MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF BYADAGI CHILLI (*CAPSICUM ANNUUM* L.)

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)"** is a bonafide record of research work done independently by **Ms. Renuka S. Karennavar (2012-11-108)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
ASTA	American Spice Trade Association
Bp	Base pair
β	Beta
CS	Capsaicin synthase
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^{0}C	Degree Celsius
cm	Centimeter
DAT	Days after transplanting
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine TetraAcetic Acid
g	Gram
GCA	General combining ability
GCV	Genotypic coefficient variation
GD	Genetic distances
a	Hectare
HCl	Hydrochloric Acid
HEGS	High efficiency genome scanning
HPLC	High-performance liquid chromatography
ISSR	Inter Simple Sequences Repeat
Kb	Kilo basepairs
KAU	Kerala Agricultural University

L	Litre
M	Molar
MAS	Marker-Assisted Selection
Mg	Milligram
Ml	Micro litre
MLM	Modified location model
µM	Micro molar
ml	Millilitre
Mg	Magnesium
$MgCl_2$	Magnesium Chloride
Mm	Milli mole
µg	Microgram
µl	Microlitre
NaCl	Sodium Chloride
ng/µl	Nanogram per micro litre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PIC	Polymorhic information content
%	Percentage
PVP	Poly vinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RNA	Ribo Nucleic acid
RNase	Ribonuclease
RFLP	Restriction Fragment Length Polymorphism
RP	Resolving power
Rpm	Revolutions per minute

SCA	Specific combining ability
SDS	Sodium dodicyl sulphate
sec	Second (s)
SIF	Small internal fruit
SHU	Scoville heat unit
SSR	Simple Sequence Repeat
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume

INTRODUCTION

1. Introduction

Chilli (*Capsicum annuum* L.), belonging to the family solanaceae is one of the most important vegetable cum spice crops. It is also called as red pepper or chili pepper and was originated in tropical America. Columbus introduced it to Europe in 15th century and it spread to rest of the globe along the spice trading routes to Africa, India, China and Japan. It was introduced to India by Portuguese in 17th century (Bosland and Votava, 2000).

Chilli is an indispensable spice essentially used in every Indian cuisine due to its pungency, colour and aroma. Chilli fruits are rich sources of vitamin C, A and E. Crystalline acrid volatile alkaloid called capsaicin present in the placenta of fruit is contributing for the pungency of chilli. Chilli oleoresin, the total flavour extract of dried and ground chillies has varied uses in food processing and beverage industries. The natural colour extract from chilli has replaced artificial colours in food items especially in the developed countries. The whole plant is also grown for aesthetic purposes as an ornamental plant in kitchen garden. Due to its refreshing aroma, palatability and medicinal properties, it is used for pickling, flavouring curries besides being used in home remedies for ailments like gastritis, arthritis and chronic indigestion problems. It is also used as a remedy to summer heat, presumably by inducing perspiration. Capsaicin is known to have anti-inflammatory and antioxidant activities, inhibits obesity by decreasing energy intake, adipose tissue weight and serum triglyceride by stimulation of lipid mobilization. Thus, chilli has diverse uses other than spice and vegetable.

India is the world's largest producer, consumer and exporter of chilli. Chilli accounts for 41.4 per cent of total Indian spices exports and earned a foreign exchange of Rs 2380.60 crores in the year 2012-13. India contributed 40 per cent of global chilli area and 39 per cent of global production. The area under chilli in India is 7.93 lakh ha with a production of 12.99 lakh tonnes (FAO stat, 2012).

Byadagi chilli is a famous chilli type grown in Karnataka. The chilli is named after the town "Byadagi" which is located in the Haveri district of Karnataka. It is known for its deep red colour and got the highest colour value (ASTA 159.9) as compared to other chilli types, which exhibited ASTA colour value of only 32.1 to 70.40 (www.indianspices.com). Byadagi chilli is characterized by wrinkles on pods with negligible or zero pungency. Two main types of Byadagi chilli grown are the Byadagi Kaddi and Byadagi Dabbi. The fruit of Byadagi Kaddi is slender, linear, light green at maturity turning to deep red on ripening. Byadagi Dabbi is suitable for green or dry chilli. Fruits of Byadagi Dabbi are medium long, curved at the apex, slightly bulged at the base of calyx. Byadagi chilli is mainly exported as oleoresin which serves as a substitute to paprika oleoresin, widely used in food industries, confectionaries, cosmetics, beverages, pharmaceuticals and even as a dye in textile industries.

The demand for Byadagi chilli has increased enormously as a source of natural red colour in food industry. Spices Board has initiated steps to protect Byadagi chilli under GI registration. Characterization of Byadagi chilli at morphological and molecular levels helps to document specific traits present in the spice which will serve as a document for protecting the chilli type. Evaluation of the performance of Byadagi chilli in a place other than its major area of cultivation throws light on the expression of specific traits like high colour value and low pungency in another geographical location. In depth molecular characterization of Byadagi specific amplicons based on molecular markers will give further insight into the high colour and low pungency present in the famous chilli type.

Keeping the above in view, the present study on "Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)" was taken up at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Thrissur, Kerala, to characterise Byadagi chilli types at morphological and molecular levels with special emphasis on colour and pungency.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The investigations on "Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)" were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University, Thrissur, with the objective of characterizing Byadagi chilli using morphological and molecular markers.

The research works included mainly the morphological and molecular characterization of two cultivars viz. Byadagi Kaddi and Byadagi Dabbi, and two Kerala Agricultural University released varieties viz. Ujwala and Anugraha. Two different molecular marker systems were used for the study which included RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequences Repeats) assays.

The relevant literature on various aspects of the investigations are reviewed in this chapter.

2.1 Origin and history

Chillies are known from pre-historic times in Peru. They are believed to have originated in tropical America. Archeological microfossils derived from pepper found in south and Central America are estimated to be up to 6000 years old (Paran and Knaap, 2007). Columbus introduced chilli to Europe in 15th century and spread to rest of the globe along the spice trading routes to Africa, India, China and Japan. It was introduced to India by Portuguese in 17th century (Bosland and Votava, 2000). Hot chilli is a traditional crop grown in the homesteads of Kerala for domestic and export purposes (Manju and Sreelathakumary, 2002).

Capsicum species are not only cultivated as vegetable and spice but also incorporated into a number of medicinal preparations in the world. Capsaicinoids have ethnopharmacological applications such as pain therapy, body temperature

regulation, anti-obesity treatments, anticancer therapy and as antioxidant and antimicrobial agents (Meghvansi *et al.*, 2010).

Peppers are grown worldwide and are highly appreciated for their spicy flavor and nutritional value (Heuvelink and Korner, 2001). Research on chilli plants has focused primarily on increasing plant yield and improving fruit quality and pungency (Blum *et al.*, 2003; Stewart *et al.*, 2005).

2.2 Species and varieties of chilli

Capsicum spp. belongs to the family Solanaceae. Chillies are the most important vegetable cum spice crop. *Capsicum* is derived from the Greek word "Kapsimo" meaning "to bite". The genus *Capsicum* consists of approximately 22 wild species and five domesticated species. *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L., and *C. Pubescens* (Bosland, 1996). Despite their vast trait differences most chilli cultivars commercially cultivated in the world belongs to the species, *C. annuum* with chromosome number 2n= 24. *Capsicum annuum* is an annual sub–shrub, the flowers of which are borne singly and fruits usually pendant, which provide red peppers, cayenne, paprika and chillies and sweet pepper (bell pepper) a mild form with large inflated fruits. Chilli types usually are classified by fruit characteristics, i.e. pungency, colour, shape, flavour, size, and their use. Several hundred chilli pod-types are grown worldwide. Types such as ancho, bell, jalapeno, pasilla, new Mexican, yellow wax, are distinct pod-types that have specific traits for processing and fresh use, flavour and pungency (Bosland, 1996). Chilli processing pod-types can be grouped into two main categories. The first consists of a fresh product that is frozen, canned, or pickled. Some of the pod-types included in this group are the pimento, jalapeno, serrano, pepperoncini, yellow wax, and the new Mexican. The second category consists of those used as a dehydrated product. Dehydrated pod-types are cayenne, ancho, pasilla, mirasol, piquin, and de arbol.

2.3 Species, varieties and cultivars of chilli

2.3.1 *Capsicum annuum*

Capsicum annuum is a domesticated species of the plant genus *Capsicum* native to southern North America and northern South America. The species encompasses a wide variety of shapes and sizes of peppers, both mild and hot, ranging from bell peppers to chili peppers.

Byadagi Kaddi, Byadagi Dabbi, Ujwala and Anugraha etc. are some promising cultivars and varieties of *C. annuum.*

The details of two Byadagi chilli cultivars viz. Byadagi Kaddi and Byadagi Dabbi and two KAU released varieties selected for the study viz Ujwala and Anugraha are as follows:

2.3.2 *Capsicum frutescens*

Capsicum frutescens can be annual or short-lived perennial plants. Flowers are white with a greenish white or greenish yellow corolla, and are either insect or self-pollinated. The fruits typically grow erect, ellipsoid-conical to lanceoloid in shape. Fruits are usually very small and pungent, growing 10-20mm long and 3-7mm in diameter (Bosland, 1996).

2.3.3. *Capsicum chinense*

Capsicum chinense is commonly known as "Yellow Lantern Chilli". *C. chinense* chillies are well known for their exceptional pungency. Within *C. chinense*, the appearance and characteristics of the plants can vary greatly (Manju and Sreelathakumary, 2002). In warm climates, it behaves as a perennial and can last for several years, but in cooler climates, *C. chinense* does not usually survive the winter (Manju and Sreelathakumary, 2002).

2.3.4. *Capsicum pubescens*

Capsicum pubescens is a species known as rocoto which is found primarily in Central and South America. It is known only in cultivation. The species name, *pubescens*, means hairy, which refers to the hairy leaves. The hairiness of the leaves, along with the black seeds, distinguishes this species from others. Of all the domesticated species of peppers, this is the least widespread and systematically farthest away from all others. A very notable feature of this species is its ability to withstand cooler temperatures than other cultivated pepper plants, but cannot withstand frost.

2.3.5 *Capsicum baccatum*

Pepper varieties in *C. baccatum* species have white or cream colored flowers, and typically have a green or gold corolla spot. The flowers are either insect or self-pollinated. The fruit pods of the baccatum species have wide variety of shapes and sizes, unlike other *Capsicum* species, which tend to have a characteristic shape. The pods typically hang down, and can have a citrus fruity flavour (Finger *et al.,* 2009).

2.3.6 Ujwala

This variety is improved by single plant selection. Fruit of Ujwala variety is 2.25-4 cm wide and 6-7 cm long with clustered linear and erect fruits which are green at immature stage and turning red after ripening. It is resistant to bacterial wilt and is a highly pungent variety. It is grown in the central zone of Kerala and areas where bacterial wilt is a problem. Average productivity of Ujwala is 18 tonnes of green chilli/ ha (Gopalkrishnan and Indira, 2000).

2.3.7 Anugraha

Anugraha is improved by advance generation selection from back cross of Ujwala and Pusa Jwala. Fruit of variety Anugraha is 2-3 cm wide and 10-12 cm

long with solitary, pendulous linear and light green fruits. It is resistant to bacterial wilt and has medium pungency. It is grown in the central zone of Kerala and areas where bacterial wilt is a problem. Average productivity of Anugraha is 27 tonnes of green chilli/ ha (Gopalkrishnan and Indira, 2000).

2.3.8 Byadagi Kaddi

Byadagi Kaddi is a famous chilli, mainly grown in Karnataka. It is named after the town Byadagi which is located in the Haveri district of Karnataka. Byadagi chilli plants begin flowering 40 days after transplanting although the majority of flowers bloom 60 to 80 days after transplanting. The fruits of Byadagi Kaddi are 12-15 cm long with slender, linear, light green at maturity turning to deep red on ripening, possessing the highest colour value and is tolerant to pest and diseases. Average productivity of Byadagi Kaddi is 12 tonnes of green chilli/ ha.

2.3.9 Byadagi Dabbi

The Byadagi Dabbi is also a famous chilli type grown in Karnataka. Plants begin flowering 40 days after transplanting although the majority of flowers bloom 60 to 80 days after transplanting. Fruits are medium long, curved at the apex, slightly bulged at the base of calyx and are susceptible to pests and diseases. Average productivity of Byadagi Kaddi is 12 tonnes of green chilli/ ha.

2.4 Chemical composition of chilli

Capsicum fruits contain fixed (fatty) oil, and steam-volatile oil, pigments and pungent principles.

The degree of pungency exhibited is the highest in chillies, moderate to mild in capsicums and virtually absent in most forms of paprika. The aroma and flavor imparted by the volatile oil is of relatively minor importance with the dried products and it is only of real significance as a quality factor with the fresh fruits which are consumed as vegetables.

Quality and pungency are determined by the oleoresin and capsaicin contents. Inner pericarps contain 90 per cent pungency (Narayanan *et al.*, 1980). The composition and yield of oleoresins obtained by extraction of the dried fruits are dependent upon the solvent and the raw material used (Szabo, 1970; Mathew *et al.*, 1971; Salzer, 1975; Bibiloni and Rovira, 1976). Maximum oleoresin content was observed in varieties like G 4 (Reddy and Murthy, 1988; Hosamani, 1993), LCA206 and Arka Lohit (Narayankutty *et al.*, 1992), Musalwadi and Co 2 (Pruthi, 1993).

2.4.1 Capsaicin

Bucholtz (1816) was the first to realize that the pungent constituents could be extracted from the pods with organic solvents. Braconnot (1817) observed that the pungent principles could form salts with alkalis. The primary pungent principle was first isolated in a crystalline state from the crude extract by Thresh (1846) who named the compound capsaicin. Micko (1898) demonstrated that capsaicin possessed hydroxyl and methoxy groups and postulated a structural relationship to vanillin. Completion of elucidation of the structure of capsaicin was accomplished by Nelson (1923) who showed it to be the amide of vanillyl amine and isodecenoic acid. The first total synthesis of the compound was carried out by Spath and Darling (1930) and later by Crombie *et al.*, (1955).

The presence of other compounds related to capsaicin in capsicum extracts was detected by Kosuge *et al.* (1961) and Friedrich and Rangoonwala (1965) using paper and thin layer chromatography methods, respectively. Kosuge *et al.* (1961) named the new compound in his sample dihydrocapsaicin, which was observed present in a ratio of 3:7 with capsaicin. Other investigations by Morrison (1967), Hollo *et al.* (1969) and Hartman (1970) also indicated the occurrence of one or more compounds related to capsaicin in extracts of *Capsicum* products.

The distribution of the pungent principles within the fruit is uneven, but it is generally agreed that the dissepiments (partitions) contain a substantial

proportion compared to their bulk. The stage of fruit maturity at harvest, the season and environment of growth and the post- harvest handling can all influence the pungency of the dried product somewhat, the greatest determinants of the pungency level are the species and cultivars grown. Deb *et al.* (1963) found the capsaicin content to differ by a factor of three among twelve cultivars of Pusa chillies grown at the same location. Capsaicin content differences found in trials of chilli and capsicum cultivars have also been reported by Balbaa *et al.* (1968), karawaya *et al.* (1969), Golifer (1973) and Sharma and Motingar (1975). Similar studies with paprika cultivars have been reported by Spanyar *et al.* (1962). These studies clearly showed the need to carefully select planting material in order to obtain a product with the desired pungency characteristics.

The hotness of chillies is measured in units called 'scovilles'; 300-600 indicates a mild variety whereas 200,000- 350,000 scovilles represent some of the hottest chillies (Dasgupta and Fowler, 1997). Capsaicinoids are produced in the placenta and it has long been known that a single dominant gene *C*, is required for pungent genotypes to produce capsaicinoids (Blum *et al.*, 2002). The content of capsaicinoids, responsible for the pungency of chilli peppers, varied between 41.8 and 65.9 mg/g dry fruit (Pino *et al.*, 2007).

2.4.2 Capsanthin

The major pigment of red forms of *capsicum annuum* is capsanthin, which was isolated in crystalline form as early as 1927 (Karrer and Jucker, 1950). In paprika powder, the proportion of red components is 70-80 per cent while the orange components comprise 20-30 per cent of the total carotenoides. The two main contributors to the red colour in *Capsicum annuum* cultivars are capsanthin and another keto-carotenoid, capsorubin. These two compounds are absent or present in very low abundance in the green fruit but increase to predominate in the ripe red fruits (Salmeron and Garrido, 1976). Three improved paprika lines namely Kt-Pl-8, Kt-Pl-9 were identified in IARI Regional station, Katrain (H.P) their colour value ranging from 178 to 233 ASTA units. At IISR, Calicut Kt-Pl-19

was found highly susceptible to bacterial wilt and fruit rot (Ravindran *et al.*, 2006).

2.4.3 Fixed oil

The distribution of the fixed (fatty) oil in the fruit is uneven, being mainly found in the seeds. As with some other constituents of the fruit, the fixed-oil content gradually increases during maturation from the green to the ripe red stage (Sievers and Mcintyre, 1921).

2.4.4 Volatile oil

The fruits of *Capsicum* species have a relatively low volatile oil content which has been reported to range from 0.1 to 2.6 per cent in paprika and similar large forms of *capsicum annuum* (Winton and Winton, 1939; Gerhardt, 1968; Szabo, 1970). The initial volatile oil content of the freshly picked fruit is dependent largely upon the species and cultivar grown and the stage of maturity at harvest. The eventual volatile oil content of the dried product however, is lower and is dependent upon the drying procedure, the duration and condition (whole or ground) of storage.

The distribution of the volatile oil in the fruit appears to be uneven, being largely found in the pericarp (Winton and Winton, 1939); and during maturation, the volatile-oil content progressively increases (Sievers and Mcintyre, 1921). The composition of the volatile oil of fresh California green bell peppers (*C. annuum* var. *grossum*) has been examined by Buttery *et al.* (1969) using gas chromatography. Twenty four components in this oil were positively identified and a further 19 were tentatively identified. One of the major components, 2-methoxy-3-isobutyltyrazine, was considered to possess and aroma characteristic of the fresh fruit and to dominate the organoleptic profile.

2.5 Genetic variability and inheritance studies in chilli

Mcleod *et al.* (1983); Pickersgill, (1988); Loaiza-Figueroa *et al.* (1989); Prince *et al.* (1992); Lefebvre *et al.* (1993) have worked on evolutionary relationships among and within the cultivated species. These studies demonstrated that the level of variation among domesticated peppers is lower than that among bell peppers and that the variation among large fruited peppers was limited compared with that among the domesticated pungent peppers.

Exotic germplasm provides a bountiful source of extraordinary genetic diversity with which to improve the commercial chillies. Assessment of different desirable traits spread over diverse genotypes is important to rapid advance in yield improvement of any crop (Eshbaugh *et al.*, 1983). The plant breeders are always interested to know the genetic divergence among the varieties available due to the reason that crosses between genetically diverse parents are likely to produce high heterotic effect (Sreelathakumary and Rajamony, 2004). Higher phenotypic and genotypic coefficients of variation were observed for fruits per plant, yield per plant, seeds per fruit and fruit weight. High estimates of heritability coupled with high genetic advance were also observed for these characters, indicating that they can be effectively improved through selection (Manju and Sreelathakumary, 2002).

Varietal performance, genetic variability and correlation studies in chilli (*Capsicum annuum* L.) were conducted by Tembhurne *et al.* (2008) where eleven elite advanced lines obtained from Chilli Research Station, Devihosur were evaluated along with KDC 1, Byadgi Dabbi and Byadgi Kaddi as checks. The differences between phenotypic and genotypic coefficient of variation were low for days to 50 per cent flowering, plant height, fruit diameter and number of fruits per plant. High genetic advance over mean coupled with higher heritability was observed for number of fruits per plant. The correlation studies indicated that the yield was positively associated with most of the traits but its magnitude was high with number of fruits per plant and fruit diameter. Similar studies were conducted

by Singh and Chaudhary (2010) revealed that high heritability coupled with high genetic advance and genetic coefficient of variation reflects that the capsaicin content, number of fruits per plant, fruit length, fruit diameter, leaf area and total fresh yield per plant are under the control of additive gene effects, and could be relied upon for yield improvement.

The capsaicinoids are not sensed by the taste buds. Heat sensation from the capsaicinoides results because of irritation of pain receptors. Capsaicinoids release a chemical messenger, substance P that signals the brain about pain. Substance P causes the nervous system to telegraph a signal to the brain to flood the nerve endings with endorphins, which are the body's natural pain killers. The release of endorphins at nerve endings gives the body a sense of pleasure. Capsaicinoides are generally not destroyed in the mouth (Bosland, 1993). It has long been known that, in the pungent types, the degree of pungency is quantitatively inherited and highly influenced by environment (Zewdie and Bosland, 2000). However, single dominant gene, C, controls the presence or absence of pungency in the fruits (Blum *et al.*, 2002).

Studies conducted so far have indicated that matured red colour is dominant over yellow colour and is controlled by a single gene (Y) and later it was reported that colour compounds (carotenoids) are under the control of four different genes (*y*, c_1 c_2 and c') with epistatic interaction (Hurtado-Hernandez and Smith,1985; Shifriss and Pilovsky, 1992). Popovsky and Paran (2000) reported mature fruit colour is under the influence of three independent pairs of genes *(c1,* c2 and *Y*). The presence of dominant alleles at these three loci results in red mature fruits, while recessive alleles give white mature fruits.

Genetic variability, heritability, genetic advance and correlation for different yield contributing characters were studied in 20 genotypes of chilli. Significant differences were observed among the genotypes for all the traits. The phenotypic coefficient of variation (PCV) was higher than genotypic coefficient of variation (GCV) for all the traits (Kumar *et. al.*, 2012).

Fifty three genotypes of *Capsicum annuum* L. collected from different parts of West Bengal were characterized for 23 characters of chilli by Datta and Das, (2013). There was a wide range of variability for all the 23 characters studied. High heritability along with higher genetic advance (as a per cent of mean) was found in capsaicin content in fruit, number of fruits per plant, yield per plant and primary branches per plant. These characters may be considered as reliable selection indices as they are possibly governed by additive gene effect.

Study on diversity of genetic resources and genetic association analyses of green and dry chillies of eastern India was conducted by Chattopadhyay *et al.* (2011). Similarly, Yatung *et al.* (2014) conducted study on genetic diversity of chilli (*Capsicum annuum* L.) genotypes of India based on morpho-chemical traits with 30 chilli (*Capsicum annuum* L.) genotypes of Indian origin. Twelve quantitative characters were taken into consideration. The analysis of variance revealed considerable variability among the genotypes for the character studied. Cluster analysis was used for grouping of 30 chilli genotypes under the study. The characters capsaicin content and ascorbic acid contents were contributed maximum towards divergence.

Six chilli cultivars belonging to three species of *Capsicum* viz. *Capsicum annuum* L. (cvs 'Meiteimorok' and 'Haomorok'), *Capsicum frutescens* L. (cvs 'Uchithi' and 'Mashingkha') and *Capsicum chinense* Jacq. (cvs 'Umorok' and 'Chiengpi') are economically important. The capsaicin content and pungency in scoville heat unit (SHU) of these six chilli cultivars were determined by high-performance liquid chromatography (HPLC). The capsaicin content and pungency of the chillies varied depending upon the genotype (Sanatombi and Sharma, 2008).

Estimation of genetic diversity in local and exotic genotypes of *Capsicum* (chilli) using SDS-PAGE analysis was done by Akbar *et al.* (2010), in which total seed proteins were extracted and separated on Polyacrylamide gel. Protein fragments of various molecular weights were separated from various *Capsicum*

genotypes. Individual protein fragments were considered as allele / loci. They also estimated genetic distances (GD) that ranged from 0 to 100 per cent Nineteen *Capsicum* genotypes were clustered in four groups. Genotypes Fehsil Bibber 1 and Ajay Bibber 1 were most distantly related to each other and hence these two genotypes could be crossed to create a segregating population with maximum genetic diversity for the improvement of chili. For chilli several genetic maps have been developed, these maps are being used for the determination of syntenic relationship, gene tagging, marker assisted selection and for gene cloning (Kumar *et al.*, 2011).

2.6 Studies on different types of markers in chilli

2.6.1 Morphological markers

According to Bhat *et al.*, (2010), morphological markers are those traits that are scored visually, or those genetic markers whose inheritance can be followed with the naked eye. The traits included in this group are plant height, disease response, photoperiod, sensitivity and shape or colour of flowers, fruits and seeds. Although they are generally scored quickly, simply and without laboratory equipments, such markers are not put much use because of the following reasons: Such markers frequently cause major alternations in the phenotype which is undesirable in breeding programmes. Dominant, recessive interactions frequently prevent distinguishing the genotypes based on morphological traits. Morphological markers mask the effect of linked minor gene, making it nearly impossible to identify desirable linkages for selection and are limited in number, influenced by environment and also specific stage of the analysis.

Morphological characterization of habanero pepper (*Capsicum chinense* Jacq.) showed that chilli fruit occasionally contains a small internal fruit (SIF) developing inside the main fruit. The small internal fruit morphology showed a normal fruit with pericarp, two locules and small reminiscent ovules joined to

placental-like tissues. Histological analysis showed that SIFs maintain physical connection with the placental tissues of the main fruit through vascular tissues. A Hardy-Weinberg analysis suggested that the frequency of SIF formation in the *Capsicum chinense* population is not the result of natural selection (Carballo-Bautista *et al.*, 2010).

