MORPHOLOGICAL AND SCAR MARKER DEVELOPMENT FOR EARLY SEX DETERMINATION IN POINTED GOURD (*TRICHOSANTHES DIOICA* ROXB.)

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By
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Thesis Abstract

Post-Graduate Degree Programme : Ph.D. (Ag.)

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Field of Research Problem : **Morphological and SCAR marker development for early sex determination in Pointed gourd (*Trichosanthes dioica* Roxb.)**

Pointed gourd (*Trichosanthes dioica* Roxb., 2n=2x=22), a perennial vegetable crop and commonly known as parwal/patal, have Indo-Malayan origin. It is said to be native of South East Asia and probably the Northern and Eastern states of India especially of West Bengal, Assam and Bihar. It has high economic value with export potential. It is mainly cultivated along the riverine belts of Bihar. Pointed gourd (*Trichosanthes dioica* Roxb) is an economically important cucurbit and is extensively propagated through vegetative means, *viz;* vine and root cuttings. The plant's dioecious in nature and its vegetative mode of propagation makes its reproduction and multiplication labour intensive. Dioecy represents an inconvenience in pointed gourd breeding since at present there are only few reports distinguishing male and female plants prior to flowering. The use of molecular marker provides a quick and reliable identification of sex types in plants. RAPD (Random amplified polymorphic DNA) has been used previously for determining the gender of plants before flowering. The SCAR marker is one of the stable markers, generally derived from RAPD increase effectiveness of RAPD marker by selecting and redesigning primers whose priming sites occur in target sequence(s) of gene or organism at optimum distance. Therefore, the present study was undertaken to identify marker associated with male and female sex expression trait in *T. dioica* Roxb. followed by development of SCAR. The screening of genomic DNA samples representative of male and female plants of pointed gourd with RAPD was used to discover sex specific PCR amplification product. A total 40 RAPD primers were used for RAPD analysis, out of which 20 primers gave good results. Among these 20 primers, OPC-04 amplified a band of 400 bp specific to female lines. This RAPD marker was eluted, sequenced and the sequence was used to design primers for SCAR marker. From the sequence, a set of two SCAR primers (N6Fn and N7Fn) was designed to allow amplification of female specific region But, only single SCAR (N7Fn/r)amplify a product size of 400bp in female specific DNA.

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Place- *Sabour, Bhagalpur* ***(Neetu Nand)***
Date:

LIST OF ABBREVIATIONS

^0C	:	Degree Centigrade
ANOVA	:	Analysis of Variance
C.V.	:	Coefficient of Variation
C.D.	:	Critical Difference
cm	:	Centimetre
df	:	Degree of Freedom
g	:	Gram
GA	:	Genetic Advance
GCV	:	Genotypic Coefficient of Variation
h^2	:	Heritability
Kg	:	Kilogram
MT	:	Metric Tonnes
ha	:	Hectare
MSS	:	Mean Sum of Squares
PCV	:	Phenotypic Coefficient of Variation
GCV	:	Genotypic coefficient of variation
S. Em (±)	:	Standard Error of mean
SS	:	Sum of Squares
Fig.	:	Figure
PV	:	Phenotypic Variance
GV	:	Genotypic Variance
µg	:	Microgram
µl	:	Microlitre
rpm	:	Revolution per Minute
TBE	:	Tris-Borate EDTA
TE	:	Tris- EDTA
UV	:	Ultraviolet
V	:	Volts
M	:	Molar
mA	:	Mili Ampere
min	:	Minutes

ml	:	Mililiter
mm	:	Milimeter
mM	:	Mili Molar
pH	:	Reciprocal of the Hydrogen ion concentration
EtBr	:	Ethidium Bromide
MgCl	:	Magnesium Chloride
ng	:	Nanogram
OD	:	Optical Density
Mol. Wt.	:	Molecular Weight
DAF/MF	:	Days to anthesis of female/ male flowers
NNFFA	:	Number of node at which first flower appears
bp	:	Base Pairs
DNA	:	Deoxyribonucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic Acid
PCR	:	Polymerase Chain Reaction
HCl	:	Hydrogen Chloride
RAPD	:	Randomly amplified polymorphic DNA
SCAR	:	Sequence Characterized Amplified Region
NPB	:	Number of primary branches

Chapter - I

Introduction

Pointed gourd (*Trichosanthesdioica*Roxb., 2n=2x=22), a perennial vegetable crop and commonly known as parwal/patal, haveIndo-Malayanorigin and includes fifteen species of which *T. dioica*and *T. anguina*are widely cultivated while theremaining species are reported to occur in wild conditions in India. Over 20 species are recorded in India of which two namely *T. anguina& T. dioica*are cultivated as vegetable. Other important species found in the world are *T. palmata, T.cordata, T.nervifolia, T. cucumerina, T. wallichiana, T.cuspida, T.incisa, T. laciniosa, T. kirilowii* etc. It is one of the most importantsummer vegetable crops of India. The genus *Trichosanthes*belongs to the tribe Trichosantheae of the family Cucurbitaceae. It is said to be native of South East Asia and probably the Northern and Eastern states of India especially of West Bengal, Assam and Bihar. It has high economic value with export potential. It is mainly cultivated along the riverine belts of Bihar. Pointed gourd is commonly grown along the banks of river Ganga, BurhiGandak, Sone, Narayani, Bagmati and Kosi. In India the Pointed gourd crop is being cultivated in the area 16 (000' hectares) with the total production of 243 (000' MT) (Anonymous,2015-16).It is one of the most nutritive cucurbit vegetables highly used in India due to its availability for about eight months in a year. The edible portion of pointed gourd fruit contains 92.0 per cent moisture, 3.1 per cent carbohydrate, 2.0 per cent protein, 3.0 per cent fibre, 0.3 per cent fat and 0.5 per cent mineral matter. The fruit is also known to be a good source of vitamins A and C, iron and protein. Besides, the fruits are reported to possess total cholesterol and blood sugar lowering properties. According to Ayurveda, leaves of the plant are used as antipyretic, diuretic, cardio tonic, laxative, antiulcer, etc. Juice of leaves of *T. dioica* is used as tonic, febrifuge, in edema, alopecia, and in sub-acute cases of enlargement of liver (Nadkarni, 1982). In Charaka Samhita, leaves and fruits find mention for treating alcoholism and jaundice (Khare, 2004).

Pointed gourd is morphologically distinct from the other cucurbitaceous species due to its well-established dioecism and vegetative means of propagation. In the plant kingdom dioecy is found only approximately in 4% of the angiosperm. Dioecism has arisen independently in different families and genera, and several distinct genetic mechanisms regulating dioecy have been found in different plant species. Dioecy in

pointed gourd is predominant sex form, while sex determination in dioecious plants may often be genetic or environmental, and only a tiny fraction of them has evolved sex chromosomes. Genetic sex determination may be due to a single locus, multiple tightly linked loci on autosomes, multiple unlinked loci on autosomes, or several genes located on heteromorphic chromosomes (Parrish *et al.* 2004). In general, three types of chromosomal sex determination mechanisms, *viz.* Active Y chromosome, X chromosome to autosomes ratio (X:A) and ZZ/ZW in dioecious plants have been documented (Heikrujam*et al.,* 2015) showed in figure below:

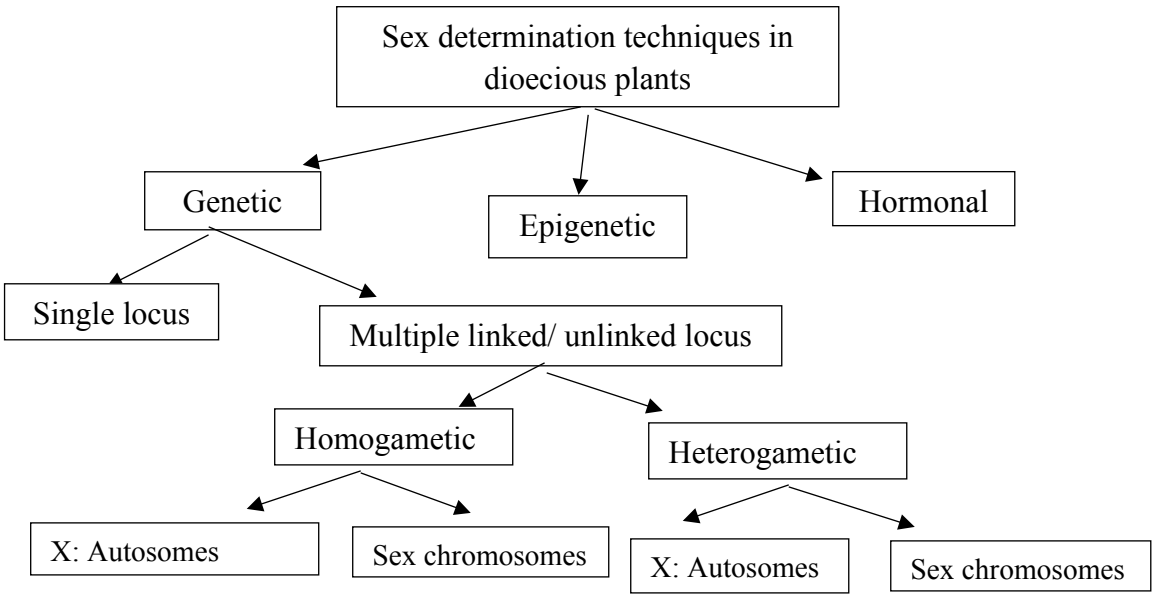

Fig. 1- Different sex determination techniques in dioecious plants

The plant's dioecious in nature and its vegetative mode of propagation makes its reproduction and multiplication labour intensive. Although each fruit contains several well-developed seeds having hard seed coat, propagation using seed is not feasible primarily due to poor germination (Kumar *et al.* 2007) and slow growth of the seed-derived plants (Nanda *et al.,* 2013). So, improvement of this crop has not yet been attempted adequately. Pointed gourd (*Trichosanthes dioica* Roxb.) is an economically important cucurbit and is extensively propagated through vegetative means, *viz*. vine and root cuttings. Because of judicious selection, considerable variability is available in pointed gourd. As the accessions are poorly characterized it is important at the beginning of a breeding program to discriminate among available genotypes to establish the level of genetic diversity and thereby, identify the most suitable materials for crossing. Breeding programmes in pointed gourd have several constraints such as poor germination, vegetative

means of propagation and dioecy. Identification of male and female at the seedling stage is a pre-requisite for the genetic improvement of this species. Dioecy represents an inconvenience in pointed gourd breeding since at present there are only few reports distinguishing male and female plants prior to flowering. A reliable method for early determination of the gender of plants before flowering would facilitate economy resources, including time and effort of the breeders.One such approach is the development of marker system. Several marker types such as biochemical, morphological, cytological and molecular markers have been developed and characterized to a certain extent that proved beneficial to differentiate male from female plants in several dioecious crops (Adhikari *et al.,* 2016). RAPD (Random amplified polymorphic DNA) has been used previously for determining the gender of plants beforeflowering.The last decade has witnessed an increasing number of research efforts directed at identifying and characterizing molecular markers and genes involved in plant dioecy (Yakubov *et al.,* 2005).

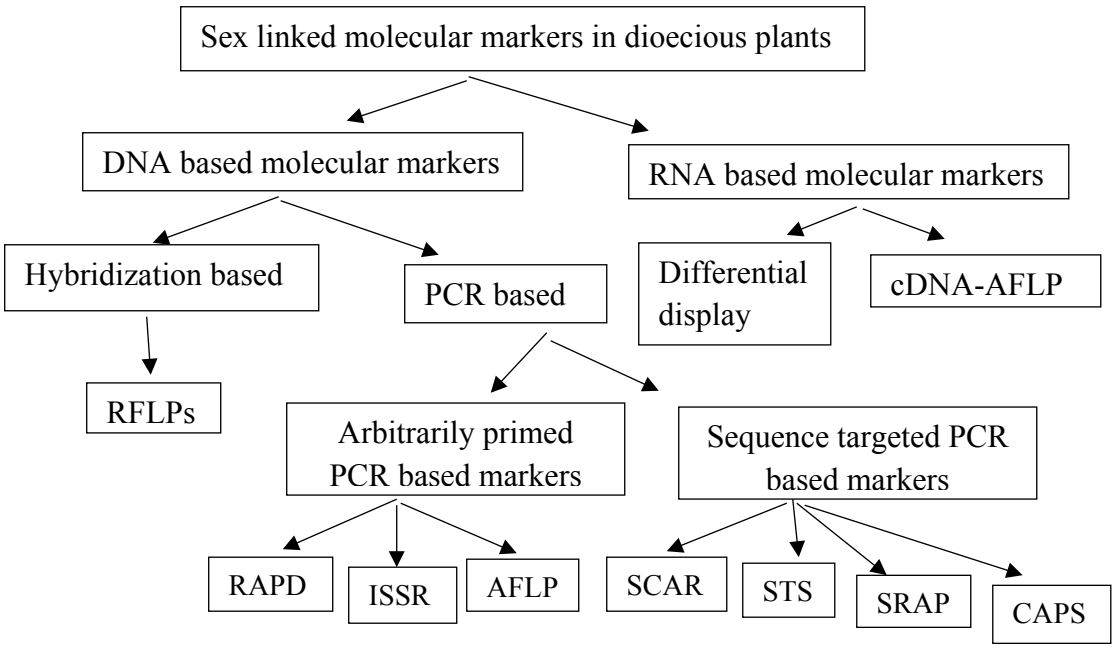

Fig. 2- Different sex linked molecular markers in dioecious plants

Several researchers have shown that random amplified polymorphic DNA (RAPD) banding patternsare linked to sex in (Esfandiyari *et al.,* 2012).RAPD is a simple dominant marker. However, concerns about the ability to compare RAPD results from one laboratory to another have not been addressed effectively. Effectiveness of RAPD can be improved by development of SCAR (sequence characterized amplified region) marker by selecting and redesigning primers whose priming sites occur in target sequence(s) of gene or organism at

optimum distance. The SCAR marker is one of the stable markers, generally derived from RAPD profile (Dayaneshwar *et al.* 2006; Li *et al.* 2010; Rajesh *et al*. 2013). SCAR markers are developed with a pair of longer primers (usually the extended sequence of a RAPD primer) that has a specific sequence of approximately 20 bases. Compared with random primers, unique primers for special regions prevent site-competition among primers, and make the results less sensitive to reaction conditions and more reproducible by increasing the specificity. The aim of this research was to develop SCAR markers that can differentiate pointed gourd sex at the seedling stage.

Keeping the above facts in view the present investigation was undertaken with following objectives:

1. Morphological characterization of pointed gourd genotypes.
2. RAPD analysis of selected male and female genotypes.
3. Development of SCAR marker based upon RAPD profile.
4. Validation of developed SCAR marker for early identification of sex.

