosses; and it would also permit their rapid backcross transfer of fertility restoration genes in elite inbred lines.

Resolving the genetics of CMS systems and identification of molecular markers that are closely linked to fertility restorer genes will represent an important step towards increasing the efficiency of breeding cytoplasmically diverse and stable male-sterile lines as well as restorer lines for the eventual development of hybrid cultivars. The present study is designed to investigate the genetics of *A4* CMS system because *A4* CMS is already an established system for commercial hybrid exploitation at ICRISAT.

Therefore, the study has following objectives:

- Development of the mapping population segregating for fertility restorer gene(s)
- Investigation of the inheritance of fertility restoration in *A4* CMS system in pigeonpea

- Identification of molecular markers linked to fertility restorer (*Fr*) gene(s) of the *A4* CMS system

II. REVIEW OF LITERATURE

The objective of this review is to provide a brief description of pigeonpea and its male-sterility systems. The nature of genetic control of fertility restoration and molecular mapping of fertility restorer genes in pigeonpea and other crops are also reviewed.

2.1 Pigeonpea

Pigeonpea is one of the major grain legumes of tropics and sub-tropics and second most important pulse crop of India. It ranks second in area, production and productivity after chickpea. India occupies more than 90% of the world's area and production in pigeonpea. Since pigeonpea is widely grown in Indian sub-continent and has a variety of end usages, special attention needs to be given to this crop for enhancing its productivity. The deep roots of pigeonpea can break the hard plough pans, to improve soil structure. It can tolerate salinity and alkalinity but not excessive acidity. It has special mechanism to use phosphorus from soil to meet its needs (Saxena 2008). Extensive ground cover by pigeonpea prevents soil erosion by wind and water and encourages filtration and minimizes sedimentation. Being a legume crop, pigeonpea fixes nitrogen and the leaf fall at maturity not only adds to the organic matter in the soil but also provides additional nitrogen for the succeeding crop. It has been estimated that around 40 kg nitrogen ha^{-1} added from the leaf fall and nitrogen fixation (Kumar Rao et al. 1981).

2.1.1 Botanical characteristics

Pigeonpea is a perennial shrub with grooved silky branches. The point on the main stem where branching starts, the number of secondary branches and the angle at which these are given off also

vary. The root consists of a deep, strong, woody tap root with well developed lateral roots in the superficial layers of the soil. Roots are nodulated by rhizobia, usually by a slow growing *Rhizobium* species. Stem is woody and straited, branching normally begins from sixth to tenth node. In spreading types the basal branches arise at an angle of 60 - 70^{o} and in erect type branching takes place at angle of 30 - 40^{o}. The first two leaves are simple, opposite and caduceus. They are narrowly ovate with a chordate to truncate base and an acute to acuminate apex. Subsequent leaves are compound, trifoliate and arranged in a two to five types of spiral. Terminal leaflets are mostly symmetrical, but the side leaflets are broader than other leaves. Terminal leaflets are usually bigger than lateral leaflets. Inflorescence is terminal or axillary racemes carried on long peduncles. Peduncles are one to eight cm long. Flowers are predominantly yellow. The calyx tube is componulate with glandular hairs. The corolla is zygomorphic and generally yellow in colour. The petals are imbricate in the bud. The standard petals are erect and spreading more or less orbicular, base clawed, biauriculate with two callosities. Stamens are 10 and diadelphous (9+1). Anthers are ellipsoid, dorsifixed and yellow in colour. The ovary is superior with two to nine ovules. The stigma is capitate and glandular-papillate. The style is long, filiform, upturned beyond the middle and glabrous. Pods are oblong, straight or sickle shaped, green at the younger stage but mature pods vary as dark purple, purple and green. Pod length varies from two to eight cm and pod width ranges from 0.4 to 1 cm. The seed shape generally varies in four namely oval, pea, square and elongate. The oval shape is most common. Seed coat colour ranges from white to black. Germination is hypogeal and cotyledons remain under ground. Under suitable conditions the seedlings appear above the ground in five to six days.

2.1.2 Pollination behaviour

Unlike most legume species, pigeonpea flowers are prone to natural out-crossing and thus it is considered as a partially cross-pollinated species. Self pollination occurs in the bud before the

flowers open while cross pollination takes place after the opening of flowers with the help of insects. In the young buds stigma lies above the level of anthers and the style is so curved at the tip that the stigmatic surface is directed towards the anthers. These are arranged around the style in two groups of five in each. As the bud develops, the filaments elongate, bringing the top five anthers dehisce in the bud a day before the flowers open. Thus, self pollination takes place. Although the stigma is completely covered with the pollen of its own flower, considerable out- crossing occurs in pigeonpea (Saxena et al. 1990). The percentage of "selfs" was negligible when flower buds were pollinated with foreign pollen without emasculation (Reddy and Mishra 1981). This indicates that foreign pollen has an advantage over native pollen in fertilization. Although anthers dehisce during the bud stage, they do not start germinating until the flowers start to wither 24 to 28 hours after dehiscence (Onim 1981). It has been found that the receptivity of stigma starts 68 hours before anthesis and continues for 20 hours after anthesis (Prasad et al. 1977). These mechanisms provide a sufficient gap for foreign pollen to be introduced onto stigma and thus favour out-crossing in pigeonpea.

2.2 Male-sterility systems

Male-sterility in plants is a phenomenon where the individuals are unable to reproduce through natural means due to their defective male-reproductive parts. Such plants reproduce only when fertile pollen from other plants is placed on the stigmatic surface of the male-sterile flowers through any mechanical means such as deliberate manual efforts, wind or insects. The phenomenon of male-sterility was recorded as early as 1763 by Kolreuter. Different kinds of male-sterility systems have been reported in plants such as genetic male-sterility (GMS), cytoplasmic-nuclear male-sterility (CMS), cytoplasmic-genetic male-sterility (CGMS), transgenic male-sterility and chemical induced male-sterility.

Genetic male-sterility is governed by nuclear genes, in most of the cases by single gene. The gene causing male sterility are ordinarily recessive (*ms*) and rarely dominant. A- line (*mm*) is genetic male

sterile line. B- line is heterozygous male fertile line (*Mm*). A- line is maintained by crossing it with B-line, the cross produces male sterile and male fertile lines in 1:1 ratio. It has been observed in wheat (Athwal et al. 1967), maize (Sjofjan et al. 1966), barley (Hermsen 1965), cotton (Richmond and Kohcl 1961) and pigeonpea (Saxena et al. 1983).

Cytoplasmic male-sterility is governed by cytoplasmic or plasma genes. Progeny of male-sterile plant is always male-sterile, as its cytoplasm is derived from female gamete. The male- sterile line is also known as A- line. The line used to maintain male-sterile line is male-fertile, B- line. The A- line is maintained by crossing it with B- line (pollinator used as recurrent parent in the backcross program), as its nuclear genotype is identical with that of A- line. The restorer (R-) line only can provide fertility in F_1. It is stable i.e. not influenced by environmental factors. It has been observed in maize, wheat, rice, sorghum, cotton and pigeonpea etc.

Cytoplasmic-genetic male-sterility is controlled by both nuclear and cytoplasmic genes. Here, nuclear genes for fertility restoration (*Fr*) are available. The fertility restorer gene is dominant and is found in certain lines of the species, or may be transferred from a related species. This gene restores male-fertility in the male-sterile line, hence it is known as restorer gene. There are commonly two types of cytoplasm, normal (F) and sterile (S). There are restorers of fertility (*Fr*) genes, which are distinct from genetic male sterility genes (Fig. 1). The *Fr* genes do not have their own expression of any kind unless sterile cytoplasm is present. It is used in commercial production of hybrid seeds in rice, maize, sorghum, pigeonpea and bajra etc. The one with cytoplasmic male-sterility would be included in the cytoplasmic genetic system as and when restorer genes for it discovered.

2.2.1 Genetic male-sterility (GMS) systems in pigeonpea

All the GMS systems reported so far in pigeonpea have come from spontaneous mutations. This happens when a male-fertility controlling dominant (*Fr*) nuclear gene mutates to its recessive form under the influence of some natural forces and with subsequent natural selfing of heterozygote (*Frfr*) the male-sterile genotypes (*frfr*) appear within the population. Such genotypes, if not cross-pollinated by fertile pollen, are eliminated from its parental population. In comparison to highly self-pollinated crops, the elimination of *frfr* genotypes is gradual in out-crossed species. Therefore, such elimination processes depend on the rate of natural out-crossing in a given population. In comparison to recessive genes, the frequency of dominant male-sterile genes in nature is very low (Kaul 1988). There are many instances where progenies of some inter-specific and inter-generic crosses have also produced male-sterile segregants. In genus *Cajanus* also, a number of such wide crosses have produced male-sterile segregants. These cases, however, were hardly pursued further (Reddy et al. 1990; Dundas 1990) for use in breeding programs.

The male-sterile mutants have also been reported in some mutagen-induced populations. In most cases such mutants could not be maintained either due to their tight association with female-sterility or reproductive abnormalities such as chromosome addition or deletion (Dundas 1990). If for some reason, a chromosome with male-fertility (*Fr*) gene is lost then male-sterility trait with *frfr* alleles will express but such plants hardly reach their maturity due to poor vigor and abnormal growth. In contrast, if the male-sterile mutant gene is dominant then it is eliminated rapidly from the population, particularly in a self-pollinated species. Therefore, most spontaneous male-sterile mutants that have been detected so far, are recessive. Relatively high occurrence of non-allelic recessive male-sterility genes in suggests that the frequency of such natural mutations is quite high and their deletion from the parental populations is rather slow. The male-sterility in legumes that is controlled by recessive genes has reported in broad bean [*Vicia faba* (L.)], grass pea [*Lathyrus sativus*

(L.)], groundnut [*Arachis hypogea* (L.)], sunhemp [*Crotalaria juncea* (L.)], soybean [*Glycine max* (L.) *Merr.*], pea [*Pisum sativum* (L.)] white clover [*Trifolium repens* (L.)], common bean [*Phaseolus vulgaris* (L.)], alfalfa [*Medicao sativa* (L.) spp. *sativa*] etc.; while dominant genetic control of male-sterility was reported in *Trifolium repens* (Kaul 1988). The first spontaneous mutant could not be maintained because of its tight linkage with female-sterility (Deshmukh 1959). A deliberate search for male-sterility in 7,216 germplasm accessions sown at ICRISAT in 1974 provided 75 single plants which remained green till the end of season and had a few pods, suggesting absence of self-fertilization of flowers to affect normal pod setting (Reddy et al. 1977). These selections were female-fertile and had different types of anthers with variable fertility levels. Among these, six plants with fully grown translucent anthers and no pollen grains were selected for further studies and use in hybrid breeding programs.

Dundas et al. (1982) reported a male-sterile mutant within a photo-insensitive pigeonpea breeding line. At about the same time yet another genetic male-sterile spontaneous mutant was selected in a breeding line B15B (Saxena et al. 1983). This mutant was characterized by brown coloured arrow-head shape anthers. Another recessive male-sterile mutant in a population of cultivar UPAS 120 had translucent anthers, sparse podding and delayed flowering (Verulkar and Singh 1997).

A genetic male-sterile mutant that was selected from an inbred population of cultivar ICPL 85010 was characterized by small light yellow anthers with no pollen grains (Saxena and Kumar 2001). Perhaps the similar male-sterile gene was linked to a characteristic of obcordate leaves (Venkateswarlu et al. 1981; Pandey et al. 1994). In a segregating population of cross between obcordate leaf genotype and cultivar HY 3C, a total of 13 obcordate leaf type plants were found with 60 - 100% pollen sterility (Venkateswarlu et al. 1981). The authors postulated a linkage between

male-sterility and obcordate leaf trait. They further observed that all the male-sterile plants had modified keel that exposed the flowers for out-crossing. Partial male-sterile plants with sparse pollen production in an F_2 population of cross MS 4A × QPL-1 showed pollen-sterility in a range of 40 – 80% and there was no intra-plant variation for pollen-sterility. The pod set on these plants varied in accordance with their pollen-fertility (Saxena et al. 1981). The anthers of the identified 11 male-sterile plants in a population of cross 0DT × ICPL 86 were small, white (later turned brown) and non-dehiscent (Gupta and Faris 1983). Authors also reported another mutant with non-dehiscent type of male-sterility where the pollen grains were released only when the mature anthers were physically ruptured. The pollen thus obtained was 70 – 80% sterile. These mutants were not studied further.

2.2.2 Cytoplasmic-nuclear male-sterility (CMS) systems in pigeonpea

The CMS systems can arise either through spontaneous mutation, intra-specific crosses, inter-specific crosses, or inter-generic crosses. The wide hybridization programs such as inter-specific and inter-generic crosses have been found to produce a greater proportion (about 75%) of CMS systems (Kaul 1988). Scanning of literature on this subject shows that in the dicots most CMS cases have arisen through inter-specific crosses, while in monocots it is the inter-generic hybrids that have yielded most CMS sources (Kaul 1988). Since the expression of CMS requires two different genetic systems, one each in cytoplasm and nucleus, to come together in a single cell; the frequency of spontaneously occurring mutants simultaneously in both the entities (i.e., nucleus and cytoplasm) is quite low. On the contrary in GMS system, only a single nuclear mutation can lead to the development of male-sterility. Unlike GMS controlling genes, the influence of environment (temperature and/or photoperiod) on CMS controlling nuclear *fr* and *Fr* genes is more prominent. This may lead to instability of the expression of male-sterility and its fertility restoration. Such unstable expressions are also sometimes influenced by the genetic background of an individual.

First attempt to breed a CMS line in pigeonpea was done by crossing a cultivated type (as female) with pollen from two different wild relatives, *Atylosia sericea* and *A. scarabaeoids* (Reddy and Faris 1981). The fertile F_1 plants of these two crosses were used as male parent to produce backcrosses with wild species as female parents. The resultant BC_1F_1 plants were male fertile while their BC_1F_2 progenies segregated for male-sterility and fertility. The maternally inherited male-sterility in these segregants was found to be tightly linked with various floral abnormalities such as petaloid anthers, free stamen or heterostyly. They also reported that these segregants had different degrees of female-sterility and could never be stabilized as pure lines therefore, could not be used in hybrid breeding programs. In order to develop CMS through chemical and physical mutagens, a GMS line with ms_2 gene, when treated with 0.025% sodium azide or 500 mg kg^{-1} of streptomycin sulphate, showed mutational changes and expressed male-sterility that was maternally inherited (Ariyanayagam et al. 1993). This male-sterility was maintained only by heterozygote sibs that raised doubts about its nature and use in hybrid breeding program. The proportion of male-sterile plants in these mutagenic progenies varied a lot and no good male-sterile line could be derived subsequently, a few CMS systems were developed in pigeonpea and these are briefly described below:

An accession of *Cajanus sericeus* (A_1) was crossed with an advanced breeding line of pigeonpea. The F_1 progeny of this cross showed partial male-sterility but in F_2 generation a few segregants expressed 100% pollen-sterility (Ariyanayagam et al. 1993). In the subsequent backcross generations, for some reasons, these male-sterile plants could not maintain their high levels of male-sterility. In addition, it was also observed that some male-sterile plants reverted back to male-fertility when local environment, particularly temperatures and photoperiods changed. To stabilize the male-sterile trait, besides conventional backcrossing, multiple cross genome transfer methodology was also implemented (Ariyanayagam et al. 1995). Both these approaches yielded certain proportion of male-sterile segregants, but the backcross derivatives were also found to be female-sterile and failed

to set any pod. The progenies derived from the genome transfer scheme also produced a few male-sterile segregants which were maintained by other pigeonpea inbred lines. These male-sterile segregants led to the development of male-sterile lines such as CMS 85010A, CMS 88034A and CMS 13091A (K. B. Saxena, unpublished). From these populations, male-sterile lines that revert back to full male-fertility under low temperature and shorter days and again to full male-sterility under high temperature and longer days were selected (Saxena 2006a).

In an attempt to develop a stable CMS line, *Cajanus scarabaeoids* (A_2) as female parent crossed with a pigeonpea line ICPL 85030. The F_1 plants were partial male-sterile. In the backcross progenies some promising male-sterile plants were identified but no stable CMS line could be bred (Ariyanayagam et al. 1993). The development of a CMS line by crossing a cultivated type with its wild relative *C. scarabaeoides* as a female parent was also reported. The resultant F_1 plant was partial male-sterile and in F_2 a number of male-sterile segregants were recovered. Subsequently, a perfect male-sterile maintainer line ICPL 288 was also identified. The fertility restoration of this male-sterile line was also found among fertile F_2 segregants (Tikka et al. 1997). This male-sterile source was used in developing experimental hybrids in Gujarat state of India. *C. scarabaeoides* as a female parent was also crossed with four pigeonpea cultivars. Among F_1s, a progeny derived from cross *C. scarabaeoides* × ICPL 88039 was completely male-sterile. To stabilize this source of male-sterility, backcrosses were made with ICPL 88039 as recurrent parent and all the plants in BC_1F_1 through BC_6 F_1 generations were male-sterile (Saxena and Kumar 2003). They also reported eight fertility restorers and six male-sterility maintainers. This allowed breeding of genetically diverse hybrids for different cropping systems. The fertility restoration in hybrids involving this CMS was not perfect and a large variation (50 – 95%) was observed for pollen fertility. This variation could be due to differential inter-genomic or cytoplasmic-genomic interactions (Saxena 2008). Differences arising

due to genes, can also yield inconsistent expressions of both male-sterility and fertility restoration (Abdalla and Hermsen 1972).

A number of male-sterile segregants with maternal inheritance from a cross involving *Cajanus volubilis* (A_3) and a cultivated type were selected (Wanjari et al. 2001). These selections, however, could not be used in any hybrid breeding program due to lack of fertility restoring genotypes.

In an attempt to develop CMS line from *Cajanus cajanifolius* (*A4*) as male parent with a GMS line as female parent, the progenies from this cross were male-fertile and could not be used further (Rathnaswamy et al. 1999). Whereas, ICPW 29 an accession of *C. cajanifolius*, a wild relative of pigeonpea, as female parent crossed with pigeonpea line ICPL 28 (Saxena et al. 2005). *C. cajanifolius* resembles with cultivated types in most morphological traits and differs by only a solitary gene (De 1974). The inter-specific F_1 hybrid plants grown in 2001 expressed variable extents of pollen-sterility and one plant with 60% pollen-sterility was backcrossed to ICPL 28. This was followed by six backcrosses to substitute the nuclear genome of wild species with that of the cultivated type. This substitution led to enhanced male-sterility that was fully maintained by its recurrent pigeonpea parent. This male-sterile source is the best among those identified so far and it was designated as ICPA 2039. It was found to be highly stable male-sterile line across environments and years and never showed any morphological deformity (Saxena 2008, Dalvi et al. 2008a). To develop diverse pigeonpea hybrids this male-sterile source has now been transferred into a number of genetic backgrounds.

A GMS line crossed with *C. acutifolius* as male parent and all the F_1 plants were male-fertile (Rathnaswamy et al. 1999). While using *C. acutifolius* as a female parent in a cross with pigeonpea

accession *Cajanus cajan* (A_5) ICP 1140, only 1.5% pod set was observed. The use of gibberellic acid (@ 50 mgL^{-1}) in backcrosses enhanced the pod set to 6% but the seeds, thus obtained, were under developed and failed to germinate (Mallikarjuna and Saxena 2002). To overcome this problem, the developing embryos were rescued and successfully cultured in artificial media. Encouraged with the success of embryo rescue technology, authors again crossed six pigeonpea cultivars as female parent with two accessions (ICPW 15613, ICPW 15605) of *C. acutifolius*. The F_1s involving pigeonpea lines ICPL 85010, ICPL 85030, and ICPL 88014 produced a few male-steriles with some plants exhibiting up to 100% pollen-sterility. The anthers of these male-sterile plants were shrunken and pale yellow in colour. Such male-steriles maintained their sterility when crossed to their respective wild relative accessions. Most of the cultivated accessions when crossed to these male-steriles restored the male-fertility of the plants. An exception to this was HPL 24, where F_1 progeny produced both male-sterile and fertile plants. This suggests the presence of both *fr* and *Fr* genes in its nuclear genome (Mallikarjuna and Saxena 2005). Further backcrossing with this line and selection for pollen-sterility helped in stabilizing the male-sterility. Interestingly, HPL 24 was bred from a cross involving *C. sericeus*, another wild species (Saxena et al. 2010c), and this suggested that besides *C. acutifolius* the *fr* genes may also be present in *C. sericeus*.

In 2002 rainy season, a naturally out-crossed partial male-sterile plant was observed in an open-pollinated population of *Cajanus lineatus* (A_6) (K.B. Saxena, unpublished) and the morphology of this plant was very different from rest of the population. The vegetative cuttings of this plant were raised in a glasshouse and out of five cuttings planted only two survived and the plants were found to be male-sterile. These were crossed with pigeonpea line ICPL 99044 and produced normal pod set. The F_1 plants grown in 2004 season were partial male-sterile. Back-crosses (BC_1F_1) were made with ICPL 99044 and out of 20 plants grown five were partial male- sterile. In BC_4F_1 generation 167 plants were examined for pollen viability and it ranged from 92 – 100%. The plants showing 100%

male-sterility were crossed with four pigeonpea lines in 2008 season. At present this CMS source is in BC_5F_1 stage with perfect male-sterility maintenance system available.

Cajanus platycarpus (A_7), a wild species in the tertiary gene pool of pigeonpea, is cross incompatible with cultivated types and, therefore, hormone- aided pollinations coupled with embryo rescue techniques were employed to obtain viable F_1 and BC_1F_1 progenies (Mallikarjuna et al. 2006). In BC_2F_1 generation a progeny (BC_2-E) with low pollen fertility was selected. Within this progeny two plants with 100% pollen sterility were selected and crossed with a set of pigeonpea cultivars. The examination of their F_1s showed that the hybrid involving cultivar ICPL 85010 maintained complete male-sterility, whereas cultivars ICPL 88014 and ICP 14444 restored male-fertility. The detailed studies on this new CMS source are in progress.

2.2.3 Cytological studies on male-sterile genotypes

A number of bio-chemical changes are responsible for the development of pollen mother cell (PMC) from the meristematic tissues. Further, it is followed by a series of developmental changes which lead to the mature pollen grains. In the determination of male-sterility in crop plants, the anther wall and in particular the tapetum, plays an important role of producing and transporting critical enzymes, hormones and nutrients that are essential for the growth of PMCs and any abnormality in the anther wall development leads to the production of defective pollen grains. During the process of meiosis any abnormality in the supply of nutrients generally leads to aberrant outputs such as large and more number of PMCs (Vasil 1967). Fusion of cells into in to multi-nuclear syncytia or abnormal vacuolization or degeneration of the tapetal layer leads to the abnormal development and separation of PMCs. The normal development of PMCs in general is arrested either pre-meiotic, during meiosis, or in post-meiotic stages of growth.