Thirty two accessions of hot chilli *(Capsicum chinense* Jacq.) were evaluated to estimate the variability, heritability and genetic advance in randomized block design with three replications. Higher phenotypic and genotypic coefficients of variation were observed for fruits per plant, yield per plant, seeds per fruit and fruit weight. High estimates of heritability coupled with high genetic advance were also observed for these characters, indicating that they can be effectively improved through selection (Manju and Sreelathakumary, 2002).

Study on genetic diversity was conducted with 45 chilli (*capsicum annuum* L.) genotypes by Farhad *et al.* (2010) in which fourteen quantitative characters viz. days to 50 per cent flowering, time between fruit set and maturity, plant height, number of primary branches per plant, number of secondary branches per plant, fruit length, fruit weight, number of fruits per plant, seeds per fruit, 100 seed weight, fruit length, vitamin C content and dry fruit yield per plant were taken into consideration. The analysis of variance revealed remarkable variability among the genotypes for the character concerned. Khan *et al.* (2012) studied the effect of different soil water levels on the physio-morphological features of ten chilli (*Capsicum annuum* L.) accessions.

Fifty six *Capsicum* spp accessions were evaluated based on 25 characters, 14 of which were morphological and 11 agronomic. Based on the qualitative characters, it was possible to identify all species together with the agronomic characters. Genotypes were indicated with potential for use in various production sectors. Five were determined as the ideal number of groups by the criteria pseudo-F and pseudo-t2. The Ward-MLM procedure allowed the differentiation of

the species *C. annuum*, *C. frutescens*, *C. baccatum*, and *C. chinense* in separate groups (Sudre *et al.*, 2010).

Datta and Das (2013) characterized fifty three genotypes of *Capsicum annuum* L. collected from different parts of West Bengal for 23 characters namely, stem colour, plant growth habit, branching habit, leaf size, leaf shape, leaf margin, leaf colour, leaf pubescence, pigment at node, flower per axil, corolla colour, anther colour, calyx margin, mature fruit colour at intermediate stage, fruit shape, fruit position, fruit adherence to the calyx, fruit shape at pedicel attachment, blossom end fruit shape, ascorbic acid content of the fruit, capsaicin in red fruit and colour value of the ripe fruit. These genotypes upon cataloguing showed distinct variations with respect to vegetative, inflorescence, fruit and quality characters. Variability studies revealed that there was a wide range of variability for all the characters studied. High heritability along with high genetic advance (as percentage of mean) was found in capsaicin content in fruit, number of fruits per plant, yield per plant and primary branches per plant. These characters may be considered as reliable selection indices as they are possibly governed by additive gene effect.

2.6.2 Biochemical markers

Isozymes being multiple forms of protein are primary gene products, variation in their structure should give reliable information about the variability in the genome as they are less susceptible to environmental influence than secondary products of metabolism which are formed as a result of enzyme activity. The technique of isozyme electrophoresis is being widely used to study genotypic variation in living organisms. Isozymes are theoretically well suited to identify closely related individuals or clones, simply by comparision of phenotypic banding patterns.

Chemical composition, the radical-scavenging and antioxidant activities of hot pepper fruits (*Capsicum annuum* L. var. *acuminatum*) differ at three maturity stages viz. small green, green and red. The first stage of maturation (small green)

showed the highest radical-scavenging activity, the methanolic extract of green pepper showed significant antioxidant activity, addition of methanolic extract of red and green pepper inhibited oxidation of linoleic acid. Methanolic extract of red pepper showed greater antioxidative potency than others. The different composition of lipophilic compounds and the various amount of phenolics, showed in the three stage of ripening of *C. annuum* var. *acuminatum* fruits, modifies antioxidant activity (Conforti *et al.*, 2007).

Prasad *et al.* (2008) studied capsaicin biosynthesis involves condensation of vanillylamine and 8-methyl nonenoic acid, brought about by capsaicin synthase (CS). They characterized CS activity correlated with genotype-specific capsaicin levels. Immunolocalization studies confirmed that CS is specifically localized to the placental tissues of *Capsicum* fruits. Western blot analysis revealed concomitant enhancement of CS levels and capsaicin accumulation during fruit development. He also determined the N-terminal amino acid sequence of purified CS, cloned the CS gene (*csy1*) and sequenced full-length cDNA.

Singh *et al.* (2009) tested the efficacy of seed protein electrophoresis over morphological and other biochemical characters, like total soluble sugars, oil, capsaicin and peroxidase activity for varietal identification of 12 chilli genotypes. The results revealed that morphological descriptors like thousand seed weight, single fruit weight, leaf area, single fruit length and width ratio were used for grouping of 12 chilli genotypes.

Morphological, biochemical and electrophoretic evaluation of chilli (*Capsicum annuum.* L) genotypes (Singh *et al.*, 2009) revealed that morphological descriptors like thousand seed weight, single fruit weight, leaf area, single fruit length and width ratio were used for grouping of 12 chilli genotypes. Biochemical tests showed that maximum TSS, oil percentage and capsaicin were present in G-4, ACG-254 and ACG-291 having values of 36.16 per cent, 53 per cent and 0.726 mg/g, respectively, whereas ACS-2000-2, ACS- 2001-1 and ACG-209 exhibited minimum TSS, oil percentage and capsaicin giving values of 19.98 per cent, 20.25

per cent and 0.261 mg/g, respectively. The differences have also been observed in peroxidase activity. The highest and the lowest activity was found in ACS-2001-4 and ACS-2000-2, respectively. SDS-PAGE of soluble seed proteins showed different banding patterns, which might be used for varietal identification.

Capsaicin and ascorbic acid contents of seven Indian pepper varieties/accessions from *Capsicum annuum* (CA 97, CCH, K1, KTPL19, Arka Abhir and Byadagi Kaddi) and *C.frutescence* (CF1) species were determined using High Performance Liquid Chromatography (HPLC), based on their pungency value. All the chilli accessions/varieties (CA 97, CCH, K1 AND CF1) were classified as highly pungent peppers. The accession CF1 showed the highest concentration of capsaicin and Arka Abhir variety showed the lowest capsaicin concentration pungency value. Similarly, Byadagi Kaddi showed the highest ascorbic content and the accession CA 97 showed the lowest ascorbic acid contents (Tilahun *et al.*, 2013).

2.6.3 Molecular markers

Molecular markers are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism than morphological and proteins based markers and constitute a new generation of genetic markers (Bostein *et al.*, 1980; Tanksley *et al.*, 1989). Hence, varietal profiling methods that directly utilize DNA could potentially address all of the limitations associated with morphological and biochemical data. Mathew (2006) reviewed the application of molecular markers for crop improvement in chilli. Many research groups have examined genetic diversity in the *Capsicum* spp by using Polymerase Chain Reaction (PCR) based techniques which makes use of random or specific primers to amplify random or specific DNA fragments from the genome. They are simple to perform, easily amenable for automation and can be used to assay a large number of samples. These include Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) (Ahmed, 2013), restriction fragment length

polymorphism (RFLP) (Prince *et al.*, 1992; Prince *et al.*, 1995), and Amplified Fragment Length Polymorphisms (AFLP) (Paran *et al.*, 1998; Toquica *et al.*, 2003) techniques for analysing genetic diversity (Sitthiwong *et al.*, 2005; Thul *et al.*, 2011), genetic distance (Sanatombi *et al.*, 2010), genetic relationship (Ince *et al.*, 2010), phylogeny (Oyama *et al.*, 2006; Adetula *et al.*, 2006) in region specific accessions and land races (Makari *et al.*, 2009).

2.6.3.1 Random Amplified Polymorphic DNA

Molecular DNA marker analysis is considered as important method for the determination of genetic diversity among genotypes (Gilbert *et al.*, 1999). Random Amplified Polymorphic DNA (RAPD) is one such method (Welsh and McClelland, 1990; Williams *et al.*, 1990) of identifying polymorphism that can be used to elicit information on molecular differences among individuals of a population between lines or accessions or any breeding material. RAPD markers are generated by the use of short (10-mer) synthetic oligonucleotides in a single strand primer (Williams *et al.*, 1990). In this technique, a decamer primer of arbitrary sequence is allowed to anneal at a relatively low temperature priming the amplification of DNA fragments distributed at random in the genome. Amplification products are visualized by separation on agarose and stained with ethidium bromide. They usually result in DNA fragment patterns that are polymorphic between genotypes, by their detecting diversity within them (Tommercup *et al.*, 1998). However, a key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation.

There are several advantages of RAPD compared to other DNA based techniques. It is simple, fast, low cost and it can be done with small amount of DNA also it does not involve radioactivity (Varghese *et al.*, 1997). Another advantage of the RAPD method is that a universal set of random primers can be used for genomic analysis of any organism (Welsch and McClelland, 1990). Short random primers have been used to reproducibly amplify segments of genomic

DNA from a wide variety of species including plants (Williams *et al.*, 1990; Quiros *et al.*, 1991; Fregene *et al.*, 1997). Polymorphisms detected by RAPD are inherited in a Mendelian fashion as dominant markers (Williams *et al.*, 1990; Welsch *et al.*, 1991). This polymorphism has been proved to be useful for identifying variations at different levels. RAPD analysis enables differentiation between very closely related organisms due to high resolution of the technique (Tommercup *et al.*, 1998). The polymorphic fragments generated by RAPD are useful as genetic markers to identify organisms (Williams *et al.*, 1990) and the relative degree of similarity between individual populations and species (Yang and Quiros, 1993; Tonukari *et al.*, 1997).

Random amplified polymorphic DNA markers have been used for detection of polymorphism and assessment of level of genetic variation within populations of chilli (Wang *et al.*, 1997). *Capsicum* germplasm was subjected to RAPD analysis and sufficient degree of polymorphism was detected to differentiate among the species. Markers based on differences in DNA sequences between individuals generally detect more polymorphisms.

A more illustrious example on the use of RAPD markers in genetic diversity estimation in genus *Capsicum* is from Mexico, which is the primary centre of origin of this genus (Vavilov, 1951). Votava (2000) has done extensive work on the characterisation of *Capsicum* accessions from that region. He was successful in assigning the accessions into different species based on RAPD markers.

Paran *et al.* (1998) used RAPD and AFLP markers to examine genetic relationships among thirty four *Capsicum annuum* L. cultivars. RAPD markers separated the large fruited sweet cultivars from small fruited pungent peppers. They reported that AFLP primers were four times more efficient than RAPD primers in detecting the polymorphism. RAPD markers have been successfully employed for determination of intra-species diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami

and Ranade, 1999). Use of SSR markers to complement tests of DUS in *Capsicum annuum* L. varieties was reported by Kwon *et al.* (2005). They observed similarity between morphological trait based clustering and dendrogram constructed based on SSR markers.

Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding and population genetics. Similarly, the diversity within *capsicum* germplasm collection from Nepal was estimated by Baral and Bosland (2002). Both morphological and RAPD markers were used for the characterization.

Prince *et al.* (1995) examined intraspecific variability among four *C. annuum* cultivars using both RFLP and RAPD. Seventeen per cent of the clones used singly in RFLP analyses were sufficient for the differentiation of these varieties, as were 12.5 per cent of the RAPD PCR amplifications. Dendrograms constructed from RFLP and RAPD analyses of the intraspecific data are similar but not identical. Southern analysis and RAPD PCR should be useful for DNA fingerprinting and the discrimination of closely related *C. annuum* genotypes.

Genetic relationships were examined among thirty four pepper (*Capsicum annuum*) cultivars of different types. Two types of PCR based markers were used, RAPD and AFLP, and their relative effectiveness were compared. The percentage of polymorphic markers were lower for AFLP than for RAPD markers (13 and 22 per cent respectively). However, AFLP primers amplified on average six times more products than RAPD markers. The average numbers of polymorphic products per primer were 1.6 and 6.5 for RAPD and AFLP primers, respectively, i.e., AFLP primers were four times more efficient than RAPD primers in their ability to detect polymorphism in pepper (Paran *et al.*, 1998).

One hundred thirty four accessions from six *Capsicum* cultivars were characterized using 110 Randomly Amplified Polymorphic DNA (RAPD)

markers (Rodriguez *et al.*, 1999). Ten pairs of potentially duplicated accessions were identified. Diagnostic RAPDs were identified which discriminate among the *Capsicum* species. The diagnostic markers were employed for improved taxonomic identification of accessions since many morphological traits used in the identification of *Capsicum* are difficult to score.

Pawar (2000) attempted to study the genetic diversity among and within the three native cultivars of chilli *viz.*, Byadgi Dabbi, Byadgi Kaddi and Sankeshwar both at morphological and molecular level. The molecular polymorphism assessed employing the RAPD analysis using five random decamer primers generated 187 RAPD loci of which 97 were polymorphic. The level of polymorphism generated was 48.5 per cent and the highest number of polymorphic bands was recorded by OPJ 10.

Genetic diversity in *C. annuum* landraces from Nepal was investigated using RAPD markers and compared with that of *C. annuum* landraces from the centre of diversity, Mexico. RAPD marker based cluster analysis of *C. annuum* clearly separated each accession. All the accessions of *C. annuum* from Nepal grouped into a single cluster at a similarity index value of 0.80; whereas, accessions from Mexico grouped into eight different clusters at the same similarity level indicating greater genetic diversity in Mexican accessions. Some Nepalese accessions had unique RAPD markers suggesting that additional sources of genetic variation are available in Nepalese germplasm (Baral and Bosland, 2002).

Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) were used to assess genetic diversity within and between five populations of a landrace of *Capsicum annuum* L., grown in a limited area in North-West Italy. 41.6 per cent genetic variation occurred between the populations and 58.4 per cent within the population (Lanteri *et al.*, 2003).

Ilbi (2003) observed that RAPD has been used in varietal identification and genetic purity test of hybrid varieties of *Capsicum annuum*. Five Jalepeno

hybrid varieties and their corresponding parents were screened for polymorphic RAPD markers with 12 arbitrary 10-mer primers. Among a total of 177 bands observed, 14 bands contributed by nine primers were polymorphic in the five pepper varieties. Furthermore, four RAPD markers were found to be cultivar specific markers for three hybrid varieties. Out of the 12 primers, six primers generated 11 useful RAPD markers to determine seed purity of all chilli varieties.

In North-West Italy, a local landrace of pepper (*Capsicum annuum* L.) known as 'Cuneo' is grown; it deserves particular attention because of its hardiness, late production and fruit quality. RAPD and AFLP markers were used to assess the effect, on genetic composition of a population, of seed sampling carried out using the selection criteria adopted by the farmer (Portis *et al.*, 2004). After two reproductive cycles using selection, it was already possible to detect loss in genetic variation and a change in allele frequencies, while no significant effect was found after two cycles of random sampling. Over this period, farmer selection pressure led to disappearance of eight low-frequency alleles, while only three alleles were lost in randomly sampled populations. The results stress the need to adopt appropriate techniques for seed sampling in order to avoid genetic erosion of local landrace.

Devi *et al.* (2005) studied intra and inter-specific genetic diversity in 33 chilli (*Capsicum* spp) accessions of north-eastern India belonging to three species using morphological characteristics and Randomly Amplified Polymorphic DNA (RAPD) markers. For RAPD analysis, 20 random oligonucleotide primers were used and only 15 showed amplification. The RAPD markers detected 83.17 per cent polymorphism among the accessions. Two distinct clusters were delineated with all the *C. frutescens* accessions in one and *C. annuum* and *C. chinense* accessions in another. However, the *C. chinense* accessions were separated from *C. annuum* at sub-cluster level.

Sugita *et al.* (2005) reported that genetic linkage map of the sweet pepper (*Capsicum annuum* L.) was developed using an intra-specific double haploid

population which was constructed by Amplified Fragment Length Polymorphism (AFLP) using the High Efficiency Genome Scanning (HEGS) system and Random Amplified Polymorphic DNA (RAPD).

Makari *et al.* (2009) studied the genetic diversity of ten different commercial varieties of chilli using Random Amplified Polymorphic DNA (RAPD) analysis. The DNA extracted by CTAB method from the leaf sample was amplified by successive PCR cycles. Using 12 different primers, the amplified DNA solution was subjected to agarose gel electrophoresis. Around 500 different bands were observed under UV light. Seven were polymorphic for a specific primer and can be used as differential markers. This effort could serve determining genetic diversity among ten commercial varieties of chilli in India.

Litoriya *et al.* (2009) studied varietal identification of ten chilli (*Capsicum annuum* L.) varieties using RAPD marker. Total 70 primers were tested for varietal identification, out of which 11 gave maximum scorable bands, in which ten primers showed polymorphism. A total of 126 bands were amplified using ten random primers, of which 64 were polymorphic.

Genetic relationships were estimated among 24 accessions belonging to 11 species of *Capsicum*, using 2,760 RAPD markers based on Touch-down Polymerase Chain Reactions (Td-RAPD-PCR). These markers were implemented in analyses of principal coordinates, unweighted pair group mean average, and 2,000 bootstrap replications. The accessions were divided into four groups: *C. annuum* complex (CA), *C. baccatum* complex (CB), *C. pubescens* complex (CP), and *C. chinense* accessions (CA/B). Their overall mean genetic similarity index was 0.487 ± 0.082, ranging from 0.88 to 0.32, based on Jaccard's coefficient. The highest genetic variation was observed among the accessions in CP; the accessions in CB had a low level of variation as judged from the standard deviations of the genetic similarity indices. Based on the Td-RAPD-PCR markers, the 24 accessions were divided into four major groups, three of which corresponded to the three distinct *Capsicum* complexes. Accessions of *C.*

chinense were found to be equally related to complexes CA, CB, and CP (Ince *et al.*, 2010).

DNA profiling of *Capsicum* landraces of Manipur were studied by Sanatombi *et al.* (2010). Seven chilli landraces of Manipur belonging to three cultivated species of *Capsicum* (*Capsicum annuum, Capsicum frutescens* and *Capsicum chinense*) form economically important food crops of the region. The genotypes were characterized using ten Random Amplified Polymorphic DNA (RAPD) markers. The cluster analysis based on Jaccard's similarity coefficient calculated by UPGMA method differentiated the genotypes into two main cluster groups. One cluster represented the *C. annuum* genotypes while the other cluster represented the *C. frutescens* and the *C. chinense* genotypes. *C. chinense* genotypes were more close to *C. frutescens* genotypes. Genetic variation between the *C. frutescens* genotypes were more than among the *C. annuum* genotypes and the *C. chinense* genotypes were the least similar ones.

Bhadragoudar and Patil (2011) subjected 45 accessions of chilli to RAPD analysis to understand the genetic homology. Among the 45 accessions, three RAPD primers (decamer) efficiently amplified genomic DNA. The dendrogram constructed from pooled data revealed 14 clusters. Two clusters were represented by a single genotype exhibiting their genetic divergence. However, one large cluster consisted of maximum of eight genotypes revealing genetic closeness which could be efficiently used in breeding programmes.

El-Domyati *et al.* (2011) used RAPD, ISSR and AFLP technologies to detect genetic diversity of the selected medicinal plants. The study showed that taxonomical locations can be distinguished for each subspecies with as low as zero to one per cent polymorphism using AMOVA analysis.

Twenty two accessions of six *Capsicum* species, namely, C. *annuum*, C. *baccatum*, C. *chinense*, C. *eximium*, C. *frutescens*, and C. *luteum* were investigated for phenotypic diversity based on flower colour and for genetic differences by molecular makers. The genetic cluster analyses of 27 RAPD and

eight ISSR primers showed genetic similarities in the ranges of 23-88 per cent and 11-96 per cent. Principal component analysis of the pooled RAPD and ISSR data further supports the genetic similarity and groupings (Thul *et al.*, 2011).

Rego and Rego (2011) evaluated the genetic diversity among 29 accessions of the germplasm bank of the Universidade Federal de Roraima by RAPD markers. The DNA was extracted of the 29 accessions and amplified by ten primers. Eight primers (UBC- 135, UBC-146, UBC-155, UBC-168, UBC-228, UBC-247, UBC-253, and UBC-296) produced polymorphic bands. The minimum distance was 0.09, between AC 06 and AC 37. The accessions were grouped in ten different groups. Group 1 was the largest group formed by accessions 75, 76, 5, 32,69, 56, 73, 6, and 67; group 2 was formed by accessions 59, 72, 43, 58, and 24; groups 3 and 4 were formed by three accessions, 61, 71 and 8, 49,74, and 22, respectively; while groups 5 and 6, were formed by two accessions, 25 and 70, 45 and 55, respectively, and other group (7 to 10) were constituted by one accession (20, 44, 40 and 3 respectively). RAPD markers were effective to detect the genetic diversity among 29 hot pepper accessions.

Bhadragoudar and Patil (2011) used RAPD markers for the assessment of genetic diversity among 45 accessions of *Capsicum annuum* L. genotypes. Among 45 accessions, three RAPD primers (decamer) efficiently amplified genomic DNA. The dendrogram constructed from pooled data revealed 14 clusters. Two clusters are represented by a single genotype exhibiting their genetic divergence. However, one large cluster consists of maximum of eight genotypes revealing genetic closeness which could be efficiently used in breeding programme.

Troconis-Torres *et al.* (2012) studied the relationship of the carotenoid and phenolic profiles with the RAPD fingerprint of three different commercial cultivars of chilli peppers of seven regions of Mexico. Through RAPD, the species of chilli were differentiated by means of different primers.

Random Amplified Polymorphic DNA (RAPD) assay was performed to estimate genetic polymorphisim in ten chilli cultivars (Uddin *et al.*, 2012). Out of

12 primers, four (OPA11, OPB03, OPB04 and OPB17) showed amplification of genomic DNA and generated 21 distinct scorable bands of which 17 (80.95%) were polymorphic. The result indicates the genetic diversity among the chilli cultivars and RAPD marker could be used for improvement of chilli varieties.

Genetic variability in ten commercial chilli pepper varieties using Random Amplified Polymorphic DNA (RAPD) markers was examined by Prasad *et al.* (2013). The primer OPAB02 showed amplification pattern in ten chilli pepper genotypes. Dendrogram generated by OPAB02 primer showed that the ten chilli pepper varieties could be grouped into four clusters. Average genetic similarity index revealed 100 per cent similarity between varieties of first cluster, 50 per cent similarity between varieties of second and fourth clusters and 32 per cent genetic similarity between varieties of third cluster.

Genetic diversity in ten commercial varieties of chilli was assessed by Makari *et al.* (2009) using RAPD markers and they reported seven polymorphic bands for a specific primer which could be used as a marker. DNA profiling of seven *Capsicum* land races of Manipur belonging to three cultivated species was attempted by Sanatombi *et al.* (2010) and they observed that genetic variation between *C. frutescens* genotypes was more than *C. annuum* genotypes. Thul *et al.* (2012) studied molecular profiling for genetic variability in *Capsicum* species based on ISSR and RAPD markers. Biochemical and molecular analyses of some commercial samples of chilli peppers from Mexico were attempted by Troconis-Torres *et al.* (2012). The study was meant to determine the relationship between molecular markers and content of carotenoids and phenolic compounds in different commercial cultivars of chilli peppers from diverse geographic origins. They observed a close relationship between carotenoids and genetic profile but such relationship was not observed with respect to phenol. But the study helped for identification of *Capsicum* spp. with respect to their geographical origin.

Genetic diversity analysis of chilli landraces comprising of *Capsicum chinense*, *Capsicum frutescens* and *Capsicum annuum* using RAPD markers was

reported by Orenthung and Changkija (2013). The RAPD analysis produced a total of 114 bands, out of which 109 bands were polymorphic indicating high level of polymorphism (95.6 %). The average PIC value of the primers was 0.75 indicating that the markers were able to distinguish a high degree of variation. Jacquard's similarity matrix constructed using software NTSYS showed a similarity coefficient ranging from 2.682-8.378, indicating high degree of variability among the chilli landraces.

Genetic diversity in 23 chilli genotypes was analyzed by RAPD markers of the 13 random primers used 9 were polymorphic. The amplification profile of these 9 primers consisted of 63 fragments used with size ranging from 391 bp to 2754 bp of which 35 were monomorphic and 28 were polymorphic with 44.4 per cent polymorphism (Bahurupe *et al.*, 2013).

Manibala (2013) used three different marker systems viz. RAPD, ISSR and SSR for DNA fingerprinting of selected chilli varieties. OPAH 06 and ISSR 07 primers showed highest polymorphism among ten RAPD and ISSR primers, respectively. Distinct bands were used to develop DNA fingerprint of chilli varieties (Ujwala, Anugraha, Jwalamukhi, Jwalasakhi, Vellayani Athulya and Vellayani Samrudhi) through RAPD, ISSR and SSR analyses. Sharing of amplicons developed for each primer with other varieties was also analyzed and demarcated with different colour codes in the fingerprints developed. Most of the amplicons were found shared among the varieties. However, the pattern of sharing was different and good enough to separate out the varieties. Combined DNA fingerprint for each variety with RAPD, ISSR and SSR data was also developed.

2.6.3.2 Inter Simple Sequence Repeats (ISSR)

Inter Simple Sequence Repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16-25 bp long, as primers in

a single primer PCR reaction targeting multiple genomic loci to amplify mainly the ISSR sequences of different sizes (Reddy *et al.*, 2002).

Inter Simple Sequence Repeat ISSR markers are effective multilocus markers for applications such as diversity analysis, fingerprinting and genome mapping, gene tagging and marker assisted selection as no prior sequence knowledge is required, they are more rapidly applied than SSR markers, and they are more reliable and robust than RAPD markers mainly due to the method of detection, and possibly also to the fact that primers are longer, and hence PCR condition are more stringent. ISSR has been successfully used to estimate the extent of genetic diversity at inter and intra specific level in a wide range of crop species.

