CHARACTERISATION OF PUMPKIN (*Cucurbita moschata* Duch.) VARIETIES THROUGH MORPHOLOGICAL AND MOLECULAR MARKERS

By

AGINA GOPAN

(2017-11-138)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(SEED SCIENCE AND TECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF SEED SCIENCE AND TECHNOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680 656

KERALA, INDIA

2019

DECLARATION

I hereby declare that this thesis entitled **'Characterisation of pumpkin (*Cucurbita moschata* Duch.) varieties through morphological and molecular markers'** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
Date: 13/08/2019

Agina Gopan
(2017-11-138)

CERTIFICATE

Certified that the thesis entitled **'Characterisation of pumpkin (*Cucurbita moschata* Duch.) varieties through morphological and molecular markers'** is a bonafide record of research work done independently by **Ms. Agina Gopan** under my guidance and supervision and that it has not been previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

13/08/2019

Place: Vellanikkara
Date: 13/08/2019

Dr. Rose Mary Francies
(Major Advisor, Advisory Committee)
Professor and Head
Department of Seed Science and Technology
College of Horticulture
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Agina Gopan (2017-11-138)**, a candidate for the degree of **Master of Science in Agriculture** with major field in Seed Science and Technology, agree that this thesis entitled **'Characterisation of pumpkin (*Cucurbita moschata* Duch.) varieties through morphological and molecular markers,'** may be submitted by **Ms. Agina Gopan** in partial fulfillment of the requirement for the degree.

Dr. Rose Mary Francies
(Chairman, Advisory Committee)
Professor and Head
Department of Seed Science and Technology
College of Horticulture, Vellanikkara

Dr. Dijee Basfian
Professor (Plant Breeding and Genetics)
Department Seed Science and Technology
College of Horticulture, Vellanikkara

Dr. Pradeepkumar. T
Professor (Hort.) and Director of Planning
Kerala Agricultural University
Vellanikkara, Thrissur

Dr. Deepu Mathew
Assistant Professor (Hort.)
Department of Plant Biotechnology
College of Horticulture, Vellanikkara

ACKNOWLEDGEMENT

First and foremost I humbly bow my head before the ***Almighty*** *who enabled me to pursue this work in to successful completion*

It is with immense pleasure I wish to express my deep sense of whole hearted gratitude, indebtedness and heartfelt thanks to my major advisor ***Dr. Rose Mary Francies****, Professor and Head, Department of Seed Science and Technology, College of Horticulture, Vellanikkara for her expert guidance, constant inspiration, affectionate advices, unreserved help, abiding patience and above all, the understanding and wholehearted co-operation rendered throughout the course of my study. This work would not have been possible without her unfailing support in the preparation of the manuscript.*

I am very much obliged and grateful to my Advisory committee members ***Dr. Dijee Bastian, Dr. Pradeepkumar. T,*** *and* ***Dr. Deepu Mathew*** *for their meticulous help, unwavering encouragement, forbearance, timely support and critical examination of the manuscript that has helped a lot for the improvement and preparation of the thesis.*

My special thanks to ***Dr. Berin Pathrose, Dr. S. Nirmala Devi, Dr. Minimol, Dr. Sindhu P.V., Dr. Ritheesh, Dr. Modha, and Dr. Gopal*** *for their valuable suggestions, critical comments and blessings showered on me throughout the course of my thesis.*

Everything went well with the presence of ***Dr. Ajinkya (chetta)*** *during my study period. I would like to express my deepest gratitude to him for making my thesis work easy and worth through his valuable suggestion.*

I owe special thanks to Librarian, College of Horticulture, ***Dr. A. T. Francis*** *and all other staff members of the Central Library. I also thankfully remember the services rendered by all staff members of the Student's computer club, Library and Office of College of Horticulture, Vellanikkara. I am thankful to the* ***Kerala Agricultural University*** *for technical and financial assistance for persuasion of my study and research programme.*

I express my sincere thanks to the ***Department of Seed Science and Technology*** *and the non-teaching staffs, especially* ***Jeena chechi, Hitha chechi, Smitha chechi*** *and* ***Uthaman chettan*** *for all the facilities, cooperation and support during the conduct of the research.*

I am deeply indebted to my father ***Mr. K Gopalakrishnan****, my mother* ***Mrs. Asha M****, my grandmother* ***Mrs. Sumathy Rajagopal*** *and my brother* ***Agin Gosh*** *for their unconditional love, affection, sacrifices, moral support, faith and prayers. Words have no power to express my love towards* ***Gokul Gopinath*** *for being in every aspect of my life. I feel happy to thank all my uncles, aunties and cousins for their everlasting love and care.*

It's my fortune to gratefully acknowledge the infinite affection, warm concern, constant encouragement and moral support of classmates ***Gayathri, Athulya, Rosna*** *and also my juniors* ***Jyothish, Riya, Milu and Harsha*** *for their assistance. I am genuinely indebted to my seniors* ***Megha, Nishidha, Aathmaja, Reshma, Bennett, Adersh, Nagendra, Vipul, Priya, Renuja*** *and* ***Lalit*** *for their words of support and guidance during the entire period of my research.*

Words seem inadequate to express my deep sense of gratitude and sincere thanks to my beloved friends, ***Nayana, Anunayana, John, Haritha, Anirudh, Anil, Anusha, Anusree, Deepak, Megha, Archita, Sreedevi, Virabhadra, Minnu, Anitta, Shilpa, Allen, Ashni*** *and* ***Sijo,*** *for their generous help and mental support rendered to me during the research work.*

Last but certain not least, thanks to my lifetime friends and well-wisher ***Raushan, Pinku, Rahul, Janki, Aswani, Lekshmi, Shana, Aditya*** *and* ***Sithin****, for their presence and the good hours spent together.*

A word of apology to those whom I forgot to mention here. Once again I express my sincere gratitude to all those who inspired, encouraged and supported me for the successful completion of my reseach.

Agina Gopan

TABLE OF CONTENTS

Chapter	Title	Page number
1	INTRODUCTION	1-3
2	REVIEW OF LITERATURE	4-22
3	MATERIALS AND METHODS	23-50
4	RESULTS AND DISCUSSION	51-100
5	SUMMARY	101-105
6	REFERENCES	i-xxv
	ABSTRACT	

LIST OF TABLES

Table no.	Title	Page no.
1	Details of six pumpkin varieties and its source	24
2	List of ISSR primers used for screening	44-45
3	List of SSR primers (with their forward and reverse sequences) used for screening	48-49
4a	Quantitative seedling, leaf, tendril, floral and plant characteristics of six pumpkin varieties	57
4b	Quantitative fruit characteristics of six pumpkin varieties	60
4c	Quantitative seed characteristics of six pumpkin varieties	65
5a	Qualitative vegetative traits of six pumpkin varieties	66
5b	Qualitative fruit and seed traits of six pumpkin varieties	66
6	Distribution of six pumpkin varieties based on morphological data	67
7	Distances between cluster centroids based on morphological data	68
8	Cluster mean values for morphological traits of six pumpkin varieties	70-71
9	Eigenvector values for principal components with Eigen value > 1	73-74
10	OD value and quantity of DNA isolated in pumpkin varieties	76
11	Particulars of ISSR primer profiling in pumpkin varieties	78-79
12	Clustering of pumpkin varieties based on ISSR profile	80

Table no.	Title	Page no.
13	Pair wise similarity between pumpkin varieties based on ISSR profile	80
14	Particulars of SSR primer profiling in pumpkin varieties	91
15	Pair wise similarity between pumpkin varieties based on SSR profile	92
16	Clustering of pumpkin varieties based on SSR profile	92
17	Combined fingerprint of variety Ambili using ISSR and SSR primers	98
18	Fingerprint of variety Saras using ISSR primers	98
19	Fingerprint of variety Suvarna using ISSR primers	98
20	Combined fingerprint of variety CO-1 using ISSR and SSR primers	99
21	Fingerprint of variety CO-2 using ISSR primers	99
22	Combined fingerprint of variety Arka Chandan using ISSR and SSR primers	100

LIST OF FIGURES

Figure no.	Title	Between pages
1	Dendrogram showing relationship among six pumpkin varieties based on quantitative and qualitative morphological data	67-68
2	Scree plot of principal component analysis in six pumpkin varieties	74-75
3	Loading plot on first two components of six pumpkin varieties in factor analysis extracted through principal components of correlation matrix	74-75
4a	Dendrogram obtained by UPGMA clustering method across six varieties of pumpkin based on ISSR markers	92-93
4b	Dendrogram obtained by UPGMA clustering method across six varieties of pumpkin based on SSR markers	92-93
5	Colour chart of ISSR primers (a) UBC-866 and (b) UBC-815	82-83
6	Colour chart of ISSR primers (a) UBC-827 and (b) UBC-807	82-83
7	Colour chart of ISSR primers (a) UBC-811 and (b) UBC-847	83-84
8	Colour chart of ISSR primers (a) UBC-868 and (b) UBC-810	83-84
9	Colour chart of ISSR primers (a) Oligo ISSR 5 and (b) UBC-855	84-85
10	Colour chart of ISSR primers (a) UBC-808 and (b) UBC-809	84-85
11	Colour chart of ISSR primers (a) UBC-812 and (b) UBC-834	85-86
12	Colour chart of ISSR primers (a) UBC-835 and (b) UBC-857	85-86

Figure no.	Title	Between pages
13	Colour chart of ISSR primers (a) UBC-836 and (b) UBC-846	86-87
14	Colour chart of ISSR primers (a) UBC-826 and (b) UBC-822	86-87
15	Colour chart of ISSR primers (a) UBC-844, (b) UBC-854 and (c) UBC-818	86-87
16	Colour chart of ISSR primers (a) UBC-816, (b) UBC-825 and (c) UBC-813	87-88
17	Colour chart of ISSR primers (a) UBC-823 and (b) UBC-841	87-88
18	Fingerprint for pumpkin variety Ambili using five ISSR marker profiles	88-89
19	Fingerprint for pumpkin variety Saras using seven ISSR marker profiles	88-89
20	Fingerprint for pumpkin variety Suvarna using three ISSR marker profiles	88-89
21	Fingerprint for pumpkin variety CO-1 using seven ISSR marker profiles	89-90
22	Fingerprint for pumpkin variety CO-2 using five ISSR marker profiles	89-90
23	Fingerprint for pumpkin variety Arka Chandan using 21 ISSR marker profiles	89-90
24	Colour chart of SSR primers (a) CMTm7, (b) CMTm20, (c) CMTm97, (d) CMTm144 and (e) CMTm232	94-95
25	Overall colour chart of six pumpkin varieties using five SSR marker profiles	95-96

LIST OF PLATES

Plate no.	Title	Between pages
1	View of experiment plot at different stages of the crop	24-25
2	Density of pubescence on leaves at 12.5 X magnification	37-38
3	Measuring the dimensions of seed (a) Seed length, (b) Seed width and (c) Seed thickness	37-38
4	Leaf characteristics of six pumpkin varieties	55-56
5	Leaf pubescence density of six pumpkin varieties	55-56
6	Fruit characteristics of six pumpkin varieties	62-63
7	Seeds characteristics of six pumpkin varieties	65-66
8	Amplification profile generated by ISSR primers UBC-866 and UBC-815	82-83
9	Amplification profile generated by ISSR primers UBC-827 and UBC-807	82-83
10	Amplification profile generated by ISSR primers UBC-811and UBC-847	83-84
11	Amplification profile generated by ISSR primer UBC-868 and UBC-810	83-84
12	Amplification profile generated by ISSR primers Oligo ISSR 5, UBC-855, UBC-808 and UBC-809	84-85
13	Amplification profile generated by ISSR primers UBC-812, UBC-834, UBC-835 and UBC-857	85-86
14	Amplification profile generated by ISSR primers UBC-836, UBC-846, UBC-826, UBC-814, UBC-822, UBC-844, UBC-854 and UBC-818	86-87
15	Amplification profile generated by ISSR primers UBC-816, UBC-825, UBC-813, UBC-823and UBC-841	87-88
16	Amplification profile generated by SSR primers in six pumpkin varieties	94-95

LIST OF ABBREVIATIONS

&	:	And
@	:	At the rate
bp	:	Base pair
cm	:	Centimeter
CTAB	:	Cetyl trimethylammonium bromide
°C	:	Degree Celsius
DAS		Days after sowing
DNA	:	Deoxyribonucleic acid
et al.	:	*et alii* (and co-workers)
etc.	:	*et cetera* (and the rest)
EDTA	:	Ethylene Diamine Tetra Acetic Acid
Fig.	:	Figure
g	:	Gram
pH	:	Hydrogen ion concentration
i.e.	:	*Id est* (that is)
KAU	:	Kerala Agricultural University
µl	:	Micro litre
µg		Microgram
µmol		Micromole
mg	:	Milligram
ml	:	Millilitre/s
mm	:	Millimeter
mM	:	Millimolar

min.	:	Minute/s
M	:	Molar
ng	:	Nano gram
nm	:	Nanometer
N	:	Normal solution
OD	:	Optical Density
p	:	Page
pp	:	Pages
/	:	Per
%	:	Per cent
pM	:	Pico molar
PCR	:	Polymerase chain reaction
PIC	:	Polymorphic Information Content
PVP	:	Polyvinylpyrrolidone
rpm	:	Revolution per minute
RNA	:	Ribonucleic acid
sec.	:	Second/s
NaCl	:	Sodium chloride
mm^2	:	Square millimeter
TAE	:	Tris-acetate-EDTA
viz.	:	*Vi delicet* (namely)
v/v	:	Volume/volume
w/v	:	Weight/volume

Introduction

1. INTRODUCTION

Pumpkin (*Cucurbita moschata* Duch.), a crop of Central Mexican origin belonging to the family Cucurbitaceae, is popularly cultivated and valued in Kerala as a vegetable. The word pumpkin originated from the Greek word '*Pepon*' which means 'large melon, something round and large'. Like other cucurbits, pumpkin is an annual crop (2n=2x=40) (Whitaker and Robinson, 1986), has a trailing habit and is highly cross pollinated owing to its monoecious nature. Pollination is entomophilous due to its large showy campanulate yellow coloured corolla. Both immature and mature fruits, along with leaves and seeds of pumpkin have culinary importance. However, in Kerala, it is commonly valued for its immature fruits. The tender, large and often round immature fruits of pumpkin with a thick, smooth to slightly ribbed skin, which is mostly deep yellow to orange in colouration is an integral part of the Kerala cuisine. Besides this, pumpkin is a valuable source of Vitamin A, rich in energy and carbohydrates (Jahan *et al.*, 2012) and its consumption could contribute to improve the nutritional security.

China and India top the pumpkin production statistics with the US, Egypt, Ukraine, Mexico, Cuba, Italy, Iran and Turkey as followers (Ferriol and Pico, 2008). In India, the total area under pumpkin cultivation, its production and productivity amounts to 98,000 hectares, 2093 thousand tonnes and 21.25 MT/ha, respectively, while in Kerala, the pumpkin production during 2018 was a meagre 47.26 thousand tonnes (GOI, 2018).

Despite its popularity in Kerala, very few high yielding varieties are in cultivation in the state. To ensure increased production of this crop, the availability of good quality seed of improved varieties or hybrids has to be ensured. Genetically pure and good quality seeds enable the farmers to exploit the full potential of a variety or hybrid along with yield hikes.

Pumpkin being a cross-pollinated crop, maintenance of varietal purity and cultivar identity is tedious and warrants the production of seeds in large isolated

plots coupled with strict adherence to other seed field requisites so as to avoid contamination. In addition, with the introduction of the Protection of Plant Varieties and Farmers' Rights Act (PPV & FRA) (GOI, 2001), the new crop varieties should necessarily be distinct from other varieties, uniform in their characteristics and genetically stable over the years. Hence, it is necessary to describe varieties of the crop with distinct qualitative and quantitative characters or descriptors that aids in varietal identification.

Morphological characterisation is the first step for describing and classifying varieties. A combination of laboratory and field testing has been advocated to determine the cultivar trueness and genetic purity of the seed sample. Traditionally, grow out test (GOT) for determination of genetic purity of a variety and Distinctness, Uniformity and Stability (DUS) testing for granting protection to varieties is carried out by evaluating the morphological or physiological traits expressed by seed, seedlings or mature plants.

Morphological characterisation *via* GOT is often inaccurate because environmental conditions which can mask the expression of specific morphological or physiological traits may mislead varietal identification. Besides this, the process entails a lot of costs and requires a full growing season that results in late entry of seeds in the market. Field testing is also land intensive and requires highly skilled personnel (Lucchese *et al.*, 1999). With an increase in the number of varieties with a narrow genetic base, morphological characters fail to characterise and identify between them (Singh *et al.*, 2008). This has forced the scientific community to explore new economic alternative or combination of alternatives to augment morphological approach.

Use of biochemical markers such as isozymes and seed storage proteins for varietal classification was found to overcome the limitations of the morphological approach only to a certain extent (Dadlani *et al.*, 1997; Mehetre and Dahat, 2001; Borle *et al.*, 2007 and Rakshit *et al.*, 2008). With the advent of PCR assay based

molecular markers and DNA fingerprinting techniques, distinguishing genotypes based on its genetic makeup has become more efficient.

Researchers contemplated the introduction of molecular markers in cultivar identification, seed quality control and DUS testing of vegetable crops owing to its high reliability, rapidness and cost-effectiveness (Garg *et al*., 2006; Liu *et al*., 2007; Aneja *et al.,* 2019). The efficacy of Inter-simple sequence repeats (ISSR) and Simple sequence repeats (SSR) markers for molecular characterisation, cultivar identification, purity testing, assessment of genetic diversity and relationship of genotypes was reported by investigators around the globe (Inan *et al.*, 2012; Xanthopoulou *et al.*, 2015; Bhaskaran and Umarani, 2016; Chaudhary *et al.*, 2018). However, resorting to a combination of morphological characterisation and molecular approaches simultaneously would help to generate more information and increase the reliability of varietal characterisation.

Considering the above, the present investigation 'Characterisation of pumpkin (*Cucurbita moschata* Duch.) varieties through morphological and molecular markers' was envisaged with following objectives:

1. Morphological characterisation of pumpkin varieties.
2. Genotyping of pumpkin varieties using molecular markers.

18

Review of literature

2. REVIEW OF LITERATURE

Cultivar identification plays crucial role in crop improvement, variety release and seed production system. Establishment of cultivar identity and genetic purity testing is pre-requisite for variety release and seed multiplication programmes. The variety attains acceptance when the farmers get genetically pure seed of high standard. For this purpose, each cultivar should be properly defined with suitable descriptors, so as to maintain its identity during seed production through field inspection and certification. Growing international and national level seed business has created an inquisitiveness in descriptive characterisation of the plant varieties for unambiguous identification of varieties in the context of its protection (Cooke, 1999). Henceforth, varietal identification has attained an extreme importance world-wide in view of plant variety protection and genetic purity maintenance in seed programmes.

Considering the above, the present study was formulated to characterise six pumpkin varieties in the seed chain using morphological and molecular markers, and to generate fingerprints or molecular ID's of the six varieties using selected polymorphic Inter-simple sequence repeats (ISSR) and Simple sequence repeats (SSR) markers. The literature related to the study is detailed below in brief under the following headings.

2.1 Relevance of establishing cultivar identity

2.2 Varietal characterisation and identification

2.2.1 Characterisation and varietal identification using morphological markers

2.2.1.1 Characterisation of seed characters

2.2.1.2 Characterisation of plant characters

20

2.2.2 Characterisation and varietal identification using molecular markers/DNA based markers

2.2.2.1 Characterisation using Inter-Simple Sequence Repeats (ISSR)

2.2.2.2 Characterisation using Simple Sequence Repeats (SSR)

2.3 DNA fingerprinting using molecular profiles

2.1 Relevance of establishing cultivar identity

To safeguard its plant varieties, India became signatory to Trade Related Aspects of Intellectual Property Rights Agreements (TRIPs) under World Trade Organisation (WTO) in 1994. India was obliged to enact legislation that brought plant varieties within the general purview of intellectual property. The Protection of Plant Varieties and Farmers' Rights Act (PPV & FRA) was enacted in October, 2001 for granting registration and legal protection to all varieties, the essential requirements are Novelty, Distinctness, Uniformity and Stability (N-DUS). A precise system for identification of varieties and parents of hybrids became a fundamental requirement to enforce this protection. Conformity to the standards of DUS test needed to be checked by examining the variety which generates a description of the variety, using its relevant characteristics. DUS testing has an imperative role in registration and identification of varieties, plant variety protection, for varietal information system and classification of varieties into different groups (Jyoti *et al.*, 2015).

The aim of both public and private seed industries is to make available good quality seeds of improved varieties to the farmers. Seed quality is a multiple concept comprising of attributes like genetic and physical purity, physiological quality and seed health, of which trueness to type or variety identity is most important since the full potential of that variety is achieved only when it is genetically pure.

21

With increasing demand for quality seeds, malpractices in seed trade is also on the rise. Seed certification, a legally sanctioned and a voluntary activity in India aiming to maintain and making available high quality seeds and propagating materials of notified kind to the public by ensuring genetic identity and purity. Seed testing deciphers the quality of seed lot. The field plot technique or grow out test (GOT) serves as a pre-control as well as a 'post-control' test for avoiding genetic deteriorations under the provisions of the Seeds Act 1966. Since GOT relies on morphological characterisation of the variety, it is of paramount importance to describe, differentiate and characterise the varieties for their correct identification and for an effective seed quality control programmes (Cooke, 2003).

2.2 Varietal characterisation and identification

Genetic markers have significant role in various breeding programmes, genetic diversity studies, fingerprinting, identification, purity assessment of cultivar *etc*. Though the idea of using genetic markers appeared in very early literatures (Semagn *et al*., 2006), choosing of marker/s from this class is objective dependent (Kordrostami and Rahimi, 2015). The classical (morphological, cytological) markers and molecular (biochemical and DNA markers) (Nadeem *et al*., 2018) are employed to discern the genetic differences between individual organisms or species. Morphological markers are visually assessable traits while, cytological are based on variations in chromosome features. The DNA markers detect differences at the genic constitution of an individual whereas, biochemical markers like isozymes elucidate the variations in gene product.

The prominence of morphological and molecular markers in the field of characterisation and purity testing of varieties has reviewed below:

2.2.1 Characterisation and varietal identification using morphological markers

Morphological characterisation is the first step in the description and classification of genetic resources (Smith and Smith, 1989). Since ancient times,

22

humans have efficaciously used various morphological markers to investigate the variation in plants for utilization in crop improvement activities. Morphological markers can distinguish important traits of plant visually and are easy to use, with no requirement of specialised technique.

Descriptors of cultivars of crop species are required for validating varietal/cultivar identity, determining varietal purity, establishing the distinctiveness of new variety from existing varieties and documentation of genetic resources (Begum and Kumar, 2011). The use of morphological descriptors in sequential fashion is an age old, universally approved method to distinguish different varieties and convenient for testing genetic purity of seed lot (Sumathi and Balamurugan, 2014). A comprehensive characterisation of a genotype during different growth phases of crop *viz.*, seed, seedling, vegetative, reproductive and maturation phases would prove beneficial for better description of a variety rather than a single morphological character.

2.2.1.1 Characterisation of seed characters

Seed characters such as seed shape, colour, texture, presence of special appendages, seed weight, seed volume, *etc.* are useful laboratory key characters for characterisation of genotypes (Reddy *et al.*, 2008).

Among *Cucurbita pepo*, *Cucurbita maxima* and *Cucurbita moschata* of *Cucurbita* genus, significant differences have been observed in length, breadth, colour and test weight of seed. Lebeda *et al.* (2009) used seed length/breadth ratio to distinguish pumpkin (*C. moschata*) varieties.

Forty different pumpkin populations were grouped into large, medium and small with respect to seed length (13.80-24.30 mm) and width (7.50-15.30 mm). The seed thickness was found to range between 1.60 and 4.70 mm. Appreciable genotypic variation in seed colour was also observed in the collections (Balkaya *et al.*, 2010a).

23

Quantitative seed traits offers an effective way for distinguishing and identifying pumpkin. Kiramana and Isutsa (2017a) reported that seed size in pumpkin ranged from intermediate, large and very large and the average seeds per fruit was highly variable (>100 and < 100).

Wide variations in seed length ranging from long (>1.60 cm) to medium (1.20-1.60 cm) and small (<1.20 cm) was observed in pumpkin cultivars (Nagar *et al.*, 2017). Similar variability was found to exist in width of seed. It varied from large (>0.90 cm); medium (0.6 0-0.90 cm) and small (< 0.60 cm).

Kiramana and Isutsa (2017b) observed ample variation in seed length, seed thickness, 100 seed weight and seed coat colour (yellow-white, cream-yellow or brown) among 155 pumpkin cultivars.

Seymen *et al.* (2012) developed seed keys for the identification of 128 edible seed pumpkin (*C. pepo* L.) varieties on the basis of seed shape and colour. They grouped 39 genotypes seeds as elliptical, 72 genotypes as wide-elliptical and three genotypes as narrow-elliptical. Seed colour also ranged from light cream, cream and dark cream.

Wide variation was observed in seed phenotypic among 160 winter squash (*C. maxima*) genotypes (Balkaya *et al.*, 2009b). Majority possessed cream (49.60 %) seeds, while, 25.60 per cent were white, 20.00 per cent were brown and 7.50 per cent tawny.

Maggs-Kolling *et al.* (2000), Tlili *et al.* (2009) and Mahla *et al.* (2014) employed seed descriptors like number of seeds per fruit, seed shape, colour and 100 seed weight for varietal discrimination of watermelon.

Quantitative seed parameters serve well for varietal classification and also during seed certification and testing in bottle gourd (*Lagenaria siceraria*). High variability in seed characters *viz.*, mean seed length, seed width and thickness of seed were used to group varieties (Mladenovic *et al.*, 2011)

24

Sidhu (2013) observed that total number of seeds/fruit play a focal role in varietal identification and can be used a DUS descriptor in bitter gourd (*Momordica charantia* L. Moench.).

Based on seed coat colour, Pandravada *et al.* (2014) classified 36 ridge gourd (*Luffa hermaphrodita*) genotypes into black and grey.

Ali-Shtayeh *et al.* (2017) found that hundred seed weight (HSW) can be employed as a morphometric marker for making varietal-identities as it differs significantly with the varieties of snake melon. Kumari *et al.* (2019) had also observed the usefulness of HSW in grouping varieties as it differed significantly among five *Luffa acutangula* and three *L. aegyptiaca* varieties, respectively.

Kalyanrao *et al.* (2016) employed HSW and seeds/fruit to group genotypes of bottle gourd.

Hamdi *et al.* (2017) classified 15 winter squash genotypes based on seed length/breath ratio into elliptical and round seeds. The seeds were white, cream to light brown in colour.

Varietal identification of 30 cucumber cultivars using seed characters *viz.*, length, breadth, cavity length and breadth and test weight was carried out by Pal *et al.* (2017). They grouped the cultivars based on seed cavity length, seed cavity breadth, seed length, seed breadth and hundred seed weight.

Orsenigo *et al.* (2018) found appreciable variation in seed dimensions, shape, colour, brightness and seed test weight in winter squash. They concluded that these characters could be used as key descriptors in cultivar characterisation and identification.

2.2.1.2 Characterisation of plant characters

Systematic study of genotypes based on most discriminating and feasible agro-morphological characters *i.e.*, vegetative, reproductive and fruit of plants has

25

been and continues to be, the most widely used approach for describing varieties *de novo*, varietal identification and monitoring purity.

Clearly visible morphological (plant, leaf and fruit) characters can be used to distinguish between different varieties and hybrids of pointed gourd and also serve in making varietal identities (Prasad and Singh, 1990; Kumar *et al.*, 1995; Hazra *et al.*, 1998, Khan *et al.*, 2007 and Ara *et al.*, 2012).

According to Rahman *et al.* (1991), fruit length and breadth along with days to 50 % flowering (female) act as a real tool for varietal characterisation and documentation.

Characterisation and evaluation of snake gourd (*Trichosanthes anguina)* genotypes pertaining to agronomic and morphological characters was carried out to delineate the genotypes. Qualitative, flowering and fruiting features unveiled wide range of variability statistically and proved useful in discerning cultivar differences (Varghese, 1991; Banik, 2003 and Ara *et al.*, 2013 and 2015).

Researchers across the world discovered that phenotypic parameters facilitated characterisation and identification of genotypes among the Cucurbitaceae family. For instance, significant difference in terms of fruit length (21.60 to 40.21 cm), fruit diameter (22.30 to 47.11 cm) and thickness of fruit flesh (11.37 to 39.95 mm) existed among 24 pumpkin genotypes. Among the genotypes, the number of fruits/plant ranged between 1.50 and 4.28 with majority (45.80 %) of fruits being elliptical (Balkaya *et al.*, 2009b).

In 115 open pollinated genotypes of winter squash (*C. maxima*), variation for morphological characters was apparent for fruit shape (globular: 49.60 %, oval: 28.20 % and transverse elliptical: 22.20%). Fruit colour of the cultivars was mainly dark green (60.70 %) and light green (20.50 %). A range of 21.20 to 55.10 mm, 26.00 to 49.80 cm and 35.10 to 56.50 cm was observed for flesh thickness, fruit length and fruit diameter, respectively (Balkaya *et al.*, 2010b).

26

Ahmed *et al.* (2011) characterised 21 pumpkin genotypes and found wide variability in leaf length, number of fruits/plant, vine length, fruit yield, fruit shape (elliptical, round and pyriform), fruit skin colour (green yellow to brown) and flesh colour (whitish, greenish, orange and deep orange). These characters were highly useful in distinguishing the cultivars.

Du *et al.* (2011) revealed existence of significant variations in leaf colour (light green to dark green), flesh colour of fruit (white to orange), fruit length (9.40 to 80.00 cm) and width (10.00 to 24.00 cm) among 39 inbred lines of pumpkin in China.

Kalyanrao *et al.* (2016) studied and documented genotypes of bottle gourd for identification and conduct of GOT *viz.*, vine, leaf, flower and fruit characters. Fifteen genotypes studied portrayed a wide range of diversity in qualitative characters including early plant vigour, stem pubescence, leaf size, fruit shape, peduncle separation from fruit, blossom end fruit shape, fruit skin colour and fruit pubescence. However, quantitative traits like length of petiole, peduncle, vine and fruit, days to 50 % flowering, fruit diameter and average fruit weight exhibited notable variation, and hence proved less useful in delineating genetic difference among genotypes. Such results were also reported earlier by Mladenovic *et al.* (2012)

Ali-Shtayeh *et al.* (2017) collected data on flower, stem and fruit of snake melon genotypes using a descriptor list with a set of predefined morphological characters adopted by International Plant Genetic Resources Institute (IPGRI). Out of the 17 traits (quantitative and qualitative) evaluated, seven were monomorphic (fruit shape, skin texture, fruit hair, sex type, ovary shape, pubescence length and hair density) and ten polymorphic. The polymorphic characters examined in the study could be used to establish a catalogue of snake melon cultivars.

Pumpkin varieties from Kenya were studied by Kiramana and Isutsa (2017a) to distinguish them into distinct groups using numerous plant characters. Early

27

vigour ranged from poor to vigouros, leaf shape was cordate in all varieties while, leaf pubescence was soft, intermediate and hard. The leaves were green to dark green in colour variation with silvery patches present in almost all the genotypes. Early appearance of yellow coloured flowers were observed in majority of the genotypes.

Kiramana and Isutsa (2017b) observed wide variations among 155 pumpkin genotypes with respect to 50 % flowering, peduncle length, (short to long) , number of fruits per vine (1.00 to 13.00 fruits), fruit shape [short (7 cm) and narrow fruits to long (36.00 cm) and wide fruits], fruit flesh thickness (10.50 to 42.50 mm), immature fruit skin colour (pale green, green and dark colour), fruit shape (globular, flattened, ovate, elliptical, pyriform pear-like, acorn), blossom and peduncle end shapes (rounded, depressed, flattened or pointed) and size of scar at blossom end (small to large). They concluded that these traits can be employed for morphological characterisation and varietal identification of pumpkin cultivars.

Agro-morphological characterisation of 15 *C. maxima* genotypes revealed significant differences in petiole length (29.03 to 48.65 cm). The male flowers appeared early compared to female flower. The genotypes had yellow coloured flowers in all cultivars and orange flesh coloured fruits. Variations were observed with respect to thickness of flesh (3.00 to 7.30 mm) and rind (0.10 to 0.30 mm), fruit weight (5.04 to 12.60 kg), fruit height (16.63 to 23.83 cm) and peduncle length (2.00 to 9.40 cm). Most of the genotypes had broad transverse fruit shape (Hamdi *et al.,* 2017).

Pal *et al.* (2017) investigated the uniqueness of 30 varieties by noting yield and its contributing traits. Substantial difference was observed for almost all quantitative parameters like node number bearing first female flower (4.53-24.07), number of primary branches (1.60-8.24), days to first harvest (46.00-76.00), average fruit weight (75.25- 310.42 g), fruit length (8.83-21.22 cm), fruit diameter (2.89-5.56cm), mesocarp thickness (7.16-13.37 cm), marketable fruits/plant (1.57-12.90), duration between first and last fruit harvest (12.00-37.67 days), vine length

28

(209.31-323.37 cm) and TSS (3.80-5.38 °B), indicating importance of above mentioned traits in grouping of varieties morphologically.

Phenotypic expression of 28 morphological traits was recorded among spine gourd (*Momordica dioica)* genotype (Bhagat, 2017). Characters of leaf like intensity of green colour, length, pubescence of surface showed limited variation. Fruit colour (immature and mature), shape, length, single fruit weight and fruit yield/plant all indeed furnished greater variability. The study revealed that fruit characters are very effective in classifying and identifying varieties from each other.

Nagaraju and Thiruvenkatasamy (2017) carried out genetic characterisation of ash gourd (*Benincasa hispida)* using yield contributing characters. From the study it was found that vine length, node at first female flower appearance, days to 50 % female flower opening, number of fruits per vine, yield per vine, average fruit weight, polar diameter, equatorial diameter, flesh thickness *etc*. are important for varietal identification.

Orsenigo *et al*. (2018) and Priori *et al*. (2018) separately diagnosed winter squash (*C. maxima*) cultivars and they demonstrated that attributes like weight, shape and colour of fruit allowed cost effective and rapid identification and classification of varieties.

An experiment on morphological characterisation of ivy gourd (22 genotypes) revealed that fruit characters *viz*., fruit length, fruit weight and fruit yield per plant were key traits useful in grouping genotypes (Saikia and Phookan, 2018).

Morphometric measurements for vegetative and reproductive parameters was collected by Kumari *et al*. (2019) as per IPGRI descriptors in five varieties *Luffa acutangula* and three varieties of *L. aegyptiaca.* Leaf length (LL) did not distinguish varieties of *L. aegyptiaca*. Leaf width, petiole length, first female flower node and skin thickness , number of fruits per plant, fruit width, fruit girth, flesh thickness remained incapable for classifying varieties of *L. acutangula,* However, these traits

29

helped grouping varieties of *L. aegyptiaca*. Neither fruit length nor peduncle length differed in varieties of both the species.

2.2.2 Characterisation and varietal identification using molecular markers/DNA based markers

Intensive modern breeding technology has resulted in the advent of numerous varieties and hybrids in crops with narrow genetic base. As they possess minimum phenotypic variation, it makes morphological and biochemical markers insufficient and difficult to use in identification. For unambiguous identification, molecular tools developed during last few decades provided an efficient analysis of genotypic variation. This is because genetic difference between individuals are encoded in their genetic material and these molecular markers provide straightforward comparison by determining the sequence polymorphism (Semagn *et al.*, 2006).

The chief breakthrough of DNA based molecular marker was driven by the discovery of Polymerase Chain Reaction (PCR) invented by Kary Mullis in 1986. Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), *etc.* are the important PCR-based markers tuned after the development of PCR technology which seems to be a best candidate for fine-scale genetic characterisations.

Characterisation, varietal identification and diversity analysis of cucurbits was executed using techniques including random amplified polymorphic DNA (RAPD) (Stachel *et al.*, 1998; Gwanama *et al.*, 2000; Staub *et al.*, 2000; Ferriol *et al.*, 2003; Dey *et al.*, 2006; Muralidhara and Narasegowda, 2014; Zhao *et al.*, 2017), amplified fragment length polymorphism (AFLP) (Garcia-Mas *et al.*, 2000; Ferriol *et al.*, 2004; Martins *et al.*, 2018) and so on. For the present study, ISSR and SSR markers were used in order to characterise six pumpkin varieties.

30

2.2.2.1 Characterisation using Inter-Simple Sequence Repeats (ISSR)

In early 1990s, several research groups (Meyer *et al.*, 1993; Gupta *et al.*, 1994; Wu *et al.*, 1994; Zietkiewicz *et al.*, 1994) independently developed the technique of ISSR markers. Inter-Simple Sequence Repeats (ISSR) are region in the genome flanked by microsatellite sequence. During single primer PCR amplification, it targets variation in the DNA between two identical, oppositely oriented microsatellite loci present at an amplifiable distance. Inter-Simple Sequence Repeats (ISSR) primer is the repetitive (Di, tri, tetra or pentanucleotide) sequence complementary to microsatellite regions, either unanchored (Meyer *et al.*, 1993 and Gupta *et al.*, 1994) or more usually anchored at 3` or 5` end with one to four degenerate bases extended into the flanking sequences (Zietkiewicz *et al.*, 1994) can be used.

The efficacy of ISSR marker technique for molecular characterisation, identification and assessment of genetic diversity and relationship of *Cucurbita* genotypes was reported by investigators around globe (Heikal *et al.*, 2008; Inan *et al.*, 2012; Xanthopoulou *et al.*, 2015). With the advantage of using arbitrarily designed primers (Joshi *et al.*, 2000), this multilocus, dominant marker with 86 per cent to 94 per cent reproducibility, have been used for cultivar identification in carrot (Briard *et al.*, 2001), groundnut (Raina *et al.*, 2001), rice (Dharmaraj *et al.*, 2018), wheat (Tungalag *et al.*, 2018), genetic purity testing in cotton, chilli *etc.* (Dongre *et al.*, 2011; Pujar *et al.*, 2017), to evaluate crop genetic diversity, fingerprinting *etc.* in crops like soybean, cashew, radish, broccoli *etc.* (Pradeep *et al.*, 2002; Archak *et al.*, 2003; Liu *et al.*, 2008; Lu *et al.*, 2009).

A study using six ISSR primers in 28 genotypes of squash (*C. pepo*) produced 147 bands. Of these 108 bands (74 %) were polymorphic indicating that the analysis with ISSR primers quickly identified and characterised squash varieties (Paris *et al.*, 2002).