The cytological studies on the fertile and sterile siblings showed that the microsporogenesis in the two genotypes was similar up to tetrad formation stage. The differences between the two emerged when the tetrads in the male-sterile plants failed to be released and leading to degeneration of tetrads through vacuolation. The tapetum continued to persist even when the tetrads degenerated. On the contrary, in the fertile plants, tapetum began to degenerate during the formation of tetrad and disappeared during male gametophyte development. In case of male-sterility the callose is synthesized due to the presence of high concentrations of cellular calcium (Worral et al. 1992). Further studies conducted on the persistence of callose and tapetum in the ms_1 type of male-sterility concluded the accumulation of callose and persistent tapetum during post-meiotic stages (Ketti et al. 1994). They further deliberated that a gradual reduction in the concentration of polysaccharides and RNA proteins in the tetrads were responsible for disorientation of cytoplasm leading to malnutrition and poor tetrad growth. The degeneration of microspores occurred at the tetrad stage through rupturing of nuclear membrane and resulting in to collapse of the outer wall (Dundas et al. 1981). While reporting a new source of GMS, in the male-sterile plants the PMCs count was almost double than their fertile counterparts (Dundas et al. 1982). The abnormal enlargement of PMCs and their number was associated with the failure of adjacent PMC walls to separate. The breakdown of microsporogensis of this male-sterile occurred at prophase I. The delayed and incomplete anther wall development appeared to be responsible for PMC degeneration. Similar observations were also reported in cotton (Murthi and Weaver 1974). In the male-sterile plants, however, PMC degeneration occurred at young tetrad stage with the rupturing of nuclear membrane and collapse of outer cell walls. The vacuoles developed in the tapetal cells metaphase I and by tetrad stage the entire cell gets vacuolated. In this case, the precocious degeneration of tapetum ending its role as a nutrient source for PMCs (Echlin 1971) could be responsible for tetrad breakdown. Similar results were also reported in *Hordeum vulgare* (Kaul and

Singh 1966); *Sorghum* (Overman and Warmke 1972) and *Pennisitum typhoides* (Reddy and Reddi 1974).

In all the three GMS systems the blockages in the microsporogenesis occured at different stages of development which also determined their anther morphology. Studies showed that if an individual plant carries two male-sterility inducing genes, then the one which expresses first and hinders the normal process of microsporogenesis, determines the phenotype of the anthers and the other genes become redundant as far as their expression is concerned (Saxena et al. 1983; Saxena and Kumar 2001). Cytological examination of sparse pollen producing flowers revealed that their tetrad formation was normal but soon after this, only a portion of microscopores collapsed. Further, the locules of anthers within individual flowers varied in the proportions of microspore degeneration (Saxena et al. 1981). The cause of this partial breakdown of microsporogeneis could not be ascertained. Ariyanayagam et al. (1995) working with a *C. sericeus* derived CMS lines, reported that meiosis in the male-sterile plants proceeded normally until the release of microspores and this was followed by vacuolation and degeneration of protoplasm. Cytological investigations with *C. acutifolius* derived CMS showed that the process of meiosis in the male-sterile plants proceeded normally till the onset of tetrad stage but their further growth was arrested and the tetrads remained inside the tapetum layer. This resulted in the loss of cell contents and collapse of the process of microsporogenesis (Mallikarjuna and Saxena 2005).

A detailed study identified two different kinds of male-sterile plants in a cross involving a cultivated pigeonpea as female parent and *C. acutifolius* as male parent (Mallikarjuna and Kalpana 2004). These two male-sterile variants had different anther morphology. In Type I, the anthers were shrivelled with brown colour, while in Type II male-steriles, the plants had pale white shrivelled

anthers. These variants also differed in their microsporogenesis. The PMCs of Type I male-sterile plants remained in prophase stage and subsequent processes of meiosis were arrested. The PMCs enlarged normally and once nucleus grew, further cell division did not take place. In these plants persistence of tapetum was also observed. In Type II plants, the anthers were translucent and microsporogenesis continued up to tetrad stage but the tetrads failed to separate and produce pollen grains. This was followed by collapse of anther development process, a sort of post-meiotic arrest of microspore development. Cytogenetic studies of *A4* CMS revealed an early breakdown of tapetum (Fig. 2). In these plants the anthers were under- developed and the male-sterility expressed at tetrad stage, where the tetrad wall failed to degenerate and resulted in the degeneration of its contents (Dalvi et al. 2008b).

It can be concluded that two primary reasons are responsible to produce GMS in pigeonpea. The first process is characterized by the development of brown and shrivelled anthers followed by pre-meiotic breakdown of PMCs. In the other process the anthers are pale white or translucent accompanied by post-meiotic breakdown of PMCs.

It has been widely assumed that the CMS trait is expressed due to impairment of pollen formation processes that result from interaction of the nuclear and the mitochondrial genomes. Pollen maturation requires great amounts of energy (Zhao et al. 2000). This is evident by the many fold increase in the number of mitochondria in the tapetal tissue and PMCs during pollen development. In sugar beet and wheat, low temperatures cause CMS like microspore disturbances as microspores and tapetum cells are more sensitive than the female reproductive organs and oxidative processes are responsible for this development (Kuranouchi et al. 2000). It is also believed that the mitochondria have a major role to play in the expression of CMS trait. In pigeonpea there is only one report (Sivaramakrishnan et al. 2002) that deals with the assessment of mitochondrial genome of the CMS plants.

2.3 Inheritance of fertility restorer genes

Fertility restoration systems can be classified as being either sporophytic or gametophytic: sporophytic systems act prior to meiosis in saprophytic tissues while gametophytic systems act post meiosis in microspores or pollen grains. These differences lead to very distinct transmission patterns. A diploid plant containing heterozygous restorer gene will produce two classes of pollen grains: one that carry restorer allele for the gene and second do not. In the case of a sporophytic restorer, both genotypic classes can restore fertility in hybrids.

2.3.1 Population genetics of restorers

Information regarding allelic frequencies of restorer genes can prove beneficial in order to understand their evolutionary origins and to search for new genes. For example, although the *Rf*1 restorer allele, which confers pollen fertility in T-cytoplasm of maize, is quite rare among maize inbred lines, the restorer allele of *Rf*2 gene for this cytoplasm is widely present in maize inbred lines, even though most of these lines have never been exposed to the T- cytoplasm. This indicates that restorer alleles of *Rf*2 gene have been maintained during evolution by selection and must therefore have a important function independent of pollen fertility restoration (Schnable and Wise 1994). The *ogu*, *pol* and *nap* cytoplasms of *B.napus* induce male-sterility in all, some and only a few cultivars respectively (Jean et al. 1997). Hence it can be inferred that the *ogu* restorer (*Rfo*) is absent from *B.napus* germplasm, *pol* restorers are rare and *nap* restorers are more abundant.

In pigeonpea *A4* CMS system is already established and commercially exploited due to high frequency of maintainers and restorers as compared to other male sterility systems detected (K.B.Saxena pers. comm.).

2.3.2 Mechanisms of fertility restoration

The mechanisms of fertility restoration are very diverse; the possible mechanisms of male-sterility maintenance male-fertility restoration have been described in several reviews (Schnable and Wise 1998; Budar et al. 2003; Hanson and Bentolila 2004). Fertility restorer genes could overcome male sterility through the following mechanisms:

I) Physical loss of CMS associated genes from mitochondria (He et al. 1995).
II) Processing of CMS associated transcripts (Wise et al. 1999).
III) Post transcriptional RNA editing. For example, editing might change the length of ORFs related to CMS by creating new start and stop codons, because most common editing in plant mitochondria is C to U.

2.3.3 Number of genes controlling fertility restoration

The diversity in restoration systems extends to the number of genes involved in restoration process. In majority of male-sterility systems one or two major restorer loci confers complete male fertility. In some male sterility systems, full male fertility restoration requires involvement of a number of genes, many of which provide only small effects. CMS system male fertility restoration is by dominant nuclear genes in commercially exploitable systems of cotton and cereals, in many case few in number. Precise identification of genetic control of sterility maintenance and fertility restoration in CMS systems is confounded due to complicated nuclear-cytoplasmic interactions with the effect of minor genes and environmental factors.

2.3.4 Inheritance of fertility restorer genes in pigeonpea

In pigeonpea GMS system with one exception, all the sources of GMS are controlled by a single recessive gene pair (Reddy et al. 1978; Saxena et al. 1983). They also reported that during microsporogenesis the ms_2 is expressed at an earlier stage than that of ms_1 gene. The male-sterility reported within ICPL 85010 population was also controlled by a single recessive gene (ms_3) and it was also non-allelic to ms_1 and ms_2 genes (Saxena and Kumar 2001). They further reported that all the three male-sterility genes were independent and when present within a plant system, expressed independently at different stages of microsporogenesis. The first to express is ms_2, followed by ms_3, and finally ms_1 gene. The translucent type of GMS was also controlled by a single recessive gene but its allelic relationship with ms_1 which also has translucent anthers was not studied (Verulkar and Singh 1997).

Among various CMS sources reported, the genetics has been reported for only *A4* type of CMS. Genetics of fertility restoration was studied in five crosses. Of these, in three crosses a single dominant gene, while in one cross two dominant genes with duplicate gene action restored the fertility. In the fifth cross also two dominant genes with complimentary action governed the fertility. Further investigation into the origin of fertility restoring lines showed that these *Fr* genes were randomly distributed in the germplasm (Dalvi et al. 2008a).

2.4 Molecular mapping of fertility restorer genes

There are no reports available on the molecular mapping of fertility restoration genes in pigeonpea and therefore examples have been taken from other crops. The marker systems employed were mostly RAPD, RFLP, AFLP, STS markers and in some cases the identified RFLP or RAPD markers were converted to PCR-based markers for further use. Most of the studies involved bulk segregant

analysis (BSA) approach based on making male-sterile or male-fertile bulks for identifying linked markers and then performing the genotyping on a sub-set of the whole population with the identified linked markers with the aim to create a localized linkage map of the *Fr* loci. A few studies reported a QTL mapping approach for identifying QTL linked to fertility restoration. *Fr*-linked molecular markers have been used in marker-assisted selection in crops like *Brassica* (Hansen et al. 1997) and to identify restorer lines having the *Rf*1 gene in rice. A few crops have seen much advanced work in order to understand the mechanisms underlying fertility restoration as evidenced by cloning of four restorer genes, *Rf2* of maize (Cui et al. 1996), *Rf* of petunia (Bentolila et al. 2002), *Rfk1* (*Rfo*) of raddish (Brown et al. 2003) and *Rf1* of rice (Komori et al. 2004). Fertility restoration patterns of F_1 hybrids developed by crossing male-sterile lines and inbred fertility restorers have been conventionally used for the classification of CMS sources in pearl millet (Burton and Athwal 1967), maize (Laughnan and Gabay-Laughnan 1983), sorghum (Schertz et al. 1989), sunflower (Miller 1996) and barley (Ahokas 1982).

Male-sterile cytoplasms have also been characterized through mtDNA restriction endonuclease profiles in maize (Levings and Pring 1976) and sorghum (Pring et al. 1982) and many other crops. Rajeshwari et al. (1994) characterized diverse pearl millet cytoplasms by Southern blot hybridization using maize mtDNA probes. Liu et al. (2002) developed a polymerase chain reaction (PCR) assay for discrimination of male-sterile cytoplasms in maize by designing PCR primers specific to the mtDNA sequences of three major classes of maize CMS cytoplasms: T, C and S.

III. MATERIALS AND METHODS

3.1 Plant material

With an objective of developing mapping populations segregating for fertility restoration genes a SSR based diversity study was conducted. A total of 159 pigeonpea lines including 37 A- (male sterile), 38 B- (maintainer) and 84 R- (restorer) lines that are in use in hybrid breeding programme at ICRISAT were used for molecular characterization (Table 1). A- lines used in present study represented different backcross generations (BC_2 to BC_9) while all the B- and R- lines used represented recombinant inbred lines.

3.2 Development of mapping populations

The present investigation comprised of two cytoplasmic-genic male-sterile (CMS) lines of pigeonpea, ICPA 2039 and ICPA 2043 with *A4* cytoplasm, derived from *Cajanus cajanifolius* (Saxena et al. 2005) and four fertility restorer lines ICPR 2432, ICPR 2447, ICPR 2671 and ICPR 3467. These lines were selected on the basis of genetic variation, diverse morphological traits and ability to restore fertility.

To study inheritance of fertility restoring genes in pigeonpea, male-sterile lines ICPA 2039 and ICPA 2043 were crossed to four known fertility restorers of different maturity groups by following the crossing scheme as illustrated in Figure 3. The early maturing A- line (ICPA 2039) was crossed to ICPR 2438 and ICPR 2447; while another male-sterile line (ICPA 2043) was crossed with two late maturing restorer lines ICPR 2671 and ICPR 3467.

3.2.1 Testing of parents, F_1, F_2 and test crosses

The inheritance of fertility restoration was studied in F_1, F_2 and BC_1F_1 generations (Fig. 3). In addition, in the late maturing cross 53 fertile F_3 progenies were also assessed for segregation. In the early maturing materials backcross seed could not be produced due to severe pod damage caused by borers (*Maruca testulalis* Geyer.).

Two rows (4m) with inter-row spacing 75cm were used for planting of parents and hybrids (F_1). Population of ~230 plants was maintained for each F_2 and ~160 for each test cross.

3.3 Phenotyping for pollen fertility

For assessing pollen fertility, 10 fully grown but un-opened floral buds were harvested from different parts of the plants between 9 and 11 A.M. to prepare slides for examination. Anthers from the sampled flowers were removed and squashed in 1% aceto-carmine solution. In each slide three different microscopic fields were studied under light microscope. The pollen grains were considered fertile if they were stained with dye and counts for fertile/sterile pollen grains were made. Within each population discrimination among the plants for male-fertility restorers and non-restorers was done on the basis of their pollen fertility data. Plants with ≥80% stained pollen grains were classified as male-fertile; while those with ≤10% pollen fertility were identified as male-steriles. The data, thus obtained, were subjected to chi-square analyses for testing their goodness of fit to different expected phenotypic ratios.

3.3.1 Goodness of fit

The goodness of fit in F_2 and test cross ratios were tested using a chi-square test (Panse and Sukhatme 1985). The confirmation of ratios obtained in F_2 segregating population was done by the ratios obtained in test crosses. When the calculated value of χ^2 is less than the table value the fit is said to be good or the assumed ratio is correct. Conversely when the calculated value is more than the table value, the fit is not good and the assumed ratio is not correct.

3.4 Diversity analysis of A-, B- and R- lines and genotyping of mapping populations

3.4.1 DNA extraction

Genomic DNA was isolated from two to three young leaves of pigeonpea genotypes and F_2 progenies of mapping populations by a Cetyl trimethyl ammonium bromide procedure mentioned in Cuc et al. (2008).

3.4.1.1 Sample preparation

- Leaves were harvested from 15 days old seedlings.
- Leaf tissue of 70-100mg was placed in 12 x 8-well strip tube with strip cap (Marsh Biomarket, USA) in a 96 deep-well plate together with two 4mm stainless steel grinding balls (Spex CertiPrep, USA).

3.4.1.2 CTAB extraction

- Each sample was mixed with 450µl of preheated (65ºC) extraction buffer (100 mM Tris-HCl (pH-8, 1.4 M NaCl, 20mM EDTA, CTAB (2-3%w/v), β- mercaptoethanol) was added to each sample and secured with eight strip caps.
- Samples were processed in a Geno Grinder 2000 (Spex CertiPrep, USA), following the manufacturers instructions, at 500 strokes/min for 5 times at 2 min interval.
- Plate was fitted into locking device and incubated at 65ºC for 10 min with shaking at periodical intervals.

3.4.1.3 Solvent extraction

- Each of the sample were mixed with 450µl of chloroform-isoamylalcohol (24:1) by inverting twice.
- Plate was centrifuged at 5500 rpm for 10 min. The aqueous layer (300µl) is transferred to fresh strip tubes (Marsh Biomarket, USA).

3.4.1.4 Initial DNA precipitation

- 0.7 vol (210µl) of isopropanol (stored at –20ºC) was added to each sample and
- Inverted once to mix.
- Plate was centrifuged at 5000 rpm for 15 min.
- Supernatant was decanted from each sample and pellet was air dried for 20 min.

3.4.1.5 RNase treatment

200µl low salt TE [10 mM Tris EDTA (pH-8)] and 3µl RNase was added to each sample and incubated at 37ºC for 30 min.

3.4.1.6 Solvent extraction

- 200µl of phenol-chloroform-isoamylalcohol (25:24:1) was added to each sample and inverted twice to mix.
- Plate was centrifuged at 5000 rpm for 5 min.
- Aqueous layer was transferred to a fresh 96 deep-well plate (Marsh Biomarket, USA).
- 200µl chloroform-isoamylalcohol (24:1) was added to each sample and inverted twice to mix.
- Plate was centrifuged at 5000 rpm for 5 min.
- Aqueous layer was transferred to a fresh 96 deep-well plate.
- 315µl ethanol-acetate solution [30ml ethanol, 1.5ml 3M NaOAc (pH-5.2)] was then added to each sample and placed in –20ºC for 5 min.
- Plate was again centrifuged at 5000 rpm for 5 min.
- Supernatant was decanted from each sample and pellet was washed with 70% ethanol.
- Plate was centrifuged at 6000 rpm for 10 min.
- Supernatant was again decanted from each sample and samples were air dried for approximately 1 hour.
- Pellet was resuspended in 100µl low-salt TE and stored at 4ºC.

3.4.2 Quantification of DNA

DNA quality was checked and quantified on 0.8% agarose gel with known concentration of uncut lambda DNA standard.

3.4.3 Polymerase Chain Reactions (PCRs)

PCRs for amplification of SSR loci were performed in a 5 µl reaction volume [0.5 µl of 10X PCR buffer, 1.0 µl of 15 mM $MgCl_2$, 0.25 µl of 2mM dNTPs, 0.50 µl of 2 pM/µl primer anchored with M13-tail (MWG-Biotech AG, Bangalore, India), 0.1 U of *Taq* polymerase (Bioline, London, UK) and 1.0 µl (5 ng/µl) of template DNA] in 96-well micro titre plate (ABgene, Rockford, IL, USA) using thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). A touch down PCR programme was used to amplify the DNA fragments: initial denaturation was for 5 min at 95°C followed by 5 cycles of denaturation for 20 sec at 94°C, annealing for 20 sec at 60°C (the annealing temperature for each cycle being reduced by 1°C per cycle) and extension for 30 sec at 72°C. Subsequently, 35 cycles of denaturation at 94°C for 20 sec followed by annealing for 20 sec at 56°C and extension for 30 sec at 72°C and 20 min of final extension at 72°C. PCR products were checked for amplification on 1.2% agarose gel.

3.4.4 Diversity analysis

For diversity analysis, marker profiles obtained on silver stained polyacrylamide gels were scored manually. For understanding relationships among parental lines of hybrids, allelic data so obtained were used to prepare dissimilarity matrix and to construct a two dimensional (2D) plot by using factorial analysis method with DARwin V5.0.128 software (Perrier et al. 2003). The polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a given germplasm, depending on the number of detectable alleles and the distribution of their frequency.

In the present study, PIC value of markers was calculated using following formula (Anderson et al. 1993)

$$\text{PIC} = 1 - \sum_{i=1}^{k} P_i^2$$

where k is the total number of alleles detected for a given marker locus and P_i is the frequency of the i^{th} allele in the lines analyzed.

3.4.5 Data recording

The amplification products obtained by using M13 tailed primer pairs together with Liz Gene Scan-500 labeled internal size standards, were analyzed on 36 cm capillaries with POP7 polymer on ABI 3730 Genetic Analyzer. Fragment analysis data were collected by the data collection software and pre-processed by the GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA, USA). SSR allele data for the population was recorded as "A" [allele of male- sterile parent (A- line)], "B" [allele of fertility restorer parent (R- line)] and "H" (alleles from both the parents "Hybrid") format.

3.4.6 Linkage mapping

Segregation data obtained for polymorphic SSR markers on the F_2 populations were used for linkage mapping using MAPMAKER/EXP 3.0 (Lander et al. 1987). Once the dataset was sorted at logarithm of odds (LOD) of 3 with a minimum recombination threshold of 37.5, each group was used to check marker orders and obtain marker statistics. The exact order of the markers within each group was determined by using the "try" command, which compares the maximum-likelihood of each marker order after keeping the markers, one by one, into every interval of the established order. Small sorting errors were removed using the "ripple" command on all groups. Kosambi mapping function was used to convert recombination frequency into map distances (Kosambi 1944).

The graphical maps of the linkage groups were constructed by using QTL Cartographer version 2.5 (Wang et al. 2007).

3.4.7 QTL identification

Genotyping and phenotyping data were analyzed for mapping QTLs by composite interval mapping (CIM) proposed by Zeng (1994) using Windows QTL Cartographer, version 2.5 (Wang et al. 2007). CIM was performed using the Model 6, scanning the genetic map and estimating the likelihood of a QTL and its corresponding effects at every 1 cM, while using significant marker cofactors to adjust the phenotypic effects associated with other positions in the genetic map. The number of marker cofactors for the background control was set by forward–backward stepwise regression. A window size of 10 cM was used and therefore cofactors within 10 cM on either side of the QTL test site were not included in the QTL model. Thresholds were determined by permutation tests (Churchill and Doerge, 1994; Doerge and Churchill, 1996) using 1,000 permutations and a significance level of 0.05. QTLs were determined significant if the corresponding likelihood ratio (LR) score was greater than 11.5 (equal to a LOD score of 2.5).The percent phenotypic variance explained (PVE) by a QTL was estimated at the highest probability peaks.

IV. RESULTS

The present study was carried out with the objective of developing mapping populations segregating for fertility restoration gene(s), resolving inheritance and identifying molecular markers for fertility restoration of *A4* CMS system. In this chapter results have been presented for each objective under following headings.

4.1 Development of mapping populations

With an objective of developing a set of diverse mapping populations of pigeonpea, segregating for fertility restoration, an elite collection of pigeonpea genotypes was analysed with SSR markers (Table 1).

4.1.1 Diversity analysis of A-, B- and R- lines with SSR markers

In order to maximize polymorphism in the mapping populations for mapping loci for fertility restoration in pigeonpea, a set of 159 pigeonpea lines (Table 1) were screened for polymorphism with 148 microsatellite or simple sequence repeat (SSR) markers (Table 2).

4.1.1.1 SSR polymorphism in A-, B- and R- lines

A total of 148 SSR markers (Table 2) were used for characterization of the 159 parental lines (Table 1) of hybrids. These lines included 37 cytoplasmic male sterile (A-) lines, 38 maintainer (B-) lines and 84 fertility restorer (R-) lines that are being used in the hybrid breeding programme at ICRISAT. As a result, 41 markers (Table 3) showed polymorphism among 159 lines analyzed. These polymorphic

markers amplified a total of 130 alleles with an average of 3.1 alleles per marker in the lines surveyed (Table 3). Majority of the markers (17) amplified 2 alleles, while a maximum of 6 alleles were amplified by two markers (CCtta011 and CCtc002). The PIC values calculated for these 41 polymorphic markers were in the range of 0.01 (CCac007 and CCac031) to 0.81 (CCtta011) with an average of 0.41 per marker (Table 3).

Among 37 CMS (A-) lines studied, the 40 polymorphic markers amplified a total of 106 alleles with a range of 2 (21 markers) to 6 (CCtta011) with an average of 2.6 alleles per marker. The PIC values, across A- lines, ranged from 0.05 (ICPM131) to 0.75 (CCtta011) with an average of 0.34. In case of 38 maintainer (B-) lines, the 34 polymorphic markers amplified a total of 94 alleles with a range of 2 (17 markers) to 6 (CCtta011) with an average of 2.7 alleles per marker. The PIC values, across B- lines, varied from 0.06 (CCgtt004) to 0.78 (CCtta011) with an average of 0.39 per marker. In case of 84 fertility restorer (R-) lines, 115 alleles were obtained by 39 polymorphic markers with a range of 2 (19 markers) to 6 (CCtta011 and CCtc002) with an average of 2.9 alleles per marker and the PIC values varied from 0.03 (CCtta008) to 0.78 (CCtta011) with an average of 0.37 per marker. Detailed results on this aspect have already been published in RK Saxena et al. (2010c).