Non anchored Inter Simple Sequence Repeats (ISSR) are arbitrary multi loci markers produced by PCR amplification with a microsatellite primer by Bornet and Branchard (2001). They are advantageous because no prior genomic information is required for their use. This technique is found stable across a wide range of PCR parameters. Polymorphisms were abundant among the seven dicot species tested with 2 tri-nucleotide and 2 tetra-nucleotide primers. Thus, non anchored ISSR markers are a good choice for molecular characterisation.

Kumar *et al.* (2001) used Inter Simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) and fluorescent ISSR-PCR (FISSR-PCR) were used for differentiating the four disputed chilli samples. A total of 17 ISSR anchored primers, which included nine di and eight tri-nucleotide primers were used for the analysis. The ISSR-PCR products were separated on a two per cent agarose gel. A total of 212 and 288 bands were resolved by seven di- and eight tri-nucleotide primers, respectively, with an average of 30 bands per primer. Five out of nine dinucleotide primers and four out of eight trinucleotide primers could unambiguously differentiate all the four disputed chilli samples. The sensitivity and informativeness of the ISSR-PCR assay were further enhanced by the use of FISSR-PCR technique.

Kochieva *et al.* (2004) used AFLP, RAPD and ISSR marker systems to determine genetic variation and phylogenetic relationships within the genus *Capsicum.* In total, 1921 bands were used in the genome analysis of 61 accessions representing 11 species of the *Capsicum* genus. The genetic data confirmed the recognition of *C. frutescens* and *C. chinense* as separate species with high bootstrap values. *C. praetermissum* as a separate species inside the *C. baccatum* complex. Molecular markers revealed high genetic similarities of *C. pubescens* with *C. eximium* and *C. cardenasii. C. tovarii* was found to be genetically closer to *C. baccatum* (AFLP, RAPD data) or *C. praetermissum* (ISSR data). Molecular data support the close relationships of *C. galapagoense* with *C. frutescens* (RAPD analysis) and *C. annuum* (ISSR analysis) and this species could therefore be considered to be a member of the *C. annuum* complex.

Ruo-lin *et al.* (2005) analyzed DNA samples of 11 native cultivars of *Capsicum* with 12 screened ISSR primers which produced polymorphisms. Out of a total of 66 amplified bands, 26 bands were divergent, accounting for 39.39 per cent, and seven of these 26 bands were cultivar-specific, that is 10.61 per cent. The genetic dendrogram among the materials constructed by the Neighbour-Joining method based on the Jaccard's coefficient showed that the eleven cultivars could be separated into two groups, one with an average genetic distance 0.15 and the other 0.134. The average genetic distance between the two groups was 0.194.

Refaat and Elgarhy (2007) studied the relationship between hybrid performance and genetic diversity based on ISSR-PCR markers in pepper (*Capsicum annuum. L.*). They measured genetic distances, using ISSR markers among parental lines, and the heterosis observed as yield, of the F1 hybrids as estimations of GCA, SCA. Heterosis was performed using seven elite lines and their F1 hybrids.

Jin-bing *et al.* (2009) analyzed genomic DNAs between sweet pepper cytoplasmic male sterile line CMST6A and its maintainer line T6B by means of ISSR technique. Thirty three primers amplified products in the two lines from 44

primers, among which the specific fragment ISSR-8_{1400} of CMST6A was found. The primer ISSR-8_{1400} was identified with sterile plants and fertile plants. The result showed the marker ISSR-8_{1400} was likely linked with male sterile gene.

Highly reproducible molecular marker assays, Inter Simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) and Fluorescent ISSR-PCR (FISSR-PCR) were used for differentiating the four disputed *Capsicum* samples (Kumar *et al.*, 2011). A total number of 17 ISSR anchored primers, which included nine di and eight tri-nucleotide primers were used for the analysis. Five out of nine dinucleotide primers and four out of eight trinucleotide primers could unambiguously differentiate all the four disputed chilli samples. The sensitivity and informativeness of the ISSR-PCR assay were further enhanced by the use of FISSR-PCR technique. These four primers could reliably distinguish all the four disputed samples unambiguously. The present report is, therefore, a step to protect the Plant Breeder's Rights by making use of reliable and modern DNA technologies.

DNA from thirteen *Capsicum* cultivars was screened using Inter Simple Sequence Repeat (ISSR) and microsatellite (SSR) markers. Five ISSR primers amplified 204 reproducible bands of which 139 were polymorphic. The percentage of polymorphic bands detected by ISSR was 100 per cent. The highest polymorphic bands were obtained by the use of primers UBC-809 (34) and UBC-66 (53). A total of 1-5 alleles were detected by six SSR primers, with an average of two alleles per primer. This study reveals the great importance of guaranteeing the differentiation of chilli cultivars and the application for certification purposes (Patel *et al.*, 2011).

Molecular profiling for genetic variability in *Capsicum* species based on ISSR and RAPD markers was reported by Thul *et al.* (2012). Twenty two accessions of six *Capsicum* species, namely, *C. annuum*, *C. baccatum*, *C. chinense*, *C. eximium*, *C. frutescens*, and *C. luteum* were investigated for phenotypic diversity based on flower color and for genetic differences by

molecular markers. The genetic cluster analyses of 27 RAPD and eight ISSR primers, respectively, revealed genetic similarities in the ranges of 23-88 per cent and 11-96 per cent. *C. annuum* accessions formed a single cluster in the molecular analysis. *C. chinense* accessions shared flower features with the accessions of *C. frutescens* and were found to be closer at genotypic level. *C. luteum* was found to be rather closer to *C. baccatum* complex, both phenotypically and genetically. The only accession of *C. eximium* presenting purple flowers fell apart from the groupings.

Genetic diversity of five cultivated pepper species using Inter Simple Sequence Repeat (ISSR) analysis was reported by Lijun and Xuexiao (2012). The amplicons of 13 out of 15 designed primers were stable, polymorphic and therefore were used as genetic biomarkers. 135 total clear bands were obtained, of which 102 were polymorphic bands with an average polymorphism rate of 75 per cent. Cluster analysis showed that they belonged to different branches: *C. annuum* L., *Capsicum chinense* Jacq. and *Capsicum frutescens* L. were in one cluster, whereas *Capsicum baccatum* L. and *Capsicum pubescens* Ruiz and Pavon were in another cluster. These results suggested that ISSR markers were valid tags in the studies on pepper genetic diversity and that cultivated species identification and gene exchange between cultivated and wild species should be strengthened in pepper breeding to improve heterosis.

Ahmed (2013) analyzed DNA samples of six hybrids of *Capsicum annuum* and *Capsicum frutescens* with ten Inter Simple Sequence Repeats (ISSR) primers, which produced 52 polymorphic bands out of 87 bands with polymorphism average of 60 per cent. ISSR patterns scored five distinguishable species-specific bands; two for *C. frutescens* and three for *C. annuum* and 16 unique bands for hybrids individually that were considered as molecular markers for Egyptian hybrids. Based on analysis performed on a similarity matrix using UPGMA, six hybrids were grouped into two main clusters. The first cluster included hybrids of *C. frutescens* and the other grouped those of *C. annuum* indicating narrow genetic base among the tested hybrids in both species.

2.6.3.3 Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al.*, 1995). In AFLP, total genomic DNA is digested with two restriction enzymes and adaptors of known sequence are then ligated to the DNA fragments. Primers complementary to the adaptors, with additional 1-3 selective nucleotides on the 3' end, are used to amplify the restriction fragments. The PCR amplified fragments can then be separated by gel electrophoresis and the banding patterns visualized. AFLP profiles require no prior DNA sequence information and the number and nature of amplified fragments are altered by the choice of primer pair. The technique also has the advantage of sampling many loci simultaneously and in addition, it is more robust than arbitrary priming techniques such as RAPD, because more stringent conditions are used in the PCR.

Amplified fragment length polymorphism markers have been used in mapping plant genomes such as barley (Becker *et al.*, 1995), tomato (Saliba-Colombani *et al.*, 2000), chromosome landing (Cnops *et al.*, 1996) and positional cloning (Simons *et al.*, 1998). Paran *et al.* (1998) evaluated the level of variation in *C. annuum* using different marker systems and the percentage of polymorphic markers was high for AFLP.

Amplified fragment length polymorphism provides a novel and very powerful tool for gene tagging technique of any origin or complexity (Vos *et al.*, 1995; Blears *et al.*, 1998). It is typically inherited in Mendelian fashion and may therefore be used for typing, identification of molecular marker, and mapping of genetic loci. Wang *et al.* (2005) evaluated genetic diversity of wild and cultivated pawpaw through AFLP and estimated high level of genetic diversity within population which indicated that conservation strategy should focus on preserving genetic resources in major population.

Yan *et al.*, (1999) compared estimates of population genetic parameters between AFLPs and RFLPs in populations of the yellow fever mosquito, to prove that the Hardy-Weinberg equilibrium is strictly followed in AFLP markers.

Based on the technique of AFLP the research advances for improvement of *Capsicum* spp. were reviewed, including genetic tagging, genetic diversity detection, variety taxonomy, variety identification and pedigree analysis (Mathew, 2006).

Polymerase chain reaction based methodologies provide an alternative method for isolation of microsatellite loci. Microsatellite loci have been developed using an AFLP-PCR approach called FIASCO (Fast Isolation by AFLP of Sequences Containing Repeats) (Zane *et al.*, 2002; Sun *et al.*, 2008; Zang *et al.*, 2008). The ability of AFLPs to distinguish among genotypes is not hindered by their bi-allelic nature (presence or absence) and thus polymorphisms can be identified between very closely related genotypes.

Amplified Fragment Length Polymorphisms (AFLP) were also used to study genetic diversity in four chilli varieties. Two wild chilies (Amashito and Ojo de cangrejo: *Capsicum annuum* var. *glabriusculum*) and two cultivated types (Jalapeno and Habanero: C. *annuum* and *Capsicum chinense* Jacq.), were used as parents in a diallel cross, with no reciprocal crosses, to produce six F1 hybrid combinations (Castanon-Najera *et al.*, 2011).

Krishnamurthy *et al.* (2013) assessed the morphological and Amplified Fragment Length Polymorphism (AFLP) marker-based genetic diversity, to estimate mid-parent heterosis and to study the possible limits of the parental divergence for the occurrence of heterosis for yield and its contributing traits in chilli hybrids.

Bulked segregant analysis with AFLP primer combination EcoACT and MseCAC was done by Thakur *et al.* (2014) using the DNA from donor parent Ujwala, susceptible parent Pusa Jwala, resistant parent Anugraha, bulked

susceptible F2 and bulked resistant F2 plants. On resolution using capillary electrophoresis system in genetic analyzer, the AFLP products have yielded three polymorphic bands (103, 117, and 161 bp) which were linked with the resistant recessive allele and three polymorphic bands (183, 296, 319 bp) linked with the dominant susceptible allele of the bacterial wilt resistance gene. The results were confirmed through co-segregation analysis in most resistant and susceptible plants of F2 segregating population.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The investigations on "Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during July 2013 - June 2014. The study was aimed to characterize Byadagi chilli using morphological and molecular markers. The experimental materials and methodologies adopted for characterizing chilli genotypes at morphological and molecular level are presented in this chapter.

3.1 Materials

3.1.1 Plant Materials

Two Byadagi chilli cultivars viz. Byadagi Kaddi and Byadagi Dabbi and two chilli varieties released from Kerala Agricultural University viz. Ujwala and Anugraha formed the experimental materials for the study. Ujwala was selected for the study for its high pungency and Anugraha for its less colour on drying. The four chilli genotypes were raised in pots with four replications and ten pots/replication/variety and maintained at CPBMB (Plate 1). The crop was managed as per Package of Practices recommendations of Kerala Agricultural University. Morphological and molecular characterization of Byadagi Kaddi and Byadagi Dabbi were attempted along with two KAU varieties Ujwala and Anugraha.

3.1.2 Laboratory chemicals, Glassware and Equipment items

The chemicals used for the study were of good quality (AR grade) procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The Taq DNA polymerase, dNTPs, Taq buffer and molecular marker (λDNA / *Hind*III+ *Eco*RI and 100 bp Ladder) were supplied by Banglore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. The

A- Byadagi Kaddi

B- Ujwala

C- Byadagi Dabbi

D- Anugraha

Plate 1 Different chilli genotypes raised in pots

decamer primers were obtained from Operon Technologies Inc., RAPD and ISSR primers were obtained from Sigma Aldrich Chemical Pvt. Ltd.

The equipment used in the study were mainly High speed refrigerated centrifuge for centrifugation (KUBOTA 6500), NanoDrop^{R} ND-1000 spectrophotometer for the estimation of quality and quantity of DNA, Veriti^{R} Thermal Cycler (Applied Biosystem, USA) for DNA amplification, horizontal gel electrophoresis system (BIO-RAD, USA) for agarose gel electrophoresis. Details of laboratory equipments used for the study are provided in Annexure I.

3.2 Methods

3.2.1 Morphological characterization

Morphological parameters of four chilli genotypes viz. Byadagi Kaddi, Byadagi Dabbi, Ujwala and Anugraha were recorded as per the minimal descriptor of Agri- Horticultural crops of NBPGR (Srivastava *et al.*, 2001). Observations on vegetative, floral and fruit characters were recorded. Yield and quality parameters like colour and pungency and incidence of pests and diseases were also recorded. Altogether, 21 qualitative and 16 quantitative characters were recorded. The minimal descriptor of chilli used for recording observations is provided in Annexure II.

3.2.2 Molecular characterization

Molecular marker analyses in four chilli genotypes (Byadagi Kaddi, Byadagi Dabbi, Ujwala and Anugraha) were carried out with two marker systems viz. RAPD and ISSR.

3.2.2.1 Genomic DNA isolation

Young, tender, green leaves were collected in ice from individual plants of four chilli genotypes. The surface was cleaned by washing with sterile water and wiping with 70 per cent alcohol. The fresh leaves were ground to a fine powder in

liquid nitrogen along with β-mercaptoethanol and PVP using ice cold mortar and pestle.

CTAB method developed by Rogers and Bendich (1994) was used for the extraction of genomic DNA.

Reagents:

I. 2X CTAB buffer:
II. 10% CTAB buffer:
III. TE buffer
IV. Chloroform : isoamyl alcohol (24:1) v/v
V. Isopropanol
VI. Ethanol 70% and 100%
VII. Sterile distilled water

Chemical composition of reagents is provided in Annexure III

Procedure

- Ground one gram of cleaned leaf tissues in a pre-chilled mortar and pestle in the presence of liquid nitrogen and β mercaptoethanol (50µl) in an okridge tube
- Added 4ml of 2X CTAB buffer
- Transferred the homogenized sample into an autoclaved 50ml centrifuge tube which contained 3ml of pre-warmed extraction buffer (Total 7ml)
- Mixed well and incubated the mixture at 65°C for 30 minutes with occasional mixing by gentle inversion.
- Added equal volume (7ml) of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify.
- Spun at 10,000 rpm for 15 minutes at 4°C
- After centrifugation, the contents got separated into three distinct phases.

  Aqueous topmost layer - DNA

Interphase - Fine particles, cell debris, and emulsified protein

Lower layer - Chloroform and pigments

- Transferred the top aqueous layer to a clean centrifuge tube and added $1/10^{th}$ volume of 10% CTAB solution and equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion
- Centrifuged at 10,000 rpm for 15 minutes at 4°C
- Transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitated. Kept at -20°C for half an hour for complete precipitation
- Centrifuged at 10,000 rpm for 15 minutes at 4°C. Gently poured off the supernatant
- Added 70% ethanol to DNA pellet
- Spun for 5 minutes at 8000rpm and discarded the ethanol
- Air dried the pellet, dissolved in 50µl of autoclaved distilled water and stored at -20°C

3.2.2.2 Purification of DNA

The DNA which had RNA as contaminant was purified by RNase treatment and further precipitated.

Reagents

I. Chilled isopropanol
II. 70% ethanol
III. TE buffer
IV. Chloroform: isoamyl alcohol (24: 1, v/v)
V. 1% RNase

One percent solution was prepared by dissolving RNase (Sigma, USA) in autoclaved distilled water. The solution was dispensed into aliquots and stored at -20°C.

Procedure

- To 100 µl DNA sample, added RNase solution (2 µl) and incubated at 37°C in dry bath for 45 minutes
- Added equal volume of chloroform: isoamyl alcohol (24: 1) mixture and mixed gently
- Centrifuged at 10,000 rpm for 20 minutes at 4°C
- Transferred the aqueous phase into a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24: 1)
- Centrifuged at 10,000 rpm for 15 minutes at 4°C
- Transferred the aqueous phase into a fresh micro centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitated. Kept at -20°C for half an hour for complete precipitation
- Centrifuged at 10,000 rpm for 15 minutes at 4°C.
- Gently poured off the supernatant and washed the DNA pellet with 70% ethanol
- Air dried the pellet, dissolved in autoclaved distilled water and stored at 20°C

3.2.2.3 Quantification of DNA

3.2.2.3.1 Assessment of quality and quantity of DNA by electrophoresis

The quantity and quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Materials used for agarose gel electrophoresis

I. Agarose
II. 50X TAE buffer (pH 8.0)
III. Tracking/Loading dye (6X)
IV. Ethidium bromide

V. Electrophoresis unit, power pack, gel casting tray and comb

VI. UV transilluminator

VII. Gel documentation and analysis system

Chemical composition of reagents is provided in Annexure IV.

Procedure

- The gel tray was prepared by sealing the ends with cello tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray
- Prepared 0.8 per cent agarose (0.8g in 100ml) in a conical flask with 100ml 1X TAE buffer. Microwaved for 45 to 60 seconds until agarose was dissolved and solution was clear
- Solution was allowed to cool to about 42 to 45°C before pouring. (Ethidium bromide was added at this point to a concentration of 0.5 µg/ml)
- Poured this warm gel solution into the tray to a depth of about 5mm and allowed the gel to solidify for about 30 to 45 minutes at room temperature
- Gently removed the comb and the cello tape used for sealing, placed the tray in electrophoresis chamber and covered (just until wells are submerged) with 1X TAE buffer
- To prepare samples for electrophoresis added 1µl of 6X gel loading dye for 5µl of DNA solution. Mixed well and loaded 6µl DNA per well. Loaded λDNA / *Hind*III+ *Eco*RI double digest (1000bp) in one lane.
- Electrophoresed at 100 volts until dye has migrated two third the length of the gel
- Intact DNA appeared as orange fluorescent bands when viewed under UV transilluminator. If degraded, it appears as a smear because of the presence of a large number of bands, which differ in one or two bases.
- The image was documented and saved in the gel documentation system.

3.2.2.3.2 Assessing the quality and quantity of DNA by Nanodrop spectrophotometer.

The purity of DNA was further checked by using NanoDrop ND-1000 spectrophotometer. Nucleic acid shows absorption maxima at 260nm whereas proteins show peak absorbance at 280nm. Absorbance was recorded at both wavelength and purity of DNA was indicated by the ratio OD_{260}/OD_{280}. The values between 1.8 and 2.0 indicate that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} is equivalent to 50µg double stranded DNA/ml sample.

1 OD at 260 nm = 50µg DNA/ml

Therefore $OD_{260} \times 50$ gives the quantity of DNA in µg/ml.

Procedure for Nanodrop spectrophotometry

- Connected the Nanodrop spectrophotometer to the System and opened the operating software ND-100.
- Selected the option Nucleic acid.
- With the sampling arm open, pipetted 2µl distilled water onto the lower measurement pedestal.
- Closed the sampling arm and initiated a spectral measurement using the operating software on the PC. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made.
- Set the reading to zero with sample blank.
- 1µl sample was pipetted onto measurement pedestal and selected measure.
- When the measurement was complete, opened the sampling arm and wiped the sample from both the upper and lower pedestals using a soft laboratory

wipe. Simple wiping prevented sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.2.2.4 Molecular markers used for the study

Two different marker systems were used for the study which included RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequences Repeats) assays. Under each marker analysis system, each of the four varieties were amplified separately with the selected primers. For both the marker systems, only reproducible bands were scored for further characterization.

3.2.2.4.1 RAPD (Random Amplified Polymorphic DNA) analysis

Good quality genomic DNA (40 to 50ng/µl) isolated from four chilli genotypes by CTAB method were subjected to RAPD analyses as per the procedure reported by Williams *et al.*, (1990). Random decamer primers with good resolving power were used for amplification of DNA. Nine decamer primers reported for chilli by Manibala (2013) viz. S12, S13, OPA10, OPA28, OPAH06, OPC08, RN07, OPK01, OPU02 and four chilli specific primers OPA11 (Baral and Bosland 2002), S07 (Ilbi, 2003), OPE18 (Troconis-Toress *et al.*, 2012), OPB04 (Uddin *et al.*, 2012 and Bahurupe *et al.*, 2013) were used for RAPD assay. The amplification was carried out in a Veriti^{R} Thermal Cycler (Applied Biosystems, USA). The PCR reaction was performed using 20µl reaction mixture.

Composition of the reaction mixture for PCR (20 µl)

Materials	Quantity (µl)
a) Genomic DNA (45 ng/ µl)	- 2.0
b) 10X Taq assay buffer B	- 2.0
c) $MgCl_2$	- 1.8
d) dNTPs (10mM/ µl)	- 1.5
e) Taq DNA polymerase (1U/ µl)	- 0.4
f) Decamer primer (10 pM)	- 2.0

g) Autoclaved distilled water	- 10.3
Total volume	- 20.0 µl

A master mix with all reagents for the required number of reactions was prepared first and aliquots were dispensed into PCR tubes followed by addition of template DNA in each tube. One tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycler and the following programme was run:

Step1:	94°C for 2 minutes	-	Initial denaturation	
Step2:	94°C for 1 minute	-	Denaturation	40 cycles
Step3:	39- 43°C for 1 minute	-	Primer annealing	
Step4:	72°C for 2 minutes	-	Primer extension	
Step5:	72°C for 10 minutes	-	Final extension	
Step6:	4°C for infinity to hold the sample			

The amplified products were run on 1.3 per cent agarose gel using 50X TAE buffer stained with ethidium bromide along with marker (100bp-3kb ladder). Electrophoresis was performed at 70 volts for two hours. The profile was visualized under UV transilluminator and documented using gel documentation system. The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb/bp of bases was recorded in comparison with the marker and using the software Quantity One.

3.2.2.4.2 ISSR (Inter Simple Sequence Repeats) analysis

Good quality genomic DNA (40 to 50 ng/µl) isolated from four chilli genotypes by CTAB method were subjected to ISSR analysis as per the procedure reported by Williams *et al.*, (1990). Primers with good resolving power were used for amplification of DNA. Seven ISSR primers reported by Manibala (2013) viz. UBC840, 2UBCS2, ISSR05, ISSR07, ISSR08, ISSR15, C- Renteria 6 and four chilli specific primers UBC823 (Patel *et al.*, 2011), UBC835 (Thul *et al.*, 2011),

HB10 (Ahmed, 2013), 17899A (Refaat and Elgarhy, 2007) were used for ISSR assay.

The amplification was carried out in a Veriti^{R} Thermal Cycler (Applied Biosystems, USA). The PCR reaction was performed using 20µl reaction mixture.

	Materials	**Quantity (µl)**
a)	Genomic DNA (45 ng/ µl)	- 2.0
b)	10X Taq assay buffer B	- 2.0
c)	$MgCl_2$	- 2.0
d)	dNTPs (10mM/ µl)	- 1.5
e)	Taq DNA polymerase (1 U/ µl)	- 0.4
f)	ISSR primer (10 pM)	- 1.5
g)	Autoclaved distilled water	- 10.6
h)	Total volume	- 20.0µl

A master mix with all reagents for the required number of reactions was prepared first and aliquots were dispensed into PCR tubes followed by addition of template DNA in each tube. One tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycle and the following programme was run:

Step1:	94°C for 4 minutes	-	Initial denaturation	
Step2:	94°C for 45 seconds	-	Denaturation	} 35 cycles
Step3:	43- 47°C for 1 minute	-	Primer annealing	
Step4:	72°C for 2 minutes	-	Primer extension	
Step5:	72°C for 10 minutes	-	Final extension	
Step6:	4°C for infinity to hold the sample			

The amplified products were run on 1.3 per cent agarose gel using 50X TAE buffer stained with ethidium bromide along with marker (100bp-3kb ladder).

Electrophoresis was performed at 70 volts for two hours. The profile was visualized under UV transilluminator and documented using gel documentation system. The documented ISSR profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb/bp of bases was recorded in comparison with the marker and using the software Quantity One.

3.2.2.5 Data analysis

Morphological characterization

The observations recorded on vegetative, flowering, fruit and yield characters were subjected to analysis of variance and the level of significance was compared between genotypes for different parameters analyzed.

Molecular characterization

Scoring of bands on agarose gel was done with the Quantity One software. Molecular weight size marker (100bp) was used for each gel alongside the DNA samples. The bands were scored as 1 and 0 for the presence and absence of bands respectively and their size recorded in relation to the molecular weight markers used. Only distinct and well resolved fragments were scored. The molecular data obtained from RAPD and ISSR profiles were transformed into data matrix as discrete variables. Jaccard's coefficient of similarity was measured and a dendrogram based on similarity coefficients was generated by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The resulting data were analyzed using the software package NTSYS (Rohlf, 2005).

Resolving power was used to identify the primers that would distinguish the varieties most efficiently. Resolving power (Rp) of a primer was calculated as the sum of 'band informativeness' of the bands produced by the primer (Prevost and Wilkinson, 1999). Band informativeness (Ib) is $1-[2\times(0.5-p)]$, where p is the proportion of varieties containing the band. Resolving power of the primer is represented as: $Rp= \Sigma Ib$.