31

Thirty one (81.60 %) of the 38 ISSR primers efficiently differentiated 44 watermelon cultivars with limited genetic diversity by identifying significant polymorphism (Levi *et al.*, 2004).

Inter-Simple Sequence Repeats (ISSR) markers reflected presence of high level of genetic variation among bitter gourd genotypes. Out of the 15 ISSR primers used, four primers (UBC 854, UBC 855, UBC 856 and UBC 861) yielded 100 per cent polymorphism. The degree of polymorphism revealed by UBC 840, UBC 854, UBC 855, UBC 856, UBC 861 and UBC 890 was sufficient to identify all the accessions examined (Singh *et al.*, 2007).

Parvathaneni *et al.* (2011) while characterising and accessing genetic diversity of 13 genotypes belonging to *Cucumis* spp with 15 ISSR primers reported UBC 825 as a highly informative ISSR primer with PIC value of 0.89.

Ninety three amplicons were obtained using 13 selected ISSR primers in 28 cultivars of ornamental pumpkin. Eighty-nine per cent of amplicons were polymorphic and the similarity coefficient ranged from 0.31 to 0.99 (Shang *et al.*, 2012).

Santos *et al.* (2012), using 15 ISSR primers to study genetic diversity among 31 pumpkin (*C. moschata)* genotypes, observed an average of 8.40 fragments per primer. In total 126 polymorphic and 11 monomorphic bands were obtained and using this characterisation among genotypes was undertaken.

Analysis of genetic diversity in 42 bottle gourd accessions and characterising them using 20 ISSR primers amplified 209 bands, of which 186 were polymorphic registering 89.50 per cent polymorphism (Abdin *et al.*, 2014).

Rathod *et al.* (2015) concluded that ISSR markers produced specific DNA fragments for identification of genotypes in an experiment to evaluate eight ridge gourd and nine sponge gourd genotypes.

32

Tomar *et al.* (2016) screened amplification profiles for the presence of polymorphism with 62 ISSR primers among 50 muskmelon genotypes. A total of 462 fragments were generated with an average of 7.50 fragments per primer.

Singh *et al.* (2016) successfully characterised and assessed diversity between 11 genotypes (parthenocarpic and monoecious) of cucumber using eight primers. Six primers yielded 49 polymorphic alleles out of 57, indicating 88.88 per cent polymorphism level.

According to Kiani and Siahchehreh (2017), eight ISSR in ten varieties yielded 73 scorable bands, out of which 45 bands were polymorphic (61.60 %) with similarity between varieties ranging from 0.14 to 0.70.

Punetha *et al.* (2017) used eight ISSR primers for screening 13 varieties of cucumber in order to characterise and measure polymorphism. A total of 52 loci were amplified using 6 primers that exhibited 92.30 per cent polymorphism. The number of alleles produced by different primers ranged from 3 (UBC 809) to 11(UBC 855 and UBC 890) with an average of eight alleles per primer. Unique bands were observed by the primers namely UBC 808 and UBC 855.

Characterisation of 43 Styrian pumpkin (*C. pepo* var. *styriac*a) cultivars using 12 ISSR markers resulted in 100 per cent polymorphism (Amiri *et al.*, 2017).

Investigation in 16 pumpkin (*C. moschata)* genotypes using five ISSR produced 79 amplicons ranging in molecular size from 130 to 2140 bp revealing the ability of ISSR markers to evaluate the genetic diversity and differences among genotypes (Abdein, 2018).

As per Soghani *et al.* (2018), in watermelon, 11 ISSR primers displayed considerable polymorphisms in 38 genotypes. UBC 812, UBC 816, UBC 825 and UBC 826 showed 88.88 per cent polymorphism and the PIC value ranged from 0.20 to 0.32.

33

Molecular characterisation of 12 Turkish bitter gourd genotypes with 15 ISSR created 113 bands and 59 (52 %) of them were polymorphic (Karaman *et al.*, 2018).

2.2.2.2 Characterisation using Simple Sequence Repeats (SSR)

Simple sequence repeats (SSR) also known as microsatellites (Litt and Lutty, 1989) belongs to the repetitive DNA family and are species specific. They are tandem repeats with short repeat motifs (1-6 bp long monomer). These sections of DNA contain repeating di, tri, tetra or penta nucleotide units that are widely distributed throughout the genome of the plants and animals (Powell *et al.*, 1996). SSR are developed as markers by deducing the sequence of flanking region and with this known sequence specific primers can be designed to amplify the microsatellite by PCR. Therefore, polymorphism or SSR allelic difference are the result of variation of the number of repeats within the microsatellite structure.

Co-dominance Mendelian inheritance, hyper variability, high genome abundance with uniform dispersion, robustness, reproducibility all adds to the strengths of SSRs (Morgante *et al.* 2002).

Cultivar identification, purity testing and diversity analysis studies using SSR were conducted in soybean (Meesang *et al.*, 2001), potato (Moisan-Thiery *et al.*, 2005), mango (Tsai *et al.*, 2013), pineapple (Lin *et al.*, 2015), rice (Bhaskaran and Umarani, 2016), maize (Chaudhary *et al.*, 2018).

Characterisation and polymorphism assessment among 104 accessions of *C. pepo* with 134 SSRs amplified a total of 418 alleles, spread among all 20 linkage groups (Gong *et al.*, 2012).

Genetic characterisation with 18 SSR markers revealed sufficient variability among 51 genotypes. Marker amplification yielded 109 alleles totally with an average number of alleles per locus of 6.06 and PIC value varied between 0.02 and 0.87 (Murovec, 2015). Similarly, ten SSRs out of 50 discriminated the landraces of *C. pepo* by revealing 68 per cent polymorphism (Ntuli *et al.*, 2015).

34

The polymorphism in the molecular analysis among 21 *C. moschata* genotypes with six SSR markers amounted to 100 per cent (Martins *et al.*, 2015). Similarly the results of work by Sim *et al.* (2015) suggested that 29 SSR markers represent highly polymorphic loci and are able to detect genetic variations between 160 elite pumpkin cultivars. The highest number of alleles was found in the CMTm7 marker (14), with ≥ 10 alleles detected by CMTm80 (13), CMTm119 (12), CMTm128 (12), CMTm18 (11), CMTm35 (10), CMTm48 (10) and CMTm162 (10). The PIC values of the markers varied considerably, ranging from 0.48 (CMTm63) to 0.89 (CMTm7) with an average of 0.69 in the 160 pumpkin.

Liu *et al.* (2013) reported that SSR marker based characterisation of 66 accessions was useful in analysing genetic diversity of *C. maxima.* The study stated that SSRs like CMTm20, CMTm144, CMTm232 with high PIC value had the potential for identification of cultivars.

Characterisation of 19 pumpkin genotypes with five SSRs amplified 14 polymorphic bands in all (Miladinovic *et al.*, 2016).

Twenty three *Cucurbita* conserved genomic SSRs used for analysis of 85 genotypes of *C. maxima* helped in detecting genetic diversity patterns and grouping of genotypes. Six markers that were tested in this study (CMTm7, CMTm19, CMTm20, CMTm48, CMTm60, and CMTm61) was suggested to be highly effective for pumpkin and squash cultivar identification (Kazminska *et al.*, 2017).

Barman and Anshumali (2018) suggested the usefulness of SSR markers in assessing genotypic polymorphism. They screened 60 North Eastern cultivars using 20 SSR molecular markers. The 20 markers together generated 323 amplicons with PIC valued ranged from 0.31 to 0.91. They detected nine SSR markers that exhibited allelic frequency of less than 5 per cent.

2.3 DNA fingerprinting using molecular profiles

It is a technology used to identify an organisms through their DNA that contains the blueprint of its characteristics.

Since its development, DNA fingerprinting has been used in diverse scientific arenas such as forensic investigations, anthropological genetics (for interpreting the origin and evolutionary history of humans), zoology (in documenting genetic variation levels both in rare and endangered species), botany (genotype identification and variations among wild and cultivated species, protection of biodiversity *etc.*) and agriculture among others to derive benefits from this profiling research (Garcial and Mino, 2017).

The DNA fingerprinting technology has opened the door for precise method of varietal identification and genetic purity analysis compared to conventional approaches. In the era of Intellectual Property Rights (IPR), patenting and piracy of genetic materials, proper and precise identification and cataloguing of germplasm is gaining utmost importance. Similarly, increasing the availability of true to type seeds by seed sector is a farmer and environment friendly option for boosting up agriculture production.

Conventionally, morphological characteristics at various growth stages of crop *via* grow out test and later on biochemical methods like isozyme analysis and seed protein electrophoresis were used for genetic purity estimation and distinguishing varieties. Along with these criteria, PCR based DNA fingerprinting offering several advantages open up new avenues and platform to establish an identity of genotype without any ambiguity (Morell *et al.*, 1995). Substantial achievements using DNA fingerprinting technique as a supplementary information of a genotype, made PPV & FR authority to take in DNA fingerprint as an additional mandatory requirements for the release of new crop varieties by Central Variety Release Committee.

36

DNA fingerprinting is identified as an effective substitute to GOT for purity estimation even at seed level as it can provide accurate, cost effective results to detect the off-types in the seed lot present either through contamination or wilful deception. It is also proved to be time-saving with which seeds can be marketed for immediate cultivation as it takes less than a week for testing thereby saving the cost of storage for next season. Seed industry make use of DNA profiling *via* molecular markers as a supportive test for GOT because the results are in parallel with morphological markers based field plot test.

Several recent studies by scientists community worldwide elicits the use of DNA fingerprinting *via* molecular markers in cultivar differentiation and purity determination in several crops (Korir *et al.,* 2013 in tomato, Bhattacharjee *et al.*, 2018 in sesame, Christ *et al*., 2018 in piper, Lee *et al.,* 2018 in onion, Pattanaik *et al.,* 2018 in cauliflower, Ragul *et al.,* 2018 in cowpea, Sultana *et al.,* 2018 in brinjal, Ishaq *et al.,* 2019 in rice, Mannino *et al.,* 2019 in pista, Raatz *et al.,* 2019 in bean).

Establishing DNA fingerprints of various crops of Cucurbitaceae family using both PCR and non- PCR based molecular markers has been done for the purpose of purity determination and varietal identification.

Alam *et al.* (2012) tried a combination of six RAPD markers on five commercial snake gourd varieties to construct unique fingerprints.

Twenty-two cultivars of *Trichosanthes dioica* Roxb. from various agro-climatic regions of India were fingerprinted using RAPD (37 nos.) and ISSR (15 nos.) markers. The study revealed that RAPD and ISSR markers could provide a practical and efficient tool in quality control of the *T. dioica*. These can prove useful to protect the plant breeder's rights as they are reliable and repeatable (Goswami and Tripathi, 2010).

Gao *et al.* (2012) constructed fingerprint database and quick response (QR) codes of 471 Chinese melon selecting 470 polymorphic SSRs to provide an

37

evidentiary support of seed quality appraisal which help in preventing homonyms and synonyms in melon seed market.

Out of 20 SSR makers used for DNA profiling of 20 bottle gourd varieties, ten primers exhibited polymorphic profiles. Of these 10 primers, five SSRs provided unique profile of all the genotypes, making it feasible to differentiate unambiguously the varieties respectively (Sarao *et al.*, 2014).

The DNA fingerprints of a bitter gourd cultivar Xiuyu 1 and its parents were analysed using ISSR primers. ISSR-845 could distinguish the male parent from F_1 hybrid and the female parent, while ISSR-891 could distinguish the female parent inbred line from Xiuyu 1. The seed purity test with these markers gave the same result as of field trials based on morphological identification (Chen *et al.*, 2015).

Fifty-two microsatellite markers were employed to fingerprint three hybrids and their parental lines in bottle gourd. Three SSR markers were found to be polymorphic across the hybrids and produced unique fingerprints for each of the three hybrids. The results indicated that microsatellites are excellent genomic tools for parentage confirmation and hybridity determination (Ghatage *et al.*, 2017).

Materials and Methods

3. MATERIALS AND METHODS

In seed production programmes, proper varietal identification plays an important role in order to maintain the genuineness and quality of seed. Hence, a proper description of varieties at the morphological and molecular level would serve as a reliable identification tool in seed production programmes. Considering the importance of establishing cultivar identity, the present investigation envisaged characterisation of six varieties of pumpkin in seed chain using morphological and molecular markers. The details of experiments, materials used and methods followed in the study are described hereunder.

3.1 Location

The experiment was conducted during December, 2018 to April, 2019 using the field and laboratory facilities of the Department of Seed Science and Technology, College of Horticulture, Kerala Agricultural University, (KAU), Vellanikkara, Thrissur, located 40 m above MSL between 10° 54' North latitude and 76° 28' East longitude.

3.2 Climatic conditions

The experimental location experiences a humid tropical climate. During the study period, relative humidity varied between 63 per cent (December 2018) and 70 per cent (April 2019). No rainfall was received during the study period, except in April 2019 (76.40 mm). The monthly mean maximum temperatures ranged from 33.00 °C in December 2018 to 36.20 °C in April 2019, while, the mean minimum temperature varied between 22.50 °C in December 2018 and 25.50 °C in April 2019.

3.3 Experiment materials

The study material comprised of six varieties of pumpkin. The details are catalogued in Table 1.

40

Table 1: Details of six pumpkin varieties and its source

Treatment	Variety	Seed source
T1	Ambili	Regional Agricultural Research Station (RARS), Pattambi
T2	Saras	Kerala Agricultural University (KAU), Thrissur
T3	Suvarna	Kerala Agricultutal University (KAU), Thrissur
T4	CO-1	Tamil Nadu Agricultural University (TNAU), Coimbatore
T5	CO-2	Tamil Nadu Agricultural University (TNAU), Coimbatore
T6	Arka Chandan	Indian Institute of Horticultural Research (IIHR), Bengaluru

3.4 Experiment details

Morphological characterisation and genotyping of pumpkin cultivars were done. The experimental details are enumerated below.

3.4.1 Experiment 1: Morphological characterisation of pumpkin varieties

The experiment was laid out in Randomised Block Design (RBD) with six treatments (varieties) and four replications in the field facility of Department of Seed Science and Technology, College of Horticulture. Pits of 60 cm diameter were aligned along the plot area. The field was divided into 24 sub-plots for the randomised application of four replications of the six treatments. A spacing of 3 m between rows and 2 m between pits was ensured in each sub-plot of size 3 m × 6 m in order to accommodate 24 plants (3 plants/pit). The seeds of six varieties were soaked separately in water overnight and sown in the respective sub-plot during the first week of December 2018. Agronomic and plant protection practices were adopted as per the Package of Practices (POP) Recommendations of Kerala Agricultural University (KAU POP, 2016) to raise a good crop (Plate 1).

41

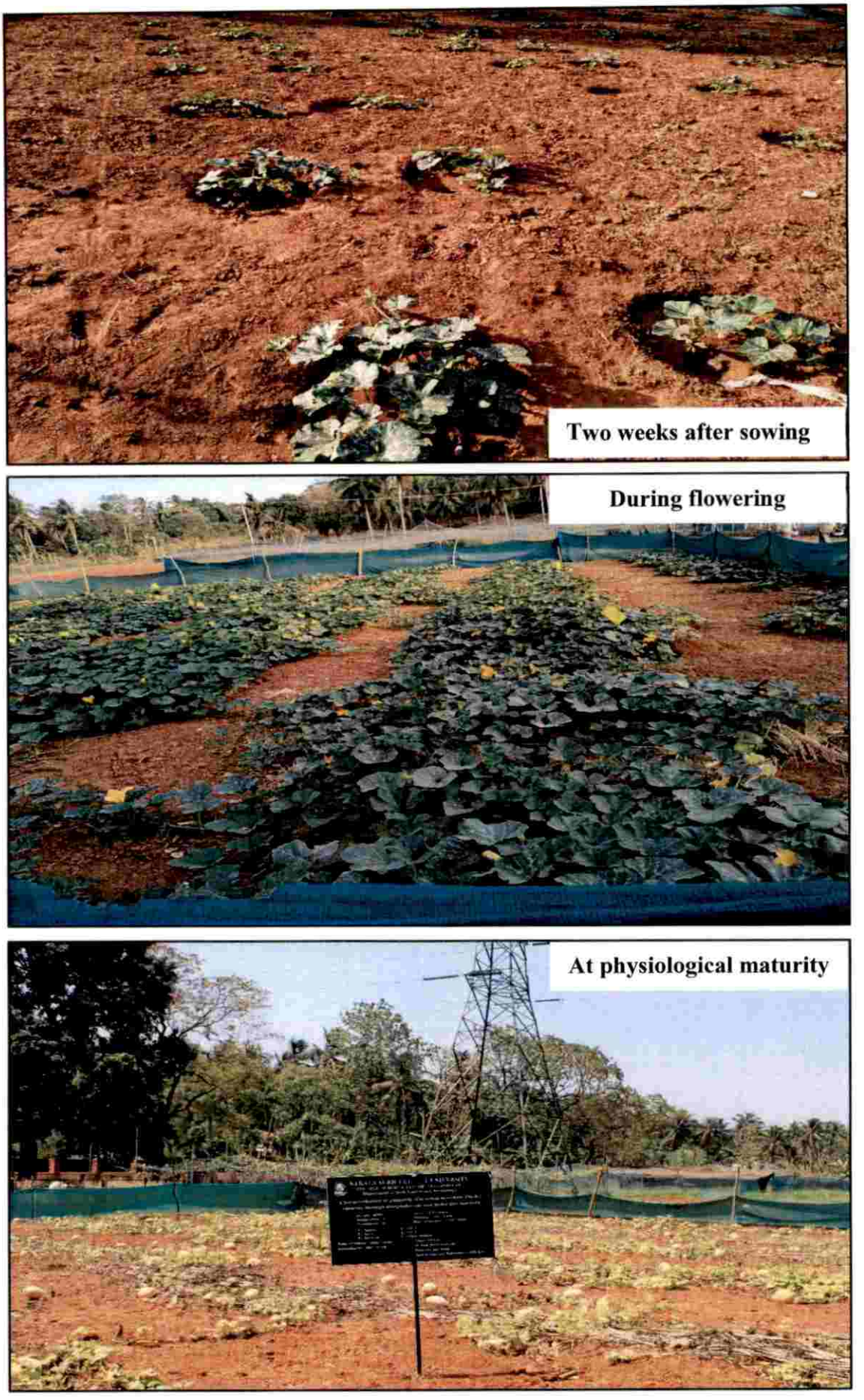


Plate 1: View of experiment plot at different stages of the crop

3.4.1.1 Morphometric observations evaluated for characterising the varieties

Observations on morphological characteristics (qualitative and quantitative) at the appropriate stage of growth in each variety were recorded as per the National Test Guidelines for Distinctness, Uniformity and Stability (PPV & FRA, 2017) and NBPGR descriptors (Srivastava *et al.,* 2001). For each metric traits, measurements were recorded on five randomly selected plants per replication in each variety and the average was computed.

3.4.1.1.1 Seedling characters

3.4.1.1.1.1 Cotyledon length (cm)

Length of completely unfolded cotyledon leaf was recorded using a meter scale in the tagged plants in each replication of a treatment. The grouping of varieties using mean value expressed in centimetres were as follow:

States	**Length**
Short	< 4.50 cm
Medium	4.50-5.50 cm
Long	> 5.50 cm

3.4.1.1.1.2 Cotyledon width (cm)

Width of fully expanded cotyledon leaf of all varieties was measured in centimetres and the average computed. Based on mean cotyledon width, varieties were classified into the following groups:

States	**Width**
Narrow	< 2.50 cm
Medium	2.50-3.50 cm
Broad	> 3.50 cm

43

3.4.1.1.2 Early plant vigour

The early plant vigour was determined based on a number of leaves scored visually after 30 days of sowing and the varieties grouped into the following classes:

Category	Note
Poor	3
Good	5
Very good	7
Others	99

3.4.1.1.3 Tendril characteristics

3.4.1.1.3.1 Tendril

During the flowering stage, presence or absence of tendril was recorded visually in each variety.

Note	Category
0	Absence
1	Presence

3.4.1.1.3.2 Tendril type

Nature of tendril was observed visually in all the six varieties during the flowering phase.

Note	Category
1	Coiled
2	Straight
99	Others

44

3.4.1.1.3.3 Tendril branching

Branching habit of the tendril in the varieties was examined during the flowering.

Note	Category
1	Unbranched
2	Branched
99	Others

3.4.1.1.3.4 Tendril stalk length (cm)

Stalk length of tendril (5 nos.) per tagged plant in each treatment was measured and the average is expressed in centimetres.

3.4.1.1.4 Leaf characteristics

3.4.1.1.4.1 Leaf blade length (cm)

It was measured in centimetres from the base of the leaf blade to the tip of the fully developed leaf between the 15^{th} and 20^{th} node in tagged plants, using a meter scale. Based on the average length of the leaf blade, varieties were grouped into three categories:

States	Length of leaf blade
Short	< 15 cm
Medium	15-20 cm
Long	> 20 cm

45

3.4.1.1.4.2 Leaf blade width (cm)

Leaf blade width in centimetres was measured from the widest end of the fully developed leaf (2 nos.) per tagged plant between the 15th and 20th node using a meter scale. Based on the average width of the leaf blade, varieties were grouped into three categories:

States	Width of leaf blade
Narrow	< 15 cm
Medium	15-20 cm
Broad	> 20 cm

3.4.1.1.4.3 Petiole length (cm)

At full foliage stage, five random leaves in the middle section of the vine in each tagged plant were selected and the length of the petiole was measured in centimetres using a meter scale. Based on petiole length, the varieties were grouped as follows:

States	Petiole length
Short	< 12 cm
Medium	12-18 cm
Long	> 18 cm

3.4.1.1.4.4 Leaf shape

Leaf shape was recorded at full foliage stage and classified as follows:

Note	Category
1	Cordate
2	Oblong

46

3	Ovate
4	Obovate
5	Orbicular
99	Others

3.4.1.1.4.5 Leaf margin

Based on leaf margin patterns during full foliage stage, the varieties were grouped as:

Note	Category
1	Entire or very weakly incised
2	Weakly incised
3	Moderately incised

3.4.1.1.4.6 Leaf blade colour (upper side)

At full foliage stage, the colour of the leaf blade of each variety was determined using the Royal Horticultural Society (RHS) colour chart. The grouping was done as:

Note	Colour
3	Light green
5	Medium green
7	Dark green

3.4.1.1.4.7 Leaf blade silver patches

Presence or absence of silvery patches on the upper surface of leaves of each variety was recorded visually. Scoring was given as 1 for presence and 0 for absence.

47

3.4.1.1.4.8 Leaf pubescence density

Leica-EZ4D stereomicroscope equipped with Leica Application Suite (LAS) image analysing software was used to observe pubescence on leaves of each variety at 12.5X magnification.

The density of pubescence on leaves was measured from a marked area of 1 mm^2 digitally using Digimizer image analysing software (Plate 2). Counts were taken from three different points on each leaf and the averages were worked out.

3.4.1.1.5 Flower characteristics

3.4.1.1.5.1 Node number at which first female flower appears

The node number from cotyledonous leaves at which the first female flower appeared was noted in the tagged plants and the mean computed.

3.4.1.1.5.2 Days to 50 per cent flowering

A number of days from sowing to the day when 50 per cent of the plants in a replication showed at least one opened female flower was noted. The data was collected in all replications of each treatment and averaged.

3.4.1.1.6 Fruit characteristics

3.4.1.1.6.1 Colour of immature fruit skin

Assessment of fruit colour during the immature stage was done in the field by referring to the RHS colour chart. Depending on the colour, the fruits were categorised as follows:

Note	Colour
1	Cream
2	Light green

48

3	Medium green
4	Dark green

3.4.1.1.6.2 Fruit shape

Fruit shape in each variety was determined by comparing with the images given in the descriptor list of pumpkin in National Test Guidelines for Distinctness, Uniformity and Stability (PPV & FRA, 2017).

Note	Shape
1	Heart shape
2	Round flat
3	Oval or oblong
4	Rectangular
5	Spherical
6	Pear shaped
7	Club shaped
8	Cylindrical
99	Others

3.4.1.1.6.3 Fruit shape at peduncle end

Fruit shape at peduncle end of each variety was determined by comparing with the images given in the descriptor list of pumpkin in National Test Guidelines for Distinctness, Uniformity and Stability (PPV & FRA, 2017).

Note	Shape at peduncle end
1	Raised
2	Flat

49

3	Moderately depressed
4	Strongly depressed

3.4.1.1.6.4 Fruit shape at blossom end

Fruit shape at the blossom end of each variety was determined by comparing with the images given in the descriptor list of pumpkin in National Test Guidelines for Distinctness, Uniformity and Stability (PPV & FRA, 2017).

Note	**Shape at blossom end**
1	Depressed
2	Flat
3	Raised

3.4.1.1.6.5 Diameter of scar at blossom end (cm)

Horizontal diameter of blossom end scar of fruits from the tagged plants was measured in centimetres and the average computed. The grouping based of blossom end diameter is described below:

States	**Diameter of scar**
Short	< 1 cm
Medium	1-2 cm
Large	> 2 cm

3.4.1.1.6.6 Peduncle length (cm)

The length of peduncle in centimetres was recorded from all the fruits in the tagged vines during the marketable stage. Based on average values, they were categorised into three groups:

50

States	Peduncle length
Short	< 5 cm
Medium	5-10 cm
Long	> 10 cm

3.4.1.1.6.7 Fruit length (mature stage) (cm)

The length of fruits in a vine was measured using a thread and further in meter scale from peduncle end (stalk end) to blossom (distal) end at physiological maturity and the average computed and categoriesed as follows:

States	Fruit length
Short	< 12 cm
Medium	12-20 cm
Long	21-30 cm
Very long	> 30 cm

3.4.1.1.6.8 Fruit circumference (mature stage) (cm)

The circumference of each fruit was measured using a thread and further in meter scale at the widest point horizontally across the fruit and the mean value was calculated and expressed in centimetres.

3.4.1.1.6.9 Number of ribs/fruit

A total number of ribs (ridges) per fruit was counted at a full mature stage and averaged.

3.4.1.1.6.10 Length of flare (cm)

Flare length of each fruit obtained from tagged plants was measured and the average tabulated in centimetres.

51

3.4.1.1.6.11 Colour of mature fruit skin

Assessment of fruit colour during the mature stage was done referring to the RHS colour chart. Depending on the colour, the categories are as follows:

Note	Colour
1	Cream
2	Green with creamy patches
3	Orange

3.4.1.1.6.12 Waxiness of mature fruit skin

Waxiness of fruit skin was determined by touching and scratching the skin and recorded either as presence or absence.

3.4.1.1.6.13 Fruit diameter (mature stage) (cm)

Fruits were cut open in the middle at the vertical axis and the diameter was measured horizontally at the widest point across the fruit and the average is expressed in centimetres.

States	Fruit diameter
Short	< 15 cm
Medium	15-30 cm
Large	> 30 cm

3.4.1.1.6.14 Seed cavity diameter (cm)

After cutting the fruit, the horizontal diameter of the seed cavity was measured using a meter scale at the widest point and the average was expressed in centimetres.

52

3.4.1.1.6.15 Rind thickness (mm)

The thickness of rind from fruits of each variety was measured using vernier calliper and the average is expressed in millimetres.

3.4.1.1.6.16 Fruit flesh thickness (cm)

Fruits were cut open longitudinally and the thickness of flesh was measured at the widest point using meter scale and the average was expressed in centimetres.

States	Flesh thickness
Thin	< 2.50 cm
Medium	2.50-4.50 cm
Thick	> 4.50 cm

3.4.1.1.6.17 Colour of fruit flesh

Colour of fruit flesh of each variety was assessed by comparing it with the RHS colour chart. Depending on the colour, variety can be grouped into creamy white, yellowish orange, greenish orange or dark orange.

3.4.1.1.6.18 Number of fruits/plant

Fruits obtained per vine in each harvest from each tagged plant in each replication were counted and added.

3.4.1.1.6.19 Fruit yield/plant (kg)

Total weight of all the fruits per vine was obtained and expressed in kilograms (kg). The data was collected from each tagged plant per replication of all the treatments and averaged to obtained fruit yield/plant.

53

3.4.1.1.7 Plant main vine length (m)

Using a measuring tape, the length of the vine in each tagged plant was measured at the end of the season from the base to the tip of the plant and the average expressed in metres.

States	Vine length
Short	< 3.00 m
Medium	3.00-4.50 m
Long	> 4.50 m

3.4.1.1.8 Seed characters

3.4.1.1.8.1 Seeds per fruit

The seeds from the harvested fruits were extracted manually and the total number of seeds per fruit was counted using seed counter and the average was worked out.

3.4.1.1.8.2 Seed length (cm)

Ten seeds were randomly picked from the seed lot of each observational fruit and the seed length was measured using digital Vernier calliper (Plate 3) and the average was expressed in centimetres.

States	Seed length
Short	< 1.20 cm
Medium	1.20-1.60 cm
Long	> 1.60 cm

54

3.4.1.1.8.3 Seed width (cm)

Ten seeds were randomly picked from the seed lot of each observational fruit and the seed width was measured using digital Vernier calliper (Plate 3) and the average was expressed in centimetres.

States	Seed width
Short	< 0.60 cm
Medium	0.60-0.90 cm
Long	> 0.90 cm

3.4.1.1.8.4 Seed thickness (cm)

Ten seeds were randomly picked from the seed lot of each observational fruit and the seed thickness was measured using digital Vernier calliper (Plate 3) and the average was expressed in centimetres.

3.4.1.1.8.5 Seed coat colour

The coat colour of seeds of each variety was assessed by comparing it with RHS colour chart. Depending on the colour, variety can be grouped into cream, yellow, white or brown.

3.4.1.1.8.6 Hundred seed weight (g)

After proper drying, the weight of 100 randomly picked seeds from each fruit in the tagged plants was recorded and the average tabulated in grams.

3.4.1.1.8.7 Seed volume (ml)

The volume of water displaced by 100 seeds was quantified thrice and the average computed as seed volume and expressed in millilitres.

55

Plate 2. Density of pubescence on leaves at 12.5 X magnification

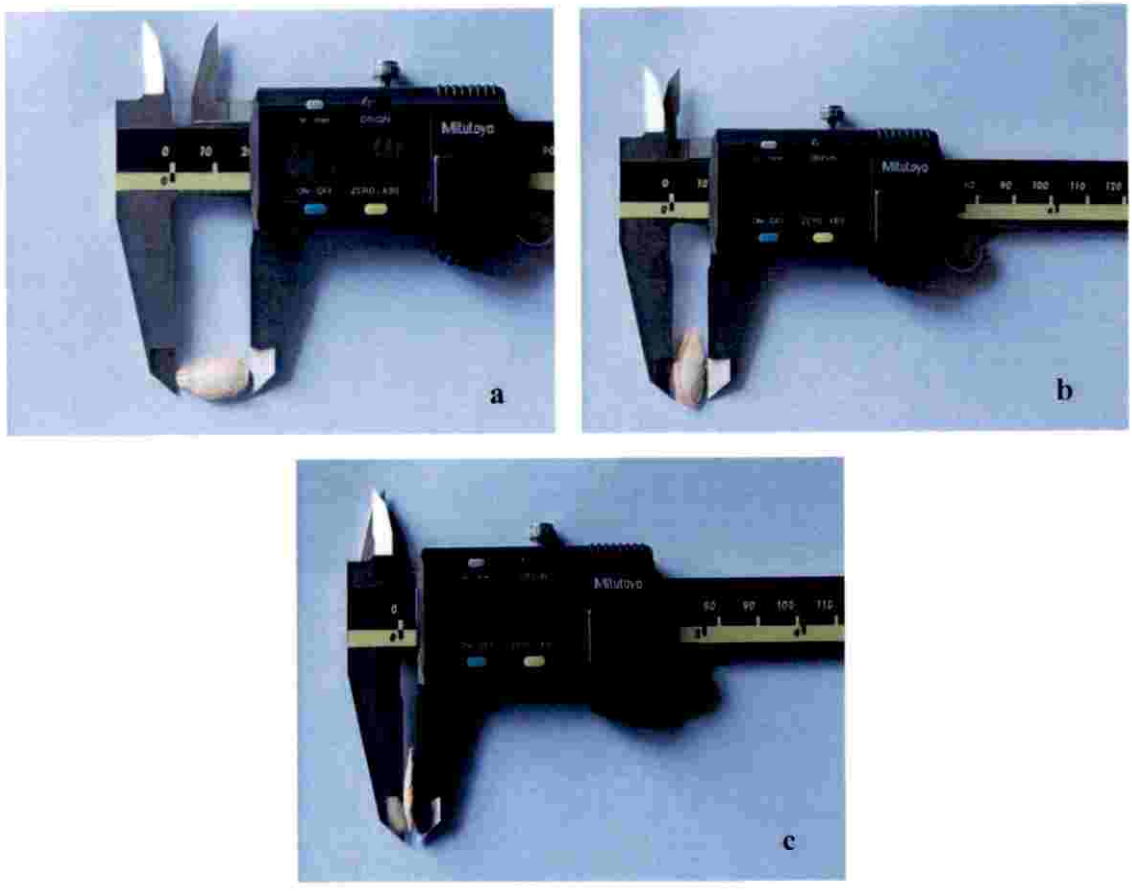


Plate 3. Measuring the dimensions of seed (a) Seed length, (b) Seed width, (c) Seed thickness

3.4.2 Experiment 2: Genotyping of pumpkin varieties using molecular markers

Molecular characterisation and DNA fingerprinting of six varieties were accomplished using Inter Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR).

3.4.2.1 Laboratory chemicals, glassware and equipment

The AR (analytical reagents) grade chemicals (extra pure) from Sisco Research Laboratories (SRL) and plastic wares from Tarson India Ltd. were used for the study. The PCR master mix was procured from Genei Pvt. Ltd., Bangalore, while, the ISSR (Inter Simple Sequence Repeats) and SSR (Simple Sequence Repeats) primers used in the study were synthesised by Sigma Aldrich Chemicals Pvt. Ltd., Bangalore.

For centrifugation, high-speed refrigerated centrifuge (Eppendorf 5804 R) was used. The DNA quality and quantity estimation were done using Nanodrop Spectrophotometer (Jenway- Genova Nano) and PCR amplification was done using Applied Biosystems Verti thermel cycler PCR machine. Horizontal gel electrophoresis unit by Bio-Rad, USA, was employed to carry out Agarose gel electrophoresis.

3.4.2.2.Genomic DNA isolation

3.4.2.2.1 Reagents used

1. CTAB extraction buffer (2 %)
    - 2 per cent CTAB (w/v)
    - 100 *mM* Tris (pH 8.0)
    - 20 *mM* EDTA (pH 8.0)
    - 1.4 *M* NaCl
2. Polyvinylpyrrolidone (1 %)

57

3. β mercaptoethanol (10 *mM*)
4. Chloroform: Isoamyl alcohol (24:1 v/v)
5. Isopropanol (100 %)
6. Ethanol (70 %)
7. Sterile autoclaved distilled water

3.4.2.2.2 Procedure for extraction of genomic DNA

The DNA was isolated by following the CTAB protocol of Doyle and Doyle (1987) with the slight modifications of buffer concentration. In about three-four weeks old plants, newly flushed tender leaves were collected early morning from the tip of the vine. The extraction of genomic DNA was done using the following protocol.

1. Young tender leaf samples from each variety were collected in an aluminium foil, marked and transported immediately to the laboratory inside an icebox.
2. Before the start of DNA extraction, the autoclaved mortar and pestle needed to be pre-chilled and the CTAB buffer (2 %) was pre-warmed by keeping in hot water bath at 60 °C.
3. From the collected leaf samples, 0.5 g was weighed out and sterilised using 70 % ethanol. Leaves were cut into small pieces using sterile scissors, ground to a fine paste in a pre-chilled mortar and pestle using 1 ml CTAB buffer along with 50 µl of β mercaptoethanol and a pinch of PVP.
4. Homogenised samples (1 ml) were transferred to autoclaved 2 ml centrifuge tube marked and incubated at 65 °C in water-bath for 40 min. with gentle inversion every 10 min. interval.
5. After incubation, the tubes were taken out and an equal volume (1 ml) of chilled chloroform: isoamyl alcohol (24:1) was added, inverted to mix and emulsify the contents. The contents were then centrifuged at 12,000 rpm for 15 min. at 4 °C.
6. After centrifugation, the contents were separated into three distinct layers.

58

a) Aqueous topmost layer: containing DNA and RNA
b) Interphase: containing fine particles and proteins
c) Lower layer: containing chloroform and some pigments

7. The tubes were taken out from the centrifuge without disturbing the three layers and the top aqueous layer was carefully transferred to a fresh centrifuge tube. To this, an equal volume of chloroform: isoamyl alcohol (24:1) mix was added.
8. The content was mixed well with gentle inversions and centrifuged at 12,000 rpm for 15 min. at 4 °C.
9. After centrifugation, the tubes were taken out and the topmost layer was carefully transferred to a new centrifuge tube. To this, 2 µl of RNase was added and incubated in the water bath at 37 °C for 15 min.
10. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 min. at 4 °C.
11. On centrifugation, the aqueous phase was carefully transferred to a new 1.5 ml centrifuge tube. About 500 µl of chilled isopropanol was added and the tubes were incubated at -20 °C for two hours.
12. After incubation, the tubes were centrifuged at 10,000 rpm at 4 °C for ten minutes.
13. Then, the supernatant was discarded and to the pellet, 700 µl of 70 per cent ethanol was added. Then the tubes were centrifuged at 10,000 rpm for five min. at 4 °C.
14. The 70 % ethanol wash was repeated. After centrifugation, the supernatant was discarded without disturbing the pellet.
15. The pellets were dried until all the ethanol got evaporated and was dissolved in 50 µl autoclaved double distilled water.
16. The tubes were gently tapped to dissolve pellet completely and then the DNA samples were stored at -20 °C.

59

3.4.2.3 Quality and quantity estimation of DNA

The purity and quantity of the DNA were estimated using a Nanodrop Spectrophotometer (Jenway- Genova Nano). Since the absorption maxima for nucleic acid and proteins are at 260 and 280 nm, respectively, absorbance was recorded at both the wavelengths and purity of the sample was estimated using the OD260/OD280 ratio. The DNA sample was considered to be pure if the OD260/OD280 value was between 1.8 and 2.0. Values below 1.8 and above 2.0 are due to contamination by protein and RNA, respectively. The concentration of DNA in the sample was estimated using the relation, 1 OD at 260 nm = 50 ng DNA/µl, hence, OD260×50 gave the quantity of DNA (ng/µl).