4.1.1.2 Genetic relationships among A-, B- and R- lines

Genetic dissimilarity among the parental lines varied from 0.03 (ICPB 2044 and ICPB 2162) to a maximum of 0.52 (ICPL 92045 and ICPA 2148) with a mean dissimilarity of 0.28. The genetic dissimilarity estimates for 159 lines were employed to generate a two dimensional distribution plot by using factorial analysis with DARwin V5.0.128 software (Perrier et al. 2003) (Fig. 4). Parental lines were classified into two main clusters ‘I’ (64) and ‘II’ (95) (Fig.

4) and could distinguish all 159 (A-, B- and R-) lines. Most of the male sterile lines (34) were grouped into cluster 'I' with 14 maintainers and 16 restorers. For instance a male sterile line ICPA 2039 and its corresponding maintainer line ICPB 2039 were clustered together in cluster 'I'. Three male sterile lines ICPA 2043, ICPA 2047 and ICPA 2092 were found to be grouped into clusters 'II'.

4.1.2 Selection of crossing parents

As the final objective of diversity study of parental lines was to select the most diverse parental combination(s) for developing the mapping populations segregating for fertility restoration gene(s), the marker polymorphism data were analyzed together with the genetic dissimilarity data. While selecting the potential parental combinations for developing the most informative mapping populations, following criteria were used: (a) high number of polymorphic markers, (b) higher genetic dissimilarity coefficient, (c) high fertility restoration and (d) stability of male sterility. However, it was very difficult if not impossible to identify the parental combinations that have higher values for all the above parameters. In such cases, more emphasis was given to fertility restoration and stability of male sterility data.

For fertility restoration, a total of 56 combinations of A- and R- lines included in the present study, being utilized for the production of hybrids in Pigeonpea Breeding Division at ICRISAT, were checked for all four parameters mentioned above (Table 4). While two parental combinations were identified in the early maturing group and two parental combinations were selected for late maturing group. In total, four parental combinations were selected for developing the mapping populations (Table 5).

4.1.3 Development of BC_1F_1/F_2 and $F_{2:3}$ mapping population

Growing of parents for crossing and rising of mapping population was carried out under nylon net coverings to avoid cross pollination through insect pollinators. All the selected combinations (ICPA 2039 × ICPR 2438, ICPA 2039 × ICPR 2447, ICPA 2043 × ICPR 2671 and ICPA 2043 × ICPR 3467) were crossed and the resultant F_1 was raised. F_2 seeds collected from a single F_1 plant were used to obtain F_2 plants and back crossed with female parent to develop BC_1F_1 population (Table 6). All the F_2 plants were selfed to obtain the $F_{2:3}$ mapping population (Fig. 3). The BC_1F_1, F_2 and $F_{2:3}$ populations were used for phenotyping for fertility restoration as described in Chapter 3 (Material and Methods).

4.2 Inheritance of fertility restorer gene(s)

Two male-sterile lines ICPA 2039 and ICPA 2043 were crossed to four known fertility restorers of different maturity groups. The early maturing A- line (ICPA 2039) was crossed to ICPR 2438 and ICPR 2447; while another male-sterile line (ICPA 2043) was crossed with two late maturing restorer lines ICPR 2671 and ICPR 3467. The inheritance of fertility restoration was studied in BC_1F_1, F_1 and F_2 generations. To validate segregation pattern obtained in F_2 generation, in addition to back cross population, 53 fertile F_3 progenies were also assessed in the late maturing cross (ICPA 2043 × ICPR 3467).

4.2.1 Early maturing crosses

Two early maturing crosses were made on ICPA 2039 using ICPR 2438 and ICPR 2447 as male parents. Both of these crosses behaved in a similar manner as far as their segregation of male fertility/sterility was concerned. In the F_1 generation, a perfect dominance of male-fertility was recorded; while in F_2 generation, the populations segregated and fit well to the expected di-genic duplicate dominant ratio of 15 fertile : 1 sterile (Table 7). In the early maturing BC_1F_1 generations

seed could not be produced due to severe pod damage caused by borers (*Maruca testulalis* Geyer.), hence there was no opportunity to test the segregation of these crosses.

4.2.2 Late maturing crosses

Two late maturing fertility restorers namely ICPR 3467 and ICPR 2671 were crossed with ICPA 2043. A complete dominance of fertility restoring genes was observed in F_1 generation, where all the hybrid plants were fully fertile with good pollen load. In F_2 generation, both the hybrids exhibited a di-genic ratio with a duplicate dominant (15:1 ratio) gene action. This hypothesis of gene action was confirmed in BC_1F_1 generation of both the crosses (Table 8), where the ratio of 3 fertile: 1 sterile was observed.

In F_3 generation of cross ICPA 2043 × ICPR 3467, out of 96 random fertile progenies grown, 53 segregated for fertility/sterility (Table 9), while 43 did not segregate. This segregation pattern fit well to the expected ratio of 8 segregating: 7 non-segregating (p = 0.72). The variation observed within 53 segregating progenies indicated the presence of two sub- groups (Table 9). The sub- group I, with 33 progenies, segregated in a di-genic ratio of 15 fertile to 1 sterile genotypes (p = 0.94), while in the sub- group II (20 progenies) the progenies segregated only for one gene, resulting in 3 fertile: 1 sterile ratio (p = 0.28) (Table 9). Overall the two sub-groups segregated in the expected ratio of 1:1 (p = 0.07).

4.3 Molecular mapping of fertility restorer gene(s)

The identification of molecular markers tightly linked to fertility restoration loci in pigeonpea would allow breeders to classify breeding lines as either maintainers (B- lines) or restorers

(R- lines) without the requirement of field evaluation of test crosses. Further, these identified linked markers would also be helpful in transferring these genes form one genetic background to other through marker assisted selection (MAS). For mapping the fertility restorer gene(s) of *A4* CMS system, two F_2 mapping populations one each from early (ICPA 2039 × ICPR 2447) and late maturing (ICPA 2043 × ICPR 2671) groups were selected. Detailed results on mapping are presented below.

4.3.1 Parental screening for marker polymorphism

The parental lines of two mapping populations (ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671) were screened for detecting polymorphism using 3,220 SSR primer pairs (Burns et al. 2001; Odeny et al. 2007; 2009; RK Saxena et al. 2010a; 2010c; Bohra et al. 2010). A total of 98 and 145 SSR markers detected polymorphism between ICPA 2039 vs ICPR 2447 (Table 10) and ICPA 2043 vs ICPR 2671 respectively (Table 11). These polymorphic markers were used for generating the segregation data for the respective polymorphic loci on F_2 mapping population consisting of 188 individuals for each of two crosses mentioned above.

4.3.2 Goodness of fit of markers and segregation distortion

The goodness of fit of segregation data generated in the study was calculated by Chi square analysis. The observed segregation pattern of the marker loci was compared with the expected 1:2:1 (A: H: B) ratio and probability was calculated. In case of ICPA 2039 × ICPR 2447 mapping population (F_2), while normal segregation ($p \geq 0.01$) was observed for segregation data for 75 out of 98 marker loci, segregation distortion was observed for the remaining 23 marker loci (Fig. 5). Similarly in the case of ICPA 2043 × ICPR 2671 mapping population, normal segregation was observed for 131 out of 145

SSR loci ($p \geq 0.01$); the segregation data for the remaining 14 SSR loci showed distorted segregation (Fig. 6).

4.3.3 Construction of the SSR-based genetic maps

The segregation data for the SSR loci showing the normal segregation in each of the above mapping population were used for constructing genetic maps for the respective mapping population by using MAPMAKER/EXP version 3.0b software (Lander et al. 1987). In summary, two different genetic linkage maps were constructed for the ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671 groups. Details about these maps are provided as below.

4.3.3.1 Genetic map based on ICPA 2039 × ICPR 2447 population

The genetic map for the ICPA 2039 × ICPR 2447 population was constructed at LOD value of 3.0 and the confidence map interval distance at less than 40 cM. A total of 82 markers were mapped on 11 linkage group (LGs) spanning 802.8 cM, however, the remaining 16 markers could not be integrated into the map (Fig. 7, Table 12). The number of markers mapped per linkage group ranged from 3 (LG 5) to 12 (LG 6). The lengths of linkage groups ranged from 6.3 cM (LG 5) to 166 cM (LG 7) with an average inter-marker locus distance of 9.7 cM across 11 linkage groups (Table 12).

4.3.3.2 Genetic map based on ICPA 2043 × ICPR 2671 population

The segregation data obtained on the ICPA 2043 × ICPR 2671 population for the polymorphic SSR loci were used to construct the map at linkage map distances less than 40 cM and LOD value 4.0. As a result, a total of 117 SSR loci were mapped on 11 linkage groups (LGs) spanning 871 cM (Fig. 8, Table 13) and the remaining 28 SSR loci remained ungrouped. The number of marker loci mapped per

linkage group ranged from 2 (LG 2) to 20 (LG 6). The lengths of linkage groups ranged from 22.4 cM (LG 2) to 165.2 cM (LG 4) with an average inter-marker locus distance of 7.4 cM across 11 linkage groups (Table 13).

4.3.4 QTL mapping for fertility restoration

The linkage maps constructed based on ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671 F_2 mapping populations were used for identification and mapping of QTL for fertility restoration. In this context, mapping data for all SSR marker loci assigned to the genetic maps were analysed together with phenotypic data for pollen fertility/ sterility obtained on each of two populations by using Windows QTL Cartographer, version 2.5 (Wang et al. 2007). CIM (composite interval mapping) analysis was performed using the Model 6, scanning the genetic map and estimating the likelihood of a QTL and its corresponding effects at every 1 cM, while using significant marker cofactors to adjust the phenotypic effects associated with other positions in the genetic map. The number of marker cofactors for the background control was set by forward–backward stepwise regression. A window size of 10 cM was used, and therefore cofactors within 10 cM on either side of the QTL test site were not included in the QTL model. QTLs were determined significant if the corresponding likelihood ratio (LR) score was greater than 11.5 (equal to a LOD score of 2.5).

4.3.4.1 QTLs for fertility restoration based on ICPA 2039 × ICPR 2447 population

QTL analysis, as mentioned above, in this mapping population showed a total of six putative QTLs on five different linkage groups (Table 14). While four QTLs detected at LOD value > 2.5 were considered as major QTLs, the remaining two QTLs on LG2 and LG9, detected at LOD value between 1 and 2, had small effects each with 4.0% phenotypic variance explained (PVE).

Of the four major QTLs, the QTL flanked by CcM2149 and CcM0468 and detected at LOD value of 44 on LG7 explained highest phenotypic variation (50%). While the other two QTLs detected at LG11 bracketed between CcM0381 and CcM2735; and CcM2735 and CcM1713 explained 22% and 25% phenotypic variation, respectively (Fig. 9 and Table 14). The remaining major QTL detected at LOD value of 4.2 and flanked by CcM1109 and CcM1522 marker loci on the top of LG3 explained 20% phenotypic variation (Fig. 9).

4.3.4.2 QTLs for fertility restoration based on ICPA 2043 × ICPR 2671 population

CIM based QTL analysis identified a total of four QTLs on four linkage groups (Table 15). However, all these QTLs except one were minor as they explained ≤10% of phenotypic variation. The solitary major QTL flanked by CcM2542 and CcM1277 marker loci and detected at LOD 5.4 on LG3 contributed 24% phenotypic variation (Fig. 10).

V. DISCUSSION

Shull (1908) was the first to describe the phenomenon of hybrid advantage in crop plants and considering its potential in enhancing yields. Subsequently, the breeders of cross - pollinated crops developed suitable breeding procedures and successfully enhanced yields by 2 – 3 folds. Various theories explained the complex phenomenon of heterosis and concluded that some complementary inter - genomic and non - allelic interactions, operating at different structural and functional levels, are responsible for the expression of hybrid vigour (Sinha and Khanna 1975; Srivastava 1981). Although pigeonpea breeding research began in the early part of 20th century, the first report of hybrid vigour was published in late 1950s (Solomon et al. 1957). Subsequently, a number of reports were published on hybrid vigour for yield and important yield components (Saxena and Sharma 1990). Earlier in the absence of CMS in pigeonpea, an attempt was made to exploit hybrid vigour using genetic male-sterility based hybrids such as ICPH 8, PPH 4, CoH 1, CoH 2 etc (Saxena et al. 1992). These hybrids exhibited 20 to 40% superiority over the respective control cultivar in farmers' fields. CMS-based hybrids exhibited standard heterosis up to 156% for yield (Kandalkar 2007) where as 50 to 100% yield advantage over the controls has also been reported (Saxena 2007).

It was in 1974 when pigeonpea breeders at ICRISAT started exploring the possibility of breeding commercial hybrids by exploiting its natural out - crossing. The major component for commercial hybrid breeding that was missing at that time was the availability of an efficient male - sterility system. Therefore, an elaborate search for male - sterility was made germplasm and two different genetic male - sterility systems were discovered (Reddy et al. 1978; Saxena et al. 1983). Initially, these two sources were used in hybrid breeding without much success at commercial level due to

limitations encountered in large - scale seed production of hybrids and their parents. Hence, the development of a CMS system became imperative.

It is a well known fact that the expression of CMS, in part, is controlled by genetic factors carried through the female parent, which are never lost or diluted in succeeding generations (Kaul 1988). This cytoplasmic factor is referred to as 'N' for male - fertile cytoplasm and 'S' for the male – sterile cytoplasm. The male - sterile (A- line) line with 'S' cytoplasm and homozygous recessive (*msms*) nuclear genes is maintained by its male – fertile maintainer (B-) line that has a normal (N) cytoplasm and homozygous recessive nuclear genes. For producing male - fertile hybrids, the A- line with 'S' cytoplasm is crossed with a male - fertile (R-) line carrying dominant fertility restoring nuclear genes (*FrFr*). To sum up, the three - line hybrid system is geared for multiplying A- line seed with the help of B- line and for producing hybrid seed the A- line is pollinated with R- line.

In pigeonpea since CMS was not available earlier, plans were made to breed for this trait by placing pigeonpea genome in to the cytoplasm of its wild relative. It was expected that the interaction of such cytoplasm and nuclear genomes would produce male - sterility that would inherit maternally. This endeavour resulted in development of an excellent CMS system that was developed by crossing a pigeonpea line with a wild species, *Cajanus cajanifolius* (*A4* cytoplam) (Saxena et al. 2005). It is the most closely related wild species of pigeonpea and is considered as the progenitor of cultivated type that differs only by a single gene (De 1974). The CMS system derived using this species is considered the best because it has a number of good maintainers and fertility restorers (Saxena et al. 2005). The F_1 hybrid plants produce excellent pollen load and pod set. The A- lines with *C. cajanifolius* cytoplasm are being used extensively in hybrid breeding programmes. Since pigeonpea is cultivated under diverse environments and cropping systems with specific maturity and plant type

requirements (Saxena 2008), the CMS trait was transferred to early (ICPA 2039) and late- maturing (ICPA 2043) lines to facilitate development of hybrids in diverse maturity groups for different agro-climatic zones.

Information on genetics of fertility restoration and molecular markers linked to this trait helps in designing strategies for breeding elite hybrid parents. This study reports genetics of fertility restoration in four crosses of two maturity groups and an attempt to establish relationships between molecular markers to fertility restoration. The findings of present study are discussed with suitable subheadings.

1. Development of mapping populations
2. Inheritance studies for fertility restoration
3. Construction of genetic linkage maps for intra-specific mapping populations
4. Identification and mapping of QTLs for fertility restoration

5.1 Development of mapping populations

The most important step in a breeding programme is the choice of parents with good performance and wide genetic base. Therefore, diversity analysis on parental genotypes, ahead of making any cross, may help breeders to concentrate their efforts only on most promising combinations. Heterosis, manifested in the crosses, is directly related to genetic divergence among the parents (Falconer 1981). Therefore, in the choice of parents to be used in hybridization, emphasis should be placed both on the cultivars performance and on its genetic distances. In this context, molecular markers based studies were conducted in several crop species. For instance, in case of soybean, genetic divergence was observed among 34 lines to identify suitable parents for breeding

programmes (Pipolo et al. 1995). Likewise, several other studies recommended the use of parents with the greatest possible divergence to maximize the heterosis in the hybrids and to enhance the probability of superior segregants in advanced generations and widen the genetic base (Amaral Junior et al. 1996; Souza 1996; RK Saxena et al. 2010b).

As pigeonpea germplasm has a narrow genetic diversity, it is important to undertake molecular characterization of the potential parental lines. Molecular diversity studies may lead to the selection of genotypes for making the crosses using genetic distance data (RK Saxena et al. 2010b). Furthermore, the identification of genotypes based on genetic divergence alone, without considering their phenotypic performance, may not be a good strategy for a breeding program. Crosses among divergent genotypes that have a superior performance for the main characteristics of agronomic importance seem to be more recommended for breeding programs. Therefore in this study, selection of parents was done not only based on molecular diversity analysis but also based on genetic distance and their performance for the desirable traits. This increased the selection pressure to select some of the contrasting genotypes for developing mapping populations for fertility restoration. The mapping populations developed in such a way should have good genetic diversity that should allow genotyping of the mapping population with a larger number of molecular markers to develop the good genetic maps, an important prerequisite of molecular mapping.

In case of pigeonpea, though, hybrid breeding programme has been developing and using a number of hybrid parental lines, no genetic diversity information was available at the time of undertaking this study. This may be attributed partly to unavailability of adequate molecular marker resources in past. Therefore, estimation of genetic diversity on parental lines should be useful for selecting crossing parental genotypes that may enhance the hybrid vigour.

The present study was undertaken on surveying the molecular diversity among 159 hybrid pigeonpea parental genotypes representing male sterile (A-), maintainer (B-) and restorer (R-) lines with 148 SSR markers. However, only 27.7% markers showed polymorphism in the set of 159 parental lines. Lower level of polymorphism observed in this study is in agreement with earlier SSR based diversity studies in pigeonpea (Burns et al. 2001; Odeny et al. 2007; RK Saxena et al. 2010a; 2010c). Lower level of diversity was also reported based on other marker systems such as amplified fragment length polymorphism (AFLP, Panguluri et al. 2006) and Diversity Array Technology (DArT, Yang et al. 2006). In contrast to lower level of diversity among A- lines as observed in the present study, Souframanien et al. (2003) reported a higher level of genetic diversity. It is, however, important to note that in the present study, all A- lines were derived from the *C. cajanifolius* (A_4) while in study of Souframanien et al. (2003), A- lines analyzed were derived from two different wild relatives *C. sericeus* and *C. scarabaeoides*.

In terms of molecular diversity, the present study grouped majority of A- lines (34) and 14 B- lines together in the major cluster 'I'. While majority of the B- lines (24) and a few A- lines (3) were grouped in the other major cluster 'II'. In an ideal condition for hybrid breeding, A- and B- lines should be homogeneous except for the male sterility but in present study majority of the A- lines used are still in early back crossing generations (BC_2 to BC_4). Therefore, these A- lines can not be considered as iso- nuclear lines with the corresponding B- lines. On the other hand, ICPA 2039 and ICPB 2039 used in the study represent BC_9 generation and these lines were found to share the maximum numbers of common alleles and were grouped together in cluster 'I' with 94% of genetic similarity. In fact, at present these lines are in BC_{11} stage and if these lines are examined for molecular diversity, higher genetic similarity in these lines is expected. Therefore, the genetic background of this particular combination of A- and B- line is satisfactory for producing

homogeneous A- line seeds. Differences that remain exist between these iso- nuclear lines are mainly due to differences in the organellar genomes while the nuclear material is expected to be almost similar after several backcrosses. Also the grouping of several A- lines together in the same cluster can be attributed to the shared pedigree of the maintainer lines from which these A- lines have been generated. On the other hand differences in the pedigree of maintainer lines for some other A- lines made them classified in the other cluster.

For developing the mapping populations for mapping fertility restoration, parental genotypes were selected based on marker genotyping data i.e. high number of polymorphic markers and higher genetic dissimilarity coefficient and phenotypic data (high diversity). By using these criteria, two parental combinations (ICPA 2039 × ICPR 2438 and ICPA 2039 × ICPR 2447) were identified for early maturing group and two parental combinations (ICPA 2043 × ICPR 2671 and ICPA 2043 × ICPR 3467) for late maturing group were selected. Four parental combinations, selected in such a way, were used for crossing and development of mapping populations. Development of such mapping populations has been suggested in some earlier studies (Pipolo et al. 1995; RK Saxena et al. 2010b).

5.2 Inheritance of fertility restoration

In the present study two A- lines having A_4 cytoplasm were crossed with four fertility restoring lines and two male-fertility restoring genes segregating independently, were identified. In the four male parents (ICPR 2438, ICPR 2447, ICPR 2671 and ICPR 3467) two dominant genes were involved in the expression of fertility restoration with duplicate dominant gene action. Similarly, in pearl millet (*Pennisetum glaucum*) 1-3 dominant genes (Yadav 2005); in faba bean (*Vicia faba*) 1-2 dominant genes (Kaul 1988); and in soybean (*Glycine max*) two dominant genes (Bai and Gai 2005) were found to be responsible for the restoration of male fertility of the respective CMS systems.

To the best of our knowledge, no study was conducted to establish relationship among various fertility restoring alleles in pigeonoea. From the present experiments, however, it appeared that the differences in the pollen load observed among the hybrid plants were linked to the number of fertility restoring genes present in the individual. In the hybrid plants with two fertility restoring genes, the pollen load in the floral buds was similar to that of pure line cultivars and the hybrids expressed greater stability across environments. In this context, it is worth mentioning that a hybrid ICPH 2671, with two dominant fertility restoring genes, has performed extremely well in farmers' fields in six states of India and three provinces of Myanmar in terms of fertility restoration and high yields under diverse environments (KB Saxena et al. 2010b). On the contrary, when a single fertility restoring gene was present, the hybrids produced relatively less amount of pollen grains. This single gene perhaps was unable to produce hybrids with stable fertility restoration (KB Saxena et al. 2010b).

It has also been observed in certain wheat (*Triticum aestivum*) lines that the restorer parents with a single gene might be responsible for their poor pollinating capacity (Hughes and Bodden 1977). Partial fertile plants in a sorghum (*Sorghum vulgare* Pers.) population segregating for fertility restoring alleles and the full pollen fertility in a genotype essentially resulted from the presence of all the major and minor genes together (Tang et al. 2007). Further, the partial male-fertility in the plants resulted due to the absence of some fertility restoring alleles at minor affect loci, which separated from the major effect restoring genes during segregation and assortment processes (Tang et al. 2007). In pigeonpea cytology studies of a partial male-fertile line showed breakdown of tapetum was irregular and there was no consistency in the extent and the site of degeneration of the tapetal tissues in different flowers and plants (Saxena et al. 1981). In the present case the micro-sporogenesis of partial male-fertile plants of pigeonpea was not studied but it appears that both the

pollen growth and their release processes were defective. In both the late maturing pigeonpea crosses, a variation for pollen production was also observed among F_2 segregants. Since, at that time the information on pollen production trait was not considered important in the expression of fertility restoration, no such data were recorded in this study. In view of present results it appears that for the production of good hybrids, selection of fertility restorers with both the dominant genes will be essential. This is likely to facilitate the development of high yielding and widely adapted hybrids.

