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THESIS

IDENTIFICATION OF MANDARIN (*Citrus reticulata* Blanco) IN BHUTAN BY USING MORPHOLOGICAL CHARACTERISTICS AND AFLP ANALYSIS

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Kinley Dorji 2011: Identification of Mandarin (*Citrus reticulata* Blanco) in Bhutan by Using Morphological Characteristics and AFLP Analysis. Masters of Science (Tropical Agriculture), Major Field: Tropical Agriculture, Interdisciplinary Graduate Program. Thesis Advisor: Mr. Chinawat Yapwattanaphun, Ph.D. 107 pages.

Citrus in Bhutan has not been identified and classified and often considered as a single variety. This has hindered the pace of development of citrus industry in Bhutan. To address this, a total of 69 accessions of mandarin (*Citrus reticulata* Blanco) from Bhutan were characterized by evaluation of morphological characteristics. Of these, 30 were unknown accessions from six major mandarin growing regions and 39 were from germplasm collection maintained at Renewable Natural Resources Research and Development Center (RNRRDC), Wengkhar, under Mongar district of Bhutan. One way ANOVA and Duncan Multiple Range Test were used separately for analysis of morphological characters. The statistical analysis of physicochemical parameters showed a highly significant difference among the groups of accessions for the characters of leaves and fruits ($p<0.001$). Among the accessions from the field, accession from Dagana was observed with desired horticulture traits. The accessions from Shumar, Kengkhar and Sodrung were superior to accessions from germplasm accessions. Further, 23 out of 30 accessions from the field were analyzed, and verified for genetic variation and diversity through AFLP marker analysis. The five primers combinations discriminated 22 accessions. A total of 126 bands were polymorphic (51.64%) out of 244 total bands generated. E-ACA+M-CAG primer combination generated the highest number of total bands of which 38 percent were polymorphic. The UPGMA dendrogram obtained categorized 23 accessions to two broad groups containing 14 and 9 accessions respectively. The similarity coefficient among the accessions ranged from 0.48 to 0.91. Accessions *Samtse4* and *Dagana2* emerged very similar with similarity coefficient of 0.91. The accessions from Zhemgang formed a separate cluster. The AFLP analysis indicates that the mandarins in Bhutan are uniquely diverse as against the assumption of single variety.

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Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

A	=	Adenine
bp	=	base pairs
cm	=	centimeter
C	=	Cytosine
°C	=	Degree Celsius
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
DMRT	=	Duncant's multiple range tests
DNase	=	Deoxyribonuclease
dNTP	=	deoxynucleotide-5/-triphosphate
EDTA	=	ethylenediamine tetraacetic acid
EtOH	=	ethanol
G	=	Guanine
g	=	gram
H	=	hour
HCl	=	Hydrochloric acid
Λ	=	lambda
ml	=	milliliter
M	=	molar
Mb	=	mega base pairs
mg	=	miligram
µl	=	microliter
µM	=	micromolar
MW	=	molecular weight
NaCl	=	sodium chloride
NaOAc	=	Sodium acetate
ng	=	nanogram
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	Polymerase chain reaction
RFLP	=	restriction fragment length polymorphism

LISTS OF ABBREVIATIONS (Continued)

RAPD	=	random amplified polymorphic DNA
RDC	=	Research and Development Center
RNA	=	ribonucleic acid
RNase	=	ribonuclease
Rpm	=	rotation per minute
sec	=	second
SSR	=	Simple Sequence Repeats
TAE	=	Tris-acetate-EDTA electrophoresis buffer solution
TBE	=	Tris-borate-EDTA electrophoresis buffer solution
T	=	Thymine
U	=	Uracil

IDENTIFICATION OF MANDARIN (*Citrus reticulata* Blanco) IN BHUTAN BY USING MORPHOLOGICAL CHARACTERISTICS AND AFLP ANALYSIS

INTRODUCTION

Citrus is the most popular and economically important fruit crop worldwide. The production and consumption of citrus has increased vigorously since 1980s. The total world citrus production in 2007 was above 115 million tons (FAOSTAT, 201010). The growth has been mainly attributed to increase in area under cultivation, improved transportation and packaging while rise in income and consumer preference for healthy foods also played a role in it.

It is one of the highest valued fruits in the international market. About 10% of total citrus production is exported as fresh fruit of which 62% of the total exports occur in northern Hemisphere. Mediterranean region play prominent role in fresh fruit export providing nearly 60% of global fresh fruit exports. In addition, the production and trade of citrus juice have also improved over time (UNCTAD, 2010).

Generally citrus is grown in countries between latitude of 35° north to 35° south. Currently more than 140 countries in the world grow citrus. About 70% of the global citrus production comes from Northern Hemisphere particularly Brazil, US and countries around Mediterranean region. Brazil is the world's leading citrus growing country followed by China with a contribution of 18.1% and 17.2% to the world production respectively while United States rank third (UNCTAD, 2010). These three countries combined turn out almost 50% of world citrus production. However, the relative importance of citrus irrespective of their proportion to the global citrus production is of significance to national economy.

Geographic Description of Bhutan

Bhutan is a small landlocked country situated between China to the north and India to the south. It falls between latitude of 26°40' and 28°15'N and longitude 88°45' and 92°10'E along the southern slopes of eastern Himalaya which is considered as the major centre of diversity for the cultivated citrus (Sharma *et al.* 2004; Das *et al.*, 2005). The total country's area is about 38,394 sq km with a total population of over 670,000 of which about 69% of the population is directly dependant on agriculture for their livelihood (National Statistical Bureau, 2010).

The elevation starts from about less than 150 m msl in the south to about 7300 m msl in the north giving rise to a wide range of agro-climatic conditions. Climatologically, the country is divided into six agro-ecological zones. South-west monsoon is the key factor for rain fed agriculture and constitutes about 75% of country's annual precipitation. Mean daily temperature ranges from 15-30°C in the southern foothills and 5-25 °C in warm inner valleys. The wide variation in climatic conditions within location has favoured citrus production in the country. The southern foothills and other low lying areas of the country present a very congenial environment for citrus cultivation. .

History and Geographic Distribution

Citrus cultivation is believed to be as old as Bhutanese culture although there is no authentic history. Citrus cultivation gained momentum in past few decades. Unlike elsewhere, the citrus industry in Bhutan is considered pro-poor. The citrus growers are identified as large farmers, medium farmers, small and backyard farmers based on size of the orchards (Dorjee *et al.*, 2007). Dagana district had the highest production with 17, 445 MT followed by Sarpang with 12,746 during 2007. Trongsa district topped the yield of citrus in the country with 56kg per tree (DoA, 2008).