3.4.2.3.1 Procedure

1. The lid of spectrophotometer was opened, and the sampling arm and the pedestal were wiped with tissue paper to remove any dust particles.
2. The reading was set to zero with a blank sample (double distilled water).
3. One µl of the test sample was loaded on to the pedestal and the measure option selected and readings recorded.
4. After the measurements, the pedestal was wiped clean with 70 per cent ethanol using a soft laboratory wipe.

3.4.2.4 Agarose gel electrophoresis

3.4.2.4.1 Reagents used

1. Agarose (0.8 %)
2. 50X TAE buffer (pH 8.0)
   a) Tris buffer (1 *M*)
   b) Glacial Acetic acid
   c) 0.5 *M* EDTA
3. Tracking/loading dye (6X)
4. Ethidium bromide (stock 10 mg/ml, working concentration 0.5 µg/ml)

60

3.4.2.4.2 Procedure

1. The gel was prepared by adding 0.8 g of agarose in 100 ml of 1X TAE buffer in a glass conical flask. The mixture was heated in a microwave oven until all the agarose particles were completely dissolved and a clear solution was obtained.
2. The gel casting tray was placed appropriately in a gel caster and the movable wall was adjusted such that the gel casting tray was closed at both ends. A comb was selected depending on the number of samples to be electrophoresed and positioned on the grooves provided on the gel casting tray.
3. The solution was allowed to cool down to 50 °C and the required the amount of ethidium bromide (1 µl/10 ml of gel) was added and mixed well. The warm gel was then poured into the gel casting tray and left to solidify for 20 min. at room temperature.
4. Special care was taken to avoid any air bubbles near the wells or on the gel.
5. Once the gel was solidified, a small amount of 1X TAE was poured on top of the gel and the comb was removed carefully without breaking the gel. The TAE solution was discarded and the gel along with the tray was kept inside the electrophoresis tank with the wells on the negative electrode side.
6. The electrophoresis tank was filled with 1X TAE sufficient enough to submerge the wells.
7. The samples to be electrophoresed were prepared by mixing 5 µl of the DNA sample with 1 µl of 6X gel loading dye. After mixing, the total volume of 6 µl was loaded into individual wells.
8. The samples were electrophoresed at 75 volts until gel tracking dye reached two third of the gel length.

3.4.2.5 Gel documentation

Documentation of the electrophoresed gel was done under UV with gel documentation system (GeNei TM- UVITEC Fire Reader, Merck, UK+ Dell computer system)

3.4.2.6 ISSR and SSR amplification

3.4.2.6.1 Preparation of reaction mixture for thermal cycling of ISSR

The reaction mixture consisted of template DNA, PCR master mix and ISSR primer. The desired number of PCR cycles, time and temperatures for denaturation, annealing and extension were standardised based on the primers used (Table 2) and the conditions were programmed and saved in the thermal cycler (model- AB Applied Biosystems Verti thermal cycler).

3.4.2.6.2 Thermal cycling of ISSR

1. PCR microcentrifuge tubes (0.2 ml) were numbered from 1 to 6.
2. 2.0 µl of template DNA from individual variety was added to each tube.
3. 16.5 µl of the master mix was added to all the tubes and was given a short spin to mix the contents.

Thermal cycling was carried out with 20 µl reaction mixture. The composition of the reaction mixture is detailed below

a.	Genomic DNA (25 ng/µl)	2.0 µl
b.	PCR master mix	16.5 µl
c.	Primer (10 *pM*)	1.5 µl
	Total reaction volume	**20.0µl**

4. The tubes were placed in the thermal cycler for 35 cycles of PCR. The PCR programme followed was as follows:

62

a.	94 °C for 4 min.	Initial denaturation	
b.	94 °C for 45 sec.	Denaturation	35 cycles
c.	50 °C to 55 °C for 1 min.	Primer annealing	
d.	72 °C for 2 min.	Primer extension	
e.	72 °C for 8 min.	Final extension	
f.	4 °C hold for infinity	Storage	

5. Samples were held at 4 °C in the thermal cycler followed by storage at -20 °C until the contents were loaded on to the gel for electrophoresis.
6. The PCR amplified products were electrophoresed on 1.8 per cent agarose gel at 70 volts. A ProxiO 100 bp DNA Ladder Plus (SRL) was used. The gel profile was visualized under UV and was saved for further analysis.

Table 2. List of ISSR primers used for screening

Sl. No.	Name	Sequence (5'-3')	Annealing temperature (°C)
1	Oligo ISSR 04	ACACACACACACACACC	53.30
2	Oligo ISSR 05	CTCTCTCTCTCTCTTG	42.40
3	Oligo ISSR 07	CTCTCTCTCTCTCTTG	42.40
4	UBC-807	AGA GAG AGA GAG AGA GT	54.00
5	UBC-808	AGAGAGAGAGAGAGAGC	54.00
6	UBC-809	AGAGAGAGAGAGAGAGG	54.00
7	UBC-810	GAG AGA GAG AGA GAG AT	52.70
8	UBC-811	GAGAGAGAGAGAGAGAC	53.00
9	UBC-812	GAGAGAGAGAGAGAGAA	51.50
10	UBC-813	CTCTCTCTCTCTCTCTT	50.50
11	UBC-814	CTCTCTCTCTCTCTCTA	49.00
12	UBC-815	CTCTCTCTCTCTCTCTG	52.70
13	UBC-816	CACACACACACACACAT	52.00
14	UBC-818	CTCTCTCTCTCTCTCTG	52.20
15	UBC-822	TCTCTCTCTCTCTCTCA	49.50

63

Table 2 continued

Sl. No.	Name	Sequence (5'-3')	Annealing temperature (°C)
16	UBC-823	TCTCTCTCTCTCTCTCC	50.50
17	UBC-825	ACACACACACACACACT	52.00
18	UBC-826	ACACACACACACACACC	45.70
19	UBC-827	ACACACACACACACACG	54.90
20	UBC-834	AGAGAGAGAGAGAGAGCT	51.00
21	UBC-835	AGAGAGAGAGAGAGAGCC	53.00
22	UBC-836	AGAGAGAGAGAGAGAGYA	54.00
23	UBC-841	GAGAGAGAGAGAGAGACC	50.50
24	UBC-844	CTCTCTCTCTCTCTCTRC	50.60
25	UBC-846	CACACACACACACACAAT	45.10
26	UBC-847	CACACACACACACACARC	46.00
27	UBC-854	TCTCTCTCTCTCTCTCRG	51.40
28	UBC-855	ACACACACACACACACYT	60.50
29	UBC-857	ACACACACACACACACYG	51.60
30	UBC-866	CTCCTCCTCCTCCTCCTC	60.50
31	UBC-868	GAAGAAGAAGAAGAAGA	47.80
32	UBC-880	GGAGAGGAGAGGAGA	49.10
33	UBC-890	AGCACTAGCGTGTGTGTGTGTGT	50.90

3.4.2.6.3 Preparation of reaction mixture for thermal cycling of SSR

The reaction mixture consisted of template DNA, PCR master mix and SSR primer (forward and reverse). The desired number of PCR cycles, time and temperatures for denaturation, annealing (AT) and extension were standardised based on the primers used (Table 3) and the conditions were programmed and saved in the thermal cycler (model- AB Applied Biosystems Verti thermal cycler).

64

3.4.2.6.4 Thermal cycling of SSR

1. PCR microcentrifuge tubes (0.2 ml) were numbered from 1 to 6.
2. 1.0 µl of template DNA from individual variety was added to each tube.
3. 7.0 µl of the master mix was added to all the tubes and was given a short spin to mix the contents.

Thermal cycling was carried out with 10 µl reaction mixture. The composition of the reaction mixture used is detailed hereunder:

a.	Genomic DNA (25 ng/µl)	1.0 µl
b.	PCR master mix	7.0 µl
c.	Primer (10 *pM*)	1.0 µl each of forward and reverse primer
	Total reaction volume	**10.0µl**

4. The tubes were placed in the thermal cycler for 30 cycles of PCR. The PCR programme followed is detailed below:

a.	94 °C for 4 min.	Initial denaturation	
b.	94 °C for 45 sec.	Denaturation	30 Cycles
c.	50 °C to 55 °C for 1 min.	Primer annealing	
d.	72 °C for 2 min.	Primer extension	
e.	72 °C for 8 min.	Final extension	
f.	4 °C hold for infinity	Storage	

5. Samples were held at 4 °C in the thermal cycler followed by storage at -20 °C until the contents were loaded on to the gel for electrophoresis.
6. The PCR amplified products were electrophoresed on 2.0 per cent agarose gel at 70 volts. A ProxiO 100 bp DNA Ladder Plus (SRL) was used. The gel profile was visualized under UV and was saved for further analysis.

65

3.4.2.7 Observations recorded

The gel profiles of individual ISSR and SSR primer were carefully observed and well resolved bands were scored for the presence or absence of band and this data was used for further analysis. The analysis of the molecular weight of PCR images was done by using Navigating 1D MAX Fire reader software, UVITECH Cambridge.

3.4.2.7.1 Nature of amplification

UVITEC Fire Reader software, gel documentation system was used to capture the image for analysing the banding pattern resolved by gel electrophoresis. Observation on the nature of the banding pattern was recorded as monomorphic or polymorphic.

3.4.2.7.2 Number of amplicons

UVITEC Fire Reader software used for gel documentation was used to count the number of amplicons resolved on the electrophoresed gel.

3.4.2.7.3 Size of amplicons

Using UVITEC Fire Reader software the size of amplicons for each marker resolved on the gel were estimated in base pairs (bp) by comparison with a known molecular weight ladder that was run along with PCR product.

3.4.2.7.4 Uniqueness of amplicons

UVITEC gel documentation system was also used for identifying the uniqueness of amplicons in terms of size in base pairs (bp) by comparing the banding pattern observed in the gel image of each primer.

66

3.4.3 Statistical analysis

3.4.3.1. Statistical analysis of morphometric data

Statistical analysis of the data on various morphological data was performed using the Web Agri Stat Package (WASP) developed by Indian Council of Agricultural Research for randomised block design and significant test by Duncan's Multiple Range Test (DMRT). Analysis of variance (Panse and Sukhatme, 1985) of the data collected from the various experiments was done to test the significance of differences among genotypes with respect to the characters and to estimate the variance components. Cluster analysis of qualitative and quantitative data and Principal component analysis (PCA) of quantitative data was performed by using Minitab 18.1.0 software.

Table 3. List of SSR primers (with their forward and reverse sequences) used for screening

Sl. No.	Primer name	Primer sequence (5'-3')	Annealing temperature (°C)
1	CMTm19	F: GCATGGGAGATGAAGGTTAG R: ATTTCCTGGTGGTATGAGATTC	55.60
2	CMTm61	F: GCCATTATTCCACTCCATGC R: TGCCTGCACCTGTTTTAGC	59.00
3	CMTm20	F: GTGGGCCATATCGATTCACT R: CGAAAGTCGCAGAGAACACA	58.70
4	CMTm60	F: TCCTCCAAAGCATACCAACTGT R:GCGCCATTTTATTGATTGGAT	59.00
5	CMTm52	F: GCTCTCCATTTTCCAGCTTC R: GACGCAGAGGGAGATTAATGA	58.00
6	CMTm88	F: CATCGACATTCGCCTCATC R: AGGCAGCTTCCAAATCAGC	59.00
7	CMTm144	F: ACATGGGCATACCTCGAATC R: CACCTGGCTGTTTTGTCTGA	58.80
8	CMTm34	F:TGAAACTACACTACATGACCTTGG R: TGGGTTGGTAGACTTGTAGTTGA	56.90

67

Table 3 continued

Sl. No.	Primer name	Primer sequence (5'-3')	Annealing temperature (°C)
9	CMTm97	F: AGGGGGAACTGATAGTCATCG R: ACCTCACCTTCCATCGTCAC	59.00
10	CMTp201	F: GTGGAAGTTACTGCGATTGG R: GCAAAGAATGTCCTCAGCAG	57.10
11	CMTm48	F: AAGCCTTTGGGGACCTTTAC R:TTGAAACCTTCAAACAAGAAATTG	57.90
12	CMTm259	F: ACCTCGAGGAAGCAAAAATG R: ATGGAGACGCGCAAGTAGA	58.00
13	CMTm9	F: TTTTTGTGTGCGTGTGTGG R:GCCCAGAAGACAAAAGTTCG	58.60
14	CMTm7	F: AACCAAACTCCGGCAAGA R: GTTCTCTCCGTTCAGGATGG	58.30
15	CMTm29	F: AGCGCAGCGACAGAAAAG R: AGCTTCTACGAAGGCGAGGT	59.00
16	CMTm232	F: AGAAAGAAATAAGGAACCCACAG R: CGTCTCGCAATTCTTCAACT	56.30
17	CMTp18	F: ACACCTTCGCTTCCGACATC R: TGACATCACTCCGGCAACTC	61.40
18	CMTm111	F: CTCCATTCCCATGGCTTC R: CCATGAGCTTGAGAGAGGTG	57.90
19	CMTm88	F: CATCGACATTCGCCTCATC R: AGGCAGCTTCCAAATCAGC	50.00
20	CMTm91	F: CCCTAGAATTAGTGGGCAAT R: TAGGCCTAAAAAGACCCAAT	50.00

3.4.3.2. Statistical analysis of molecular data

The data generated from the molecular weight analysis of all ISSR and SSR primers were compiled together to form a data sheet for cluster analysis. The ISSR and SSR primers across the six varieties were scored. For the presence of each band 1 code has been used, while, for its absence, 0 code has been allotted for each primer. Pair-wise similarity coefficient matrix was generated by Jaccard's coefficient of similarity by using NTSYS-PC (Numerical Taxonomy and

68

Multivariate Analysis System) version 2.10. The cluster analysis was performed from the distance matrix using Jaccard's similarity coefficient. Distance matrix and dendrogram were constructed based on diversity coefficient generated from pooled data by using the unweighted pair group method of arithmetic means (UPGMA), a computer programme for distance estimation.

A PIC (Polymorphic Information Content) were calculated using the following formulas. A PIC of each primer was determined using PIC calculator (Jan, 2002).

$$\text{PIC} = \frac{\text{Total no. of bands} - \text{Highest allelic Frequency}}{\text{Total no. of bands}}$$

3.4.4 DNA fingerprinting

Out of 33 ISSR and 20 SSR, the markers which produced unique bands were selected by visualising the gel images of six varieties under each marker system for making fingerprints. The DNA amplification pattern of selected primers was depicted in tabular form using different colour codes.

69

Results and discussion

4. RESULTS AND DISCUSSION

Characterisation and identification of varieties are essential pre-requisites in seed quality control programs. It ensures access to quality seed material of specific variety. The present study was implemented in the Department of Seed Science and Technology, Kerala Agricultural University (KAU) during 2018-2019 with the objective of characterising pumpkin varieties through morphological and molecular markers. The results obtained are enumerated and discussed below.

4.1 Experiment 1: Morphological characterisation of pumpkin varieties

Morphological characters plays a vital role in describing and identifying a genotype. The result of characterisation of six pumpkin varieties with respect to the 28 quantitative and 16 qualitative traits are enumerated and discussed further.

4.1.1 Evaluation and grouping of varieties based on morphological traits

4.1.1.1 Analysis of variance

Test of analysis of variance (ANOVA) for different quantitative parameters revealed significant differences in all the measured traits except for length of seed, indicating the existence of wide variability among the six pumpkin varieties. The mean values for the quantitative traits are presented in Table 4a, 4b and 4c.

4.1.1.2 Seedling characters (cotyledon length and width)

Cotyledon length was found to vary between 5.07 cm in Arka Chandan and 8.16 cm in CO-2. Cotyledons of Arka Chandan belonged to medium length category compared to long cotyledons in the other varieties.

Results revealed that the cotyledon width ranged from 2.42 cm (Arka Chandan) to 4.02 cm (CO-2) with an average of 3.41 cm. The cotyledon width of CO-1 (3.86 cm) was found to be on par with CO-2. Based on this trait, the cotyledon of varieties CO-1 and CO-2 was classified as broad compared to the narrow ones

found in variety Arka Chandan. Varieties Ambili, Saras and Suvarna were found to possess cotyledons of medium width.

Significant variations observed in seedling characters *viz.*, cotyledon length and width stressed its utility as a descriptor for characterisation of the studied varieties. The usefulness of cotyledon length and width in identifying and characterising *Cucumis melo* genotypes were confirmed earlier by workers (Yildiz *et al.*, 2014; Gocmen *et al.*, 2017).

4.1.1.3 Early plant vigour

Initial plant growth vigour varied among the varieties. At 30 DAS, early plant vigour varied from poor to vigouros. among varieties. Variety Arka Chandan possessed poor vigour, while Suvarna, CO-1 and CO-2 were vigouros. Ambili and Saras exhibited intermediate vigour habit (Table 5a).

Kiramana and Isutsa (2017a) classified 155 pumpkin cultivars based on early plant growth habit as either poor, intermediate or vigouros. The probable reason for the difference in early growth habit between varieties was attributed to the variation in seed size. Seed size has been observed to exhibit a direct effect on amount of food reserve and hence in germination, early seedling and plant vigour. Studies by Ndoro *et al.* (2012) in pumpkin also emphasised the importance of seed morphological parameters on early plant vigour.

4.1.1.4 Tendril characteristics

All the six varieties had coiled, branched tendrils (Table 5a). The tendril stalk length varied from 2.19 cm in variety CO-2 to 6.34 cm in Saras. Varieties CO-2 and Suvarna (2.50 cm) were found to be on par with each other.

72

4.1.1.5 Leaf characteristics

4.1.1.5.1 Leaf dimensions

Leaf blade length was highest for Ambili (16.78 cm) and lowest in Saras (15.51 cm). However, leaf blade length of Suvarna (16.29 cm), CO-1 (16.37 cm), CO-2 (16.65 cm) and Arka Chandan (16.37 cm) were on par with Ambili. However, all the six varieties were categorised into medium class based length of leaf blade. Grubben and Ngwerume (2004) described that leaf length of *Cucurbita moschata* genotypes were 9.00 to 24.00 cm long.

Leaf blade width ranged from 23.14 cm (Ambili) to 21.03 cm (CO-1) with a mean of 21.73 cm. On the basis of leaf blade width observed, all the six varieties were grouped under broad category.

Petiole length ranged from 21.87 cm to 18.97 cm. Variety Ambili was observed to have longer petioles (21.87 cm) and was found to be on par with CO-2 (21.05 cm). Petiole length was the least in Arka Chandan (18.97 cm). It was on par with Saras (19.50 cm). All the six pumpkin varieties grouped under long category on the basis of petiole length. Kiran and Ranjith (2018) observed significant variation in petiole length among 30 pumpkin genotypes.

Although variations in *per se* dimensions of leaf *i.e.* blade length and width along with petiole length was observed among the varieties, all of them grouped into the same category (broad and medium long leaves with long petioles). The result was found to be in concurrence with the findings of Bhagat (2017), Gocmen *et al.* (2017) and Kumari *et al.* (2019). They observed the ineffectiveness of metric leaf and petiole parameters for distinguishing and grouping melon, spine gourd and *Luffa aegyptiaca* genotypes. Unlike this, Pornsuriya *et al.* (2011) in bitter gourd, Kalyanrao *et al.* (2016) in bottle gourd and Nagar *et al.* (2017) in pumpkin found that leaf dimensions played an essential tool in characterisation of genotypes into groups to aid in identification.

73

4.1.1.5.2 Leaf shape and colour

The shape of leaves in all the varieties was cordate (Plate 4). Agbagwa *et al.* (2007), Ajuru and Okoli (2013) and Kiramana and Isutsa (2017a) had also observed absence of variability in leaf shape among various pumpkin genotypes.

Results revealed that leaf blade margin was weakly incised in varieties Ambili, Saras, Suvarna, CO-1 and CO-2 and moderately incised in Arka Chandan. A study by Nagar *et al.* (2017) also revealed that genotypes could be grouped into either weakly incised (53 genotypes) or very weakly incised (22 genotypes) category depending on leaf margin.

Out of six varieties examined for upper side leaf blade colour, varieties Ambili, Suvarna and CO-2 possessed dark green leaf blade, while in the remaining three varieties *i.e.,* Saras, CO-1 and Arka Chandan, the green colour on the upper side of leaf was of medium intensity (Table 5a).

Hence, according to the above, leaf incision (blade margin) and intensity of green colour on the upper side of leaf could be used as diagnostic feature in grouping and characterisation of pumpkin genotypes. The results are in concurrence with the previous studies, reporting the significant importance of leaf colour as a diagnostic trait in grouping genotypes of pumpkin (Lebeda *et al.,* 2009; Du *et al.*, 2011; Ajuru and Okoli, 2013; Nagar *et al.*, 2017 and Kiramana and Isutsa, 2017a), bitter gourd (Dey *et al.*, 2006), spine gourd (Bhagat, 2017).

4.1.1.5.3 Leaf blade silver patches and pubescence density

All the varieties except Arka Chandan had silver patches on leaf blade (Plate 4). Nagar *et al.* (2017) grouped 76 genotypes of pumpkin based on the presence (75 Nos.) or absence (1 No.) of silvery patches. Paris and Brown (2005) stated that the presence of silvery greyish patches on upper leaf axil is controlled by dominant gene *M* and absence by the recessive gene *m.* Presence of air spaces within the

74

palisade cell layer and between this layer and epidermis can lead to formation of silvery patches on upper leaf surface (Brown, 2002).

With respect to pubescence density on adaxial surface of leaf (Plate 5), the highest density was found to exist in variety Ambili (14.60/mm^2). It was found to be on par with variety CO-1 (14.00/mm^2). Minimum pubescence density was recorded in Suvarna (5.27/mm^2). The varieties were grouped into soft, intermediate and dense pubescence category, respectively. Kiramana and Isutsa (2017a) had employed leaf pubescence density to distinguish and assign distinct groups for easy and effective identification of pumpkin cultivars.

4.1.1.6 Plant main vine length

Vine length was longest in CO-2 (4.99 m), followed by Suvarna (4.13 m) and Ambili (3.92 m), whereas, Saras (3.22 m) registered the least length of vine at time of harvest. Saras was found to be on par with Arka Chandan (3.37 m). The result is in line with the findings of Ahmed *et al.* (2011) who reported that vine length of pumpkin genotypes ranged from 169.00 cm to 400.00 cm.

Based on main vine length, Ambili, Saras, Suvarna, CO-1 and Arka Chandan were grouped into medium category (2.50-4.50 m), while CO-2 belonged to the long vine (> 4.50 m). Nagar *et al.* (2017) could classify 75 genotypes during DUS testing in a similar fashion.

4.1.1.7 Flower characteristics

4.1.1.7.1 Node number at which first female flower appears

The node at which the first female flower appeared ranged from 13.73 (CO-1) to 17.08 (Suvarna). Early node number of female flower is considered as a desirable trait for crop improvement. Similar variations in this trait and its use in crop improvement has been reported earlier (Kumar *et al.*, 2011; Selvi *et al.*, 2012; Muralidhara and Narasegowda, 2014; Nagar *et al.*, 2017; Kiran and Ranjith, 2018)

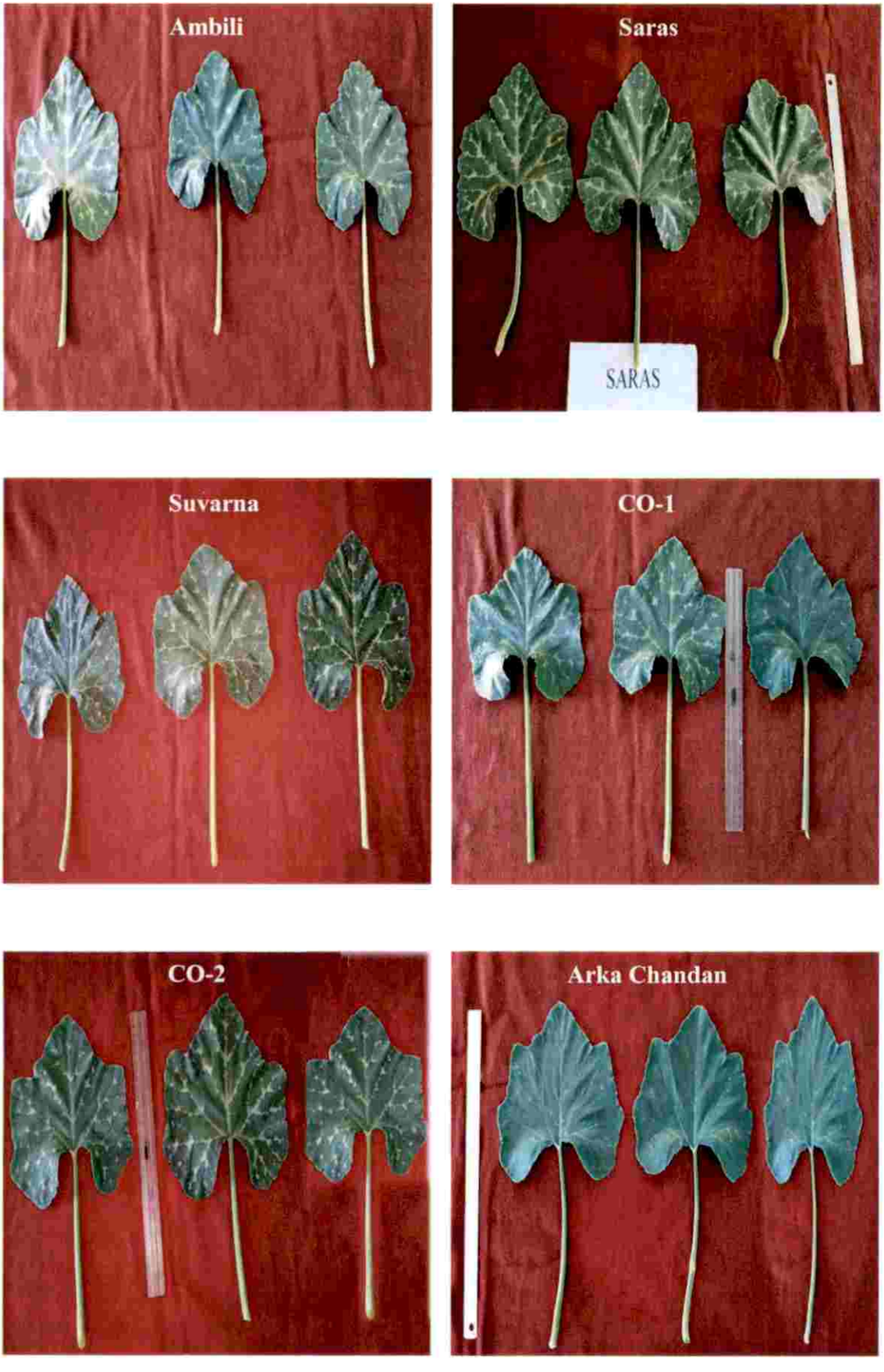


Plate 4. Leaf characteristics of six pumpkin varieties

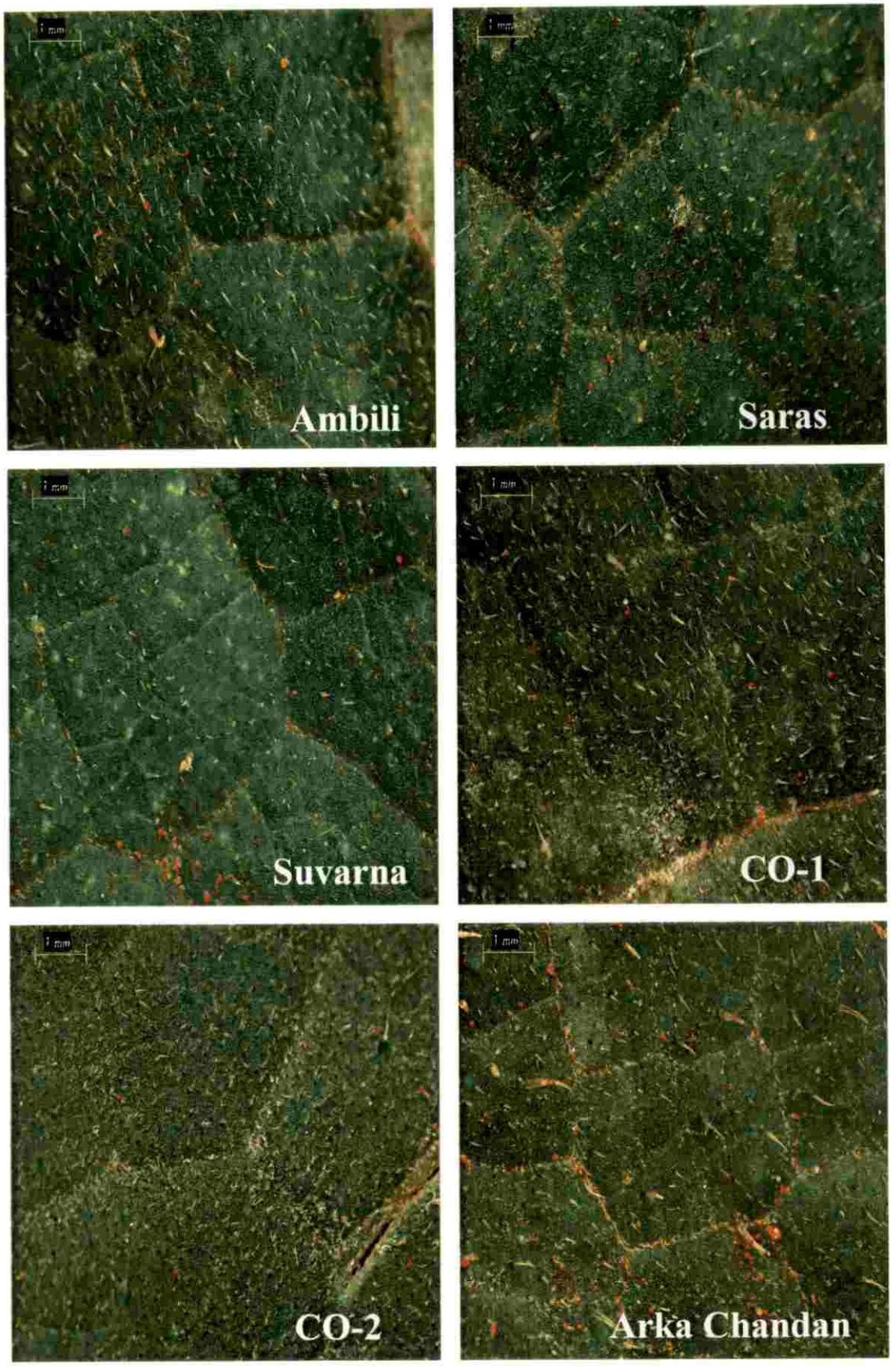


Plate 5. Leaf pubescence density of six pumpkin varieties

in various genotypes of pumpkin pointing to its utility in morphological characterisation and varietal identification.

A study by Tamilselvi and Jansirani (2016) reported that the average node number of first female flower appearance in Ambili was 25.62 and 21.12 in CO-2. This was contradictory to the observations in the present study (Ambili and CO-2: 14.80). However, the probable reason for the variation can be attributed to the influence of various environmental factors that prevailed earlier to or present at the time of crop growth. The variation shown for this character by same varieties in different experimental season/years confirms the strong influence of environment on trait expression thereby pointing to its limitation in accurate characterisation.

4.1.1.7.2 Days to 50 per cent flowering

Among the six varieties, days to 50 % flowering ranged from 49.25 days to 68.00 days. Variety Ambili had flowered the earliest (49.25 days), followed by Saras (55.50 days), CO-1 (55.25 days) and CO-2 (56.25 days). Variety Arka Chandan had taken more days for 50 % flowering *i.e.*, 68.00 days. However, in a study conducted for diversity analysis of pumpkin genotypes, Muralidhara and Narasegowda (2014) found that variety CO-2 and Arka Chandan took 41.67 and 41.00 days, respectively to attain for 50 % flowering, contradicting the results of the present research.

Ahmed *et al.* (2011) on working with 20 pumpkin accessions reported that days to 50 % flowering ranged from 55.00 days to 73.70 days. Significant variation in days to 50 % flowering was attributed to the genetic difference between varieties, similar to that stated by Mladenovic *et al.* (2012). Hence, this trait can act as an important tool in varietal classification and identification of any crop. The conclusion confirms the findings of Ara *et al.* (2015) in snake gourd, Kalyanrao *et al.* (2016) in bottle gourd, Bairwa *et al.* (2018) in ash gourd and Lakshmi *et al.* (2018) in rice.

78

Table 4a. Quantitative seedling, leaf, tendril, floral and plant characteristics of six pumpkin varieties

Characters/ Varieties	Cotyledon length (cm)	Cotyledon width (cm)	Tendril stalk length (cm)	Leaf blade length (cm)	Leaf blade width (cm)	Petiole length (cm)	Leaf pubescence density per mm^2	Plant vine length (m)	Node number of first female flower	Days to 50% flowering
Ambili	6.23^{c}	3.40^{b}	3.43^{c}	16.78^{a}	23.14^{a}	21.87^{a}	14.60^{a}	3.92^{bc}	14.80^{b}	49.25^{d}
Saras	6.53^{bc}	3.28^{b}	6.34^{a}	15.51^{b}	21.21^{bc}	19.50^{c}	8.53^{d}	3.22^{d}	14.00^{cd}	55.50^{c}
Suvarna	7.04^{b}	3.45^{b}	2.50^{d}	16.29^{a}	22.02^{b}	20.53^{b}	5.27^{e}	4.13^{b}	17.08^{a}	59.00^{b}
CO-1	6.99^{b}	3.86^{a}	4.30^{b}	16.37^{a}	21.03^{c}	20.59^{b}	14.00^{ab}	3.53^{cd}	13.73^{d}	55.25^{c}
CO-2	8.16^{a}	4.02^{a}	2.19^{d}	16.65^{a}	21.49^{bc}	21.05^{ab}	13.33^{b}	4.99^{a}	14.80^{b}	56.25^{c}
Arka Chandan	5.07^{d}	2.42^{c}	3.42^{c}	16.37^{a}	21.47^{bc}	18.97^{c}	11.93^{c}	3.37^{d}	14.68^{bc}	68.00^{a}
CD (0.05)	0.55	0.37	0.34	0.53	0.85	0.89	1.15	0.52	0.77	2.33
CV (%)	5.49	7.18	6.13	2.15	2.58	2.91	6.79	9.02	3.43	2.7

4.1.1.8 Fruit characteristics

4.1.1.8.1 Colour of fruit and waxiness of mature fruit skin

On determining the fruit skin colour at immature stage, the six pumpkin varieties were grouped into three categories *i.e.*, dark green (Ambili), medium green (Saras, Suvarna, CO-1 and CO-2) and light green (Arka Chandan).

At physiological maturity, the colour of fruit skin was observed and compared with RHS colour chart. The colour of fruit skin in varieties Ambili, Suvarna and CO-1 was cream brown, yellowish brown for variety Saras, whereas CO-2 and Arka Chandan possessed orange fruit skin (Plate 6).

The flesh colour of fruits of variety Ambili, Suvarna and CO-1 was found to be orange yellow colour, while that of Saras was orange. The flesh of CO-2 was orange with a green overcast and in Arka Chandan it was dark orange in colour (Plate 6).

As the mature fruit skin in all the six varieties exhibited waxiness, this character was not effective in grouping the pumpkin varieties. However, the study indicated that the colour of immature skin, colour of fruit skin at physiological maturity and the flesh colour of fruits can be effectively used for characterisation and varietal identification

4.1.1.8.2 Fruit shape and size

Fruit shape at peduncle end was found to be raised (Saras), flat (CO-1 and Arka Chandan) and moderately depressed (Ambili, Suvarna and CO-2).

With respect to shape of fruit at blossom end, the six varieties could be grouped as raised (Saras), flat (CO-1 and Ambili) and depressed (Suvarna, CO-2 and Arka Chandan) (Table 5b).

80

Overall fruit shape of Ambili, Suvarna and CO-2 was round flat. Fruit shape of Saras was elongate/oblong, whereas, the fruit of CO-1 was club shaped. The fruits of Arka Chandan were flattish round (Plate 6).

Diameter of blossom end scar was found to vary significantly from 0.94 cm in Saras to 2.48 cm in Arka Chandan. Based on scar diameter, the varieties were grouped as small with less than 1.00 cm diameter (Saras), medium having diameter ranging from 1.00-2.00 cm (Ambili, Suvarna, CO-1 and CO-2) and large with more than 2.00 cm diameter (Arka Chandan).

It was evident that the overall fruit shape and its shape at both peduncle end and at blossom end as well as the diameter of blossom end scar could prove useful for varietal identification.

4.1.1.8.3 Fruit dimensions

With respect to the length of peduncle, significant difference was observed between varieties. Long peduncle were observed in CO-2 (11.27 cm), whereas Arka Chandan had the shortest peduncle length of 4.90 cm. Varieties were grouped as short (Arka Chandan), medium (Ambili, Saras, Suvarna and CO-1) and long (CO-2) on the basis of peduncle length.

Average fruit length at maturity varied from 27.76 cm (Suvarna) to 19.79 cm (Arka Chandan). Length of fruit in Suvarna was on par with Saras (27.73 cm). The varieties Ambili, Saras, Suvarna, CO-1 and CO-2 grouped into long fruit length category, while, Arka Chandan fell into the medium category based on fruit length.

Suvarna was observed to have the largest fruit diameter (19.08 cm) at maturity and was also found to be on par with CO-2 (18.14 cm) and Ambili (17.95 cm), while, short fruit diameter was observed in Arka Chandan (12.25 cm). It was found to be on par with Saras (12.43 cm). Ambili, Suvarna, CO-1 and CO-2 grouped into medium (15.00-30.00 cm) category and the remaining two in short (> 15.00 cm) category.

81

The fruit circumference at maturity in Suvarna was 65.68 cm, which was on par with Ambili (64.56 cm). Variety Saras (44.09 cm) was observed to have the shortest fruit circumference. It was found to be on par with Arka Chandan (44.72 cm).

Flared length in fruit was found to vary between 2.16 cm (CO-2) and 1.49 cm (Arka Chandan) with a mean value of 1.84 cm. Suvarna (1.99 cm) was on par with CO-2, while Saras (1.64 cm) was on par with Arka Chandan that had registered the least value for this trait.

With respect to rind thickness, variety CO-2 was found to have the thickest rind (3.48 mm) and was on par with that observed in Suvarna (3.39 mm), followed by CO-1 (2.94 mm), Ambili (2.80 mm) and Arka Chandan (2.63 mm). The least rind thickness was observed in variety Saras (1.46 mm).

The fruit flesh thickness ranged from 2.17 cm (Saras) to 2.91 cm (CO-2). Variety Suvarna (2.63 cm) was found to be on par with CO-2. Based on measurements of fruit flesh thickness, the varieties Ambili, Saras and Arka Chandan could be grouped under the thin flesh class (< 2.50 cm) and the remaining three varieties *viz*., Suvarna, CO-1 and CO-2 belonged to medium flesh thickness class (2.50-4.50 cm).