5.3 Construction of genetic linkage map

After screening of a total of 3,220 markers including 148 SSR markers, available in public domain (Burns et al. 2001; Odeny et al. 2007; 2009; RK Saxena et al. 2010a; 2010c) as well as 3072 unpublished SSR markers (Bohra et al. 2010) on the parental genotypes of the two mapping population provide 98 markers polymorphic in ICPA 2039 × ICPR 2447 and 145 in ICPA 2043 × ICPR 2671 population. The very low level of polymorphism (3.03% in ICPA 2039 × ICPR 2447 and 4.49% in ICPA 2043 × ICPR 2671) observed in the present study is not unexpected, as lower level of polymorphism in the cultivated gene pool of pigeonpea observed in several other studies (Burns et al. 2001; Odeny et al. 2007; 2009; RK Saxena et al. 2010a; 2010c). Low level of genetic polymorphism in cultivated pigeonpea has been attributed to the breeding methodologies used in past. However, additional contributing factors to the low levels of molecular polymorphism observed to date could be due to marker techniques used. Indeed, development of SSR markers, from longer SSR-enriched libraries, BAC-end sequences and SNP (single nucleotide polymorphism) markers using next generation sequencing technologies is underway in several laboratories (Varshney et al. 2010).

All identified polymorphic markers were used for genotyping 188 progenies of F_2 mapping populations for each of two crosses. On checking the genotyping data obtained for all polymorphic

loci for segregation ratio, 76.53% and 90.34% marker loci were found in normal segregation (1A:2H:1B) in ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671 respectively. These SSR loci, showing the normal segregation were used for constructing the genetic map. However, the remaining SSR loci did not conform to the expected segregation ratio and most of these distorted markers could not be mapped on the linkage maps. A number of reasons are responsible for segregation distortion such as rearrangement in genome, allele inducing gametic or zygotic selection (Lu et al. 2002), parental reproductive differences and the presence of lethal genes (Blanco et al. 1998).

Development of a framework linkage map is an important pre-requisite for identification of QTL(s) for marker-assisted selection in any crop improvement programme. One of the reasons for slow progress in the molecular breeding of the important legume crop like pigeonpea has been attributed to the limited availability of molecular markers and non-availability of the linkage map so far. Although, tremendous progress has been made in terms of availability of genomic resources in this orphan legume crop in recent times (Varshney et al. 2010), availability of a framework linkage map was still lacking at the time of undertaking the study. To the best of our knowledge, this is the first genetic map of pigeonpea based on mapping population developed from cultivated genotypes and segregating for fertility restoration. The linkage map obtained for the ICPA 2039 × ICPR 2447 population is comprised of 82 SSR marker loci and spans 802.8 cM map distance with an average of 9.7 cM. Whereas, in case of ICPA 2043 × ICPR 2671 mapping population, the genetic map could be developed for a total 117 SSR marker loci spanning 871 cM map distance with an average of 7.4 cM. Interestingly, a total of 32 common markers could be mapped in both the linkage maps. The locus order of these common markers was highly conserved in both the linkage maps and was sufficiently reliable for use as a reference to define various linkage groups.

5.4 Identification of QTLs for fertility restoration

Breeders must select both maintainer and restorer lines for extreme phenotypes to assure a completely sterile female parent, but a fully fertile hybrid. Moreover, breeding is complicated by interactions between modifier genes and environmental effects. In order to assist selection through molecular approaches, a more detailed genetic analysis of the suspected major and minor restorer genes must be performed using QTL approach. In various studies, fertility restoration was checked as a quantitative or a qualitative trait and was reported to be governed by dominant alleles at one or two nuclear (*Fr*) genes or by a major dominant allele with modifier gene. For instance, by using bulked segregant analysis in pepper of the extreme plants from an F_2 progeny, two RAPD markers were identified tightly linked to a major restorer gene (Zhang et al. 2000). However, the F_2 segregation was quantitative and the intermediate phenotypes (i.e. partially sterile) were not taken into consideration. Intermediate phenotypes can either result from interactions with the environment or from genetic control.

To the best of our knowledge, this study is the first report on identification of QTLs for fertility restoration of CMS in pigeonpea. Genetic analysis of two pigeonpea mapping populations provided a total of five major QTLs and five minor QTLs. While two QTL regions on the LG11 were found overlapping and together contributing 47% of the phenotypic variation. In the similar population, two additional major QTLs were identified on LG3 and LG7 that contributing 20% and 50% of phenotypic variations respectively. On the other hand, in the other population (ICPA 2043 × ICPR 2671), only one major QTL was detected on the LG3. This QTL explained 24% of the phenotypic variation in the population. Although LG3 in both mapping populations contained at least one major QTL, unavailability of common markers on the genetic maps of the two mapping populations could not conclude whether the same QTL is responsible for fertility restoration in both mapping populations.

Although in case of the pigeonpea, this is the first study on mapping of QTLs, quantitative restoration of CMS was subjected to QTL analysis in some other crops like maize (Tie et al. 2006), pepper (Wang et al. 2004), coffee (Coulibaly et al. 2003), suger beet (Hjerdin-Panagopoulos et al. 2002), wheat (Ahmed et al. 2001), winter rye (Miedaner et al. 2000). In case of winter rye, the major QTL determined more than 50% of the phenotypic variation (Miedaner et al. 2000). Similarly, in case of the pepper, one major QTL on chromosome P6 was identified which shows phenotypic variation up to 69% (Wang et al. 2004).

As reported in the present study, the minor QTLs were also present in the case of sugar beet. In this case, one minor fertility restorer allele was also detected in the maintainer parent and displays an epistatic effect with the major QTL (Hjerdin-Panagopoulos et al. 2002). In pepper, complete sterility and high fertility also found to be dependent on the minor QTLs which may differ in distinct restorer lines, and whose expression is environment dependent (Wang et al. 2004). While major QTLs for fertility restoration will be very useful for the breeders, minor QTLs certainly pose a challenge for harnessing then in hybrid breeding.

VI. SUMMARY

Pigeonpea, because of its special traits such as drought tolerance and ability to recover from various biotic and abiotic stresses makes it a favourite crop to farmers of the semi-arid tropic regions. In view of reducing land holdings, increasing population pressure and potential forces of climate changes; the diversification of cropping systems has become inevitable. Under these circumstances, breeding high yielding early maturing hybrids are likely to play an important role in the diversification of cropping systems. In this context, the present study entitled "Study of inheritance and identification of molecular markers for fertility restorers in pigeonpea [*Cajanus cajan* (L.) Millspaugh]" was conducted with the following objectives – (1) Development of mapping populations segregating for fertility restoration, (2) Investigation of the inheritance of fertility restoration in *A4* CMS system in pigeonpea, and (3) Identification of molecular markers linked to fertility restorer (*Fr*) gene(s) of the *A4* CMS system. The results and conclusions for each of these objectives are briefly summarized below.

I. Development of mapping populations segregating for fertility restoration

1. Molecular diversity among 159 hybrid pigeonpea parental genotypes representing 37 male sterile (A-), 38 maintainer (B-) and 84 restorer (R-) lines was estimated with 148 simple sequence repeat (SSR) markers.
2. In total, 41 (27.7%) SSR markers showed polymorphism with 2 to 6 (average 2.6) alleles and 0.01 to 0.81 (average 0.34) polymorphism information content (PIC) value across the 159 lines surveyed.

3. On the basis of SSR diversity data together with phenotypic data (efficiency of fertility restoration of R- lines and stability of male sterility in case of A- lines), two parental combinations (ICPA 2039 × ICPR 2438 and ICPA 2039 × ICPR 2447) were identified in the early maturing group and two parental combinations (ICPA 2043 × ICPR 2671 and ICPA 2043 × ICPR 3467) were selected for late maturing group.

II. Investigation of the inheritance of fertility restoration

1. The F_1 hybrids produced from A × R for all the four crosses had all plants fully fertile, indicating that male fertility is dominant over male sterility.
2. The segregation patterns of male fertile and male sterile plants were studied in BC_1F_1 [A × (A × R)], F_2 and $F_{2:3}$ populations produced from A × R crosses.
3. A good χ^2 fit to the expected di-genic ratios of 15 fertile: 1 sterile in the F_2 and 3 fertile: 1 sterile in the BC_1F_1 mapping populations was observed that may be a result of gene action of two duplicate dominant genes.
4. In order to validate di-genic ratios obtained, $F_{2:3}$ population derived from one late maturing cross (ICPA 2043 × ICPR 3467) was used. From random fertile progenies grown, fertile and sterile progenies fitted well in to the expected ratio of 8 segregating: 7 non-segregating.
5. The variation observed within fertile segregating progenies indicated the presence of two sub-groups. The sub- group I, segregated in a di-hybrid ratio of 15 fertile to 1 sterile genotypes, while in the sub- group II, the progenies segregated only for one gene, resulting in 3 fertile: 1 sterile ratio. Overall the two sub-groups also fitted into expected ratio of 1 fertile: 1 sterile.

III. Identification of molecular markers linked to fertility restorer (*Fr*) gene(s)

A. Linkage map construction

1. In order to construct linkage maps in pigeonpea, two F_2 mapping populations one each from early (ICPA 2039 × ICPR 2447) and late maturing (ICPA 2043 × ICPR 2671) groups were selected.
2. A total of 98 and 145 SSR markers detected polymorphism between ICPA 2039 vs ICPR 2447 and ICPA 2043 vs ICPR 2671 respectively.
3. These polymorphic markers were used for generating the segregation data for the respective polymorphic loci on F_2 mapping population consisting of 188 individuals for each of two crosses mentioned above.
4. On checking the genotyping data obtained for all polymorphic loci for segregation ratio, 76.53% and 90.34% marker loci were found in normal segregation (1A:2H:1B) in F_2 populations for crosses ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671, respectively.
5. In case of ICPA 2039 × ICPR 2447 mapping population, a total of 82 markers were mapped on 11 linkage groups (LGs) spanning 802.8 cM with an average inter-marker locus distance of 9.7 cM.
6. In case of ICPA 2043 × ICPR 2671 mapping population, a total of 117 SSR loci were mapped on 11 linkage groups (LGs) spanning 871 cM with an average inter-marker locus distance of 7.4 cM.

B. QTL identification

1. The molecular marker analysis together with phenotyping data of the F_2 mapping population based on the cross ICPA 2039 × ICPR 2447 detected six putative QTLs (four major and two minor) involved in the fertility restoration.

2. Four putative QTLs (one major and three minor) were detected to be involved in the fertility restoration of the F_2 mapping population based on the cross ICPA 2043 × ICPR 2671.

In summary, this study has generated; (i) molecular diversity information on parental lines of hybrids, (ii) four mapping populations segregating for fertility restoration, (iii) two duplicate dominant genes were identified responsible for fertility restoration, (iv) two genetic maps based on ICPA 2039 × ICPR 2447 and ICPA 2043 × ICPR 2671 mapping populations with 82 and 117 SSR loci, respectively, and (v) 10 QTLs including five major QTLs and linked SSR markers for fertility restoration. These results should have important implications for accelerating pigeonpea genetics and breeding especially in the area of hybrid technology. For instance, molecular diversity information generated on hybrid parental lines will help in identification of diverse parental combinations for harnessing the full potential of CMS-based hybrid technology. Similarly, molecular markers linked with major QTLs for fertility restoration will facilitate selection of suitable parental lines containing fertility restorer genes. Furthermore, mapping of fertility restoration trait can be extended to cloning of major QTLs for fertility restoration through map based cloning, which in turn can help in understanding the molecular basis of fertility restoration in pigeonpea.

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Table 1: List of hybrid parental genotypes used for SSR based diversity analysis

Genotype	Growth habit	Days to flowering (50%)	Days to maturation (80%)	Plant height (cm)
A- lines				
ICPA 2039	DT*	80	122	110
ICPA 2040	DT	52	90	65
ICPA 2041	DT	86	125	145
ICPA 2042	NDT#	92	142	250
ICPA 2043	NDT	105	176	235
ICPA 2044	NDT	116	189	250
ICPA 2045	NDT	115	160	175
ICPA 2047	NDT	116	181	250
ICPA 2048	NDT	108	178	270
ICPA 2050	NDT	116	178	250
ICPA 2051	NDT	107	178	248
ICPA 2076	DT	50	95	75
ICPA 2078	DT	100	150	107
ICPA 2079	DT	58	95	90
ICPA 2080	DT	53	95	63
ICPA 2085	DT	74	125	108
ICPA 2087	DT	72	122	120
ICPA 2089	NDT	60	113	145
ICPA 2090	NDT	90	147	192
ICPA 2091	NDT	140	205	275
ICPA 2092	NDT	121	162	250
ICPA 2098	NDT	119	165	263
ICPA 2101	DT	113	150	165
ICPA 2102	NDT	118	155	160
ICPA 2148	DT	62	105	100
ICPA 2149	DT	72	110	120
ICPA 2155	NDT	72	110	170
ICPA2156	NDT	69	110	165
ICPA 2157	NDT	73	120	120
ICPA 2158	DT	72	112	110
ICPA 2160	DT	85	135	110
ICPA 2161	DT	107	157	150
ICPA 2162	DT	68	115	115
ICPA 2163	DT	68	100	105
ICPA 2164	DT	64	105	72
ICPA 2165	DT	65	110	95
ICPA 11376	NDT	125	210	280
B- lines				
ICPB 2039	DT	75	122	115

ICPB 2040	DT	59	90	67
ICPB 2041	DT	78	115	125
ICPB 2042	NDT	90	142	252
ICPB 2043	NDT	120	176	235
ICPB 2044	NDT	123	170	258
ICPB 2045	NDT	125	162	248
ICPB 2046	NDT	135	178	257
ICPB 2047	NDT	128	181	252
ICPB 2048	NDT	130	178	272
ICPB 2049	NDT	115	165	240
ICPB 2050	NDT	120	178	252
ICPB 2051	NDT	115	165	248
ICPB 2076	DT	52	105	90
ICPB 2078	DT	95	150	107
ICPB 2079	DT	58	95	76
ICPB 2080	DT	50	95	69
ICPB 2085	DT	74	125	137
ICPB 2086	DT	70	130	137
ICPB 2087	DT	72	120	148
ICPB 2089-24	NDT	60	113	145
ICPB 2090	NDT	90	147	192
ICPB 2091	NDT	140	200	275
ICPB 2092	NDT	121	178	250
ICPB 2098	NDT	119	165	263
ICPB 2101	DT	113	145	165
ICPB 2102	NDT	118	163	260
ICPB 2148	DT	62	114	69
ICPB 2149	DT	72	110	115
ICPB 2155	NDT	76	119	157
ICPB 2156	NDT	67	105	145
ICPB 2158	DT	72	112	110
ICPB 2159	NDT	68	100	104
ICPB 2160	DT	85	135	142
ICPB 2161	DT	107	147	150
ICPB 2162	DT	69	115	95
ICPB 2163	DT	68	110	86
ICPB 2164	DT	64	105	71
R- lines				
BDN 1	NDT	112	168	230
BSMR 853	NDT	138	180	230
C 11	NDT	116	170	235
HPL 21-3	NDT	105	185	170
HPL 24-47	NDT	123	185	230
HPL 24-63	NDT	124	180	225

ICP 10897	NDT	65	115	39
ICP 10907	NDT	77	145	150
ICP 10934	NDT	120	172	110
ICP 11378	NDT	91	136	155
ICP 12320-1-3	NDT	138	190	220
ICP 13186	NDT	84	105	160
ICP 14282	NDT	120	201	250
ICP 14479	NDT	64	105	110
ICP 8094	NDT	140	200	280
ICP 8744	NDT	72	130	155
ICP 9939	NDT	112	182	243
ICPL 10650	NDT	120	190	165
ICPL 13991-2-10	NDT	144	206	257
ICPL 13991-2-5	NDT	144	215	285
ICPL 149	NDT	95	137	170
ICPL 150	NDT	80	120	180
ICPL 161	NDT	92	135	180
ICPL 20058	NDT	100	172	243
ICPL 20093	NDT	130	183	190
ICPL 20094	NDT	132	185	170
ICPL 20095	NDT	120	181	190
ICPL 20096	NDT	127	185	190
ICPL 20098	NDT	132	184	170
ICPL 20099	NDT	128	184	180
ICPL 20102	NDT	130	181	195
ICPL 20104	NDT	130	182	205
ICPL 20106	NDT	126	182	205
ICPL 20107	NDT	130	185	160
ICPL 20108	NDT	128	181	180
ICPL 20110	NDT	135	186	200
ICPL 20111	NDT	130	183	180
ICPL 20112	NDT	126	182	185
ICPL 20113	NDT	133	185	190
ICPL 20115	NDT	126	181	170
ICPL 20116	NDT	122	181	170
ICPL 20117	NDT	130	189	195
ICPL 20118	NDT	126	182	170
ICPL 20121	NDT	125	181	175
ICPL 20122	NDT	123	180	160
ICPL 20123	NDT	126	179	175
ICPL 20125	NDT	128	190	180
ICPL 20126_1	NDT	130	183	170
ICPL 20126	NDT	130	183	170
ICPL 20127	NDT	122	183	150

ICPL 20128	NDT	128	182	170
ICPL 20129	NDT	130	185	185
ICPL 20132	NDT	122	184	180
ICPL 20135	NDT	132	179	175
ICPL 20136	NDT	130	184	170
ICPL 366	NDT	145	220	180
ICPL 81-3	NDT	70	120	155
ICPL 86022	NDT	78	120	125
ICPL 87	DT	69	125	105
ICPL 87119	NDT	130	180	210
ICPL 88034	NDT	69	130	165
ICPL 88039	NDT	66	110	105
ICPL 89	NDT	67	120	140
ICPL 90030	NDT	62	108	125
ICPL 90036	NDT	64	118	146
ICPL 90048	NDT	67	110	150
ICPL 92042	NDT	59	101	105
ICPL 92043	NDT	69	105	130
ICPL 92045	NDT	75	115	145
ICPL 92047	NDT	79	120	155
ICPL 93101	NDT	67	105	140
ICPL 93103	NDT	100	110	185
ICPL 93105	NDT	82	140	180
ICPL 93107	NDT	67	105	140
ICPL 94068	NDT	136	189	233
ICPL 96053	NDT	120	174	175
ICPL 96058	NDT	118	171	185
ICPL 96061	NDT	115	174	170
ICPL 97249	NDT	119	173	135
MA 3	NDT	125	190	260
MA 6	NDT	135	205	250
MAL 9	NDT	140	192	240
MARUTI	NDT	106	155	230
UPAS 120	NDT	88	110	140

“*”- Determinate; “#”- Non determinate

Table 2: Details on 148 microsatellite markers used for diversity analysis

*Marker name	Primer sequence	Repeats in cloned allele	Ta °C	Reference
CCB1	F- AAGGGTTGTATCTCCGCGTG R- GCAAAGCAGCAATCATTTCG	$(CA)_{10}$	57	Burns et al. 2001
CCB2	F- CCATAATCCAATCCAAATCC R- AGAAGGCTTTCATGTAACGC	$(CA)_{21}$	57	Burns et al. 2001
CCB3	F- TCACAAAAACAAGTTGCCAC R- ATGACCATGATTACGCCAAG	$(CA)_{10}$	52	Burns et al. 2001
CCB4	F- GGAGCTATGTTGGAGGATGA R- CCTTTTTGCATGGGTTGTAT	$(CA)_{31}$	57	Burns et al. 2001
CCB5	F- GACAATTTTGCATGCATTGC R- TTGCAAAAACACTTGGTTGG	$(CT)_{22}$	57	Burns et al. 2001
CCB6	F- ACAATGCTAGGGAACACCGC R- TACCTTAACCCACAATGGCC	$(CA)_{6}$	57	Burns et al. 2001
CCB7	F- CAACATTTGGACTAAAAACTG R- AGGTATCCAATATCCAACTTG	$(CT)_{16}$	55	Burns et al. 2001
CCB8	F- TGCGTTTGTAAGCATTCTTCA R- ACTTGAGGCTGAATGGATTTG	$(CT)_{30}$	52	Burns et al. 2001
CCB9	F- CACTTGGTTGGCTCAAGAAC R- GCCAATGAACTCACATCCTTC	$(CT)_{22}$	55	Burns et al. 2001
CCB10	F- CCTTCTTAAGGTGAAATGCAAGC R- CATAACAATAAAAGACCTTGAATGC	$(CA)_{15}$	50	Burns et al. 2001
CCac001	F- CTGGGCCTCTAGCATAGCAA R- AAACTTCTGGACGCAAAATGA	$(TG)_{6}$	58	Odeny et al. 2007

CCac002	F- GCGGGATTCTCTTGCTTAC R- TCACAAAACAATTTGGCACA	$(CA)_8$	48	Odeny et al. 2009
CCac003	F- TGCTTCAAGTTGCCTACCAG R- TCAAGGGAGGTGGACTACAAA	$(CA)_8$	48	Odeny et al. 2009
CCac004	F- TCTTAGCATGTCCTCTATTTTCGT R- AGTACATTTCAAATCCACACATCC	$(TA)_5(TG)_7ta(TG)_4$	48	Odeny et al. 2009
CCac007	F- AGGCTTTCTCCCTTCAATCC R- GCCTTTTCAAACTTTTCTCACA	$(CA)_7$	54	Odeny et al. 2009
CCac008	F- ACATGTGTGGCGTAGTGTGA R- GCAAAACCGTTCCATAAAAA	$(CA)_{10}cg(CA)_6$	48	Odeny et al. 2009
CCac009	F- GGGAAACTCACCTATATTACCAA R- CACTACCGTCTACAGCCATCTC	$(TG)(TC)_2(TG)_7$	48	Odeny et al. 2009
CCac010	F- GATAGCACACACACACACAACA R- TACCTTAGGGTCACCAACGA	$(CA)_7aca(TA)_3$	54	Odeny et al. 2009
CCac011	F- AAGTTGCCTACTGGGGGTTC R- AAATAGAGCTGTCAGGGGAGGT	$(CA)_8$	54	Odeny et al. 2009
CCac012	F- ACCTTGCTTGTTTCGCTTTT R- AAGGGAGGTGGACTACAAGGA	$(CA)_7$	58	Odeny et al. 2009
CCac013	F- GTGAGTGAGAGTGAGTGTATTTGTG R- GCTCTGATGCCAAATGTTGA	$(GT)_7$	58	Odeny et al. 2009
CCac014	F- CATCATAATCATACATGTCAATGCTA R- GGTTTTATCTTTGTCTCCAATTCTG	$(TG)_6n(GT)_{11}n(TG)_6$	58	Odeny et al. 2009
CCac015	F- TGGGAAACAAAATATCCCCTAA R- AGAGGGGTGTGATGAAGCAG	$(AC)_{10}c(CA)_{74}$	48	Odeny et al. 2009
CCac018	F- CAGGTCTGCTACTGCCATCA R- AGCCCACTTCTGCATCACTC	$(TG)_6(AGTG)_3$	48	Odeny et al. 2009