Citrus is grown in 17 out of 20 districts and more commercially in districts of subtropical region. Citrus is the major fruit crop and important source of income for

rural subsistent farmers. There exists enormous potential to enhance the livelihood through increase in yield and productivity. The crop is a major source of income for most of the subsistent farmers. Citrus is grown between the altitude ranges of 300 meters and 1650 meters mean sea level (msl) in Bhutan. The annual production is over 72000 metric tons covering an approximate area of 7000 hectares (DoA, 2008). Over 66% of the total production is exported as fresh fruits annually besides processed products (Connellan *et al*., 2008). It has a substantial contribution to the income of subsistent farm economy apart from generating employments in various corporative and processing industries. It is estimated that at least 60% of the population are involved in citrus industry (Dorjee *et al*., 2007).

Trend and Socio-economic Importance

The steady increase in overall production over the years could be due to an increase in average yield of citrus 32kg /tree in 2004 to 36kg/tree in 2007. However, a survey on age composition of citrus trees (Connellan *et al*., 2008) reported 46% of the trees in age range of 10-20 years, 32% above 20 years, about 5% between age of 7-9 years. And the rest (17%) of the trees were below six years of age. The lower proportion of younger tree composition indicates that the increase would continue only for some time and the citrus industry in Bhutan would be at stake in near future. The decline of citrus in Himalayan region was also reported (Das *et al*., 2005) as a result of inapplicability of production technology and narrowing of genetic variability.

Bhutanese mandarin is mainly exported to Bangladesh and India. About 85% of the country's total export goes to Bangladesh owing to a relatively higher market price over India. The average annual export price to Bangladesh during the period 1999-2004 was 0.26 USD/kg while it was 0.19 USD/kg to India (Dorjee *et al*., 2007). The top export commodities of Bhutan are shown below in Figure 3.

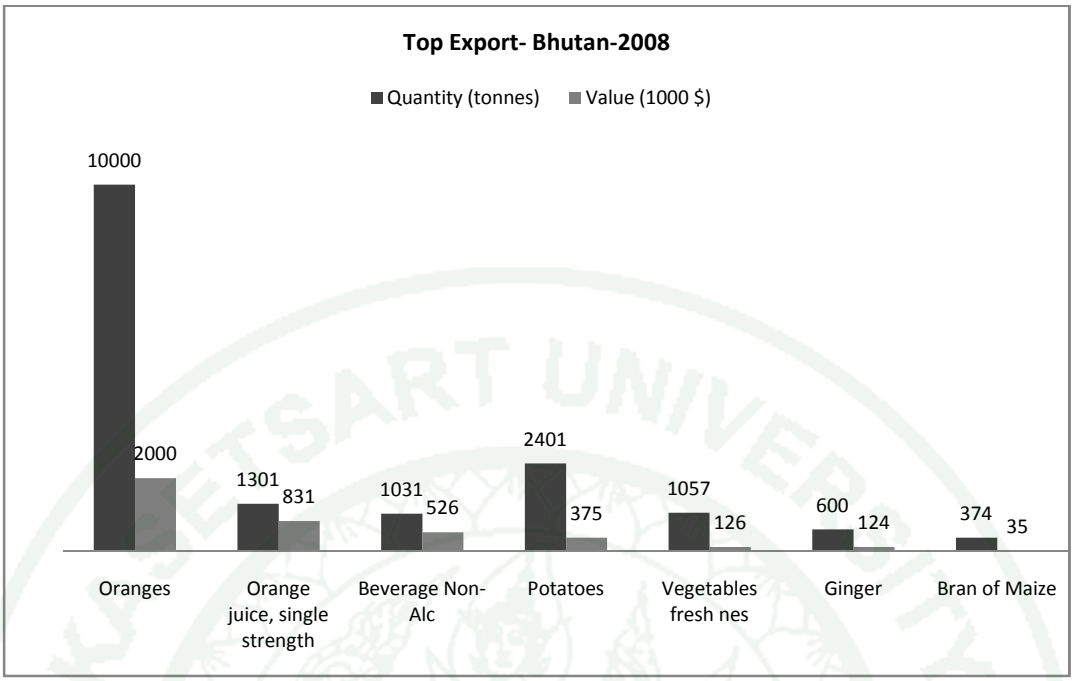


Figure 1 Top export commodities of Bhutan in the year 2008

Source: FAOSTAT, 2010.

Citrus Research and Cultivars in Bhutan

Citrus in Bhutan is predominantly local mandarin (*Citrus reticulata* Blanco) while lime (*Citrus aurantifolia*) and lemons (*Citrus limon*) are also produced in very small quantities (Connellan *et al*., 2008). About 66% of the total mandarin production is being exported to neighboring countries annually as fresh fruit. Rest of the production is absorbed in domestic market either as a fresh fruits or as processed products (Dorjee *et al*., 2007). Other citrus such as lime and lemon are consumed in domestic market and the processing industry within the country.

Ever since the inception of a plan for strategic citrus development for sustainable citrus production in the country, the Renewable Natural Resource Development Center (RNRDC), *Wengkhar*, has collected local mandarin accessions from across the country. So far, RNRDC has a germplasm collection of more than 140 local mandarin accessions. The center has also identified two superior accessions

based on yield performance evaluation. Similarly, in the West Central Region, RNRDC, Bajo, Wangduephodrang also maintains accessions from different parts of region. In addition, the centre also has many introduced accessions under evaluation.

The Renewable Natural Resource Development Centre (RNRDC), *Wengkhar*, conducts a number of performance evaluation studies on citrus. The centre maintains a germplasm of local mandarins to save from devastating citrus greening disease. Two superior lines have been identified based on yield performance evaluation for the accessions at germplasm collection. However, the germplasm is still in its infancy. The actual genetic diversity and genetic relationship is not understood as morphological analysis is not conducted except for yield parameters. As such, no variety has been registered or released so far for Bhutanese mandarin. Although, the mandarin differs both in quality and market value, no study has been carried out yet for varietal identification and characterization.

Mandarin Production and Classification

Mandarin cultivation gained momentum in the last few decades. The total annual production was over 72 thousand metric tons in 2007 (DoA, 2008). However, the average yield is 36 kg per tree which is below national production benchmark set at 47 kg per tree (Connellan *et al*., 2008). This is mainly due to poor citrus orchard management, unfavorable landscape, poor soil condition, pest and diseases. The lack of nursery for certified disease free seedlings was reported as another constraint. Above all, there is lack of commercial and location specific varieties identified for rejuvenation and promotion (National Plant Protection Center, 2007).