Chapter-II

Review of Literature

Pointed gourd is morphologically distinct from the other cucurbitaceous species due to its well-established dioecism and vegetative means of propagation. Identification of male and female at the seedling stage is a pre-requisite for the genetic improvement of this species. Sex determination in dioecious plants may often be genetic or environmental, and only a tiny fraction of them has evolved sex chromosomes. A reliable method for determining thegender of pointed gourd before flowering would facilitate breeding programs. Genetic diversity among individuals or populations can be determined using morphological and molecular markers. The research towards determining sex in *Trichosanthes*is not pursued in huge way till date. The literature pertinent to the present investigation has been reviewed under the following sections.

2.1Molecular Marker

The molecular markers have acted as versatile tools and have found application in various fields like taxonomy, physiology, embryology, genetics and plant breeding. This facilitated the development of marker-based gene tagging, map-based cloning of genes for agronomic importance, variability studies, phylogenic analysis, synteny mapping, marker-assisted selection of desirable genotypes etc. DNA markers developed during the last two decades of molecular biology research have become useful tools to provide a relatively unbiased estimation of genetic diversity in plants (Clegg, 1990).

2.1.1 RAPD:

A male sex associated sequence of 400 bp was found by Mandolino*et al.*(1999)using a random decamer RAPD primer OPA08 (5'-GTGACGTAGG-3') in 14 dioecious cultivars and accessions of hemp (*Cannabis sativa*L.). RAPD analysis were performed with 20 decamer primers out of which only one primer was sex specific. They cloned OPA8400 band and different transformants were recovered, with inserts from three different size classes. The Cloned bands of each class were used as hybridization probes on Southern blots of OPA8-amplified DNA from different male and female plants. Only one inserts (coded class A_3, with an approximate size of 440 bp) gave a strong band at 400 bp and two minor bands, thus confirming that the clone contained the male specific fragment.

Singh *et al.* (2002) screened hundred decamer primers for differences in male and female cultivars. In this way 5 primers were identified which produced probable female-related bands. In the next stage, the 5 selected primers were used to confirm the presence and absence of bands in all the male and female entries, individually. In this stage 567 bp band amplified by the OPC07primer from the genomic DNA of all the female entries was absent in the PCR (Polymeric Chain Reaction) products of the DNAs from all the male entries. Thus within the limits of male and female genotypes available in this study, the RAPD band OPC07567 appeared to be the female sex-related DNA marker in *T. dioica*.

Bedoya and Nunez (2007) screened 32 arbitrary 10-mer Operon primers, they identified the OP-Y7 (5'-AGAGCCGTCA- 3') as being able to differentiate sex type, when tested on three Colombian papaya cultivars (Catira, ILS 647 and ILS 649). This primer generated a 900 bp band ($OP\text{-}Y7_{900}$) present in male samples and absent in female plants and hermaphrodites. From this sequence a 20-mer SCAR primer was designed using the Oligo software. This primer amplified a 369 bp fragment from male and hermaphrodite but not in female papayaplants.

Li Zheng*et al.* (2008) identified eight markers linking to the *M/m* locus by combining the BSA (Bulked Segregants Analysis) and the SRAP (Sequence-Related Amplified Polymorphism) technology and by the chromosome walking method using the cucumber genomic BAC(Bacterial Artificial chromosome) library.

Liu XiaoHong *et al.* (2008) amplified genome DNA of different sexual cucumber inbred lines by several RAPD, ISSR primers and sequence-specific primers which were designated by Primer Premier 5.00 from DNA sequence of ethylene biosynthesis and ethylene signal transduction pathway, sex determining protein, AGMOUS homologues with the identity of sex organ.

Oyama*et al.* (2009) developed a sex-linked sequence-characterizedamplified region (SCAR) marker for *T. dioica*and sequenced it for individuals representing the full geographic range of the species from Scotland to North Africa.

QiuXiang *et al.* (2009) developed segregating cucumber BC_1 population using the nearly isogenic lines WI1983G (gynoecious, *FFMM*) as donor and WI1983H (hermaphroditic, *FFMM*) as recurrent parent. With screening of polymorphism of one SCAR and, 2 112 SSR markers, three polymorphic co-

dominant markers SSR23487, SCAR123 and SSR19914 were found to be tightly linking to the *M/m* gene, with a distance of 0.28, 0.94 and 3.20 cM, respectively.

Khan *et al.* (2009) investigated genetic variation in 64 pointed gourd accessions using RAPD and found that out of 45 random primers screened five were selected, which gave 38 clear and bright fragments, out of which 30 (79.5%) fragments were considered polymorphic. The proportion of polymorphic loci across all loci was 96%.

Kumar *et al.* (2011) used 41random decamer primers from which 509 amplification products were obtained, of which five were associated with sex expression. A 1000 bp amplification product from the primer OPC05 was found to be present only in males and a 400 bp amplification product from the primer OPC14 was found to be present only in female individuals. The two RAPD markers, the males specific $OPC05_{1000}$and the female-specificmarker $OPC14_{400}$together can reliably differentiate male and female plants of *Trichosanthesdioica*long before the plants attain maturity and flowering.

Patil *et al.* (2012)utilizedrandom amplification of polymorphic DNA (RAPD) and sequence-characterized amplified region (SCAR) markers to search specific markers linked to the sex locus. A total of 50 random decamer primers were used for screening of specific RAPD markers in male and female populations. Only one primer, OPA-15, amplified genomic DNA in different patterns in the male genotypes. The sex-specific band $OPA\text{-}15_{1500}$ was cloned and sequenced. Based on the RAPD sequence, a pair of SCAR primers MSSM-01F and MSSM-01R was developed. These SCAR primers amplified a single 1501 bp DNA band only in male populations.These SCAR primers should be useful in identifyingmarkers associated with sex determination in *Momordicadioica*Roxb.

To distinguish between males and females of jojoba (*Simmondisiachinensis)*Al-Obaidi*et al.* (2012) used eight different RAPD primers and out of eight one primer (C5) showed amplification and represent a mean of distinguishing between the two sexes. This primer gave difference between the female and male sample at the presence of two bands (about 1.5 and 0.85 kbp) in the female and absence of these bands for male. Also, this primer showed that the band was around 1.0 kbp for the male sample and absent for this band for the female sample. Thus (C5) primer could be recognized as a putative sex-linked marker for four jojoba tissue culture cultivars.

Random amplified polymorphic DNA primers were tested on dioeceious and hermaphrodite plant of *Simaroubaglauca* by Vaidya and Naik (2014). They screened a set of eighty five RAPD primers andfive primers OPU-10 (5ACCTCGGCAC3), OPD

19(5CTGGGGACTT3), OPU-19 (5GTCAGTGCGG3), OPS-05(5TTTGGGGCCT3) and OPW-03 (5GTCCGGAGTG3) produced unique amplicon for sex differentiation.Among these five primers three primers, OPU-10, OPD-19, and OPU-19 showed sex specificity of male, female and hermaphrodite respectively. The primer OPU-10 produced a unique band in male which was absent in femaleand hermaphrodite and OPD-19primer produced 350 bpuniqueamplicon in female that was completely absent in male and hermaphrodite. OPS-05 primer was specific to both female and hermaphrodite and was absent in male plant and OPW-03 is specific to both male and hermaphrodite and was absent in female plants. This could help farmers to select the best seedlings and maintain an optimum sex ratio in plantations aswell as save time and costs in *Simaroubaglauca*breeding programs.

2.1.2Conversion of RAPD/ISSR marker into SCAR marker

Haymes *et al.* (2000) constructedtwo dominant sequence characterized amplified region (SCAR) markers(linked at 3.0 cM, coupling phase) for the strawberry (*Fragaria*×*ananassa*Duch.) gene *Rpf1* which confers resistance to red stele root rot, caused by the soil-borne fungus *Phytophthorafragaria*e var. *fragaria*e. They developed SCAR markers originally from the sequence of RAPD OPO-16C (438)that is linked in repulsion phase to the *Rpf1* allele. This SCAR primer set produced multiple bands in the resistant test progeny and in some of the susceptible progeny and these new SCARs were linked in coupling phase to the *Rpf1*allele and mapped to the same location as the original RAPD OPO-16C (438). The SCAR markers and RAPD markers known to be linked to *Rpf1*, that are highly conserved in linkage to the gene based on examination of 133 European and North American *Fragaria*L. sp. cultivars and breeding selections. These flanking. RAPD and SCAR markers can be used in breeding programs for the selection of red stele (*Rpf1*) resistance.

The male-associated DNA was analysed in a dioecious plant *Schisandranigra* by Jung *et al.* (2001). They tested 120 primers out of which only one primer OP-17 (5' GACCGCTTGT 3') produced segment 800 bp which is male specific. The SCAR primers was designed with a forward sequence of (5' GGAATTGAGTTCACCTCATC 3') and a reverse sequence of (5' CACTTGGTCTCTTGAGTTCC 3') and specificity of the primer pair was tested with genomic DNA from 12 male plants and 12 female plants and only the DNA from male plants gave a PCR product (~0.5 kb) with this primer pair.

Bulked DNA samples of male, female and hermaphrodite plants were amplified by Niroshini*et al.* (2008) using (RAPD-PCR)using 100 random primers and they obtained two

primers OPC09-1.7 kb and OPE03-0.4 kb were considered as associated with maleness and hermaphrodism of papaya. Two SCAR primers, C09/20FP(CTCACCGTCCATTTTAATTA)and C09/20RP (CTCACCGTCCGCGGCATCAATGTA) amplified two fragments of length 1.7 kb and a 978 bp in both male and hermaphrodite plants that are absent in females confirming the male and hermaphrodite specific RAPD-PCR markers in papaya.

20 random primers selected by Jiang *et al.* (2009) from 80 RAPD primers to identify the genetic relationships among Kava, Pepper and its wild relatives. These primers generated a total of 170 bands ranging in size from roughly 300 bp to 2000 bp.Out of 170 bands, 20 (12%) were polymorphic for one or more species. For increasing the specificity and reproducibility of RAPD markers, they converted the two RAPD markers (OPQ-02_{562}, OPQ-03_{355}) to SCARs. The SCAR primers designed for OPQ-02_{562} with a forward sequence of 5'-TCTGTCGGTCGTGAACAAAAAGAATG-3' and a reverse sequence of 5'-TCTGTCGGT CATTTAATTGGTTAATTGT-3' and for OPQ-03_{355} forward sequence of 5'-GGTCACCTCAAACCAAGCTTAATCAAG-3' and a reverse sequence of 5'-GGTCACCTC ATAATACAAACTTGCAAG C-3'. The validity of these two SCAR markers were confirmed by PCR amplification of 28 DNA samples.The SCAR primer pairs designed for OPQ-02_{562}, OPQ-03_{355} amplified the target fragments (562 bp, 355 bp) exclusively, which indicated that the two SCAR primers were kava-specific SCAR markers which could be used for the molecular identification of Kava germplasm resources.

For differentiating staminate and pistillate flower of dioecious *Hippophaesalicifolia*(Willow-Leaved Sea Buckthorn)genotypes, 31decamer RAPD primers were used by Rana*et. al.* 2009 out of which 27primers amplified genomic DNA fall 10 genotypes and 25 primers were found to be polymorphic. They got 118 reproducible bands out of that 95 bands are found to be polymorphic. Maximum number of polymorphic bands (8) was obtained from primer OPF-01, and OPF-14 but only one primer OPF-11 having sequence 5' TTGGTACCCC 3' produced an 1190 bp band which was female specific and this female specific RAPD marker can be used in future for SCAR development.

About 250 random primers were screened by Prasanthi*et al.* (2010)to identify the regions associated with sex linked marker. Specific amplification with 900bp band in males and andromonoecious (male dominated bisexual flowers) was produced by RAPD

primer OPA 8 and from this sequence of 915bp, two SCAR primers were designed with 12, and 14 bp. SCAR 12f/r with consistent results was utilized in screening of all *Simaroubaglauca* lines and gave specific band at 915bp in males and andromonoecious plants only.

Sixteen individuals were used by Yuskianti and Shiraishi (2010) as materials for selecting polymorphic RAPD fragments and for developing SCAR marker whereas confirmation of polymorphisms of the SCARs was conducted using 24 materials from Candiroto Seed Orchard, Indonesia. They obtained forty-eight polymorphic fragments from screening 288 RAPD primers and they successfully developed forty-six out of 48 SCAR primers. After examining newly-designed of SCAR primer using 24 *Paraseriathesfalcataria*L.(Moliccan albizia) from Candiroto Seed Orchard, Indonesia they found 5 polymorphic SCAR primers which are considered as useful marker for genotype and clone identification within *P. Falcataria*L.

Chinese fir (*Cunninghamialanceolata*(Lamb.) Hook.) and their clones having similar morphological characteristics which make their exact identification very difficult. So Shen*et al.* (2011) collected and analysed 20 *C. lanceolata*clones using nine polymorphic RAPD bands (RACu90, RACu511, RACu2059_S, RACu2059_X, RACu63, RACu1002, RACu1015, RACu88, andRACu500) which were cloned and sequenced. Six pairs of specific SCAR primers (SCAR1,5,6,7,8,9) were designed from six sequenced polymorphic RAPD bands showed polymorphism detected by RAPD among the 20 studied *C. lanceolata*clones, while SCAR 2,3,4 designed using three RAPD markers (RACu511, RACu2059_S, and RACu2059_X) were not successfully converted into specific SCAR markers and they failed to detect polymorphism among 20 *C. lanceolata*clones.

Sangwijit*et al.* (2012)used a HAT-RAPD (High Annealing Temperature-RAPD) analysis to investigate the genome of 7 mutants of rice (EPOS1, EPOS2, EPOS3, TPOS1, TPOS2, EBPOS1 and EBPOS3). And they found that from a total of 110 arbitrary primers, one primer named OPAA14 produced a unique candidate fragment at a molecular size of 700 bp and presented only in the EBPOS3 mutant. This fragment was selected for sequencing and conversion to SCAR marker. PCR amplification using the SCAR marker revealed a 300 bp fragment only in the EBPOS3 mutant. Thus the use of SCAR marker is a useful tool for identification purposes for right protection. This technique is a cost-effective and morphologically independent way to identify such mutants.

Bulked genomic DNA samples from ten male (XY) and ten female (XX) plants was screened by Ii *et al.* (2012) using 188 decamer random RAPD primers and only one primer T35R54 (5'-TTCACGGTGG-3') distinguished the sexes. The 1600-bp fragment was observed in all ten males, but not in the ten females and the haploid. They concluded from the result that T35R54-1600 might be located on the Y chromosome in asparagus, and has potential utility as a sex-related marker among asparagus cultivars.