CCac019	F- CCACATCCCTCAACCCATAC R- GAAAAGCCCTTGATGACACC	$(AC)_7(CA)_3$	48	Odeny et al. 2009
CCac020	F- GGGAAACAAAATATCCCCTAATC R- TAATCACACACATCACACCTAGCA	$(AC)_4aa(AC)_{38}c(CA)_7$	48	Odeny et al. 2009
CCac021	F- CACGATTCCATTGGTGGAG R- ACGGTTTCTGGGAGGGTCTA	$(AC)_6aag(CTAA)_3$	48	Odeny et al. 2009
CCac022	F- GACTAGAAAATTCACCTCCGTCTG R- TTACAAAGGCTACATTGATGAGAAC	$(A)_5(CA)_6c(A)_4$	48	Odeny et al. 2009
CCac024	F- TCTTTCAGACGCAATGACCTT R- CACTTATTTGTGGGGACCATC	$(AC)_6a$	48	Odeny et al. 2009
CCac025	F- CAAGGAATCACTTAAAAACCAAGC R- AGATGGCCAAGATTCCACAAC	$(TG)_6$	48	Odeny et al. 2009
CCac027	F- GCCTTTTCAAACTTTTCTCA R- CATATGCTTTAAGTGCTTTCCT	$(GT)_6$	55	Odeny et al. 2009
CCac028	F- TGTATGTTCGTTTAGAGGCTTCC R- GCCCCTTTTCACTTTTCTCA	$(AC)_6$	55	Odeny et al. 2009
CCac029	F- TGCCTACTAGGGGTTTCGTG R- TGAACTATCCAGGGAGGTGAG	$(CA)_6c$	48	Odeny et al. 2009
CCac030	F- TGATTTGTGCTTGTGCCTTG R- GTCTTGCTTACGCGTGGACT	$(TGT)(TTG)_2(TG)_7$	48	Odeny et al. 2009
CCac031	F- AACGATGAAATTCCCAAACG R- TGTTAGATGCTCAACCCAAGG	$(CA)_8$	48	Odeny et al. 2009
CCac032	F- AGCCACTTAATAACCAAGCCTTTT R- GTGTATGCTTTACTTGCTTTCCTTT	$(TG)_7$	48	Odeny et al. 2009
CCac033	F- AAATTCACCACCATGATCCAA R- TCTTCACTTCCGAGACACAACT	$(GT)_7$	56	Odeny et al. 2009

CCac034	F- CAAGAAAGCACCCCTCGTAG R- ATAGGAGCATCCGTCGACAA	$(ATG)_4$	54	Odeny et al. 2009
CCac035	F- TGAGAGGCAATGATGTTGGA R- TCTACAGGCACCCTTTGAAAAT	$(AC)_7$	48	Odeny et al. 2009
CCac036	F- ATCGGCTTTTGTCTTGATGA R- AAGCTACAAGGGATACACATGC	$(CATA)_3ta(TG)_6$	48	Odeny et al. 2009
CCac038	F- GACGTGGTCATTGAAAGTAGCA R- AGACAAAAACTACACGCACTCAAG	$(TG)_7$	48	Odeny et al. 2009
CCac039	F- TTAGGGTCACCAGTGATGATATGT R- TTTCAGGTGCAGAAATAAAGGTTAG	$(TG)_8$	48	Odeny et al. 2009
CCac040	F- CGTGGACTAATCATCCCGTAA R- ATAATGCCAAAGGGGGAGAA	$(CAA)(CA)_6caa$	48	Odeny et al. 2009
CCat001	F- CTTCCCCCAACTAAGATCCA R- GTTCGTTCTCTTTAATTGACTTGC	$(TA)_8$	48	Odeny et al. 2007
CCat002	F- TTTCCTGAGCCATCAGTCG R- AAGCATCAACGTACCAGCAA	$(TA)_{10}(TG)_9$	54	Odeny et al. 2007
CCat003	F- TGAATTGCTGAGAGGACGTTT R- CTGTTCCAATTCCACGGTTT	$(TA)_{11}$	54	Odeny et al. 2007
CCat004	F- CTACAATCCCAGGGAAAAGG R- AACAAACGTAATCTGTGTTGATCTC	$(TA)_4(GATAG)(AT)_4$	48	Odeny et al. 2007
CCat005	F- TGAATTGCTGAGAGGACGTTT R- CTGTTCCAATTCCACGGTTT	$(TA)_{11}$	48	Odeny et al. 2009
CCat008	F- CTTCCCCCAACTAAGATCCA R- GTTCGTTCTCTTTAATTGACTTGC	$(TA)_4(GATAG)(AT)_4$	48	Odeny et al. 2009
CCat009	F- TTTCCTGAGCCATCAGTCG	$(TA)_{10}(TG)_9$	48	Odeny et al. 2009

	R- AAGCATCAACGTACCAGCAA			
CCat010	F- TCGTGGGAATGCTCTACAAC R- AACCACAAGTACACCCACACC	$(AT)_6$	48	Odeny et al. 2009
CCat011	F- TGCTCTAATGGCTAGTTCATCC R- AAACACTCATGGGTTAGATTCTCC	$(TA)_7(CA)_6$	48	Odeny et al. 2009
CCat012	F- CCACAAGTACACCCACACCA R- TTCGTGGGAATGCTCTACAA	$(AT)_6$	48	Odeny et al. 2009
CCcat001	F- TGATAGGGACCACAACGACA R- AGCGTTGACTCCTCCCTCTT	$(CAT)_4$	58	Odeny et al. 2007
CCcat002	F- ACGGTGCCTTGTTGATTGTA R- CGGAACAGGAGGAAAAGGTC	$(CAT)_6$	48	Odeny et al. 2009
CCcct004	F- ATCCTCCAAAAGTTCCACCA R- CAAAGGAGGATTTCCACCAA	$(CTC)_4$	48	Odeny et al. 2009
CCcta001	F- TGGGCATGGTAGAGGAAGTT R- CGTCATGAAGCAACAGGAGA	$(GAT)_5(TCT)(GAT)_4$	48	Odeny et al. 2009
CCcta002	F- ATCCCAGACTTCATAGGGAGATAG R- GTCTAGTCCCAGGTACAAAGAGGT	$(ACT)_4$	48	Odeny et al. 2009
CCcta003	F- GAGGAGGAGGAAGAAGAAGAAGA R- TCGTCGCCGTATCACTACAA	$(TGA)_{11}$	48	Odeny et al. 2009
CCcta004	F- TAGTATGGGCGTGGTAGAGGA R- CGTGACAGAGTCAATCAGAAGC	$(GAT)_4$	48	Odeny et al. 2009
CCcttc001	F- TAAGGAAATGGCTGGGGTTG R- CACATAAATTTGGGGGTTCG	$(CTTC)_4$	48	Odeny et al. 2007
CCgaaa002	F- GGACTTGTTACTGGGGCACT R- AATTCCCATGGTCATTCG	$(CTTT)_4$	48	Odeny et al. 2009
CCgaaaaa001	F- CTTTGTTCAGAGCGGAGCAT	$(GAAAAA)_5$	48	Odeny et al. 2009

	R- TTTTTAGGACATTGGGAAGCA			
CCggc001	F- CCATTGTGCGTCTTTGTGTT R- GCTTTTCCTCTTCCTTTCTCG	$(GGC)_4$	54	Odeny et al. 2007
CCgggaga001	F- GAGAAATATGAGAGGCAGAGAGAGA R- AAGATAATTCATTAGGGGGTGGA	$(GGGAGA)_4$	54	Odeny et al. 2009
CCggt001	F- ACGCTTCTGATGCTGTGTTG R- CATCAGCATCATCGTTACCC	$(GGT)_4$	54	Odeny et al. 2007
CCgtt001	F- ATAGGCCCATCTCCAGGTTC R- TTAATGCCCAGCCAATTCTT	$(AAC)_4$	54	Odeny et al. 2007
Ccgtt002	F- ATAGGCCCATCTCCAGGTTC R- TTAATGCCCAGCCAATTCTT	$(AAC)_4$	54	Odeny et al. 2009
CCgtt003	F- TGGGCTGTGATCGATGAAT R- CGACAACAACAACACCGACT	$(TGT)_4$	54	Odeny et al. 2009
CCgtt004	F- GTTCTTCTTGTTGTTGTTGTTG R- AATTCGTGGAGTTCATTGG	$(TTG)_5(TTC)_7$	48	Odeny et al. 2009
CCtacccg001	F- GTCGGGGCGTGTAAGTCATA R- CCGAAATAAGGATGGCAAAT	$(TACCCG)_4$	48	Odeny et al. 2007
CCtacccg002	F- GTCTTTGAGGGACGGAACC R- GGGGCGGGGAAAGTACATA	$(CGGGTA)_4$	48	Odeny et al. 2007
CCtc001	F- GACTCTTCACCTCACACTCATCAC R- ACCTCATACAACAACCCTAAGCAC	$(CT)_6tt(CT)_2$	48	Odeny et al. 2007
CCtc002	F- GACTCTTCACCTCACACTCATCAC R- ACCTCATACAACAACCCTAAGCAC	$(GA)_{12}$	48	Odeny et al. 2007
CCtc004	F- GGAAAACCCCGAGACAAAAG R- GGGCAACCCATAAACCCTAA	$(GA)_{12}$	48	Odeny et al. 2009
CCtc006	F- GCGCTAAGGGAAAACAAAAA	$(TC)_8$	48	Odeny et al. 2009

	R- AACTCCCTTGTTGTCATATGGTG			
CCtc007	F- CATTTATTTCTCTCTGGCATTCAC R- CGAGCTGCAAGCATAAACG	$(TC)_8$	48	Odeny et al. 2009
CCtc008	F- TGCACAGATTCGAAGGTTCC R- CCTCAAGATTCCTCTTTCTCTCA	$(AG)_{20}$	48	Odeny et al. 2009
CCtc012	F- GAGGATTGCACCAAGCAACT R- GCACTGCTGGCCTTACCATA	$(TC)_7$	48	Odeny et al. 2009
CCtc013	F- CTTCTCCCTGCCTCTTTTCC R- CAAGTGGAGGGGAGTGAAGA	$(TC)_6$	48	Odeny et al. 2009
CCtc014	F- GCGAAGAGGGTAAAGGGAAA R- CCGGTCACGAGAAATGTGTA	$(AG)_5aac(GA)_4$	48	Odeny et al. 2009
CCtc016	F- ATCATCAGATTCTTCAGCCGTA R- GGTTAGACCAATCCAATCAAGC	$(GA)_4ca(GA)_4n(GA)_8$	48	Odeny et al. 2009
CCtc018	F- ACAAATCCGGTGACCCATAA R- CCGAGAACAAAAACATTGAACA	$(TC)_6$	48	Odeny et al. 2009
CCtc020	F- CTAGGCCCTCGAGCTACATT R- TCTTTTAGAGGTGCGCTGTG	$(TC)_{13}$	48	Odeny et al. 2009
CCtta001	F- TTCTGGATCCCTTTCATTTTTC R- TGACACCCTTCTACCCCATAA	$(TC)_6$	48	Odeny et al. 2007
CCtta002	F- CCCATTTAGTGAGGGTTAAT R- GACTACTCCAGGTCAAACACG	$(TAT)_9$	48	Odeny et al. 2007
CCtta003	F- CCCATTTAGTGAGGGTTAAT R- GACTACTCCAGGTCAAACACG	$(TTA)_4$	54	Odeny et al. 2007
CCtta004	F- CCAAGAAAAGGTGCTCCAAGT R- TTGCTTCTTTTCTCGCTTGC	$(TTA)_4$	58	Odeny et al. 2007
CCtta005	F- TCTTCCATTGCATGGTGTT	$(AAT)_4$	54	Odeny et al. 2007

	R- GCATGATATGAGATGATGACGA			
CCtta007	F- ACCCATTATTGATTTGGGTA R- CCAAATTTCACCCAAGAAA	$(ATT)_4$	48	Odeny et al. 2009
CCtta008	F- TCTTCCATTGCATGGTGTT R- GCATGATATGAGATGATGACGA	$(AAT)_4$	48	Odeny et al. 2009
CCtta011	F- TCAGGGGTAAATGCGGTATC R- GAATTGCTTTTTGCTTCCTCA	$(ATT)_{21}$	48	Odeny et al. 2009
CCtta013	F- CAGGATTTTAATGGATTCTGCAA R- GGGTGAATACTATTTAAAAGGATAGG	$(ATT)_4$	48	Odeny et al. 2009
CCtta015	F- AACACGCACCTCAATTCCA R- GAATGAGGAATGAAGGGACAAA	$(AAT)_4$	48	Odeny et al. 2009
CCttat001	F- TACAGCAGCCACATCAAAGC R- TGAACCGTGAAAGTGGGATT	$(TTAT)_4$	54	Odeny et al. 2007
CCttc001	F- CGGGCTTCCTTTTCTTCTCT R- AAAACCCCGAAAACACCATT	$(TTC)_5$	54	Odeny et al. 2009
CCttc002	F- GGGAATTTTGTTGGGGTTTT R- TGCTTACGCGTGGACTAATG	$(GAA)_5g(GAA)_5$	48	Odeny et al. 2009
CCttc003	F- ACACCACCATGCTAAAGAACAAG R- CCAAGCAAGACACGAGTAATCATA	$(GAA)_5g(GAA)_5$	48	Odeny et al. 2009
CCttc005	F- ATCGCTTTGCATCCTTATC R- CTTCACGTACATTTTCGTTT	$(GAA)_{11}n(GAA)_5n(GAA)_{17}$	48	Odeny et al. 2009
CCttc006	F- GTAGAGGAGGTTCCAAATGACATA R- ATCTGTCTGGTGTTTTAGTGTGCT	$(GAA)_{11}gag(GAA)_5n(GAA)_{17}$	48	Odeny et al. 2009
CCttc007	F- CTCTTGCTTACGCGTGGACT R- CTTTTGCTTTTGCGTGCTT	$(GAA)_{16}$	48	Odeny et al. 2009

CCttc008	F- TCACAGAGGACCACACGAAG R- TGGACTAGACATTGCGTGAAG	$(AGA)_5$	48	Odeny et al. 2009
CCttc010	F- AGAGGGAAAGGGAAGAGAAGA R- TCAAGCAACTCCAAGAAATTCA	$(AGA)_4$	54	Odeny et al. 2009
CCttc011	F- AAGGCTTTTCAACAAATAGGG R- AGAAGAGAAAAAGCATAAAACTTCA	$(CTT)_4$	54	Odeny et al. 2009
CCttc012	F- TAATCCCATTCCGTTGTCGT R- CCCAGGAAGAGATGAGACCA	$(CTT)_4$	54	Odeny et al. 2009
CCttc015	F- TGTTCCGTTTCAAGTGGTCA R- CGACATTTACCCACTCGTTCA	$(AGA)_4$	54	Odeny et al. 2009
CCttc016	F- TAGAGCGTTGTCCCTTTTCTG R- TCGAAGGACAACTCAAGCATT	$(TTC)_7$	54	Odeny et al. 2009
CCttc018	F- ATGGGCATGGTAGAGGAGGT R- CGCTCATCATCGTCATCAAA	$(AGA)_{10}$	58	Odeny et al. 2009
CCttc019	F- AGTCGATGTGGAACATGAGGA R- TGTTGTAAGCCGTGGGTAGG	$(TTC)_4tgc(TTC)_3$	48	Odeny et al. 2009
CCttc020	F- AGGTGCAAAGGAAGCACTAAT R- CAGCTCCACTGTCTTCAACG	$(GAA)_2n(GAA)_4n(GAA)_2$	48	Odeny et al. 2009
CCttc022	F- TGGACTACCAAACGCAGACA R- TCGTAGCTGCAGAGCATTTT	$(GAA)_6$	48	Odeny et al. 2009
CCttc023	F- CGGCCCCTTCTATACTGTCA R- GAAAAGAGAAAAGAAGGAAAGAGGA	$(TCT)_4$	48	Odeny et al. 2009
CCttc025	F- TGGGCATGGTAGAGGAAGTT R- TCAGAAGTCGATGGCAAGTG	$(AGA)_{11}(GGAG)(GAA)_4ga(GGA)_3a(GAA)_{16}$	48	Odeny et al. 2009
CCttc030	F- ACAATTACTCAAATGCTCTCAACG R- TAAATGTCGCTTCCTATGATAGACC	$(AGA)_5$	48	Odeny et al. 2009

CCttc031	F- TGAAATGAACAAACCTCAATGG R- TGTATTGCACATTGACTTGGCTA	$(AAG)_{13}$	48	Odeny et al. 2009
CCttc033	F- ATTCCCTCTCTATCTCAGACTTTT R- TCGTGATGGAACTCAAGATACACT	$(CTT)_8$	48	Odeny et al. 2009
ICPM101	F- GGCCAAGTCACTGTCGAATC R- TGTAGTCCACGCGTAAGCAA	$(AC)_8(AT)_7$acat	48	Saxena et al. 2010
ICPM102	F- GCGGTGAAGATGGATGGAT R- CTCTTGCTTACGCGTGGACT	$(AG)_8$	48	Saxena et al. 2010
ICPM103	F- ATCCCGTAATGCACCTTTTG R- TTGGTCTGAATTGTGGCCTAT	$(AAC)_4$	48	Saxena et al. 2010
ICPM104	F- CGTCTATGGAGGGTTTTCAG R- AGACATTTATCAATCCAAGGTG	$(CA)_9$	48	Saxena et al. 2010
ICPM105	F- GCCATTACTTGAGTGTGAGTTTTG R- GTGTGTGTGTGTGTGTGTGTGT	$(AC)_{53}$	48	Saxena et al. 2010
ICPM106	F- CCTATCGGAAGGAGAAAAACATT R- TCGCTAAAGTCTTGGTAGATAATGG	$(GAC)_4$	48	Saxena et al. 2010
ICPM107	F- CCGGTTTAGGGTTTAGGGTTT R- GGTTGGAGGGTTTAGGGTTG	$(AAACCC)_{43}$	48	Saxena et al. 2010
ICPM108	F- TCTTCATCCTCACTCTTCCCCTAA R- GAGGTGCCCAAGGAAGATAG	$(TTTC)_4$	48	Saxena et al. 2010
ICPM109	F- CCTTTTCTTTGTCGGAATCACTAA R- CGGAGGCTGTTGGATCTAGTATTT	$(TC)_{13}$	48	Saxena et al. 2010
ICPM110	F- GGGGTGAATGGTAGTGGAAA R- TCCCTCTCTCCTCCCCTTAT	$(AGG)_4$	48	Saxena et al. 2010
ICPM111	F- CCAGCCGGATCGTTACACTA R- TGGTAGATTTTCTCGTGACTGC	$(TCT)_{29}$	48	Saxena et al. 2010

ICPM112	F- GCAGTCACGAGAAAATCTACCAC R- GGTTGATTATCGAATGAAATGGAG	$(ATA)_4$	48	Saxena et al. 2010
ICPM113	F- TGGGCATGGTAGAGGAAGTT R- CATCATAATCGTCTTCATCACTTG	$(GAA)_{47}$	48	Saxena et al. 2010
ICPM114	F- AAAAATTTCGTCCAAAGCTCCT R- GGAAGATTGAATTACATACCTCTCG	$(TC)_7$	48	Saxena et al. 2010
ICPM115	F- TGGGCATGGTAGAGGAAGTT R- CCCACCATTACCAAGCAAGT	$(AGA)_9$gaaagaa	48	Saxena et al. 2010
ICPM116	F- TCCTCTCTCCTCTTGTCTTGTC R- ATGGAGAAGTGAAAGGGATATGT	$(CTT)_4$	48	Saxena et al. 2010
ICPM117	F- TGCATGATATGAGATGATGGAGA R- CCCTTTTCACCCAAAAATACAA	$(ATT)_5$	48	Saxena et al. 2010
ICPM118	F- CATCCATTGGGTTGTTCTCA R- GGATTAAAGCGCACCATCAT	$(TTC)_5$	48	Saxena et al. 2010
ICPM119	F- CATGCGTATTGAATGAATTG R- TCTCGTCTGAGTGGGAGTGT	$(AC)_{53}$	48	Saxena et al. 2010
ICPM120	F- GCCCCTCTTACACCTTTTCTT R- CTCTTGCTTACGCGTGGACT	$(GT)_8$	48	Saxena et al. 2010
ICPM121	F- TTACCTGACGTGAAGTGAATGG R- CGTGCGACAGGACTACAATG	$(TTCCC)(TTC)_3$tcc$(TTC)_4$taca$(TCT)_7$	48	Saxena et al. 2010
ICPM122	F- TTGTCCGTAGCTCTCGTTTCT R- GCTATGCAGCGGTAAGTGTG	$(TTC)_4$	48	Saxena et al. 2010
ICPM123	F- TTGGGAAATGAAGGTTGAGC R- GCGTCGAGTAATCCATGAAAA	$(AG)_6$g$(C)_9$	48	Saxena et al. 2010
ICPM124	F- CATCAGGCGTTAGGAACTCTC R- TTGTGGATTGTGTTATGTGTGC	$(CA)_6(TA)_6(CA)_3$	48	Saxena et al. 2010

ICPM125	F- TCTTCGCTTTGAGGGGACTA R- GGGAATTTTGTTGGGGTTT	$(AGA)_6$	48	Saxena et al. 2010
ICPM126	F- TCAACACCTGATTAAGATTTGTTCC R- AGGGTTTCTCAAGTGGTAAGGTTT	$(CAA)g(CA)_5cg(CA)(TA)_2$	48	Saxena et al. 2010
ICPM127	F- CGAGCTCGAATTGACCCTAT R- TTTGTTTTTGGGCTCATTCC	$(AT)_{16}$	48	Saxena et al. 2010
ICPM128	F- CCAATCCTGGGCAGTTTCT R- GCGGGCTTCATGACAACTT	_	48	Saxena et al. 2010
ICPM129	F- TTGTTTTGGACCTTATTTTGTACTT R- CCCATTTTCCTTCTCTTCTAACC	$(TAT)_4$	48	Saxena et al. 2010
ICPM130	F- CAACATGACATCCTCCTCCA R- CTACGCCCCAAGAAACACAA	$(GTT)_4$	48	Saxena et al. 2010
ICPM131	F- CTACCTTGGCCAACCATTCT R- GGCACAGTTCTTCCACCATT	$(AAT)_4$	48	Saxena et al. 2010
ICPM132	F- TGAAAAGGTTCTCATGATCTCT R- GGCTCACTATAGGGCGAATTA	$(TC)_6$	48	Saxena et al. 2010

[#] Markers in bold faces were found polymorphic across 159 (A-, B- and R-) lines.