Polymorphic Information Content (PIC) value of a primer is represented as: PIC= $1-\Sigma pi^2$, where *pi* is the frequency of *ith* allele, thus confirming the suitability of the primers selected for DNA fingerprinting. The PIC value of a marker detects polymorphism within a population depending on the number of detectable alleles and their frequency (Hollman *et al.*, 2005).

RESULTS

4. RESULTS

The study on "Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2013 - March 2014. The results of various aspects of the study are described in this chapter.

The research works included mainly morphological and molecular characterization of two cultivars (Byadagi Kaddi and Byadagi Dabbi) along with two released varieties of KAU (Ujwala and Anugraha). Two molecular marker systems viz. RAPD and ISSR were used in the investigations to characterize chilli genotypes.

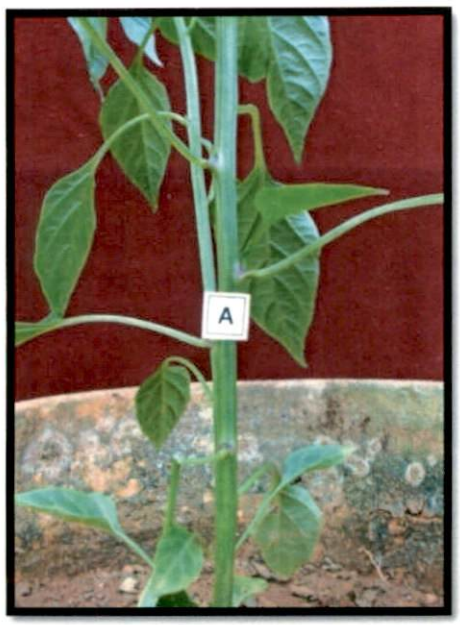
4.1 Morphological Characterization

Morphological characterization of chilli genotypes was attempted as per the minimal descriptor published from NBPGR, New Delhi. Twenty one qualitative and 16 quantitative characters were recorded as per the minimal descriptor.

4.1.1 Vegetative Characters

Vegetative characters like stem colour, plant height, canopy width, growth habit, branching habit, leaf size, shape, colour and pigmentation at node recorded at full foliage stage are presented in Table 1. Life cycle of all the four genotypes studied was annual.

Stem colour was green with purple stripes in both the Byadagi cultivars and two released varieties (Plate 2). The plant height recorded ranged from 61.5 to 70.9 cm in the four genotypes studied. However, no significant variation was observed in plant height among genotypes observed. Significant variation in plant canopy width was noticed in the varieties and cultivars. Plant canopy width recorded was highest



A- Byadagi Kaddi

B- Ujwala

C- Byadagi Dabbi

D- Anugraha

Plate 2 Stem colour in different chilli genotypes

Table 1. Vegetative characters in different chilli genotypes

Sl. no	Chilli genotypes	Life cycle	Stem colour	Plant height (cm)	Plant canopy width (cm)	Plant growth habit	Branching habit	Leaf size	Leaf area $(cm)^2$	Leaf shape	Leaf colour	Pigmentation at node
1	Byadagi Kaddi	Annual	Green with purple stripes	66.47	27.47	Erect	Intermediate	Medium	20.25	Ovate	Dark green	Present
2	Byadagi Dabbi	Annual	Green with purple stripes	69.47	30.42	Erect	Intermediate	Medium	27.25	Ovate	Dark green	Present
3	Ujwala	Annual	Green with purple stripes	61.57	26.72	Erect	Intermediate	Large	31.15	Ovate	Green	Present
4	Anugraha	Annual	Green with purple stripes	70.95	47.50	Erect	Intermediate	Small	17.50	Ovate	Green	Present
5	CD (0.05)			NS	5.36				5.68			

(47.57 cm) for the variety Anugraha and which was significantly superior to all other genotypes and lowest (26.725 cm) for the variety Ujwala which was statistically on par with Byadagi Kaddi and Byadagi Dabbi.

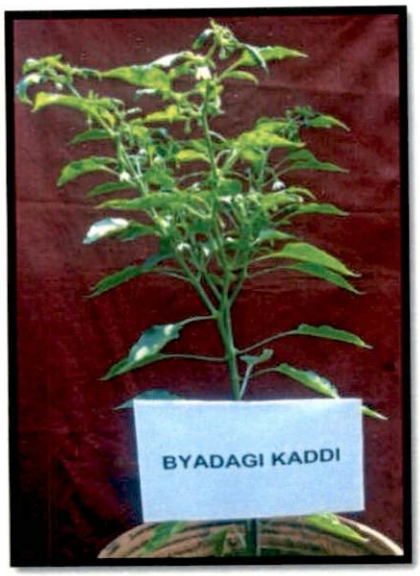
Plant growth habit was observed at fruit maturity stage and it was erect in all the four genotypes studied (Plate 3). Branching habit was observed when plants have ceased its growth and it was intermediate in all the four genotypes studied (Plate 3). Leaf size was calculated using graphical method at full foliage stage and it was large in Ujwala where as small in Anugraha and are medium in both Byadagi Kaddi and Byadagi Dabbi (Plate 4).

Significant variation in leaf area was noticed in the cultivars and varieties studied. Leaf area recorded was highest (31.15 cm) in the varirty Ujwala which was statistically on par with cultivar Byadagi Dabbi. Leaf area was lowest (17.5 cm) in the variety Anugraha which was statistically on par with cultivar Byadagi Kaddi. Also statistically, Byadagi Kaddi was on par with Byadagi Dabbi with respect to leaf area.

Leaf shape was noticed ovate in all the four genotypes (Plate 4) which was also observed at full foliage stage. Leaf colour was dark green in cultivars Byadagi Kaddi and Byadagi Dabbi, while it was green in Ujwala and Anugraha (Plate 4). Purple colour pigmentation at node was seen in all the four genotypes (Plate 5).

4.1.2 Flowering and fruit characters

Flowering and fruit characters like days to 50 per cent flowering, number of flowers per axil, corolla colour, anther colour, calyx margin, days to 50 per cent fruiting, mature fruit colour, ripe fruit colour, fruit shape, fruit length, fruit width, fruit position adherence of calyx to fruit, fruit pedicel length, fruit shape at pedicel attachment, blossom end fruit shape, fruit surface, placenta length and organoleptic test were recorded at different stages as per the minimal descriptor of Agri-



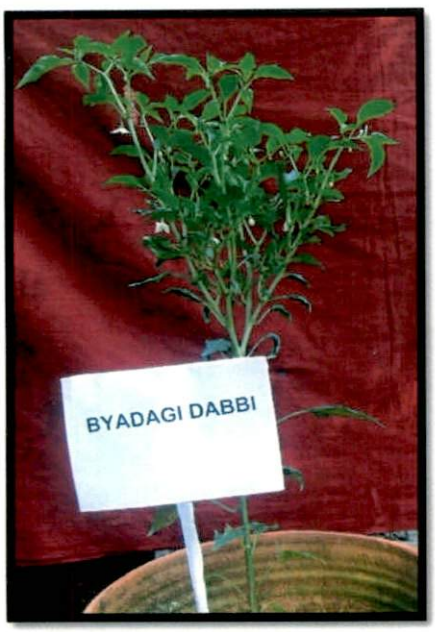


Plate 3 Plant growth and branching habit in different chilli genotypes

A- Byadagi Kaddi, **B-** Ujwala, **C-** Byadagi Dabbi, **D-** Anugraha

Plate 4 Leaf size, shape and colour indifferent chilli genotypes

Horticultural crops of NBPGR (Srivastava *et al.*, 2001) and data are presented in Table 2.

Days to 50 per cent flowering was observed 82 days after transplanting in cultivar Byadagi Kaddi where as in Byadagi Dabbi and variety Anugraha it was observed 55 days after transplanting. In variety Ujwala, 50 per cent flowering was observed 65 days after transplanting.

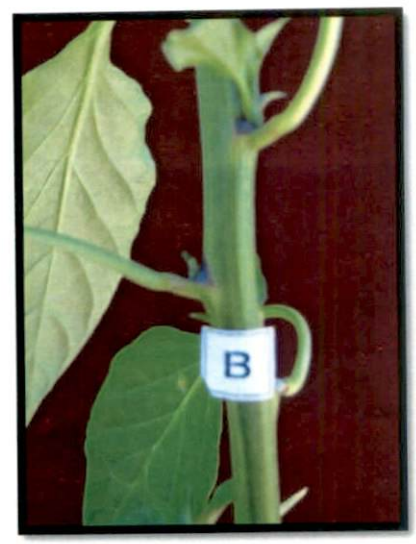
Number of flowers per axil varied from 1-4.4 and was observed as one in Byadagi Kaddi, Byadagi Dabbi and Anugraha and more than three (4.4) in Ujwala. Corolla colour was white in all the four genotypes studied which was observed immediately after blooming where as anther colour was observed after blooming but before anthesis and it was pale blue in all the four genotypes (Plate 6). Calyx margin was observed at fully blossom stage and it was dentate in all the four genotypes (Plate 7).

Days to 50 per cent fruiting was noticed 91 days after transplanting in Byadagi Kaddi. In Ujwala, 83 days after transplanting and in Anugraha 72 days of transplanting.

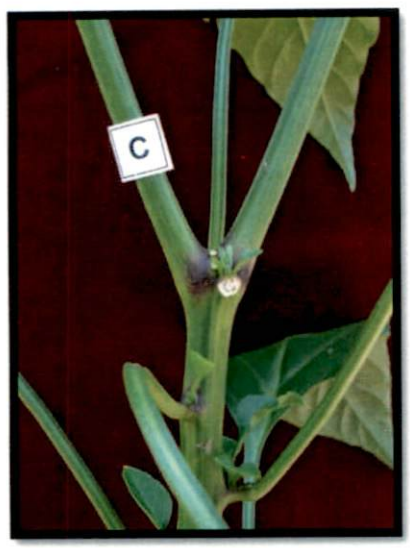
Mature fruit colour was green in three genotypes viz, Byadagi Kaddi, Byadagi Dabbi and variety Ujwala where as it was pale green in variety Anugraha (Plate 8a). Ripe fruit colour was dark red in Byadagi Kaddi and Byadagi Dabbi and it was red in Ujwala and Anugraha (Plate 8b). Fruit shape recorded at mature fruit stage was elongate in the four genotypes studied. In Byadagi Kaddi fruit was slender and linear where as in Byadagi Dabbi fruits were medium long and curved at the apex.

Significant variation in fruit length was noticed in the cultivars and varieties studied. Fruit length ranged from 5-13.02 cm in the genotypes. Fruit length was highest (13.02 cm) in Byadgi kaddi where as it was lowest (5.0 cm) in the variety Ujwala.

A- Byadagi Kaddi



B- Ujwala



C- Byadagi Dabbi

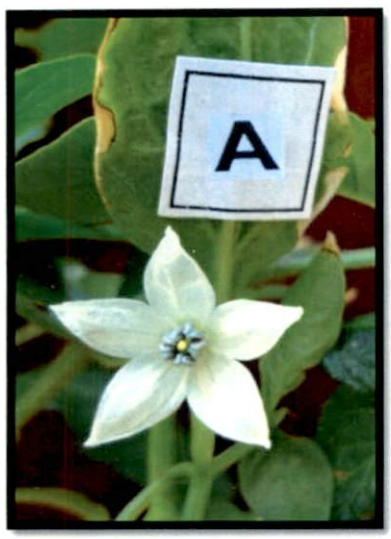
D- Anugraha

Plate 5 Pigmentation at node in different chilli genotypes

Table 2. Flowering and fruit characters in different chilli genotypes

Sl no	Chilli genotypes	Days to 50% flowering	No. of flowers/axil	Corolla colour	Anther colour	Calyx margin	Days to 50% fruiting	Mature fruit colour	Ripe fruit colour	Fruit shape	Fruit length (cm)	Fruit width (cm)	Fruit position	Adherence of calyx to fruit	Fruit pedicel length (cm)	Fruit shape at pedicel attachment	Blossom end fruit shape	Fruit surface	Placenta length (cm)
1	Byadagi Kaddi	82 DAT *	1	White	Pale blue	Dentate	91 DAT	Green	Dark red	Elongate	13.02	1.15	Pendent	Semi hard	3.27	Obtuse	Pointed	Wrinkled	10.82
2	Byadagi Dabbi	55 DAT	1	White	Pale blue	Dentate	83 DAT	Green	Dark red	Elongate	8.57	1.77	Pendent	Hard	3.00	Cordate	Blunt	Semi wrinkled	6.62
3	Ujwala	65 DAT	>3	White	Pale blue	Dentate	83 DAT	Green	Red	Elongate	5.00	0.92	Erect	Hard	2.92	Obtuse	Pointed	Smooth	3.95
4	Anugraha	55 DAT	1	White	Pale blue	Dentate	72 DAT	Pale green	Red	Elongate	7.15	0.82	Pendent	Semi hard	2.80	Acute	Pointed	Smooth	4.37
5	CD (0.05)										0.51	0.13			NS				1.04

*- Days after transplanting



A- Byadagi Kaddi

B- Ujwala

C- Byadagi Dabbi

D- Anugraha

Plate 6 Corolla and anther colour in different chilli genotypes

Significant variation was noticed in fruit width in the cultivars and varieties studied. Fruit width was highest (1.77 cm) in Byadagi Dabbi where as it was lowest (0.82 cm) in the variety Anugraha.

Fruit position was pendant in Byadagi Kaddi, Byadagi Dabbi and Anugraha where as it was erect in variety Ujwala (Plate 7). Adherence of calyx to fruit recorded during fruit maturity stage was semi hard in Byadagi Kaddi and variety Anugraha, and it was hard in Byadagi Dabbi and variety Ujwala (Plate 7). No significant variation was noticed among genotypes in fruit pedicel length.

Fruit shape at pedicel attachment, blossom end fruit shape and fruit surface were recorded at fruit maturity stage. Fruit shape at pedicel attachment was obtuse in both Byadagi Kaddi and variety Ujwala where as it was cordate and acute in Byadagi Dabbi and variety Anugraha respectively (Plate 7). Blossom end fruit shape was observed pointed in three genotypes viz. Byadagi Kaddi, Ujwala and Anugraha where as it was blunt in Byadagi Dabbi (Plate 9). Fruit surface was smooth in varieties Ujwala and Anugraha and it was wrinkled and semi wrinkled in Byadagi Kaddi and Byadagi Dabbi respectively.

Placental length was also recorded at fully mature stage of the fruit. Significant variation was noticed in placental length. Length of placenta was highest (10.82 cm) in Byadagi Kaddi and it was lowest (3.95 cm) in variety Ujwala. Statistically, the variety Ujwala was on par with variety Anugraha with respect to placental length.

4.1.3 Yield characters

Yield contributing characters like number of fruits per plant, fruit weight and fruit yield per plant and observations on seed colour, number of seeds per fruit and 100 seed weight and organoleptic characters were observed and recorded at different

A- Byadagi Kaddi

B- Ujwala

C- Byadagi Dabbi

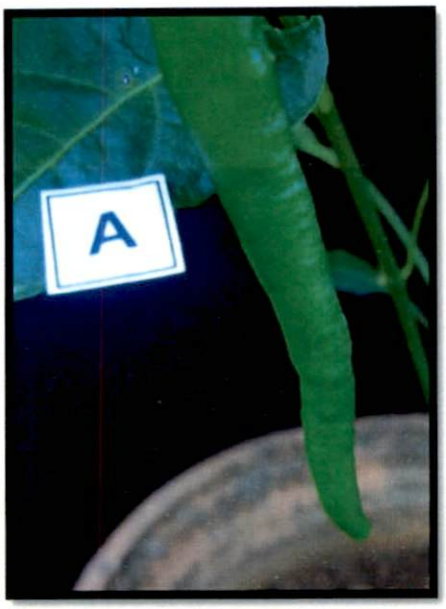
D- Anugraha

Plate 7 Calyx margin, fruit position, adheresence of calyx to fruit and fruit shape at pedicel attachment in different chilli genotypes

a. Mature fruit colour

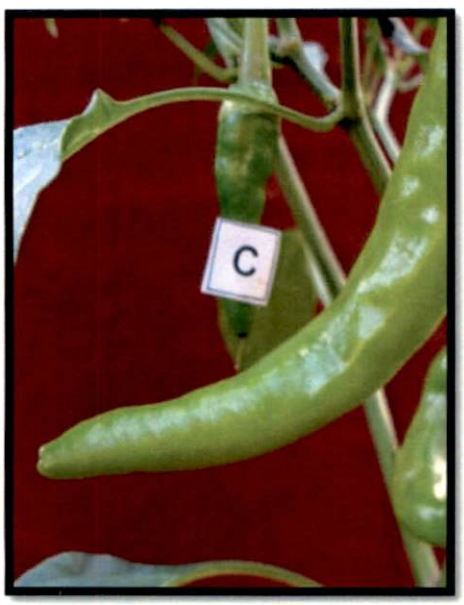
b. Ripe fruit colour

Plate 8 Fruit colour in different chilli genotypes

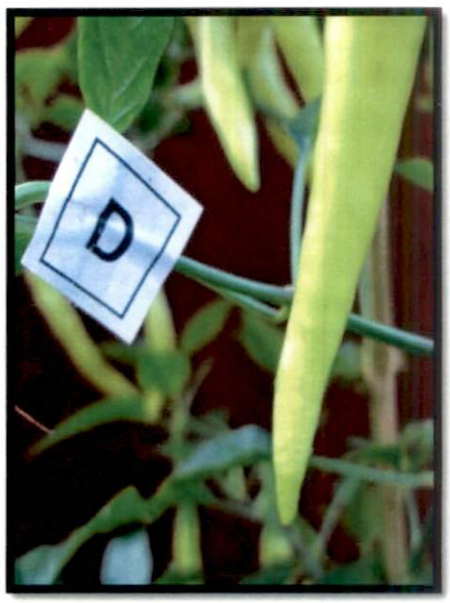


A- Byadagi Kaddi

B- Ujwala



C- Byadagi Dabbi



D- Anugraha

Plate 9 Blossom end fruit shape in different chilli genotypes

stages as per the minimal descriptor of Agri- Horticultural crops of NBPGR (Srivastava *et al.*, 2001) and data are presented in Table 3.

Significant variation was observed in number of fruits per plant in the genotypes studied. Number of fruits per plant recorded was highest (89 fruits) in variety Anugraha and it was lowest (15.67 fruits) in Byadagi Kaddi. Statistically the variety Ujwala was on par with variety Anugraha with respect to number of fruits, similarly Byadagi Dabbi was on par with Byadagi Kaddi.

Fruit yield per plant also exhibited significant variation. Fruit yield per plant varied from 82.13 g to 166.28 g. Fruit yield/plant was highest (166.28 g) in variety Anugraha where as it was lowest (82.13 g) in cultivar Byadagi Kaddi. Statistically Byadagi Dabbi, Ujwala and Anugraha were on par with respect to fruit yield/plant.

Significant variation was noticed in fruit weight in the genotypes studied. Fruit weight was highest (7.23 g) in Byadagi Dabbi and it was lowest (1.64 g) in variety Ujwala. Statistically, the variety Ujwala was on par with variety Anugraha in terms of fruit weight.

Seed colour was observed at dry seed stage. Seed colour was yellow in Byadagi Dabbi and variety Anugraha and it was light yellow and deep yellow in Byadagi Kaddi and variety Ujwala respectively (Plate 10).

Number of seeds per fruit was recorded at ripened fruit stage. Significant variation was noticed in number of seeds per plant in the genotypes studied. Number of seeds per fruit was highest (98.15) in Byadagi Kaddi and it was lowest (58.45) in variety Anugraha. Statistically Byadagi Kaddi was on par with Byadagi Dabbi.

Significant variation was also noticed in 100 seed weight in the genotypes studied. The seed weight was highest (0.55 g) in Byadagi Dabbi and it was lowest

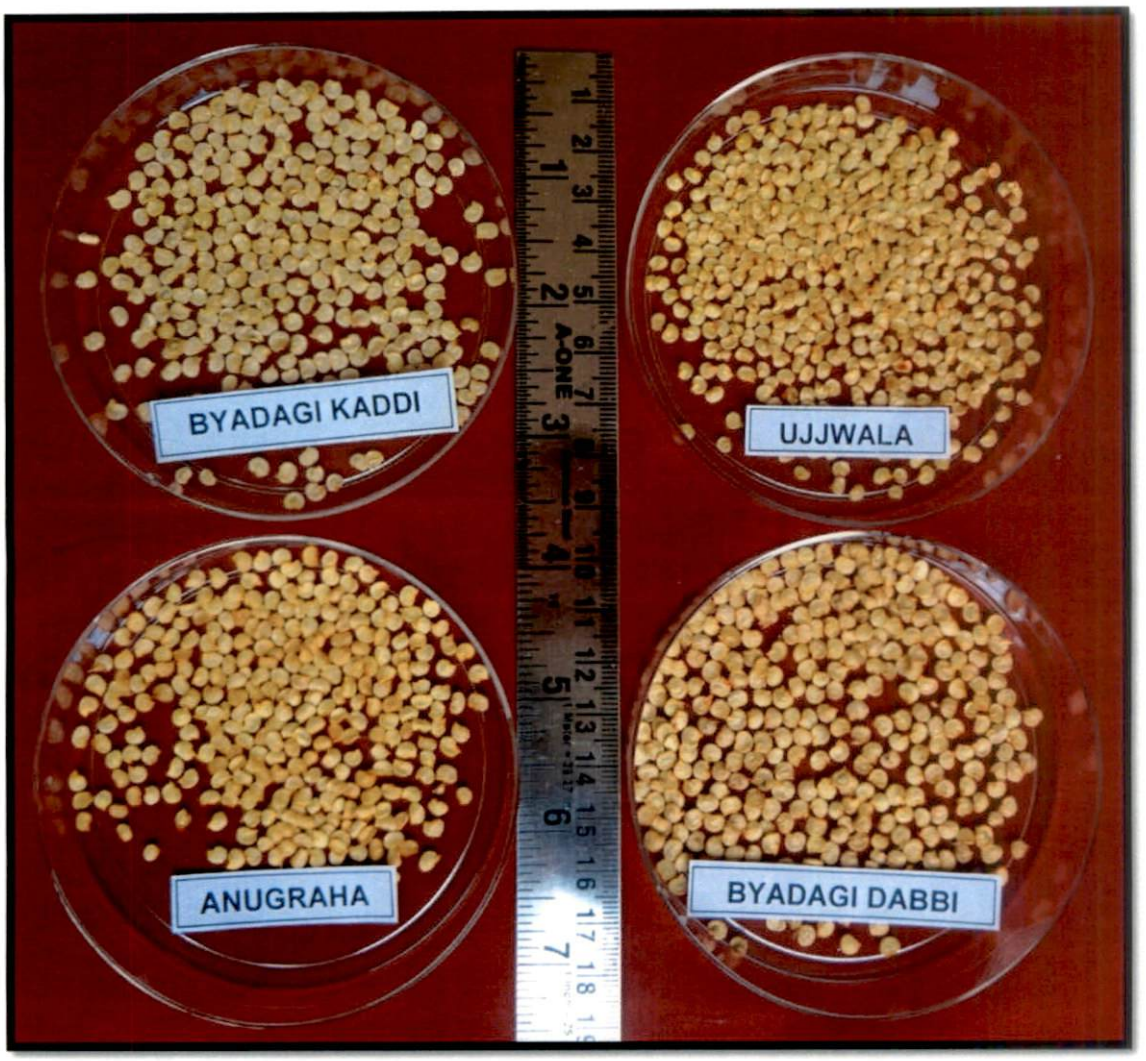


Plate 10 Seed colour in different chilli genotypes

Table 3. Yield parameters in different chilli genotypes

Sl.no	Chilli genotypes	No.of fruits/plant	Fruit yield/plant(g)	Fruit weight (g)	Seed colour	No.of seeds/fruit	100 seed weight (g)
1	Byadagi Kaddi	15.67	82.13	5.30	Light yellow	98.15	0.43
2	Byadagi Dabbi	18.75	118.12	7.23	Yellow	96.82	0.55
3	Ujwala	87.17	138.52	1.64	Deep yellow	73.47	0.37
4	Anugraha	89.00	166.28	1.91	Yellow	58.45	0.45
	CD (0.05)	18.28	40.76	0.64		8.72	0.02

(0.37 g) in Ujwala. Statistically Ujwala was on par with Byadagi Kaddi and Anugraha.

4.1.4 Quality parameters

The colour and pungency of four chilli genotypes were analysed at Spices Board Quality Evaluation Laborattory, Kochi, India and data are presented in Table 4. The ASTA colour value was found highest in cultivar Byadagi Dabbi (120.4) and lowest in KAU varieties Ujwala (58.5) and Anugraha (59.7), both of them were found on par. Byadagi kaddi recorded an ASTA colour value of 97.45. Capsaicin content of variety Ujwala was found highest recording 0.39 per cent (7750 SHU) and lowest was noticed in Byadagi cultivars, Byadagi Dabbi with 0.04 per cent (6300 SHU) and Byadagi Kaddi with 0.05 per cent.

4.1.5 Pests and disease incidence/ susceptibility to biotic stress

Pest and disease incidence and susceptibility to biotic stresses like leaf curl and fusarium wilt diseases were observed and recorded during the crop season and data are presented in Table 5.

Reactions of four chilli genotypes to leaf curl and fusarium wilt diseases were recorded. Leaf curl incidence was more in Byadagi Kaddi and Byadagi Dabbi (51.2 and 42.2 per cent respectively), while Fusarium wilt incidence was seen in the KAU released varieties Ujwala and Anugraha (2.5 and 5 per cent respectively). There was no incidence of Fusarium wilt in Byadagi genotypes.