Given the country's long history of cultivation, the wide spread presence of mandarin, even in some of the most remote and poorly accessible parts of country strongly suggests that mandarin in Bhutan are unique and diverse. The evidence is that morphological differences in fruit quality characters have emerged over the period of time in different parts of the country. The consumers in the local market are

able to differentiate between produce of different locations. Perhaps, Bhutanese mandarin might have independent evolutionary significance.

Mandarin is one of the single most important groups of *Citrus* that constitute more than 50% of total citrus production in the world. China is the leading producer of mandarin in the world contributing to more than half of the world's mandarin production. Generally, mandarin is considered as a group even though it consists of several different species and biotypes. Indeed mandarin group consists of about 13 groups including its relatives and hybrids (Anonymous, 2009).

The mandarin in Himalayan range is claimed to have excellent quality (Ghosh, 1993) and their high quality and distinct ecotypes have been reported by Das *et al.* (2005). Particularly, the excellence about quality of Bhutanese mandarin was described by Connellan *et al*. (2008). However, cross-pollinating nature of the mandarin coupled with zygotic twins (Das *et al*., 2005) has resulted in wide variations in the plant types and non-uniformity of fruit quality. Such non-uniformity has a disadvantage in export of Bhutanese mandarin and fetches a lower price.

The present germplasm repositories over world have collected most of the commercial local cultivars, varieties and wild species. A link between the present commercial local cultivars and the wild species is said to be in backyard farm orchards.) The presence of backyard orchards with less than 50 mandarin trees and small-farm orchards with number of mandarin trees between 50-120 owners' accounted to 75% of total citrus growers in the country. In contrast, 50% of the total production comes from 10% of large farm orchards (Connellan *et al.*, 2008). Thus, majority of citrus growers own citrus orchards in backyard or in small farm orchards this might possess diverse genetic resources.

Despite the several different types of mandarins observed in Bhutan, it is collectively known as "local mandarin" (*Citrus reticulata* Blanco) which is considered as single variety (Dorjee *et al.*, 2007; National Plant Protection Center, 2007). They also mention that Bhutanese mandarins are of two types: *Khasi* (type in

Khasi hills of Meghalaya state in India) and *Sikkim* (Indian state towards east of Bhutan). In fact, there is dearth of information on the number of varieties or types about mandarin in Bhutan

The present germplasm repositories over world have collected most of the commercial local cultivars, varieties and wild species. A link between the present commercial local cultivars and the wild species is said to be in backyard farm orchards.) The presence of backyard orchards with less than 50 mandarin trees and small-farm orchards with number of mandarin trees between 50-120 owners' accounted to 75% of total citrus growers in the country. In contrast, 50% of the total production comes from 10% of large farm orchards (Connellan *et al.,* 2008). Thus, majority of citrus growers own citrus orchards in backyard or in small farm orchards this might possess diverse genetic resources.

The lack of proper documentation and understanding on varietal types, diversity and also the differences among mandarin from different localities questions the diverse mandarin in Bhutan. The poor understanding on varieties and genetic diversity has been the greatest hurdle for the citrus improvement program and citrus industry in Bhutan. Thus, identification has become increasingly important to extend the export of products gradually beyond neighboring countries and also for maintenance of uniformity in fruit quality.

Therefore, this study was aimed at identifying and characterizing local mandarin accessions in the field and the germplasm accessions. The identification was based on morphology and AFLP marker analysis. The study was expected to reveal some basic information about the diversity of mandarin accessions and further provides a genetic relationship among Bhutanese mandarin accessions. The outcome of this study is expected to be useful for initiation of citrus breeding programs and registration of promising cultivars for uniformity of fruit quality.

OBJECTIVES

The objectives of this study were:

1. To characterize the mandarin cultivars (*C. reticulata* Blanco) in Bhutan using morphological characteristics.

2. To elucidate and identify mandarin cultivars (*C. reticulata* Blanco) in Bhutan by Amplified Length Fragment Polymorphism (AFLP) technique.

LITERATURE REVIEW

Citrus production has flourished across the globe for almost three decades now. The increase in production was tremendous in last few decades with an average increase of about 2.5% annually until 2003 from just over 20,000 MT in 1961 to a little less than 1,20,000 MT in 2003. However, the rate of increase in production has slowed down over the years (UNCTAD, 2010). Mandarin is very important commodity of citrus. Mandarins with orange constitute more than 50% of total world's citrus production. The share of mandarin in global citrus is shown below (Figure 1).

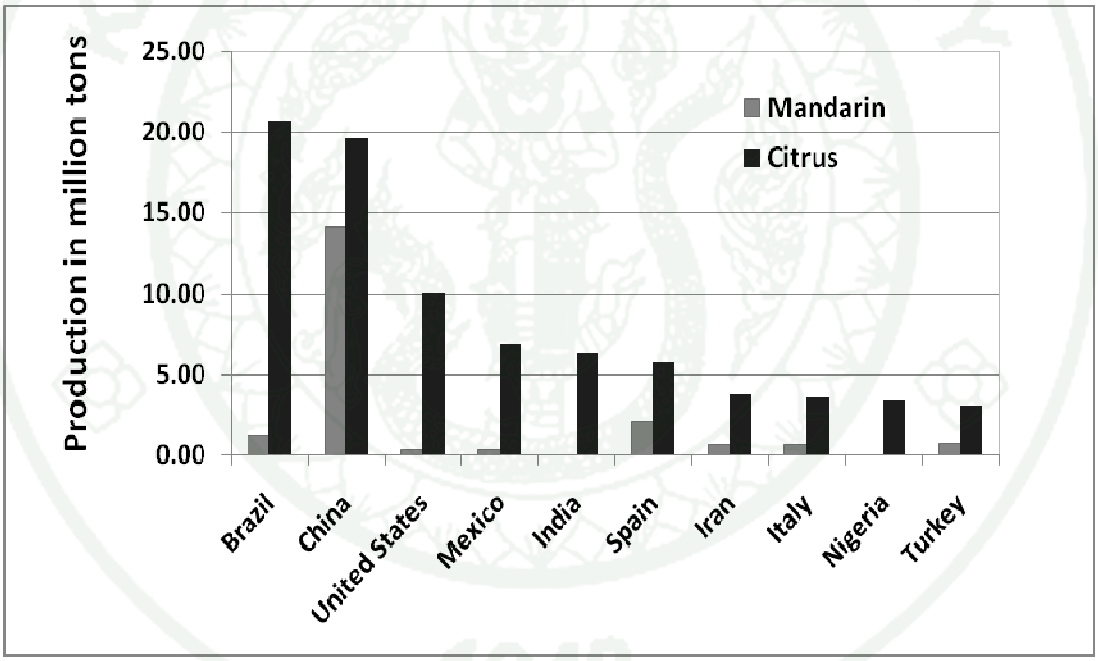


Figure 2 Share of mandarin in world citrus production 2007

Source: FAOSTAT (2010)

Genetic systems in citrus

Citrus in general is a cross pollinated, however, only few varieties of *Citrus* are usually self-fertile (requiring only a bee to move pollen within the same flower) or

parthenocarpic (not requiring pollination and therefore seedless, such as the Satsuma). 'Blossoms from the Dancy' cultivar is one such exception. They are self-sterile, and therefore must have a pollenizer variety to supply pollen, and a high bee population to make a good crop. Citrus species are highly heterozygous and hybrids produced by inter or intra-specific crosses reveal huge variations in characters in F_1 generation (Fatima, 2004).