Table 3: SSR polymorphism among male sterile (A-), maintainer (B-) and restorer (R-) lines

SSR markers	A- lines		B- lines		R- lines		Across all lines	
	PIC value	No. of alleles	PIC value	No. of alleles	PIC value	No. of alleles	PIC value	No. of alleles
CCac003	0.40	2	0.33	2	0.37	2	0.37	2
CCac007	0.06	2	0.00	1	0.00	1	0.01	2
CCac012	0.69	3	0.66	3	0.36	3	0.56	4
CCac013	0.29	4	0.62	4	0.66	4	0.63	4
CCac020	0.49	2	0.40	2	0.31	2	0.43	2
CCac021	0.10	2	0.45	2	0.48	2	0.42	2
CCac022	0.16	2	0.47	2	0.44	4	0.51	4
CCac027	0.00	1	0.00	1	0.05	2	0.03	2
CCac031	0.06	2	0.00	1	0.00	1	0.01	2
CCac035	0.49	2	0.50	2	0.35	2	0.46	2
CCac036	0.40	2	0.41	3	0.37	2	0.39	3
CCac039	0.11	2	0.00	1	0.15	2	0.11	2
CCat005	0.36	2	0.07	2	0.05	2	0.10	3
CCat009	0.47	2	0.36	2	0.21	2	0.32	2
CCat011	0.49	2	0.46	2	0.46	2	0.47	2
CCB1	0.48	3	0.46	3	0.54	3	0.52	3
CCB10	0.44	4	0.72	5	0.63	5	0.71	5
CCB4	0.55	4	0.61	3	0.66	3	0.66	4
CCgaaaaa001	0.29	3	0.46	2	0.13	2	0.42	3
CCggt001	0.23	2	0.00	1	0.26	2	0.44	2
CCgtt002	0.12	2	0.15	2	0.37	3	0.29	3

CCgtt004	0.17	2	0.06	2	0.03	2	0.08	2
CCtc002	0.54	3	0.24	2	0.10	6	0.26	6
CCtc006	0.23	2	0.49	2	0.45	2	0.50	2
CCtc013	0.65	4	0.62	4	0.54	4	0.60	4
CCtc018	0.53	3	0.57	4	0.62	5	0.59	5
CCtta008	0.23	3	0.00	1	0.03	2	0.08	4
CCtta011	0.75	6	0.78	6	0.78	6	0.81	6
CCtta015	0.11	2	0.23	2	0.21	2	0.19	2
CCttc001	0.31	3	0.66	3	0.56	3	0.63	3
CCttc003	0.29	3	0.48	3	0.64	3	0.60	3
CCttc006	0.67	3	0.60	3	0.68	3	0.68	5
CCttc007	0.29	3	0.61	3	0.48	4	0.57	5
CCttc008	0.57	3	0.63	3	0.60	3	0.60	3
CCttc016	0.08	2	0.00	1	0.29	2	0.20	2
CCttc030	0.16	2	0.48	2	0.30	2	0.48	2
CCttc033	0.52	3	0.52	3	0.37	3	0.45	4
ICPM103	0.31	2	0.35	2	0.12	2	0.24	2
ICPM127	0.40	3	0.38	2	0.40	3	0.40	3
ICPM128	0.46	3	0.61	3	0.63	4	0.64	4
ICPM131	0.05	2	0.52	4	0.56	5	0.49	5
Maximum	0.75	6	0.78	6	0.78	6	0.80	6
Minimum	0.00	1	0.00	1	0.00	1	0.01	2
Mean	0.34	2.6	0.39	2.7	0.37	2.9	0.41	3.1

Table 4: Polymorphism features among the parental combinations of 56 hybrids

A- line	Hybrid no.	No. of poly. markers between A- and R- lines	Dissimilarity
ICPA 2039	ICPR 2364	19	0.34
ICPA 2039	ICPR 2447	22	0.40
ICPA 2039	ICPR 2429	21	0.35
ICPA 2039	ICPR 2460	20	0.41
ICPA 2039	ICPR 2463	24	0.40
ICPA 2039	ICPR 2469	21	0.33
ICPA 2039	ICPR 2384	19	0.28
ICPA 2039	ICPR 2438	22	0.37
ICPA 2039	ICPR 2441	18	0.28
ICPA 2039	ICPR 3310	13	0.21
ICPA 2039	ICPR 2533	11	0.20
ICPA 2039	ICPR 3301	12	0.19
ICPA 2039	ICPR 2459	19	0.33
ICPA 2042	ICPR 3507	18	0.35
ICPA 2042	ICPR 3525	20	0.40
ICPA 2042	ICPR 3522	20	0.41
ICPA 2042	ICPR 3513	19	0.35
ICPA 2042	ICPR 3514	17	0.30
ICPA 2042	ICPR 3516	16	0.28
ICPA 2042	ICPR 3519	18	0.34
ICPA 2042	ICPR 3521	19	0.27

ICPA 2042	ICPR 3524	18	0.38
ICPA 2042	ICPR 3510	22	0.20
ICPA 2043	ICPR 2671	18	0.26
ICPA 2043	ICPR 3468	17	0.25
ICPA 2043	ICPR 3472	16	0.27
ICPA 2043	ICPR 3475	21	0.37
ICPA 2043	ICPR 2691	22	0.37
ICPA 2043	ICPR 3466	15	0.26
ICPA 2043	ICPR 3462	16	0.26
ICPA 2043	ICPR 3338	15	0.24
ICPA 2043	ICPR 3337	16	0.22
ICPA 2043	ICPR 3467	21	0.31
ICPA 2043	ICPR 3470	14	0.21
ICPA 2043	ICPR 3471	17	0.25
ICPA 2043	ICPR 3473	17	0.25
ICPA 2043	ICPR 3756	13	0.20
ICPA 2043	ICPR 3469	14	0.26
ICPA 2043	ICPR 3340	14	0.20
ICPA 2043	ICPR 3341	11	0.14
ICPA 2043	ICPR 2681	17	0.27
ICPA 2043	ICPR 3812	17	0.24
ICPA 2043	ICPR 2698	15	0.22
ICPA 2043	ICPR 2669	20	0.30
ICPA 2043	ICPR 2674	18	0.39
ICPA 2045	ICPR 3374	16	0.28

ICPA 2047	ICPR 3352	25	0.40
ICPA 2047	ICPR 3477	8	0.14
ICPA 2047	ICPR 3344	13	0.26
ICPA 2047	ICPR 3351	12	0.19
ICPA 2048	ICPR 3494	18	0.37
ICPA 2048	ICPR 3369	14	0.27
ICPA 2048	ICPR 3366	19	0.32
ICPA 2051	ICPR 3386	21	0.39
ICPA 2051	ICPR 3407	21	0.37
ICPA 2051	ICPR 3394	18	0.31

Table5: Features of parental genotypes selected for developing mapping populations segregating for fertility restoration

Parental genotype	**Growth habit**	**Flower colour**	**Pod colour**	**No of polymorphic markers**	**Dissimilarity**
Parental combinations for early maturing group					
ICPA 2039	[a]DT	Yellow	[c]GPS	22	0.37
ICPR 2438	[b]NDT	Yellow	GPS		
ICPA 2039	DT	Yellow	GPS	22	0.40
ICPR 2447	NDT	Yellow	GPS		
Parental combinations for late maturing group					
ICPA 2043	NDT	Light Yellow	Green	18	0.26
ICPR 2671	NDT	Red with streaks	Purple		
ICPA 2043	NDT	Light Yellow	Green	21	0.31
ICPR 3467	NDT	Yellow with streaks	[d]GS		

[a]Determinate, [b]Non determinate, [c]Green with purple streaks, [d]Green with streaks

Table 6: Segregating populations produced/evaluated for inheritance study

Crosses	**Generation**	**No. of progenies**
Early maturing group		
ICPA 2039 × ICPR 2438	F_2	225
ICPA 2039 × ICPR 2447	F_2	222
Late maturing group		
ICPA 2043 × ICPR 2671	F_2	238
	BC_1F_1	138
ICPA 2043 × ICPR 3467	F_2	230
	BC_1F_1	189

Table 7: Segregation for male-fertility and sterility in F_1 and F_2 generations of early maturing crosses and their chi-square tests

Crosses	**Generation**	**Number of plants**			**Expected ratio**	**χ^2 calculated**	***p* value**
		Total	**Fertile**	**Sterile**			
ICPA 2039 × ICPR 2438	F_1	175	175	0	01:00	-	-
	F_2	225	212	13	15:01	0.09	0.77
ICPA 2039 × ICPR 2447	F_1	170	170	0	01:00	-	-
	F_2	222	210	12	15:01	0.27	0.60

Table 8: Segregation for male-fertility and sterility in F_1, F_2 and BC_1F_1 generations of late maturing crosses and their chi-square tests

Crosses	**Generation**	**Number of plants**			**Expected ratio**	**χ^2 calculated**	***p* value**
		Total	**Fertile**	**Sterile**			
ICPA 2043 × ICPR 2671	F_1	138	138	0	1:0	-	-
	F_2	238	223	15	15:1	0	0.97
	BC_1F_1	138	98	40	3:1	1.17	0.28
ICPA 2043 × ICPR 3467	F_1	90	90	0	1:0	-	-
	F_2	230	216	14	15:1	0.01	0.92
	BC_1F_1	189	140	49	3:1	0.09	0.77

Table 9: Segregation for male fertility and sterility in F_3 fertile progenies derived from cross ICPA 2043 × ICPR 3467

Progeny number	**Number of plants**			χ^2	***p* value**
	Total	**Fertile**	**Sterile**	**Calculated**	
Group1 (15:1 ratio)					
3	35	34	1	0.69	0.41
5	22	21	1	0.11	0.74
8	31	29	2	0.00	0.96
9	31	29	2	0.00	0.96
16	33	32	1	0.58	0.44
20	36	35	1	0.74	0.39
22	29	28	1	0.39	0.53
23	35	34	1	0.69	0.41
24	20	18	2	0.48	0.49
25	31	30	1	0.48	0.49
27	37	35	2	0.05	0.83
29	19	17	2	0.59	0.44
30	19	17	2	0.59	0.44
31	31	29	2	0.00	0.96
34	33	31	2	0.00	0.96
35	34	31	3	0.38	0.54
42	28	27	1	0.34	0.56
47	34	32	2	0.01	0.93
50	33	32	1	0.58	0.44
53	30	27	3	0.72	0.40
54	28	27	1	0.34	0.56

55	18	17	1	0.01	0.90
56	16	15	1	0.00	1.00
58	29	26	3	0.83	0.36
59	23	22	1	0.14	0.71
63	18	16	2	0.73	0.39
67	30	27	3	0.72	0.40
72	24	22	2	0.18	0.67
73	18	17	1	0.01	0.90
79	14	13	1	0.02	0.89
89	18	16	2	0.73	0.39
90	22	21	1	0.11	0.74
95	29	26	3	0.83	0.36
Pooled (n =33)	888	833	55	0.00	0.94
Group II (3:1 ratio)					
11	24	18	6	0.00	1.00
12	37	31	6	1.52	0.22
13	33	24	9	0.09	0.76
14	26	15	11	4.15	0.04
18	32	24	8	0.00	1.00
32	33	28	5	1.71	0.19
33	33	27	6	0.82	0.37
36	32	20	12	2.67	0.10
37	30	23	7	0.04	0.83
38	33	25	8	0.01	0.92
41	44	33	11	0.00	1.00
45	34	27	7	0.35	0.55

49	33	25	8	0.01	0.92
65	28	24	4	1.71	0.19
68	21	18	3	1.29	0.26
70	27	21	6	0.11	0.74
76	21	18	3	1.29	0.26
81	16	13	3	0.33	0.56
92	18	14	4	0.07	0.79
94	18	13	5	0.07	0.79
Pooled (n =20)	573	441	132	1.18	0.28

χ^2 calculated between two segregating groups (15:1 *vs* 3:1) for 1:1 ratio is 3.19 (p = 0.07).

Table 10: Polymorphic SSR markers and their goodness of fit in F_2 mapping population derived from cross ICPA 2039 × ICPR 2447

Polymorphic marker	**Allele size in (bp)**		**Number of**			χ^2	***p* value**
	ICPA 2039	**ICPR 2447**	**A**	**B**	**H**		
CcM0008	193	195	48	47	89	0.21	0.90
CcM0047	176	183	55	39	90	2.87	0.24
CcM0057	283	287	42	61	73	9.22	0.01
CcM0095	224	228	50	43	86	0.82	0.66
CcM0121	285	278	51	43	89	0.84	0.66
CcM0126	240	237	13	48	122	33.72	0.00
CcM0133	204	197	37	48	86	1.42	0.49
CcM0179	205	209	29	66	86	15.57	0.00
CcM0181	292	290	41	53	93	1.55	0.46
CcM0183	252	254	48	29	73	4.92	0.09
CcM0195	237	234	49	44	93	0.27	0.87
CcM0246	250	248	4	42	141	63.71	0.00
CcM0252	246	256	48	45	92	0.10	0.95
CcM0257	254	258	47	46	94	0.02	0.99
CcM0268	217	223	48	54	81	2.80	0.25
CcM0293	250	247	43	53	75	3.75	0.15
CcM0322	292	306	58	40	72	7.79	0.02
CcM0381	267	266	46	51	77	2.59	0.27
CcM0407	213	215	51	44	91	0.61	0.74
CcM0431	139	137	46	49	85	0.66	0.72
CcM0443	260	274	31	24	43	2.47	0.29
CcM0445	249	247	49	41	93	0.75	0.69

CcM0468	208	206	24	26	40	1.20	0.55
CcM0522	199	203	23	32	40	4.07	0.13
CcM0596	249	247	17	29	48	3.11	0.21
CcM0602	223	229	19	24	50	1.06	0.59
CcM0603	244	238	24	23	48	0.03	0.98
CcM0611	280	274	55	27	104	11.03	0.00
CcM0673	291	289	50	39	97	1.65	0.44
CcM0710	291	294	26	33	37	6.06	0.05
CcM0810	213	211	26	30	31	7.55	0.02
CcM0843	291	277	18	15	15	7.13	0.03
CcM0974	178	182	128	0	6	355.61	0.00
CcM0978	300	302	106	34	2	207.13	0.00
CcM0988	247	251	58	48	60	13.95	0.00
CcM1001	278	268	39	87	52	56.65	0.00
CcM1045	279	281	21	48	99	14.04	0.00
CcM1108	268	275	145	13	19	306.04	0.00
CcM1109	229	226	40	42	91	0.51	0.77
CcM1143	226	228	33	19	54	3.74	0.15
CcM1235	242	248	57	40	90	3.35	0.19
CcM1246	207	213	46	57	85	3.01	0.22
CcM1251	230	241	47	49	74	2.89	0.24
CcM1263	245	247	34	35	102	6.38	0.04
CcM1282	274	268	34	66	62	21.56	0.00
CcM1366	307	297	19	38	39	10.90	0.00
CcM1398	247	253	41	44	96	0.77	0.68
CcM1438	235	232	27	56	105	11.52	0.00

CcM1493	177	179	46	50	92	0.26	0.88
CcM1503	198	191	39	70	64	22.82	0.00
CcM1522	226	232	49	34	82	2.73	0.25
CcM1565	296	304	42	51	87	1.10	0.58
CcM1611	260	256	58	49	81	4.46	0.11
CcM1616	231	235	45	51	85	1.07	0.59
CcM1651	247	249	40	44	77	0.50	0.78
CcM1680	294	292	11	15	10	8.00	0.02
CcM1713	189	194	43	51	86	1.07	0.59
CcM1725	235	245	79	19	1	167.77	0.00
CcM1735	158	164	47	37	99	2.32	0.31
CcM1809	286	288	44	47	95	0.18	0.91
CcM1818	174	175	44	30	13	47.28	0.00
CcM1821	227	224	44	40	94	0.74	0.69
CcM1837	271	273	51	41	95	1.12	0.57
CcM1886	265	262	41	62	80	7.71	0.02
CcM1895	286	284	41	41	105	2.83	0.24
CcM1935	208	204	50	43	88	0.68	0.71
CcM1976	204	200	35	14	43	9.98	0.01
CcM1984	304	295	38	45	84	0.59	0.74
CcM2046	271	273	50	40	97	1.33	0.51
CcM2066	122	120	50	45	90	0.41	0.82
CcM2097	217	213	44	92	50	64.54	0.00
CcM2126	253	256	39	48	99	1.65	0.44
CcM2128	230	227	49	50	83	1.42	0.49
CcM2149	262	256	9	80	95	54.99	0.00

CcM2164	200	202	42	62	80	7.48	0.02
CcM2221	215	221	41	43	103	1.97	0.37
CcM2228	291	293	43	47	92	0.20	0.91
CcM2241	147	145	49	46	89	0.29	0.86
CcM2283	182	177	42	67	65	18.31	0.00
CcM2296	210	214	37	49	61	6.21	0.04
CcM2332	254	251	54	44	89	1.50	0.47
CcM2380	275	257	93	17	66	76.64	0.00
CcM2413	279	277	41	64	81	8.78	0.01
CcM2460	296	298	45	47	83	0.51	0.78
CcM2463	230	224	50	45	92	0.32	0.85
CcM2530	261	257	46	49	87	0.45	0.80
CcM2565	285	288	43	44	96	0.45	0.80
CcM2639	156	148	35	68	83	13.86	0.00
CcM2704	147	145	46	38	103	2.61	0.27
CcM2707	250	252	32	30	122	19.61	0.00
CcM2735	225	223	54	50	84	2.30	0.32
CcM2751	225	223	23	44	100	11.80	0.00
CcM2753	290	293	41	45	91	0.32	0.85
CcM2802	290	287	50	49	87	0.78	0.68
CcM2855	298	289	38	51	76	3.07	0.22
CcM2871	210	208	49	51	83	1.62	0.44
CcM2891	229	231	41	44	100	1.31	0.52
CcM2909	180	184	49	45	84	0.74	0.69

Table 11: Polymorphic SSR markers and their goodness of fit in F_2 mapping population derived from cross ICPA 2043 × ICPR 2671

Polymorphic marker	**Allele size in (bp)**		**Number of**			χ^2	***p* value**
	ICPA 2043	**ICPR 2671**	**A**	**B**	**H**		
CCac009	248	250	66	95	8	148.47	0.00
CCB4	221	229	48	31	84	3.70	0.16
CcM0008	197	199	41	52	68	5.39	0.07
CcM0021	296	299	39	35	93	2.35	0.31
CcM0030	231	233	45	45	73	1.77	0.41
CcM0051	290	284	22	31	33	6.53	0.04
CcM0080	196	190	19	23	34	1.26	0.53
CcM0093	277	284	45	43	88	0.05	0.98
CcM0121	284	288	32	40	78	1.09	0.58
CcM0126	249	237	46	41	91	0.37	0.83
CcM0133	197	205	42	40	77	0.21	0.90
CcM0176	254	252	44	40	99	1.40	0.50
CcM0185	252	248	22	43	85	8.55	0.01
CcM0195	238	236	42	54	67	6.93	0.03
CcM0207	249	251	31	42	69	1.82	0.40
CcM0246	250	242	49	45	89	0.31	0.86
CcM0248	297	292	33	26	31	9.80	0.01
CcM0252	254	250	15	31	42	6.00	0.05
CcM0257	256	250	30	30	82	3.41	0.18
CcM0268	223	229	41	38	75	0.22	0.90
CcM0374	182	181	45	43	76	0.93	0.63
CcM0381	277	275	45	49	71	3.40	0.18

CcM0392	164	162	50	43	82	1.25	0.53
CcM0399	271	263	38	36	85	0.81	0.67
CcM0413	270	273	31	45	79	2.59	0.27
CcM0444	206	203	39	67	67	17.86	0.00
CcM0445	247	249	12	24	54	6.80	0.03
CcM0468	205	199	29	49	92	5.86	0.05
CcM0471	280	273	11	24	49	6.36	0.04
CcM0476	253	255	41	45	82	0.29	0.87
CcM0484	255	251	35	42	63	2.10	0.35
CcM0494	127	134	51	31	90	5.02	0.08
CcM0502	261	255	50	48	58	10.31	0.01
CcM0516	204	208	43	40	85	0.13	0.94
CcM0522	205	209	47	34	76	2.31	0.31
CcM0588	296	293	51	37	77	3.11	0.21
CcM0602	227	232	47	44	72	2.33	0.31
CcM0611	274	276	38	57	86	4.44	0.11
CcM0624	284	281	44	44	83	0.15	0.93
CcM0627	296	294	32	50	98	5.02	0.08
CcM0637	184	176	37	53	81	3.47	0.18
CcM0721	190	186	46	42	79	0.68	0.71
CcM0737	189	193	46	37	84	0.98	0.61
CcM0743	268	266	28	50	92	6.85	0.03
CcM0752	244	242	31	43	63	2.99	0.22
CcM0810	217	219	39	43	69	1.33	0.51
CcM0820	185	198	56	42	62	10.55	0.01
CcM0824	190	188	40	64	57	20.88	0.00

CcM0834	282	280	49	38	90	1.42	0.49
CcM0849	281	283	34	39	99	4.22	0.12
CcM0859	257	260	39	32	61	1.50	0.47
CcM0859	257	260	19	19	27	1.86	0.39
CcM0882	294	282	14	29	38	5.86	0.05
CcM0887	187	185	29	50	85	5.60	0.06
CcM0922	218	231	30	20	25	11.00	0.00
CcM0956	231	239	39	51	82	2.05	0.36
CcM0978	297	304	32	40	89	2.59	0.27
CcM0988	247	251	47	44	87	0.19	0.91
CcM1079	287	289	41	48	83	0.78	0.68
CcM1105	253	249	65	21	73	25.42	0.00
CcM1110	253	256	21	16	48	2.01	0.37
CcM1139	220	217	41	37	90	1.05	0.59
CcM1146	191	189	15	19	35	0.48	0.79
CcM1207	246	244	39	52	68	5.45	0.07
CcM1232	284	286	33	37	96	4.27	0.12
CcM1238	244	246	128	0	48	222.55	0.00
CcM1246	207	209	46	38	72	1.74	0.42
CcM1258	280	278	18	26	36	2.40	0.30
CcM1263	245	241	36	47	75	1.94	0.38
CcM1266	197	202	47	36	94	2.05	0.36
CcM1277	276	263	39	52	77	3.18	0.20
CcM1278	268	280	14	25	37	3.24	0.20
CcM1282	267	273	9	20	44	6.40	0.04
CcM1313	272	274	40	45	79	0.52	0.77

CcM1357	286	294	38	38	102	3.80	0.15
CcM1366	312	295	39	47	80	0.99	0.61
CcM1392	215	208	29	47	91	5.23	0.07
CcM1398	250	247	43	39	80	0.22	0.89
CcM1447	282	285	162	1	6	452.61	0.00
CcM1459	197	190	32	30	99	8.55	0.01
CcM1503	210	196	49	52	77	3.34	0.19
CcM1506	289	295	41	47	81	0.72	0.70
CcM1522	232	224	43	54	78	3.45	0.18
CcM1559	275	273	37	45	96	1.82	0.40
CcM1565	282	304	57	28	71	12.04	0.00
CcM1584	282	270	40	48	72	2.40	0.30
CcM1597	241	257	32	42	105	6.49	0.04
CcM1602	263	265	42	41	80	0.07	0.97
CcM1609	232	225	42	46	83	0.33	0.85
CcM1615	261	253	26	37	33	11.90	0.00
CcM1616	249	237	11	145	25	293.22	0.00
CcM1635	272	289	47	46	82	0.70	0.70
CcM1647	271	269	39	39	72	0.24	0.89
CcM1688	263	255	42	5	41	31.52	0.00
CcM1707	294	296	48	38	93	1.39	0.50
CcM1744	240	242	41	46	77	0.91	0.63
CcM1770	183	180	20	24	40	0.57	0.75
CcM1837	271	275	41	46	81	0.51	0.77
CcM1886	286	289	48	47	86	0.46	0.80
CcM1895	284	288	41	53	78	3.16	0.21

CcM1962	234	230	40	37	80	0.17	0.92
CcM1976	204	200	63	0	12	140.52	0.00
CcM1982	263	265	45	29	80	3.56	0.17
CcM1984	304	298	34	39	91	2.28	0.32
CcM1991	215	219	42	42	84	0.00	1.00
CcM1997	227	236	37	41	63	1.82	0.40
CcM1999	183	177	45	40	91	0.49	0.78
CcM2012	262	239	28	48	86	5.56	0.06
CcM2060	284	288	44	53	82	2.16	0.34
CcM2095	229	247	57	34	81	6.73	0.03
CcM2097	217	211	51	47	84	1.25	0.53
CcM2149	250	252	41	44	93	0.46	0.79
CcM2176	272	275	48	54	79	3.32	0.19
CcM2228	295	293	42	43	76	0.52	0.77
CcM2237	242	246	18	24	42	0.86	0.65
CcM2280	210	206	40	37	69	0.56	0.76
CcM2281	242	244	47	38	89	1.02	0.60
CcM2283	184	182	45	43	77	0.78	0.68
CcM2296	210	216	42	47	88	0.29	0.87
CcM2314	318	310	32	38	85	1.92	0.38
CcM2341	286	289	38	45	96	1.49	0.47
CcM2370	286	294	28	136	17	248.27	0.00
CcM2371	292	297	42	55	82	3.15	0.21
CcM2379	166	168	41	43	73	0.82	0.66
CcM2380	260	266	18	18	53	3.25	0.20
CcM2394	277	279	36	49	95	2.43	0.30