After screening of three representatives sorghum samples using 8 primers which were selected from 163 primers by Zhang *et. al.* 2013using RAPD-PCR electropherogram amplification of the three samples with eight different primers. The RAPD marker band of *Sorghumhalepense* specific was cloned in vector pMD18-T and competent DH5α Escherichia coli cells were transformed and mini-prep DNA from white colonies was sequenced with M13 universal primers flanking the insert. The sequence data was used to design and synthesize two specific primers of *S. Halepense*each containing 18-19 nucleotides including the sequence of the original RAPD primer. The SCAR primers SH1 (5'-AGATTGAGTCTCAGGTGC-3') and SH2 (5'-GAGTCTCAGGGTATGATCT-3')were verified by 65 sorghum DNA samples which showed that these SCAR primers SH1/SH2 can be used to distinguish *S.halepense* and its relatives *S. almum*, *S. nitidum*, *S. propinquum*, *S. sudanense*, and *S. bicolor*, which were difficult to identify morphologically.

45 individual plants (25 female and 20 male)belonging to different varieties of date palm was subjected to PCR amplification using 100 random amplified polymorphic DNA (RAPDby Dhawan*et al.* (2013). Only one RAPD primer, OPA-02, amplified a fragment of 1031bp in all the individual samples of male genotypes. This male-specific fragment was cloned and sequenced (Gene Bank accession no. JN123357), and a sequence-characterized amplified region (SCAR) primer pair was designed that amplified a 406-bp fragment in both female and male genotypes and a unique fragment of 354 bp in only male genotypes.

Joseph *et al.* 2014 differentiated male and female samples of *Garciniamorella using* 150 decamer primers out of that they got only one primer OPN-15 (5' CAGCGACTGT 3') that differentiated both samples and is found to be male specific. Based on the sequence of this unique male specific RAPD fragment, a pair of SCAR primers MOR-634 specifically amplifying the 634 bp fragment was designed and the reproducibility and reliability of the designed SCAR primers was verified by analysing 24 samples with known sexes that could identify the male and female samples correctly.

Sequencing of three cloned RAPD fragments by Cheng *et al.* (2015) revealed that the clone L7-16 consisted of 222 nucleotides, clone L9-6 consisted of 648 nucleotides and clone L11-26 consisted of 369 nucleotides. Then, specific primers for SCAR markers L7-16, L9-6, and L11-26 were designed and synthesized by them. After PCR amplification performed by them using the DNA templates from the 24 different samples, including 6 samples of *Litchi chinensis* and other plants and they found that the SCAR marker L9-6 was specific for all of the *L. chinensis* samples, the SCAR marker L11-26 specific for *five L. Chinensis*samples. Thus they concluded that combined use of RAPD and SCAR markers provides us a simple and reliable tool for both sex determination as well as genetic characterization of plant species.

Shah *et al.* (2015) obtained RAPD profiles with 104 primers using 10 male and 44 female plants for presence/absence of specific bands. Two sex-specific fragments, $OPC05_{1000}$ and $OPK07_{300}$, associated with maleness and femaleness, respectively, were converted in SCAR markers. Based on the sequence of $OPC5_{1000}$ and $OPK\text{-}07_{300}$, five and one pair of specific forward and reverse SCAR markers were designed, respectively. Among five male specific SCAR, only one marker (SCF/R-3628) discriminated both sexes.

Parmar*et al.*(2015) had done a specific detection of *Trichodermaharzanium and Trichodermaviride*among other species.A total 305 bands were produced by 21 RAPD primers with an average frequency of 14 bands per primer. They obtained total 305 polymorphic bands out of which 266 were polymorphic and shared between at least two individuals, and 39 bands were polymorphic and unique. Primer OPC-05 generated unique band of *T. koningii*(500 bp) and *T. viride*(900 bp), OPA-16 generated unique band of *T. harzianum*(220 bp) and *S. rolfsii*(500 bp) and OPA-17 generated unique band of *T. koningii*(900 bp.) and *S. rolfsii*(500bp) which was purified and used to develop SCAR marker for particular species. The two RAPD products (NBAII Th 1) which obtained using primer OPA-16 for *T.harzanium*and(NBAII Tv 2) from primer OPC-05 for *T. viride* and these RAPD products were sequenced. On the basis of sequence obtained one primer was designed, out of which a primer pair HAR220FP5 (CTTTTGGTTTGACACGGTTCT and HAR220RP5 (AAGCTTTGAAGTTGCGAGGA) amplified a sequence of 220 bp which was specific to *T. harzianum* and VIRI900FP7 (TACGCTCCAGGCTACCACTT) VIRI900RP7 (GAGATGAGCTCCTTGCTGCT) amplified a sequence of 900 bp. specific to *T. viride*. Then they tested marker specificity against sixisolates of *Trichoderma*species

and they found that these SCAR markers were sensitive and could detect small quantities of *Trichoderma*DNA as low as 25 to 30 ng with high efficiency.

Miziaet *al.* (2016) tested30 RAPD primers out of which only 18 primers yield reproducible band profiles in *ujaponicas* plants. Only two primers (OPU-08 and OPJ-09) produced male-specific bands both in *H. Japonicus* and *H. lupulus*. RAPD profiles produced by OPJ-09 primer showed one male-specific fragment in *H. lupulus*(~1.2 kb) and two in *H. Japonicus*(~1.6 kb and ~700 bp). OPU- 08 generated one male-specific product both in *H. Lupulus*(~1.4 kb)and *H. japonicus* (~1 kb). 8 out of 11 male-specific *H. Japonicus*RAPD products were cloned and sequenced, and respective SCAR primers were designed. Testing reactions with these primers showed that only one marker HJY09(5'-TCACCACTTTGAACTCGCTG-3'), based on the shorter OPJ-09 fragment was highly male-specific in *H. Japonicus*plants but ineffective in *H. lupulus* males.

2.1.3 SCAR marker development from other molecular markers

Thirty-five ISSR primers were used by Khadkeet *al.* (2012) out of which they found three putative sex-linked primers, viz. ISSR-10, UBC-852 and ISSR-23 which showed sex-specific banding pattern. The primers ISSR-10 (5' GAGAGAGAGAGACC 3') and UBC-852 (5' TCTCTCTCTCTCTCTCRA 3') amplified unique 459 bp fragment in the 10 individual male DNA, that was absent in female DNA while primer ISSR-23 (5'GACAGACAGACACC3') amplified 636 bp fragment unique in 10 individual females that was absent in male DNA. Out of these three primers ISSR-23 had shown very clear and consistent co-segregation with female sex, so it was selected by them for converting it to SCAR marker. From the sequence, female-specific SCAR primers SCAR-23-F (5'GACAGACAGACACCAAGTTCAAGC3') and SCAR-23-R (5'ATATATTTAGTGGTGTCTGTCTGTCA3') which are able to amplify a single unique 636 base-pair fragment are designed and these primers are efficient in identification of female plants in betel vine.

Twenty SSR primers were used by Shivkumaret *al.* (2014) out of which 16 primers showed polymorphism giving average polymorphic percentage of eighty. The SCAR marker used for the validation of sex types in the collected papaya cultivars gave a band size of 369bp for male and hermaphrodite plants and no band was obtained for female plants. Similarly (Deputy *et al*., 2002) developed RAPD-SCARS for sex determination in

papaya like SCAR T12 and SCAR W11 in male and hermaphrodite plants which is tightly linked to sex gene that determines male and female plants in papaya.

Male and female DNA pools of twenty accessions of pointed gourd were screened with 42 ISSR primers for sex-specific polymorphisms and reproducibility by Adhikari*et al.* (2014) Of all the 42 primers tested, only one primer, ISSR-6, was found to show sex specificity in bulk analysis. Primer ISSR-6 (5'GACAGACAGACAGACA 3') produced a unique ~550 base-pair fragment in male bulk DNA, and this band was absent in female bulk DNA.

100 ISSR DNA markers were screened for sex determination of papaya by Tomar*et al.* (2015) and four primers with amplicon size UBC-880 (600 bp), UBC- 812 (600 bp), UBC-817 (600 bp) and UBC-857 (1200 bp) were selected for gene cloning. Clones were sequenced and new primers derived from these sequences were used to obtain Sequence Characterized Amplified Region (SCAR) fragments. Four SCAR markers with product size of 410 bp form cps-1, 782 bp from cps-2, 427 bp from cps-3 and 487 bp from cps-4 were obtained. These primers were validated in the individual Male and Female plants of "Madhubindu" variety of *Carica papaya* and were able to detect sexuality of papaya at seedling stage before flowering.

Al-Ameri*et al.* (2016)studied gene expression in male and female date palm trees at flowering stage and they found that the unique band was amplified in male trees (size: 478 bp) using SCoT primer-3 (5′-CAACAATGGCTACCACCG-3′), whereas it was not amplified in female trees. This male specific band was cloned and sequenced and they designed new SCAR primers for the amplification of cDNA of both male and female date palm samples. The unique band was appeared in male plant samples of size 253 bp whereas it was absent in female plant samples. Further, this designed SCAR marker was validated on independently collected samples (MC_1, MC_2; male); and (FC_1, FC_2; female) from which total transcripts were extracted from flowers and found same results as obtained from the samples used in the development of SCAR marker.

A brief summary of RAPD primers linked to sex determination reported by different scientists is presented hereunder:

Table 2.1 List of RAPD primers linked to sex determination in different crops

Primer	Size of Fragment (bp)	Specificity	Plant Species	Reported by	Total primer screened
OPC-05	1000	Male	*Trichosanthes dioica*Roxb.	Kumar *et al*. (2012)	41
OPC-14	400	Female			
OPN-01		Male and Female			
OPC-07	567	Female		Singh *et al.* (2002)	100
OPA-08	440	Male	*Cannabis sativa* L.	Mandolino *et al*. (1999)	20
OPD-20	911	Male	*Hippophae Rhamnoides*L.	Sharma *et al*. (2010)	35
OPB-01	686	Male	*Carica papaya*L.	Gangopadh Yay*et al.* (2007)	
OPB-05	2048	Female			
OP-Y7	900	Male		Bedoya and Nunez (2007)	32
OPC-09	1.7 kb	Male		Niroshini*et al.* (2008)	100
OPA-15			*Momordica dioica*Roxb	Patil*et al.* (2012)	50
OPJ-09	700	Male	*Humulus japonicus* siebold&zucc.)	Mizia *et al.* (2016)	30
OPU-10	1 kb	Male	*Simarouba glauca*	Vaidya and Naik (2014)	85
OPD-19	350	Female			
OPC 05	1 kb	Male	*Simmondisiachinensis*	Al-Obaidi*et al.* (2012)	8
OPA-10	908	Male	*Piper longum*L.	Banerjee *et al*. (1999)	40
OPQ-02	562	Male		Jiang *et al.* (2009)	80
OPC- 17	800	Male	*Schisandranigra*	Jung *et al.* (2001)	120
OPF-11	1190	Female	*Hippophoesalicifolia*	Rana*et. al.* 2009	31
OPA-08	915	Male	*Simarouba glauca*	Prasanthi *et al.* (2010)	250

T35R54	1600	Male	*Asparagus officinalis*	Ii *et al.* (2012)	188
OPN-15	634	Male	*Garcinia Morella*	Joseph *et al.* 2014	150
OPA-15	1500	Male	*Momordicadioica*Roxb.	Patil *et al.* (2012)	50
OPC05	1000	Male	*Trichosanthesdioaca*	Shah *et al.* (2015)	104
OPK07	300	Female			
OPU-08	1 kb	Male	*Humulusjaponicus*	Mizia *et al.* (2016)	30
OPJ-09	1.4 kb	Male	*Humuluslupulus*		

Chapter-III

Materials and Methods

An investigation entitled '**Morphological and SCAR marker development for early sex determination in pointed gourd (*Trichosanthes dioica* Roxb.)'** using morphological and molecular markerswas carried out during the year, 2015-2016. Materials used, experimental procedures followed and techniques employed in the present investigation have been described in this chapter.

3.1LOCATION OF EXPERIMENTAL SITES:

The field experiment of this investigation was conducted on Vegetable Research Farm,Department of Horticulture (Vegetable and floriculture) at Bihar Agricultural College, BAU, Sabour, farm during the year 2015-16and the Laboratory works were done in the Departmentof Plant Breeding and Genetics, Bihar Agricultural College, Sabour, Bhagalpur. Sabour is located about 10 km east of Bhagalpur city in Bihar state of India. It is situated at longitude 87º2'72" east and latitude 25º15'40" North at an altitude of 46 meters above mean sea level in the heart of the vast Indo-Gangetic plain of North India, South of river Ganga.

3.2 CLIMATE AND WEATHER CONDITION

Weather data from September' 2015to October' 2016

Months	Temperature (^{0}C)		Rainfall	Relative humidity (%)	
	Maximum	Minimum		7 AM	2 PM
September'2015	32.7	24.8	243.7	89	73
October'2015	28.4	14.6	11.4	85	59
November'2015	28.7	15.1	0.0	90	61
December'2015	23.5	9.5	0.0	89	66
January'2016	22.4	7.6	24.4	95	61
February'2016	27.1	10.7	3.4	88	49
March'2016	32.3	18.4	2.4	82	44
April'2016	38.6	21.4	23.2	67.7	35.4
May'2016	35.2	23.1	106.0	85.3	56.1

June'2016	34.0	25.5	116.2	84.0	58.8
July'2016	31.9	25.4	309.8	89.5	79.9
August'2016	32.5	25.9	353.3	86.9	75.6
September'2016	31.2	24.7	320.9	89.8	80.2
October'2016	31.6	21.7	31.2	89	68.4

3.3. EXPERIMENTAL DETAIL

Male and female individuals of twenty five cultivated accessions of pointed gourd were procured randomly from isolated areas of Bihar and U.P., India for their analyses.