Table 4. Quality parameters in different chilli genotypes

Sl.No	Chili genotypes	Capsaicin (%)	ASTA colour value
1	Byadagi Kaddi	0.05	97.45
2	Byadagi Dabbi	0.04	120.4
3	Ujwala	0.39	58.5
4	Anugraha	0.25	59.7
CD (0.05)			1.65

Table 5. Susceptibility of chilli genotypes to leaf curl and Fusarium wilt diseases

Sl.no	Genotypes	Biotic stress susceptibility *	
		Leaf curl	Fusarium wilt
1	Byadagi Kaddi	High (7)	Very low (1)
2	Byadagi Dabbi	High (7)	Very low (1)
3	Ujwala	Low (3)	Low (3)
4	Anugraha	Low (3)	Low (3)

***Biotic stress susceptibility scale**

1	Very low or no visible sign of susceptibility
3	Low
5	Intermediate
7	High
9	Very high

4.2 Genomic DNA isolation from different chilli genotypes

4.2.1 Source of DNA

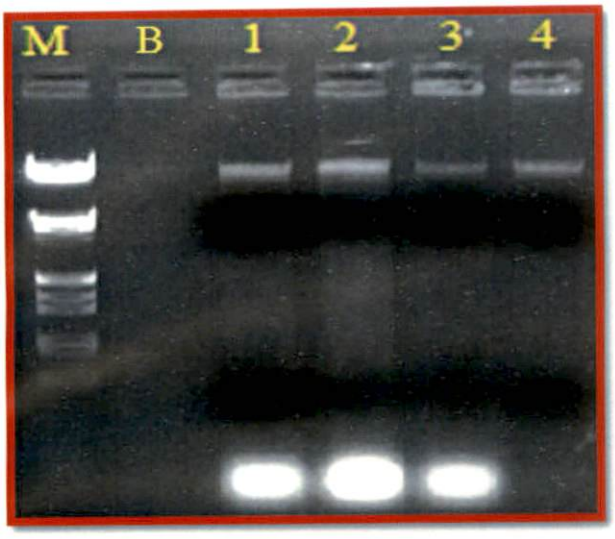
For isolation of genomic DNA, leaf samples were collected from potted plants maintained at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara. As reported in many other crops, young, tender green coloured leaves were found best for the recovery of good quality DNA in sufficient quantity from chilli.

4.2.2 Isolation and purification of DNA

Genomic DNA isolated through the CTAB method reported by Roger and Bendich (1994) was not pure and had RNA contamination (Plate 11a). However, RNAse treatment after the DNA isolation resulted in good quality DNA (Plate 11b).

4.3 Quantification of DNA

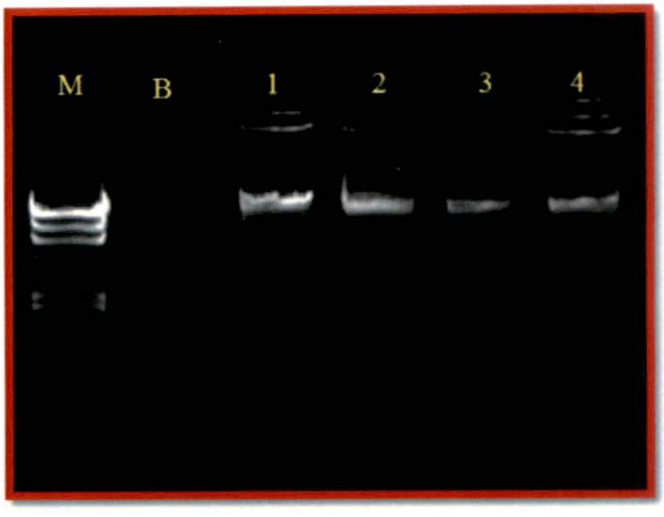
The quality and quantity of isolated DNA was analysed using both electrophoresis and NanoDrop^{R} ND-1000 spectrophotometer. Intact clear bands indicated that DNA extracted was non-degraded and was of good quality. The ratio of absorbance for the DNA isolated ranged from 1.5 to 2.1, which indicated that the quality of DNA was good (Table 6). The DNA after appropriate dilutions was used as template for RAPD and ISSR analyses.



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000bp)

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Isolated DNA



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000bp)

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Isolated DNA after RNase treatment

Plate 11. Isolated DNA from different chilli genotypes

Table 6. Assessment of quality and quantity of DNA isolated from chilli genotypes by Nanodrop spectrophotometer

Genotypes	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
Byadagi Kaddi	87.33	49.88	1.75	4366.96
Ujwala	65.12	31.47	2.07	3256.12
Byadagi Dabbi	51.97	24.25	2.14	2598.74
Anugraha	90.69	60.14	1.51	4534.58

4.4 Molecular Characterization

Two marker systems viz., RAPD and ISSR were used for molecular characterization. RAPD and ISSR analyses were done with already reported chilli specific primers, amplification of which was checked using bulked genomic DNA from four chilli genotypes.

4.4.1 Random Amplified Polymorphic DNA (RAPD) analysis

The RAPD analysis with thermal settings described in the section 3.2.2.4.1 gave good amplification. List of RAPD primers used for the study is given in Table 7. Based on the previous study conducted at CPBMB by Manibala (2013), 14 chilli specific decamer primers were selected for RAPD assay of chilli genotypes which were S 12, S 13, OPA 10, OPA 28, OPAH 06, OPU 02, OPC 08, OPA 11, RN 07, OPK 01, S 07, OPE 18, OPB 04 and OPP 17 (Table 7). Finally the analysis of RAPD data was carried out using NTSYS.

4.4.2 Inter Simple Sequence Repeats (ISSR) analysis

The ISSR analysis with thermal settings described in the section 3.2.2.4.2 gave good amplification. Based on the previous study conducted at CPBMB by Manibala (2013), 11 chilli specific primers were selected for ISSR assay of chilli genotypes which are UBC 835, UBC 840, 2 UBC S2, ISSR 05, ISSR 07, ISSR 08, ISSR 15, UBC 823, HB 10, C- Renteria 6, 17899A (Table 8). Finally the analysis of ISSR data was carried out using NTSYS.

Table 7. Details of RAPD primers used for characterizing chilli genotypes

Sl. No	Primer	Sequence
1	S 12	5'-CCTTGACGCA-3'
2	S 13	5'-TTCCCCCGCT-3'
3	OPA 10	5'-GTGATCGCAG-3'
4	OPA 28	5'-GTGACGTAGG-3'
5	OPAH 06	5'-GTAAGCCCCT-3'
6	OPU 02	5'-CTGAGGTCTC-3'
7	OPC 08	5'-TGGACCGGTG-3'
8	OPA11	5'-CAATCGCCGT-3'
9	RN 07	5'-CAGCCCAGAG-3'
10	OPK 01	5'-TGGCGACCTG-3'
11	S07	5'-TCCGGATGCTG-3'
12	OPE18	5'-CGGCCCACGT-3'
13	OPB04	5'-GGACTGGAGT-3'
14	OPP17	5'-TGACCCGCCT-3'

Table 8. Details of ISSR primers used for characterizing chilli genotypes

Sl. No	Primers	Sequence
1	UBC 835	5'-AGAGAGAGAGAGAGAGYC-3'
2	UBC 840	5'-GAGAGAGAGAGAGAGAYT-3'
3	2 UBC S2	5'-CTCTCTCTCGTGTGTGTG-3'
4	ISSR 05	5'-ATTATTGTTGTTGTTTTC-3'
5	ISSR 07	5'-ATTATTGTTGTTGTTGTA-3'
6	ISSR 08	5'-ATTATTATTATTATTGTA-3'
7	UBC823	5'-TCTCTCTCTCTCTCTCC-3'
8	ISSR 15	5'-TCCTCCTCCTCCTCC-3'
9	HB10	5'-GAGAGAGAGAGACC-3'
10	C-Renteria 6	5'-GTGTGTGTGTGTCC-3'
11	17899A	5'-CACACACACACAAG-3'

4.5 Molecular characterization in different chili genotypes

4.5.1 Molecular characterization in different chili genotypes using RAPD primers

Amplification of DNA from different chilli genotypes was carried out using already reported chilli specific primers for RAPD marker system. The details of amplification with the 14 RAPD primers are provided in Table 9. Observations were as follows:

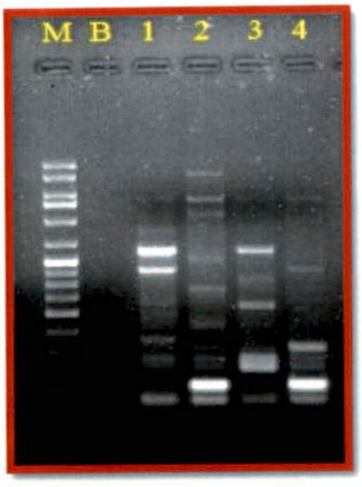
S12

A total of seventeen amplicons ranged in size 100 bp to 2500 bp were produced by the primer S12. They were clear, distinct and reproducible. It could generate fourteen polymorphic bands out of seventeen amplicons (Plate 12a.) and the percentage polymorphism was 82.35.

Two loci of size 650 bp and 1000 bp were found in cultivars Byadagi Kaddi and Byadagi Dabbi. Two loci of size 400 and 2000 bp were found in varieties Ujwala and Anugraha. Six loci of size 500 bp, 700 bp, 950 bp, 1500 bp, 2000 bp and 2500 bp were found only in variety Ujwala. Two loci of size 450 bp and 1092 bp were present only in Byadagi Kaddi cultivar. One loci of size 150 bp present in all the genotypes except Byadagi Kaddi and one loci at 800 bp size was present only in Byadagi Dabbi.

S13

The primer S13 could generate a total of eleven clear, distinct and reproducible bands (400 bp to 2500 bp) out of which only nine were polymorphic. The percentage polymorphism was 81.81. The amplification profile is given in Plate 12b.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer S12

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer S13

Plate 12. Amplification pattern in chilli genotypes with RAPD primers S12 and S13

Two loci of size 500 bp and 2500 bp were found in Byadagi Kaddi and Byadagi Dabbi. Four loci of size 400 bp, 800 bp, 900 bp and 1092 bp were found in variety Ujwala and Anugraha. One loci of size 450 bp was present only in Byadgi Kaddi and absent in other three genotypes. One loci of size 1342 bp was present in all the genotypes except Byadagi Kaddi and one loci of 2000 bp was present in Byadagi Kaddi and Anugraha.

OPA 10

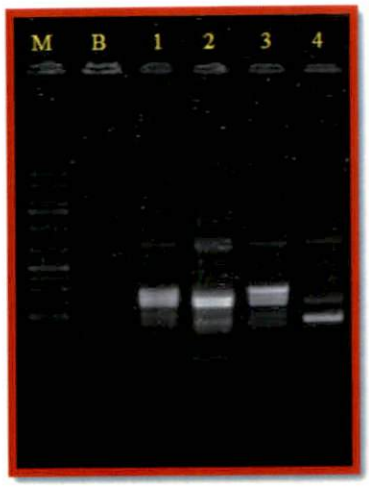
Ten clear, distinct and reproducible amplicons were produced by the primer OPA 10 (Plate 13a.) ranged in size 300 bp to 1815 bp. It could detect seven polymorphic amplicons out of ten amplicons. The polymorphism percentage calculated was 70 per cent.

Two loci of size 800 bp and 900 bp were present in Byadagi Kaddi and Byadagi Dabbi and one loci of size 600 bp was found in variety Ujwala and Anugraha. Three loci of size 500 bp, 1000 bp and 1815 bp were found only in variety Ujwala.

OPA 28

OPA 28 generated twelve clear, distinct and reproducible amplicons ranged in size from 200 bp to 1815 bp and ten of them were polymorphic (Plate 13b). The percentage polymorphism was 83.33.

Two loci of size 800 bp and 1000 bp were found in Ujwala and Anugraha varieties and they were absent in Byadagi cultivars. One loci of size 400 bp was present only in Byadagi Kaddi and one loci of size 1815 bp was found only in variety Anugraha and it was absent in rest of the genotypes. Three loci of size 200 bp 1185 bp and 1500 bp were present only in variety Ujwala and two loci of size 300 bp and



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer OPA10

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer OPA28

Plate 13. Amplification pattern in chilli genotypes with RAPD primers OPA10 and OPA28

1092 bp were present in all the genotypes except variety Ujwala and Byadgi Dabbi respectively. One loci size of 400 bp was present only in Byadagi Kaddi.

OPAH 06

Amplification of four chilli genotypes with the selected primer OPAH 06 produced thirteen clear, distinct and reproducible amplicons ranged in size 200 bp to 2500 bp (Plate 14a). Seven amplicons were polymorphic and the percentage polymorphism was 53.84.

One loci of size 200 bp was found in Byadagi Kaddi and Byadgi Dabbi. Two loci of size 400 bp and 500 bp were present only in Byadagi Dabbi and one loci of size 2500 bp was present only in variety Ujwala. Two loci of size 600 bp and 800 bp were found in all the genotypes except variety Anugraha and one loci of size 1815 bp was present in Ujwala and Byadagi Dabbi.

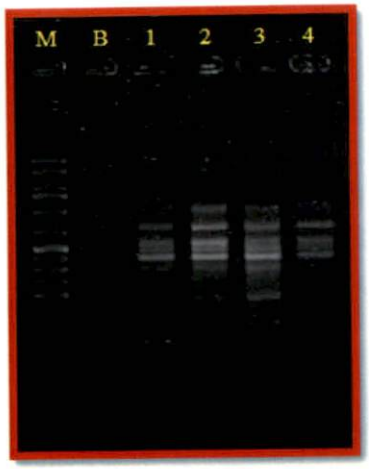
OPC 08

The primer OPC 08 was able to generate fourteen clear, distinct and reproducible amplicons (Plate 14b). The amplicons were ranged in size 300 bp to 2250 bp. seven amplicons were polymorphic giving 50 per cent polymorphism.

Three loci of size 1185 bp, 1815 bp and 2000 bp were present in Ujwala and Anugraha. Two loci of size 300 bp and 500 bp were present in all the four genotypes except Byadagi Kaddi and two loci of size 1092 bp and 2250 bp were found only in variety Ujwala.

RN 07

The decamer primer RN 07 could generate eleven clear, distinct and reproducible amplicons ranged in size 300 bp to 2000 bp, seven of them were polymorphic (Plate 15a). The percentage polymorphism was 63.63.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer OPAH 06

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer OPC 08

Plate 14. Amplification pattern in chilli genotypes with RAPD primers OPAH 06 and OPC 08

One loci of size 400 bp was present in variety Ujwala and Anugraha and three loci of size 500 bp, 600bp and 2000 bp were present in all the genotypes except Byadagi Dabbi. Two loci of size 800 bp and 1000 bp were found only in variety Ujwala and one loci of size 300 bp was present in Byadgi Kaddi and Ujwala.

OPK 01

Eleven clear, distinct and reproducible amplicons were produced by the primer OPK 01 (Plate 15b) ranged in size 300 bp to 1657 bp, out of eleven five were polymorphic and polymorphism per centage was 45.45.

One loci of size 700 bp was present in variety Ujwala and Anugraha. Two loci of size 300 bp and 500 bp were present only in variety Anugraha where as loci of size 1500 bp was present in all the genotypes except variety Anugraha and one loci of size 1657 bp was present in Byadagi Kaddi and Ujwala.

OPU 02

OPU 02 could generate a total of eleven clear, distinct and reproducible amplicons (300 bp to 1815 bp) and out of which six were polymorphic (Plate 16a). The polymorphism percentage recorded was 54.54.

One loci of size 400 bp was present in all the genotypes except variety Ujwala where as two loci of size 500 bp and 800 bp were present only in variety Ujwala and absent in rest of the genotypes. Two loci of size 700 bp and 1500 bp were present only in Byadagi Kaddi and variety Anugraha respectively and one loci of size 1342 bp was present in all the genotypes except Byadagi Kaddi.

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer RN 07

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer OPK 01

Plate 15. Amplification pattern in chilli genotypes with RAPD primers RN 07 and OPK 01

OPA 11

Eleven amplicons were produced by OPA 11 (300 bp to 2000 bp) and five of them were polymorphic. The bands were clear, distinct and reproducible (Plate 16b). The polymorphism percentage calculated was 71.42.

Three loci of size 600 bp, 700 bp and 800 bp were present in Byadagi Kaddi and Byadagi Dabbi. One loci of size 2000 bp was present in variety Ujwala and Anugraha and one loci of 300 bp was found only in Byadagi Kaddi.

S 07

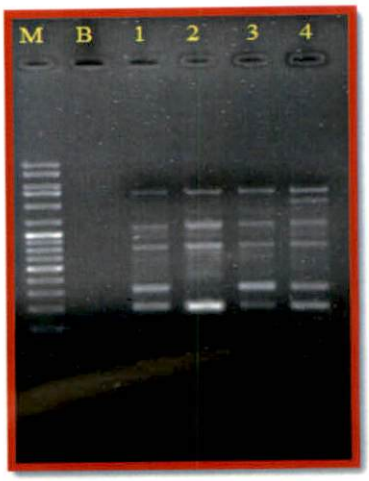
The primer S 07 was able to generate nine amplicons ranged in size 200 bp to 1500 bp, five of them were polymorphic. The bands were clear, distinct and reproducible (Plate 17a). The polymorphism percentage was 55.55.

Three loci of size 1000 bp, 1185 bp and 1500 was found only in variety Ujwala and absent in other three genotypes where as loci of size 1092 bp was present in all the genotypes except variety Ujwala and one loci of size 500 bp was preent in Byadagi Kaddi and Ujwala.

OPE 18

OPE 18 produced eleven clear, distinct and reproducible amplicons ranged from 400 bp to 2250 bp (Plate 17b). The polymorphism per centage was less (38.46) as it could detect only four polymorphic amplicons out of eleven amplicons.

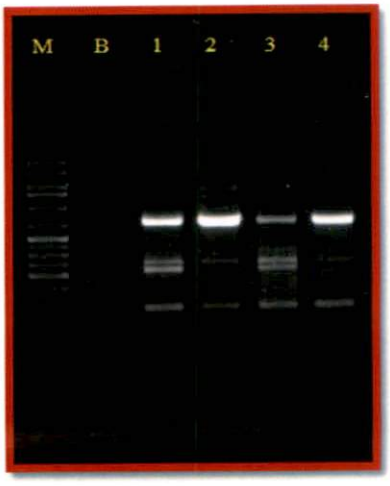
Two loci of size 1185 bp and 2250 bp were present in variety Ujwala and Anugraha. One loci of size 600 bp was found in Byadagi cultivars and one loci of size 700 bp was present in all the genotypes except Byadagi Dabbi.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer OPU 02

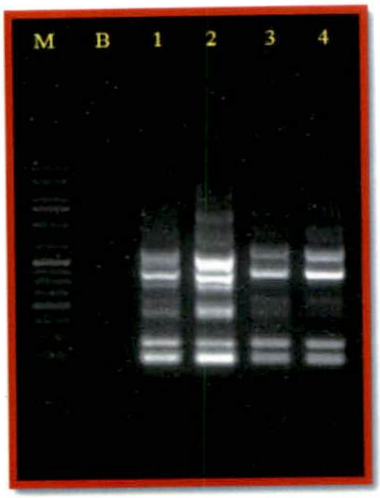


M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer OPA 11

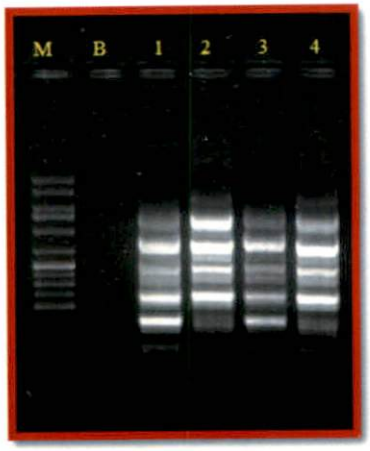
Plate 16. Amplification pattern in chilli genotypes with RAPD primers OPU 02 and OPA 11



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer S 07



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer OPE 18

Plate 17. Amplification pattern in chilli genotypes with RAPD primers S 07 and OPE 18

OPB 04

A total of ten amplicons ranged in size 200 bp to 3000 bp were produced by the primer OPB 04. They were clear, distinct and reproducible. It could generate eight polymorphic bands out of ten amplicons (Plate 18a.) and the percentage polymorphism was 80.

Two loci of size 400 bp and 3000 bp could present in Byadagi Kaddi and Anugraha where as one loci of size 2500 bp was present in Ujwala and Byadagi Dabbi. One loci of size 200 bp was present in all the genotypes except Byadagi Dabbi where as one loci of size 1500 bp and two loci of size 1000 bp and 1092 bp were present only in Byadagi Dabbi and variety Ujwala respectively and one loci of size 900 bp was found only in Byadagi Kaddi.

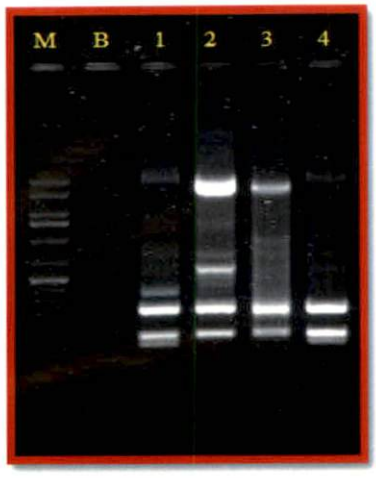
OPP 17

The primer OPP 17 could generate a total of eight clear, distinct and reproducible bands (300 bp to 1500 bp) out of which only six were polymorphic. The percentage polymorphism was 75. The amplification profile is given in Plate 18b.

One loci of size 700 bp was present in variety Ujwala and Anugraha. Three loci of size 300 bp, 500 bp and 1500 bp were present only in variety Anugraha where as a loci of size 1185 bp was present in all the genotypes except variety Anugraha and one loci of size 400 bp was present in all the genotypesd except Ujwala.

4.5.1.1 Amplicons specific to chilli genotypes in RAPD assay

Amplicons specific to Byadagi Kaddi and Byadagi Dabbi in RAPD assay are presented in Table 10. Eight amplicons resolved with five primers in RAPD marker system were found specific to Byadagi Kaddi and Byadagi Dabbi. High molecular weight amplicons were found specific when resolved with S12 (1000 bp) and S13 (2500 bp).



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer OPB 04

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer OPP 17

Plate 18. Amplification pattern in chilli genotypes with RAPD primers OPB 04 and OPP 17

4.5.1.2 RAPD data analysis

Reproducible, well resolved fragments were scored using Quantity one software (Biorad) and each scorable band was scored for presence (1) or absence (0). RAPD analysis using fourteen selected primers produced a total of one fifty five markers in the four chilli genotypes (Table 9). The number of scorable markers produced by each primer ranged from eight (OPP 17) to seventeen (S 12) with an average of 11.07 markers per primer. The molecular weight of these markers ranged 100 bp to 3000 bp. The polymorphic bands were 64.51 per cent of the total, each primer detecting on an average 7.14 polymorphic bands.

The numerical series for the presence or absence of bands was entered into a binary data matrix and used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993). The dendrogram generated using NTSYS is given in Figure 1.

Dendrogram separated the four chilli genotypes into two major clusters with a Jaccard's similarity coefficient of 0.51 to 0.66. The overall variability observed among the four genotypes studied was 49 per cent. The first cluster included the two Byadagi cultivars which were sixty six per cent similar and were closely related to each other. The second cluster included the varieties Ujwala and Anugraha which were also showed similarity of 64 per cent. Highest similarity of 66 per cent was observed between Byadagi Kaddi and Byadagi Dabbi.

When KAU varieties were compared with Byadagi cultivars they exhibited on an average 51 per cent similarity. Of the two Byadagi cultivars, Byadagi Dabbi and Ujwala showed only 47 per cent similarity.

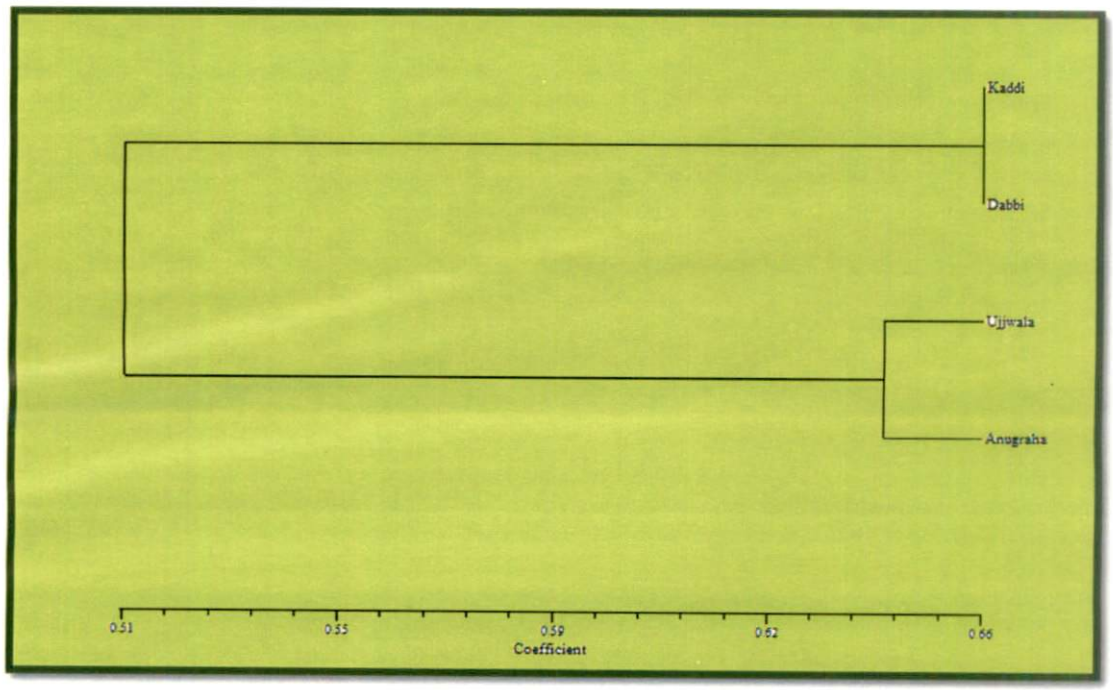


Fig. 1 Dendrogram generated with RAPD profile in chilli genotypes

Jacard's similarity coefficient with RAPD data of chilli genotypes is presented in Table 11. The pairwise similarity coefficient values varied between 0.47 and 0.65 indicating variability of 35-53 per cent in the chilli genotypes studied.