CcM2505	219	216	41	38	89	0.70	0.70
CcM2517	239	236	42	34	84	1.20	0.55
CcM2542	300	290	35	58	80	7.09	0.03
CcM2639	146	155	41	34	76	0.66	0.72
CcM2704	145	143	43	41	93	0.50	0.78
CcM2707	246	252	60	47	73	8.30	0.02
CcM2751	225	221	31	31	81	2.52	0.28
CcM2781	236	239	49	33	90	3.35	0.19
CcM2802	284	287	44	41	87	0.13	0.94
CcM2852	241	245	32	43	87	2.38	0.30
CcM2855	292	289	31	51	80	4.96	0.08
CcM2871	208	210	52	34	89	3.75	0.15
CcM2891	233	231	169	0	8	469.17	0.00
CcM2895	260	262	45	43	86	0.07	0.97
CcM2898	260	262	38	43	81	0.31	0.86
CcM2906	219	240	37	36	80	0.33	0.85
CcM2948	231	234	52	47	78	2.77	0.25
CcM2955	226	234	12	22	41	3.32	0.19
CCttc006	295	301	39	48	64	4.58	0.10

Table 12: Characteristics of ICPA 2039 × ICPR 2447 linkage map of pigeonpea

Linkage group	**No. of markers**	**Length (cM)**	**Density (marker/cM)**
LG1	8	98.1	12.26
LG2	8	63.8	7.98
LG3	9	95.8	10.64
LG4	8	76.7	9.59
LG5	3	6.3	2.1
LG6	12	54.6	4.55
LG7	10	166.2	16.62
LG8	4	33.9	8.48
LG9	5	30.1	6.02
LG10	9	103.2	11.47
LG11	6	74.1	12.35
Total	82	802.8	9.79

Table 13: Characteristics of ICPA 2043 × ICPR 2671 linkage map of pigeonpea

Linkage group	**No. of markers**	**Length (cM)**	**Density (marker/cM)**
LG1	10	64.7	6.47
LG2	2	22.4	11.2
LG3	16	64.7	4.04
LG4	17	165.2	9.72
LG5	7	32.1	4.59
LG6	20	120.8	6.04
LG7	12	121.1	10.09
LG8	10	86.5	8.65
LG9	10	72.1	7.21
LG10	9	40.8	4.53
LG11	4	80.6	20.15
Total	117	871	7.44

Table 14: Details on QTLs identified for fertility restoration in ICPA 2039 × ICPR 2447 F_2 mapping population

Linkage group	LOD value	Phenotypic variation (%)	Flanking markers
LG3	**4.2**	**20**	**CcM1109 - CcM1522**
LG2	1.0	4	CcM2463 – CcM1809
LG7	**44.0**	**50**	**CcM2149 - CcM0468**
LG9	1.4	4	CcM0431 - CcM1611
LG11	**5.7**	**25**	**CcM0381 - CcM2735**
LG11	**5.0**	**22**	**CcM2735 - CcM1713**

Table 15: Details on QTLs identified for fertility restoration in ICPA 2043 × ICPR 2671 F_2 mapping population

Linkage group	**LOD value**	**Phenotypic variation (%)**	**Flanking markers**
LG1	1.8	3	CcM1079 - CcM1635
LG3	**5.4**	**24**	**CcM2542 - CcM1277**
LG4	1.8	5	CcM2898 - CcM0627
LG6	**2.6**	**10**	**CcM0824 - CcM0392**

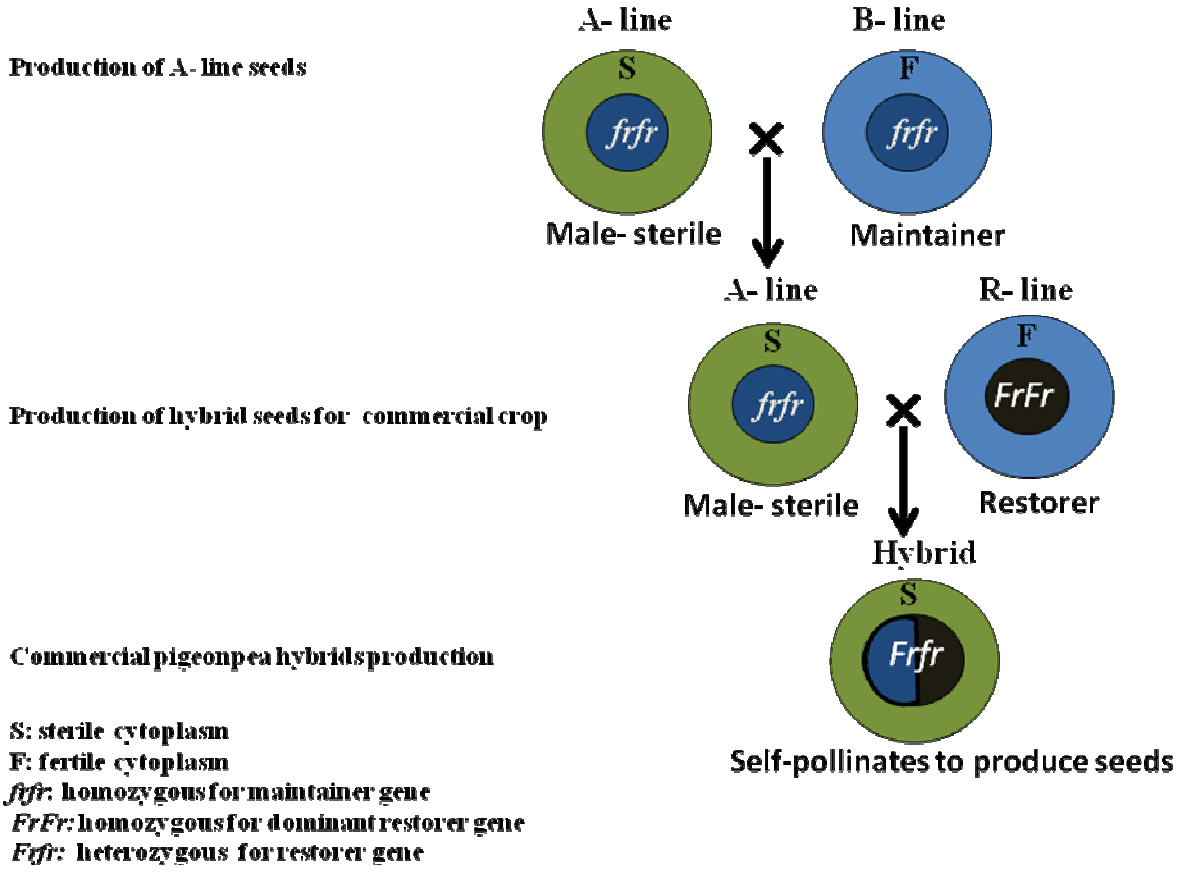


Figure 1: A general methodology for the production of commercial hybrids by using cytoplasmic male sterility system

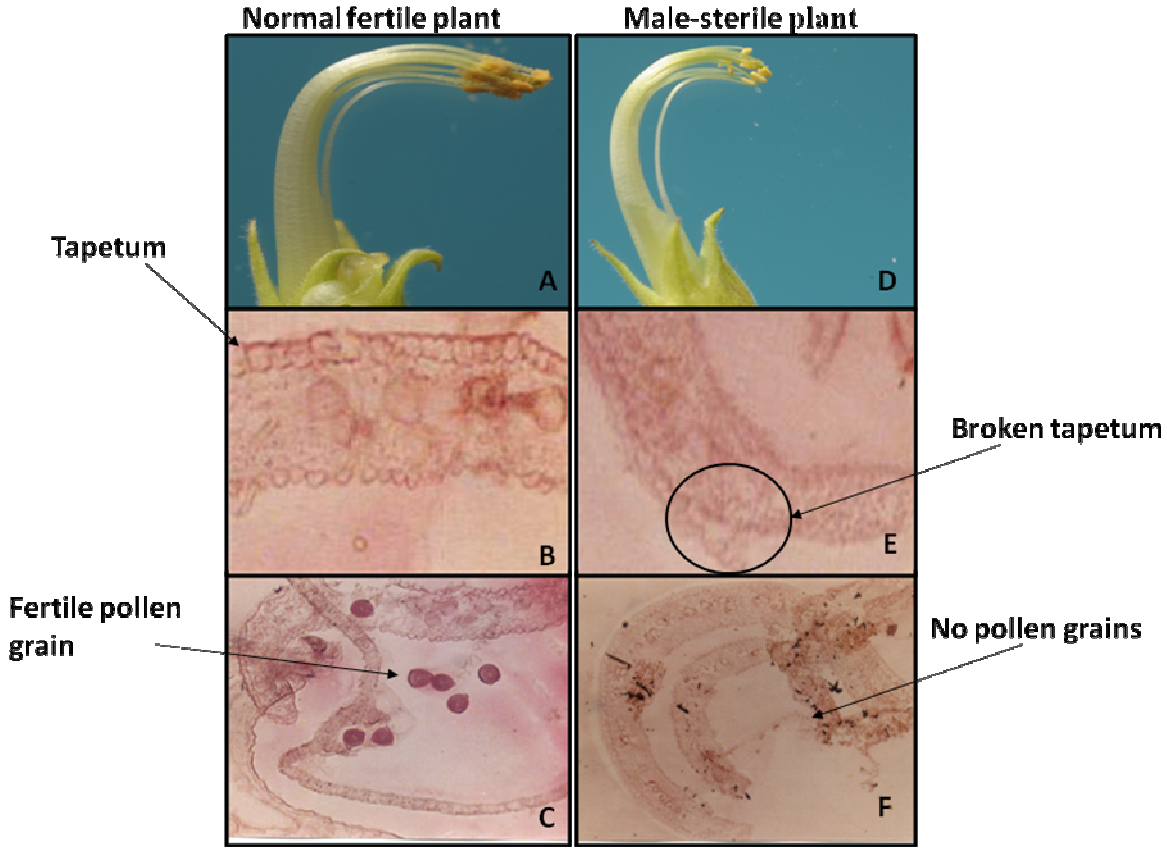


Figure 2: Evidences of CMS in pigeonpea. (A) Anther of male fertile flower containing full load of pollen grains. (B) Transverse section of a normal fertile anther, it shows intact tapetum. (C) Acetocarmine staining visualized stained normal fertile pollen grains. (D) Anther of male sterile flower containing no pollen grains. (E) Transverse section of a male sterile anther, it shows broken tapetum. (F) Fully male sterile having no stained pollen grains

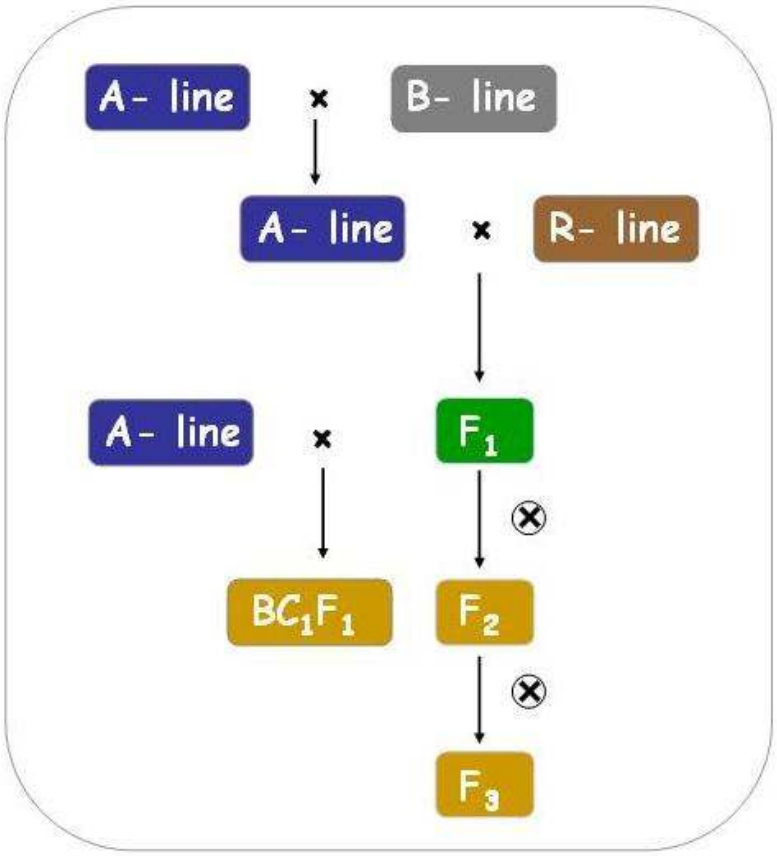


Figure 3: Crossing scheme for inheritance studies

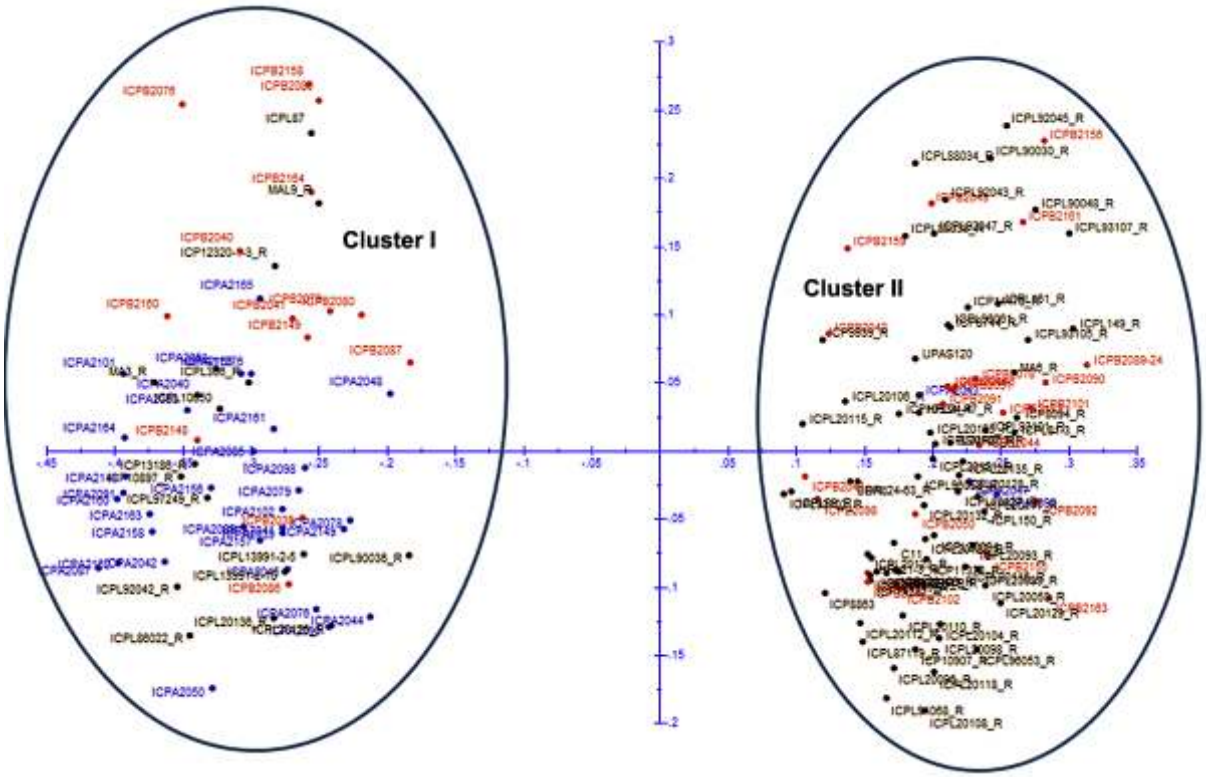


Figure 4: Diversity analysis among 159 (37 A-, 38 B-, and 84 R-) lines of pigeonpea.

The figure shows A-, B- and R- lines in blue, red and black colours, respectively

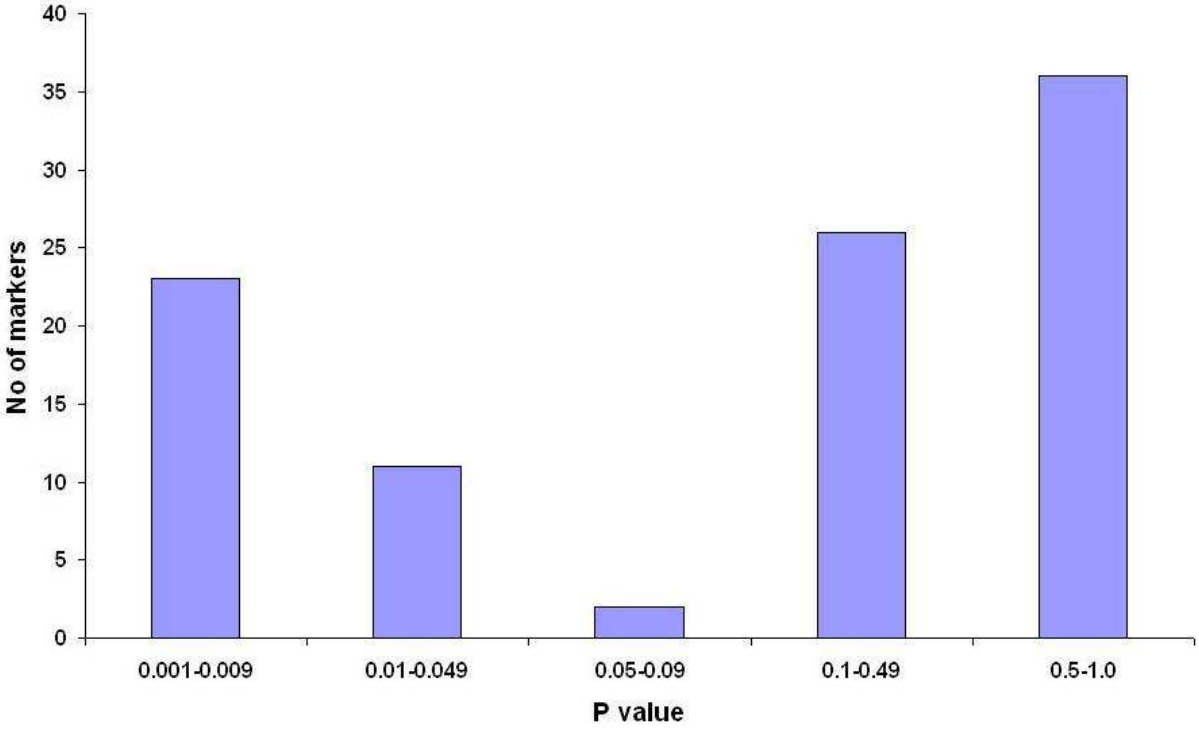


Figure 5: Distribution of segregation of 98 polymorphic SSR markers on 188 F_2s derived from cross ICPA 2039 × ICPR 2447

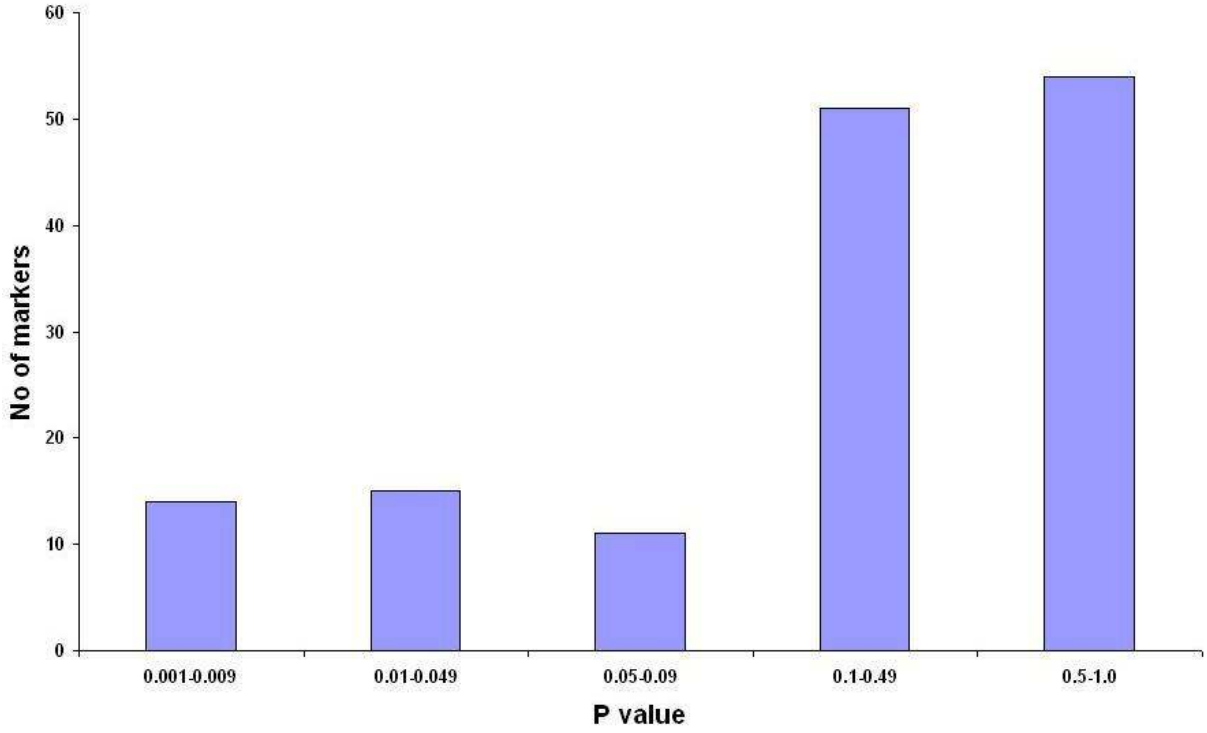
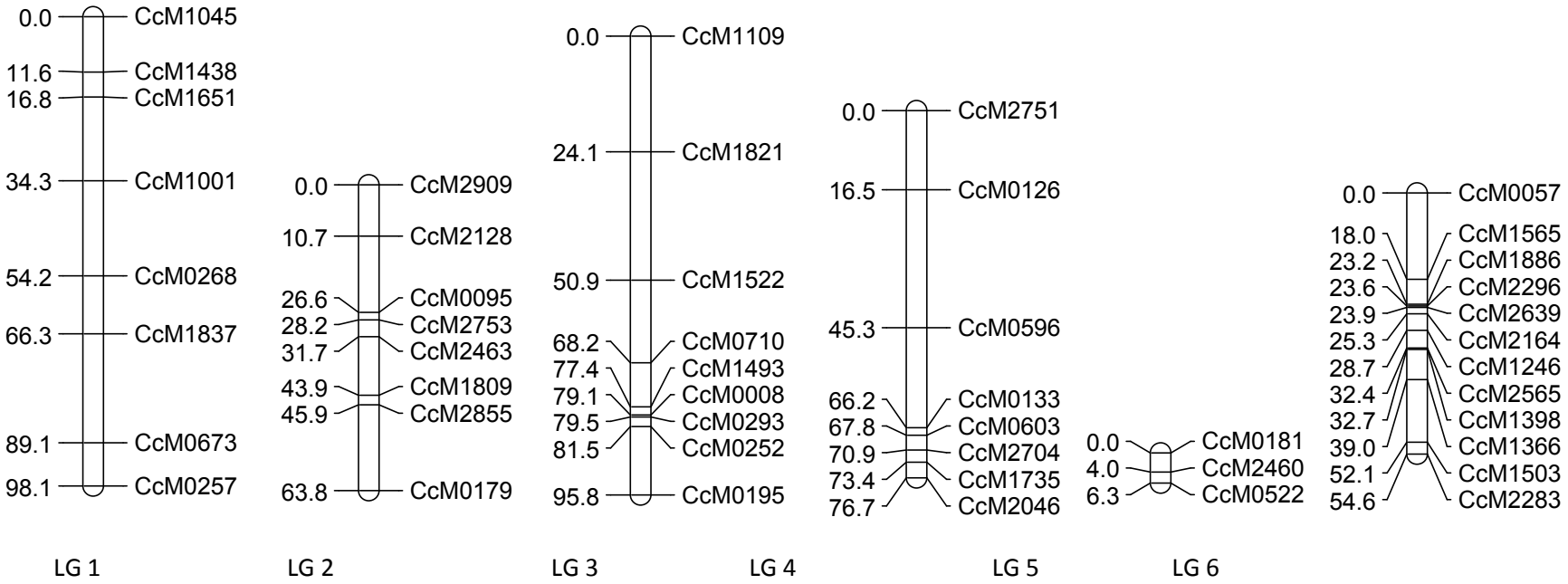