The average values for seed cavity diameter in the varieties studied ranged from 8.10 cm (Arka Chandan) to 13.78 cm (Suvarna). Ambili (13.23 cm) was found to be on par with Suvarna. Variety Saras with a seed cavity diameter of 8.85 cm was on par with Arka Chandan, which had registered the least value for this trait.

It was evident that the fruit dimensions such as length of peduncle, fruit length, diameter and circumference, flared length, thickness of rind and flesh, and diameter of seed cavity had showed great variation because varieties studied had different fruit shape. Hence, these traits can prove useful in easy identification and establishing the trueness of the cultivars. Similar results were also shared by the other researchers in *Cucurbita* genus (Paris *et al*., 2006 and Seymen *et al*., 2012).

82

4.1.1.8.4 Number of ribs per fruit, number of fruits and fruit yield per plant

Ribs were present on fruits of all the varieties studied. However, the number of ribs/fruit exhibited sufficient variation. An average of 19.00 ribs per fruit was observed in CO-2, which was on par with Ambili (18.80 ribs/fruit) and Suvarna (18.83 ribs/fruit). An average 14.33 ribs/fruit was observed in Arka Chandan.

The number of fruits per vine (plant) varied from 1.00 (Arka Chandan) to 1.47 (Ambili). Ambili was on par with CO- 2 (1.33) and was followed by Suvarna (1.13) and CO-1 (1.13). Saras with 1.07 fruits per vine was on par with variety Arka Chandan that had registered the least value for this trait.

Suvarna recorded the highest fruit yield, 2.09 kg, followed by CO-2 (1.99 kg) and Ambili (1.86 kg). The least fruit yield per plant was observed in Arka Chandan (1.06 kg).

The above results pointed that the number of ribs on fruit could be useful along with quantitative traits like number of fruits and fruit yield per vine in grouping the varieties and varietal characterisation.

Qualitative and quantitative fruit traits studied in this experiment except for presence of waxiness in fruit skin showed high level of variations among the six varieties. This emphasises the key role of fruit characters in analysing diversity, characterisation and identification among any genotypes. Different experiments of Balkaya *et al*., (2009a), Balkaya *et al*. (2010b), Ahmed *et al.* (2011), Hamdi *et al*. (2017), Kiramana and Isutsa (2017a), Priori *et al*. (2018), Kirmi and Isutsa (2018) and Orsenigo *et al*. (2018) in different species of *Cucurbita* genus supports the results of present research.

83

Table 4b. Quantitative fruit characteristics of six pumpkin varieties

Characters/ Varieties	Diameter of scar at blossom end(cm)	Peduncle length (cm)	Fruit length (cm)	Fruit diameter (cm)	Fruit circumference (cm)	Flare length (cm)
Ambili	1.40^{bc}	8.57^{c}	25.25^{bc}	17.95^{a}	64.56^{a}	1.86^{b}
Saras	0.94^{d}	8.56^{c}	27.73^{a}	12.43^{c}	44.09^{d}	1.64^{c}
Suvarna	1.29^{bcd}	9.95^{b}	27.76^{a}	19.08^{a}	65.68^{a}	1.99^{ab}
CO-1	1.05^{cd}	9.89^{b}	26.51^{ab}	15.55^{b}	57.22^{c}	1.90^{b}
CO-2	1.46^{b}	11.27^{a}	24.47^{c}	18.14^{a}	60.25^{b}	2.16^{a}
Arka Chandan	2.48^{a}	4.90^{d}	19.79^{d}	12.25^{c}	44.72^{d}	1.49^{c}
CD (0.05)	0.36	0.93	1.30	1.30	2.88	0.20
CV (%)	16.76	7.12	3.42	5.43	3.41	7.31

Table 4b continued

Characters/ Varieties	Rind thickness (mm)	Fruit flesh thickness (cm)	Seed cavity diameter (cm)	Number of ribs/fruit	Number of fruits/plant	Fruit yield/plant (kg)
Ambili	2.80^{b}	2.25^{c}	13.23^{ab}	18.80^{a}	1.47^{a}	1.86^{ab}
Saras	1.46^{c}	2.17^{c}	8.85^{d}	17.00^{b}	1.07^{c}	1.25^{c}
Suvarna	3.39^{a}	2.63^{ab}	13.78^{a}	18.83^{a}	1.13^{bc}	2.09^{a}
CO-1	2.94^{b}	2.59^{b}	11.28^{c}	17.88^{ab}	1.13^{bc}	1.76^{b}
CO-2	3.48^{a}	2.91^{a}	12.60^{b}	19.00^{a}	1.33^{ab}	1.99^{ab}
Arka Chandan	2.63^{b}	2.47^{bc}	8.10^{d}	14.33^{c}	1.00^{c}	1.06^{c}
CD (0.05)	0.39	0.31	0.85	1.13	0.25	0.31
CV (%)	9.23	8.18	5.01	4.25	14.11	12.18

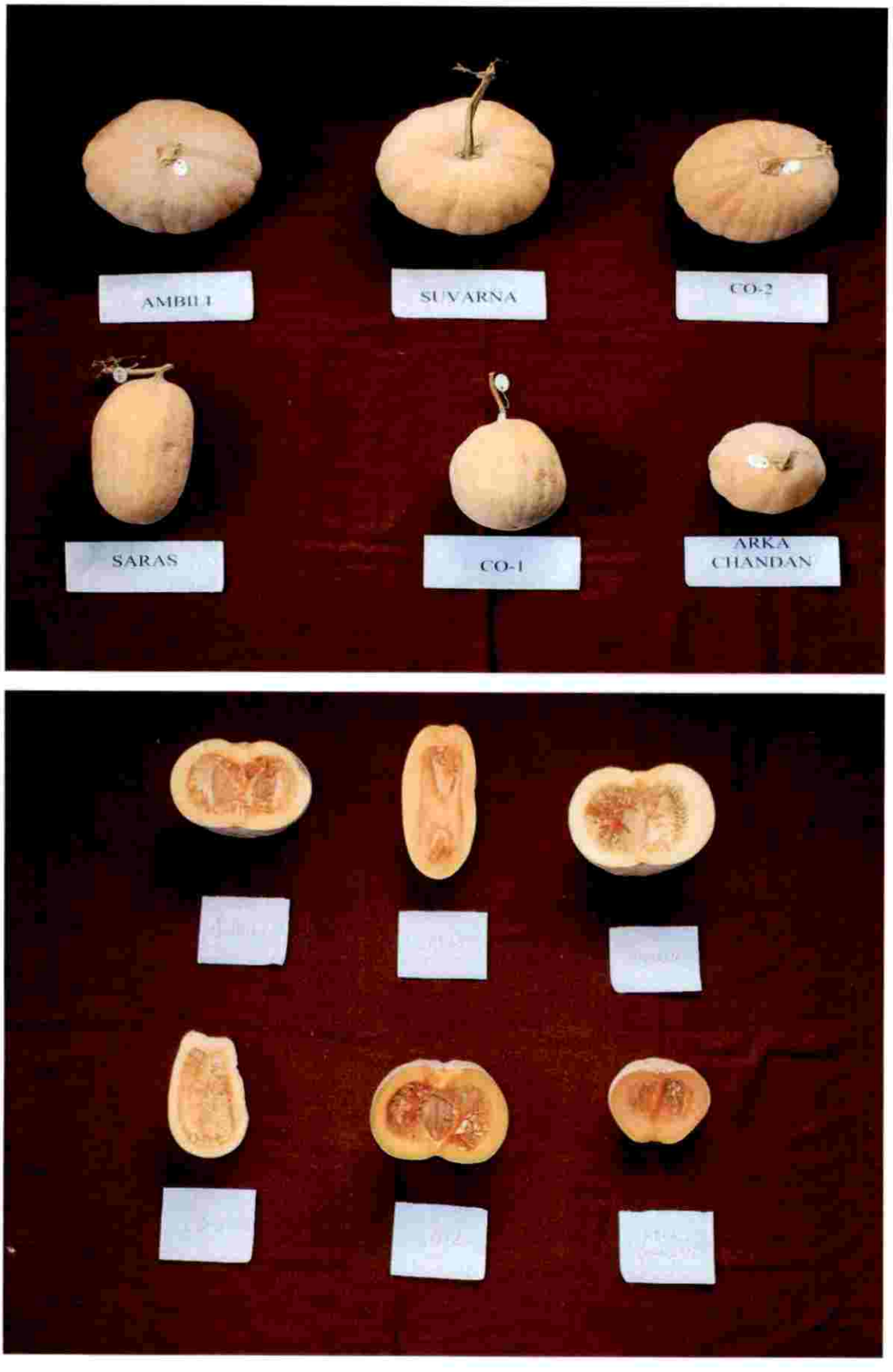


Plate 6. Fruit characteristics of six pumpkin varieties

4.1.1.9 Seed characteristics

4.1.1.9.1 Seed dimensions

The varieties did not exhibit significant difference on seed length. However, using the estimates of seed length, the varieties were grouped into two categories *i.e.*, medium (1.20-1.60 cm) in varieties Ambili and Arka Chandan and long (> 1.60 cm) in varieties Saras, Suvarna, CO-1 and CO-2. None of the varieties grouped under the short category.

Significant variations were found among the six varieties for seed width. Seed width was high in CO-1 (0.91 cm) and CO-2 (0.91 cm), while a minimum value of 0.58 cm was observed in Arka Chandan. Based on seed width, the six varieties grouped into three categories *i.e.*, small (< 0.60 cm: Arka Chandan), medium (0.60-0.90 cm: Ambili, Saras and Suvarna) and large (> 0.90 cm: CO-1 and CO-2).

Variety CO-1 (0.34 cm) recorded the thickest seed and found to be on par with CO-2 (0.33 cm) and Suvarna (0.33 cm), followed by Ambili (0.30 cm), while thin seeds was observed in Arka Chandan (0.18 cm).

4.1.1.9.2 Seeds per fruit

Average number of seeds per fruit ranged from 292.23 (Arka Chandan) to 467.88 (CO-2). Similar to CO-2, higher number of seeds per fruit was also observed in CO-1 (438.15) and Suvarna (398.68) and these varieties were found to be on par with each other. The least number of seeds was recorded in Arka Chandan (292.23). It was on par with variety Ambili (303.57).

4.1.1.9.3 Hundred seed weight and seed volume

Significant variation was found between varieties for hundred seed weight. High test weight was recorded in CO-1 (13.73 g), followed by CO-2 (13.34 g), while the least test weight was observed in Arka Chandan (6.47 g).

86

Volume of 100 seeds varied from 5.81 ml (Arka Chandan) to 15.42 ml (CO-2). The seed volume of CO-1 (13.29 ml), Suvarna (12.25 ml) and Ambili (11.82 ml) was on par with each other.

4.1.1.9.4 Seed coat colour

The seed coat of varieties Ambili, Saras and Suvarna was cream in colour while in CO-1, CO-2 and Arka Chandan, it was creamish brown in colour (Plate 7).

Seed morphological traits varied significantly among the studied varieties and play a key role in conducting grow out test of varieties and characterisation of genotypes in laboratory conditions. Numerous studies done for characterisation of various genotypes in pumpkin (Kiramana and Isutsa, 2017b and Nagar *et al.*, 2017), summer squash (Seymen *et al.*, 2012), winter squash (Hamdi *et al.*, 2017 and Orsenigo *et al.*, 2018), watermelon (Mahla *et al.*, 2014), cucumber (Pal *et al.*, 2017), bitter gourd (Sidhu and Pathak, 2016), *etc.* also provided evidence that seed characters can aid in varietal classification.

Although the *per se* estimates of seed length was non-significant, the seeds could be grouped into different classes. Thus it can be concluded that the qualitative character *i.e.,* seed coat colour and qualitative characters *viz.,* seed length, width and thickness, number of seeds per fruit, and hundred seed weight and volume can serve as keys for varietal identification.

Hence, on analysing the various quantitative and qualitative characters in six pumpkin varieties, it could be pointed out that fruit and seed traits have considerable implications in charactersing and categorising the six varieties into specific groups. The potentiality of these traits in evaluating, characterising and distinguishing genotypes was identified in different horticultural crops (Solmaz *et al.*, 2010; Das *et al.*, 2017; Kiramana and Isutsa, 2017a; Bhagwat *et al.*, 2018; Salim *et al.*, 2018).

87

Table 4c. Quantitative seed characteristics of six pumpkin varieties

Characters/ Varieties	100 seed weight (g)	Seed length (cm)	Seed volume (ml)	Seed width (cm)	Seed thickness (cm)	Seed count/fruit
Ambili	11.74^{d}	1.54	11.82^{b}	0.83^{b}	0.30^{b}	303.57^{c}
Saras	8.04^{e}	1.61	7.34^{c}	0.72^{d}	0.26^{c}	390.78^{b}
Suvarna	12.29^{c}	1.62	12.25^{b}	0.79^{c}	0.33^{ab}	398.68^{ab}
CO-1	13.73^{a}	1.65	13.29^{b}	0.91^{a}	0.34^{a}	438.15^{ab}
CO-2	13.34^{b}	1.64	15.42^{a}	0.91^{a}	0.33^{ab}	467.88^{a}
Arka Chandan	6.47^{f}	1.41	5.81^{c}	0.58^{e}	0.18^{d}	292.23^{c}
CD (0.05)	0.34	NS	1.59	0.04	0.04	75.90
CV (%)	2.04	10.16	9.63	3.64	9.09	13.19

Ambili

Saras

CO-2

CO-1

Suvarna

Arka Chandan

Plate 7. Seeds characteristics of six pumpkin varieties

Table 5a. Qualitative vegetative traits of six pumpkin varieties

Varieties/ Characters	Early plant vigour	Tendril	Tendril type	Tendril branching	Leaf shape	Leaf blade margin	Leaf blade colour (upper side)	Leaf blade silver patches
Ambili	Intermediate	Present	Coiled	Branched	Cordate	Weakly incised	Dark green	Present
Saras	Intermediate	Present	Coiled	Branched	Cordate	Weakly incised	Medium green	Present
Suvarna	Vigouros	Present	Coiled	Branched	Cordate	Weakly incised	Dark green	Present
CO-1	Vigouros	Present	Coiled	Branched	Cordate	Weakly incised	Medium green	Present
CO-2	Vigouros	Present	Coiled	Branched	Cordate	Weakly incised	Dark green	Present
Arka Chandan	Poor	Present	Coiled	Branched	Cordate	Moderately incised	Medium green	Absent

Table 5b. Qualitative fruit and seed traits of six pumpkin varieties

Varieties/ Characters	Colour of immature fruit	Fruit shape at peduncle end	Fruit shape at blossom end	Fruit shape	Colour of mature fruit	Waxiness of mature fruit skin	Colour of fruit flesh	Colour of seed coat
Ambili	Dark green	Moderately depressed	Depressed	Round flat	Cream brown	Present	Orange yellow	Cream
Saras	Medium green	Raised	Raised	Elongate/ oblong	Yellowish brown	Present	Orange	Cream
Suvarna	Medium green	Moderately depressed	Depressed	Round flat	Cream brown	Present	Orange yellow	Cream
CO-1	Medium green	Flat	Flat	Club shape	Cream brown	Present	Orange yellow	Creamish brown
CO-2	Medium green	Moderately depressed	Depressed	Round flat	Orange	Present	Orange with green overcast	Creamish brown
Arka Chandan	Light green	Flat	Depressed	Flattish round	Orange	Present	Dark orange	Creamish brown

4.1.2 Multivariate analysis

4.1.2.1 Cluster analysis and dendrogram of morphological characters

Cluster analysis takes into account the dissimilarity in several traits among the studied cultivars and delineates them into groups by displaying similarity or differences between pairs of subjects (Goda *et al.*, 2007).

Cluster analysis was performed using both quantitative and qualitative characters and the dendrogram generated is presented in Fig. 1. The similarity coefficient ranged from 0.37 to 0.89. Maximum similarity of 86.13 % was observed between varieties CO-1 and CO-2, whereas minimal similarity of 36.87 % was found to exist between varieties Ambili and Arka Chandan. Number of varieties in each cluster and distance between clusters are shown in Table 6 and 7. The maximum inter-cluster distances was recorded between the cluster IV and V (212.25) followed by the distance between cluster II and cluster V (169.97), indicating distinct genetic variation among the varieties in these three clusters. The lowest inter-cluster distance was observed between cluster II and IV (65.16) followed by cluster I and cluster II (90.69) suggesting comparatively close relationship among the varieties in these three clusters.

Table 6. Distribution of six pumpkin varieties based on morphological data

	Number of observations	Cluster members
Cluster1	1	Ambili
Cluster2	1	Saras
Cluster3	1	Suvarna
Cluster4	2	CO-1, CO-2
Cluster5	1	Arka Chandan

91

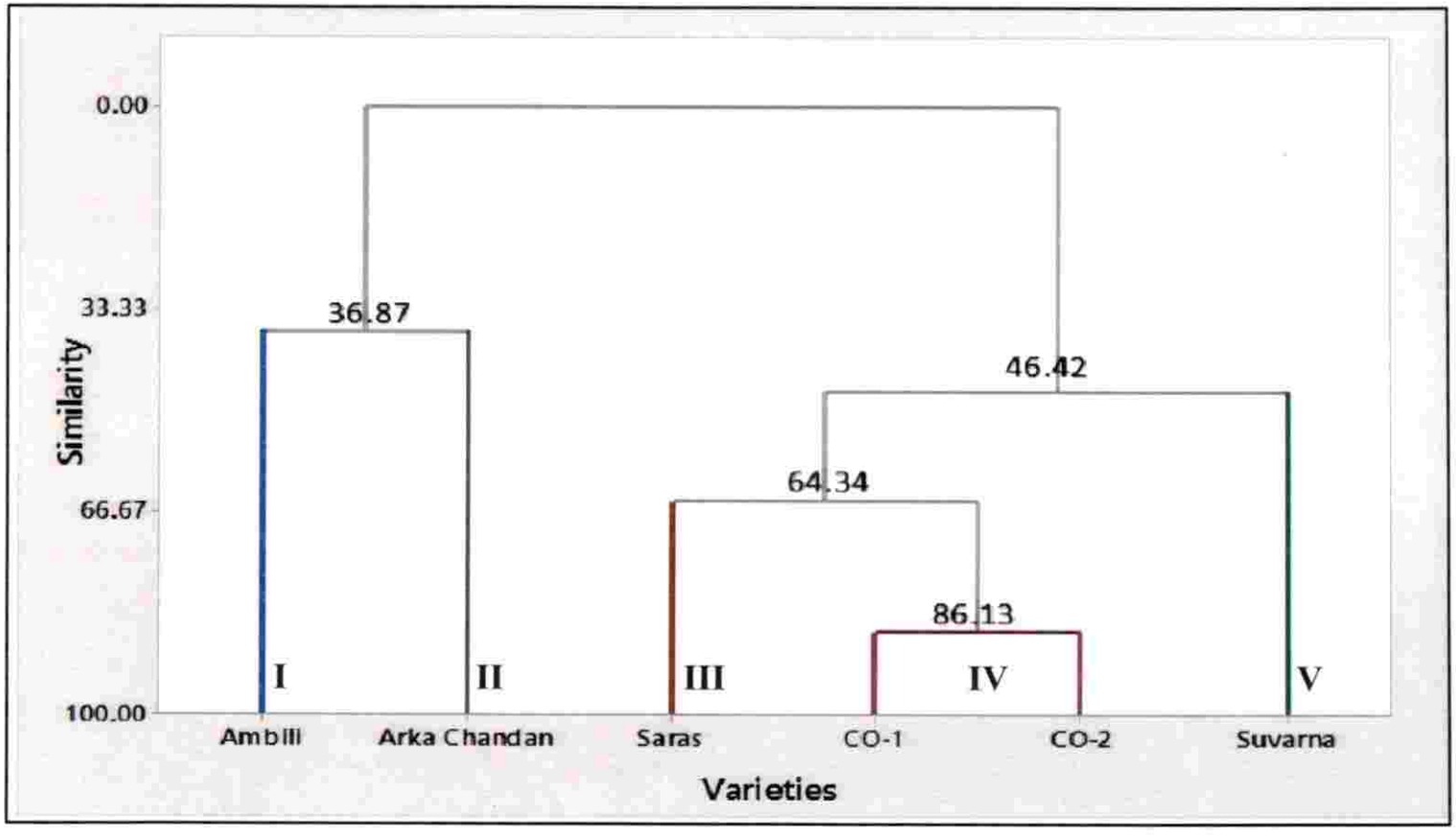


Fig 1. Dendrogram showing relationship among six pumpkin varieties based on quantitative and qualitative morphological data

Table 7. Distances between cluster centroids based on morphological data

	Cluster1	Cluster2	Cluster3	Cluster4	Cluster5
Cluster1	-				
Cluster2	90.69	-			
Cluster3	137.33	101.60	-		
Cluster4	149.82	65.16	112.81	-	
Cluster5	142.07	169.97	147.24	212.25	-

4.1.2.1.1 Cluster mean values for morphological traits

Differences were observed in cluster means for almost all the characters studied (Table 8). Cluster I was monogenic with a single variety *i.e.*, Ambili. The variety showed maximum mean value for leaf blade length (16.78 cm), leaf blade width (23.14 cm), leaf pubescence density (14.60/mm^2), petiole length (21.87 cm), earliness in 50 % flowering (49.25 days) and number of fruits per plant (1.47). This cluster registered the least mean values for plant main vine length (3.15 m). Variety Ambili had exhibited intermediate early plant growth and possessed dark green leaves as well as immature fruit.

Cluster II was also monogenic comprising of one variety Saras. The variety ranked first for tendril stalk length (6.55 cm) and second for fruit length (27.73 cm), while it possessed the shortest leaf blade length (15.51 cm) and width (21.21 cm), the least estimate for first female flower node (14.00), fruit circumference (44.09 cm), blossom scar diameter (0.94 cm) and rind thickness (1.46 mm) and fruit flesh thickness (2.17 cm). Elongate/oblong fruit shape was the characteristic feature of this cluster.

Cluster III was also monogenic with variety Suvarna. This cluster registered the least estimates for leaf pubescence density (5.27/mm^2) and tendril stalk length (2.41 cm). But it scored first rank for node of first female flower (17.08), fruit length (27.76 cm), fruit diameter (19.08 cm), fruit circumference (65.68 cm), number of

93

ribs/ fruit (18.83), rind thickness (3.39 mm), seed cavity diameter (13.78 cm) and hundred seed weight (12.19 g), all of which are desirable horticultural traits.

Cluster IV comprised of two varieties (CO-1 and CO-2) and this cluster registered the highest mean value for majority of traits *viz.,* cotyledon length (7.12 cm), cotyledon width (5.88 cm), peduncle length (10.58 cm), flared length (2.03 cm), fruit flesh thickness (2.75 cm), seed length (1.65 cm), seed width (0.88 cm), seed thickness (0.34 cm), seed volume (14.36 ml) and seed count per fruit (453.01), in addition to plant main vine length (4.32 m). Though the members of this cluster differed for a few traits, results indicate that these varieties were genetically more similar than the others. This may be due to their same geographic origin.

Cluster V contained only one variety, Arka Chandan. Though this cluster stood highest for blossom scar diameter (2.48 cm), it registered the least value for cotyledon length, cotyledon width, length of petiole, peduncle and fruit, fruit diameter, flare length, number of ribs per fruit, number of fruits per plant, fruit yield per plant, seed length, seed width, seed thickness, seed volume, seed count per fruit, seed cavity diameter and hundred seed weight. Poor early plant vigour, absence of silvery patches on upper leaf surface, uniqueness in immature fruit colour, fruit shape and flesh colour were the peculiar features of the member of cluster V.

Due to its ability to produce desirable compact clusters, cluster analysis using quantitative and qualitative characters provided wide scope to discriminate between the six pumpkin varieties studied.

The result that emphasise the importance of cluster analysis in grouping varieties/genotypes of this study are in accordance with findings of Mohsin *et al.* (2016); Oliveira *et al.* (2016); Kiramana and Isutsa (2017a), Kirimi and Isutsa (2018) and Krishnamoorthy and Sampath (2019). They also opined that clustering using morphological characters did not group the genotypes according to their geographical origin, but on the basis of dissimilarity of morphological traits.

Table 8. Cluster mean values for morphological traits of six pumpkin varieties

Variable	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Cotyledon length (cm)	6.27	6.54	6.92	7.12	5.22
Cotyledon width (cm)	3.36	3.28	3.44	3.88	2.42
Leaf blade length (cm)	16.78	15.51	16.29	16.51	16.37
Leaf blade width (cm)	23.14	21.21	22.02	21.26	21.47
Leaf pubescence density (per mm^2)	14.60	8.53	5.27	13.67	11.93
Petiole length (cm)	21.87	19.50	20.53	20.82	18.97
Node number of first female flower	14.80	14.00	17.08	14.26	14.68
Days to 50% flowering	49.25	55.50	59.00	55.75	68.00
Peduncle length (cm)	8.57	8.56	9.95	10.58	4.90
Tendril stalk length (cm)	3.86	6.55	2.41	3.26	3.18
Fruit length (cm)	25.25	27.73	27.76	25.49	19.79
Fruit diameter (cm)	17.95	12.43	19.08	16.85	12.25
Fruit circumference (cm)	64.56	44.10	65.68	58.74	44.72
Number of ribs/fruit	18.80	17.00	18.83	18.44	14.33
Rind thickness (mm)	2.80	1.46	3.39	3.21	2.63
Diameter of scar at blossom end (cm)	1.40	0.94	1.29	1.25	2.48
Fruit flesh thickness (cm)	2.25	2.17	2.63	2.75	2.47
Seed cavity diameter (cm)	13.23	8.85	13.78	11.94	8.10
100 seed weight (g)	10.30	8.42	12.19	11.42	6.74
Seed length (cm)	1.54	1.61	1.62	1.65	1.41
Seed width (cm)	0.83	0.72	0.79	0.88	0.63
Seed thickness (cm)	0.30	0.26	0.33	0.34	0.18
Seed count/fruit	303.57	390.78	398.68	453.01	292.23
Seed volume (ml)	11.82	7.34	12.25	14.36	5.81

95

Table 8 continued

Variable	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Number of fruits/plant	1.47	1.07	1.13	1.23	1.00
Fruit yield/plant (kg)	1.86	1.25	2.09	1.88	1.06
Plant main vine length (m)	3.15	3.34	4.05	4.32	3.36
Flared length (cm)	1.86	1.64	1.99	2.03	1.49
Leaf blade silver patches	9.00	9.00	9.00	9.00	1.00
Leaf blade colour (upper side)	7.00	5.00	7.00	5.00	5.00
Colour of immature fruit	4.00	3.00	3.00	3.00	2.00
Fruit shape at peduncle end	3.00	1.00	3.00	2.50	2.00
Fruit shape at blossom end	1.00	3.00	1.00	1.50	1.00
Fruit shape	2.00	3.00	2.00	4.50	99.00
Colour of mature fruit	1.00	1.00	1.00	2.00	3.00
Colour of fruit flesh	2.00	4.00	2.00	2.50	5.00
Colour of seed coat	1.00	1.00	99.00	1.00	99.00
Leaf blade margin	2.00	2.00	2.00	2.00	3.00
Early plant vigour	5.00	5.00	7.00	7.00	3.00

4.1.2.2 Principal component analysis using quantitative traits

Principal component analysis (PCA) discloses variation patterns and decreases redundancy in univariate analysis when dimensionality of data set involving muliticollinear variables (Maji and Shaibu, 2012). Eigen value one criterion (or Kaiser criterion) was used to retain and estimate the relative contribution of each Principal Component (PC) to the total variance of all the variables. A component with Eigen value > 1.00 accounted for a greater amount of variation and vice versa.

Principal Component Analysis (PCA) was performed with Minitab 18.1.0 software with a purpose to obtain a small number of linear combinations of 28 quantitative variables in six pumpkin varieties and to project the analysed varieties on the axis.

First four components, out of 28 trait components retained a cumulative Eigen value of more than 1.00. Contribution of characters towards divergence in the four PC of the varieties is summarised in Table 9. The sign on loadings or weights depicts the direction of relationship between the trait measured and the PC.

Cotyledon length (0.22), cotyledon width (0.22), petiole length (0.20), peduncle length (0.23), flared length (0.24), fruit diameter (0.22), fruit circumference (0.21), number of ribs per fruit (0.23), fruit yield (0.24), seed cavity diameter (0.22), 100 seed weight (0.22), seed width (0.21), seed thickness (0.23) and seed volume (0.24) loaded positive in PC1 which had registered an Eigen value 16.78 and accounted for 58 per cent of the total variations.

PC2 with an Eigen value of 5.19 and aggregating 18 per cent of the variation, had high coefficient scores for tendril stalk length, fruit length and seed length, but had negative load for leaf blade length, leaf blade width, rind thickness and diameter of scar at blossom end. Traits such as days to 50 per cent flowering (0.34), fruit flesh thickness (0.35), plant vine length (0.32) and seed count per fruit (0.29) were the major contributors to PC3. PC3 had an Eigen value of 3.86 that explained 13 per cent variability. PC4 with an Eigen value of 2.48 and accounted for 9 per cent of the variation and had high positive load for fruit length (0.25) and first female flower node (0.52). Characters with Eigen vector equal to or greater than 0.5 show large contribution effect to variation (Balkaya *et al.*, 2010 a and b).

The first four PCs together explained substantial amount of variations *i.e.*, 98 per cent. Similar to the study, Onyishi *et al.* (2013) found that the first four PCs explained very high variability among *C. maxima* accessions (98.60 per cent).

97

Table 9. Eigenvector values for principal components with Eigen value > 1

Variable	PC1	PC2	PC3	PC4
Cotyledon length (cm)	0.22	0.18	0.11	0.01
Cotyledon width (cm)	0.22	0.16	0.04	-0.14
Leaf blade length (cm)	0.12	-0.34	-0.11	-0.21
Leaf blade width (cm)	0.06	-0.21	-0.40	0.19
Leaf pubescence density per mm^2	0.02	-0.10	-0.18	-0.58
Petiole length (cm)	0.20	-0.07	-0.27	-0.08
Node of first female flower	0.08	-0.19	0.07	0.52
Days to 50 % flowering	-0.15	-0.17	0.34	0.08
Peduncle length (cm)	0.23	0.14	0.07	-0.01
Length of tendril (cm)	-0.11	0.36	-0.17	-0.04
Fruit length (cm)	0.13	0.31	-0.07	0.25
Fruit diameter (cm)	0.22	-0.14	-0.07	0.15
Fruit circumference (cm)	0.21	-0.14	-0.13	0.13
Rind thickness (mm)	0.17	-0.29	0.13	-0.02
Number of ribs/fruit	0.23	0.07	-0.12	0.08
Diameter of scar at blossom end	-0.14	-0.35	0.09	-0.07
Fruit flesh thickness (cm)	0.14	-0.16	0.35	-0.13
Seed cavity diameter (cm)	0.22	-0.10	-0.12	0.16
100 seed weight (g)	0.22	-0.06	0.08	0.10
Seed length (cm)	0.19	0.26	0.09	0.02
Seed width (cm)	0.21	0.09	-0.08	-0.21
Seed thickness (cm)	0.23	0.11	-0.02	-0.02
Seed count/fruit	0.16	0.19	0.29	-0.11
Seed volume (ml)	0.24	-0.03	0.03	-0.13
Number of fruits/plant	0.16	-0.11	-0.31	-0.13
Fruit yield/plant (kg)	0.24	-0.06	-0.03	0.11

98

Table 9 continued

Variable	PC1	PC2	PC3	PC4
Plant main vine length (m)	0.16	-0.07	0.32	-0.06
Flared length (cm)	0.24	-0.03	0.07	-0.03
Eigenvalue	16.78	5.19	3.86	2.48
Proportion	0.58	0.18	0.13	0.09
Cumulative	0.58	0.76	0.89	0.98

To explain the per cent of variance associated with each PC, scree plot was laid out by drawing a graph between Eigen values and PC (Fig 2). Semi curve line obtained after third PC suggested the importance of PC1, PC2 and PC3 for reflecting most of variation patterns. This also shows the association and usefulness of characters in differentiating the six pumpkin varieties. These results are corroborated with findings of Guei *et al.* (2005) and Balkaya *et al.* (2010b). Loading plot constructed between PC1 and PC2 is presented in Fig 3.

Results of the present study revealed the application of PCA in characterisation and estimation of extent of genetic variability among pumpkin varieties. The high scores for cotyledon length and cotyledon width, tendril length, petiole length, peduncle length, node of first female flower, days to 50 % flowering, flared length, fruit length, fruit diameter, fruit circumference, number of ribs per fruit, fruit yield, seed cavity diameter, seed count per fruit, hundred seed weight, seed length, seed width, seed thickness and plant main vine length in various PCs, indicated the importance of these traits for efficient characterisation and varietal identification. The findings are in conformity with the reports of Liu *et al.* (2013), Mohsin *et al.* (2016), Kiramana and Isutsa (2017b), Nagar *et al.* (2017), Kirimi and Isutsa (2018) and Krishnamoorthy and Sampath (2019) in pumpkin, Gichimu *et al.* (2009) in watermelon, Shankar *et al.* (2009) in bittergourd, Nagaraju and Thiruvenkatasamy (2017) in ashgourd.

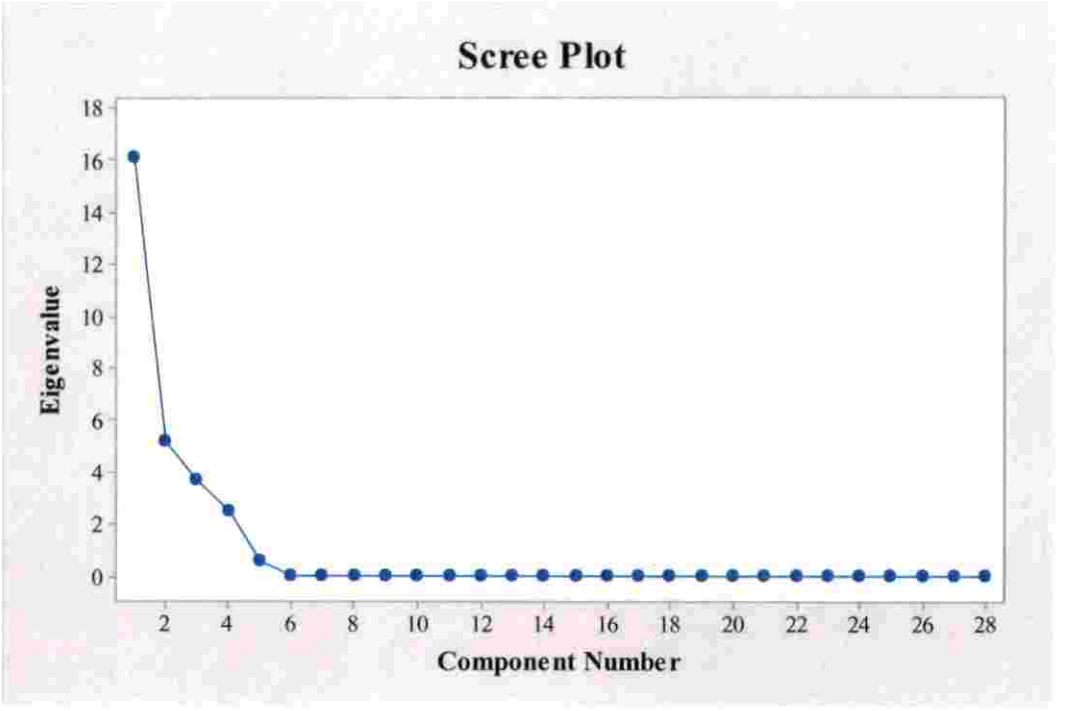


Fig 2. Scree plot of principal component analysis in six pumpkin varieties

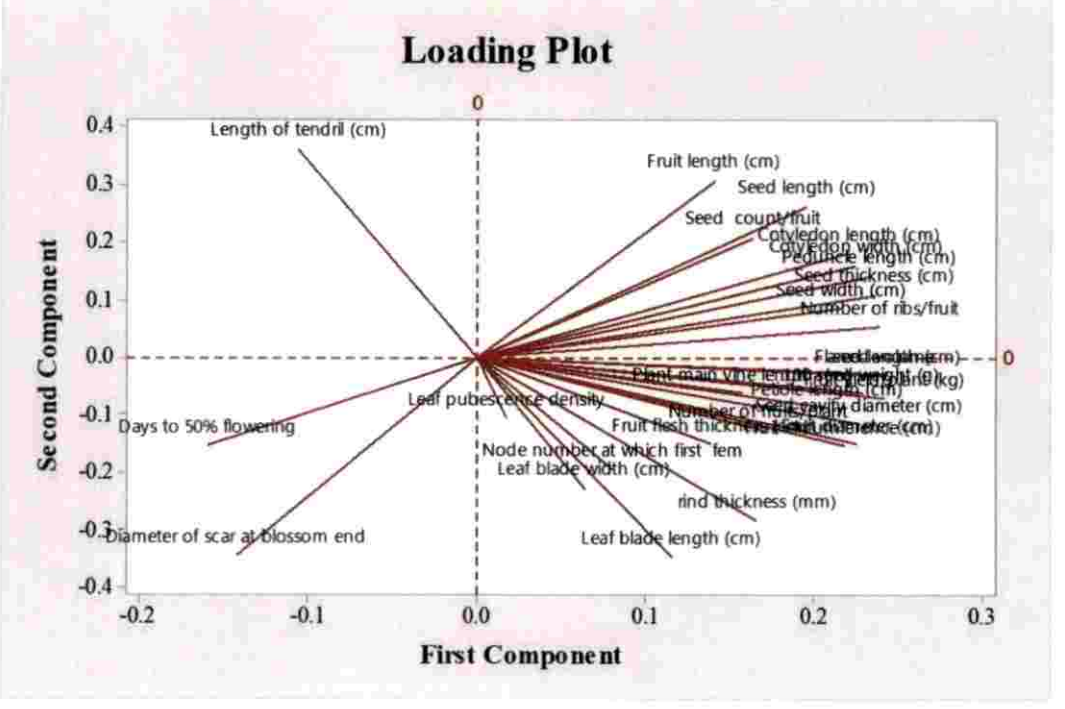


Fig 3. Loading plot on first two components of six pumpkin varieties in factor analysis extracted through principal components of correlation matrix

100

4.2 Experiment 2: Genotyping of pumpkin varieties using molecular markers

Cultivar identification and grow out test were conducted traditionally by morphological descriptors specific to a crop under consideration.