Most of the varieties under this genus are usually polyembryonic in nature. This is due to development of embryos from nucellar cells in addition to zygotic embryos. It is known that pollination triggers the development of nucellar embryo and develops prior to zygotic embryos by almost 4 weeks (Das *et al*., 2007). Therefore nucellar seedlings are more vigorous. Thus polyembryony has enormous significance in breeding of citrus rootstock as it retains parental characters. On the other hand, polyembryony is nuisance as it serves as barrier to conventional breeding.

Within polyembryony, some species of citrus are also monoembryonic for example Pummelo, Tangelo and Clementine varieties of mandarin. Therefore the monembryonic nature of citrus like any other species produces only hybrid seeds. Other monoembryonic citrus types include:

1. *Citrus indica*
2. *Citrus japonica*
3. *Citrus maxima*
4. *Citrus medica*

Thus these citrus species provide scope for development of hybrids which is acted upon by evolutionary forces in the process of speciation. These citrus types are highly variable. Thus this complex genetic system of the genus has not only resulted in many different systems of classification but also hampered the progress in citrus improvement in general. The impediment of research on citrus genetics was also reported by Guo and Deng (2001).

Origin of Citrus and Route of Dispersal

Citrus taxonomists base their conclusions on morphological and geographical data which might have lead to differences in views. Citrus is known to exist before recorded history therefore center of origin and diversity cannot be ascertained. However, Southeast Asia, especially east India, north Burma and southwest China is considered as center of origin. The exact routes of dispersal from its origin are unknown. However, according to Webber and his friends, the history of spread of citrus was compared to a romance. In fact the citron was first member of the group to be known for European civilization which dates back to 310 BC by Theophrastus (Webber *et al*., 1967). The detail of subsequent spread and possible routes as presented by Natural History Museum, London is reproduced in Figure 2.

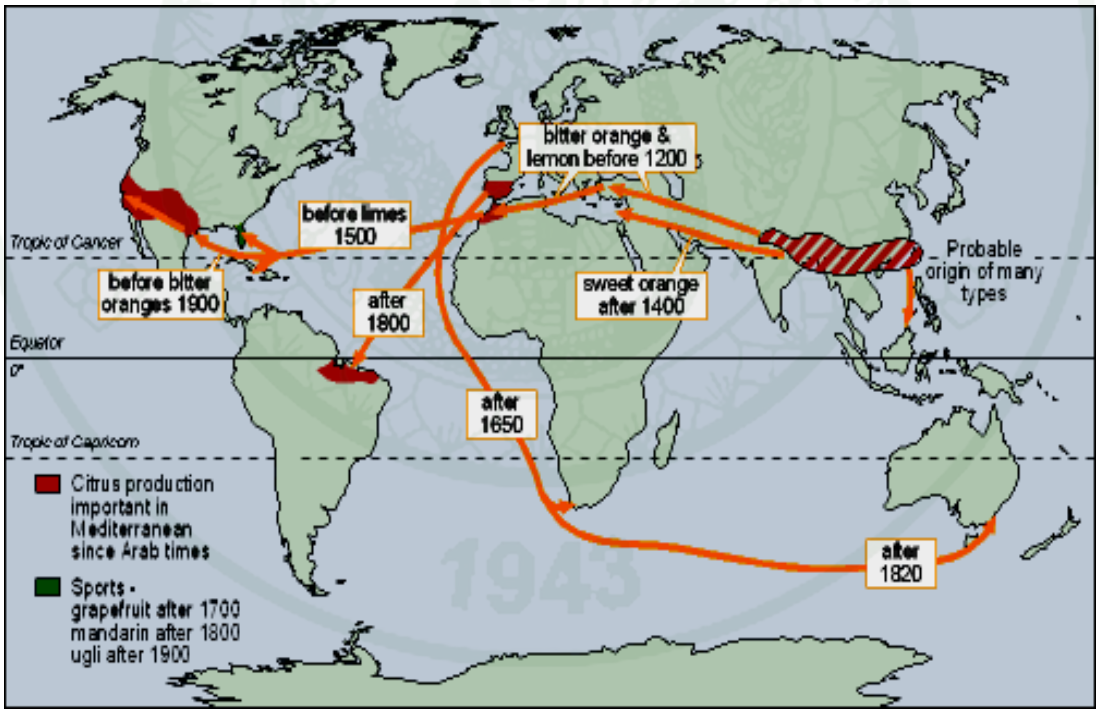


Figure 3 The probable origin and routes of dispersal for citrus

Source: Natural History Museum (2010)

Taxonomy of Citrus

The taxonomy and systematic of citrus is complex and precise number of natural species are unclear as most of named species are clonally propagated hybrids. Evidence suggests that some of the wild true breeding species are of hybrid origin (Nicolosi *et al.*, 2000). The cultivated species are believed to have derived from as few as four ancestral species.

The term Citrus refers to a member of *Rutaceae* family. It consists of several different species such as mandarins (*C. reticulata* Blanco), oranges (*Citrus sinensis* L. *Osbeck*), grapefruit (*C. paradisi* Macf.), lemons (*C. limon* L. Burmf.) and limes (*C. aurantifolia* Swingle). There are also some minor categories of citrus like tangerines, pummelos (*C. grandis* Linn.), tangelos (mandarin X grapefruit) and some small fruit distant relatives of citrus such as calamondin (*Citrus madurensis* Loureiro), kumquat (*Fortunella* spp.).

There are two main systems of Citrus taxonomy that are widely used; Swingle 1943 (revised by Reece in 1967) and Tanaka (1977). Swingle systems recognized up to 16 species within two subgenera (*Citrus* containing ten specie and *Papeda* with six species) while Tanaka (1977) systems included 162 species under two subgenera *Archicitrus* and *Metacitrus.* The two systems differed in their basic concepts in assigning of species level. The subsequent classifications of Citrus by various authors were based on these two systems of classifications with little modifications.