Design of experiment : RBD

Treatments : 25

Replications : 3

Plot size : 4 m × 2 m

Spacing : 2 m × 1 m

3.4 TREATMENTS DETAILS

The experimental material comprising 25 genotypes along with their sources are as follows:

S.N.	Treatment	Genotype	Source
1	T_1	BRPGM-13-1 (Male)	Collected from farmer's field of Bhagalpur district (Bihar) adjoining the basin of Ganga river.
2	T_2	BRPGM13-2 (Male)	Collected from farmer's field of Faizabad (U.P) adjoining the basin of Ghaghra river.
3	T_3	BRPGM-13-3 (Male)	Collected from farmer's field of Faizabad (U.P) adjoining the basin of Saryu river.
4	T_4	BRPGM13-4 (Male)	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
5	T_5	BRPG-12-1	Collected from farmer's field of Katihar district (Bihar) adjoining the basin of Kosi river.
6	T_6	BRPG-12-7	Collected from farmer's field of Bhagalpur district (Bihar) adjoining the basin of Ganga river.
7	T_7	BRPG-12-8	Collected from farmer's field of Bhagalpur district (Bihar) adjoining the basin of Ganga river.
8	T_8	BRPG-12-9	Collected from farmer's field of Katihar district (Bihar) adjoining the basin of Kosi river
9	T_9	BRPG-12-10	Collected from farmer's field of Katihar district (Bihar) adjoining the basin of Kosi river

10	T_{10}	BRPG-12-11	Collected from farmer's field of Katihar district (Bihar) adjoining the basin of Kosi river
11	T_{11}	BRPG-12-13	Collected from farmer's field of Bhagalpur district (Bihar) adjoining the basin of Ganga river.
12	T_{12}	Rajendra Parwal-1	R.A.U,Pusa, Bihar
13	T_{13}	BRPG-13-5	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
14	T_{14}	BRPG-13-10	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
15	T_{15}	BRPG-13-16	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
16	T_{16}	BRPG-13-29	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
17	T_{17}	BRPG-13-19	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
18	T_{18}	BRPG-13-20	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
19	T_{19}	BRPG-13-22	Collected from farmer's field of Samastipur district (Bihar) adjoining the basin of BurhiGandak river.
20	T_{20}	BRPG-13-25	Collected from farmer's field of Faizabad (U.P) adjoining the basin of Saryu river.
21	T_{21}	BRPG-13-26	Collected from farmer's field of Faizabad (U.P) adjoining the basin of Saryu river.
22	T_{22}	BRPG-13-27	Collected from farmer field of Kushinagar district (U.P)
23	T_{23}	BRPG-13 -28	Collected from farmer's field of Faizabad (U.P) adjoining the basin of Ghaghra river.
24	T_{24}	Nimia	Collected from farmer field of Gaya district (Bihar) adjoining the basin of Falgu river.
25	T_{25}	Rajendra Parwal-2	R.A.U,Pusa, Bihar

3.5 OBSERVATIONS RECORDED

Observations were recorded on five randomly selected plants from each genotype in every replication, summed up and divided by five to get mean value. The procedure is described under the respective sub-heads.

3.5.1 MORPHOLOGICAL CHARACTERISTICS

3.5.1.1 Node number at which first flower appears:The number of node at which first flowers appears on mature plant was recorded.

3.5.1.2 Number of primary branches: The number of branches was counted during peak fruiting stage of the tagged plant and the average was recorded.

3.5.1.3 Leaf shape: Cordate/oblong/ovate/obovate/orbicular/others

3.5.1.4 Vine shape: Rounded/angular/other

3.5.1.5 Leaf margin: Entire/serrate/multifid

3.5.1.6 Leaf size: Small/medium/large

3.5.1.7 Leaf pubescence density: No hairs/sparse/intermediate/dense

3.5.1.8 Stem pubescence: Smooth/pubescent

3.5.1.9 Tendril branching: Branched/unbranched

3.5.1.10 Tendril type: Coiled/straight

3.5.1.11Inter nodal distance (cm): Inter nodal distancewas measured with the help of a scale of fully developed plants and the average was recorded.

3.5.1.12Petiole length (cm): Length of petiole was measured with the help of a scale of fully developed leaves and the average was recorded.

3.5.1.13Days to anthesis of male/female flower: Days to anthesis was considered as a number of days from the date of planting to the opening of first flower on the plants.

3.5.1.14 Vine girth: Vine girth was recorded in mature selected plant between 10^{th} and 11^{th} node.

3.6 Statistical methods for the analysis of experimental data

The experimental data for various characters, recorded in course of this investigation were subjected to statistical analysis using suitable technique for different characters. The technique of analysis of variance for Randomised Block Design was adopted, as suggested by Panse and Sukhatme (1967).

3.6.1 Phenotypic, Genotypic variance

3.6.1.1 Phenotypic variance

Phenotypic variance was calculated by adding genotypic variance to error mean sum of square as suggested by Comstock and Robinson (1952).

$$V_{ph} = V_g + E$$

Where,

'Vph' is phenotypic variance

'Vg' is genotypic variance

'E' is error mean sum of square.

3.6.1.2 Genotypic Variance

Genotypic variance was calculated by subtracting the error mean sum of square from the treatment mean sum of square and dividing it by the number of replications as suggested by Comstock and Robinson (1952).

$V_g = V\text{-}E/r$

Where,

'V' is the treatment mean sum of square

'E' is error mean sum of square

'Vg' is genotypic variance

'r' is number of replications.

In the present study, the genotypic variance has been expressed as percentage of phenotypic variance. The error mean sum of square was taken as environmental variance *i.e.* Ve= E, it has been expressed as percentage of phenotypic variance in the present study.

3.6.1.3 Coefficient of Variation

It is the ratio of standard deviation of a sample to its mean and expressed in percentage.

$$CV\ (\%) = \frac{Standard\ Deviation}{Mean} X\ 100$$

Phenotypic and Genotypic coefficients of variation were calculated by the method suggested by Burton and Devane (1953):

Phenotypic coefficient of variation

$$PCV = \frac{Phenotypic\ standard\ deviation}{General\ mean} \times 100$$

$$PCV = \frac{\sqrt{\sigma_p^2}}{\overline{X}}$$

Where, σ^2_p = Phenotypic variance

$\overline{X}$= General mean

Genotypic coefficient of variation

$$GCV = \frac{Genotypic\ standard\ deviation}{General\ mean} \times 100$$

$$GCV = \frac{\sqrt{\sigma_g^2}}{\overline{X}}$$

Where, σ^2_g = Genotypic variance

$\overline{X}$= General mean

PCV and GCV were classified as low, moderate or high by **Sivasubramanian and Menon (1973)** as given below:

0 – 10 %	:	Low
10 – 20 %	:	Moderate
20 % and above	:	High

3.6.1.4 Heritability

Heritability in broad sense is the ratio of genotypic variance to the total variance and is calculated by the formula given by Lush (1940).

$$h^2 = \frac{\sigma_g^2}{\sigma_p^2}$$

Where, H = Heritability in broad sense

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance = $\sigma^2_g + \sigma^2_e$ (MSE)

The heritability % was categorized by Johnson *et al*. (1955) as:

0 – 30 %	: Low
30 – 60 %	: Moderate
60 % and above	: High

3.6.1.5 Genetic Advance

Genetic advance is the improvement in mean genotypic value of selected plants over the parental population. The estimates of genetic advance were obtained by the formula given by Lush (1949) and Johnson *et al.* (1955).

GA = K. σ_p. H

Where, K= Constant selection differential at 5% level intensity (2.06)

σ_p= Phenotypic standard deviation

H= Heritability in broad sense

Genetic advance as percentage of mean was calculated by the following formula:

$$Genetic\ Advance\ as\ Percentage\ of\ Mean = \left(\frac{GA}{\overline{X}}\right) x\ 100$$

Where, GA = Expected genetic advance

$\overline{X}$ = General mean of the character in the population

The GA as per cent of mean was categorized by Johnson *et al.* (1955) as:

0 – 10 %	: Low	
10 – 20 %	: Moderate	
20 % and above	: High	

3.7 MOLECULAR CHARACTERIZATION

3.7.1 Composition of DNA extraction buffer

Genomic DNA of pointed gourd was isolated from new young leaves by following CTAB (CetylTrimethyl Ammonium Bromide)method (Singh *et al*., 2002).

3.7.2 Solutions:

A. 2X CTAB buffer:

2% CTAB (w/v)

100 mMTris-Cl, pH 8.0

20 mM EDTA, pH8.0

1.4M NaCl

1% PVP [Mr: 40,000]

B. 5% CTAB:

5% CTAB

0.35 M NaCl

C. CTAB precipitation buffer:

1% CTAB

50 mMTris-Cl, pH 8.0

10 mM EDTA, pH 8.0

D. High salt TE buffer:

10 mMTris-Cl, pH 8.0

1 mM EDTA, pH 8.0

1M NaCl

F. 0.1 × TE buffer:

1mM Tris-Cl, pH 8.0

0.1 mM EDTA, pH 8.0

G. Chloroform:Isoamyl alcohol [24:1]

For 50 ml-

Chloroform: 48ml

Isoamyl alcohol: 2ml

3.7.3 Procedure of DNA isolation:

Freshly harvested young and tender or old leaf samples (0.5 g) were ground by adding 1.5ml of 2XCTAB extraction buffer (warm 65^0C) using mortar and pestle. Approximately, 0.7 ml of the liquid were transferred in to 1.5 ml of microcentrifuge and equal volume (W/V) (350 µl) of ice cold chloroform: isoamyl alcohol (24:1) was added, mixed well by inversion and centrifuged at 12,000 rpm for 5 min. The top (aqueous) phase was collected into a new microcentrifuge tube and 1/5^{th} volume of 5% CTAB solution was added mixed well by gentle inversion. Again, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed well by inversion and centrifuged at 12,000 rpm for 5 min. The top (aqueous) phase was collected using cut tips into a new micro centrifuge tube and equal volume of CTAB precipitation buffer was added and incubated on ice for 5 min. After incubation, microcentrifuge tube was centrifuged at 12,000 rpm for 5 min at 4^0Cand supernatant was discarded. Fifty microlitres of high salt solution was added into the microcentrifuge to dissolve the pellet. DNA was precipitated by addition of 2.5 vol. of (the supernatent) ice cold 70% Ethanol and mix gently by inversion. Microcentrifuge tubes were centrifuged at 12,000 rpm for 15 min and supernatant was discarded. DNA pellet was washed with 70% ethanol, air dried, dissolved in 25 µl of 0.1 × TE buffer.

3.7.4 Amplification of isolated genomic DNA

3.7.4.1 RAPD analysis:

RAPD analysis of 25 pointed gourd genotypes was conducted by using 10 decamer arbitrary primers obtained from Operon Technologies, California. Two bulk DNA samples (male and female) were prepared by pooling an equal amount of DNA from 10 individual (4 male and 6 female) cultivars. These bulks were amplified with 60 RAPD primers. Once polymorphism was detected with a particular primer, then the primer was used to amplify the DNA samples from individual plants for sex-typing. PCR was repeated twice to ensure reproducibility and consistency of the banding patterns for male and female accessions. One primer, which was discriminating the male and female plants with consistent banding patterns were selected for development of the SCAR marker. The polymorphic markers were verified for their consistency and reproducibility using DNAs from 4 random male plants and 6 female plants from field. Putative sex-linked markers which differentiated the male and female bulks as well as the individuals of each sex are used for developing the SCAR marker. RAPD amplification was performed in 25 µl volume containing 1X Taq DNA Polymerase buffer, 200 µMdNTPs mixture, 0.5 µM primer, 25 ng of template DNA and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a thermal cycler. The reagents were mixed thoroughly and then placed on a thermal cycler for cyclic amplification and the conditions for amplification were programmed as follows:

Programme of polymerase chain reaction (PCR)

Cycle 1 : 94^0C for 5 min

Cycle 2 : 94^0C for 1 min

Cycle 3 : 34^0C for 1 min

Cycle 4 : 72^0C for 2 min

Cycle 5 : 72^0C for 7 min for final extraction

Cycles 2, 3 and 4 were repeated for 40 times. Amplification products were then subjected to electrophoresis in 1.2 per cent Agarose gel using 1X TBEbuffer and detected by ethidium bromide staining, viewed under ultraviolet light and photographed with Gel documentation system. Band size was estimated by comparison to 1 kb ladder DNA standard.

3.7.4.2Cloning and sequencing of sex specific fragments:

To convert the sex-diagnostic RAPD band to a SCAR marker, the bands were excised from 1.2% Agarose gel. Elution and purification of PCR fragment was performed using Qiagen-gel extraction and purification according to manufactures protocol. The purified PCR productwas quantified by ethidium bromide spotting method as described by Sambrook and Russell (2001).The eluted DNA was re-amplified with the corresponding RAPD primers to verify whether the fragment size was amplified consistently. Finally, for cloning of eluted amplicon into pJET 2.0 cloning vector.

3.7.4.3Cloning of PCR product

The purified DNA fragments were ligated into a PCR cloning vector pJET 2.0 vector using the cloning kit (Invitrogen) according to the manufacturer directions. The components of ligation mixture were mixed into a 0.5 ml micro-centrifuge tube and incubated overnight at 27˚C in incubator. A control ligation reaction was performed using control PCR fragment provided in the kit. The chimeric plasmid was transformed to *E. coli*strain DH5ά competent (Sambrook and Russell, 2001).

3.8. Transformation of *E. coli* DH5∞ with recombinant clone

3.8.1 Preparation of competent cells

The competent cells of *E. coli* DH5∞ were prepared following the protocol mentioned by Sambrook and Russell (2001) as described below:

An isolated colony from *E. coli* DH5∞ plate was inoculated into 5 ml Luria broth and incubated at 37˚C overnight at 200 rpm. After 12 hours, the culture was diluted to 1:100 using Luria broth (0.5 ml of culture + 49.5 ml of Luria broth), andincubated for 2 to 3 hours to attain the OD of 0.4 - 0.6 at 600 nm. The culture was kept on ice for 30 min and 25 ml of culture was dispensed into two tubes each with 50 ml of Luria broth. It was incubated for 2 to 3 hours to attain the OD of 0.4 - 0.6 at 600 nm. Later on, the cells were pelleted by centrifugation at 4000 rpm at 4^0C for 4min, the supernatant was discarded again add 0.1 M $CaCl_2$. $2H_2O$ and keep it for 15 minutes then mix it gently and again spin itat 4000 rpm for 4 minutes at 4^0C, the supernatant was again discarded, the process was repeated again after adding 0.1 M $CaCl_2$. $2H_2O$ and finally the pellets were re-suspended in 100-200µl of ice-cold 0.1M $CaCl_2$and to this 20 % Glycerol were added. About 200 µl of cells were distributed to each pre- chilled 1.5 ml eppendorf tubes and store it at -80^0C tubes for further used.

3.8.2 Transformation into *E. coli* DH5α

Competent cells of *E. coli* DH5α were used for transformation with plasmid DNA. About 100 µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 µl of ligated mixture was added into the tube and mixed gently. The mixture was kept on ice for 45 min. Later, heat shock was given by keeping the chilled mixture in water bath at 42˚C temperature for 2 minutes. Immediately, it was kept on ice for 5 min. To this, 900 µl of Luria broth was added and incubated in thermo shaker at 37˚C at 200 rpm for 45 min, to allow bacteria recover and express the antibiotic marker encoded by the plasmid. The culture was centrifuged at 13,000 rpm for 1 min and supernatant was discarded and the pellet was dissolved in the remaining supernatant and spreaded on Luria agar plates containing ampicillin (100µg/ml) followed by incubation at 37˚C for overnight. The recombinant cells were identified by white colony and white colonies having recombinant vectors were picked up and streaked on plates having Luria agar with $Ampicilin_{100}$ incubated at 37˚C overnight for confirmation and further use.

3.8.3 Sequencing of the fragment

Similarity searches on nucleotide and amino acid sequences were carried out using BLAST at the National Center for Biotechnology Information GenBank database. A phylogenetic tree was constructed by the neighbor-joining method based on the genetic distance.

3.8.3.1 Sequencing Analysis software

Similarity searches on nucleotide and amino acid sequences were carried out using BLAST at the National Center for Biotechnology Information (NCBI) GenBank database or Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) database. Top hits will be used to construct phylogenetic tree using neighbour-joining (NJ) method based on the genetic distance of the protein sequences using MEGA 7 (http://www.megasoftware.net/) tool.