With the advent of biotechnology, in addition to morphological characterisation, use of fast and reliable molecular marker analysis have been employed to characterise and identify varieties.

4.2.1 Marker analysis for characterisation

PCR based ISSR and SSR markers were used to characterise six pumpkin varieties. ISSR and SSR markers that produced unique amplicons were then used for making DNA fingerprints or ID's of each varieties.

4.2.2 Quality and quantity of DNA isolated

The isolation of high quality DNA is important for all molecular biological analyses, because contaminants (proteins, polyphenols, *etc.*) can interfere with the end result.

Genomic DNA isolated from young tender leaves of each variety using Doyle and Doyle (1987) protocol and were checked for its quality and quantity (Table 10). The extracted DNA was confirmed to be of good quality through Agarose gel electrophoresis as well as through computation of the OD value (ratio between absorbance at 260 nm and 280 nm). The quantity (ng/g of leaf sample) of DNA was also found to be significant enough to aid easy analysis .

101

Table 10. OD value and quantity of DNA isolated in pumpkin varieties

Varieties	OD value (260/280)	Quantity (ng/g of leaf sample)
Ambili	1.94	69897.00
Saras	1.87	44068.00
Suvarna	1.98	73585.00
CO-1	1.83	97432.00
CO-2	2.06	87978.00
Arka Chandan	2.09	104100.00

4.2.3 Genotyping with ISSR markers

To characterise and measure the extent of variation between the six varieties, DNA of each pumpkin varieties were subjected to PCR amplification using 33 ISSR primers mentioned earlier in Table 2. Out of 33 ISSRs, only 28 markers showed polymorphic bands. The total number of amplicons, number of polymorphic amplicons, number of unique bands, range of amplicon size, PIC (Polymorphic Information Content) value and PP (Percent Polymorphism) of 28 polymorphic ISSR markers are tabulated (Table 11)

In the ISSR investigations, a total of 279 amplicons were produced, and among these, 68 were found to be polymorphic between all six varieties of pumpkin. The total number of amplicons detected by an individual primer ranged from 4 (UBC-818) to 18 (UBC-847). The results are similar with the findings of Santos *et al.* (2012), who screened 15 ISSR primers among 31 pumpkin genotypes and observed a range of 3-13 amplicons per primer. Inan *et al.* (2012) had observed the number of fragments detected in ISSR analysis varied from 4 to 15 per primer also substantiate the present result.

Polymorphic amplicons produced per primer varied from 1 (UBC-818) to 15 (UBC-847), while, the number of unique amplicons ranged from 1 (UBC-809,

102

UBC-810, UBC-813, UBC-818, UBC-835, UBC-841, UBC-846, UBC-854) to 7 (UBC-866).

The overall amplicon size varied from 156 bp (UBC-809: 156 bp-1134 bp) to 2178 bp (UBC-822: 328 bp-2178 bp). It was found to be within the range of results (343 bp to 3379 bp) reported by Khalil and Hassan (2016). Similar results have been reported by Behera *et al.* (2008) in bitter gourd accession. Haung *et al.* (2010) had observed 125 bands ranging in size from 150 bp to 2700 bp in bitter gourd accession on using 15 ISSRs.

The per cent polymorphism ranged from 30 % (UBC-809) to 100 % (UBC-841) with an average of 66.01 % across 28 ISSR primers across the six varieties of pumpkin studied. This was less than that reported in other studies on pumpkin (89 % Shang *et al.*, 2012; 100 % by Amiri *et al.*, 2017; 91.97 % by Abdein, 2018) but more than that reported by Kiani and Siahchehreh (2017) in pumpkin (61.60 %).

Polymorphic Information Content (PIC) provides an estimate of the discriminatory power of a marker to differentiate genotypes based on both the number of alleles expressed and their relative frequency (Naga *et al.*, 2012). Markers with PICs more than 0.50 are generally assumed to be efficient and useful in genotype discrimination and also for measuring degree of polymorphism at a given locus (De Woody *et al.*, 1995).

The PIC value was calculated for 28 ISSR primers and was found to vary between 0.64 (UBC-818) and 0.88 (UBC-809) with an average of 0.81. UBC-808 with a PIC value of 0.87 was next best to UBC-809.

Results (Table 11) pointed out that out of 28 ISSR markers, 18 markers registered a PIC value of more than 0.80. This indicated high discriminatory and differentiation power of those markers. Varshney *et al.* (2004) stated that high PIC value indicates high polymorphism and the presence of a rare allele or alleles at one marker locus and shows the high discriminatory and differentiation power of that marker.

Table 11. Particulars of ISSR primer profiling in pumpkin varieties

Sl. No.	Primer	No. of amplicons	No. of polymorphic amplicons	No. of unique bands	Amplicon size (bp)	PIC	PP
1	Oligo ISSR 05	6	5	2	531-1264	0.75	83.33
2	UBC807	9	7	3	1218-339	0.83	77.78
3	UBC808	12	8	2	1348-289	0.87	66.67
4	UBC809	10	3	1	1134-156	0.88	30.00
5	UBC810	9	7	1	1483-271	0.86	77.78
6	UBC811	8	7	2	1200-322	0.82	87.50
7	UBC812	11	7	3	1310-242	0.86	63.64
8	UBC813	5	3	1	1187-351	0.73	60.00
9	UBC815	10	7	4	1887-343	0.83	70.00
10	UBC816	7	5	2	1792-495	0.80	71.43
11	UBC818	4	1	1	682-338	0.64	25.00
12	UBC822	15	14	5	2178-328	0.86	93.33
13	UBC823	9	4	2	1161-442	0.84	44.44
14	UBC825	6	5	2	1838-565	0.75	83.33
15	UBC826	9	3	2	1070-362	0.85	33.33
16	UBC827	6	2	2	1606-357	0.75	33.33
17	UBC834	11	10	3	1560-621	0.86	90.91
18	UBC835	9	4	1	1630-382	0.86	44.44
19	UBC836	11	4	2	1821-179	0.86	36.36
20	UBC841	6	6	1	1274-595	0.78	100.00
21	UBC844	8	3	3	1364-494	0.82	37.50
21	UBC844	8	3	3	1364-494	0.82	37.50
22	UBC846	7	5	1	1443-619	0.81	71.43
23	UBC847	18	15	5	1595-300	0.86	83.33
24	UBC854	6	3	1	1613-406	0.77	50.00
25	UBC855	6	5	4	1544-428	0.67	83.33

10H

Table 11 continued

Sl. No.	Primer	No. of amplicons	No. of polymorphic amplicons	No. of unique bands	Amplicon size (bp)	PIC	PP
26	UBC857	7	5	3	1775-535	0.77	71.43
27	UBC866	14	13	7	1483-343	0.85	92.86
28	UBC868	7	6	2	1539-394	0.80	85.71

4.2.3.1 Cluster analysis and dendrogram construction using ISSR data

Cluster analysis using ISSR profile revealed the presence of high genetic variation among all varieties studied. The dendrogram based on Jaccard's similarity coefficients was constructed using UPGMA after analysis of banding patterns generated by 28 polymorphic primers across the six varieties.

A UPGMA-based dendrogram separated the six pumpkin varieties into four clusters when truncated at 85 per cent similarity (Table 12, Fig. 4a). Cluster I was the largest cluster, with three varieties (Ambili, Saras and Suvarna) and was divided into two, sub-clusters IA with Ambili and sub-cluster IB with Saras and Suvarna, whereas, the remaining three clusters were monogenic *i.e.,* with one variety each. The results indicated that varieties Ambili, Saras and Suvarna are inherently similar at genomic regions which have been amplified by the 28 ISSR primers used in the present study.

The Jaccard's similarity coefficient values obtained are presented in Table 13. The genetic similarity indices estimated on the basis of 28 ISSR primers between the six varieties of pumpkin ranged from 0.57 to 0.89, which implies the existence of a moderate level of variation between the studied varieties. This may be due to use of varieties from same sources rather than diverse ones. Similar range of Jaccard's similarity coefficient values (0.41 to 0.85) was found during genetic diversity analysis of bottle gourd genotypes (Bhawna *et al.*, 2014).

105

Table 12. Clustering of pumpkin varieties based on ISSR profile

Cluster	Number of members	Members of cluster
I	3	Ambili, Saras and Suvarna
II	1	CO-2
III	1	CO-1
IV	1	Arka Chandan

Varieties Saras and Suvarna, registered the highest Jaccard's similarity value among the studied varieties with a genetic coefficient of 0.89, indicating higher similarity in genetic composition among them, followed by Ambili and Saras (0.86). Minimum similarity was exhibited by Ambili and Arka Chandan (0.57), which indicated the existence of considerable genetic differences between these two varieties. Similarity coefficient values help the breeder to select diverse parents for hybridisation programme that leads to creation of modern pumpkin hybrids.

Table 13. Pair wise similarity between pumpkin varieties based on ISSR profile

	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
Ambili	-					
Saras	0.86	-				
Suvarna	0.86	0.89	-			
CO-1	0.65	0.65	0.67	-		
CO-2	0.74	0.75	0.81	0.69	-	
Arka Chandan	0.57	0.58	0.62	0.58	0. 68	-

To summarise, the 28 ISSR primers can be effectively used for differentiation, identification of cultivar and varieties and genetic diversity analysis of pumpkin varieties. This further indicated the capability of these PCR based ISSR

106

markers in fingerprinting as it examines and detects variation in genomic sequence among genotypes neutrally without any bias. The present investigation was in confirmation with earlier studies conducted in pumpkin and squashes (Paris *et al.*, 2002; Inan *et al.*, 2012; Santos *et al.*, 2012; Shang *et al.*, 2012; Esmailnia *et al.*, 2015 and Amiri *et al.*, 2017), muskmelon (Tomar *et al.*, 2016), cucumber (Punetha *et al.*, 2017), watermelon (Soghani *et al.*, 2018), *etc.*

4.2.3.2 Comparison between clustering pattern generated by ISSR and morphological data

Both morphological and ISSR data were efficient in clustering the varieties. However, no consistency in grouping was observed between the two. Morphological data based clustering grouped the six varieties into five clusters with varieties CO-1 and CO-2 grouping under the same cluster (Cluster IV). However CO-1 and CO-2 were disbursed into two different clusters based on ISSR data. Variety Saras and Suvarna shared a common sub cluster (IB) based on 28 ISSR marker data, while, these two varieties belonged to two different clusters based on the evaluated morphological data. Arka Chandan and Ambili belonged to different cluster in both ISSR and morphological data cluster analysis.

Such difference may arise since environmental influence on trait expression cannot be negated. In addition, ISSRs are repeat sequences and are capable of amplifying several loci along the genomic DNA.

4.2.4 DNA fingerprinting using polymorphic ISSR

To develop ISSR fingerprints of six pumpkin varieties used in the study, the primers that produced at least one variety specific unique band was selected. Twenty eight primers were selected for fingerprinting and are detailed below. The number of amplicons generated and the range of molecular band size is tabulated (Table 11).

4.2.4.1 UBC-866

Amplification of DNA of six varieties using primer UBC-866 (Plate 8) produced unique bands in CO-1 at 1235 bp, 944 bp and 791 bp, in CO-2 at 1483 bp and at 853 bp, 631 bp and 568 bp in Arka Chandan (Fig. 5a). Hence, UBC-866 can be effectively used for distinguishing these three varieties.

4.2.4.2 UBC-815

UBC-815 generated amplification pattern is shown in Plate 8. In variety CO-2, a unique band was obtained at 426 bp and in variety Arka Chandan, distinct amplicons at 1110 bp, 575 bp and 343 bp were obtained (Fig. 5b) revealing its discriminatory power. Hence, it can prove to be an ideal primer for unequivocal identification of these two varieties.

4.2.4.3 UBC-827

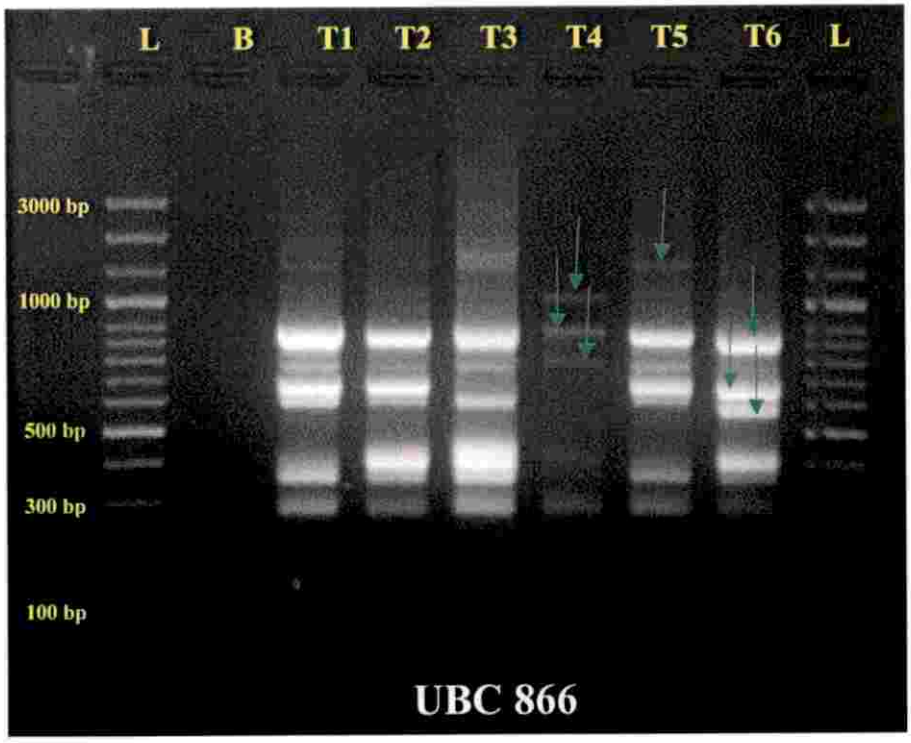
The banding pattern generated by UBC-827 (Plate 9) was carefully scored. This primer yielded unique amplicon at 700 bp for Saras and at 623 bp for Suvarna (Fig. 6a), and hence, it can be used distinguish between these varieties as well from other studied varieties.

4.2.4.4 UBC-807

Plate 9 shows the amplification pattern of ISSR primer UBC-807. The primer produced unique amplicons (Fig. 6b) at 543 bp for Suvarna and at 751 bp and 581 bp for Arka Chandan, making it suitable for identifying these two varieties.

4.2.4.5 UBC-811

Scoring of amplication pattern of six pumpkin generated by the primer UBC-811 (Plate 10) yielded unique bands at 927 bp and 340 bp in variety Arka Chandan (Fig. 7a).



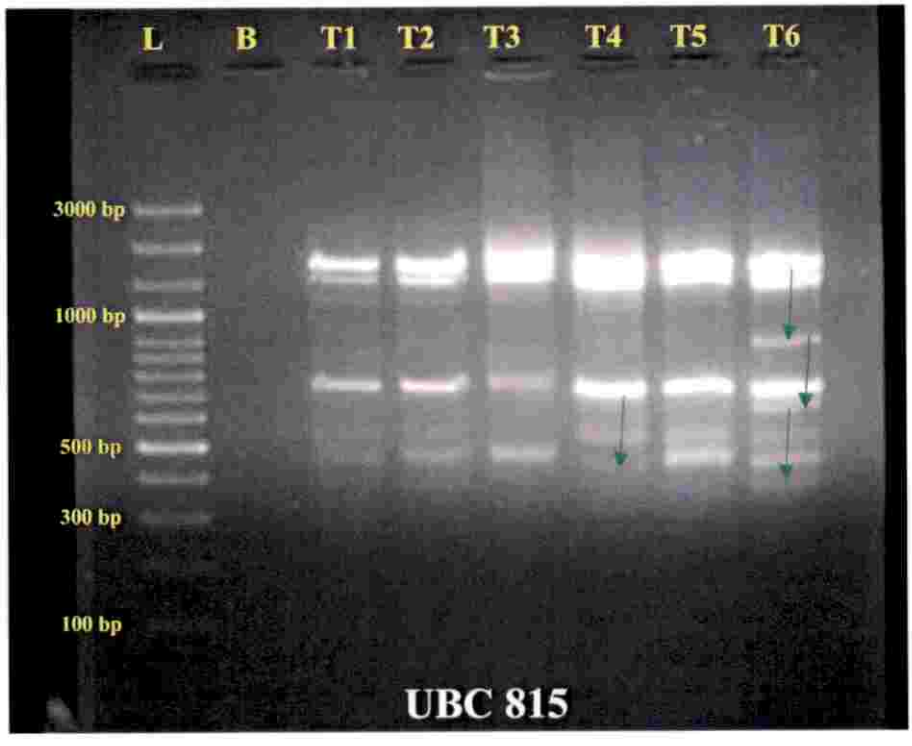


Plate 8: Amplification profile generated by ISSR primers UBC-866 and UBC-815

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1483						
1235						
944						
900						
853						
791						
750						
665						
631						
600						
568						
405						
365						
295						

a. Colour chart of UBC-866

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1887						
1594						
1110						
766						
575						
483						
443						
426						
396						
343						

b. Colour chart of UBC-815

Colour code for shared amplicons among genotypes

1 2 3 4 5 6

Fig. 5. Colour chart of ISSR primers (a) UBC-866 and (b) UBC-815

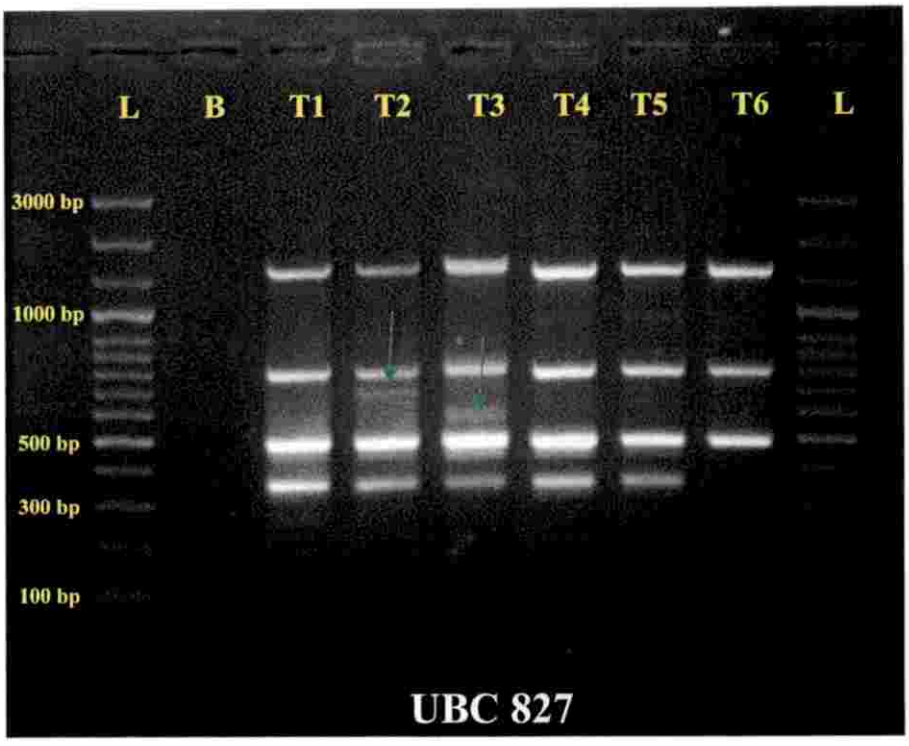


Plate 9: Amplification profile generated by ISSR primers UBC-827 and UBC-807

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1606						
800						
700						
623						
493						
357						

a. Colour chart of UBC-827

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1218						
847						
779						
751						
609						
581						
543						
367						
339						

b. Colour chart of UBC-807

Colour code for shared amplicons among genotypes

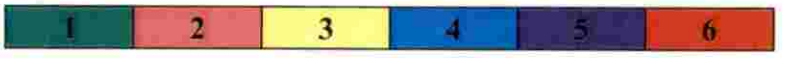


Fig. 6. Colour chart of ISSR primers (a) UBC-827 and (b) UBC-807

4.2.4.6 UBC-847

The primer UBC-847 generated unique amplicons (Fig. 7b and Plate 10) in variety Ambili (586 bp), Saras (1595 bp) and Arka Chandan (1500 bp, 1415 bp and 1185 bp). Thus UBC-847 can serve as an ideal primer for identifying varieties Ambili, Saras and Arka Chandan.

4.2.4.7 UBC-868

Six pumpkin varieties DNA samples were amplified using primer UBC-868 and the gel image with amplification pattern is shown (Plate 11). Distinct and unique amplicons were obtained at 1100 bp and 1020 bp (Fig. 8a) in variety Arka Chandan.

4.2.4.8 UBC-810

UBC-810 generated amplification pattern in the studied varieties (six) is shown in Plate 11. In variety Arka Chandan, unique band at 600 bp was obtained (Fig. 8b) revealing the discriminatory power of the marker. It can be used as an ideal primer for uniequovocal identification of the variety.

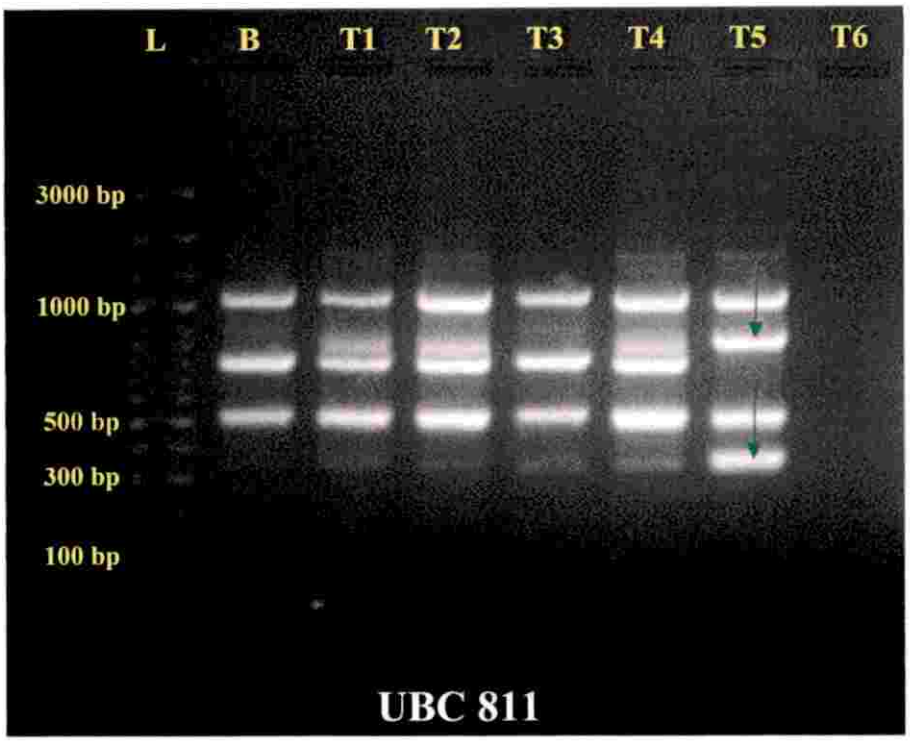
4.2.4.9 Oligo ISSR 5

Oligo ISSR 5 generated amplication pattern is shown in Plate 12. Unique amplicons at 1264 bp and 822 bp (Fig. 9a) was obtained in Arka Chandan. The primer can be used to generate the fingerprint for the variety and hence can help in varietal discrimination.

4.2.4.10 UBC-855

DNA amplification of six varieties using primer UBC-855 (Plate 12) produced unique bands in variety Ambili at 810 bp and 428 bp, in variety CO-2 at 1544 bp and at 700 bp in variety Arka Chandan (Fig. 9b). UBC-855 can be effectively used for distinguishing these three varieties.

113



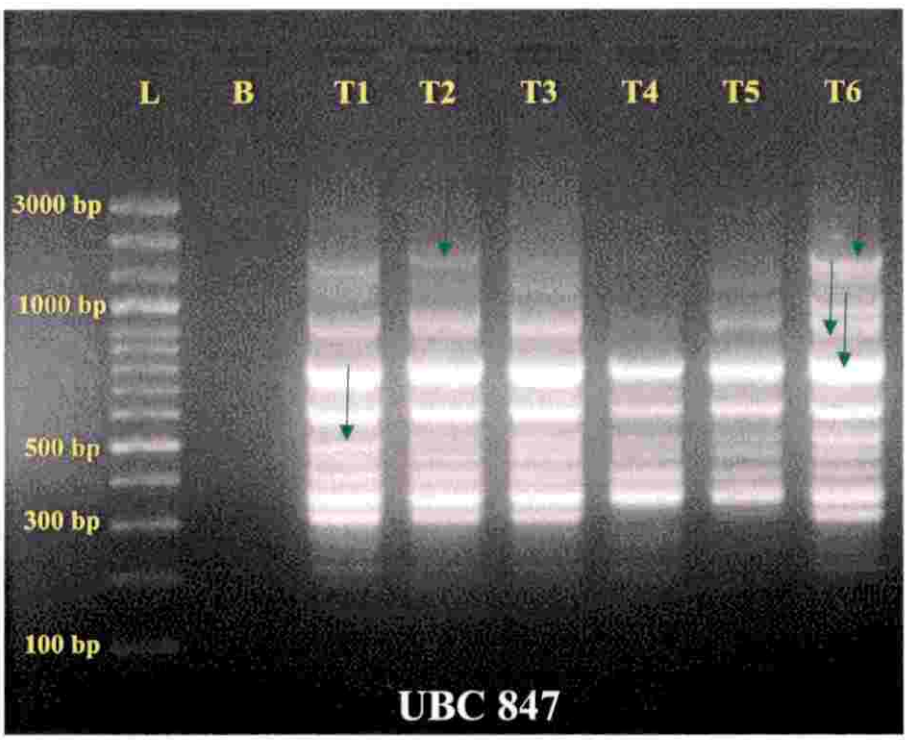


Plate 10: Amplification profile generated by ISSR primers UBC-811 and UBC-847

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1200						
1177						
927						
884						
785						
527						
340						
322						

a. Colour chart of UBC-811

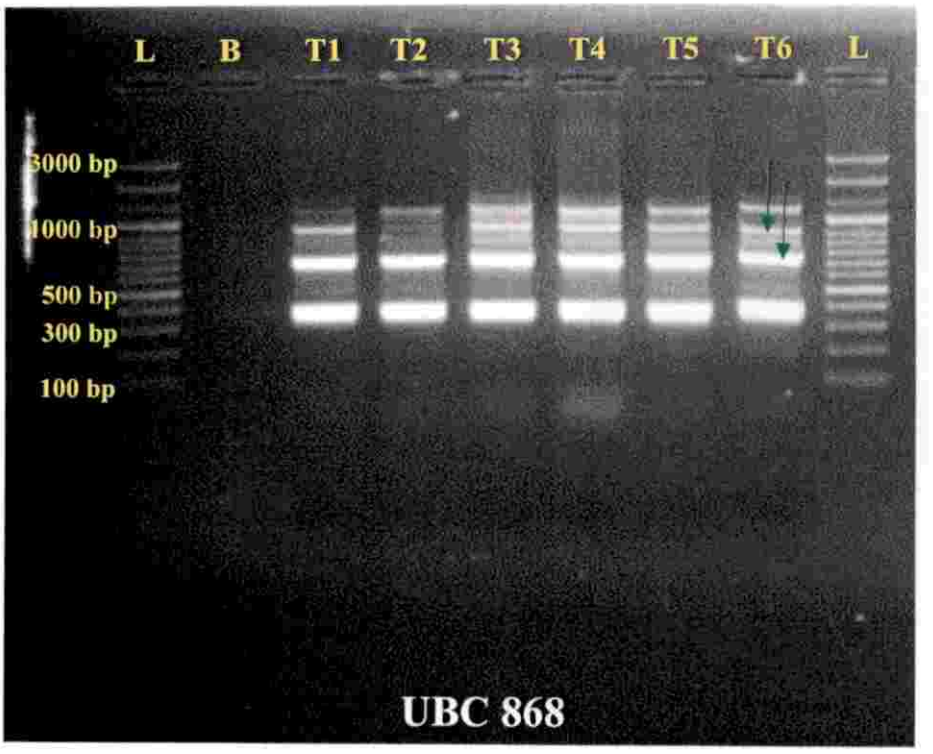
	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1595						
1500						
1415						
1331						
1185						
1039						
1000						
773						
755						
626						
608						
586						
500						
466						
394						
361						
340						
300						

b. Colour chart of UBC-847

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 7. Colour chart of ISSR primers (a) UBC-811 and (b) UBC-847



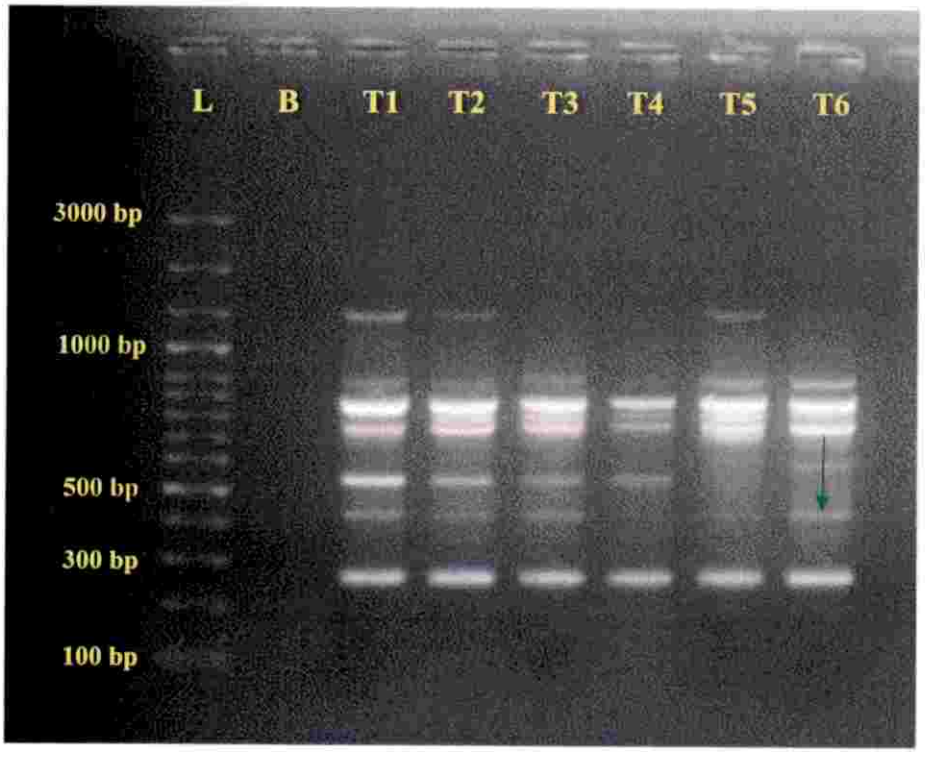


Plate 11: Amplification profile generated by ISSR primer UBC-868 and UBC-810

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1539						
1160						
1100						
1020						
844						
747						
394						

a. Colour chart of UBC-868

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1483						
994						
877						
821						
746						
600						
539						
427						
271						

b. Colour chart of UBC-810

Colour code for shared amplicons among genotypes

Fig. 8. Colour chart of ISSR primers (a) UBC-868 and (b) UBC-810

4.2.4.11 UBC-808

Scoring of amplication pattern in six pumpkin generated by the primer UBC-808 (Plate 12) yielded unique bands at 289 bp in variety Suvarna and at 814 bp in variety Arka Chandan (Fig. 10a). Hence, these two varieties can be discriminated from each as well as from other studied varieties using UBC-808.

4.2.4.12 UBC-809

The PCR amplification pattern of six pumpkin varieties using ISSR primer UBC-809 (Plate 12) was scored. A unique amplicon at 421 bp was obtained (Fig. 10b) for variety Saras. This primer was hence found to be suitable for identification of the variety from a mixture.

4.2.4.13 UBC-812

Plate 13 shows the amplification pattern of ISSR primer UBC-812. At 753 bp for variety Ambili and at 289 bp and 947 bp in CO-2, the primer produced unique amplicons (Fig. 11a) making it suitable for distinguishing these two varieties.

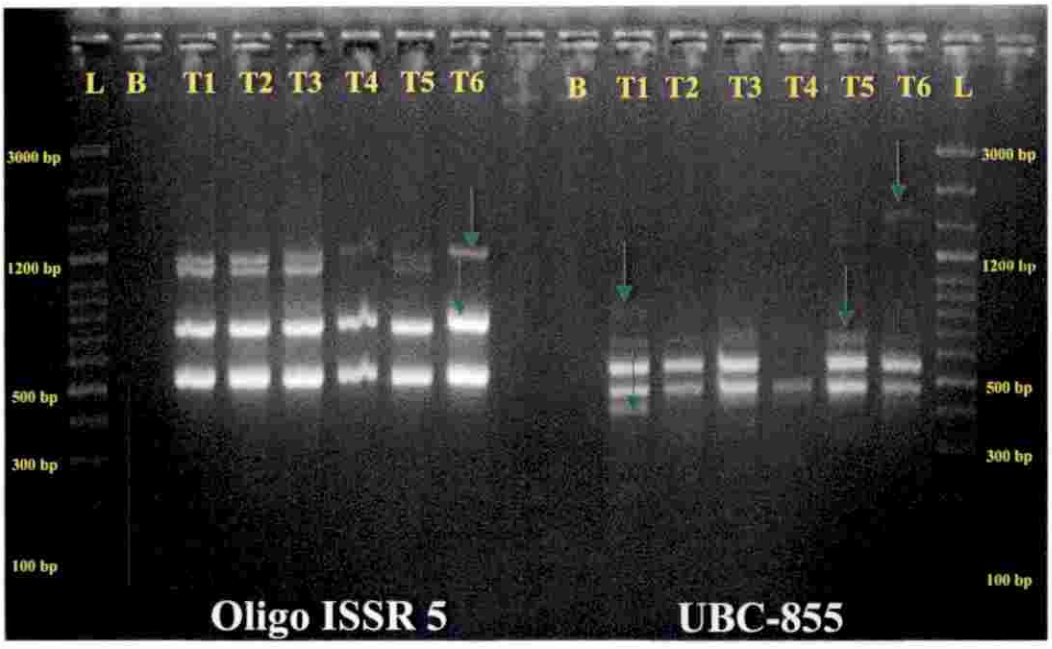
4.2.4.14 UBC-834

The banding pattern generated by UBC-834 (Plate 13) was carefully scored. This primer yielded unique amplicon at 1200 bp for CO-1, at 776 bp and 1133 bp in Arka Chandan (Fig. 11b). Thus, this primer can be used for identification of these varieties.

4.2.4.15 UBC-835

UBC-835 generated amplification pattern (Plate 13) produced polymorphic bands in CO-1 at 1059 bp (Fig 12a). Thus, primer UBC-835 can be an ideal primer for identification of variety CO-1.

118



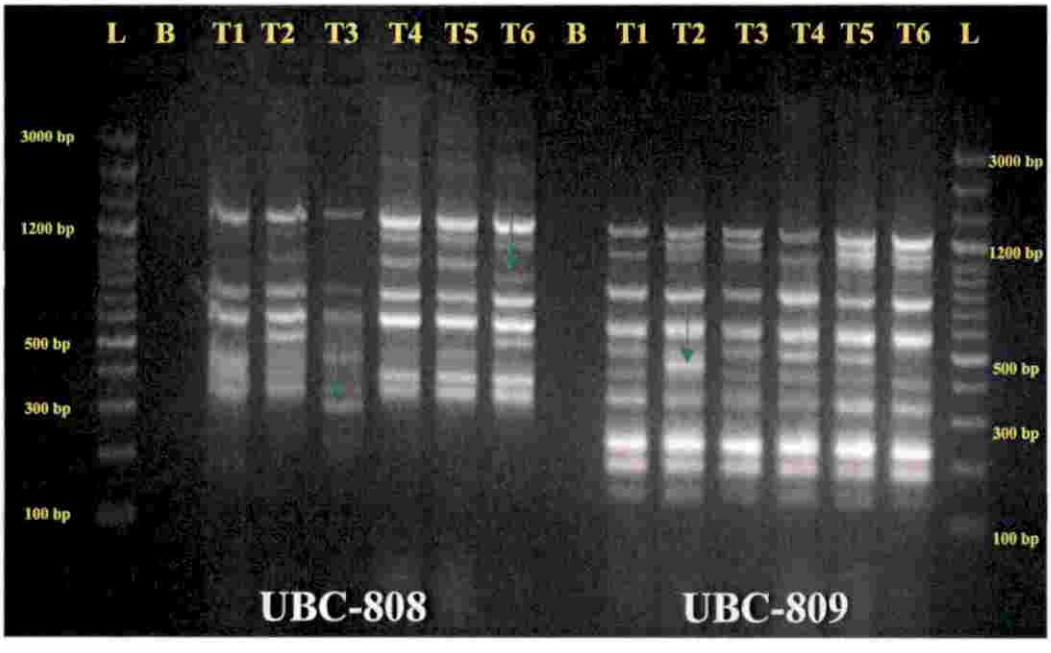


Plate 12: Amplification profile generated by ISSR primers Oligo ISSR 5, UBC-855, UBC-808 and UBC-809

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1264						
1186						
1076						
822						
735						
531						

a. Colour chart of Oligo ISSR 5

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1544						
810						
700						
587						
494						
428						

b. Colour chart of UBC-855

Colour code for shared amplicons among genotypes

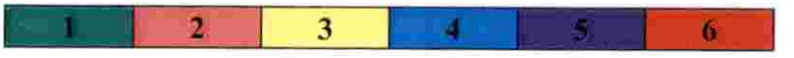


Fig. 9. Colour chart of ISSR primers (a) Oligo ISSR 5 and (b) UBC-855

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1348						
1246						
1073						
915						
814						
733						
600						
500						
416						
371						
331						
289						

a. Colour chart of UBC-808

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1134						
1000						
929						
551						
451						
421						
388						
315						
212						
156						

b. Colour chart of UBC-809

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 10. Colour chart of ISSR primers (a) UBC-808 and (b) UBC-809

4.2.4.16 UBC-857

Careful scoring of amplification pattern obtained using the primer UBC-857 (Plate 13) revealed the presence of three unique bands at 700 bp, 1111 bp and 1775 bp in variety Arka Chandan (Fig. 12b). Thus, UBC-857 can be effectively utilised for identifying the said variety.

4.2.4.17 UBC-836

The banding pattern generated by UBC-836 (Plate 14) was scored to detect unique amplicons. This primer yielded unique amplicon at 1821 bp in Saras and at 462 bp in Arka Chandan (Fig. 13a). Hence, it can be used for identification of varieties.