Figure 6: Distribution of segregation of 145 polymorphic SSR markers on 188 F_2s derived from cross ICPA 2043 × ICPR 2671


0.0 CcM1045
11.6 CcM1438
16.8 CcM1651
34.3 CcM1001
54.2 CcM0268
66.3 CcM1837
89.1 CcM0673
98.1 CcM0257
LG 1
0.0 CcM2909
10.7 CcM2128
26.6 CcM0095
28.2 CcM2753
31.7 CcM2463
43.9 CcM1809
45.9 CcM2855
63.8 CcM0179
LG 2
0.0 CcM1109
24.1 CcM1821
50.9 CcM1522
68.2 CcM0710
77.4 CcM1493
79.1 CcM0008
79.5 CcM0293
81.5 CcM0252
95.8 CcM0195
LG 3
0.0 CcM2751
16.5 CcM0126
45.3 CcM0596
66.2 CcM0133
67.8 CcM0603
70.9 CcM2704
73.4 CcM1735
76.7 CcM2046
LG 4
LG 5
0.0 CcM0181
4.0 CcM2460
6.3 CcM0522
LG 6
0.0 CcM0057
18.0 CcM1565
23.2 CcM1886
23.6 CcM2296
23.9 CcM2639
25.3 CcM2164
28.7 CcM1246
32.4 CcM2565
32.7 CcM1398
39.0 CcM1366
52.1 CcM1503
54.6 CcM2283

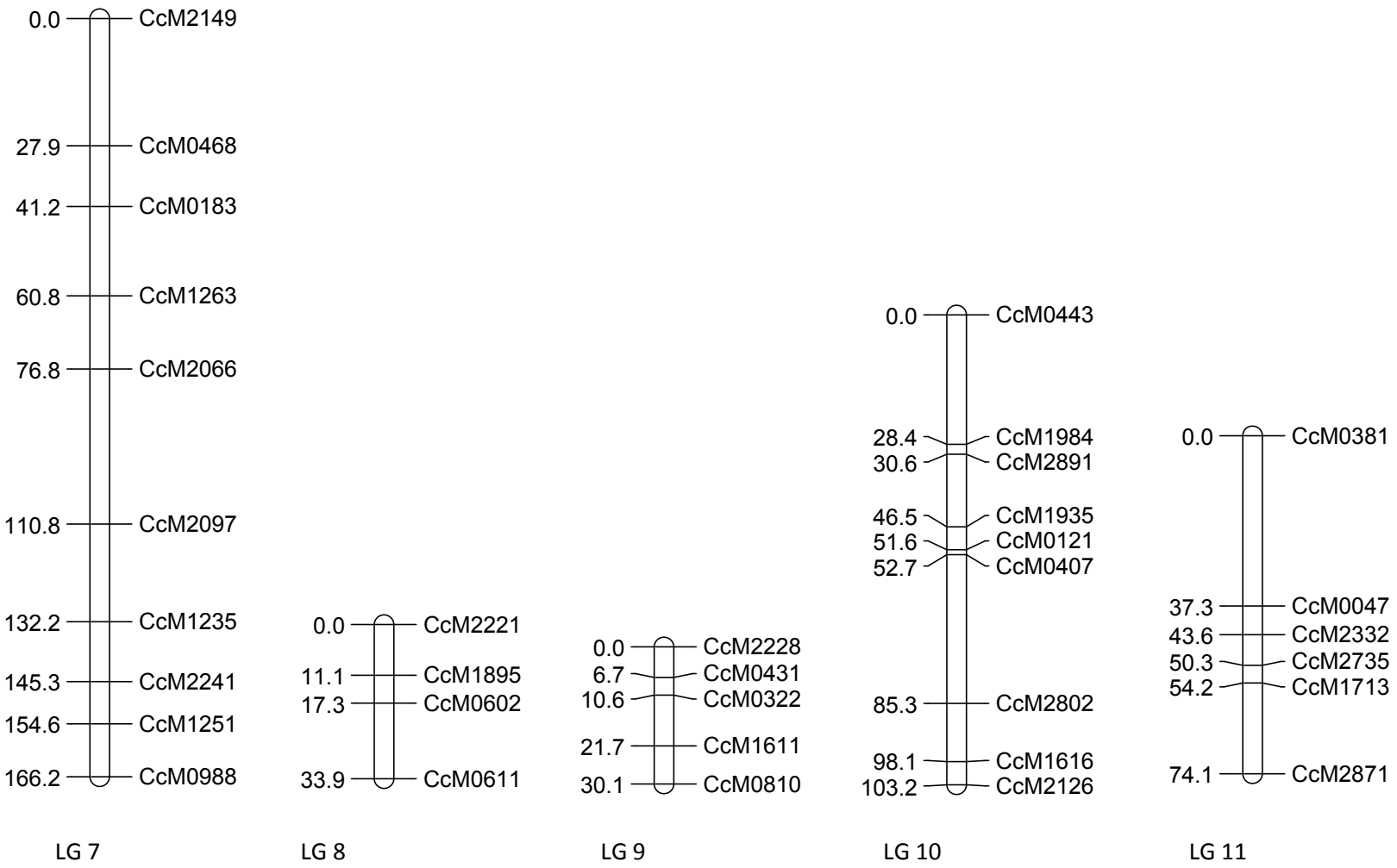
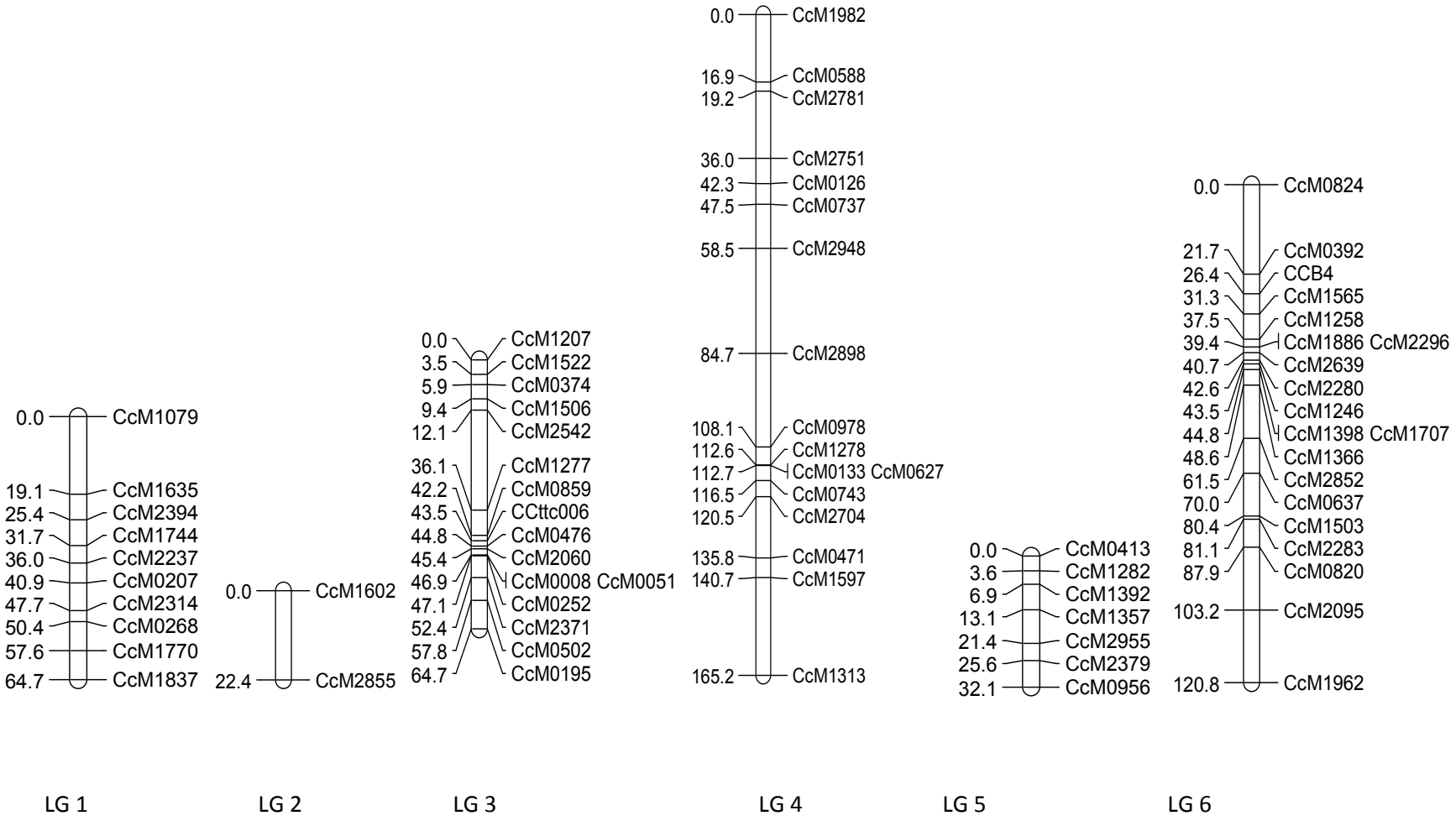


Figure 7: Pigeonpea linkage map using 82 polymorphic SSR markers on 188 F_2s derived from cross ICPA 2039 × ICPR 2447; name of markers are on the right side, while the cumulative genetic distances (cM) are on the left side of the map


0.0 CcM1079
19.1 CcM1635
25.4 CcM2394
31.7 CcM1744
36.0 CcM2237
40.9 CcM0207
47.7 CcM2314
50.4 CcM0268
57.6 CcM1770
64.7 CcM1837
0.0 CcM1602
22.4 CcM2855
0.0 CcM1207
3.5 CcM1522
5.9 CcM0374
9.4 CcM1506
12.1 CcM2542
36.1 CcM1277
42.2 CcM0859
43.5 CCttc006
44.8 CcM0476
45.4 CcM2060
46.9 CcM0008 CcM0051
47.1 CcM0252
52.4 CcM2371
57.8 CcM0502
64.7 CcM0195
0.0 CcM1982
16.9 CcM0588
19.2 CcM2781
36.0 CcM2751
42.3 CcM0126
47.5 CcM0737
58.5 CcM2948
84.7 CcM2898
108.1 CcM0978
112.6 CcM1278
112.7 CcM0133 CcM0627
116.5 CcM0743
120.5 CcM2704
135.8 CcM0471
140.7 CcM1597
165.2 CcM1313
0.0 CcM0413
3.6 CcM1282
6.9 CcM1392
13.1 CcM1357
21.4 CcM2955
25.6 CcM2379
32.1 CcM0956
0.0 CcM0824
21.7 CcM0392
26.4 CCB4
31.3 CcM1565
37.5 CcM1258
39.4 CcM1886 CcM2296
40.7 CcM2639
42.6 CcM2280
43.5 CcM1246
44.8 CcM1398 CcM1707
48.6 CcM1366
61.5 CcM2852
70.0 CcM0637
80.4 CcM1503
81.1 CcM2283
87.9 CcM0820
103.2 CcM2095
120.8 CcM1962
LG 1
LG 2
LG 3
LG 4
LG 5
LG 6

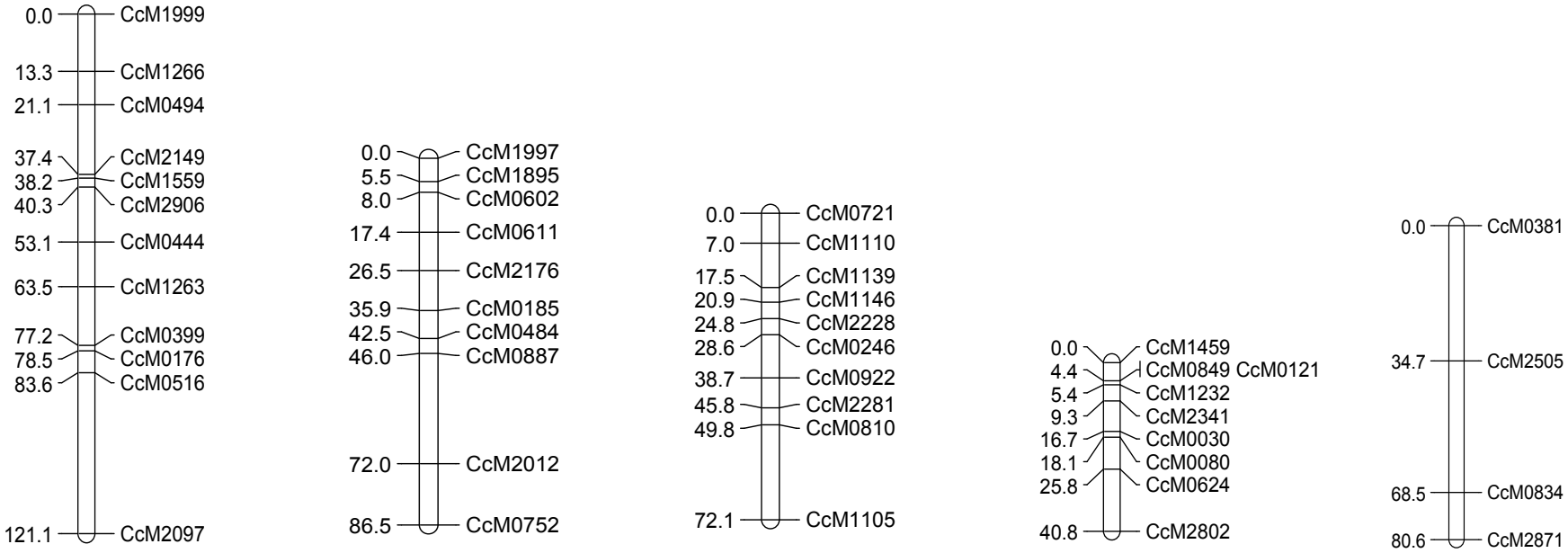


Figure 8: Pigeonpea linkage map using 117 polymorphic SSR markers on 188 F_2s derived from cross ICPA 2043 × ICPR 2671; name of markers are on the right side, while the cumulative genetic distances (cM) are on the left side of the map

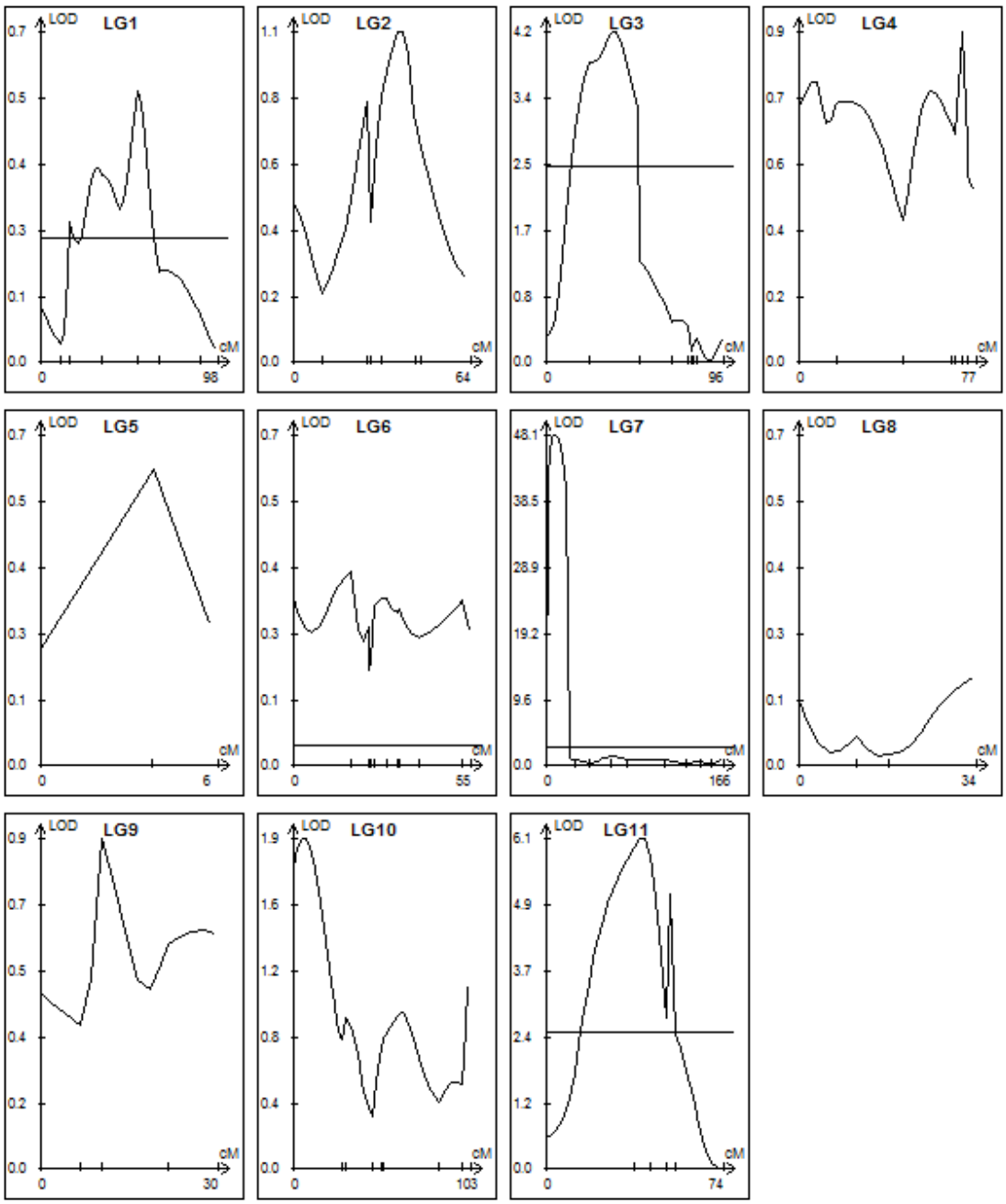


Figure 9: QTLs identified in 188 F_2s derived from cross ICPA 2039 × ICPR 2447

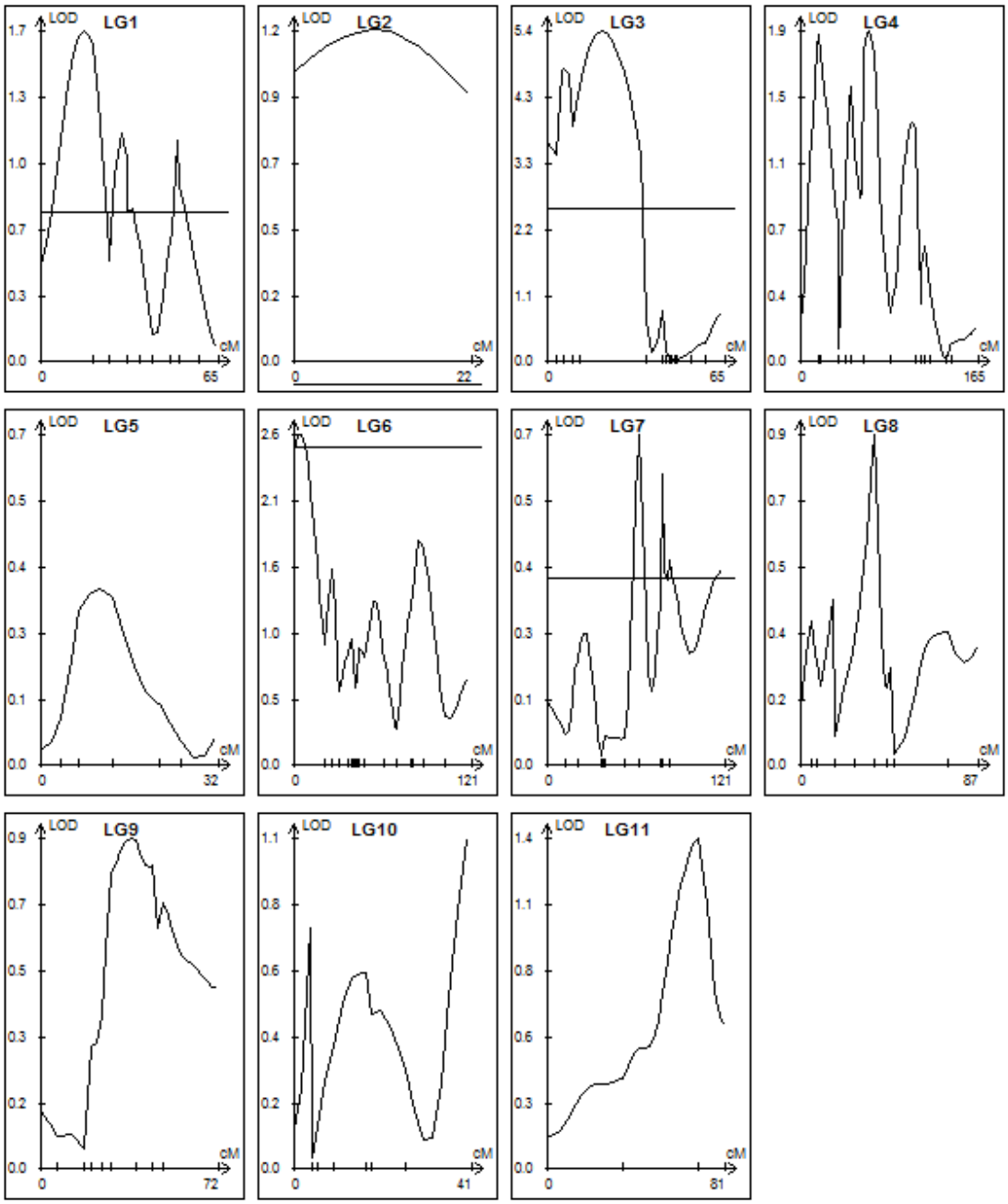


Figure 10: QTLs identified in 188 F_2s derived from cross ICPA 2043 × ICPR 2671