The natural and commercially cultivated citrus include orange, grapefruit, lemon, lime and tangerine. It is now understood that there are as few as three true or basal species for edible citrus (Abkenar *et al*., 2004; Jena *et al.*, 2009; Uzun *et al*., 2009). They are citron (*C. medica* L), Pummelo (*C. maxima* Burm.) and mandarin (*C. reticulata* Blanco). Among which mandarin group is the most heterogeneous with many species hybrids and subtypes.

Classification of Mandarins

Mandarin together with its hybrids and relatives form the largest group in the genus. Mandarin group consists of numerous inter-generic species and inter-specific hybrids making it phenotypically the most heterogeneous of the three true species. In fact, this genus includes some of the most complicated species to improve genetically (Campos *et al*., 2005). The compatibility across the genus and related genera is a major drawback. Thus relationship and classification of these species are difficult to assign based on morphological characters alone. Moreover, the phenotypic characters are affected largely by environmental factors. It was also reported that grafting of scion on rootstock in citrus produce graft-induced variants (Ebtissam *et al.,* 2003). Overall, the classification of mandarin is more complicated than other citrus species.

Grouping of all mandarins into three groups as that of American taxonomist Swingle 1967 was too arbitrary while regrouping of all mandarins into 36 species and subtypes by Tanaka was too elaborate. However Robert Hodgsons has grouped the mandarins into four species based on their origin, distribution and characteristics was widely used for long time (Webber *et al*., 1967). They are *C. deliciosa* (Mediterranean mandarin), *C. nobilis* (King type), *C. unshiu* (Satsumas) and *C. reticulata* (common mandarin). Species reticulata also included all other types and hybrids. This method was widely used over a time in the past (Webber *et al*., 1967).

Later mandarin was divided into 13 groups consisting of seven mandarin types and its hybrid into six types (Anonymous, 2009). This system divided *C. nobilis* into two subgroups of mandarin and common mandarin. Other groups were Clementines, Tangerines, Satsumas, Mediterranean willow leaf mandarin and King mandarin.

The most common mandarin groups include Ponkan (*Citrus reticulata* Blanco) which is cultivated extensively in China, India, other South Asian countries and Southeast Asian countries under different names. The fruits of this mandarin group are seeded, easy peeler and have good eating quality. The fruit do not orange coloration in tropical regions and puff when over matured. The acidity of the fruit was

observed to decrease both on tree and under storage. The fruit shape and quality varies with soil types and climate. The fruits are obovate, collared with neck in tropical conditions while the fruit develop orange color in subtropical conditions with ovate to globose fruit shape in cooler dry climates (Ladayina, 2008c).

Markers for Identification and Diversity Study

Broadly, the markers are of two types: morphological marker and non morphological markers. Non morphological markers are molecular markers (Bhat *et al.,* 2010). Morphological marker refers to those characters that can be visually scored. Otherwise, morphological markers are genetic markers whose inheritance can be followed with naked eye. However, Campos *et al.* (2005) commented that such markers are environmentally affected and liable to subjective evaluation. Dominant recessive interactions often pose difficulty in their identification.

Characterization of any species has traditionally been relied on evaluation of morphological characteristics. In fact, morphological and geographical evaluations serve as initial step in gene bank collection as morphological characters are reliable, easy to study, relatively cost effective to evaluate and are visually observed. Also, morphological descriptors are potentially useful in clonal propagation and genetic distance estimation. On the other hand, the phenotypic plasticity of the genotypes makes phenotypic characterization a complex process.

Morphological Markers

In many situations, the most easily obtained assessment of genetic variation is by measuring the morphological or phenotypic variation. The sharing of phenotypic characters is interpreted as a clue of relatedness. However, morphological characters are often influenced by environmental conditions and this in turn may affect the estimation of genetic variation, relatedness and identification (Bhat *et al*., 2010). Consequently, morphological measurements are carried out on plant material that is grown in comparative trials. Such trails besides being expensive and time consuming

are impossible to accomplish for some species that are very difficult to grow. However, when the morphological characters are shown to be heritable, they invariably reflect the genetic structure within plant material (Perrson, 2001)

In general these mandarins are small tree with or without thorns. Leaves are unifoliate, alternate or spiral, most often with petiole wings. Flowers are either solitary or cymose inflorescence with two to four flowers at axial or in terminal. The flower consists of five sepals and petals with 18-23 stamens fascicle or connate filament. The fruits are berry with *hesperidium* type. The number of seeds varies from being seedless to as high as 30 seeds per fruits (Pattanapakdee, 2002).

Morphological analysis was applied for identification and study of diversity of Korean and Turkish watermelon germplasm (Huh *et al.*,2008), study of phenotypic diversity in sweet potato (Elameen *et al.*, 2010; Karuri *et al.*, 2010), identification of potential rootstocks (Jaskani *et al.*, 2008) and variation in kinnow and rough lemon (Altaf and Khan, 2008). The characterization of citrus in north-eastern India was surveyed and reported using morphology uncovered highly valuable genetic resources of citrus (Sharma *et al.*, 2004).

Molecular Markers

Molecular markers are also known as non-morphological markers. Any kind of molecules that indicate the presence of a chemical or physical process is known as molecular markers. These include biochemical constituents (secondary metabolites in plant) and macromolecules (proteins and deoxyribo-nucleic acids) which easily express differences in form of polymorphism. The first molecular markers used were protein based markers. The concept is central dogma where protein synthesis is directed by genes. In other words, protein is primary product of structural genes.

Isozymes were one such earliest protein based markers. Isozymes analysis was found to be unequivocal markers in fingerprinting and molecular characterization. Thus it was widely used in study of citrus in recent past. The genetic analysis with

Isozymes was conducted for citron cultivars (Rahman *et al*., 1994, 2001; Protopadakis and Papanikolaou, 1998; Elisiario *et al*., 1999). Similarly, leaf isozymes of citrus were used to distinguish nucellar seedlings from zygotic origin depending on genotypes of particular parents with different level of efficiency (Torres *et al.*, 1982). However, a relative low number limits isozyme discrimination power (Cabrita *et al*., 2001) in addition its phenological variations and environmental influence.

Several molecular marker systems have been used for identification of cultivars in last few decades. With the advent of polymerase chain reaction (PCR) technology (Williams *et al*., 1990), several new markers have been identified. The choice of molecular marker depends on its suitability to answer a particular ecological question. For this purpose, the main difference among molecular markers is their degree of dominancy. Co-dominant markers enable easy estimation of allelic frequencies (Bhat *et al*., 2010).

Consequently, co-dominant markers are suitable to estimate gene flow between populations or in study of dispersal. On the other hand, dominant markers are used as fingerprints and are useful in identification of clones or closely related species. More increasingly, citrus phylogeny and genetic origin of important species are being investigated using molecular markers (Nicolosi *et al*., 2000).