4.2.4.18 UBC-846

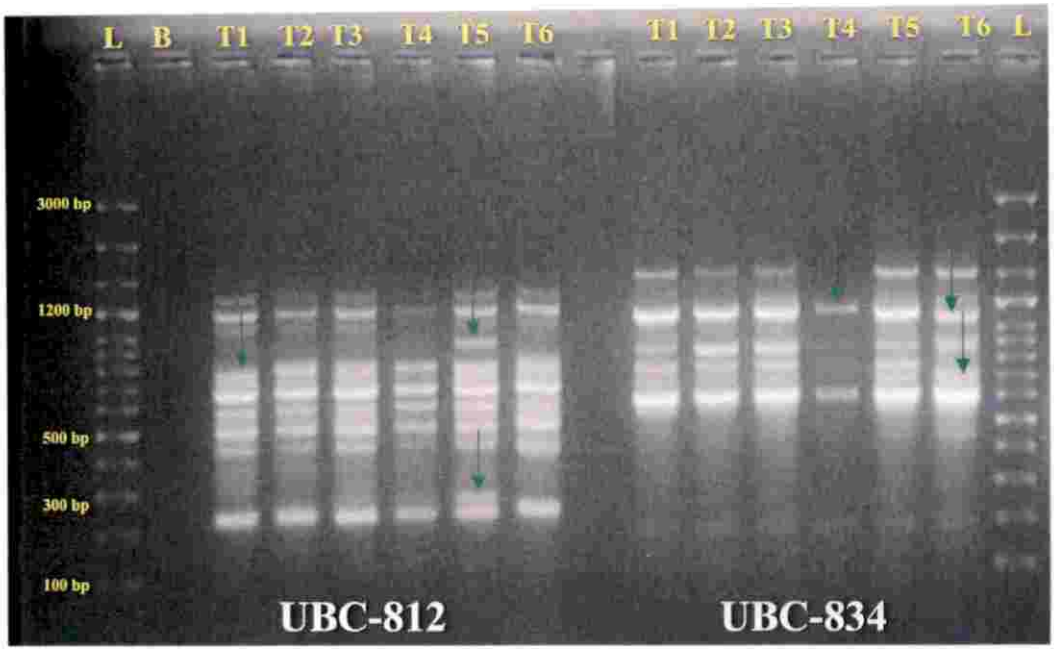
By examining the gel image of DNA amplification pattern obtained by UBC-846 of six pumpkin varieties (Plate 14), unique and distinct band at 1443 bp was obtained in Arka Chandan (Fig. 13b).

4.2.4.19 UBC-826

The amplification pattern of six varieties of pumpkin in study generated by UBC-826 (Plate 14) was scored to identify unique amplicons. At 1070 bp and 864 bp, unique band was present for the variety CO-1 (Fig. 14a). Hence, this primer can prove useful in varietal identification and making fingerprint.

4.2.4.20 UBC-822

On careful examination of gel image of the amplification pattern generated by UBC-822 in the six varieties under study (Plate 14), one unique amplicon was observed at 473 bp, 552 bp and 517 bp respectively in varieties Ambili, Saras and Arka Chandan. Two unique amplicons were observed in variety CO-1 at 353 bp



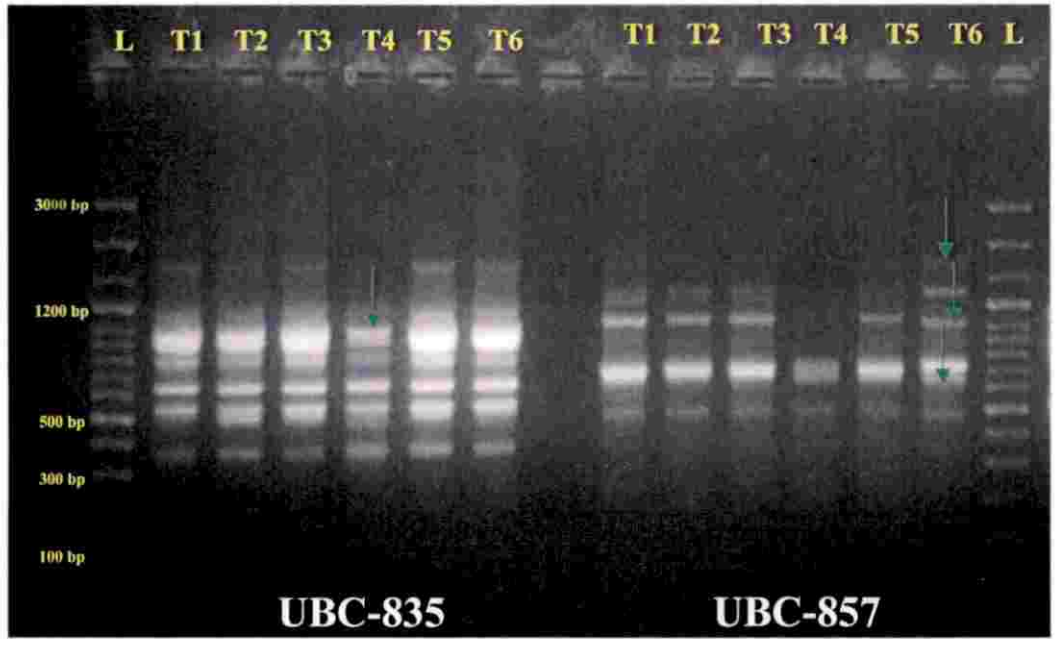


Plate 13. Amplification profile generated by ISSR primers UBC-812, UBC-834, UBC-835 and UBC-857

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1310						
1186						
947						
810						
753						
660						
600						
524						
460						
289						
242						

a. Colour chart of UBC-812

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1560						
1540						
1200						
1186						
1133						
922						
891						
800						
776						
720						
621						

b. Colour chart of UBC-834

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 11. Colour chart of ISSR primers (a) UBC-812 and (b) UBC-834

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1630						
1157						
1059						
949						
782						
644						
542						
507						
382						

a. Colour chart of UBC-835

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1775						
1311						
1134						
1111						
737						
700						
535						

b. Colour chart of UBC-857

Colour code for shared amplicons among genotypes

Fig. 12. Colour chart of ISSR primers (a) UBC-835 and (b) UBC-857

and 1403 bp (Fig. 14b). This makes UBC-822 effective in distinguishing these varieties from each other.

4.2.4.21 UBC-844

The PCR amplification pattern of six pumpkin varieties using ISSR primer UBC-844 (Plate 14) was scored. One unique amplicon at 691 bp, 783 bp and 742 bp respectively was obtained in variety Ambili, Saras and Arka Chandan, (Fig. 15a) respectively, making this primer suitable for identification of these varieties from a mixture.

4.2.4.22 UBC 854

Scoring of amplication pattern of six pumpkin generated by the primer UBC-854 (Plate 14) yielded unique band at 444 bp in variety Saras (Fig. 15b).

4.2.4.23 UBC 818

From the amplification pattern generated by UBC-818 (Plate 14 and Fig. 15c), variety Arka Chandan had a unique amplicon at 506 bp. The ISSR primer UBC-818 can thus be used for fingerprinting and identifying the said variety.

4.2.4.24 UBC-816

Plate 15 shows the amplification pattern generated by ISSR primer UBC-816. The primer produced unique amplicons at 1385 bp in variety in CO-1 and at 1792 bp in Arka Chandan (Fig. 16a) making it suitable for identifying the varieties

4.2.4.25 UBC-825

UBC-825 generated amplification pattern (Plate 15) produced two unique bands in Arka Chandan, at 791 bp and 1244 bp (Fig. 16b). Thus, the primer UBC-835 is an ideal primer for distinguishing this variety from others.

126

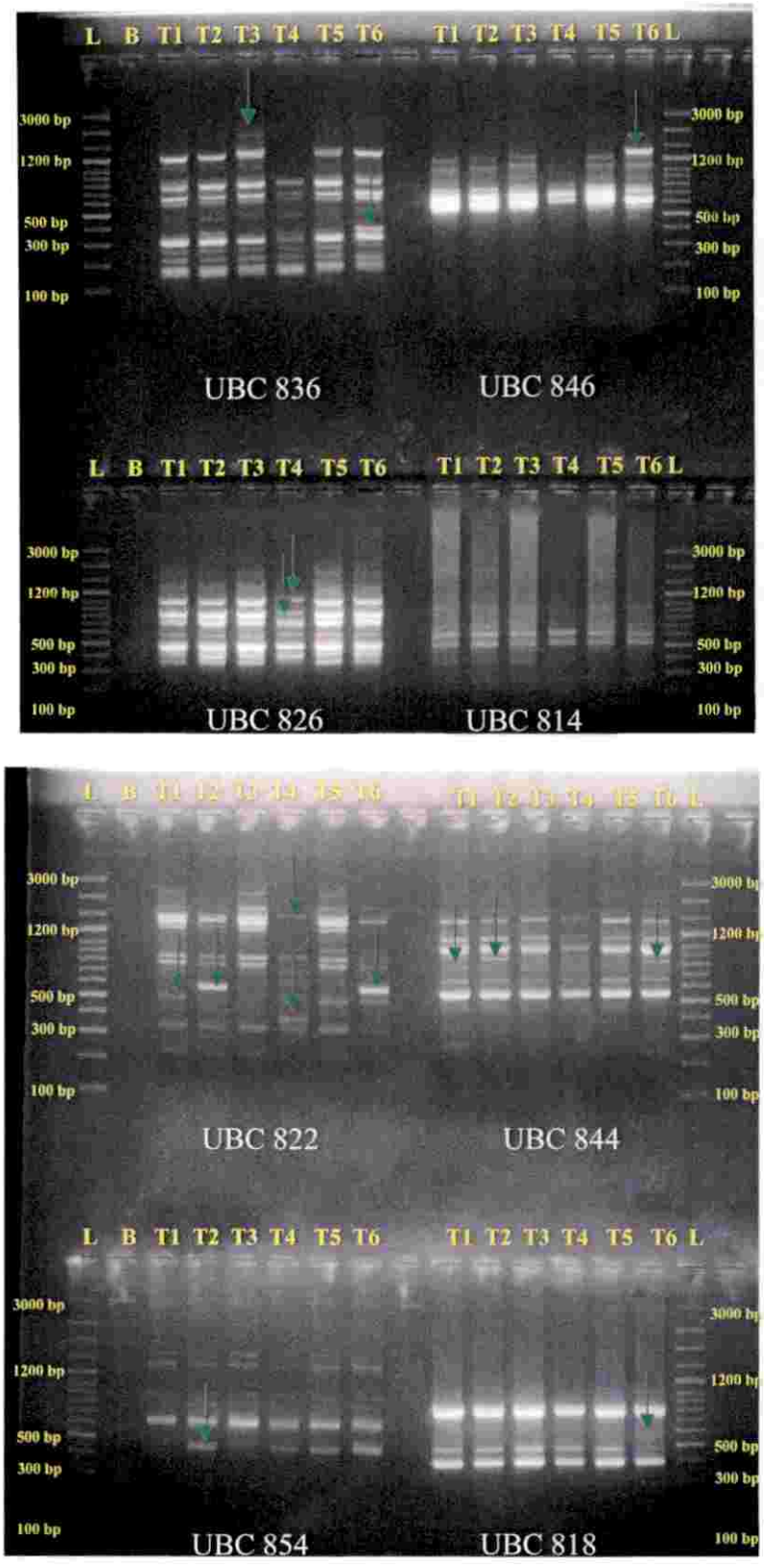


Plate 14. Amplification profile generated by ISSR primers UBC-836, UBC-846, UBC-826, UBC-814, UBC-822, UBC-844, UBC-854 and UBC-818

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1821						
1522						
1354						
922						
872						
747						
462						
375						
286						
227						
179						

a. Colour chart of UBC-836

	1	2	3	4	5	6
Amplicon size(bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1443						
1263						
1107						
856						
736						
659						
619						

b. Colour chart of UBC-846

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 13. Colour chart of ISSR primers (a) UBC-836 and (b) UBC-846

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1070						
1013						
864						
792						
700						
553						
465						
400						
362						

a. Colour chart of UBC-826

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
2178						
1552						
1403						
1364						
1280						
1200						
900						
875						
755						
552						
517						
473						
440						
353						
328						

b. Colour chart of UBC-822

Colour code for shared amplicons among genotypes

Fig. 14. Colour chart of ISSR primers (a) UBC-826 and (b) UBC-822

	1	2	3	4	5	6
Amplicon size(bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1364						
1027						
881						
783						
742						
691						
582						
494						

a. Colour chart of UBC-844

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1613						
1338						
848						
587						
444						
406						

b. Colour chart of UBC-854

	1	2	3	4	5	6
Amplicon size(bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
682						
506						
405						
338						

c. Colour chart of UBC-818

Colour code for shared amplicons among genotypes

Fig. 15. Colour chart of ISSR primers (a) UBC-844, (b) UBC-854 and (c) UBC-818

4.2.4.26 UBC-813

The banding pattern generated by UBC-813 (Plate 15) was scored to detect unique amplicons. This primer yielded an unique amplicon at 436 bp in Arka Chandan (Fig. 16c), proving its utility in fingerprinting and identification of the variety.

4.2.4.27 UBC-823

By examining the gel image of DNA amplification pattern obtained by UBC-823 of six pumpkin varieties (Plate 15), unique and distinct bands at 1161 bp in CO-2 and 1085 bp in Arka Chandan were obtained (Fig. 17a). Hence, primer UBC-823 can be used for identification of the above varieties.

4.2.4.28 UBC-841

The PCR amplification pattern of six pumpkin varieties using ISSR primer UBC-841 (Plate 15) was scored. One unique amplicon at 1034 bp was obtained (Fig. 17b) in variety CO-1, making this primer suitable for identification of CO-1 variety from a varietal mixture.

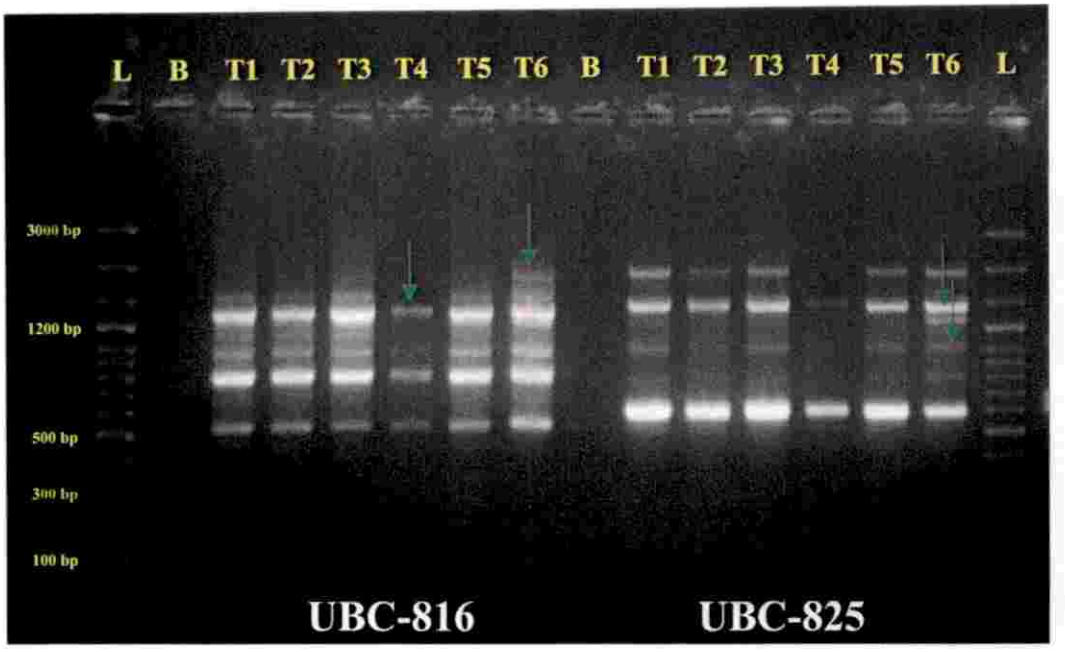
4.2.5 DNA fingerprinting of individual pumpkin varieties using ISSR profile

The data obtained using analyses with selected 28 ISSR primers were further utilised for locating useful unique amplicons in each variety. A consolidated amplicon colour chart was developed. This can be highly useful in distinguishing these varieties from the others. Variety-wise DNA fingerprint details are described below.

4.2.5.1 Ambili

On examination of amplication pattern produced by 28 selected ISSR primers, it was observed that five primers *viz*., UBC-847 at 586 bp, UBC-855 at 810 and 428 bp, UBC-812 at 753 bp, UBC-822 at 473 bp and UBC 844 at 691 bp

131



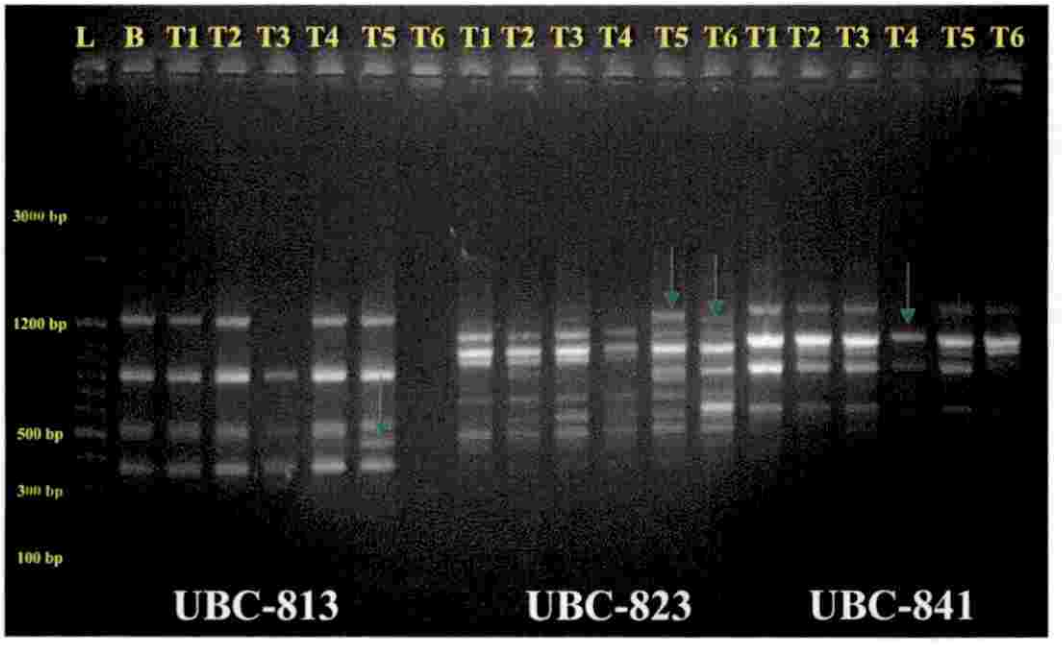


Plate 15. Amplification profile generated by ISSR primers UBC-816, UBC-825, UBC-813, UBC-823and UBC-841

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1792						
1500						
1385						
1305						
914						
774						
495						

a. Colour chart of UBC-816

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1838						
1418						
1244						
964						
791						
565						

b. Colour chart of UBC-825

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1187						
776						
506						
436						
351						

c. Colour chart of UBC-813

Colour code for shared amplicons among genotypes

Fig. 16. Colour chart of ISSR primers (a) UBC-816, (b) UBC-825 and (c) UBC-813

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1161						
1085						
1000						
900						
828						
769						
600						
506						
442						

a. Colour chart of UBC-823

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
1274						
1034						
1000						
959						
858						
595						

b. Colour chart of UBC-841

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 17. Colour chart of ISSR primers (a) UBC-823 and (b) UBC-841

produced unique and distinct bands in the variety Ambili. Amplicon size generated by these five primers ranged from 242 bp to 2178 bp. Fingerprint developed in variety Ambili using distinct bands with the five primers is provided in Fig. 18.

4.2.5.2 Saras

From the amplification pattern observed for variety Saras using 28 ISSR primers, seven were selected to make the fingerprint of the variety (Fig. 19).

At 700 bp (UBC-827), 1595 bp (UBC-847), 421 bp (UBC-809), 1821 bp (UBC-836), 552 bp (UBC-822), 783 bp (UBC-844) and 444 bp (UBC-854) unique amplicons were obtained for the variety. The amplicon sizes obtained by these seven primers ranged from 156 bp to 821 bp. Maximum amplicons was generated by ISSR primer UBC-809 (10) and minimum number by UBC-827 and UBC-854 (5).

4.2.5.3 Suvarna

Three out of 28 ISSR primers produced distinct and unique amplicons in variety Suvarna (Fig. 20). The size of all amplicons generated by UBC-827, UBC-807 and UBC-808 in this variety ranged from 289 bp to 1606 bp. The number of amplicons generated varies from 5 (UBC-827 and UBC-807) to 7 (UBC-808). It was observed that the unique bands produced at 289 bp, 543 bp and 623 bp, by markers UBC-808, UBC-807 and UBC-827, respectively, can be used for making the fingerprint of this variety.

4.2.5.4 CO-1

Fingerprint was developed based on unique bands produced by seven primers out of 28 ISSR primers. The number of bands amplified ranged from 2 (UBC-834 and UBC-841) to 8 (UBC-826) in this variety and the amplicons ranged in size from 1403 bp to 295 bp. Primers UBC-866 yielded 3 unique amplicons at 1235 bp, 944 bp and 791 bp, UBC-816 at 1385 bp, UBC-835 at 1059 bp, UBC-834

Primer	UBC-847	UBC-855	UBC-812	UBC-822	UBC-844
Amplicon size (bp)					
2178					
1552					
1364					
1310					
1280					
1186					
1027					
1000					
881					
875					
810					
755					
753					
691					
660					
600					
587					
586					
582					
524					
500					
494					
473					
466					
460					
428					
394					
340					
328					
300					
242					

Colour code for shared amplicons among genotypes

Fig. 18. Fingerprint for pumpkin variety Ambili using five ISSR marker profiles

Primer	UBC-827	UBC-847	UBC-809	UBC-836	UBC-822	UBC-844	UBC-854
Amplicon size (bp)							
156							
179							
212							
227							
286							
300							
315							
328							
340							
357							
375							
388							
394							
421							
444							
451							
466							
493							
494							
500							
551							
552							
582							
587							
608							
700							
747							
755							
783							
800							
848							
872							
875							
881							
922							
929							
1000							
1027							
1134							
1200							

Primer	UBC-827	UBC-847	UBC-809	UBC-836	UBC-822	UBC-844	UBC-854
Amplicon size (bp)							
1280							
1338							
1354							
1364							
1595							
1606							
1613							
1821							

Fig. 19. Fingerprint for pumpkin variety Saras using seven ISSR marker profiles

Primer	UBC-827	UBC-807	UBC-808
Amplicon size (bp)			
1606			
1348			
1218			
800			
779			
733			
623			
609			
600			
543			
493			
416			
371			
357			
339			
331			
289			

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 20. Fingerprint for pumpkin variety Suvarna using three ISSR marker profiles

at 1200 bp, UBC-826 at 1070 and 864 bp, UBC-822 at 1403 and 353 bp and UBC-841 at 1034 bp. These were used to develop the fingerprint for this variety (Fig. 21).

4.2.5.5 CO-2

From the amplification pattern observed for the genomic DNA of variety CO-2 using 28 ISSR primers, five were selected to make fingerprint.

The amplicon sizes obtained by these five primers ranged from 242 bp to 1887 bp. Maximum amplicons was generated by ISSR primer UBC-812 (10) while, it was only three by primer UBC-855. Two polymorphic bands were produced by UBC-812 at 947 bp and 289 bp, whereas, primers UBC 815, UBC 866, UBC 855 and UBC 823 produced unique amplicons at 426 bp, 1483 bp, 700 bp and 1161 bp, respectively (Fig. 22). These were used to generate the fingerprint of this variety.

4.2.5.6 Arka Chandan

Twenty one out of 28 ISSR primers yielded unique amplicons that help in distinguishing Arka Chandan from other six varieties studied. Hence, DNA fingerprint can be made from these 21 primers. Fig. 23 depicts the amplicon size of unique bands produced by the 21 ISSR primers. Totally 31 unique amplicons were produced by the 21 ISSR primers and the number of unique amplicons detected by each primer ranged from 1 to 3.

From the above it was evident that a combination of several ISSRs that produced unique amplicons could be helpful in establishing varietal identity and distinguishing it from a varietal mixture. However, a single ISSR marker that could distinguish all the six pumpkin varieties *per se* was not found. But, ISSR primer UBC-822 developed DNA fingerprints in varieties Ambili (473 bp), Saras (552 bp), CO-1 (1403 bp) and Arka Chandan (517 bp) and could distinguish between these four varieties and can help identify them appropriately.

Primer	UBC-866	UBC-834	UBC-835	UBC-826	UBC-822	UBC-816	UBC-841
Amplicon size (bp)							
1403							
1385							
1235							
1200							
1070							
1059							
1034							
949							
944							
900							
875							
864							
858							
792							
791							
782							
774							
700							
644							
621							
553							
542							
495							
465							
400							
382							
362							
353							
328							
295							

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 21. Fingerprint for pumpkin variety CO-1 using seven ISSR marker profiles

Primer	UBC-815	UBC-866	UBC-855	UBC-812	UBC-823
Amplicon size (bp)					
1887					
1594					
1483					
1310					
1186					
1161					
1000					
947					
900					
810					
769					
766					
750					
700					
665					
660					
600					
587					
524					
506					
494					
483					
460					
442					
426					
365					
295					
289					
242					

Colour code for shared amplicons among genotypes

1	2	3	4	5	6

Fig. 22. Fingerprint for pumpkin variety CO-2 using five ISSR marker profiles

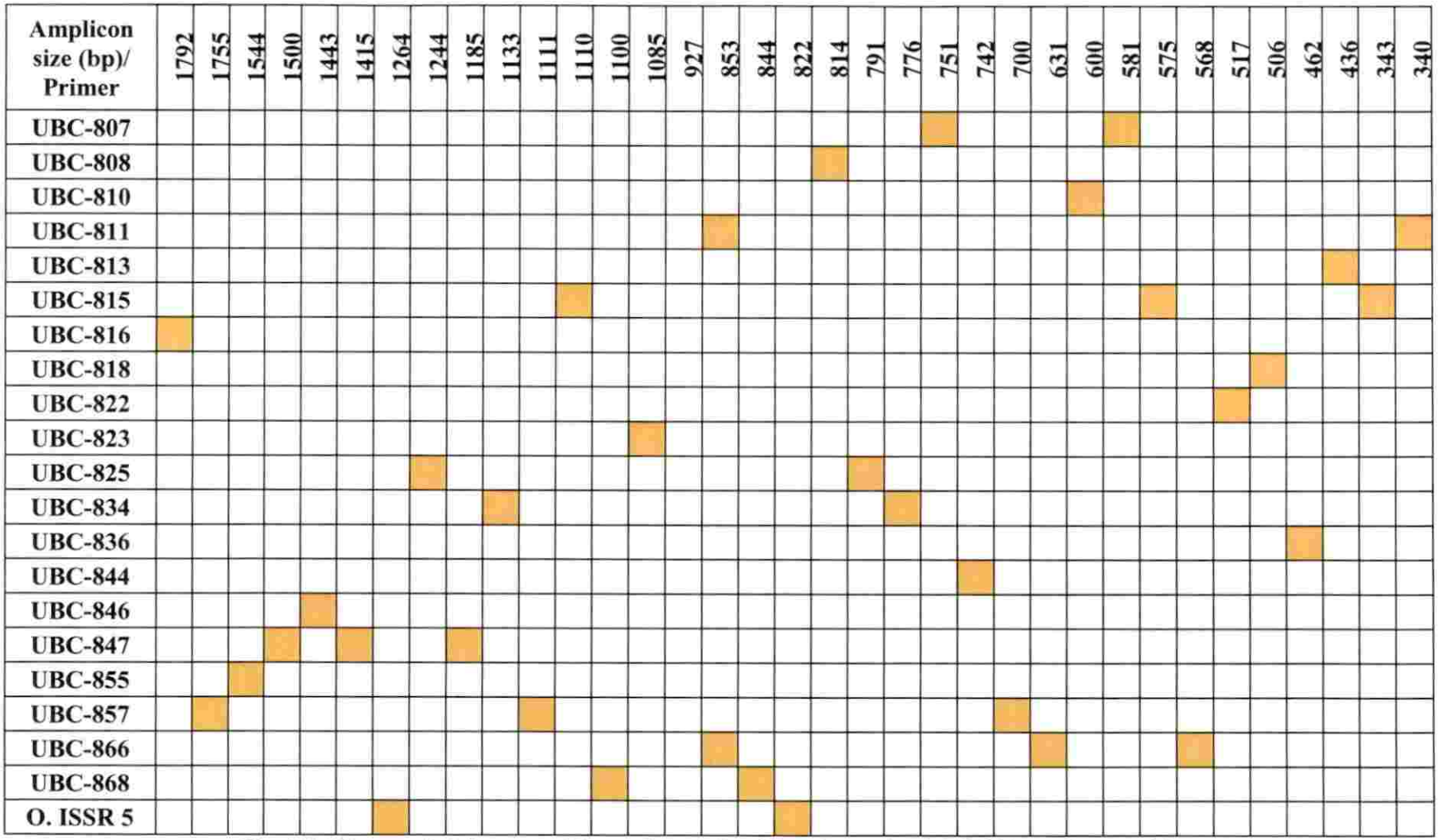


Fig. 23. Fingerprint for pumpkin variety Arka Chandan using 21 ISSR marker profiles

4.2.6 Genotyping with SSR markers

The DNA samples of six varieties were taken for SSR analysis using a set of 20 primers. Out of 20 SSR markers, only five produced polymorphic bands (Table 14). The number of amplicons detected varied from 2 to 3. It is possible that the number of alleles detected would increase with increase in sample size. Studies by Stift *et al.* (2004) found 2-6 alleles on 26 genotypes using 22 primers, whereas, number of alleles ranged from 4-10 on 26 accessions of squashes (Barzegar *et al.*, 2013).

The highest number of alleles was found in the CMTm7, CMTm20 and CMTm232 marker (3). This is contradictory to the results of Sim *et al.* (2015), who observed 14 alleles detected by CMTm7 while working with pumpkin genotypes.

One unique band each was produced by SSR primer CMTm7, CMTm97 and CMTm144, while, two unique bands was produced by CMTm20 and CMTm232, respectively.

The average polymorphism per cent across the five SSR primers among the six varieties of pumpkin was 100 per cent, which was similar to that reported in other studies on pumpkin (Ntuli *et al.*, 2015; Martins *et al.*, 2015; Kiramana *et al.*, 2017).

To identify informative markers for cultivar identification, PIC value was calculated and it ranged from 0.24 (CMTm97 and CMTm144) to 0.47 (CMTm7) with an average of 0.37. The PIC value of CMTm7 and CMTm20 was almost similar *i.e.*, 0.45 and 0.46 as reported by Kazminska *et al.* (2017). However, the PIC value of CMTm144, CMTm97 and CMTm232 was very low in comparison with earlier reports (Barzegar *et al.*, 2013 and Kong *et al.*, 2014).

Based on PIC values, it may be inferred that SSR primers CMTm7, CMTm20 and CMTm232 are more useful in distinguishing the varieties as well as highly helpful in establishing the uniqueness of a variety.

Table 14. Particulars of SSR primer profiling in pumpkin varieties

Sl. No.	Primer	No. of amplic-ons	No. of polymorp-hic amplicons	No of unique bands	Amplico-n size (bp)	PIC	PP
1	CMTm7	3	3	1	56-200	0.47	100
2	CMTm20	3	3	2	100-149	0.45	100
3	CMTm144	2	2	1	168-181	0.24	100
4	CMTm232	3	3	2	207-263	0.45	100
5	CMTm97	2	2	1	168-200	0.24	100

4.2.6.1 Cluster analysis and dendrogram construction using SSR data

In six pumpkin varieties, the polymorphic bands of each SSR markers were scored as binary characters and using SIMQUAL subprogram of NTSYS-PC version 2.10 and in the subprogram SAHN, cluster analysis was performed using UPGMA.

Using Jaccard's similarity coefficient, genetic similarity was calculated for all the six pumpkin varieties (Table 15). The highest Jaccard's similarity value (1.00) was observed between Saras and Suvarna. The most dissimilar varieties were 'Saras-Arka Chandan' and Suvarna-Arka Chandan with a similarity value of 0.12 each. The obtained range of genetic similarity values (0.12-1.00) concurred with the range (0.13-1.00) obtained by Kwon *et al.* (2010) in watermelon.

The UPGMA clustering algorithm grouped the varieties into four clusters according to dendrogram (Table 16, Fig. 4b). Cluster I and IV were monogenic, whereas, cluster II and III had two members each. Similarly, 26 accessions of *C. pepo* were clustered into 4 groups using 14 SSRs (Barzegar *et al.*, 2013).

Table 15. Pair wise similarity between pumpkin varieties based on SSR profile

	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
Ambili	-					
Saras	0.56	-				
Suvarna	0.56	1.00	-			
CO-1	0.78	0.78	0.78	-		
CO-2	0.67	0.67	0.67	0.89	-	
Arka Chandan	0.34	0.12	0.12	0.33	0.44	-

Table16. Clustering of pumpkin varieties based on SSR profile

Cluster	Number of members	Members of cluster
I	1	Ambili
II	2	CO-1 CO-2
III	2	Saras Suvarna
IV	1	Arka chandan

4.2.6.2 Comparison between clustering pattern generated by SSR and morphological data

Both morphological and SSR data were efficient to cluster the varieties. Better correspondence was observed between clustering of varieties using morphological and SSR data than that observed in grouping varieties based on morphological traits and ISSR analysis. Clustering of varieties based on morphological traits and SSR data indicated that varieties CO-1 and CO-2 grouped under the same cluster, while, Arka Chandan and Ambili belonged to different clusters. However, variety Saras and Suvarna shared a common cluster based on five SSR marker data, while, these two varieties belonged to two different clusters based on evaluated morphological data.

145

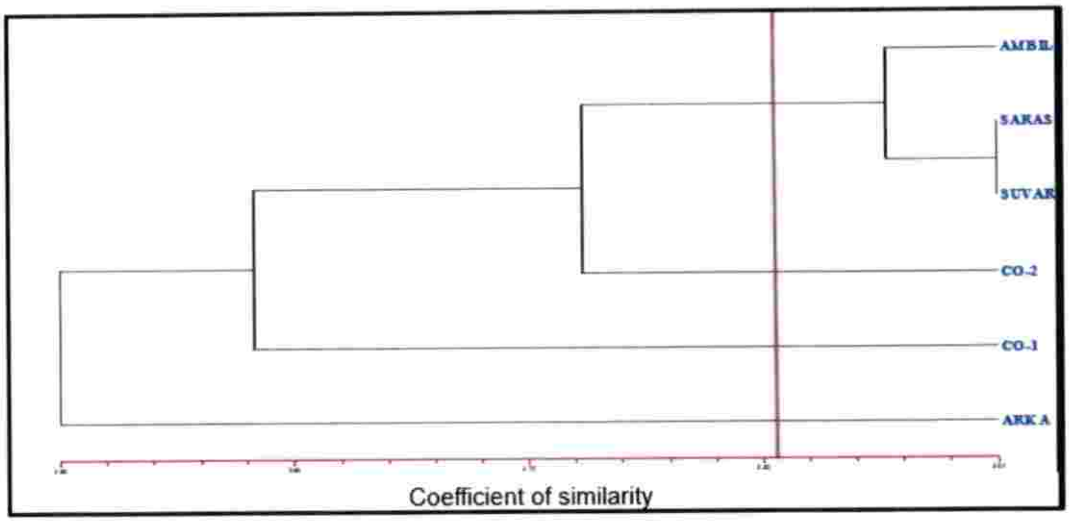


Fig. 4a. Dendrogram obtained by UPGMA clustering method across six varieties of pumpkin based on ISSR markers.

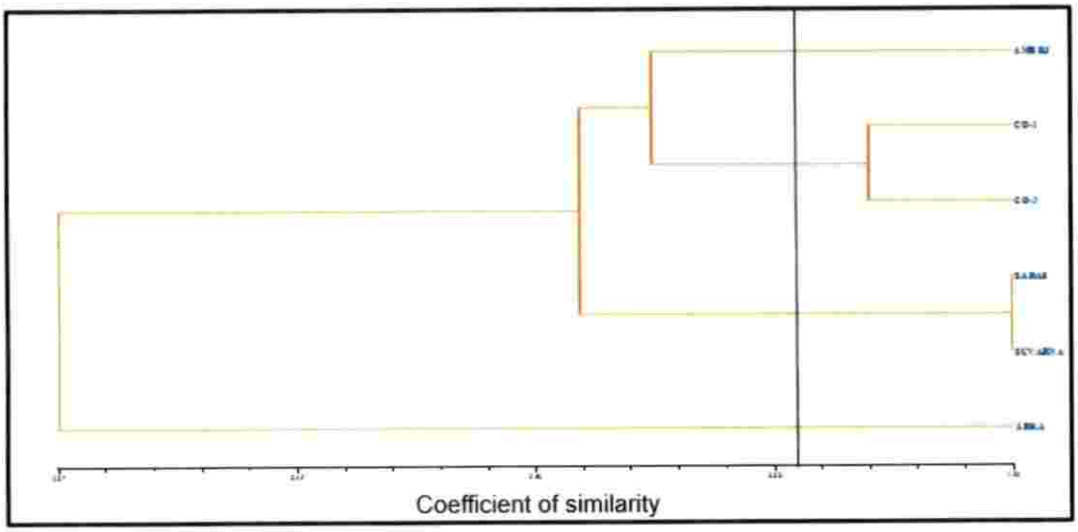


Fig. 4b. Dendrogram obtained by UPGMA clustering method across six varieties of pumpkin based on SSR markers

In general, it can be concluded that the correlation that existed between morphological and molecular assessments was of medium magnitude. The absence of high consensus between the assessments should not be considered a limitation of these tools to characterise and quantify variability. It only indicates that both morphological and molecular characterisation are important and play a complementary role in providing a better understanding and differentiation of the pumpkin varieties. Sudre *et al.*, 2010; Oh *et al.*, 2012; Carvalho *et al.*, 2017 have also opined the same.

Both SSR and ISSR markers can characterise and detect variation between genotypes, and hence prove more reliable than morphological marker analysis. This is because SSRs detect variation at pre-determinate sequence sites, whereas ISSRs quickly scan the whole genome, while, morphological evaluation is based only on phenotypic appearance which is influenced by both genotype and environment. Similar conclusion was arrived at by earlier workers (Kaur *et al.*, 2017; Serra *et al.*, 2007; Vianna *et al.*, 2019).