Table 9: Amplification pattern in chilli genotypes with RAPD primers

Sl. No.	Primer	Total no. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)	Size of amplicons (range-bp)	PIC value	Resolving power
1	S 12	17	14	3	82.35	100-2500	0.41	16.5
2	S13	11	9	2	81.81	400-2500	0.47	14.0
3	OPA 10	10	7	3	70.00	300-1815	0.44	11.5
4	OPA 28	12	10	2	83.33	200-1815	0.41	12.5
5	OPAH 06	13	7	6	53.84	200-2500	0.41	18.5
6	OPC 08	14	7	7	50.00	300-2250	0.42	21.0
7	RN 07	11	7	4	63.63	300-2000	0.42	16.0
8	OPK 01	11	5	6	45.45	300-1657	0.42	16.5
9	OPU 02	11	6	5	54.54	300-1815	0.37	15.0
10	OPA11	7	5	2	71.42	300-2000	0.47	8.5
11	S 07	9	5	4	55.55	200-1500	0.40	12.0
12	OPE 18	11	4	7	36.36	400-2250	0.46	18.5
13	OPB 04	10	8	2	80.00	200-3000	0.42	10.5
14	OPP 17	8	6	2	75.00	300-1500	0.39	9.5
Total		155	100	55			5.91	200.5
Average		11.07	7.14	3.92	65.23		0.42	14.32

Table 10. Details of amplicons specific to different chilli genotypes in RAPD assay

Primer name	B. Kaddi		B. Dabbi		Ujwala		Anugraha		B. Kaddi & Dabbi		Ujwala & Anugraha	
	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)
S 12	2	450, 1092	-	-	5	500, 700, 950, 1500, 2500	-	-	2	650, 1000	2	400, 2000
S 13	1	450	-	-	-	-	-	-	2	500, 2500	3	400, 900, 1185,
OPA 10	-	-	-	-	3	500, 1000, 1815	-	-	2	800, 900	1	600,
OPA 28	1	400	-	-	1	1500	1	1815	-	-	2	800, 1000
OPAH 06	-	-	2	400, 500	1	2500	-	-	1	200	-	-
OPU 02	1	700	-	-	2	500, 800	1	1500	-	-	-	-
OPC 08	-	-	2	1092, 2500	-	-	-	-	-	-	3	1185, 1815, 2000
OPA11	1	300	-	-	-	-	-	-	-	-	1	2000
RN 07	-	-	-	-	1	1000	-	-	-	-	1	400
OPK 01	-	-	-	-	-	-	2	300, 500	-	-	1	700
S07	-	-	-	-	3	1000, 1185, 1500	-	-	-	-	-	-
OPE18	-	-	-	-	-	-	-	-	1	600	2	1185, 2000
OPB04	1	900	1	1500	2	1185, 1342	-	-	-	-	-	-
OPP17	-	-	-	--	-	-	3	300, 500, 1500	-	-	1	700
Total amplicons	7		5		13		7		8		17	
Mean weight of amplicons		613.1		1198.4		1690.5		916.42		893.7		1133.5

Table 11. Jaccard's similarity coefficient matrix with RAPD profile in chilli genotypes

	Byadagi Kaddi	Ujwala	Byadagi Dabbi	Anugraha
Byadagi Kaddi	1.0000			
Ujwala	0.5035	1.0000		
Byadagi Dabbi	0.6577	0.4783	1.0000	
Anugraha	0.5354	0.6412	0.5328	1.0000

4.5.2 Molecular characterization in different chilli genotypes using ISSR primers

Amplification of DNA from individual genotypes was carried out using already reported chilli specific primers for ISSR marker system. The details of amplification with the 11 primers are provided in Table 12. Observations were as follows:

2UBC S2

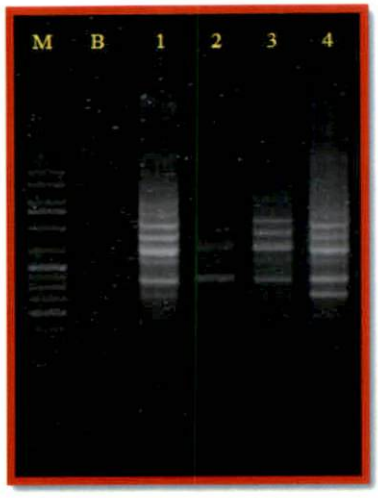
A total of ten amplicons ranged in size 500 bp to 2500 bp were produced by the primer 2UBC S2. They were clear, distinct and reproducible. It could generate seven polymorphic bands out of ten amplicons (Plate 19a) and the percentage polymorphism was 70.

Four loci of size 500 bp, 700 bp, 1185 bp and 1815 bp were present in Byadagi Kaddi and Anugraha, one loci of size 1000 bp was present in all the genotypes except variety Ujwala and one loci of size 2000 bp was present in cultivars Byadagi Kaddi and Byadagi Dabbi. One loci of size 2500 was present only in Byadagi Kaddi.

C-Renteria 6

The primer C-Renteria 6 could generate a total of seven clear, distinct and reproducible bands (400 bp to 1185 bp) out of which five were polymorphic. The percentage polymorphism was 71.42. The amplification profile is given in Plate 19b.

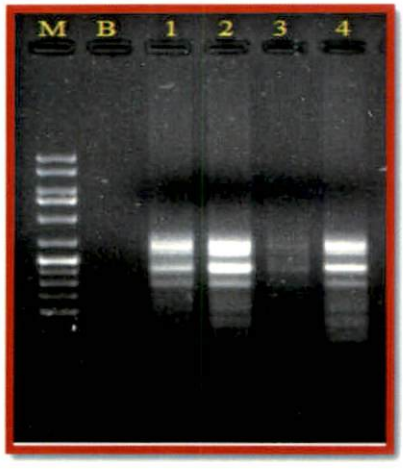
Two amplicons of size 400 bp and 1185 bp were present in all the genotypes except Byadagi Dabbi. Three loci of size 500 bp, 600bp and 1000 bp were present in both varieties viz. Ujwala and Anugraha.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer 2 UBC S2



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer C-Renteria 6

Plate 19. Amplification pattern in chilli genotypes with ISSR primers 2 UBC S2 and C- Renteria 6

ISSR 05

ISSR 05 generated eight clear, distinct and reproducible amplicons ranged in size 300 bp to 1000 bp and five of them were polymorphic (Plate 20a). The per cent polymorphism was 62.5.

Two amplicons of size 500 bp and 550 bp were present only in variety Ujwala and absent in rest of the genotypes. One loci of size 400 bp was present in cultivar Byadagi Kaddi and Byadagi Dabbi and one loci of size 900 bp was present in variety Ujwala and Anugraha. One loci of size 1000 bp was present in Byadagi Dabbi and Anugraha.

ISSR 07

Six clear, distinct and reproducible amplicons were produced by this primer (Plate 20b) ranged in size 400 bp to 1500 bp. Out of which only two were polymorphic and the polymorphism percentage calculated was 33.33.

Two loci of size 400 bp and 1500 bp was present only in all the genotypes except Byadagi Dabbi

ISSR 08

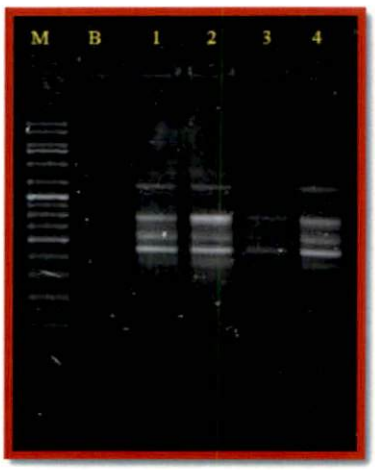
Amplification of four chilli genotypes with the selected primer ISSR 08 produced ten clear, distinct and reproducible amplicons ranged in size 400 bp to 1500 bp (Plate 21a) eight amplicons were polymorphic and the percentage polymorphism calculated was 80.

Three loci of size 400 bp, 600 bp and 700 bp were present only in Byadagi Kaddi and absent in rest of the genotypes, where as amplicon of size 1185 bp was present in all the genotypes except Byadagi Kaddi. Two amplicons of size 900 bp and 1500 bp were present in Ujwala and Anugraha. One loci of size 950 bp was present in

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer ISSR 05



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer ISSR 07

Plate 20. Amplification pattern in chilli genotypes with ISSR primers ISSR 05 and ISSR 07

Byadagi Kaddi and Anugraha and one loci of size 500 bp was present in both the Byadagi cultivars.

ISSR 15

The primer ISSR 15 was able to generate seven clear, distinct and reproducible amplicons (Plate 21b). The amplicons were ranged in size 500 bp to 1185 bp. three of them were polymorphic giving 42.85 per cent polymorphism.

Two amplicons of size 600 bp and 700 bp were present in Byadagi Kaddi and Byadagi Dabbi and one loci of size 900 bp was present in all the genotypes except Byadagi Dabbi.

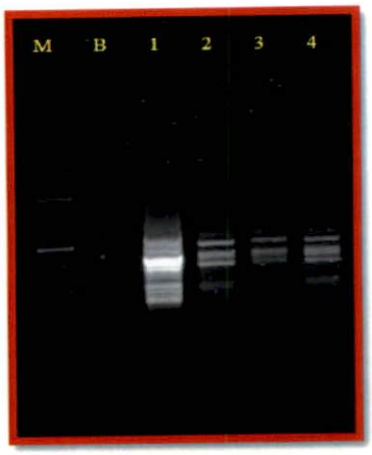
UBC 840

UBC 840 generated ten clear, distinct and reproducible amplicons ranged in size 200 bp to 1000 bp and seven of them were polymorphic (Plate 22a). The per cent polymorphism was 70.

Three loci of size 300 bp, 500 bp and 800 bp were present in Byadagi Kaddi and Byadagi Dabbi. One loci of size 750 bp was present in variety Ujwala and Anugraha. Two loci of size 200 bp and 900 bp were present only in Byadagi Kaddi and Ujwala respectively.one loci of size 550 bp was present in all the genotypes except Byadagi Dabbi.

UBC 835

Fourteen clear, distinct and reproducible amplicons were produced by the primer UBC 835 (Plate 22b) ranged in size 300 bp to 3000 bp. It could detect ten polymorphic amplicons out of fourteen. The polymorphism percentage calculated was 71.42.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

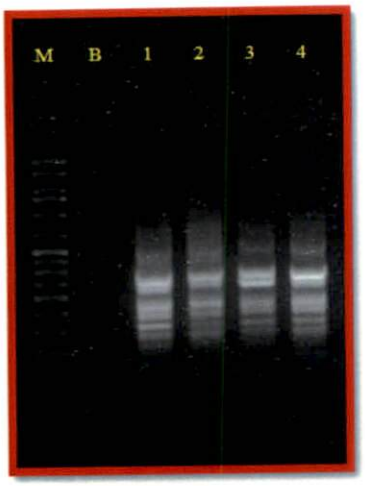
a. Primer ISSR 08

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer ISSR 15

Plate 21. Amplification pattern in chilli genotypes with ISSR primers ISSR 08 and ISSR 15



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer UBC 840

M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer UBC 835

Plate 22. Amplification pattern in chilli genotypes with ISSR primers UBC 840 and UBC 835

Three amplicons of size 300 bp, 400 bp and 550 bp were present only in Byadagi Kaddi and absent in rest of the genotypes. Five loci of size 500 bp, 700 bp, 800 bp, 1000 bp and 1815 bp were present in all the genotypes except Byadagi dabbi. Two loci of size 900 bp and 3000 bp were present in variety Ujwala and Anugraha.

HB 10

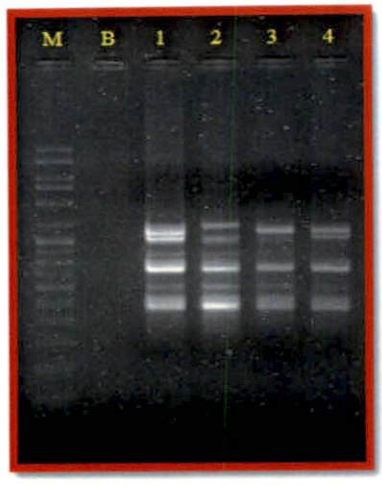
HB 10 could generate a total of eight clear, distinct and reproducible amplicons (500 bp to 1185 bp), of which three were polymorphic (Plate 23a). The polymorphism percentage recorded was 37.5.

One loci of size 1092 bp was present in all the genotypes except variety Ujwala where as one loci of size 1185 bp was present only in variety Ujwala and absent in other three genotypes and one loci of size 900 bp was present in Byadagi Kaddi and Anugraha.

17899A

17899 A produced fifteen clear, distinct and reproducible amplicons (300 bp to 1815 bp) and eleven amplicons were found polymorphic giving 73.33 per cent polymorphism. The profile showing the amplification pattern of 17899 A is presented in Plate 23b.

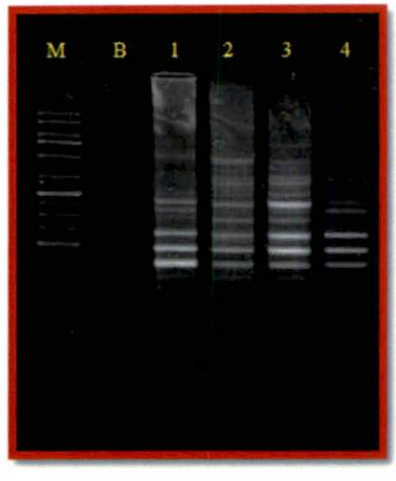
Four amplicons of size 800 bp, 1185 bp and two amplicons were present in range of 1185-1500 bp present in Ujwala and Byadagi Dabbi. Two loci of size 400 bp and 1815 were present in Byadagi Kaddi and Byadagi Dabbi. Two loci of size 850 bp and 1657 bp were present in all the genotypes except Ujwala and Byadagi Kaddi respectively, also two loci of size 300 bp and 1000 bp were present in all the genotypes except variety Anugraha. One loci of size 1500 was present in all the genotypes except Byadagi Kaddi.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

a. Primer HB 10



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

b. Primer 17899A

Plate 23. Amplification pattern in chilli genotypes with ISSR primers HB 10 and 17899A

UBC 823

The primer UBC 823 generated a total of eight clear, distinct and reproducible loci (Plate 24) out of which five were polymorphic and the percentage of polymorphism was 62.5 and amplicons ranged from 500 to 2000 bp.

Two loci of size 700 bp and 800 bp were present only in Byadagi Kaddi and absent in rest of the genotypes. One loci of size 1000 bp was present in all the genotypes except Byadagi Dabbi and two loci of size 1500 bp and 2000 bp were present in Byadagi Kaddi and Byadagi Dabbi.

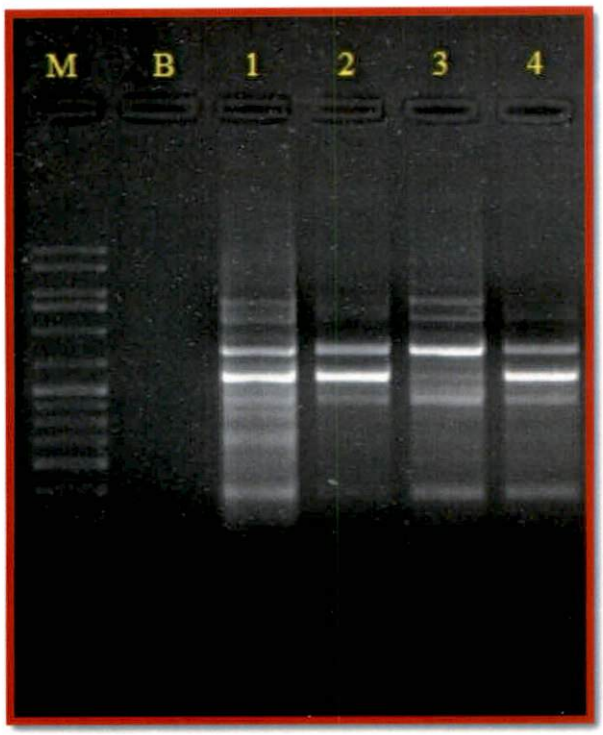
4.5.2.1 Amplicons specific to chilli genotypes in ISSR assay

Amplicons specific to Byadagi Kaddi and Byadagi Dabbi in ISSR assay are presented in Table 13. Twelve amplicons resolved with seven primers in ISSR marker system were found specific to Byadagi Kaddi and Byadagi Dabbi. High molecular weight amplicons were found specific when resolved with primer 2 UBC S2 (1000 bp), UBC 823 (1500 bp and 2000 bp), 17899A (1815 bp).

4.5.2.2 ISSR Data analysis

Reproducible, well resolved fragments were scored using Quantity one software (BIORAD) and each scorable band was scored and assigned the series of one for presence and zero for absence.

ISSR analysis using eleven selected primers produced a total of 103 markers in four chilli genotypes (Table 12). The number of scorable markers produced by each primer ranged from six (ISSR 07) to fifteen (17899A) with an average of 9.3 markers per primer. The molecular weight of the markers ranged from 200 bp to 3000 bp. The overall mean of polymorphic bands was 61.35 per cent, each primer detecting on an average six polymorphic bands.



M: 100 bp (3 kb ladder), B: Blank

1: Byadagi Kaddi, 2: Ujwala, 3: Byadagi Dabbi, 4: Anugraha

Plate 24. Amplification pattern in chilli genotypes with ISSR primers UBC 823

The presence or absence of bands was entered into a binary data matrix and used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993). The dendrogram generated using NTSYS is given in Figure 2.

Dendrogram generated using NTSYS separated the four chilli genotypes into two major clusters with a Jaccard's similarity coefficient of 0.47 to 0.72 showing variability of 28 to 53 per cent among genotypes studied. The first cluster included Byadagi Kaddi and Byadgi Dabbi. The cultivars Byadagi Kaddi and Byadgi Dabbi are closely related to each other with a similarity of 57 per cent. The second cluster included variety Ujwala and Anugraha and they were very closely related to each other showing 72 per cent similarity.

A genetic similarity matrix of different groups of chilli genotypes based on the proportion of shared ISSR fragments was also generated (Table 14). The pairwise similarity coefficient values varied between 0.47 and 0.72 indicating 47 per cent variability in chilli genotypes.

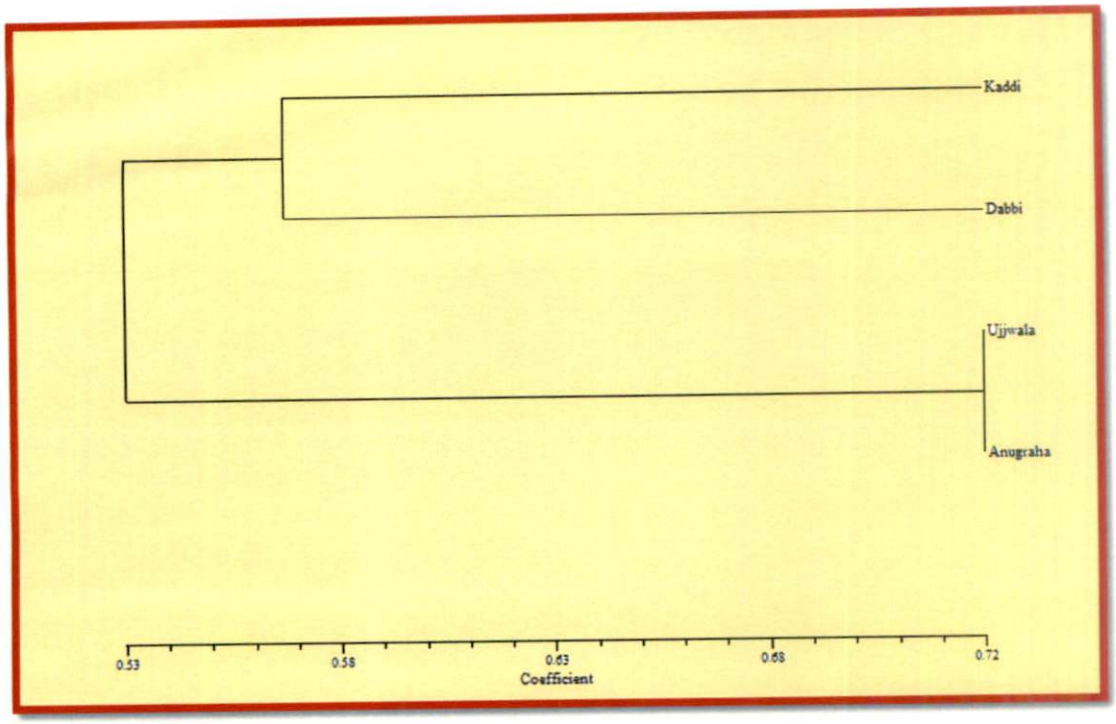


Fig. 2 Dendrogram generated with ISSR profile in chilli genotypes

Table 12: Amplification pattern in chilli genotypes with ISSR primers

Primer name	Total no. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)	Size of amplicons (range-bp)	PIC value	Resolving power
2 UBC S2	10	7	3	70.00	500-2500	0.46	13.0
C Renteria 6	7	5	2	71.42	400-1185	0.45	10.0
ISSR 05	8	5	3	62.50	300-1000	0.45	10.0
ISSR 07	6	2	4	33.33	400-1500	0.37	11.0
ISSR 08	10	8	2	80.00	400-1500	0.43	11.0
ISSR 15	7	3	4	42.85	500-1185	0.45	11.5
UBC 840	10	7	3	70.00	200-1000	0.44	12.5
UBC 835	14	10	4	71.42	300-3000	0.40	19.0
HB 10	8	3	5	37.50	500-1185	0.41	13.0
17899A	15	11	4	73.33	300-1815	0.44	20.5
UBC 823	8	5	3	62.50	500-2000	0.42	10.5
Total	103	66	37			4.72	142.0
Mean	9.36	6.0	3.36	61.35		0.42	12.90

Table 13. Details of amplicons specific to different chilli genotypes in ISSR assay

Primer name	B. Kaddi		B. Dabbi		Ujwala		Anugraha		B. Kaddi & Dabbi		Ujwala & Anugraha	
	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)	No. of bands	Mol wt (bp)
2 UBC S2	1	2500	-	-	-	-	-	-	1	1000	-	-
C Renteria 6	-	-	-	-	-	-	-	-	-	-	3	500, 600, 1000
ISSR 05	-	-	-	-	2	500, 600	-	-	1	400	1	900
ISSR 07	-	-	-	-	-	-	-	-	-	-	-	-
ISSR 08	3	400, 600, 700	-	-	-	-	-	-	1	500	-	-
ISSR 15	-	-	-	-	-	-	-	-	2	600, 700	-	-
UBC 840	-	-	-	-	1	900	-	-	3	300, 500, 800	1	750
UBC 835	3	300, 400, 550	-	-	-	-	-	-	-	-	2	900, 3000
HB 10	-	-	-	-	1	1185	-	-	-	-	-	-
17899A	1	1500	-	-	-	-	-	-	2	400, 1815	-	-
UBC 823	2	700, 800	-	-	-	-	-	-	2	1500, 2000	-	-
Total amplicons	10		0		4		0		12		7	
Mean weight of amplicons		845		-		398.1		-		876.2		1092.8

Table 14. Jaccard's similarity coefficient matrix with ISSR profile in chilli genotypes

	Byadagi Kaddi	Ujwala	Byadagi Dabbi	Anugraha
Byadagi Kaddi	1.0000			
Ujwala	0.4902	1.0000		
Byadagi Dabbi	0.5652	0.5349	1.0000	
Anugraha	0.6129	0.7250	0.4773	1.0000

4.5.3 Variability analysis in different chilli genotypes using combined RAPD and ISSR data

Amplification of four chilli genotypes produced a total of 258 markers by the RAPD and ISSR assay with an average of 10.32 markers per each primer. The total polymorphic bands were 64.34 per cent, each primer detecting on an average 6.64 polymorphic bands per primer.

The RAPD and ISSR binary data matrix were combined; the NTSYS pc version 2.02i was used for UPGMA analysis.

Based on the proximity matrix obtained from Jaccard's coefficients, Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering was done using Unweighted Pair Group Method with Arithmetic averages (UPGMA) method. The dendrogram generated using NTSYS is given in Figure 3.