3.8.4 Design of SCAR primers and PCR amplification conditions

The sequences from both ends of cloned RAPD fragment were used to design pairs of oligonucleotide primers with the help of clone manager software and designed primers were procured from Eurofin Genomics Pvt.Ltd., Bangalore. Genomic DNA was amplified with SCAR primers in PCR tube containing, 10x Taq- polymerase (Finnzymes) in a total volume of 25 µl. The amplification of the SCAR primer was carried out at 52˚C annealing temperature. The PCR conditions used for amplification are described below.

The amplified products were resolved electrophoretically in 1.2% agarose gel. In order to validate this primer, DNA isolated from all accessions comprising male and female plant was used. PCR conditions for SCAR consisted of the following steps: at 90˚C for 5 min, followed by 30 cycles with 1 min at 90˚C, 1 min at 53˚C and 4 min at 60˚C with the final extension of 7 min at 72˚C.

Table 3.1 RAPD primers used in the study for screening

S.No.	Name of Primer	Sequence	S.No.	Name of Primer	Sequence

1	OPA 01	CAGGCCCTTC	31	OPB 11	GTAGACCCGT
2	OPA 02	TGCCGAGCTG	32	OPB 12	CCTTGACGCA
3	OPA 03	AGTCAGCCAC	33	OPB 13	TTCCCCCGCT
4	OPA 04	AATCGGGCTG	34	OPB 14	TCCGCTCTGG
5	OPA 05	AGGGGTCTTG	35	OPB 15	GGAGGGTGTT
6	OPA 06	GGTCCCTGAC	36	OPB 16	TTTGCCCGGA
7	OPA 07	GAAACGGGTG	37	OPB 17	AGGGAACGAG
8	OPA 08	GTGACGTAGG	38	OPB 18	CCACAGCAGT
9	OPA 09	GGGTAACGCC	39	OPB 19	ACCCCCGAAG
10	OPA 10	GTGATCGCAG	40	OPB 20	GGACCCTTAC
11	OPA 11	CAATCGCCGT	41	OPC 01	TTCGAGCCAG
12	OPA 12	TCGGCGATAG	42	OPC 02	GTGAGGCGTC
13	OPA 13	CAGCACCCAC	43	OPC 03	GGGGGTCTTT
14	OPA 14	TCTGTGCTGG	44	OPC 04	CCGCATCTAC
15	OPA 15	TTCCGAACCC	45	OPC 05	GATGACCGCC
16	OPA 16	AGCCAGCGAA	46	OPC 06	GAACGGACTC
17	OPA 17	GACCGCTTGT	47	OPC 07	GTCCCGACGA
18	OPA 18	AGGTGACCGT	48	OPC 08	TGGACCGGTG
19	OPA 19	CAAACGTCGG	49	OPC 09	CTCACCGTCC
2	OPA 20	GTTGCGATCC	50	OPC 10	TGTCTGGGTG
21	OPB 01	GTTTCGCTCC	51	OPC 11	AAAGCTGCGG
22	OPB 02	TGATCCCTGG	52	OPC 12	TGTCATCCCC
23	OPB 03	CATCCCCCTG	53	OPC 13	AAGCCTCGTC
24	OPB 04	GGACTGGAGT	54	OPC 14	TGCGTGCTTG
25	OPB 05	TGCGCCCTTC	55	OPC 15	GACGGATCAG
26	OPB 06	TGCTCTGCCC	56	OPC 16	CACACTCCAG
27	OPB 07	GGTGACGCAG	57	OPC 17	TTCCCCCCAG
28	OPB 08	GTCCACACGG	58	OPC 18	TGAGTGGGTG
29	OPB 09	TGGGGGACTC	59	OPC 19	GTTGCCAGCC
30	OPB 10	CTGCTGGGAC	60	OPC 20	ACTTCGCCAC

3.9 Calculation of parameters of genetic diversity

Various parameters like components, number of monomorphic and polymorphicloci, Polymorphism Information Content (PIC) and polymorphism percentage werecalculated. Polymorphism Information Content (PIC) was calculated according toformula described by Joshi *et al*. (2000).

PIC=1-Σf^2 where f is the frequency of i^{th} allele.

Chapter-IV

Experimental findings

DNA markers have been reliably used in cultivar identification, for diversity analysis, and for construction of genetic maps and tagging of agronomically important genes (Ravi *et al*., 2003).

The present investigation was carried out to assess early sex determination in pointed gourd. The information revealed from unique molecular markers distinguishing to sexes was used for designing SCAR markers to discriminate male and female plants, comprising 25 accessions of pointed gourd. The primers were designed from distinct bands by sequencing these products and validation of these SCAR primers was performed using the male and female lines used in the present study. The results obtained from the experiments conducted in the present study are presented under the following subheadings.

4.1 Analysis of Variance-

Mean data for 6 characters were subjected to analysis of variance for the RBD. The mean sum of squares due to genotypes were significant for all the 6 characters under study (Table 4.1.) indicated that the genotypes included in the study were genetically diverse and considerable amount of variability were present. Hence, there is ample scope for inclusion of promising genotypes in breeding programme for yield and its component traits.

Table- 4.1: Analysis of variance for 6 quantitative characters in pointed gourd

	Mean sum of squares		
Characters	Repl. (df-02)	Tr. (df-24)	Error (df-48)
Vine girth (mm)	4.083	28.627**	5.743
Internodal distance (cm)	3.328	6.435**	1.306
Petiole length (cm)	0.703	1.524**	0.042
NNFA	10.818	15.948**	0.767
NPB	0.21	2.56**	0.074
DAF/MF	36.467	105.401**	4.601

** Significant at 1 % level of significance

4.2 Mean performance

The mean performances of genotypes for all the characters under study have been presented in Table-4.2. In general a wide range of mean values within the genotypes were found for the respective characters. Character wise mean performance of the genotypes are being discussed here.

4.2.1 GROWTH CHARACTERS

4.2.1.1 Vine girth (mm)-

Data pertaining to plant height has been presented in Table-4.2 and depicted graphically in figure 4.1. It would be evident from the data that the maximum vine girth (27.733 mm) was recorded in BRPG 12-8 which showed statistical parity with Rajendra Parwal-1 (21.267 mm). However, the minimum vine girth was obtained in genotype BRPG 12-9 (9.47 mm) which was statistically at par of BRPG 13-5 (15.6 mm).

4.2.1.2 Inter-nodal distance (cm)-

The inter-nodal distance of different genotypes provided in Table-4.2 and illustrated pictorially in figure 4.2 indicated that among the genotypes, the highest inter-nodal distance (12.75 cm) was observed in BRPG 13-19 followed by BRPG 13-5 (12.22 cm), BRPG 12-7 (11.97 cm), BRPG M 13-4 (11.79 cm) and BRPG 13-28 (11.44 cm). While, the lowest inter-nodal distance (7.56 cm) was produced by genotype BRPG 13-27 but it was statistically alike to BRPG 13-29, BRPG 13-10, BRPG 12-9 and BRPG 13-25 having the inter-nodal distance of 8.25 cm, 8.30 cm, 8.39 cm and 8.64 cm, respectively.

4.2.1.3 Petiole length (cm)

From the data regarding the petiole length presented in Table-4.2 and depicted graphically in figure 4.3. It was found that the genotypes which gave significantly maximum number of primary branches per plant are BRPG 13-16 (4.56 cm) and BRPG 12-1 (4.41 cm). However, the lowest petiole length was noticed in Rajendra Parwal-2 (1.72 cm) and BRPG 12-7 (2.13 cm).

4.2.1.4 Number of node at which first flower appeared

It is obvious from the data regarding number of node at which first flower appeared which is presented in Table-4.2 and illustrated pictorially in figure 4.4 that the in male plants the highest number of node at which first flower appeared was recorded in BRPG M 13-3 (11.51) and lowest was recorded in BRPG M 13-4 (11.27) and other two males BRPG M 13-1 and BRPG M 13-2 are statistically at par with these two male plants i.e. 11.43 and 11.33, respectively. Among females, highest number of node at which first flower appeared was recorded in Rajendra Parwal-2 (18.45) which was statistically at par with BRPG 12-8 (17.09). While, the lowest number of node at which first flower appeared was showed by BRPG 13-26 (8.65) which showed statistical at par with genotypes BRPG 12-11(9.28) and Rajendra Parwal-1 (9.75).

4.2.1.5 Number of primary branches

From the data regarding the number of primary branches per plant presented in Table-4.2. and depicted graphically in figure 4.5. It was found that the genotypes which gave significantly maximum number of primary branches per plant are BRPG 13-5 and RPG 13-27 i.e. 9.39, which was found to be at par with genotype BRPG 12-13 (9.33). However, the lowest number of primary branches per plant (6.09) was noticed in Rajendra Parwal-2 which was almost statistically similar to BRPG 13-22, BRPG 13-27 and Rajendra Parwal-1 i.e. 6.24, 6.56 and 6.63, respectively.

4.2.1.6 Days to anthesis of first male/female flower

Data pertaining days to anthesis of first male/female flower which is given in Table-4.2 and depicted graphically in figure 4.6, it was evident that the number of days taken from transplanting to of first male/female flower was recorded minimum in case of male in BRPG M13-4 (179.51) and maximum recorded in BRPG M13-3 (191.44). While in case of female plants it was recorded minimum in genotype BRPG 13-22 (175.11) however, it was statistically at par with BRPG 12-9 (177.81), BRPG 12-13 (178.35), Rajendra Parwal-2 (178.45) and BRPG 13-25 (179.57). The genotype BRPG 13-28 took the highest number of days from transplanting to days to anthesis

of first female flowering (198.18) however, it was statistically at par with genotypes BRPG 12-8 (194.8), BRPG 13-16 (193.12) and BRPG 13-5 (192.17)

4.3 STUDIES ON COMPONENT OF VARIABILITY

4.3.1 Analysis of variance

The analysis of variance (ANOVA) for all the characters indicated highly significant variations among the genotypes for all the characters under investigation. Highly significant difference among different genotypes observed for vine girth, inter-nodal length, petiole length, number of node at which first flower appears, primary branches and days to anthesis of male/female flowers.

4.3.2 Range and mean

The range of variation for different characters and their mean have been presented in Table 4.3. From the table, it is apparent that the characters expressed a high range of variation. . The days to anthesis of first male/female flower exhibited the highest range of variation varying from 175.106 to 198.176 with the general mean of 184.512. The vine girth was the next character which varied from 9.4667 mm to 27.733 mm with the general mean of 18.305 mm. The petiole length had however the lowest ranges of variation (1.723 cm to 4.556 cm) with the general mean of 2.841 cm which is followed by number of primary branches i.e. from 6.087 to 9.393 with the general mean of 7.724. The remaining two characters *viz;* number of node at which first flower appears and inter-nodal distance also showed substantial variability in descending order of magnitude during investigation.

Table - 4.3: Range and Mean table

	Range		
Characters	Low	High	Mean
Vine girth	9.4667	27.733	18.305
Inter-nodal distance	7.556	12.753	10.071
Petiole length	1.723	4.556	2.841

NNFFA	8.65	18.45	12.575
NPB	6.087	9.393	7.724
DAF/MF	175.106	198.176	184.512

Table-4.2: Mean performance of 25 genotypes for growth characters.

Genotypes	Vine girth (mm)	Inter-nodal distance (cm)	Petiole length (cm)	NNFFA	NPB	DAF/MF
M13-1	15.8333	8.9733	2.59	11.43	8.6467	183.803
M13-2	20.1	8.7667	2.32	11.33	7.873	180.967
M13-3	18.3	10.286	3.733	11.51	6.9233	191.44
M13-4	16.967	11.787	3.843	11.267	8.6	179.51
BRPG 12-8	27.733	10.31	3.223	17.086	7.726	194.803
BRPG 12-10	18.667	10.167	2.527	12.077	7.803	179.51
BRPG 12-1	18.8	11.083	4.413	10.57	6.903	182.397
BRPG 13-29	16.967	8.253	3.03	13.35	7.257	182.663
BRPG 13-16	16.1	11.35	4.557	16.29	7.923	193.116
BRPG 13-10	17.733	8.3	2.41	12.31	8.013	183.48
BRPG 12-11	18.667	9.797	2.227	9.28	7.34	187.207
BRPG 13-19	16.833	12.753	3.45	12.47	8.343	187.917
BRPG 13-20	19.267	9.83	3.547	12.533	7.557	180.073
BRPG 12-9	9.467	8.393	2.59	12.21	7.66	177.813
BRPG 12-13	18.567	11.3	2.36	13.52	9.33	178.35
BRPG 13-26	18.067	9.327	2.59	8.65	7.277	181.843
BRPG 13-5	15.6	12.217	2.373	12.35	9.393	192.177
NIMIA	17.6	11.38	2.773	13.61	8.247	187.08
RP2	19.5	8.68	1.723	18.45	6.087	178.447
RP1	21.267	8.687	2.576	9.747	6.63	187.303
BRPG 12-7	20.133	11.967	2.127	11.707	6.883	183.503
BRPG 13-22	22.1	7.557	2.48	11.56	6.243	175.107

BRPG 13-27	18.6	10.54	2.427	12.097	9.393	186.543
BRPG 13-25	18.533	8.643	2.383	15.603	6.56	179.57
BRPG 13-28	16.233	11.44	2.757	13.39	8.323	198.177
Mean	18.305	10.071	2.841	12.575	7.724	184.512
C.V.	13.091	11.349	7.233	6.968	3.54	1.162
F ratio	4.984	4.925	36.082	20.768	34.225	22.909
S.E.	1.383	0.66	0.118	0.55	0.157	1.328
C.D. 5%	3.934	1.876	0.337	1.438	0.449	3.521
C.D. 1%	5.248	2.503	0.45	1.919	0.599	4.697

4.3.3 Phenotypic variance

The data in Table- 4.4 depicted a wide range of phenotypic variance for majority of characters under study. The characters like days to anthesis of male and female flowers (38.201) showed high phenotypic variance and vine girth (13.372) exhibited moderate range of variability whereas number of node at which first flower appear (5.828), inter nodal distance (3.016), number of primary branches (0.903) and petiole length (0.536) showed narrow range of phenotypic variance.

4.3.4 Genotypic variance

It is apparent from the data presented in Table- 4.4 that the days to anthesis of male and female flowers (33.600) showed high genotypic variance. However, narrow range of genotypic variance was recorded in vine girth (7.628), number of node at which first flower appear (5.060), inter nodal distance (1.710), number of primary branches (0.829) and petiole length (0.494) in descending order.

4.3.5 Coefficient of variation

4.3.5.1 Phenotypic coefficient of variation

The analysed data concerning phenotypic coefficient variation have been depicted in Table- 4.4 and illustrated pictorially in figure 4.7 which revealed that petiole length showed the highest coefficient of variation (25.772 %). In present study, the PCV was moderate for vine girth (19.976 %), number of node at which first flower appear (19.197 %), inter nodal distance (17.245 %) and number of primary branches (12.304 %). While,

days to anthesis of male and female flower (3.350 %) showed lowest phenotypic coefficient variation.