4.2.7 DNA fingerprinting using polymorphic SSR

Fingerprinting with DNA markers provide quick and reliable method for cultivar identification and purity test in crops by using a smaller amount of DNA and therefore can assist to deduce reliable information about crop genotype.

To develop SSR fingerprints of six pumpkin varieties used in the study, the primers that produced at least one polymorphic amplicon was selected. Five primers were selected for fingerprinting and are detailed below. The number of amplicons generated and the range of molecular band size is tabulated (Table 14).

4.2.7.1 CMTm7

Six pumpkin varieties DNA samples were amplified using SSR primer CMTm7 and the gel image with amplification pattern is shown (Plate 16). The

amplicon obtained at 56 bp was unique (Fig. 24a) and can be used to generate fingerprint of Arka Chandan.

4.2.7.2 CMTm20

The amplification pattern of six varieties of pumpkin in study generated by CMTm20 (Plate 16) was scored to identify unique amplicons. At 149 bp, a unique band was present in variety CO-1, while in Arka Chandan, a unique band was observed at 100 bp (Fig. 24b). Hence, this primer can prove useful in varietal identification and making fingerprint of these varieties.

4.2.7.3 CMTm97

By examining the gel image of DNA amplification pattern obtained by CMTm97 of six pumpkin varieties (Plate 16), unique and distinct band at 168 bp was obtained for Arka Chandan (Fig. 24c). This information can be used for identification of above variety.

4.2.7.4 CMTm144

CMTm144 generated amplification pattern for the studied varieties of pumpkin is shown in Plate 16. In variety Arka Chandan, unique band at 168 bp was obtained (Fig. 24d) revealing its discriminatory power. The marker can thus be considered ideal for unequivocal identification of the variety.

4.2.7.5 CMTm232

Plate 16 shows the amplification pattern of SSR primer CMTm232. The primer produces unique amplicons at 232 bp in variety Ambili and at 207 bp in Arka Chandan, (Fig. 24e), making it suitable for identifying these two varieties.

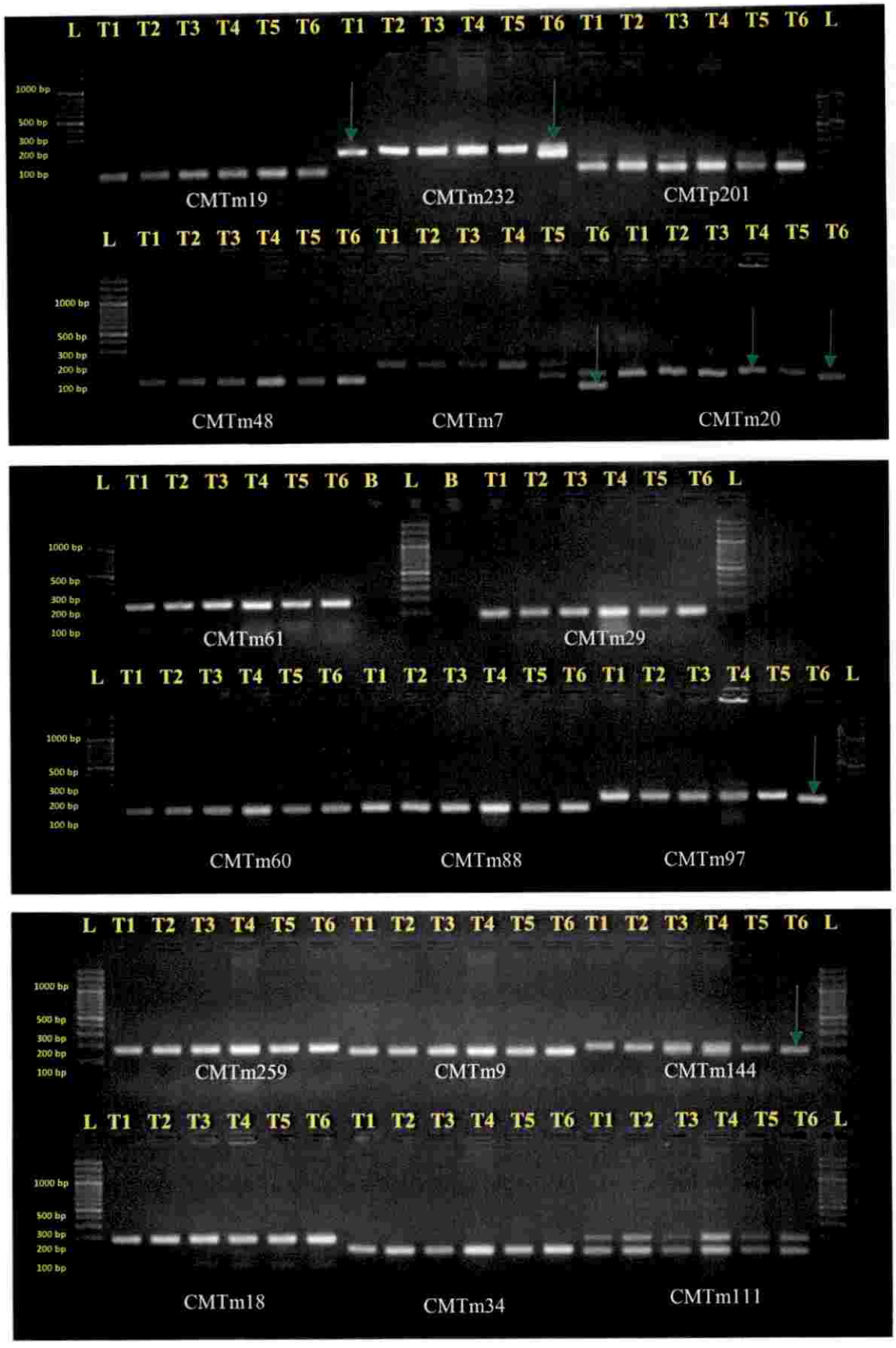


Plate 16: Amplification profile generated by SSR primers in six pumpkin varieties

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
200						
124						
56						

a. Colour chart for CMTm7

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
149						
124						
100						

b. Colour chart for CMTm20

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
200						
168						

c. Colour chart for CMTm97

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
181						
168						

d. Colour chart for CMTm144

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	Ambili	Saras	Suvarna	CO-1	CO-2	Arka Chandan
263						
238						
207						

e. Colour chart for CMTm232

Colour code for shared amplicons among genotypes

Fig. 24. Colour chart of SSR primers (a) CMTm7, (b) CMTm20, (c) CMTm97, (d) CMTm144 and (e) CMTm232

4.2.8 Fingerprinting data using five SSR primers in six varieties

The unique bands produced by the five SSR primer pairs individually were insufficient to prepare fingerprint data for individual varieties. Hence, a DNA fingerprint data of six varieties was created by combining the information obtained across the various SSRs used for analysis (Fig. 25).

The molecular size of DNA fingerprints thus generated ranged between 56 bp and 263 bp. In total, ten amplicons were produced out of which seven were unique.

Unique DNA fingerprints generated by the primers CMTm232 at 238 bp (Ambili) and 420 bp (Arka Chandan) were specific fingerprint of the respective varieties. Primer CMTm97 and CMTm144 individually produced unique amplicon at 168 bp for variety Arka Chandan. Primer CMTm20 generated an unique amplicon for variety CO-1 at 149 bp and for variety Arka Chandan at 100 bp. At 56 bp, for variety Arka Chandan, SSR primer CMTm7 generated a unique amplicon.

All five selected SSR primers can be efficiently used for identification and distinguishing Arka Chandan from a mixed population of the studied varieties and could also be recommended to Seed Testing agencies and companies for detecting the genetic purity of this variety. Sim *et al.* (2015) and Kazminska *et al.* (2017) also concluded the usefulness of markers CMTm7, CMTm20, CMTm232 and CMTm144 as core primers in cultivar identification assay, seed purity determination and for facilitating DUS testing of pumpkin.

Due to usage of finite number of SSRs, differentiation between all the varieties was not possible. Usage of limited number of SSR primers provided less information regarding characterisation and identification of several varieties of watermelon (Kwon *et al.*, 2010).

	1	2	3	4	5	6
Amplicon size (bp)/ Varieties	**Ambili**	**Saras**	**Suvarna**	**CO-1**	**CO-2**	**Arka Chandan**
263		CMTm232	CMTm232	CMTm232	CMTm232	
238	CMTm232					
207						CMTm232
200	CMTm7	CMTm7	CMTm7	CMTm7	CMTm7	
	CMTm97	CMTm97	CMTm97	CMTm97	CMTm97	
181	CMTm144	CMTm144	CMTm144	CMTm144	CMTm144	
168						CMTm97
						CMTm144
149				CMTm20		
124	CMTm20	CMTm20	CMTm20		CMTm20	CMTm7
					CMTm7	
100						CMTm20
56						CMTm7

Colour code for shared amplicons among genotypes

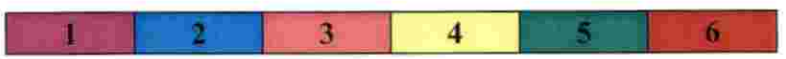


Fig. 25. Overall colour chart of six pumpkin varieties using five SSR marker profiles

4.2.9 Developing varietal fingerprints combining ISSR and SSR marker profiles

Constructing DNA fingerprints or molecular identities for the panel of six varieties studied in this experiment using ISSR and SSR markers was found to be effective (Table 17 to Table 22). This further unveiled the reliability of ISSR and SSR markers for variety identification and seed purity analysis. However, due to usage of more number of ISSR markers, fingerprinting map by ISSR was more saturated and informative in identifying and discriminating between the six varieties.

Liu *et al.* (2012) had also stated the efficacy and superiority of ISSR markers in fingerprinting sugar beet lines. Ogras *et al.* (2017) and Panwar *et al.* (2018) reported that ISSR markers produce reproducible fingerprint profiles in field and horticultural crops. SSR markers were found to be effective in discriminating and fingerprinting some varieties, but, however as the study incorporated less number of varieties and SSR primers pairs, the level of discrimination detected was low. Zhang *et al.* (2015) suggested that to construct specific DNA fingerprint profile for a genotype either the number of tested materials or SSR primers need to be increased.

The fingerprint developed through ISSR and SSR marker along with its combination for the six varieties through this study (Table 17 to Table 22) can be used by the public and private seed sector for precise assessment of genetic purity and detect contaminants so as to ensure timely supply of good quality seeds of these six varieties to the farming community. Though the use of DNA fingerprint technique is nascent in GOT for purity analysis through cultivar identification, this method is gaining popularity as expenditure for DNA testing is decline and it was also opined by Yigezu *et al.* (2019).

Agro-morphological traits are vulnerable to environment, cultural practice and subjectivity. The absence of high consensus between the morphological and

molecular characterisation should not be considered a limitation of these tools to characterise and quantify variability. It only indicates that both morphological and molecular characterisation is important and play a complementary role in providing a better understanding and differentiation of the pumpkin varieties. Even though discrepancies exist between morphological and molecular characterisation, these shortcomings can be circumvented by accompanying field test with molecular techniques as supportive means for accurate estimation of purity of commercial seed lots of the six pumpkin varieties studied. The fingerprinting map developed through studied ISSR and SSR markers will not only give impressive results during purity estimation of these six varieties but also saves time, money and overcome problems of field detection caused by human and environmental factors. Similar conclusions for purity assay were previously reported by Li *et al.* (2018) in pumpkin, Lu *et al.* (2018) in watermelon, Pattanaik *et al.* (2018) in cauliflower, *etc.*

By increasing the number of markers for screening and fingerprinting, effective core set of markers (both ISSR and SSR) can be identified for these varieties which can be used for identifying off types and other contaminants and can completely replace the traditionally conducted GOT using morphological descriptors. These markers can also be used for varietal identification and DUS testing.

155

Table 17. Combined fingerprint of variety Ambili using ISSR and SSR primers

Ambili		
	Primer	**Unique band size (bp)**
ISSR	UBC 812	753
	UBC-822	473
	UBC-844	691
	UBC-847	586
	UBC-855	810
		428
SSR	CMTm232	238

Table 18. Fingerprint of variety Saras using ISSR primers

Saras		
	Primer	**Unique band size (bp)**
ISSR	UBC-809	421
	UBC-822	552
	UBC-827	700
	UBC-836	1821
	UBC-844	783
	UBC-847	1595
	UBC-854	444

Table 19. Fingerprint of variety Suvarna using ISSR primers

Suvarna		
	Primer	**Unique band size (bp)**
ISSR	UBC-807	543
	UBC-808	289
	UBC-827	623

156

Table 20. Combined fingerprint of variety CO-1 using ISSR and SSR primers

CO-1		
	Primer	**Unique band size (bp)**
ISSR	UBC-816	1385
	UBC-822	1403
		353
	UBC-826	1070
		864
	UBC-834	1200
	UBC-835	1059
	UBC-841	1034
	UBC-866	1235
		944
		791
SSR	CMTm20	149

Table 21. Fingerprint of variety CO-2 using ISSR primers

CO-2		
	Primer	**Unique band size (bp)**
ISSR	UBC-812	947
		289
	UBC-815	426
	UBC-823	1161
	UBC-855	700
	UBC-866	1483

157

Table 22. Combined fingerprint of variety Arka Chandan using ISSR and SSR primers

Arka Chandan		
	Primer	**Unique band size (bp)**
ISSR	Oligo ISSR 5	1264
		822
	UBC-807	751
		581
	UBC-808	814
	UBC-810	600
	UBC-811	927
		340
	UBC-813	436
	UBC-815	1110
		575
		343
	UBC-816	1792
	UBC-818	506
	UBC-822	517
	UBC-823	1085
	UBC-825	1244
		791
	UBC-834	1133
		776
	UBC-836	462
	UBC-844	742
	UBC-846	1443
	UBC-847	1500
		1415
		1185
	UBC-855	1544
	UBC-857	1775
		1111
		700
	UBC-866	853
		631
		568
	UBC-868	1100
		844
SSR	CMTm7	56
	CMTm20	100
		149
	CMTm97	168
	CMTm144	
	CMTm232	207

Summary

5. SUMMARY

The research programme 'Characterisation of pumpkin (*Cucurbita moschata* Duch.) varieties through morphological and molecular markers' was carried out between 2018 and 2019 in the Department of Seed Science and Technology, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur. The study envisaged the characterisation of six varieties of pumpkin (Ambili, Saras, Suvarna, CO-1, CO-2 and Arka Chnadan) in the seed supply chain using morphological and molecular markers in order to ascertain their genetic purity. The results of the study are summarised below.

I. Morphological characterisation of pumpkin varieties

Characterisation of pumpkin varieties was done using 28 quantitative and 16 qualitative traits was based on DUS and NBPGR descriptors. The salient findings were as follows:

- Significant difference was found to exist among the six varieties for all the 28 quantitative traits studied. The exception being length of seed.
- No variability existed among the six varieties for six out of 16 qualitative traits recorded. It was observed that all the varieties possessed tendrils which were coiled and branched, cordate leaves. The mature fruit skin in all varieties had waxy coating on them. Hence, these traits did not prove useful in distinguishing between the varieties studied.
- Cotyledons of Arka Chandan were of medium length compared to long ones in the other varieties
- The varieties CO-1 and CO-2 possessed broad cotyledons compared to narrow ones in variety Arka Chandan. Varieties Ambili, Saras and Suvarna were found to possess cotyledons of medium width.

- Early plant vigour varied from poor to vigouros. among varieties. Variety Arka Chandan possessed poor vigour, while Suvarna, CO-1 and CO-2 were vigouros. Ambili and Saras exhibited intermediate vigour habit.

- Except stalk length of the tendrils, all the six varieties possessed similar tendril characteristics.
- Although leaf dimensions *i.e.,* blade length, blade width and petiole length exhibited variations *per se*, these were not useful for grouping the six varieties because all the varieties could be grouped into medium broad leaves class with long petiole.
- Although leaf shape was cordate in all six varieties, the upper side colour of leaf (dark green and medium green) and blade margin pattern (weakly and moderately incised) differed among them. Hence, the shape of leaves may not be useful in distinguishing between the varieties studied.
- All the studied varieties except Arka Chandan had silver patches on upper leaf surface.
- Pubescence density on adaxial leaf side was effective for distinct grouping among the varieties.
- Reproductive characters (node at which the first female flower appeared and days to 50 % flowering) desirable for determining varietal earliness showed significant variation between the six varieties.
- Variation shown by qualitative traits and quantitative yield contributing fruit traits were helpful in grouping the varieties studied. This implied the importance of fruit characters as key descriptors in characterisation and identification of pumpkin varieties.
- As observed in fruit traits, seed dimensions and appearance proved efficient in grouping and identification of the six pumpkin varieties although the *per se* estimates did not significantly vary between the genotypes.
- Cluster analysis distributed six varieties into five clusters based on morphological similarities indicating their distinctness from each other.

161

- Principal component analysis using quantitative data estimated extent of genetic variability among six varieties. It also indicated the importance of fruit and seed traits (cotyledon length and width, tendril length, petiole length, peduncle length, node of first female flower, days to 50 % flowering, flared length, fruit length, fruit diameter, fruit circumference, number of ribs per fruit, fruit yield, seed cavity diameter, seed count per fruit, hundred seed weight, seed length, seed width, seed thickness and plant main vine length) for efficient characterisation and varietal identification.

II. Genotyping of pumpkin varieties using molecular markers

Genotyping using ISSR and SSR markers was done to elucidate allelic diversity and to characterise six pumpkin varieties.

- DNA isolated from young leaf of each variety was confirmed to be of good quality and in sufficient quantity to aid precise analysis.

II a. Genotyping with ISSR markers

- Out of 33 ISSR markers screened, 28 showed polymorphism.
- The total number of amplicons detected ranged from 4 (UBC-818) to 18 (UBC-847) while, the number of polymorphic amplicons ranged from 1 (UBC-818) to 15 (UBC-847).
- UBC-809 recorded highest PIC (Polymorphic Information Content) value. Eighteen ISSR markers registered PIC values above 0.80, indicating its high discriminatory power.
- Cluster analysis based on ISSR profiles, separated the six pumpkin varieties into four clusters when truncated at 85 per cent similarity.
- Cluster I was the largest with three varieties (Ambili, Saras and Suvarna), whereas, the remaining three clusters were monogenic *i.e.,* with one variety each. Genetic similarity indices ranged from 0.57 to 0.89 implying moderate level of variation.

162

- As environmental influence on trait expression cannot be negated, no consistency in grouping was observed between the clustering pattern obtained using morphological and ISSR data.
- A combination of several ISSRs that produced unique amplicons could be helpful in establishing varietal identity and distinguishing it from a varietal mixture. Twenty eight out of the 33 ISSR primers used proved to be useful in generating fingerprints of the six pumpkin varieties studied
- A single ISSR marker that could distinguish all the six pumpkin varieties *per se* was not found. But, ISSR primer UBC-822 developed DNA fingerprints in varieties Ambili (473 bp), Saras (552 bp), CO-1 (1403 bp) and Arka Chandan (517 bp) and could distinguish between these four varieties and help identify them appropriately.

II b. Genotyping with SSR markers

- Five out of 20 screened SSR markers produced polymorphic bands. Total amplicons generated varied only from 2 to 3 due to usage of less number of samples.
- Based on PIC value SSR primers CMTm7, CMTm20, CMTm232 were found to be useful in distinguishing the varieties and establishing their uniqueness.
- The UPGMA clustering algorithm grouped the varieties into four clusters. Cluster I and IV were monogenic, whereas, cluster II and III had two members each.
- The clustering pattern of varieties using morphological data and SSR profile were near similar except for one dissimilarity. Variety Saras and Suvarna shared a common cluster based on SSR marker profiles while, these two varieties had belonged to two different clusters when evaluated on the basis of morphological data.
- Unique amplicons generated by the five polymorphic SSR primers were useful in generating finegrprints for the three varieties- Ambili, CO-1 and Arka Chandan.

163

- All five selected SSR primers can be efficiently used for identification and distinguishing Arka Chandan from a mixed population of the studied varieties and could also be recommended to Seed Testing agencies and Seed companies for detecting the genetic purity of this variety.
- In general, it can be concluded that the correlation that existed between morphological and molecular assessments was of medium magnitude. The absence of high consensus between the assessments should not be considered a limitation of these tools to characterise and quantify variability. It only indicated that both morphological and molecular characterisation are important and play a complementary role in providing a better understanding and differentiation of the pumpkin varieties.

References

6. REFERENCES

Abdein, M. A. E. H. 2018. Genetic diversity between pumpkin accessions growing in the northern border region in Saudi Arabia based on biochemical and molecular parameters. *Egypt. J. Bot.* 58(3): 463-476.

Abdin, M. Z., Arya, L., Saha, D., Sureja, A. K., Pandey, C., and Verma, M. 2014. Population structure and genetic diversity in bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] germplasm from India assessed by ISSR markers. *Plant Syst. Evol.* 300(4): 767-773.

Agbagwa, I. O., Ndukwu, B. C., and Mensah, S. I. 2007. Floral biology, breeding system, and pollination ecology of *Cucurbita moschata* (Duch. ex. Lam.) Duch. ex. Poir. varieties (Cucurbitaceae) from parts of the Niger Delta, Nigeria. *Turk. J. Bot.* 31(5): 451-458.

Ahmed, K. U., Akhter, B., Islam, M. R., Ara, N., and Humauan, M. R. 2011. An assessment of morphology and yield characteristics of pumpkin (*Cucurbita moschata*) genotypes in northern Bangladesh. *Trop. Agric. Res. Ext.* 14(1): 7-11.

Ajuru, M. G. and Okoli, B. E. 2013. The morphological characterisation of the melon species in the family Cucurbitaceae Juss., and their utilization in Nigeria. *Int. J. Mod. Bot.* 3(2): 15-19.

Alam, S. S., Jahan, N., Habib, M. A., and Islam, M. N. 2012. Cytogenetical and molecular characterisation of five commercial varieties in *Trichosanthes anguina* L. *Cytologia*. 77(2): 155-162.

Ali-Shtayeh, M. S., Jamous, R. M., Shtaya, M. J., Mallah, O. B., Eid, I. S., and Zaitoun, S. Y. A. 2017. Morphological characterization of snake melon (*Cucumis melo* var. *flexuosus*) populations from Palestine. *Genet. Resour. Crop Evol.* 64(1): 7-22.

166

Amiri, P., Ismaili, A., and Hadian, J. 2017. Evaluation of genetic diversity of styrian pumpkin (*Cucurbita pepo* var. *styriaca*) populations, using ISSR molecular markers. *J. Plant Genet. Res.* 4(2): 17-28.

Aneja, B., Sharma, V., Yadav, N. R., and Yadav, R. C. 2019. Application of molecular markers in characterization of vegetable crops. *Acta Sci. Agric.* 3(2): 149-152.

Ara, N., Bashar, M. K., Hossain, M. F., and Islam, M. R. 2012. Characterization and evaluation of hybrid pointed gourd genotypes. *Bull. Inst. Trop. Agric. Kyushu Uni.* 35(1): 53-60.

Ara, N., Hossain, M. F., and Akhter, B. 2013. Characterization and evaluation of snake gourd (*Trichasanthes anguina* L) genotypes. *Wudpecker J. Agric. Res.* 2(12): 330-334.

Ara, N., Hiossain, J., and Choudhuary, R. U. 2015. Study of physio-morphological characters of snake gourd germplasm. *Bull. Inst. Trop. Agric. Kyushu Univ.* 38(1): 25-30.

Archak, S., Gaikwad, A. B., Gautam, D., Rao, E. V. V. B., Swamy, K. M., and Karihaloo, J. L. 2003. DNA fingerprinting of Indian cashew (*Anacardium occidentale* L.) varieties using RAPD and ISSR techniques. *Euphytica.* 130(3): 397-404.

Bairwa, P. L., Dixit, A., Sharma, T. K., Tripathy, B., and Kumar, L. 2018. Evaluation of ash gourd [*Benincasa hispida* (Thunb.) Cogn.] genotypes. *Int. J. Curr. Microbiol. App. Sci.* 6: 289-295.

Balkaya, A., Kurtar E. S., and Yanmaz, R. 2009a. Evaluation and selection of suitable pumpkin (*Cucurbita moschata* Duchense) types for the black sea region, Turkey. *Acta Hortic.* 830: 55-62.

Balkaya, A., Yanmaz, R., and Ozbakir, M. 2009b. Evaluation of variation in seed characters of Turkish winter squash (*Cucurbita maxima*) populations. *New Zealand J. Crop Hortic. Sci.* 37(3): 167-178.

167

Balkaya, A., Ozbakır, M., and Karaagac, O. 2010a. Pattern of variation for seed characteristics in Turkish populations of *Cucurbita moschata* Duch. *Afr. J. Agric. Res.* 5(10): 1068-1076.

Balkaya, A., Ozbakir, M., and Kurtar, E. S. 2010b. Phenotypic diversity and fruit characterization of winter squash (*Cucurbita maxima*) populations from the black sea region of Turkey. *Afr. J. Biotech.* 9(2): 152-162.

Banik, R. B. 2003. Variability, gene action and heterosis in snake gourd (*Trichosanthes anguina* L.). Ph.D. Dissertation, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, 60p.

Barman, A. and Anshumali. 2018. Genetic diversity estimates in landraces of pumpkin (*Cucurbita moschata*) from north eastern Himalayan region of India using agro-morphological and molecular traits. *Indian J. Agric. Sci.* 88(1): 79-85.

Barzegar, R., Peyvast, G., Ahadi, A. M., Rabiei, B., Ebadi, A. A., and Babagolzadeh, A. 2013. Biochemical systematic, population structure and genetic variability studies among Iranian Cucurbita (*Cucurbita pepo* L.) accessions, using genomic SSRs and implications for their breeding potential. *Biochem. Syst. Ecol.* 50: 187-198.

Begum, T. and Kumar, D. 2011. Usefulness of morphological characteristics for DUS testing of jute (*Corchorus olitorius* L. and *C. capsularis* L.). *Span. J. Agric. Res.* 9(2): 473-483.

Behera, T. K., Singh, A. K., and Staub, J. E. 2008. Comparative analysis of genetic diversity in Indian bitter gourd (*Momordica charantia* L.) using RAPD and ISSR markers for developing crop improvement strategies. *Sci. Hortic.* 115(3): 209-217.

Bhagat, S. 2017. Morphological characterization, seed germination tests and character association analysis for fruit yield in spine gourd (*Momordica dioica* Roxb.). M.Sc. (Ag.) thesis, Indira Gandhi Krishi Vishwavidyalaya, Raipur, 124p.

168

Bhagwat, A., Srinivasa, V., Bhammanakati, S., and Shubha, A.S. 2018. Evaluation of cucumber (*Cucumis sativus* L.) genotypes under hill zone of Karnataka, India. *Int. J. Curr. Microbiol. App. Sci.* 7(9): 837-842.

Bhaskaran, M. and Umarani, R. 2016. Testing seed genetic purity in rice using SSR markers. *Int. J. Chem. Stud.* 4(1): 141-143.

Bhattacharjee, M., Prakash, S. H., Roy, S., Begum, T., and Dasgupta, T. 2018. DNA fingerprinting of CUMS 17 (Suprava): a newly developed variety of sesame. *Int. J. Pure Appl. Biosci.* 6(5): 161-166.

Bhawna, A. M. Z., Arya, L., Saha, D., Sureja, A.K., Pandey, C., and Verma, M. 2014. Population structure and genetic diversity in bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] germplasm from India assessed by ISSR markers. *Plant Syst. Evol.* 300(4): 767-773.

Borle, U. M., Mehetre S. S., and Harer P. N. 2007. Seed protein banding pattern studies on different cytosteriles, their maintainers, fertility restores and hybrids of upland cotton. *J. Maharashtra Agric. Univ.* 32: 208-210.

Briard, M., Le Clerc, V., Mausset, A. E., and Veret, A. 2001. A comparative study on the use of ISSR, microsatellites and RAPD markers for varietal identification of carrot genotypes. *Acta Hortic.* 546: 377-385.

Brown, R. N. 2002. Traditional and molecular approaches to zucchini yellow mosaic virus resistance in *Cucurbita*. Ph.D. (Hort.) thesis, Oregon State University, 201p.

Carvalho, S. I. C., Bianchetti, L. B., Ragassi, C. F., Ribeiro, C. S. C., Reifschneider, F. J. B., Buso, G. S. C., and Faleiro, F. G. 2017. Genetic variability of a Brazilian *Capsicum frutescens* germplasm collection using morphological characteristics and SSR markers. *Genet. Mol. Res.* 16(3): 1-18.

Chaudhary, S., Yashveer, S., Dinesh, Prajapati, D. R., Kharb, P., and Kamboj, M. C. 2018. Utilization of SSR markers for seed purity testing in popular maize hybrids. *Int. J. Curr. Microbiol. App. Sci.* 7(4): 1117-1126.

169

Chen, L., Xu, H., Song, B., Zhang, H., Kuang, Y., and Yuan, X. 2015. Identification of genetic purity of bitter gourd hybrid by ISSR markers. *Agric. Sci. Technol.* 16(4): 649-652.

Christ, J. A., Hollunder, R. K., Carvalho, M. S., Ferreira, M. F. D. S., Garbin, M. L., and Carrijo, T. T. 2018. DNA fingerprinting based on SSR amplification profiles for piper species identification (Piperaceae). *Acta Bot. Bras.* 32(4): 511-520.

Cooke, R. J. 1999. New approaches to potato variety identification. *Potato Res.* 42(3-4): 529-539.

Cooke, R. J. 2003. *Biochemical and molecular techniques for DUS testing*. Division of Seed Science and Technology, Indian Agricultural Research Institute, New Delhi, p. 39.

Dadlani, M., Vashist, V., Singh, D. P., and Varrier A. 1997. A comparison of field grow out test and electrophoresis methods for testing genetic purity of cotton hybrid seed. *Seed Res*. 24: 160-167.

Das, A., Pandit, M. K., Pal, S., Muthaiah, K., and Layek, S. 2017. Characterization of brinjal genotypes for growth, yield and morphological traits. *Res. J. Agric. Sci.* 8(4): 789-796.

DeWoody, J. A., Honeycutt, R. L., and Skow, L. C. 1995. Microsatellite markers in white-tailed deer. *J. Hered.* 86(4): 317-319.

Dey, S. S., Singh, A. K., Chandal, D., and Behera, T. K. 2006. Genetic diversity of bitter gourd (*Momordica charantia* L.) genotypes revealed by RAPD markers and agronomic traits. *Sci. Hortic.* 109(1): 21-28.

Dharmaraj, K., Ezhilkumar, S., Dinesh, R., and Ananadan, R. 2018. Studies on varietal identification of rice genotypes using ISSR markers. *J. Pharmacogn. Phytochem*. 7(1): 2808-2812.

170

Dongre, A. B., Raut, M. P., Bhandarkar, M. R., and Meshram, K. J. 2011. Identification and genetic purity testing of cotton F 1 hybrid using molecular markers. *Indian J. Biotechnol.* 10(3): 301-306.

Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

Du, X., Sun, Y., Li, X., Zhou, J., and Li, X. 2011. Genetic divergence among inbred lines in *Cucurbita moschata* from China. *Sci. Hortic.* 127(3): 207-213.

Esmailnia, E., Arefrad, M., Shabani, S., Karimi, M., Vafadar, F., and Dehestani, A. 2015. Genetic diversity and phylogenetic relationship of Iranian indigenous cucurbits investigated by Inter Simple Sequence Repeat (ISSR) markers. *Biharean Biol.* 9(1): 47-54.

Ferriol, M., Pico, M. B., and Nuez, F. 2003. Genetic diversity of some accessions of Cucurbita maxima from Spain using RAPD and SBAP markers. *Genet. Resour. Crop Evol.* 50(3): 227-238.

Ferriol, M., Pico, B., de Cordova, P. F., and Nuez, F. 2004. Molecular diversity of a germplasm collection of squash (*Cucurbita moschata*) determined by SRAP and AFLP markers. *Crop Sci.* 44(2): 653-664.

Ferriol, M. and Pico, B. 2008. Pumpkin and Winter Squash. In: Prohens, J. and Nuez, F. (eds.), Handbook of Plant Breeding Vegetables I Part 4, Volume 1, Springer, Berlin, pp. 317-349.

Gao, P., Ma, H., Luan, F., and Song, H. 2012. DNA fingerprinting of Chinese melon provides evidentiary support of seed quality appraisal. *PloS One.* 7(12): 1-10.

Garcial, D. and Mino, K. 2017. DNA fingerprinting. *Bionatura.* 2(4): 477-480.

Garcia-Mas, J., Oliver, M., Gomez-Paniagua, H., and de Vicente, M. C. 2000. Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon. *Theor. Appl. Genet.* 101(5-6): 860-864.

171

Garg, A., Singh, A. K., Parbhu, K. V., Mohapatra, T., Tyagi, N. K., Nandkumar, N., Singh, R., and Zaman, F. U. 2006. Utility of a fertility restorer gene linked marker for testing genetic purity of hybrid seeds in rice (*Oryza sativa* L.). *Seed Sci. Technol.* 34(1): 9-18.

Ghatage, T., Dhurwe, S., Mishra, R., Das, P., and Verma, A.K. 2017. Fingerprinting of bottle gourd (*Lagenaria siceraria*) hybrids and their parental lines using microsatellite markers and their utilization in genetic purity assessment. *Int. J. Curr. Biotechnol.* 5(5): 1-5.

Gichimu, B. M., Owuor, B. O., Mwai, G. N., and Dida, M. M. 2009. Morphological characterization of some wild and cultivated watermelon (*Citrullus* sp.) accessions in Kenya. *ARPN J. Agric. Biol. Sci.* 4(2): 10-18.

Gocmen, M., Aydin, E., Simsek, I., Sari, N., Solmaz, I., and Gokseven, A. 2017. Characterization of some agronomic traits and β-carotene contents of orange fleshed altinbas melon dihaploid lines. *Ekin J. Crop Breed. Genet.* 3(1): 12-18.

Goda, M., Weibull, J., and Ibrahim, E. T. 2007. Diversity of local genetic resources of watermelon, *Citrullus lanatus* (Thunb.) Matsum and Nakai, in Sudan. Master thesis, Swedish Biodiversity Centre, Uppsala, 46p.

GOI (Government of India). 2001. *The Protection of Plant Varieties and Farmers' Rights Act, 2001.* The Gazette of India 2(1), The Ministry of Law, Justice and Company Affairs, Government of India, New Delhi, 50p.

GOI (Government of India). 2018. *Horticultural Statistics at a Glance, 2018.* Horticulture Statistics Division, Department of Agriculture, Cooperation & Farmers' Welfare, Ministry of Agriculture & Farmers' Welfare, Government of India, New Delhi, 458p.

Gong, L., Paris, H. S., Nee, M. H., Stift, G., Pachner, M., Vollmann, J., and Lelley, T. 2012. Genetic relationships and evolution in *Cucurbita pepo* (pumpkin, squash, gourd) as revealed by simple sequence repeat polymorphisms. *Theor. Appl. Genet.* 124(5): 875-891.

172

Goswami, S. and Tripathi, V. 2010. The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among *Trichosanthes dioica* Roxb. cultivars. *Int. J. Biodiversity Conserv.* 2(12): 405-413.

Grubben G. J. H. and Ngwerume, F. C. 2004. *Cucurbita moschata* Duchesne. In: Grubben, G. J. H. and Denton, O. A. (eds.), Prota 2: Vegetables/Legumes, Wageningen, Netherlands.

Guei, R. G., Sanni, K. A., and Fawole, A. F. J. 2005. Genetic diversity of rice (*O. sativa* L.). *Agron. Afr.* 5: 17-28.

Gupta, M., Chyi, Y. S, Romero-Severson, J., and Owen, J. L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. *Theor. Appl. Genet.* 89(7-8): 998-1006.

Gwanama, C., Labuschagne, M. T., and Botha, A. M. 2000. Analysis of genetic variation in *Cucurbita moschata* by random amplified polymorphic DNA (RAPD) markers. *Euphytica.* 113(1): 19-24.

Hamdi, K., Ben-Amor, J., Mokrani, K., Mezghanni, N., and Tarchoun, N. 2017. Assessment of the genetic diversity of some local squash (*Cucurbita maxima* Duchesne) populations revealed by agro-morphological and chemical traits. *J. New Sci.* 42(5): 2306-2317.

Hazra, P. R. G., Sahoo, P., and Som, M. G. 1998. Characterisation of pointed gourd (*Tricosanthes dioica)* in Diara areas of Bihar. *Hortic. J.* 8(2): 165-168.

Heikal, A., Hadia, H. S., Abdel-Razzak, and Hafez, E. E. 2008. Assessment of genetic relationships among and within *Cucurbita* species using RAPD and ISSR markers. *J. Appl. Sci. Res.* 4: 515-525.

Huang, C. H., Wang, C. J., and Chyuan, J. H. 2010. Analysis of the genetic diversity and variety identification of bitter gourd (*Momordica charantia* L.) by ISSR markers. *Hualien Dist. Agric. Improv. Bull.* 28: 21-33.

173

Inan, N., Yildiz, M., Sensoy, S., Kafkas, S., and Abak, K. 2012. Efficacy of ISSR and SRAP techniques for molecular characterization of some Cucurbita genotypes including naked (hull-less) seed pumpkin. *J. Anim. Plant Sci.* 22(1): 126-136.

Ishaq, M. N., Ehirim, B. O., Nwanyanwu, G. C., and Abubaka, R. I. 2019. Simple sequence repeat (SSR) marker-based DNA fingerprinting of some varieties of rice (*Oryza sativa* L.) released in Nigeria. *Afr. J. Biotechnol.* 18(11): 242-248.

Jahan, T. A., Islam, A. K. M. A., Rasul, M. G., Mian, M. A. K., and Haque, M. M. 2012. Heterosis of qualitative and quantitative characters in sweet gourd (*Cucurbita moschata* Duch. ex Poir). *Afr. J. Food Agric. Nutri. Dev.* 12(3): 6186-6199.

Jan, S. J. K. 2002. PIC calculator. Available: https://www.liverpool.ac.uk/~kempsj/pic.html

Joshi, S. P., Gupta, V. S., Aggarwal, R. K., Ranjekar, P. K., and Brar, D. S. 2000. Genetic diversity and phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theor. Appl Genet.* 100(8): 1311-1320.

Jyoti, B., Usha, and Singh, P. 2015. Role of DUS testing in registration of plant varieties under PPV & FR Act, 2001. *Rashtriya Krishi*. 10(2): 5-6.

Kalyanrao, B. T., Tomar, B. S., Singh, B., and Aher, B. 2016. Morphological characterization of parental lines and cultivated genotypes of bottle gourd (*Lagenaria siceraria*). *Indian J. Agric. Sci.* 86(1): 65-70.