Dendrogram separated the four chilli genotypes into two major clusters with a Jaccard's similarity coefficient of 0.52 to 0.67 indicating an overall variability of 48 per cent among chilli genotypes. The first cluster included the cultivars Byadagi Kaddi and Byadagi Dabbi. The combined dendrogram revealed that the cultivar Byadagi Kaddi and Byadagi Dabbi was most closely related to each other indicating 62 per cent similarity. The second cluster included the varieties Ujwala and Anugraha. Highest similarity of 67 per cent was observed between the varieties Ujwala and Anugraha.

A genetic similarity matrix of different groups of chilli genotypes based on RAPD and ISSR profiles were also generated (Table 15). The pair wise coefficient values varied between 0.49 and 0.67.

Table 15. Jaccard's similarity coefficient matrix with RAPD and ISSR profile in chilli genotypes

	Byadagi Kaddi	Ujwala	Byadagi Dabbi	Anugraha
Byadagi Kaddi	1.0000			
Ujwala	0.4979	1.0000		
Byadagi Dabbi	0.6158	0.5000	1.0000	
Anugraha	0.5682	0.6730	0.5095	1.0000

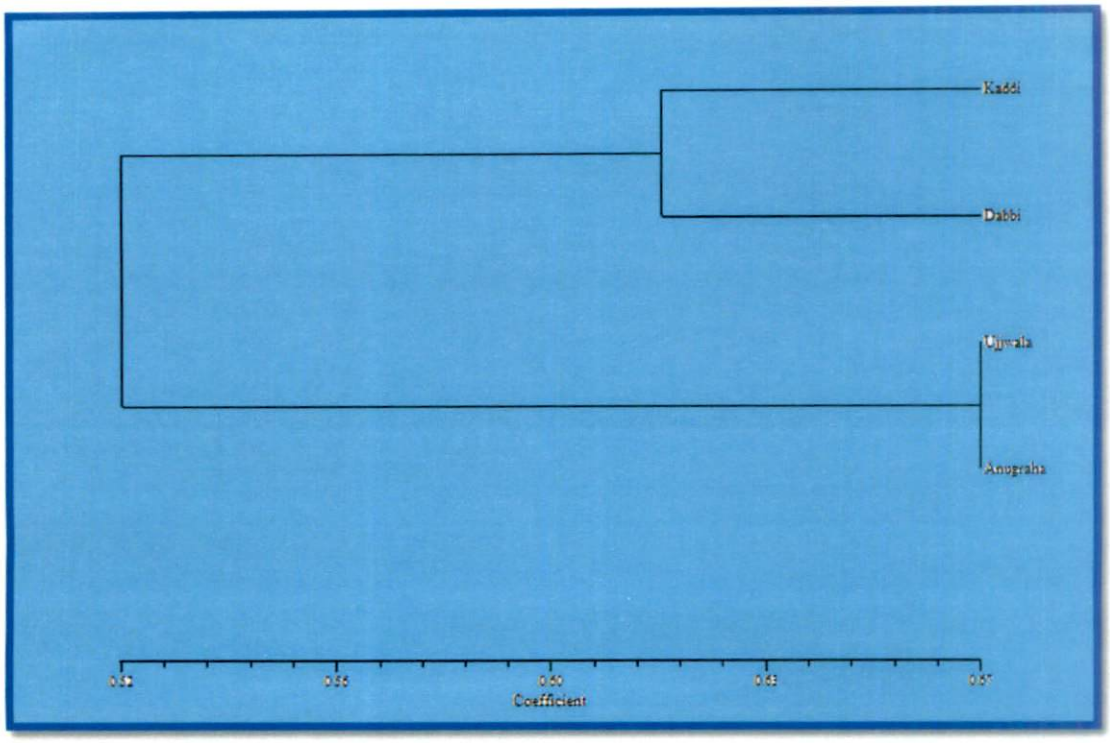


Fig. 3 Dendrogram generated with RAPD and ISSR profiles in chilli genotypes

4.6 Resolving power of RAPD and ISSR primers

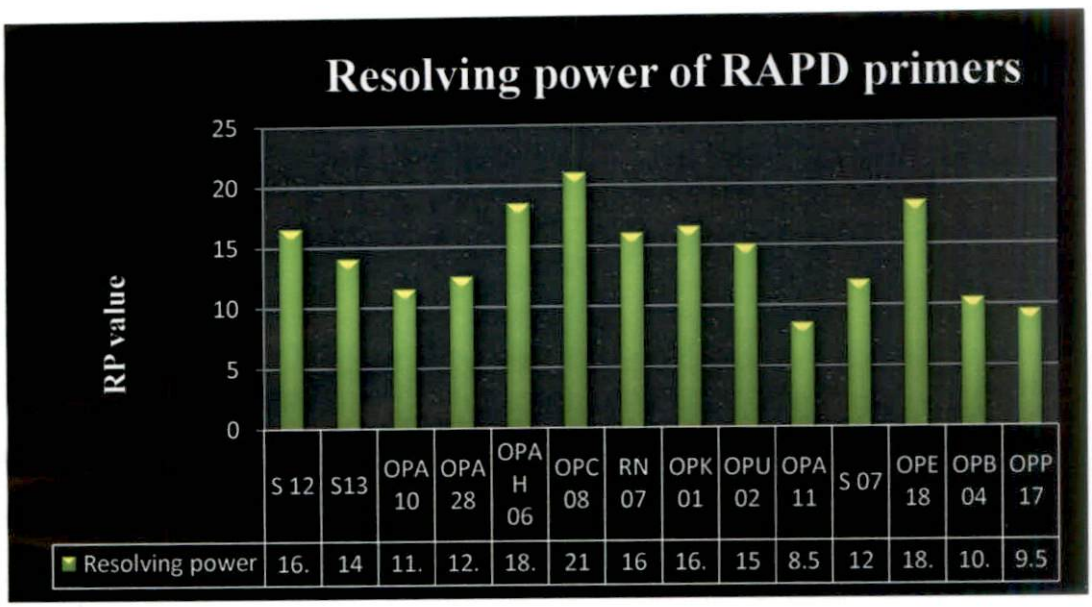
The Resolving power (Rp) was calculated for the fourteen RAPD primers and data are presented in Figure 4a and Table 9. It ranged from 8.5 (OPA 11) to 21 (OPC 08) with an average of 14.3. The highest resolving power was recorded by primer OPC 08 followed by OPE 18, OPAH 06 and S12, OPK 01. Primer OPA 11 recorded the lowest resolving power.

The resolving power was also calculated for the eleven ISSR primers and data are presented in Figure 4b and Table 12. Resolving power ranged from 10 (C-Renteria6 and ISSR 05) to 20.5 (17899A) with an average of 12.9. The highest resolving power was recorded by primer 17899A followed by UBC 835. Primer C-Renteria6 and ISSR 05 recorded lowest resolving power.

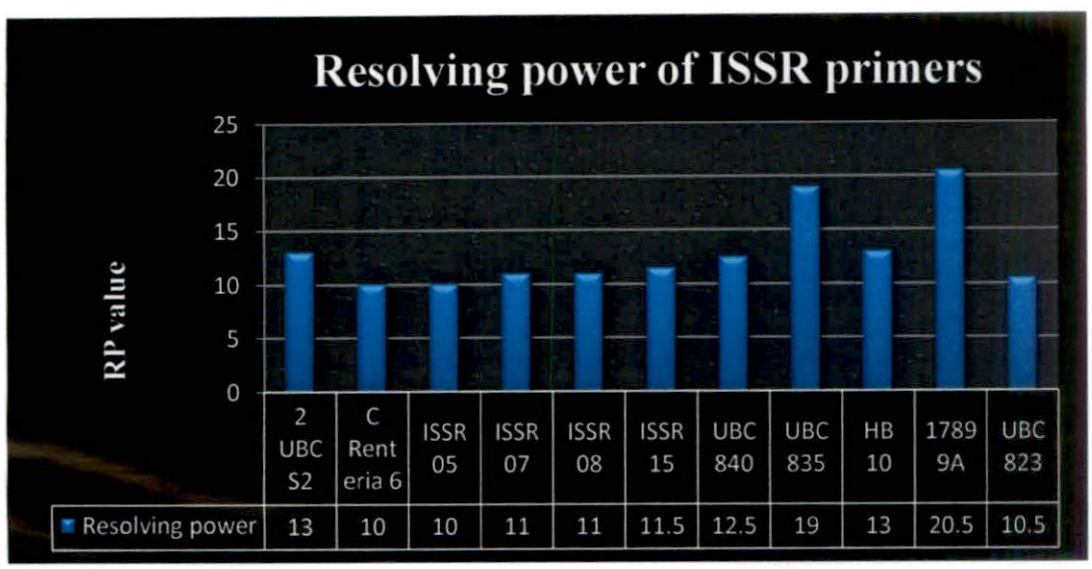
4.6.1 Polymorphic Information Content (PIC) of selected RAPD and ISSR primers

The polymorphic information content (PIC) value calculated for the 14 selected RAPD primers are depicted in Figure 5a and Table 9 and it varied from 0.37 (OPU 02) to 0.47 (S 13 and OPA 11) with an average of 0.42. The highest Polymorphic Information Content was recorded by primer S 13 and OPA 11 followed by OPE 18. Primer OPU 02 recorded the lowest Polymorphic Information Content value (0.37).

The polymorphic information content (PIC) value calculated for the 11 selected ISSR primers are depicted in Figure 5b and Table 12 and varied from 0.37 (ISSR 07) to 0.46 (2UBC S2) with an average of 0.42. The highest Polymorphic Information Content recorded by primer 2UBC S2 followed by C-Renteria 6, ISSR 05 and ISSR 15. ISSR 07 recorded the lowest Polymorphic Information Content value (0.37).

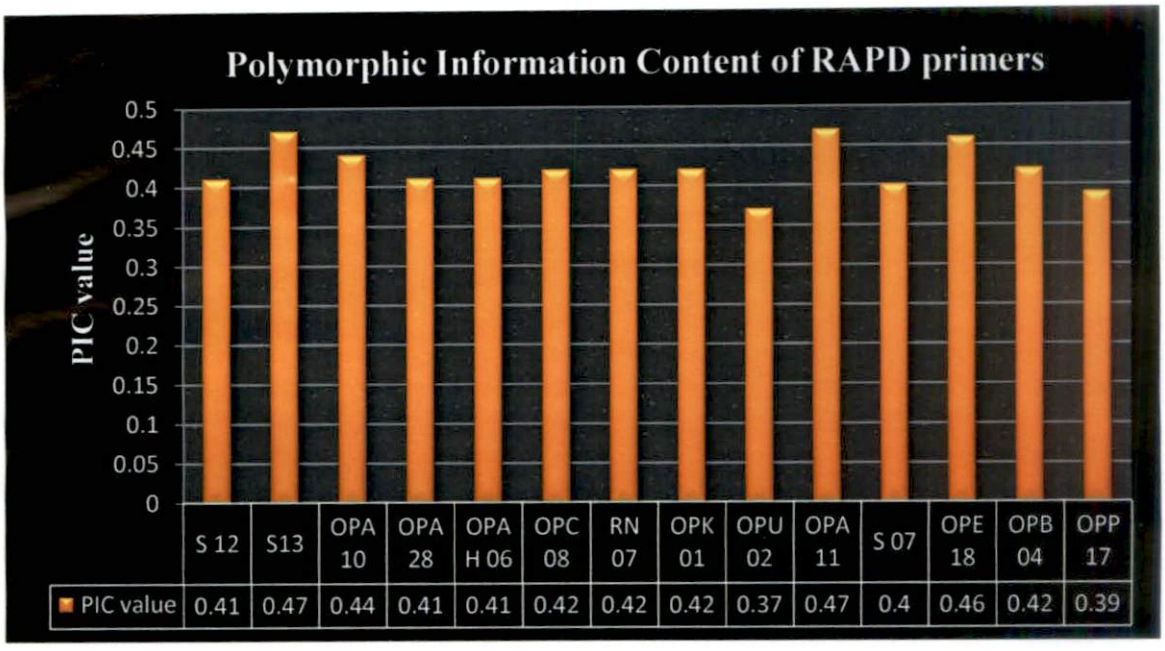


a. RAPD primers

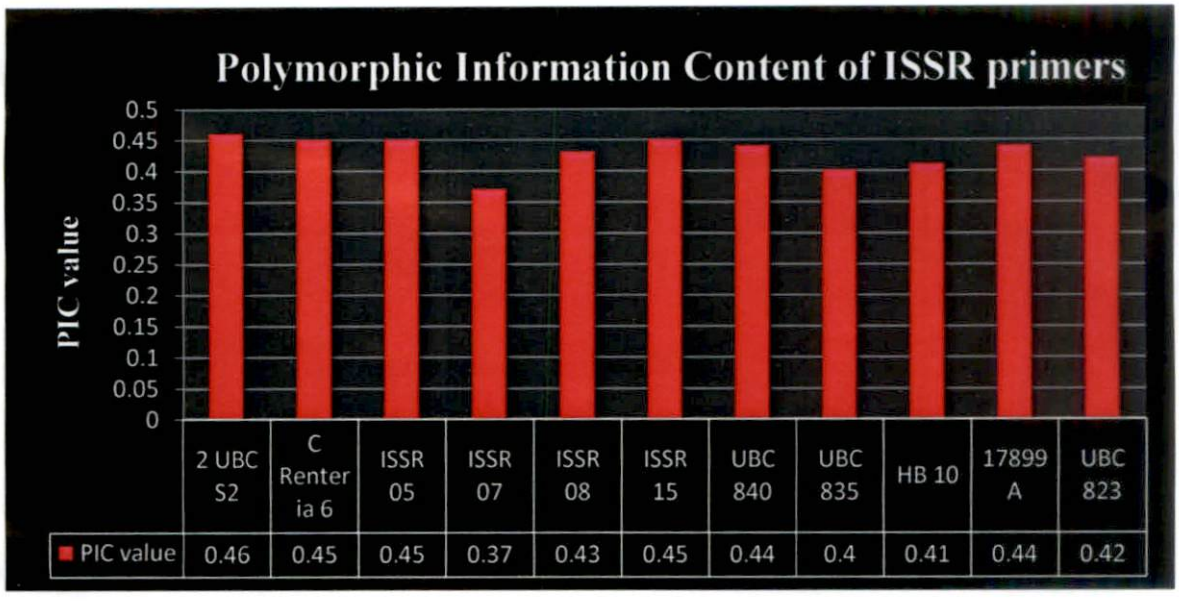


b. ISSR primers

Fig. 4 Resolving power (Rp) of selected RAPD and ISSR primers



a. RAPD primers



b. ISSR primers

Fig. 5 Polymorphic Information Content (PIC) with selected RAPD and ISSR primers

DISCUSSION

5. DISCUSSION

The genus *Capsicum* includes the most valuable and economically important spice crop, chilli, the 'Wonder Spice'. It is grown throughout the world. It is also called as hot pepper, cayenne pepper. Tropical America is the primary centre of origin. Chilli is said to be the first ever domesticated crop in America. The three species *C. annuum*, *C. frutescens* and *C. chinense* evolved from a common ancestor located in the North of the Amazon basin (NW-Brazil, Columbia). Further, *C. annuum* and *C. frutescens* were brought to Central America, where they were finally domesticated in Mexico and Panama, respectively, whereas *C. chinense* moved to the West and was first put to cultivation in Peru. Two other species were first cultivated in Western South America, *C. baccatum* in the Peruvian lowlands and *C. pubescens* at higher elevations, in the Andes (Peru, Bolivia, Ecuador) (Manju and Sreelathakumary, 2002). Hot chilli is a traditional crop grown in the homesteads of Kerala for domestic and export purposes.

Natural variability available in the crop is very high. Selection and habituation has resulted in evolving large numbers of cultivars and over 400 such genotypes are available globally with the farmers for cultivation. Efforts have been made to characterize these cultivars and varieties through molecular tools (Bhadragouder and Patil, 2011; Patel *et al.*, 2011; Thul *et al.*, 2012); based on which similarity/divergence among many of the genotypes have been worked out.

In chilli, efforts have been made to develop varieties with high yield and quality through selection as well as hybridization. Kerala Agricultural University has released six chilli varieties, namely Anugraha, Ujwala, Jwalamukhi, Jwalasakhi, Vellayani Athulya and Vellayani Samrudhi and among these Ujwala is highly pungent.

Byadagi is a famous chilli type grown in Karnataka and is known for its deep red colour and negligible or zero pungency. Byadagi chilli is mainly exported as oleoresin which serves as a substitute to paprika oleoresin, widely used in food industries, confectionaries, cosmetics, beverages, pharmaceuticals and even as a dye in textile industries. The demand for Byadagi chilli has increased enormously as a source of natural red colour in food industry. Spices Board has initiated steps to protect Byadagi chilli under GI registration. Even though germplasm collection and evaluation studies were reported by Sandeep (2007) in Byadagi chilli, reports on characterization using molecular markers are scanty.

Capsicum spp. has a high level of polymorphism indicating extensive genetic variation in the Indian germplasm. It is also reported that there is increased divergence among landraces, compared to the advanced cultivars, though a few are of hybrid origin. Morphologically divergent intra specific variants occur both in wild forms and in cultivated varieties.

Many of the reports on molecular characterization include a combination of two or more molecular markers for overcoming the drawback of individual systems and thus to generate wholesome information about the material under study (Paran *et al.*, 1997; Papavo *et al.*, 1998). In the present investigations, a combination of two molecular marker systems *viz.*, RAPD and ISSR analyses were used to characterize chilli genotypes at molecular level especially for colour and pungency.

5.1 Morphological characterization

Morphological characterization of the two Byadagi cultivars and two released varieties of chilli was done in the present investigations. Twenty one qualitative and 16 quantitative characters were recorded and morphological characters were found to vary in different genotypes studied. The intra and interspecific variability in chilli are tremendous and many characters exhibit parallel variation across the species

(Pickersgill, 1971) which often makes morphology based classification difficult. Devi *et al.* (2005) studied morphological variability in thirty three chilli accessions using Principal Component Analysis and identified twenty eight minimum morphological characters for quick characterization of chilli germplasm. Similar studies on morphological characterization were under taken by Sudre *et al.* (2010).

5.1.1 Vegetative Characters

Vegetative characters such as dark green leaves were present in cultivars Byadagi Kaddi and Byadagi Dabbi, where as variety Ujwala and Anugraha had green colour leaves. Variety Ujwala had dwarf plant stature, less canopy width, large leaf size and highest leaf area compared to other genotypes and Anugraha had small leaves, more plant height and canopy width.

Significant variation was observed in plant canopy width and leaf area. Farhad *et al.* (2010) Datta and Das (2013) also found that all the cultivars and varieties of chilli differed significantly in plant height, plant growth habit, branching habit, leaf size, leaf shape, leaf margin and leaf colour.

5.1.2 Flowering and fruit characters

Significant variation was observed in the flowering and fruit characters such as fruit length, fruit width and placental length of chilli genotypes.

Flowering and fruit characters such as days to 50 per cent flowering, number of flowers/axil, days to 50 per cent fruiting, mature and ripe fruit colour, fruit length, fruit width, fruit position, adherence of calyx to fruit, fruit pedicel length, fruit shape at pedicel attachment, blossom end fruit shape, fruit surface and placenta length had variation among the four chilli genotypes. White colour corolla and pale blue colour anther, dentate calyx margin and elongate fruit shape are same in all the four chilli genotypes studied.

Experiment conducted by Farhad *et al.* (2010) showed that a wide range of variation was observed among the chilli genotypes for several morphological, fruit and quality characters. Among the different characters, all genotypes showed white corolla colour followed by single flower per axil, pigmentation at node and green fruit colour. Predominance of single descriptor state was found in more than 50 per cent genotypes for 15 characters. Variability studies conducted by Datta and Das (2013) revealed that there was a wide range of variability for all the characters studied in chilli genotypes. High heritability along with higher genetic advance was found in capsaicin content in fruit, number of fruits per plant, yield per plant and primary branches per plant. These characters may be considered as reliable selection indices as they are possibly governed by additive gene effect.

5.1.3 Yield and quality parameters in chilli genotypes

Significant variation was also observed in the yield and quality parameters such as number of fruits/plant, fruit yield/plant, fruit weight, number of seeds/fruit, 100 seed weight in chilli genotypes and ASTA colour value.

Farhad *et al.* (2010); Sudre *et al.* (2010); Datta and Das (2013) and Khan *et al.* (2012) also found that all the chilli cultivars and varieties differed significantly in fruit length, fruit weight, number of fruits per plant, seeds per fruit, 100 seed weight etc.

Capsaicin analysis showed that cultivars Byadagi Kaddi and Byadagi Dabbi had lower pungency with 0.045 per cent and high colour value of 108.92 ASTA, where as varities Ujwala and Anugraha showed high pungency (0.32 per cent capsaicin) and colour value of 59.1 ASTA. Similar studies conducted by Tilahun *et. al.*, (2013) in chilli also found that Arka Abhir variety showed the lowest capsaicin content and pungency value. The distribution of the pungent principles within the fruit is uneven, but it is generally agreed that the dissepiments contain a substantial

proportion compared to their bulk. The stage of fruit maturity at harvest, the season and environment of growth and the post- harvest handling can all influence the pungency of the dried product somewhat the greatest determinants of the pungency level are the species and cultivars grown. Deb *et al.* (1963) found that the capsaicin content was found to differ by a factor of three among twelve cultivars of Pusa chillies grown at the same location. Difference in capsaicin content in capsicum cultivars was also reported by Balbaa *et al.* (1968), Karawaya *et al.* (1969), Golifer (1973) and Sharma and Motingar (1975).

5.2 Molecular characterization

5.2.1 Isolation, Purification and Quantification of DNA

The samples used for DNA isolation were young leaves from four chilli genotypes. The method used for isolation of DNA is CTAB method suggested by Roger and Bendich (1994) was used to isolate the DNA, which yielded good quality of DNA. The electrophoresed DNA showed distinct bands without shearing.

Grinding in liquid nitrogen was found to improve the quality of DNA isolated. The addition of antioxidant like β-mercaptoethanol during isolation of DNA was found effective. This prevents the phenolic oxidation.

The detergent used in the extraction buffer of CTAB (Cetyl Trimethyl Ammonium Bromide), helps in the disruption of the cell membrane thereby releasing nucleic acid into the extraction buffer and prevents co-precipitation of polysaccharides with nucleic acid by acting as a selective precipitant of nucleic acids. CTAB is a cationic detergent, which solubilises membranes and forms a complex with DNA (Sghaier and Mohammed, 2005).

The advantageous effect of the CTAB along with PVP on the quality of DNA was also reported by Gallego and Martinez (1996) and Sreenath *et al.*, (1992). It

effectively disrupts the cell membrane and together with NaCl, separates the polysaccharides. The EDTA in the extraction buffer protects the DNA from endonuclease by chelating the Mg^{2+} ions of DNA. Double treatment with chloroform: isoamylalcohol mixture and centrifugation effectively removes the pigments and proteins. The addition of chilled isopropanol precipitates the DNA and washing the pellet with 70 percent alcohol followed by absolute alcohol removes the traces of CTAB. TE buffer rehydrates the DNA and dissolves it (Wettasinghe and Peffley, 1998; Babu, 2000).

A DNA sample was reported high in quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasinghe and Peffley, 1998). RNase treatment was given in order to remove RNA contamination from the isolated DNA samples. Use of RNase was reported by several workers (Raval *et al.*, 1998), Wettasinghe and Peffley (1998) and Gallego and Martinez (1996). In the present investigation, the RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicated that the DNA below the well was of good quality.

The absorbance ratio was calculated as OD at 260/280, for the various samples, using spectrophotometer. Those samples with ratio between 1.8 and 2.0 were considered to be of high quality DNA. If the value goes beyond 2.0, it indicates RNA contamination and if less than 1.8, it indicated protein contamination. All molecular analyses was done with high quality DNA.

5.2.2 Molecular Marker Analysis

Molecular marker technology provides novel tools for characterization studies. Most of the molecular markers are developed by the PCR (Polymerase Chain Reaction) technology and amplifies unique regions on the genomic DNA based on

the primers designed for DNA amplification. Two such PCR based marker systems (RAPD and ISSR) were utilized for characterization of chilli genotypes.

5.2.2.1 RAPD analysis

The RAPD technique is a simple technique (Parani *et al.*, 1997) and was developed by Williams *et al.* (1990). The technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary decamer primers. In RAPD markers, polymorphism results from the changes in the sequence of the primer binding site. RAPD markers are dominant in nature (Waugh and Powell, 1992) because polymorphisms are detected as the presence or absence of bands. Technical problems associated with application of RAPD technique in the field of genetic variation research have been reported by many workers (Lynch and Milligan, 1994; Rajput *et al.*, 2006). Use of high quality DNA is shown to be a key factor in obtaining reproducible RAPDs bands (Penner *et al.*, 1993). In the present study the use of high quality DNA helped in getting reproducible bands using the standardized conditions for the thermal cycler.

However, the advantages of RAPD include simplicity, rapidity, requirement for only a small quantity of DNA, and ability to generate numerous polymorphisms (Cheng *et al.*, 2007). The RAPD amplification can be classified into two types: constant (monomorphic) and vriable (polymorphic) between the genotypes. These differences can be used to examine and establish systematic relationship (Hadrys *et al.*, 1992).