4.3.5.2 Genotypic coefficient of variation

Data pertaining to genotypic coefficient of variation presented in Table- 4.4 and depicted graphically in figure 4.7 indicated a wide spectrum of GCV. The maximum GCV was noted for petiole length (24.736 %). While, the characters like vine girth (15.088 %), number of node at which first flower appear (17.887 %), inter-nodal distance (12.983 %) and number of primary branches (11.783 %) exhibited moderate genotypic coefficient of variations. However, lowest genotypic coefficient of variation was recorded in days to anthesis of male and female flower (3.142 %).

Table-4.4: Estimates of phenotypic, genotypic variances and coefficient of variation

Characters	V_g	V_p	GCV	PCV
Vine girth	7.628	13.372	15.088	19.976
Inter-nodal distance	1.71	3.016	12.983	17.245
Petiole length	0.494	0.536	24.736	25.772
NNFFA	5.06	5.828	17.887	19.197
NPB	0.829	0.903	11.783	12.304
DAF/MF	33.6	38.201	3.142	3.35

4.4 Heritability

The table of estimates of heritability for all the characters under study are presented in Table- 4.5 and illustrated pictorially in figure 4.8 revealed that the majority of the characters were highly heritable in nature and the highest estimate of heritability (92.1 %) was observed for petiole length closely followed by number of primary branches (91.7 %), days to anthesis of male and female flower (88.0 %), number of node at which first flower appear (86.8 %). While, vine girth (57.0 %) and inter-nodal distance (56.7 %) had moderate heritability.

4.4.1 Genetic Advance

The data with respect to genetic advance among the different characters under investigation presented in Table-4.5 and depicted graphically in figure 4.8 indicated that the days to anthesis of male and female flower (14.352 %) showed moderate genetic

advance. While, rest of characters like number of node at which first flower appear (5.534 %), vine girth (5.507 %), inter-nodal distance (2.599 %), number of primary branches (2.301 %) and petiole length (1.781%) noticed low genetic advance.

4.4.2. Genetic advance as percentage of mean

Since the genetic advance as percentage of mean gives a better picture of genetic gain among the characters under study than genetic advance, therefore, it was calculated and provided in Table-4.5 and depicted graphically in figure 4.8 which revealed that genetic advance as percentage of mean ranged from 62.679 % to 7.778 % for different characters. The highest genetic advance as percentage of mean (62.679 %) was obtained for petiole length followed by number of node at which first flower appear (44.002 %), vine girth (30.086 %), number of primary branches (29.792 %), inter-nodal distance (25.805 %). However, the character days to anthesis of male and female flower (7.778 %) had low amount of genetic advance as percentage of mean.

Table-4.5: Estimates of heritability, genetic advance and genetic advance as percent of mean

Characters	Heritability	GA	GA%
Vine girth	0.57	5.507	30.086
Inter-nodal distance	0.567	2.599	25.805
Petiole length	0.921	1.781	62.679
NNFFA	0.868	5.534	44.002
NPB	0.917	2.301	29.792
DAF/MF	0.88	14.352	7.778

4.5. RAPD marker analysis-

Number of amplified bands by different decamers ranged from 3 (OPA 13) TO 13 (OPA-12, OPA-14, OPB-02, OPB-03, OPB-10, OPB-13, OPC-04, OPC-20). RAPD revealed polymorphism ranges from 37.5 (OPA-16, OPA-19, OPA-20) to 92.3 (OPC-04) and provided in Table 4.6.

S. No.	Primer	Sequence	Total number of amplified fragments	Monomorphic bands	Polymorphic bands	Polymorphism (%)
1	OPA1	CAGGCCCTTC	10	4	6	60

2	OPA6	GGTCCCTGAC	6	2	4	66.7
3	OPA11	CAATCGCCGT	6	3	3	50
4	OPA12	TCGGCGATAG	13	2	11	84.6
5	OPA13	CAGCACCCAC	3	1	2	66.7
6	OPA14	TCTGTGCTGG	13	2	11	84.6
7	OPA15	TTCCGAACCC	6	1	5	83.3
8	OPA16	AGCCAGCGAA	8	5	3	37.5
9	OPA17	GACCGCTTGT	7	3	4	57.1
10	OPA19	CAAACGTCGG	8	5	3	37.5
11	OPA20	GTTGCGATCC	8	5	3	37.5
12	OPB1	GTTTCGCTCC	6	1	5	83.3
13	OPB2	TGATCCCTGG	13	8	5	38.5
14	OPB3	CATCCCCCTG	13	3	10	76.9
15	OPB4	GGACTGGAGT	6	2	4	66.7
16	OPB6	TGCTCTGCCC	6	2	4	66.7
17	OPB7	GGTGACGCAG	7	2	5	71.4
18	OPB10	CTGCTGGGAC	13	4	9	69.2
19	OPB12	CCTTGACGCA	5	1	4	80
20	OPB13	TTCCCCCGCT	13	7	6	46.2
21	OPB17	AGGGAACGAG	6	2	4	66.7
22	OPB19	ACCCCCGAAG	6	1	5	83.3
23	OPC4	CCGCATCTAC	13	1	12	92.3
24	OPC5	GATGACCGCC	6	1	5	83.3
25	OPC10	TGTCTGGGTG	11	1	10	90.9
26	OPC12	TGTCATCCCC	6	3	3	50
27	OPC14	TGCGTGCTTG	6	1	5	83.3
28	OPC19	GTTGCCAGCC	7	1	6	85.7
29	OPC20	ACTTCGCCAC	13	4	9	69.2

Table 4.6 List of primer, polymorphic bands, Total amplified fragments, percentage polymorphism

4.6 Identification of male and female RAPD markers

Among 29 RAPD primers finalized for genetic diversity analysis, only one primer OPC-04 detected genotype specific bands of ≈ 400 bp which was presented in figure 4.9. Further analysis revealed that, these amplified products were specific to female.

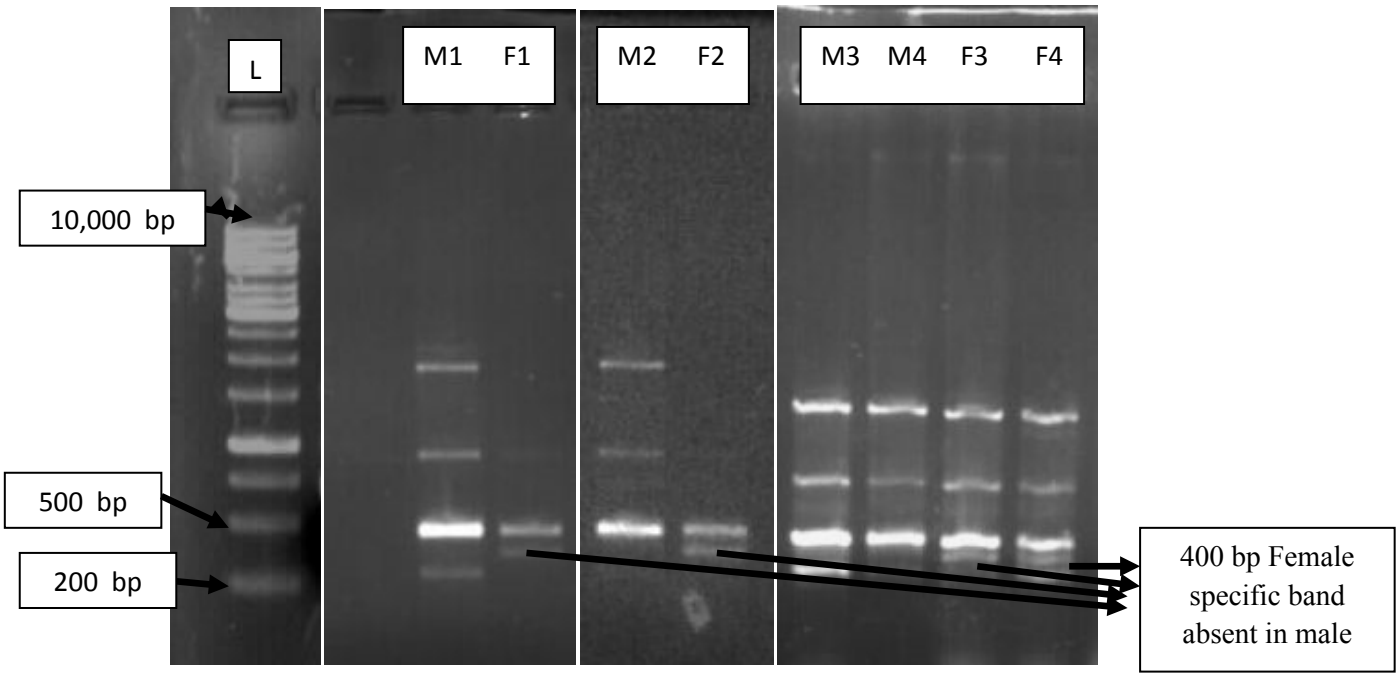

Fig 4.9 Female specific band with primer OPC -04(**M1**- BRPG M13-1, **M2**- BRPG M13-2, **M3**- BRPG M13-3, **M4**- BRPG M13-4, **F1**-BRPG RP-1, **F2**- RP-2, **F3**- BRPG 12-1, **F4**- BRPG 12-8)

RAPD amplification was performed separately for 4 male accessions and 4 female genotypes using OPC-04. The amplified products were electrophoresed in 1.2 % agarose gel. The analysis was repeated for five times using not only individuals but also pooled samples of genomic DNA corresponding to male and female accessions. Similar results were obtained in each repetition. This result paved a way to discriminate male and female plants.

4.7 Development of SCAR marker and validation of them in male and female

To confirm the result, the PCR product was electrophoresed in 1.2 % agarose gel which revealed that a 400 bp female specific product were consistently amplified by OPC-04. The elution of PCR product amplified by OPC-04 (female specific) was carried out using kit from Quiagen. The PCR product was eluted and purified and then subjected to confirmation whether same size of product is amplified or not. The eluted products were purified and quantified using Nano drop v2.02, for preparation of templates for ligation of the products in pJET 1.2 vector (Fig. 4.10)

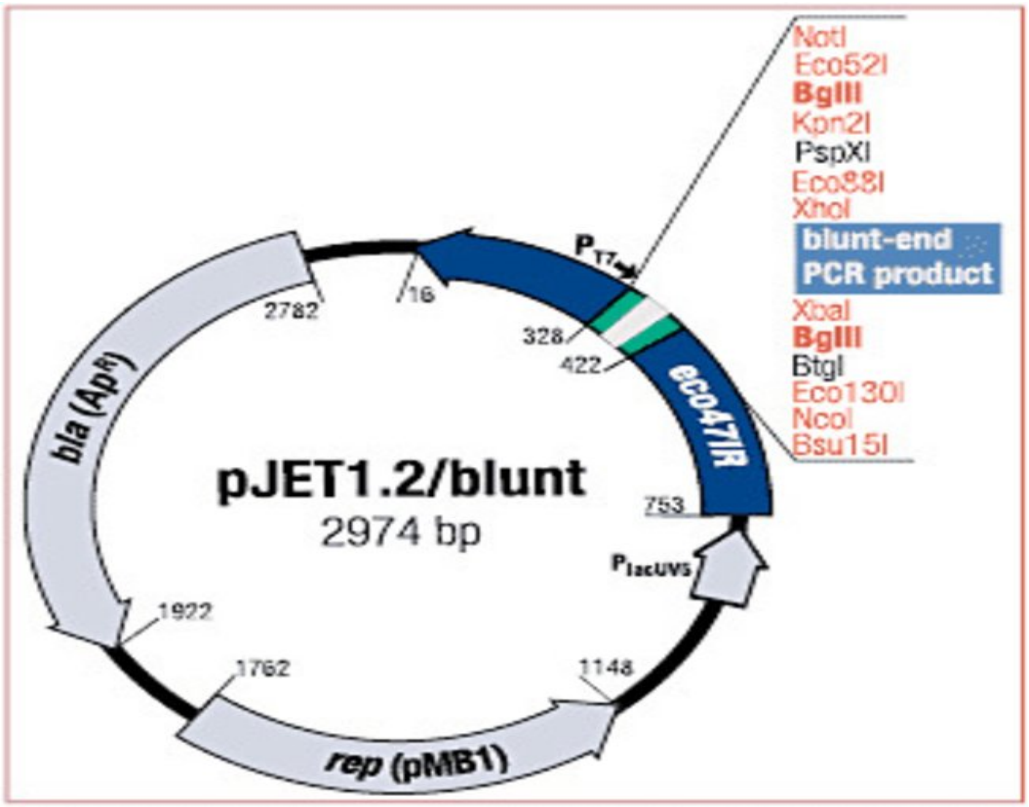

Fig. 4.10 Vector pJET1.2 with insert sequences of 2974 bp size.

4.8 Cloning, Transformation and sequencing

4.8.1 Cloning of sex specific PCR Products

Before cloning, the ligation of the PCR product into pJET 1.2 vector was performed using cloning kit procured from Quiagen. The ligated products were transformed into chemically competent *E.coli* DH5ά cells. Transformation of ligated products into DH5ά cells was confirmed by white colonies (Fig. 4.11). The white colonies containing transformed vectors were picked and used for colony PCR. The colony PCR discriminated clearly male and female specific desirable clones from undesirable clones (Figure 4.12). Then, plasmid elution was performed to extract plasmid for sequencing of PCR insert product from male and female specific desirable clones. The eluted plasmid was used as a template DNA for sequencing.