Different Types of Molecular Markers

Molecular markers are either DNA sequences at specific positions on a chromosome, or their immediate products like enzyme molecules. These markers are inherited in a Mendelian manner and are used as landmarks for genome analysis. Biochemical markers like isozymes reveal polymorphisms at the protein level and have been used for studying genetic variation in large number of species.

Isozyme markers are generally codominant, i.e. heterozygous individuals can be distinguished from homozygotes. But since these markers only detect variation in protein coding loci, they may reveal only a small amount of the variation present in

the individual or population. Moreover, the expression of isozyme markers is dependent on the developmental stage of the plant and their expression is influenced by the environment. DNA markers are numerous and they cover the entire genome. They are not influenced by developmental stage or environment, and allow selection of individuals as early as seedling stage during which the plants are large enough to yield sufficient DNA.

Among the molecular techniques available for characterization of the variation, RFLP (restriction fragment length polymorphism) is one such techniques of hybridization-based methodology which use locus-specific probes. The PCR (polymerase chain reaction) based DNA markers are RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and microsatellite markers.

RFLP (restriction fragment length polymorphism) is generally considered to be a reliable method although the technique is labor-intensive, time-consuming and requires large amounts of DNA. On the other hand most of the PCR-based techniques are easy to perform and require only small amounts of DNA. PCR based techniques are able to reveal a virtually unlimited number of markers. Under certain circumstances, RAPD or AFLP is suitable than STMS (sequence tagged microsatellite sites) analysis as it requires no prior knowledge of the DNA sequence. Unspecific primers are used to amplify non-coding as well as coding regions of the DNA.

RAPD marker technique was popular for identification, genetic linkage mapping and in diversity studies. Genetic linkage map of rose was constructed using RAPD (Debener *et al*., 1999). Another study (Dehesdtani *et al.*, 2007) assessed genetic diversity of navel sweet orange cultivars using RADP markers. RAPD was deployed in study of segregation patterns of several morphological characters for interspecific hybrids of *Dianthus giganteus* and *D. carthusianorum* (Lee *et al.*, 2005).

The RAPD analysis method is fast and easy to perform. It was used in study of phylogenetic relationships among citrus and related genera in combination with RFLP

technique (Federici *et al.*, 1998). Furthermore, mandarin hybrid was also assessed using RAPD (Elisiario *et al*., 1999). RAPD marker has a problem with reproducibility and competitive is reported remains more serious problem (Hallden *et al.* 1996).

Subsequently, many such PCR-based marker systems have been developed. This includes microsatellite-based markers and AFLP. Unlike the Isozymes marker, RAPDs or RFLPs, AFLPs provide rapid and reliable assays of more than 50 potentially polymorphic sites in a single experiment. Thus, AFLP is found superior over SSRs (Simple Sequence Repeats or other microsatellite markers) in this study for diversity and identification.

AFLP Markers

AFLP markers (Vos *et al.*, 1995) are extensively used in study of wide range of fields: from population genetics to phylogeography to phylogenetics. Amplified Length Fragment Polymorphism (AFLP) is accepted as efficient molecular tool as it provides large amount of information with high degree of stability and reproducibility (Liang *et al.*, 2007). AFLPs have been used for assessing genetic variability and constructing genetic maps in many species. As a method of multilocus fingerprinting, Arnau *et al.* (2001) found AFLP to be very promising in plant variety identification.

A comparing study conducted to assess suitability of isozyme, RAPD and AFLP markers to assess genetic differences and relatedness among fig (*Ficus carica* L.) clones (Cabrita *et al*., 2001) reported that Isozymes as well as RAPD and AFLP markers distinguished the clones genetically with different level of discrimination power. They also suggested that AFLP technique was more powerful tools that should be considered for discrimination of closely related species (between clones or varieties). A similar study (Garcia *et al*., 2004) on comparison of RAPD, RFLP, AFLP and SSR markers in tropical inbred lines of maize supported the suitability of AFLP markers for fingerprinting and assessment of genetic relationships with high accuracy.

Further, a study on the utility of AFLP in phylogenetics: a comparison of homology within and between genomes (Althoff *et al*., 2007) revealed that the method is remarkably reliable and consistent. The technique is adaptable to new taxas and has wide applicability as it does not require previous information. Besides, AFLP also surveys whole genomes and generate many potential phylogenetic markers. Thus, AFLP is the most common technique in phylogenetics although it is limited by assumption of size homology (anonymous fragments of same size with similar sequence) (Simmons *et al*., 2007).

AFLP was used for characterization and identification of many crop species. The technique was also exploited to study inheritance and genetic diversity analysis in wild and domesticated pawpaw (*Asimina triloba* L.) by Wang *et al.* (2005) and in characterization of white sapote (*Casimorea edulis* Llave and Lex.) by Yonemoto *et al*. (2007) and in genetic mapping of *Pinus radiata* D. Don (Cato *et al*., 1999). AFLP was also used for fingerprinting in hop plant for analysis of genetic variability (Fleischer *et al.*, 2004) and preliminary study of diversity in cultivated *Laminaria japonica* Sporophyte (Yi *et al.*, n. d.), and analysis for opium poppy (Saunders *et al*., 2001). Further, AFLP was found to distinguish and draw the genetic relation between apricot (*Prunus armeniaca*) and related species (Hagen *et al*., 2001). Based on AFLP and microsatellite markers, genetic linkage maps of two apple cultivars (Malus domestica Borkh.) were constructed by (Kenis and Keulemans, 2005).

In citrus, AFLP marker was used in determination of phylogenetic relationships within *Citrus* and its related genera: 29 genotypes belonging to *Citrus, Poncirus, Eremocitrus, Atlantia* and *Severinia.* The findings were in line with three basic species concept (Pang *et al*., 2007). AFLP analysis was employed in citrus taxonomical classification (Liang *et al*., 2007) and in survey of plant diversity (Mba *et al*., 2005). The inheritance of major gene which is closely linked and essential for nucellar embryony in cross of *Citrus maxima* and *Poncirus trifoliata* was explored (Kepiro and Roose, 2009). In addition, SSR and modified AFLP markers were used to identify zygotic plantlets in backcrosses of lemon cybrids (2n and 4n) and a diploid clone of lemon (*Citrus limon* L. Burm. F.).

Identification of Mandarin

The word “mandarin” is synonymously referred to as Tangerines. Specifically reddish-orange mandarin cultivars are known as tangerines while all mandarin types and its hybrids are referred as Tangerine in North America. The mandarin is a small citrus tree (*Citrus reticulata* Blanco) with fruit resembling other oranges. The tree is more drought-tolerant while the fruit is susceptible to cold. It can be grown in tropical and subtropical areas. The fruit is rather oblate than spherical. Mandarin oranges are usually eaten plain or in fruit salads.