Nine decamer primers reported for chilli by Manibala (2013) viz. S12, S13, OPA10, OPA28, OPAH06, OPC08, RN07, OPK01, OPU02 and four chilli specific primers OPA11 (Baral and Bosland 2002), S07 (Ilbi, 2003), OPE18 (Troconis-Toress *et al.*, 2012), OPB04 (Uddin *et al.*, 2012 and Bahurupe *et al.*, 2013) with good resolving power were used for amplification of DNA. Different sizes of

amplicons were produced by different primers and the results are presented in Table 10. Five primers gave eight bands which were unique to Byadagi cultivars viz. Kaddi and Dabbi are S12 (650, bp 1000 bp), S13 (500 bp and 2500 bp), OPA10 (800 bp and 900 bp), OPAH 06 (200 bp), OPE18 (600 bp). At least few of these loci may be representing for high colour value and low pungency in Byadagi cultivars as these amplicons were present only in Byadagi Kaddi and Byadagi Dabbi and absent in variety Ujwala and Anugraha.

Total thirteen amplicons were found only in Ujwala from eight RAPD primers at different molecular weight are S12 (500 bp, 700 bp, 950 bp, 1500 bp and 2500 bp), OPA 10 (500 bp, 1000 bp and 1815 bp), OPA 28 at 1500 bp, OPAH 06 at 2500 bp, OPU 02 (500 bp and 800 bp), RN 07 at 1000 bp, S07 (1000 bp, 1185 bp and 1500 bp) and OPB 04 at 1185 bp and 1342 bp. These amplicons may responsible for the higher pungency in variety Ujwala as these thirteen bands were present only in variety Ujwala and were absent in rest of the genotypes.

Seven bands were found present only in Byadagi Kaddi those are S12 (450 bp and 1092 bp), S13 (450 bp), OPA 28 (400 bp), OPU 02 (700 bp), OPA 11 (300 bp) and OPB 04 (900 bp). Five bands from primer OPAH 06 (400 bp and 500 bp), OPC 08 (1092 bp and 2500 bp), OPB 04 (1500 bp) were found only in Byadagi Dabbi. Variety Anugraha alone had seven bands in different primers of RAPD viz. OPA 28 (1815 bp), OPU 02 (1500 bp) OPK 01 (300 bp and 500 bp) and OPP 17 (300 bp, 500bp, 1500 bp).

Variety Ujwala and Anugraha had seventeen unique bands from ten primers viz. S12 (400 bp and 2000 bp), S13 (400 bp, 900 bp and 1185 bp), OPA 10 at 600 bp, OPA 28 (800 bp and 1000 bp), OPC 08 (1185 bp, 1815 bp and 2000 bp), OPA 11, RN 07, OPK 01, OPP 17 at 2000 bp, 400 bp, 700 bp and 700 bp respectively and OPE 18 at 1185 bp and 2000 bp.

Eight amplicons resolved with five primers in RAPD marker system were found specific to Byadagi Kaddi and Byadagi Dabbi. High molecular weight amplicons (1000 bp, 2500 bp) were found specific when resolved with S12 and S13. Bands can be selected based on intensity and molecular weight to get more sequence information of specific trait. These high molecular weight specific bands identified in the study can be further cloned and sequenced to know the functional annotation using bioinformatics tools like BLAST, multiple sequence alignment and expasy.

The suitability of the primers selected for analyses were evaluated by calculating the resolving power (Rp) and Polymorphic Information Content (PIC). The Rp values ranged from 8.5 to 21 for the RAPD primers. The Rp values reported for variability analysis in various crops is in conformity to the values observed in the present study (Prevost and Wilkinson, 1999; Keshavachandran *et al.*, 2005 and Sarla *et al.*, 2005).

The PIC values ranged between 0.37 and 0.47 for RAPD primers. The PIC values indicated the extent of polymorphism detected by the primer among the varieties studied. The PIC values reported in various other crops for the primers used for charcterisation is in conformity to the values observed in the present study (Karihaloo *et al.*, 2003; Kelley *et al.*, 2004; Dongre and Kharbikar, 2004 and Hollman *et al.*, 2005), thus confirming the suitability of the primers selected for characterization of chilli genotypes in the present study.

5.2.2.2 ISSR analysis

The choice of a molecular marker technique depends on its reproducibility and simplicity. The best marker for genome mapping, marker assisted selection, phylogenetic studies, and crop conservation should have low cost and labour requirements and high reliability. Since 1994, a molecular marker technique called Inter Simple Sequence Repeats (ISSR) has been available and is being exploited

(Zietkiewicz *et al.*, 1994). ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Like RAPD, ISSR markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers.

ISSR is a multilocus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation and has been successfully applied in genetic and evolutionary studies of many species, including chilli. The ISSR marker requires small amounts of DNA and does not require information on DNA sequences. ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.*, 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Oshborn *et al.*, 2005).

Primers with good resolving power were used for amplification of DNA. Seven ISSR primers reported by Manibala (2013) viz. UBC840, 2UBCS2, ISSR05, ISSR07, ISSR08, ISSR15, C- Renteria 6 and four chilli specific primers UBC823 (Patel *et al.*, 2011), UBC835 (Thul *et al.*, 2011), HB10 (Ahmed, 2013), 17899A (Refaat and Elgarhy, 2007) were used for ISSR assay. Different sizes of amplicons were produced by different primers and the results are presented in Table 13. Twelve bands present specific to cultivar Byadagi Kaddi and Byadagi Dabbi are 2UBC S2 (1000 bp), ISSR 05, ISSR 08 (400 bp and 500 bp respectively), ISSR 15 (600 bp and 700 bp), UBC840 (300 bp, 500 bp and 800 bp), 17899A (400 bp and 1815 bp) and UBC 823 (1500 bp and 2000 bp). These bands or few of these bands may be representing for the high colour value and low pungency in Byadagi cultivars.

Variety Ujwala and Anugraha had presence of seven bands in primer C-Renteria6 (500 bp, 600 bp and 1000 bp), ISSR 05 (900 bp), UBC 840 at 750 bp and UBC 835 at 900 bp and 3000 bp.

In Byadagi Kaddi there were ten bands from different primers viz. 2UBC S2 (2500 bp), ISSR 08 (400 bp, 600 bp and 700 bp), UBC 835 (300 bp, 400 bp and 550 bp), 17899A (1500 bp) and UBC 823 (700 bp and 800 bp) where as four bands ISSR 05 (500 bp and 600 bp), UBC 840 (900 bp) and HB10 (1185 bp) were found only in variety Ujwala.

Twelve amplicons resolved with seven primers in ISSR marker system were found specific to Byadagi Kaddi and Byadagi Dabbi. High molecular weight amplicons (1000 bp, 1500 bp, 1815 bp and 2000 bp) were found specific when resolved with primer 2 UBC S2, UBC 823, 17899A.

The Rp values ranged from 10 to 20.5 for ISSR primers. The Rp values reported for variability analysis in various crops is in conformity to the values observed in the present study. The PIC values ranged between 0.37 and 0.46 for ISSR primers. The PIC values indicate the extent of polymorphism detected by the primer among the genotypes studied.

5.3 Genetic variability analysis

The amplification pattern observed in RAPD and ISSR analyses was scored and analysed for relatedness/ variability among the cultivars and varieties. The computer package NTSYS-PC (Rohlf, 2005) was used for cluster analysis.

Similarity coefficient in RAPD assay ranged from 0.51 to 0.66 with a highest similarity between variety Ujwala and Anugraha and variability ranged from 34 - 49 per cent between chilli genotypes in case of vegetative, flowering and fruit, yield and quality parameters. Similarity coefficient in ISSR assay was from 0.53 to 0.72 with a maximum similarity of 72 per cent between two released varieties Ujwala and Anugraha. Variability percentage observed in ISSR analyses ranged between 28 and 47. Combined RAPD and ISSR analyses data showed the similarity coefficient of 0.52 to 0.67 with a variability of 33 to 48 per cent. Similar reports were made by

Baral and Bosland (2002) in chilli; they reported a similarity coefficient of 0.59 to 0.80 where they studied in chilli genotypes. In the analysis of genetic relatedness in *Capsicum* spp. Thul *et al.* (2012) observed a similarity range from 0.53 to 0.96 per cent in *C. annuum, C. baccatum, C. chinense, C. eximium, C. frutescens,* and *C. luteum.*

The present investigations done to characterize Byadagi chilli using morphological and molecular markers revealed that Byadagi chili could be distinguished both at the morphological and molecular levels.

The most distinguishing character of Byadagi chilli is the dark red ripened fruits with wrinkled surface and higher fruit weight. Quality analysis of dried fruit at Quality Evaluation Laboratory, Spices Board, Kochi showed the high colour value and low pungency of Byadagi cultivars.

At molecular level also, Byadagi cultivars could be distinguished with the specific amplicons. Eight amplicons resolved with five primers (S12, S13, OPA10, OPAH 06 and OPE18) in RAPD marker systems were found specific to Byadagi chilli. Similarly in ISSR marker system, twelve amplicons resolved with seven primers (2UBC S2, ISSR 05, ISSR 08, ISSR 15, UBC840, 17899A and UBC 823) were found specific to Byadagi chilli.

The specific traits of Byadagi chilli is the deep red colour and negligible, or zero pungency. The chilli has got the highest colour value of ASTA 159.9 with negligible or zero pungency while other chilli types had ASTA colour value of 32.1-70.4 (www.indianspices.com). Spices Board has taken steps to protect the famous Byadagi chilli of Karnataka through GI registration.

The most striking feature of the present investigations is that the specific traits of Byadagi chilli like high colour and low pungency are expressed in a similar fashion under Kerala conditions also. Of the two cultivars, Byadagi Dabbi recorded

higher colour value of ASTA 120.4 which is near to the reported value of 159.9. However, the incidence of leaf curl was found very high for the cultivars under Kerala conditions. Evaluation of Byadagi cultivars for different seasons will give further information on the expression of specific traits under Kerala conditions.

In depth investigations of the high molecular weight specific amplicons (1000 bp, 2500 bp in RAPD with primers S12 and S13 respectively, and 1000 bp, 1500 bp, 1815 bp and 2000 bp in ISSR with primers 2UBC S2, UBC 823 and 17899 A) will give further insight in to the genes involved in colour of Byadagi chilli. Similarly investigations on the Ujwala specific amplicons may be helpful for isolation of genes involved in pungency of chilli.

Testing of Byadagi specific amplicons identified in the present study in other capsicum species like *Capsicum frutesence* and *Capsicum chinense* will pave way for isolation of genes involved in colour and pungency of chilli. Such information on the specific amplicons/ genes involved in the colour and pungency could be further utilized in Marker Assisted Selection, metabolite pathway engineering and secondary metabolite production.

Recently whole genome sequencing of hot pepper was completed by the combined effort of a group of 28 scientists from various institutes (Kim *et al.*, 2014). The genome size of hot pepper is approximately four times larger than tomato and consists of 34,903 genes. Hope the genome sequence published by the group will serve as an important genomic resource for studying the specific traits of Byadagi chilli.

SUMMARY

6. SUMMARY

The study entitled "Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)" was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture during the period from July 2013 - June 2014.

The research works included mainly the morphological and molecular characterization of Byadagi chilli along with two KAU released varieties Ujwala and Anugraha. Morphological observations were recorded for 21 qualitative and 16 quantitative characters as per the minimal descriptor of Agri- Horticultural crops of NBPGR (Srivastava *et al.*, 2001). Two molecular marker systems viz., RAPD and ISSR were used to characterize chilli genotypes.

The salient findings of the present study are as follows:-

1. Morphological characters could distinguish Byadagi chilli cultivars from KAU released varieties.

2. Byadagi cultivars possessed dark green medium sized leaves, dark red ripened fruits with wrinkled surface, higher fruit weight (6.27 g), less number of fruits per plant (17.21), more number of seeds per fruit (97.49) and lesser fruit yield (100.12 g). KAU varieties viz. Ujwala and Anugraha recorded more number of fruits per plant (88.08) with less fruit weight (1.79 g) and higher fruit yield (152.4 g) with red coloured ripened fruits with smooth surface.

3. The dried fruits were analysed for colour and pungency at Spices Board quality evaluation laboratory, Kochi. Byadagi cultivars recorded higher colour value (108.92 ASTA) and lower pungency (0.045 per cent capsaicin) while KAU varieties viz. Ujwala and Anugraha possessed lower colour value (59.1 ASTA) and higher pungency (0.32 per cent capsaicin).

4. The protocol suggested by Rogers and Bendich (1994) was found good for isolation of DNA from young and immature leaves of chilli genotypes. The RNA contamination was completely removed through RNase treatment.

5. The quality and quantity of DNA were analyzed by NanoDrop® ND-1000 spectrophotometer. The absorbance ratio ranged from 1.5-2.1, which indicated good quality DNA. The recovery of DNA was high and was suitable for RAPD and ISSR analyses.

6. Fourteen chilli specific RAPD primers (Manibala, 2013; Baral and Bosland 2002; Ilbi, 2003; Troconis-Toress *et.al.*, 2012 and Uddin *et.al.*, 2012 and Bahurupe *et.al.*,2013) and 11 ISSR (Manibala, 2013; Patel *et.al.*, 2011; Thul *et.al.*, 2011; Ahmed, 2013) primers were subjected to RAPD and ISSR analyses.

7. The Resolving Power (Rp) of the RAPD and ISSR primers was calculated and the values ranged between 8.5 and 21 for RAPD primers and 10 to 20.5 for ISSR primers. The RAPD primer OPAH06, OPE18 and ISSR primer 17899A showed high resolving power.

8. The Polymorphic Information Content (PIC) calculated ranged between 0.37 to 0.47 for RAPD primers and 0.37 to 0.46 for ISSR primers indicating the suitability of primers to detect polymorphism. RAPD primers S13 and OPA11 and ISSR primers 2UBCS2 reported highest PIC values.

9. The RAPD marker system could generate a total of 155 amplicons with fourteen primers exhibiting an average polymorphism of 65.23 per cent and 7.14 polymorphic amplicons/ primer.

10. Eight amplicons resolved with five primers (S12, S13, OPA06, OPA10 and OPE18) in RAPD marker system were found specific to Byadagi Kaddi and Byadagi Dabbi.

11. The ISSR marker system could generate a total of 103 amplicons with eleven primers exhibiting an average polymorphism of 61.35 per cent with six polymorphic bands per primer.

12. Twelve amplicons resolved with seven primers (2UBCS2, ISSR05, ISSR08, ISSR15, UBC823, UBC840 and 17899A) in ISSR marker system were found specific to Byadagi Kaddi and Byadagi Dabbi.

13. The dendrogram generated with RAPD, ISSR and combined profiles grouped Byadagi cultivars and KAU varieties in separate clusters. The varieties Ujwala and Anugraha were found closer with 67 per cent similarity and Byadagi Kaddi and Byadagi Dabbi with 61 per cent similarity.

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7. REFERENCES

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ANNEXURE

ANNEXURE I

Details of laboratory equipment used for the study

1)	High speed refrigerated centrifuge	: Kubota 6550, Japan
2)	Horizontal electrophoresis system	: BIO-RAD, USA
3)	Thermal Cycler	: Applied Biosystem, Viriti
4)	NanoDrop$^{R.}$ ND-1000 spectrophotometer USA	: NanoDrop$^{R.}$ Technologies Inc.
5)	Gel documentation BIORAD, USA	: Gel Documentation System,
6)	Water purification system	: Milipore, Germany
7)	Ice flaking machine	: F100 Compact, Ice matics
8)	Laminar Air Flow	: HML- 104, Thermadyne

ANNEXURE II

Minimal descriptor of chilli (Srivastava *et al.*, 2001)

Chilli *(Capsicum annuum* L.)

1. Life cycle

1 Annual

2 Biennial

3 Perennial

2. Stem colour

To be recorded at full foliage stage

1 Green

2 Green with purple stripes

3 Purple

99 Others (Specify in the 'Remarks' descriptor)

3. Plant height (cm)

To be recorded as average of 5-10 random plants when the first fruit in 50% of the plants began to ripe

Quantitative

4. Plant canopy width (cm)

To be recorded simultaneously with height

Quantitative

5. Plant growth habit

To be recorded at fruit maturity

3 Prostrate

5 Intermediate

7 Erect

99 Others (Specify in the 'Remarks' descriptor)

6. Branching habit

To be recorded when plants have ceased its growth

3 Sparse

5 Intermediate

7 Dense

99 Others (Specify in the 'Remarks' descriptor)

7. Leaf size

To be recorded at full foliage stage

3 Small

5 Medium

7 Large

99 Others (Specify in the 'Remarks' descriptor)

8. Leaf shape

To be recorded at full foliage stage

1 Deltoid

2 Ovate

3 Lanceolate

99 Others (Specify in the 'Remarks' descriptor)

9. Leaf margin

To be recorded at full foliage stage

1 Entire

2 Undulate

3 Ciliate

99 Others (Specify in the 'Remarks' descriptor)

10. Leaf colour

To be recorded at full foliage stage

1 Green

2 Dark green

3 Purple

99 Others (Specify in the 'Remarks' descriptor)

11. Leaf pubescence

To be observed on the youngest mature leaf

0 Absent

3 Sparse

5 Intermediate

7 Dense

99 Others (Specify in the 'Remarks' descriptor)

12. Pigmentation at node

To be observed on the youngest mature leaf

0 Absent

1 Present

13. Days to 50% flowering

To be recorded as number of days from date of transplanting to date when at least 50% plants show first flower open

Quantitative

14. Number of flowers per axil

To be observed as average of 5-10 random axils at flowering stage

1 One

2 Two

3 Three or more

99 Others (Specify in the 'Remarks' descriptor)

15. Corolla colour

To be recorded immediately after blooming

1 White

2 Yellow

3 Purple

99 Others (Specify in the 'Remarks' descriptor)

16. Anther colour

To be observed immediately after blooming but before anthesis

1 White

2 Yellow

3 Pale blue

4 Blue

5 Bluish yellow

6 Purple .

99 Others (Specify in the 'Remarks' descriptor)

17. Male sterility

To be observed immediately after blooming

0 Absent

1 Present

18. Calyx margin

To be recorded on fully blossom stage

1 Entire

2 Intermediate

3 Dentate

99 Others (Specify in the 'Remarks' descriptor)

19. Days to 50% fruiting

To be recorded as number of days from the date of transplanting to the date when at least 50% plants bear fruiting

Quantitative

20. Mature fruit colour

To be recorded at mature fruit stage

1 White

2 Yellow

3 Green

4 Orange

5 Purple

6 Deep purple

7 Black

99 Others (Specify in the 'Remarks' descriptor)

21. Ripe fruit colour

To be recorded on ripe fruits

1 White

2 Lemon yellow

3 Pale orange yellow

4 Orange yellow

5 Pale orange

6 Orange

7 Light red

8 Red

9 Dark red

10 Brown

11 Purple

12 Black

99 Others (Specify in the 'Remarks' descriptor)

22. Fruit shape

To be recorded at mature fruit stage

1 Long

2 Very long

3 Tapering

4 Conical

5 Oval

99 Others (Specify in the 'Remarks' descriptor)

23. Fruit length (cm)

To be recorded as average of 5-10 random fruits

Quantitative

24. Fruit width (cm)

To be recorded as average of same 5-10 fruits

Quantitative

25. Fruit position

To be recorded at mature fruit stage

3 Pendent

5 Semi pendent

7 Erect

99 Others (Specify in the 'Remarks' descriptor)

26. Adherence of calyx to fruit

To be recorded at mature fruit stage

3 Loose

5 Semi hard

7 Hard

99 Others (Specify in the 'Remarks' descriptor)

27. Fruit pedicel length (cm)

To be recorded as average of same 5-10 fruits at marketable stage

Quantitative

28. Fruit shape at pedicel attachment

To be recorded at mature fruit stage

1 Acute

2 Obtuse

3 Truncate

4 Cordate

5 Lobate

99 Others (Specify' in the 'Remarks' descriptor)

29. Blossom-end fruit shape

To be recorded at mature fruit stage

1 Pointed

2 Blunt

3 Shrunken

4 Shrunken and painted

99 Others (Specify in the 'Remarks' descriptor)

30. Fruit surface

To be recorded at mature fruit stage

1 Smooth

2 Semi wrinkled

3 Wrinkled

99 Others (Specify in the 'Remarks' descriptor)

31. Placenta length (cm)

To be recorded as average of same 5-10 fruits at fully matured stage

Quantitative

32. Organoleptic test

To be recorded on fully matured fruits

3 Mild pungent

5 Intermediate

7 Pungent

9 Highly pungent

99 Others (Specify in the 'Remarks' descriptor)

33. Number of fruits per plant

To be recorded as average of same 5-10 plants

Quantitative

34. Fruit yield per plant (kg)

To be recorded as average of cumulative yield of all pickings at mature green fruit stage of same 5-10 plants

Quantitative

35. Fruit weight (g)

To be calculated on the basis of fruit yield and number of fruits per plant

Quantitative

36. Seed colour

To be recorded at dry seed stage

1 Light yellow

2 Deep yellow

3 Brown

4 Black

99 Others (Specify in the 'Remarks' descriptor)

37. Number of seeds per fruit

To be recorded as average number 5-10 'random fruits at ripen stage

Quantitative

38. 1000 seed weight (g)

To be recorded on dry seeds

Quantitative

36. Seed colour

To be recorded at dry seed stage

1 Light yellow

2 Deep yellow

3 Brown

4 Black

99 Others (Specify in the 'Remarks' descriptor)

37. Number of seeds per fruit

To be recorded as average number 5-10 'random fruits at ripen stage

Quantitative

38. 1000 seed weight (g)

To be recorded on dry seeds

Quantitative

ANNEXURE III

Reagents for DNA isolation by CTAB method as per Rogers and Bendich (1994)

Reagents:

A. 2X CTAB Buffer:

- 2% CTAB (w/v)
- 100mM Tris (pH 8)
- 0.5 M EDTA (pH 8)
- 1.4 M Nacl

B. 10% CTAB solution:

- 10% CTAB (w/v)
- 0.7 M NaCl.

C. TE buffer:

- 10mM Tris (pH 8)
- 1mM EDTA (pH 8)

ANNEXURE IV

Composition of buffers and dyes used for agarose gel electrophoresis

1. 50X TAE buffer (pH 8.0)

- 242g Tris base
- 57.1 ml glacial acetic acid
- 100ml 0.5 Mm EDTA (pH 8.0)

2. Tracking / loading dye (6X)

- 0.25% Bromophenol blue
- 0.25% Xylene cyanol
- 30% Glycerol in water

3. Ethidium bromide

- The dye was prepared as a stock solution of 10mg/ml in water and was stored at room temperature in dark bottle.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF BYADAGI CHILLI (*CAPSICUM ANNUUM* L.)

By

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ABSTRACT OF THESIS

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ABSTRACT

Byadagi is a famous chilli type grown in Karnataka and is named after the town "Byadagi" which is located in Haveri district of Karnataka. This chilli is known for its deep red colour and negligible or zero pungency. The demand for Byadagi chilli has increased enormously as a source of natural red colour in food industry. Spices Board has taken steps to protect Byadagi chilli under GI registration.

The investigations on "Morphological and molecular characterization of Byadagi chilli (*Capsicum annuum* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during July 2013 - June 2014. The study was aimed to characterize Byadagi chilli using morphological and molecular markers with special emphasis on colour and pungency.

Two Byadagi chilli cultivars viz. Byadagi Kaddi and Byadagi Dabbi and two KAU released varieties viz. Ujwala and Anugraha were characterized in the study. The variety Ujwala was selected for its high pungency and variety Anugraha for its less colour on drying. Morphological characterization was done as per NBPGR minimal descriptor for chilli. Molecular characterization was done using two different marker systems viz. RAPD and ISSR.

Twenty one qualitative and sixteen quantitative characters were recorded as per minimal descriptor. Byadagi cultivars possessed dark green medium sized leaves, dark red ripened fruits with wrinkled surface, higher fruit weight (6.27 g), less number of fruits per plant (17.21), more number of seeds per fruit (97.49) and lesser fruit yield (100.12 g). KAU varieties viz. Ujwala and Anugraha recorded more number of fruits per plant (88.08) with less fruit weight (1.79 g) and higher fruit yield (152.4 g) with red coloured ripened fruits with smooth surface.

The dried fruits were analysed for colour and pungency at Spices Board quality evaluation laboratory, Kochi. Byadagi cultivars recorded higher colour value (108.92 ASTA) and lower pungency (0.045 per cent capsaicin) while KAU varieties viz. Ujwala and Anugraha possessed lower colour value (59.1 ASTA) and higher pungency (0.32 per cent capsaicin).

The RAPD marker system could generate a total of 155 amplicons with fourteen primers exhibiting an average polymorphism of 65.23 per cent and 7.14 polymorphic amplicons/ primer. The ISSR marker system could generate a total of 103 amplicons with eleven primers exhibiting an average polymorphism of 61.35 per cent with six polymorphic bands per primer. The dendrogram generated with RAPD, ISSR and combined profiles grouped Byadagi cultivars and KAU varieties in separate clusters. The varieties Ujwala and Anugraha were found more closer with 67 per cent similarity and Byadagi Kaddi and Byadagi Dabbi with 61 per cent similarity.

Eight amplicons resolved with six primers (S12, S13, OPA06, OPA10 and OPE18) in RAPD marker system and twelve amplicons resolved with seven primers (2UBCS2, ISSR05, ISSR08, ISSR15, UBC823, UBC840 and 17899A) in ISSR marker system were found specific to Byadagi Kaddi and Byadagi Dabbi.

The morphological and molecular characterization attempted in the present study could distinguish the Byadagi cultivars from the KAU varieties , Ujwala and Anugraha with respect to colour and pungency. The specific traits of Byadagi cultivars like high colour and low pungency are expressed in a similar fashion under Kerala conditions also. In depth investigations on the unique amplicons identified specific to Byadagi cultivars will give further insight into the genes involved in colour and pungency of chilli, which could be utilized further in marker assisted selection, metabolic pathway engineering and secondary metabolite production.