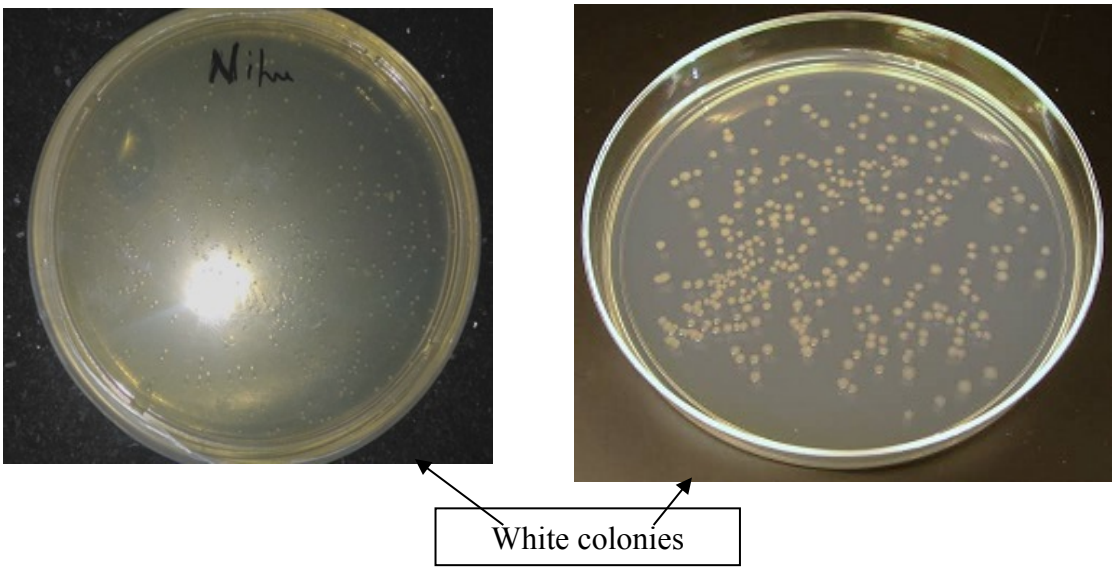

Fig. 4.11 White colonies after transformation of ligated product

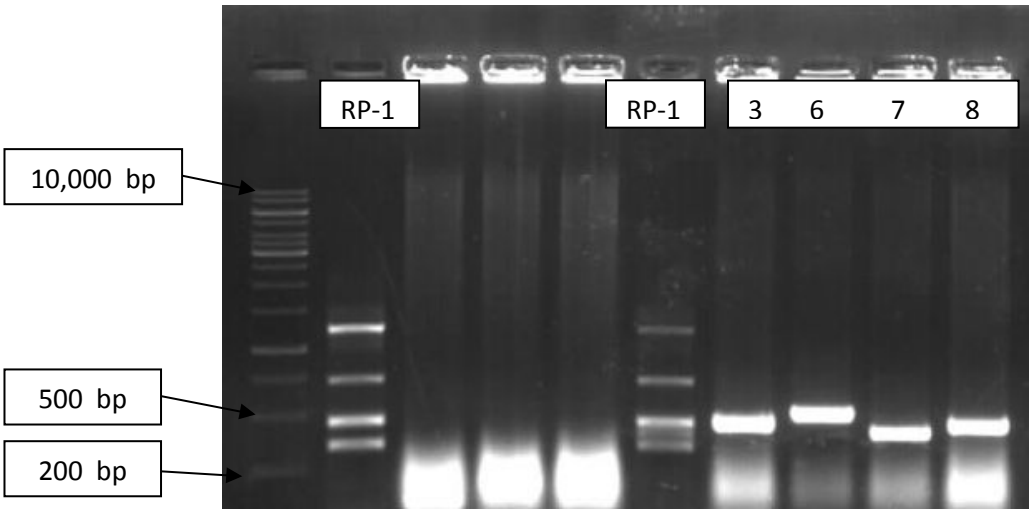

Fig. 4.12 Confirmation of the cloning of insert by PCR of colony number 3, 6,7,8 with female genotype RP-1 using vector specific primers

4.8.2 Sequencing of female specific products

The plasmid isolated from female specific clones was used as template DNA to perform sequencing using vector specific forward and reverse primers. The sequence of female (Figure 4.13) were aligned using BioEdit software. The aligned sequence was subjected to BLAST analysis. Similarity searches on nucleotide and amino acid sequences were carried out using BLAST at the National Center for Biotechnology Information (NCBI) GenBank database or Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) database. Top hits will be used to construct

phylogenetic tree using neighbour-joining (NJ) method based on the genetic distance of the protein sequences using MEGA 7 (http://www.megasoftware.net/) tool.

4.8.4 Development of SCAR marker from male and female specific sequences.

The female specific sequences were used to design forward and reverse primers for development of SCAR marker(s) using clone manager software. The details of primers are given in Table 4.7. Two SCAR primers were designed from female specific sequence (N6Fn/r and N7Fn/r) that discriminate male and female genotypes. Out of this two primers only one SCAR primer N7Fn/r was able to discriminate male and female sexes and produced about 300 bp female specific band in all female genotypes (Fig. 4.14) but other one fails to do this may be due to crossing over of male linked gene from Y chromosome with X chromosome or amplification of unspecific product in female may also occur due to presence of some sequence similarity.

Table 4.7 Sequence of SCAR primer used for female identification

Gene ID	**N6Fn/r**	**N7Fn/r**
(Fp)	**5'GATTCTTCTAGAAAGTCCGCATCTAC 3'**	**5'TCTTCTAGACGATCCGCATCTAC 3'**
(Rp.)	**5'AACCCACATCTATATCATCTCTCG 3'**	**5'AGCAGTGCCCATGGCTGCCCAC 3'**

4.8.5 Utility of newly designed Female specific SCAR marker

The newly designed SCAR marker can be used to discriminate female plants from male plants at early stages of development. This reduces the time and effort of breeders from differentiating male and female plants which depends on the evaluation of morphological characters. This makes genetic improvement of this crop easy. The marker can also be used to discriminate male and female plants produced at commercial scales in tissue culture laboratories at seedling stage.

TATCTACTGTTGATATTGCATTTTGATGAATCTTCTAGACGATCCGCATCTACG
GCGCATTTAGACGTACCGTGGCAACT
CATGCCACCCAAACCAAGTTTGTCACCTCCACCACTACTCAAATATTAAACTCA
AGCCTTTCACCTAGAAAACACATAAT

CAAGATGCTTTCCACAAACAAGAGGGGGTCAAGGTGAGATATCTCACCAACTG
AGGAAGAAATGCACAATACAGAGCCAA
GACCACCACAAATTGATGAACATGTGGCTCAAGAGGATGTCCCCATAGTTGAG
TGGGCAGCCATGGGCACTGCTAAAAAG
GCCAAGGCTGCAGAGGTTGGTGAGAAACTCTGATCTTGCTGAAAAACTCGAGC
CATCCGGAAGATCTGGCGGCCGCTCTC
CCTATAGTGAGTCGTATTACGCCGGATGGATATGGTGTTCAGGCACAAGTGTT
AAAGCAGTTGATTTTATTCACTATGAT
GAAAAAAACAATGAATGGAACCTGCTCCAAGTTAAAAATAGAGATAATACCG
AAAACTCATCGAGTAGTAAGATTAGAGA
TAATACAACAATAAAAAAATGGTTTAGAACTTACTCACAGCGTGATGCTACTA
ATTGGGACAATTTTCCAGATGAAGTAT
CATCTAAGAATTTAAATGAAGAAGACTTCAGAGCTTTTGTTAAAAATTATTTGG
CAAAAATAATATAATTCGGCTGCAGG
GGCGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTT
TCTTAGACGTCAGGTGGCACTTTTCG
CGGAAATGTGCGCGGAACCCCTATTGTTTATTTTCTAAATACATTCAATATGTA
TCCGCTCATGAGACAATAACCCTGAT
AATGCTTCATATATTGAAAAGGAAGAGTATGAGTATCCAACATTTCGTGTCGC
CTTATCCCATTATGCGCAATTTTGCCT
TCCTGATTTTGCTCACCCAGAACGCTGGGAAGGTAAAGATGCTGACAT

Fig. 4.13 Female specific sequence of pointed gourd

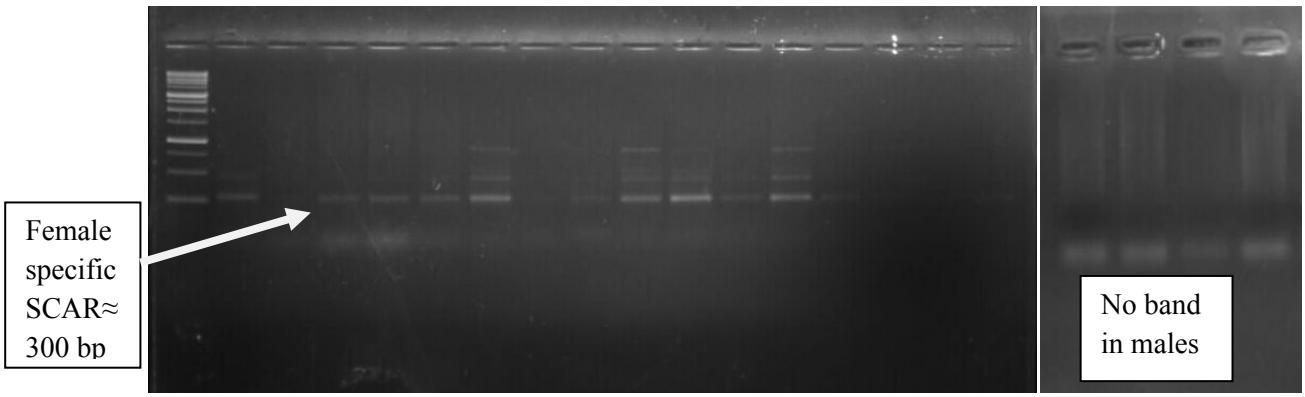

Fig.4.14 SCAR primer amplification of female specificity

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Chapter-V

Discussion

Pointed gourd is perennial vegetable crop mainly reproduce through vegetative means i.e. through root and stem cutting. It is not propagated through seed due to poor germination and viability of seeds. This plant dioecious nature and its vegetative means of propagation makes its reproduction and multiplication labour intensive and act as constraint in carrying breeding programmes in pointed gourd. So, it was very necessary to discriminate the male and female genotypes at the beginning of breeding programme to carry out crossing programmes. Identification of sex at the seedling stage is a pre requisite for genetic improvement of this crop and for maintaining proper sex ratio. However, there is little or no substantial morphological difference between male and female of *T. dioica* to identify the sex type before reproductive time.

A reliable method for early sex determination of gender before flowering is use of molecular marker which reduce time and effort of breeders. In molecular marker mostly RAPD marker is used for determining the gender of plants before flowering but due to its dominant nature it is less effective. So, its effectiveness should be increased by development of SCAR marker which was designed from RAPD profile. SCAR marker are developed with a pair of longer primers (extended sequence of a RAPD primer) that has specific sequence of approx 20 bases, which prevent site competition among primers and make result less sensitive to reaction conditions and more reproducible by increasing the specificity.

The results of the present investigation were elaborated in the preceding chapter. In this chapter scientific and logical interpretation of results obtained are discussed for better understanding. Efforts have also been made to correlate the effect of cultivars with reasoning put forth through literatures available so far and comparing the direct results of the present studies with those of past results reported by various scientists working within the country or abroad.

Morphological characters-

The efficacy of selection programme depends on the magnitude of variability present in the material under selection and the extent to which the desirable characters are heritable. This suggests the need to partition the total variability into its heritable and non heritable components. Genetic parameters *viz*; genetic coefficient of variation, heritability estimates, genetic advance are useful in differentiating of heritable and non-heritable components.

The selection of desirable genotype is primarily based on mean performance. However, for success of any breeding programme, it is very necessary to have a thorough knowledge of variability present in population. In the present investigation, significant differences were observed among the 25 lines for all the eight characters denote to substantial scope of improvement in pointed gourd. The variation among the genotypes might have occurred due to differences in genes carried by different lines, differences in the environmental factors to which these lines were exposed to and by interaction between genotypes and environment.

High phenotypic variance was recorded for days to anthesis of male and female and vine girth exhibited moderate range of variability whereas number of node at which first flower appear inter nodal distance, number of primary branches and petiole length showed narrow range of phenotypic variance. These results are in consonance with the findings of Jena *et al.* (2017), Verma *et al.* (2017) Malek *et al*. (2007), Khan *et al.* (2009). However, in case of genotypic variance only days to anthesis of male and female flowers showed high genotypic variance while all other characters like vine girth, number of node at which first flower appear, inter nodal distance , number of primary branches and petiole length exhibited low range of genotypic variance. The differences between these two values were quite less in most of the characters suggesting a negligible influence of environment on such characters which agrees with that of Rabbani *et al.* (2012), Hanumegowda *et al*. (2011) and Koppad *et al. (*2015).

Phenotypic coefficient of variance was higher than the genotypic and environmental coefficient of variances. In the pooled analysis, the highest value of phenotypic coefficient of variance was recorded for petiole length. While, the PCV was moderate for vine girth, numbers of node at which first flower appear, inter nodal distance and number of primary branches. While, days to anthesis of male and female flower

showed lowest phenotypic coefficient variation. This result has been correlated with Dora *et al.* (2003), Khan *et al.* (2009) in pointed gourd, Bharathi *et al.* (2011) in spine gourd, Hanumegowda *et al*. (2011) and Rabbani *et al.* (2012) in ridge gourd. However, the effectiveness of selection for any character depends not only on the amount of phenotypic and genotypic variability, but also on estimates of broad sense heritability.

Heritability in broad sense may be defined as it is the ratio of genotypic variance by phenotypic variance. Heritability is important genetical parameters since it provides a measure of overall importance of hereditary determination of a character and permits the prediction of progress in a selection scheme for a given character. It is a property not only of a character but also of a population and environment under which the plants were grown (Falconer, 1981) and Selection unit (Hanson, 1956).

The index of transmissibility of a character interpreted by heritability is act as a measure of selection for a particular character in various genotypes. The magnitude of heritability estimates in broad sense revealed that the majority of the characters were highly heritable in nature and the highest estimate of heritability was observed for petiole length closely followed by number of primary branches, days to anthesis of male and female flower, number of node at which first flower appear. While, vine girth and inter-nodal distance had moderate heritability. The results of the present investigation are in a line with the observations of Srivastava *et al.* (2005), Khan *et al.* (2009), Jena *et al.* (2017) and Verma *et al.* (2017) in pointed gourd, Rajkumar and Karuppaiah (2007) in snake gourd, Panigrahi *et al.* (2015) in ivy gourd.

The degree of success through selection is realised more efficiently in those characters which have high heritability along with high genetic advance. Panse and Sukhatme (1957) reported that high heritability along with high genetic advance is an indication of additive gene effects and high heritability correlation with low genetic advance is an indication of dominance and epistatic effects. The genetic advance being the function of heritability, selection intensity and phenotypic standard deviation, indicates the magnitude of improvement in the desired direction that can be expected in a particular character by selecting a certain portion of the population.

In the present investigation, genetic advance among the different characters indicates that that the days to anthesis of male and female flower showed moderate genetic advance. While, rest of characters like number of node at which first flower

appear, vine girth, inter-nodal distance, number of primary branches and petiole length noticed low genetic advance.

The high genetic advance as percentage of mean along with high heritability was obtained for petiole length, number of node at which first flower appear, vine girth, number of primary branches, inter-nodal distance. Thus, these characters were under additive gene effect and could be improved by simple selection schemes. However, the character days to anthesis of male and female flower had low amount of genetic advance as percentage of mean but high heritability. The high heritability is being exhibited due to favourable influence of environment rather than genotype and selection for such trait may not be rewarding. The findings are in accordance with Choudhary *et al.* (2014), Jena *et al.* (2017), Khan *et al.* (2009).

Molecular Analysis

From the morphological characterisation there were no any features that differentiate male and female sexes at the early stage. So, it was reliable to proceed towards molecular analysis. SCAR marker was most efficient and reproducible marker for sex identification. But for designing SCAR there was a pre requisite of RAPD analysis on basis of which SCAR primer was designed.