Karaman, K., Dalda-Sekerci, A., Yetisir, H., Gulsen, O., and Coskun, O. F. 2018. Molecular, morphological and biochemical characterization of some Turkish bitter melon (*Momordica charantia* L.) genotypes. *Ind. Crops Prod.* 123(1): 93-99.

174

KAU POP [Kerala Agricultural University]. 2016. *Package of Practices Recommendations: Crops (16th Ed.)*. Kerala Agricultural University, Thrissur, 360p.

Kaur, R., Toor, A. K., Bassi, G., and Bains, T. S. 2017. Characterization of mungbean (*Vigna radiata* L. Wilczek) varieties using morphological and molecular descriptors. *Int. J. Curr. Microbiol. App. Sci*. 6(6): 1609-1618.

Kazminska, K., Sobieszek, K., Targonska-Karasek, M., Korzeniewska, A., Niemirowicz-Szczytt, K., and Bartoszewski, G. 2017. Genetic diversity assessment of a winter squash and pumpkin (*Cucurbita maxima* Duchesne) germplasm collection based on genomic *Cucurbita*-conserved SSR markers. *Sci. Hortic.* 219: 37-44.

Khalil, R. M. and Hassan, A. 2016. Genetic analysis in some Cucurbitaceae plants. *Egypt. J. Genet. Cytol.* 42(2): 345-364.

Khan, A. S. M. M. R., Rabbani, M. G., Siddique, M. A., and Islam, M. A. 2007. Characterisation and evaluation of pointed gourd germplasm. *Bangladesh J. Agric. Res.* 32(1): 117-134.

Kiani, G. and Siahchehreh, M. 2017. Diversity in squash varieties assessed by ISSR markers. *Int. J. Veg. Sci.* 23(5): 430-437.

Kiramana, J. K. and Isutsa, D. K. 2017a. First detailed morphological characterisation of qualitative traits of extensive naturalized pumpkin germplasm in Kenya. *Int. J. Dev. Sustain.* 6(7): 500-525.

Kiramana, J. K. and Isutsa, D. K. 2017b. Morphological characterization of naturalised pumpkin (*Cucurbita moschata* (Lam.) Poir.) accessions in Kenya. *Afr. J. Hortic. Sci.* 12: 61-83.

Kiramana, J. K., Isutsa, D. K. and Nyende, A. B. 2017. Fluorescent SSR markers and capillary electrophoresis reveal significant genetic diversity in naturalized pumpkin accessions in Kenya. *Glob. J. Biosci. Biotechnol.* 6(1): 34-45.

Kiran, T. R. and Ranjit, C. 2018. Pumpkin (*Cucurbita moschata* Duch. ex Poir.) genotypes collected from different parts of India and their performance under eastern Himalayan region. *Int. J. Agric. Sci.* 10(6): 5431-5436.

Kirimi, J. K. and Isutsa, D. K. 2018. Genetic diversity of pumpkin accessions in Kenya revealed using morphological characters, diversity index, CATPCA and factor analysis. *Int. J. Sci. Res.* 3(1): 57-79.

Kong, Q., Chen, J., Liu, Y., Ma, Y., Liu, P., Wu, S., Huang, Y., and Bie, Z. 2014. Genetic diversity of *Cucurbita* rootstock germplasm as assessed using simple sequence repeat markers. *Sci. Hortic.* 175: 150-155.

Kordrostami, M. and Rahimi, M. 2015. Molecular markers in plants: concepts and applications. *G3M.* 13: 4024-4031.

Korir, N. K., Li, X. Y., Leng, X. P., Wu, Z., Wang, C., and Fang, J. G. 2013. A novel and efficient strategy for practical identification of tomato (*Solanum lycopersicon*) varieties using modified RAPD fingerprints. *Genet. Mol. Res.* 12(2): 1816-1828.

Krishnamoorthy, V. and Sampath, S. 2019. Genetic diversity analysis in pumpkin (*Cucurbita moschata* Duch. ex Poir.). *Int. J. Chem. Stud.* 7(3): 3672-3676.

Kumar, R., Brahmachari, V. S., and Kumar, R. 1995. Varietals assessment of parwal (*Tricosanthes dioica*). *Indian J. Hortic.* 47: 537-540.

Kumar, J., Singh, R.K., and Pal, K. 2011. Variability and character association in pumpkin (*Cucurbita moschata* Duch. ex Poir.). *Indian J. Agric. Res.* 45(1): 87-90.

Kumari, S. A. S. M., Nakandala, N. D. U. S., Nawanjana, P. W. I., Rathnayake, R. M. S. K., Senavirathna, H. M. T. N., Senevirathna, R. W. K. M., Wijesundara, W. M. D. A., Ranaweera, L. T., Mannanayake, M. A. D. K., Weebadde, C. K., and Sooriyapathirana, S. D. S. S. 2019. The establishment of the species-delimits and varietal-identities of the cultivated germplasm of *Luffa acutangula* and *Luffa aegyptiaca* in Sri Lanka using morphometric, organoleptic and phylogenetic approaches. *Plos One.* 14(4): 1-22.

Kwon, Y. S., Oh, Y. H., Yi, S. I., Kim, H. Y., An, J. M., Yang, S. G., Ok, S. H., and Shin, J. S. 2010. Informative SSR markers for commercial variety discrimination in watermelon (*Citrullus lanatus*). *Genes Genomics.* 32(2): 115-122.

Lakshmi, V. G. I., Gireesh, C., Sreedhar, M., Vanisri, S., Basavaraj, P. S., Muralidhara, B., Anantha, M. S., Padmavathi, G., Fiyaz, A. R., Jyothi, B., Rani, C. S., Mandal, B., and Rao, L. V. S. 2018. Characterisation of african rice germplasm for morphological and yield attributing traits. *Int. J. Curr. Microbiol. App. Sci.* 7(12): 1288-1303.

Lebeda, A., Kristkova, E., and Paris, H. S. 2009. Variation for morphological traits within and among *Cucurbita pepo* genotypes. *Acta Hortic.* 871: 211-218.

Lee, J. H., Robin, A., Natarajan, S., Jung, H. J., and Nou, I. S. 2018. Varietal identification of open-pollinated onion cultivars using a nanofluidic array of single nucleotide polymorphism (SNP) markers. *Agronomy.* 8(9): 179.

Levi, A., Thomas, C. E., Newman, M., Reddy, O. U. K., Zhang, X., and Xu, Y. 2004. ISSR and AFLP markers differ among American watermelon cultivars with limited genetic diversity. *J. Am. Soc. Hortic. Sci.* 129(4): 553-558.

Li, Q., Wang, D., and Yang, J. 2018. Identification and detection analysis of SSR purities of three tender pumpkin cultivars. *Mol. Plant Breed.* 9(9): 68-72.

Lin, Y. S., Kuan, C. S., Weng, I. S., and Tsai, C. C. 2015. Cultivar identification and genetic relationship of pineapple (*Ananas comosus*) cultivars using SSR markers. *Genet. Mol. Res.* 14(4): 15035-15043.

Litt, M. and Luty, J. A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* 44(3): 397-401.

Liu, L. W., Wanga, Y., Gonga, Y. Q., Zhaob, T. M., Liua, G., Lia, X. Y., and Yuc, F. M. 2007. Assessment of genetic purity of tomato (*Lycopersicon esculentum* L.) hybrid using molecular markers. *Sci. Hortic.* 115(1): 7-12.

Liu, L. W., Zhao, L. P., Gong, Y. Q., Wang, M. X., Chen, L. M., Yang, J. L., Wang, Y., Yu, F. M., and Wang, L. Z. 2008. DNA fingerprinting and genetic diversity analysis of late-bolting radish cultivars with RAPD, ISSR and SRAP markers. *Sci. Hortic.* 116(3): 240-247.

Liu, Q., Cheng, D., Yang, L., Luo, C., Zhang, H., Wu, Y., Liu, N., and Zhou, Q. 2012. Identification of DNA fingerprinting and cluster analysis using ISSR markers for 13 sugar beet cultivars (lines) from China and Holland. In: International conference on biomedical engineering and biotechnology, pp. 325-328.

Liu, C., Ge, Y., Wang, D. J., Li, X., Yang, X. X., Cui, C. S., and Qu, S. P. 2013. Morphological and molecular diversity in a germplasm collection of seed pumpkin. *Sci. Hortic.* 154: 8-16.

Lu, X. J., Liu, L. W., Gong, Y. Q, Zhao, L. P, Song, X. Y., and Zhu, X. W. 2009. Cultivar identification and genetic diversity analysis of broccoli and its related species with RAPD and ISSR markers. *Sci. Hortic.* 122(4): 645-648.

Lu, X., Adedze, Y. M. N., Chofong, G. N., Gandeka, M., Deng, Z., Teng, L., Zhang, X., Sun, G., Si, L., and Li, W. 2018. Identification of high-efficiency SSR markers for assessing watermelon genetic purity. *J. Genet.* 97(5): 1295-1306.

Lucchese, C., Dinelli, G., Miggiano, A., and Lovato. 1999. Identification of pepper (*Capsicum* spp.) cultivars by field and electrophoresis tests. *Seed Sci. Technol.* 27: 37-47.

Maggs-Kolling, G. L., Madsen, S., and Christiansen, J. L. 2000. A phenetic analysis of morphological variation in *Citrullus lanatus* in Namibia. *Genet. Res. Crop Evol.* 47(4): 385-393.

Mahla, H. R., Singh, J. P., and Roy, M. M., 2014. *Seed purpose watermelon in arid zone.* Central Arid Zone Research Institute, Jodhpur, 44p.

Maji, A. T. and Shaibu, A. A. 2012. Application of principal component analysis for rice germplasm characterization and evaluation. *J. Plant Breed. Crop Sci.* 4(6): 87-93.

Mannino, G., Gentile, C., and Maffei, M. E. 2019. Chemical partitioning and DNA fingerprinting of some pistachio (*Pistacia vera* L.) varieties of different geographical origin. *Phytochemistry*. 160: 40-47.

Martins, S., Carnide, O. P., de Carvalho, C. R., and Carnide, V. 2015. Assessing genetic diversity in landraces of *Cucurbita* spp. using a morphological and molecular approaches. *Procedia Environ. Sci.* 29: 68-69.

Martins, L. H. P., Lopes, M. T. G., Noda, H., Neto, P. D. Q. C., Martins, A. L. U., de Almeida, F. V., and Demosthenes, L. C. R. 2018. Genetic variability of pumpkin landraces from Brazilian Amazon. *Agric. Sci.* 9(4): 511.

Meesang, N., Ranamukhaarachchi, S. L., Petersen, M. J., and Andersen, S. B. 2001. Soybean cultivar identification and genetic purity analysis using microsatellite DNA markers. *Seed Sci. Technol.* 29(3): 637-646.

Mehetre, S. and Dahat, D. 2001. Page of seed proteins as a technique for varietal identification of *Gossypium* spp.: A review. *J. Maharashtra Agric. Univ.* 26: 233-238.

Meyer, W., Mitchell, T. G., Freedman, E. Z., and Vilgays, R. 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformes*. *J. Clin. Microbiol.* 31: 2274-2280.

Miladinovic, D., Dimitrijevic, A., Brdar-Jokanovic, M., Imerovski, I., Sikora, V., Marjanovic Jeromela, A., and Jocic, S. 2016. Molecular analysis of NS *Cucurbita moschata* collection. In: *Food Technology, Quality and Safety.* Proceedings of the III International Congress, Novi Sad, pp. 6-9.

Mladenovic, E., Berenji, J., Ognjanov, V., Kraljevic-Balalic, M., Ljubojevic, M., and Cukanovic, J. 2011. Conservation and morphological characterization

of bottle gourd for ornamental use. In: *46th Croatian and 6th International Symposium on Agriculture.* Opatija, Croatia, pp. 550-553.

Mladenovic, E., Berenji, J., Ognjanov, V., Ljubojevic, M. I. R. J. A. N. A., and Cukanovic, J. E. L. E. N. A. 2012. Genetic variability of bottle gourd (*Lagenaria siceraria* (Mol.) Standley) and its morphological characterization by multivariate analysis. *Arch. Biol. Sci.* 64(2): 573-583.

Mohsin, G. M., Islam, M. S., Rahman, M. S., Hasanuzzaman, M., and Biswas, B. K. 2016. Genetic divergence in pumpkin (*Cucurbita Moschata* Duch. ex Poir.). *Adv. Plants Agric. Res.* 4(5): 374-377.

Moisan-Thiery, M., Marhadour, S., Kerlan, M. C., Dessenne, N., Perramant, M., Gokelaere, T., and Le Hingrat, Y. 2005. Potato cultivar identification using simple sequence repeats markers (SSR). *Potato Res.* 48(3-4): 191-200.

Morell, M. K., Peakall, R., Appels, R., Preston, L. R., and Lloyd, H. L. 1995. DNA profiling techniques for plant variety identification. *Aust. J. Exp. Agric.* 35(6): 807-819.

Morgante, M., Hanafey, H., and Powell, W. 2002. Microsatellites are preferentially associated with nonrepetitive DNA in plant genome. *Nat. Genet.* 30(2): 194-200.

Mullis, K., Faloona, F., Scharf, S., Saiki, R. K., Horn, G. T., and Erlich, H. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* 51: 263-273.

Muralidhara, M. S. and Narasegowda, N. C. 2014. Genetic diversity analysis of pumpkin genotypes (*Cucurbita moschat* Duch. ex Poir.) using morphological and RAPD markers. *Asian J. Bio Sci.* 9(2): 188-194.

Murovec, J. 2015. Phenotypic and genetic diversity in pumpkin accessions with mutated seed coats. *HortScience.* 50(2): 211-217.

Nadeem, M. A., Nawaz, M. A., Shahid, M. Q., Dogan, Y., Comertpay, G., Yıldız, M., Hatipoglu, R., Ahmad, F., Alsaleh, A., Labhane, N., and Ozkan, H.

2018. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. *Biotechnol. Biotechnol. Equip.* 32(2): 261-285.

Naga, B. L. R. I., Mangamoori, L. N., and Subramanyam, S. 2012. Identification and characterization of EST-SSRs in finger millet (*Eleusine coracana* (L.) Gaertn.). *J. Crop Sci. Biotechnol.* 15(1): 9-16.

Nagar, A., Sureja, A. K., and Munshi, A. D. 2017. DUS characterization of pumpkin (*Cucurbita moschata*) genotypes. *Indian J. Agric. Sci.* 87(6): 776-784.

Nagar, A., Sureja, A. K., Kumar, S., Munshi, A. D., Gopalakrishnan, S., and Bhardwaj, R. 2017. Genetic variability and principal component analysis for yield and its attributing traits in pumpkin (*Cucurbita moschata* Duchesne Ex Poir.). *Vegetos*. 30(1): 81-86.

Nagaraju, K. and Thiruvenkatasamy, S. 2017. Genetic characterisation of ash gourd [*Benincasa hispida* (Thunb.) Moench.] germplasm for yield and quality traits through principal component analysis. *J. Pharmacogn. Phytochem.* 6(5): 1250-1255.

Ndoro, E., Dauda, N. S., and Chammang, H. B. 2012. Effect of germination media and seed size on germination and seedling vigour of fluted pumpkin (*Telferia occidentalis*) Hook. F. *Int. J. Agri. Sci.* 2(3): 113-115.

Ntuli, N. R., Tongoona, P. B., and Zobolo, A. M. 2015. Genetic diversity in *Cucurbita pepo* landraces revealed by RAPD and SSR markers. *Sci. Hortic.* 189: 192-200.

Ogras, T., Bastanlar, E. K., Metin, O. K., Kandemir, I., and Ozcelik, H. 2017. Assessment of genetic diversity of rose genotypes using ISSR markers. *Turk. J. Bot.* 41(4): 347-355.

Oh, S. J., Song, J. Y., Lee, J., and Lee, G. A. 2012. Evaluation of genetic diversity of red pepper landraces (*Capsicum annuum* L.) from Bulgaria using SSR markers. *Korean J. Int. Agric.* 24(5): 547-556.

181

Oliveira, D. R. L., Goncalves, L. S. A., Rodrigues, R., Baba, V. Y., Sudre, C. P., dos Santos, M. H., and Aranha, F. M. 2016. Genetic divergence among pumpkin landraces. *Semin.: Cienc. Agrar.* 37(2): 547-556.

Onyishi, G. C., Ngwuta, A. A., Onwuteaka, C., and Okporie, E. O. 2013. Assessment of genetic variation in twelve accessions of tropical pumpkin (*Cucurbita maxima*) of south eastern Nigeria. *World Appl. Sci. J.* 24(2): 252-255.

Orsenigo, S., Abeli, T., Schiavi, M., Cauzzi, P., Guzzon, F., Ardenghi, N. M., Rossi, G., and Vagge, I. 2018. Morphological characterisation of *Cucurbita maxima* Duchesne (Cucurbitaceae) landraces from the Po Valley (Northern Italy). *Ital. J. Agron.* 13(4): 338-342.

Pal, S., Sharma, H., and Yadav, N. 2017. Evaluation of cucumber genotypes for yield and quality traits. *J. Hill Agric.* 8(2): 144-150.

Pandravada, S. R., Sivaraj, N., Jairam, R., Sunil, N., Begum, H., Reddy, M. T., Chakrabarty, S. K., Bisht, I. S., and Bansal, K. C. 2014. *Luffa hermaphrodita*: first report of its distribution and cultivation in Adilabad, Andhra Pradesh, South India. *Asian Agri Hist.* 18(2): 123-132.

Panse, V. S. and Sukhatme, P. V. 1985. *Statistical methods for Agricultural workers* (4th Ed.) Indian Council of Agricultural Research, New Delhi, 361p.

Panwar, S., Singh, K. P., Namita, T. J., Janakiram, T., Sonah, H., and Sharma, T. R. 2018. DNA fingerprinting in African marigold (*Tagetes erecta* L.) genotypes using ISSR and URP markers. *Indian J. Hortic.* 75(1): 105-110.

Paris, H. S., Portnoy, V., Mozes-Daube, N., Tzuri, G., Katzir, N., and Yonash, N. 2002. AFLP, ISSR and SSR polymorphisms are in accordance with botanical and cultivated plant taxonomies of the highly polymorphic *Cucurbita pepo*. *Acta Hortic.* 634: 167-173.

Paris, H. S. and Brown, R. N. 2005. The genes of pumpkin and squash. *Am. Soc. Hortic. Sci.* 40(6): 1620-1630.

182

Paris, H. S., Burger, Y., and Schaffer, A. A. 2006. Genetic variability and introgression of horticulturally valuable traits in squash and pumpkins of *Cucurbita pepo*. *Isr. J. Plant Sci.* 54(3): 223-231.

Parvathaneni, R. K., Natesan, S., Devaraj, A. A., Muthuraja, R., Venkatachalam, R., Subramani, A. P., and Laxmanan, P. 2011. Fingerprinting in cucumber and melon (*Cucumis* spp.) genotypes using morphological and ISSR markers. *J. Crop Sci. Biotechnol.* 14(1): 39-43.

Pattanaik, A., Reddy, D. L., Ramesh, S., and Chennareddy, A. 2018. Comparison of traditional grow-out test and DNA-based PCR assay to estimate F1 hybrid purity in cauliflower. *Curr. Sci.* 115(11): 2095-2102.

Pornsuriya, P., Pramot, Pornsuriya, Pornthip, and Numuen, C. 2011. Phenotypic diversity and classification of Thai bitter melon (*Momordica charantia* L.) landraces from three provinces in central region of Thailand. *J. Agric. Technol.* 7(3): 849-856.

Powell, W., Machray, G. C., and Provan, J. 1996. Polymorphism revealed by simple sequence repeats. *Tren. Plant Sci.* 1: 215-222.

PPV & FRA [Protection of Plant varieties and Farmers' Rights]. 2017. DUS test guidelines (Crop: Pumpkin). [on line]. Available: http://www.plantauthority.gov.in/crop- guidelines.htm [12 Dec. 2017].

Pradeep, R. M., Sarla, N., and Siddiq, E. A. 2002. Inter simple sequence repeat ISSR polymorphism and its application in plant breeding. *Euphytica.* 128(1): 9-17.

Prasad, V. S. R. K. and Singh, D. P. 1990. Studies on morphological and agronomical components of pointed gourd (*Trichosanthes dioica* Roxb.). *Indian J. Hortic.* 47(3): 337-340.

Priori, D., Barbieri, R. L., Mistura, C. C., and Villela, J. C. B. 2018. Morphological characterization of pumpkin landraces (*Cucurbita maxima*) from southern Brazil. *Rev. Ceres*. 65(4): 337-345.

183

Pujar, U. U., Shantappa, T., Jagadeesha, R. C., and Sandhyarani, N. 2017. Hybrid purity testing of chilli hybrid (Pusa Jwala × Arka Lohit) through RAPD and ISSR Molecular markers. *Int. J. Curr. Microbiol. App. Sci.* 6(11): 2079-2086.

Punetha, S., Singh, D. K., and Singh, N. K. 2017. Research note genetic diversity assessment of cucumber (*Cucumis sativus* L.) genotypes using molecular markers. *Electr. J. Plant Breed.* 8(3): 986-991.

Raatz, B., Mukankusi, C., Lobaton, J. D., Male, A., Chisale, V., Amsalu, B., Fourie, D., Mukamuhirwa, F., Muimui, K., Mutari, B., and Nchimbi-Msolla, S. 2019. Analyses of African common bean (*Phaseolus vulgaris* L.) germplasm using a SNP fingerprinting platform: diversity, quality control and molecular breeding. *Genet. Resour. Crop Evol.* 66(3): 707-722.

Ragul, S., Manivannan, N., Mahalingam, A., Babu, R., and Lakshmi, S. 2018. SSR marker based DNA fingerprinting for cowpea varieties of Tamil Nadu [Vigna unguiculata (L.) Walp.]. *Int. J. Curr. Microbiol. App. Sci.* 7(4): 641-647.

Rahman, M. M., Dey, S. K., and Wazuddin, M. 1991. Study of yield, yield components and vine characters of some cucurbit genotypes [Bangladesh]. Bangladesh Agricultural University Research Progress, no. 5.

Raina, S. N., Rani, V., Kojima, T., Ogihara, Y., Singh, K. P., and Devarumath, R. M. 2001. RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome*. 44(5): 763-772.

Rakshit, A., Vashisht, V., Rakshit, S., and Dadlani, M. 2008. Electrophoresis technique for varietal identification and genetic purity in hybrid cotton (*Gossypium hirsutum* L.). *Seed Res.* 36: 28-32.

Rathod, R. R., Mehta, D. R., Gajera, H. P., and Delvadiya, N. A. 2015. Molecular characterization of ridge gourd (*Luffa acutangula* L.) and sponge gourd *(Luffa cylindrica* L.) genotypes through PCR based molecular markers. *Int. J. Agric. Environ. Biotechnol.* 8(3): 521-530.

Reddy, R. N., Mohan, S. M., Madhusudhana, R., Umakanth, A. V., Satish, K., and Srinivas, G. 2008. Inheritance of morphological characters in sorghum. *J. SAT Agric. Res.* 6: 1-3.

Saikia, J. and Phookan, D. B. 2018. Evaluation of ivy gourd (*Coccinia grandis* L. Voigt) genotypes in N.E. region of India. *Int. J. Agric. Sci.* 10(6): 5642-5644.

Salim, M. M. R., Rashid, M. H., Hossain, M. M., and Zakaria, M. 2018. Morphological characterization of tomato (*Solanum lycopersicum* L.) genotypes. *J. Saudi Soc. Agric. Sci.* pp. 1-8.

Santos, M. H. D., Rodrigues, R., Gonçalves, L. S. A., Sudre, C. P., and Pereira, M. G. 2012. Agrobiodiversity in *Cucurbita* spp. landraces collected in Rio de Janeiro assessed by molecular markers. *Crop Breed. Appl. Biotechnol.* 12(2): 96-103.

Sarao, N. K., Pathak, M., and Kaur, N. 2014. Microsatellite-based DNA fingerprinting and genetic diversity of bottle gourd genotypes. *Plant Genet. Resour.* 12(1): 156-159.

Selvi, N. A. T., Jansirani, P., Pugalendhi, L., and Nirmalakumar, A. 2012. Per se performance of genotypes and correlation analysis in pumpkin (*Cucurbita moschata* Duch. ex Poir.). *Electr. J. Plant Breed.* 3(4): 987-994.

Semagn, K., Bjornstad, A., and Ndjiondjop, M. N. 2006. An overview of molecular marker methods for plants. *Afr. J. Biotechnol.* 5(25): 2540-2568.

Serra, I. A., Procaccini, G., Intrieri, M. C., Migliaccio, M., Mazzuca, S., and Innocenti, A. M. 2007. Comparison of ISSR and SSR markers for analysis of genetic diversity in the seagrass *Posidonia oceanica*. *Mar. Ecol. Prog. Ser.* 338: 71-79.

185

Seymen, M., Turkmen, O., Paksoy, M., and Fidan, S. 2012. Determination of some morphological characteristics of edible seed pumpkin (*Cucurbita pepo* L.) genotypes. In: Sari, Solmaz, and Aras (eds.), *Cucurbitaceae.* Proceedings of the Xth EUCARPIA meeting on genetics and breeding of Cucurbitaceae, Antalya, Turkey, pp. 739-749.

Shang, Q. L., Song, M., Xiao, Z. M., and Wang, Z. M. 2012. Molecular evaluation on genetic diversity of ornamental pumpkin and gourd germplasm. *J. Plant Genet. Resour.* 13(3): 406-413.

Shankar, R., Bagle, B. G., and More, T. A. 2009. Diversity analysis of bitter gourd (*Momordica charantia* L.) germplasm from tribal belts of India. *Asian Australas. J. Plant Sci. Biotechnol.* 3(1): 21-25.

Sidhu, G. K. 2013. Evaluation and assessment of genetic diversity among bitter gourd *(Momordica charantia L. Moench.) germplasm*. M.Sc. (Ag.) thesis, Punjab Agricultural University, Ludhiana, 76p.

Sidhu G. K. and Pathak, M., 2016. Genetic diversity analysis in bitter gourd (*Momordica charantia* L.) using morphological traits. *Int. J. Agric. Innov. Res*. 5(1): 59-63.

Sim, S. C., Hong, J. H., and Kwon, Y. S. 2015. DNA profiling of commercial pumpkin cultivars using simple sequence repeat polymorphisms. *Hortic. Environ. Biotechnol.* 56(6): 811-820.

Singh, A. K., Behera, T. K., Chandel, D., Sharma, P., and Singh, N. K. 2007. Assessing genetic relationships among bitter gourd (*Momordica charantia* L.) accessions using inter-simple sequence repeat (ISSR) markers. *J. Hortic. Sci. Biotechnol.* 82(2): 217-222.

Singh, D., Mace, E. S., Godwin, I. D., Mathur, P. N., Okpul, T., Taylor, M., Hunter, D., Kambuou, R., Rao, V. R., and Jackson, G. 2008. Assessment and rationalization of genetic diversity of Papua New Guinea taro (*Colocasia esculenta*) using SSR DNA fingerprinting. *Genet. Resour. Crop Evol.* 55(6): 811-822.

186

Singh, D. K., Tewari, R., Singh, N. K., and Singh, S. S. 2016. Genetic diversity cucumber using inter simple sequence repeats (ISSR). *Transcriptomics*. 4(1): 1-4.

Smith, J. S. C. and Smith, O. S. 1989. The description and assessment of distances between inbred lines of maize: the utility of morphological, biochemical and genetic descriptors and a scheme for the testing of distinctiveness between inbred lines. *Maydica*. 34: 151-161.

Soghani, Z. N., Rahimi, M., Nasab, M. A., and Maleki, M. 2018. Grouping and genetic diversity of different watermelon ecotypes based on agro-morphological traits and ISSR marker. *Iheringia. Serie Bot.* 73(1): 53-59.

Solmaz, I., Sari, N., Mendi, Y. Y., Kacar, Y. A., Kasapoglu, S., Gursoy, I., Suyum, K., Killi, O., Serce, S., and Yildirim, E. 2010. Characterization of some melon genotypes collected from eastern and central Anatolia region of Turkey. *Acta Hortic.* 871: 187-196.

Srivastava, U., Mahajan, R. K., Gangopadhyay, K. K., Singh, M., and Dhillon, B. S. 2001. *Minimal Descriptors of Agri-Horticultural Crops, Part II: Vegetable Crops.* National Bureau of Plant Genetic Resources, New Delhi, pp. 74-84.

Stachel, M., Csanadi, G., Vollmann, J., and Lelley, T. 1998. Genetic diversity in pumpkins (*Cucurbita pepo* L.) as revealed in inbred lines using RAPD markers. *Rep. Cucurbit Genet. Coop.* 21: 48-50.

Staub, J. E., Danin-Poleg, Y., Fazio, G., and Horejsi, T. 2000. Comparative analysis of cultivated melon groups (*Cucumis melo* L.) using random amplified polymorphic DNA and simple sequence repeat markers. *Euphytica.* 115(3): 225-241.

Stift, G., Zraidi, A., and Lelley, T. 2004. Development and characterization of microsatellite markers (SSR) in *Cucurbita* species. Cucurbit genetics cooperative report. 27: 61-64.

187

Sudre, C. P., Goncalves, L. S. A., Rodrigues, R., and do Amaral, A. T. 2010. Genetic variability in domesticated *Capsicum* spp as assessed by morphological and agronomic data in mixed statistical analysis. *Genet. Mol. Res*. 9(1): 283-294.

Sultana, S., Islam, M. N., and Hoque, M. E. 2018. DNA fingerprinting and molecular diversity analysis for the improvement of brinjal (*Solanum melongena* L.) cultivars. *J. Adv. Biotechnol. Exp. Ther.* 1(1): 1-6.

Sumathi, S. and Balamurugan, P. 2014. Usefulness of morphological characters for varietal identification in oats (*Avena sativa* L.). *Int. J. Plant Sci.* 9(1): 7-12.

Tamilselvi, N. A. and Jansirani, P. 2016. Heterosis in pumpkin for earliness, yield, and yield-related characters. *Int.J. Veg. Sci.* 22(2): 170-182.

Tlili, I., Hdider, C., Ilahy, R., and Jebari, H. 2009. Assessing agronomic characteristics and physicochemical properties of selected watermelon varieties grown in Tunisia. *Afr. J. Plant Sci. Biotechnol.* 3(1): 7-11.

Tomar, R. S., Parakhia, M. V., Rathod, V. M., Thakkar, J. R., Kothari, V. V., Acharya, R. R., and Golakiya, B. A. 2016. Assessment of genetic variability among Muskmelon (*Cucumis melo* L.) genotypes through biometrical traits and molecular markers. *Electr. J. Plant Breed.* 7(2): 215-225.

Tsai, C. C., Chen, Y. K. H., Chen, C. H., Weng, I. S., Tsai, C. M., Lee, S. R., Lin, Y. S., and Chiang, Y. C. 2013. Cultivar identification and genetic relationship of mango *(Mangifera indica*) in Taiwan using 37 SSR markers. *Sci. Hortic.* 64: 196-201.

Tungalag, M., Ariungerel, M., Otgonbayar, B., and Ya, M. 2018. Varietal identification study of six wheat varieties using ISSR markers. *Mong. J. Agric. Sci.* 23(1): 14-17.

Varghese, P. 1991. Heterosis in snake gourd (*Trichosanthes anguina* L). M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 130p.

188

Varshney, R. K., Prasad, M., and Graner, A. 2004. Molecular marker maps of barley: a resource for intra-and interspecific genomics. In: Lorz, H. and Wenzel, G. (eds.), *Molecular marker systems in plant breeding and crop improvement*. Biotechnology in agriculture and forestry, Berlin, Heidelberg, pp. 229-243.

Vianna, L. S., Pereira, T. N. S., Santos, E. A., Viana, A. P., Pereira, M. G., Ramos, H. C. C., and Rossi, A. A. B. 2019. SSR and SSR markers for determining genetic relationships among three wild species of *Passiflora*. *Genet. Mol. Res*. 18(1): 1-10.

Whitaker, T. W. and Robinson, R. W. 1986. Squash breeding. In: Bassett, M. J. (ed.), *Breeding vegetable crops*. Westport, pp. 209-242.

Wu, K. S., Jones, R., Danneberger, L., and Scolnik, P. A. 1994. Detection of microsatellite polymorphisms without cloning. *Nucleic Acids Res.* 22(15): 3257-3258.

Xanthopoulou, A., Ganopoulos, I., Kalivas, A., Nianiou-Obeidat, I., Ralli, P., Moysiadis, T., Tsaftaris, A., and Madesis, P. 2015. Comparative analysis of genetic diversity in Greek genebank collection of summer squash (*Cucurbita pepo*) landraces using start codon targeted (SCoT) polymorphism and ISSR markers. *Aust. J. Crop Sci.* 9(1): 14-21.

Yigezu, Y. A., Alwang, J., Rahman, M. W., Mollah, M. B. R., El-Shater, T., Aw-Hassan, A., and Sarker, A. 2019. Is DNA fingerprinting the gold standard for estimation of adoption and impacts of improved lentil varieties? *Food Policy*. 83: 48-59.

Yildiz, M., Akgul, N., and Sensoy, S. 2014. Morphological and molecular characterization of Turkish landraces of *Cucumis melo* L. *Not. Bot. Horti Agrobot. Cluj-Napoca*. 42(1): 51-58.

Zhang, L., Cai, R., Yuan, M., Tao, A., Xu, J., Lin, L., Fang, P., and Qi, J. 2015. Genetic diversity and DNA fingerprinting in jute (*Corchorus* spp.) based on SSR markers. *Crop J.* 3(5): 416-422.

189

Zhao, D., Wen, L., Bi, H., Zhu, Z., Liu, J., Zhang, J., Shi, Q., You, H., Dong, D., and Liu, Q. 2017. Genetic diversity of *Cucurbita maxima* assessed using morphological characteristics and random-amplified polymorphic DNA markers in China. *Acta Agric. Scand.* 67(2): 155-163.

Zietkiewicz, E., Rafalski, A., and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*. 20(2): 176-183.

190

CHARACTERISATION OF PUMPKIN (*Cucurbita moschata* Duch.) VARIETIES THROUGH MORPHOLOGICAL AND MOLECULAR MARKERS

By
AGINA GOPAN
(2017-11-138)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

(SEED SCIENCE AND TECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF SEED SCIENCE AND TECHNOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2019

191

ABSTRACT

Pumpkin (*Cucurbita moschata* Duch.), a crop of Central Mexican origin belonging to the family Cucurbitaceae, is popularly cultivated and valued in Kerala as a vegetable. The tender, large and often round immature fruits of pumpkin with a thick, smooth to slightly ribbed skin, which is mostly deep yellow to orange in colour, is an integral part of the Kerala cuisine. Despite its popularity in the state, few high yielding varieties are in cultivation. To ensure increased production, availability of high quality seeds of improved varieties or hybrids has to be guaranteed. Pumpkin being a cross pollinated crop, occurrence of cross contamination during its seed programme cannot be overruled. Hence, ensuring the purity and identity of seeds of the variety before sale becomes inevitable.

Considering the importance of varietal identification in maintaining the genuineness and quality of seeds in seed production programmes, the present investigation envisaged to characterise six pumpkin varieties in the seed chain using morphological and molecular markers, and to generate fingerprints or molecular ID's of the six varieties using selected polymorphic Inter-simple sequence repeats (ISSR) and Simple sequence repeats (SSR) markers.

Characterisation of pumpkin varieties based on 28 quantitative and 16 qualitative traits was done using DUS and NBPGR descriptors. Qualitative vegetative traits like tendril characteristics (presence or absence of tendril, nature of coiling and branching) and leaf shape were not useful for grouping the varieties. Similarly, among the qualitative fruit characteristics, waxiness of mature fruit skin also proved insufficient to distinguish the varieties.

Fruit shape was round flat in varieties Ambili, Suvarna and CO-2, while it was elongate/oblong in Saras, club shaped in CO-1 and flattish round in Arka Chandan. Based on qualitative traits, variety Arka Chandan could be clearly distinguished from the other varieties based on poor early growth vigour, moderately incised leaf blade margin, absence of silver patches on leaf blade,

flattish round fruit shape, light green immature fruits and dark orange fruit flesh colour. In addition, the seeds of the Arka Chandan had a characteristic marking on the dorso-ventral surfaces unlike other varieties.

Quantitative traits proved to be more useful than the qualitative traits for effective identification and categorisation of varieties. Results revealed that among the quantitative traits studied, leaf dimensions (blade length and width) and length of petiole could not be employed for distinguishing the six varieties.

Variety Ambili flowered the earliest (49.25 days) and also possessed highly pubescent leaves, while variety Arka Chandan was late flowering (68.00 days). Peduncle length, fruit length and most of the seed dimensions (seed count per fruit, 100 seed weight, width and thickness of seed) was the least in this variety. In general, the size of seeds in varieties CO-1 and CO-2 was higher than those of others. Cluster analysis grouped Arka Chandan (Cluster V) and varieties CO-1 and CO-2 (Cluster IV) the farthest with an inter-cluster distance of 212.25. Principal component (PC) analysis indicated that trait components in PC1 registered an Eigen value of 16.79 and the traits in PC1 contributed 58 per cent to the variability among the varieties, emphasising their utility in identification of varieties.

Among the 33 ISSR markers, 28 exhibited polymorphism. The total number of amplicons detected by an individual primer ranged from 4 in UBC-818 to 18 in UBC-847. High polymorphic information content (PIC) value was observed in UBC-809, whereas, low PIC was recorded in UBC-818. The six varieties grouped into four clusters based on ISSR binary data.

Out of 20 SSR markers used for genotyping, only five showed polymorphism. The highest Jaccard's similarity value (1.00) was observed between Saras and Suvarna. The most dissimilar varieties were Saras and Arka Chandan, and Suvarna and Arka Chandan, with a similarity coefficient of 0.12 each. The clustering algorithm grouped the varieties into four clusters.

The polymorphic SSRs could be efficiently utilised for distinguishing Arka Chandan and therefore can prove useful for testing the genetic purity of this variety. Unique bands producing ISSR markers were used to generate variety specific DNA fingerprints. No single primer *per se* proved useful in distinguishing all six pumpkin varieties. However, ISSR primer UBC-822 could distinguish four out of six varieties studied. It produced unique amplicons of size 473 bp, 552 bp, 1403 bp and 517 bp, respectively in Ambili, Saras, CO-1 and Arka Chandan, proving its utility in testing for genuiness and purity of seed lot.

In general, it can be concluded that the correlation that existed between morphological and molecular assessments was of medium magnitude. The absence of high consensus between the assessments should not be considered a limitation of these tools to characterize and quantify variability. It only indicates that both morphological and molecular characterisation is important and play a complementary role in providing a better understanding and differentiation of the pumpkin varieties.