Most of the recent studies have combined both morphological and molecular study to obtain a complete information and accurate identification. Mandarin in north-eastern India was studied using morphological and RAPD technique (Das *et al.*, 2005). Koehler-Santos *et al*. (2003) in an attempt to characterize mandarin citrus germplasm from Southern Brazil by morphological and molecular analyses (microsatellite markers) grouped 37 mandarin cultivars into three main groups in dendrogram obtained from 18 quantitative and qualitative characters. The microsatellite findings were in agreement with previous authors (Federici *et al*., 1998) that mandarins constitute a single species with numerous varieties and hybrids with narrow genetic base.

In another related study, Satsuma mandarin (*Citrus unshiu*) cultivars were identified using AFLP markers (Chao *et al.*, 2004). About 19 individual Satsuma mandarins were identified as one major group with a within group similarity values of more than 83 percent. Identification of Ponkan mandarin (*Citrus reticulata* Blanco) linked to seedlessness in and conversion to SCAR (Sequence characterized amplified region) markers was carried out using AFLP fragments (JinPing *et al*., 2009). Further, in characterization of mandarin (*Citrus* spp.) using morphological and AFLP analysis revealed both morphological and molecular markers to show high degree of variation among the mandarin accessions analyzed. They suggested that morphological diversity was independent from molecular diversity (Campos *et al.*, 2005) which

implies that molecular analysis is not a replacement to morphological analysis but rather a complementary technique.

Thus, considering complexity of citrus biology as a whole and the wide variability within the heterogeneous mandarin group, understanding of the intra-specific variations is all the more significant to identify superior genotype(s). The rough morphological characterization with due consideration to desired horticulture characters is elucidated with DNA fingerprinting using AFLP markers analysis for possible improvement of promising and registration to release as new cultivars.

MATERIALS AND METHODS

Sampling and Survey

The first part of the study deals with morphological characterization of the sampled mandarin accessions. Survey and sampling of mandarin accessions was conducted in six districts of major citrus growing regions of Bhutan. In addition accessions from local germplasm collection at Research and Development Center (RDC) Wengkhar in Mongar District in eastern Bhutan were included. The surveyed districts and RDC Wengkhar are shown in Figure 4.

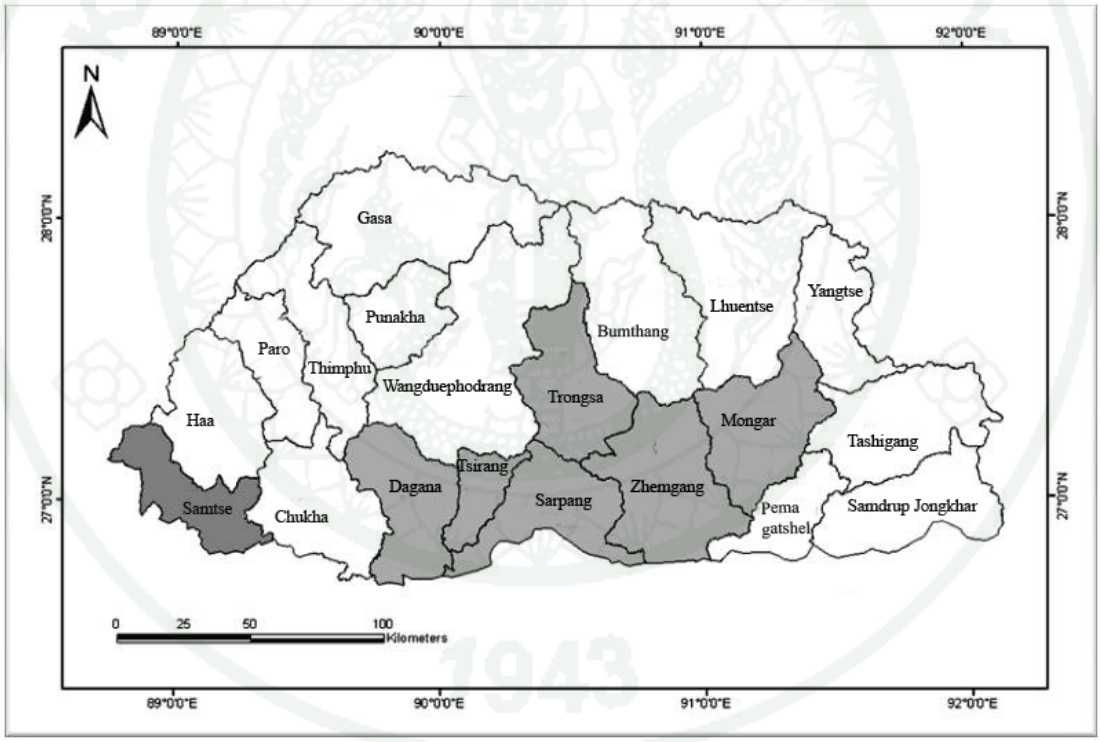


Figure 4 The sampling areas (Samtse, Sarpang, Tsirang, Dagana, Trongsa, Zhemgang and Mongar) for the mandarin in Bhutan

The samples consisted of 30 mandarin accessions from six districts and 39 accessions from the local mandarin germplasm maintained at the RDC *Wengkhar*, The surveyed areas represent diverse agro-ecological conditions with an altitude ranging from little

less than 400 to 1650 meters above msl with a highly varying temperature, temperature diurnal fluctuation and mean annual rainfall (DoE, 2010).

Agro-Ecological Condition of Surveyed Districts

Among the surveyed districts, Samtse is at a comparatively lower in elevation, Sarpang has the highest mean annual maximum temperature of 27.3°C and highest diurnal fluctuation in temperature is recorded in Trongsa (9.7°C) whereas it is lowest (2.8°C) in case of Zhemgang. Diurnal temperature fluctuation is also observed to be highest in Mongar next only to Trongsa with 7.9°C.

About 80% of mean annual precipitation occurs in summer season (June to August). Sarpang receives the highest mean annual rainfall of 4478.9 mm followed by Samtse with while Mongar experience the lowest mean annual rainfall of 922.3 mm. Dagana also receive fairly high mean annual precipitation with 3369.7 mm whereas a lesser annual precipitation range of 1133.9 to 1369 mm takes place in Tsirang, Trongsa and Zhemgang (Table 1).