RAPD marker analysis

About 29 random primers were screened to select marker showing good amplifications which were further used to identify regions associated with sex linked marker.

Number of amplified bands by different decamers ranged from 3 to 13. RAPD revealed polymorphism ranges from 37.5 to 92.3. Specific amplification with 400bp band was produced by OPC-04. Reproducibility of the primer was tested by repeating the PCR amplification thrice under similar conditions. A major problem associated with RAPD technology is the reproducibility of the profiles (Melotto, *et al.* 1996, Zhag and Stommet, 2001). The amplified products were separated on 1.2% agarose gel and were analyzed by comparing them with 1Kb DNA ladder. From the sequence of 400bp, two SCAR primers were designed N6Fn/r and N7Fn/r.

SCAR marker development

To ameliorate the utility of RAPDs, SCARs that have greater reliability than RAPDs were developed. SCARs have several advantages over RAPD markers in marker assisted selection. Because more stringent reaction conditions are used, SCAR markers are

generally more allele specific and SCAR amplifications are more stable and reliable and more easily reproduced in different laboratories with various thermal cycles (Yuskianti and Shiraishi, 2010, Kevalkumar *et al.* 2015, Mizia *et al.* 2016). Thus OPC-04 was converted to a SCAR for increasing reproducibility. This may help in detection of sex at seedling stage. SCAR developed from RAPD markers have the advantages of cost effectiveness and technical simplicity. Two pairs of SCAR marker N6Fn/r and N7Fn/r were designed with 26 mers (forward), 24 mers (reverse) and 23 mers (forward), 22 mers (reverse) in length respectively. Out of this two primer only one primer i.e. N7Fn/r produced female specific bands of about 300 bp which differentiate male and female sexes. Thus, sex prediction can be done at any development stage of plant growth. Knowledge of the sex of pointed gourd is important in selecting parents for use in hybridization work. In addition for micro propagation, the early detection or identification of the sex type of a particular seedling would be advantageous, since the desired sex type can be selected prior to micro propagation. This will ensure that the resulting micro propagated plants are 100% female (Al-Ameri *et al.* 2016, Prasanthi *et al.* 2010, Jiang *et al.* 2009).

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Chapter-VI

Summary and Conclusion

An investigation entitled '**Morphological and SCAR marker development for early sex determination in pointed gourd (*Trichosanthes dioica* Roxb.)'** was carried out at Vegetable Research Farm of the Department of Horticulture (Vegetable & Floriculture), Bihar Agricultural University, Sabour, Bhagalpur (Bihar) during the year, 2015-2016. All the 25 genotypes were grown in Randomized Block Design with three replications. The recommended agronomic practices were adopted for raising a good pointed gourd crop. Observations were recorded on six economically important traits *viz*; vine girth, inter-nodal length, petiole length, number of primary branches, numbers of node at which first flower appears and days to anthesis of male and female flowers. Highly significant difference among different genotypes observed for all the characters under investigation. The characters like days to anthesis of male and female flowers showed high phenotypic and genotypic variance. The maximum GCV, PCV, heritability and genetic advance as percent of mean was noted for petiole length, so additive gene action is present. The majority of the characters were highly heritable in nature. petiole length, number of primary branches, days to anthesis of male and female flower and number of node at which first flower appear. Hence, there is ample scope for a plant breeder for further improvement in these characters. However, the character days to anthesis of male and female flower had low amount of genetic advance as percentage of mean but high heritability indicating non-additive gene action.

The pointed gourd (*Trichosanthes dioica* Roxb.) is one of the most important and choicest cucurbitaceous vegetable crop in India. It has high economic value with export potential, but cultivated mainly on Daira lands of major rivers *viz*; Ganga, Saryu, Kosi, Burhi, Gandak. This plant dioecious nature and its vegetative means of propagation makes its reproduction and multiplication labour intensive and act as constraint in carrying breeding programmes in pointed gourd. Hence, determination of sex in pointed gourd is of utmost importance in the view of commercial agriculture. To date, several molecular markers for sex type determination in dioecious plants, including pointed gourd, have been reported (Kumar *et al*., 2012).

In the present study, molecular markers namely RAPD and conversion of RAPD marker to SCAR were carried out for sex determination. The experimental material comprised 4 male and 21 female genotypes of pointed gourd. Total 50 RAPD primers were used for screening to identify sex linked gene in *T. dioica* Roxb. Among 50 RAPD primers screened, 29 RAPD primers gave good results.

One band/ allele (of 400 bp) unique to female plants in the present study are not found at all in any of the male plants. The primer OPC-04 proved to be highly effective for the determination of male *T. dioica* Roxb. Sex specific band was eluted, cloned (using pJET 1.2 vector) and sequenced. The SCAR marker generated from the OPC-04 distinguished female plants from male plants. Two SCAR primers were designed from male specific RAPD sequence. Out of two SCAR primers only one N7Fn/r primer gave a unique 300 bp amplicon in all the female plants tested and did not give any amplification in other male plants.

This newly designed SCAR primer can be used to differentiate female plants from male plants at early stages of development. This reduces the time consuming methods of discriminating male and female plants which depends on the evaluation of morphological characters. This marker can also be used to discriminate male and female plants produced at commercial scales in tissue culture laboratories at seedling stage. New finding come out first time from this study was, N7Fn/r SCAR primer identify female plant at seedling stage before flowering.

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APPENDICES

Appendix -1: ANOVA table of each quantitative character of 25 genotypes

ANOVA for Vine girth (mm)

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability
Replicate	2	8.167469	4.083735	0.7110	0.4962
Treatments	24	687.071227	28.627968	4.9846	0.0000 ***
Error	48	275.679191	5.743316		
General Mean		18.3053	(1.3836)	95%	99%
S.E.Diff.		1.9568	Critical Diff.	3.9343	5.2484
S.E.Diff from Mean		1.3557	Critical Diff.	2.7258	3.6362
Var Environmental		5.7433	ECV	13.0919 %	
Var Genotypical		7.6282	GCV	15.0881 %	
Var Phenotypical		13.3715	PCV	19.9762 %	
h^2 (Broad Sense)		0.5705	S.E. of h^2	0.7286	
Genetic Advancement		4.2973	5.5073		
Gen.Adv as % of Mean		23.4759 %	30.0856 %(selection intensity at 5% & 1%)		

ANOVA for Internodal distance (cm)

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability
Replicate	2	6.656044	3.328022	2.5470	0.0889
Treatments	24	154.461475	6.435895	4.9254	0.0000 ***
Error	48	62.719826	1.306663		
General Mean		10.0715	(0.6600)	95%	99%
S.E.Diff.		0.9333	Critical Diff.	1.8766	2.5034
S.E.Diff from Mean		0.6466	Critical Diff.	1.3001	1.7344
Var Environmental		1.3067	ECV	11.3498 %	
Var Genotypical		1.7097	GCV	12.9829 %	
Var Phenotypical		3.0164	PCV	17.2446 %	
h^2 (Broad Sense)		0.5668	S.E. of h^2	0.7281	
Genetic Advancement		2.0279	2.5989		
Gen.Adv as % of Mean		20.1354 %	25.8046 %(selection intensity at 5% & 1%)		

ANOVA for Petiole length (cm)

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability
Replicate	2	1.407272	0.703636	16.6591	0.0000 ***
Treatments	24	36.577123	1.524047	36.0829	0.0000 ***
Error	48	2.027395	0.042237		
General Mean		2.8412	(0.1187)	95%	99%
S.E.Diff.		0.1678	Critical Diff.	0.3374	0.4501
S.E.Diff from Mean		0.1163	Critical Diff.	0.2338	0.3118
Var Environmental		0.0422	ECV	7.2335 %	
Var Genotypical		0.4939	GCV	24.7362 %	
Var Phenotypical		0.5362	PCV	25.7722 %	
h^2 (Broad Sense)		0.9212	S.E. of h^2	0.7745	
Genetic Advancement		1.3896	1.7808		
Gen.Adv as % of Mean		48.9084 %	62.6788 %(selection intensity at 5% & 1%)		

ANOVA for NNFFA

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability	
Replicate	2	21.636300	10.818150	14.0873	0.0000	***
Treatments	24	382.767703	15.948654	20.7683	0.0000	***
Error	48	36.860828	0.767934			
General Mean		12.5759	(0.5059)	95%	99%	
S.E.Diff.		0.7155	Critical Diff.	1.4386	1.9191	
S.E.Diff from Mean		0.4957	Critical Diff.	0.9967	1.3296	
Var Environmental		0.7679	ECV	6.9683 %		
Var Genotypical		5.0602	GCV	17.8874 %		
Var Phenotypical		5.8282	PCV	19.1968 %		
h^2 (Broad Sense)		0.8682	S.E. of h^2	0.7677		
Genetic Advancement		4.3179	5.5336			
Gen.Adv as % of Mean		34.3348 %	44.0018 %(selection intensity at 5% & 1%)			

ANOVA for NPB

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability	
Replicate	2	0.420403	0.210201	2.8098	0.0701	
Treatments	24	61.448806	2.560367	34.2251	0.0000	***
Error	48	3.590865	0.074810			
General Mean		7.7247	(0.1579)	95%	99%	
S.E.Diff.		0.2233	Critical Diff.	0.4490	0.5990	
S.E.Diff from Mean		0.1547	Critical Diff.	0.3111	0.4150	
Var Environmental		0.0748	ECV	3.5408 %		
Var Genotypical		0.8285	GCV	11.7834 %		
Var Phenotypical		0.9033	PCV	12.3039 %		
h^2 (Broad Sense)		0.9172	S.E. of h^2	0.7740		
Genetic Advancement		1.7958	2.3014			
Gen.Adv as % of Mean		23.2470 %	29.7923 %(selection intensity at 5% & 1%)			

ANOVA for DAF/MF (right data)

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability	
Replicate	2	72.933090	36.466550	7.9261	0.0011	**
Treatments	24	2529.626481	105.401103	22.9093	0.0000	***
Error	48	220.838164	4.600795			
General Mean		184.5120	(1.2384)	95%	99%	
S.E.Diff.		1.7513	Critical Diff.	3.5213	4.6974	
S.E.Diff from Mean		1.2134	Critical Diff.	2.4396	3.2545	
Var Environmental		4.6008	ECV	1.1625 %		
Var Genotypical		33.6001	GCV	3.1416 %		
Var Phenotypical		38.2009	PCV	3.3497 %		
h^2 (Broad Sense)		0.8796	S.E. of h^2	0.7692		
Genetic Advancement		11.1988	14.3518			
Gen.Adv as % of Mean		6.0694 %	7.7783 %(selection intensity at 5% & 1%)			

Appendix 2: Qualitative characters of all 25 genotypes

S. No.	Genotypes	Vine shape	Stem pubescence	Tendril branhing	Leaf shape	Leaf margin
1	M13-2	Angular	Pubescent	Unbranched	Cordate	Dentate
2	M13-1	Angular	Pubescent	Unbranched	Cordate	Serrate
3	M13-3	Angular	Pubescent	Unbranched	Cordate	Serrate
4	M13-4	Angular	Pubescent	Unbranched	Cordate	Dentate
5	BRPG 12-8	Angular	Pubescent	Unbranched	Sagittate	Dentate
6	BRPG 12-10	Angular	Pubescent	Unbranched	Cordate	Serrate
7	BRPG 12-1	Angular	Pubescent	Unbranched	Cordate	Serrate
8	BRPG 13-29	Angular	Pubescent	Unbranched	Sagittate	Dentate
9	BRPG 13-16	Angular	Pubescent	Unbranched	Sagittate	Serrate
10	BRPG 13-10	Angular	Pubescent	Unbranched	Sagittate	Serrate
11	BRPG 12-11	Angular	Pubescent	Unbranched	Cordate	Dentate
12	BRPG 13-19	Angular	Pubescent	Unbranched	Sagittate	Dentate
13	BRPG 13-20	Angular	Pubescent	Unbranched	Cordate	Dentate
14	BRPG 12-9	Angular	Pubescent	Unbranched	Cordate	Dentate
15	BRPG 12-13	Angular	Pubescent	Unbranched	Cordate	Serrate
16	BRPG 13-26	Angular	Pubescent	Unbranched	Cordate	Serrate
17	BRPG 13-5	Angular	Pubescent	Unbranched	Sagittate	Serrate
18	NIMIA	Angular	Pubescent	Unbranched	Cordate	Serrate
19	RP2	Angular	Pubescent	Unbranched	Sagittate	Dentate
20	RP1	Angular	Pubescent	Unbranched	Cordate	Serrate
21	BRPG 12-7	Angular	Pubescent	Unbranched	Cordate	Dentate
22	BRPG 13-22	Angular	Pubescent	Unbranched	Cordate	Serrate
23	BRPG 13-27	Angular	Pubescent	Unbranched	Sagittate	Serrate
24	BRPG 13-25	Angular	Pubescent	Unbranched	Sagittate	Dentate
25	BRPG 13-28	Angular	Pubescent	Unbranched	Sagittate	Serrate

Appendix 2: contd.

S. No.	Genotypes	Leaf colour	Leaf size	Leaf puescent density	Tendril type
1	M13-2	Dark green	Large	Dense	Straight
2	M13-1	Green	Medium	Sparse	Straight
3	M13-3	Dark green	Large	Sparse	Straight
4	M13-4	Dark green	Medium	Dense	Straight
5	BRPG 12-8	Dark green	Medium	Dense	Coiled
6	BRPG 12-10	Dark green	Small	Sparse	Coiled
7	BRPG 12-1	Light green	Large	Dense	Coiled
8	BRPG 13-29	Dark green	Medium	Intermediate	Coiled
9	BRPG 13-16	Dark green	Large	Dense	Straight
10	BRPG 13-10	Dark green	Large	Intermediate	Straight
11	BRPG 12-11	Green	Small	Sparse	Coiled
12	BRPG 13-19	Green	Medium	Sparse	Coiled
13	BRPG 13-20	Dark green	Medium	Sparse	Coiled
14	BRPG 12-9	Green	Medium	Sparse	Coiled
15	BRPG 12-13	Green	Large	Dense	Coiled
16	BRPG 13-26	Green	Small	Intermediate	Coiled
17	BRPG 13-5	Dark green	Medium	Sparse	Straight
18	NIMIA	Green	Small	Intermediate	Coiled
19	RP2	Green	Medium	Intermediate	Coiled
20	RP1	Green	Large	Intermediate	Coiled
21	BRPG 12-7	Dark green	Large	Sparse	Coiled
22	BRPG 13-22	Dark green	Large	Dense	Coiled
23	BRPG 13-27	Dark green	Medium	Intermediate	Coiled
24	BRPG 13-25	Dark green	Small	Sparse	Straight
25	BRPG 13-28	Dark green	Medium	Sparse	Coiled