Table 1 Annual Mean Temperatures (Maximum and Minimum) and Annual Mean Rainfall for different districts

Districts	Temperature (°C)		Rainfall
	Maximum	Minimum	(mm)
Samtse	25.8	20.2	3991.4
Sarpang	27.3	19.3	4478.9
Tsirang	19.0	12.5	1369.0
Dagana	19.2	12.6	3369.7
Trongsa	21.7	12.0	1133.9
Zhemgang	14.0	11.2	1302.1
Mongar[a]	21.5	13.6	922.3

Materials

Plant Materials

The sample material from each tree consisted of 15 matured leaves, five fully opened flowers and 15 matured fruits, which were randomly picked. The materials were collected from six districts: Samtse, Sarpang, Tsirang, Dagana, Trongsa and Zhemgang. However the samples from germplasm collections in RDC Wengkhar included 10 leaves, five flowers and 10 fruits per accessions. The detail of the accessions and their sources are shown below in the Table 2.

Table 2 Accessions and their sources of collection

Sl. No	Source of Collection	Samples	No. of trees
1	Field (Unknown)	Tsirang	5
2	Germplasm	Tsirang	6
3	Field (Unknown)	Samtse	5
4	Germplasm	Samtse	2
5	Field (Unknown)	Sarpang	6
6	Field (Unknown)	Dagana	5
7	Germplasm	Dagana	6
8	Field (Unknown)	Trongsa	5
9	Germplasm	Trongsa	1
10	Field (Unknown)	Zhemgang	4
11	Germplasm	Shumar, Pemagatshel	2
12	Germplasm	Kengkhar, Mongar	10
13	Germplasm	Narang, Mongar	4
14	Germplasm	Yadi, Mongar	2
15	Germplasm	Sodrung	2
16	Germplasm	Samdrupjongkhar	1
	Total		69

Equipments and Chemicals for Morphological Study

1. Cool box
2. Geographical Positioning System (GPS) Garmin
3. Digital camera (Olympus)
4. Packaging materials
5. Sampling kit
6. Digital caliper (Mitutoyo CD-15)
7. Digital balance/ Analytical balance
8. Royal Horticulture Society Color Chart (5^{th} Edition)

Equipments and Chemicals for Determining TSS and Total Acidity

1. 50 ml burette
2. Burette stand and clamp
3. 10mL pipette and pump
4. 1 L volumetric flask
5. 0.1 M Sodium Hydroxide
6. 0.1 M HCl
7. 1% phenolphthalein
8. 100ml conical flsk
9. Hand refractrometer
10. Distilled water
11. 6 % sucrose solution
12. Fresh juice from samples
13. Other safety accessories

Equipments and Chemicals for AFLP Analysis

1. PCR Machine (Biometra)
2. Horizontal electrophoresis set and accessories
3. Vertical polyacrylamide gel electrophoresis set

4. UV Transilluminator (G Box)
5. Refrigerated centrifuge (MIKRO 22R)
6. Thermostatic water bath
7. pH meter
8. Fine pipettes and accessories

Plant Materials for DNA Extraction and AFLP Analysis

1. Fully expanded young healthy leaves
2. AFLP Analysis System I (Invitrogen) version B
3. AFLP Starter Primer Kit (Invitrogen) version B

The details of chemicals and their functions (Appendix Table C1, C2, C3 and C4)

Methods

Experiment I Morphological Characterization

In this part of the study, 69 mandarin accessions (30 from farmers' field and 39 from germplasm block) were studied for phenotypic and physicochemical parameters. The selected plants of mandarin accessions were studied for tree canopy shape/growth habit, its location, fruit characteristics, leaf and floral morphology. Altogether, 20 qualitative characters and 14 quantitative characters were evaluated for each plant with respect to tree shape, fruit characteristics (including some of the desired horticultural characters), leaf and flower (Table 3)

Survey and Sampling

The survey was conducted during flowering and fruit maturing season. The sampling for phenotypic study was carried out in two rounds of survey depending on the phenological stages of mandarin stretching from October, 2009 to April, 2010.

The maturity of fruits was determined by taste at desired level of total acidity rather than appearance. The samples of leaves and fruits were then packed, labeled and brought to laboratory. The second round of survey which coincided with flowering season started from February to April, 2010. A minimum of five flowers were sampled from each tree and evaluated for floral characteristics.

Morphological Characteristics

Although, morphological characterization does not portray actual genetic diversity, it is still an important component for varietal identification (Compos *et al*., 2005). Each selected plants were evaluated for tree characters, fruit morphology (including agronomic characters), and leaf and floral morphology. The selection of the morphological characters was based on IPGRI Descriptor for *Citrus* spp., 1999.

Table 3 Morphological characters evaluated and their codes

Code	Characters	Code	Characters
FW	Fruit weight	L/W	Leaf length to leaf width
FL	Fruit length	LAS	Leaf Apex Shape
FD	Fruit diameter	PWS	Petiole wing shape
WE	Width of epicarp	LM	Leaf margin
NS	No. of seeds	LD	Leaf division
TSS	Total Soluble Solids	LCI	Leaf color intensity
FS	Fruit shape	LLA	Leaf lamina attachment
FBS	Fruit base shape	NP	No. of petals
FAS	Fruit apex shape	PL	Petal length
FSC	Fruit skin color	PW	Petal width
AA	Attachment of albedo	PdL	Pedicel length
FST	Fruit surface texture	A/S	Relative length of filament to
SSh	Seed shape		Stigma
SSf	Seed surface	FT	Flower type
SC	Seed color	FC	Flower color
LL	Leaf length	AC	Anther color
LW	Leaf width	FM	Flowering month

Data Collection

The morphological characters evaluated for each accession were:

1. Tree characteristics
2. Leaf characteristics
3. Flower characteristics
4. Fruit characteristics

The tree characters included were:

1. Tree shape (erect, spreading and drooping)
2. Branch density (sparse, medium and dense)
3. Branch angle (narrow, medium and wide)
4. Shoot tip color (green, purple and others)
5. Shoot tip surface (glabrous, intermediate and pubescent)
6. Spine density on adult tree (absent, low, medium and high)

Minimum of 15 leaves and five flowers, 10-15 fruits per tree were evaluated. The evaluated leaf characteristics

1. Leaf apex shape
2. Leaf lamina shape
3. Leaf margin
4. Leaf division
5. Leaf color intensity
6. Leaf lamina attachment
7. Petiole wing shape
8. Leaf length (mm)
9. Leaf width (mm)
10. Leaf length/width ratio

Floral Characteristics

1. No. of petals
2. No. of anthers
3. Length of petals (mm)
4. Width of petals (mm)
5. Length of pedicel (mm)
6. Relative length of stamens to stigma
7. Color of anthers

8. Color of open flowers
9. Flower type
10. Flowering month

Fruit Characteristics

1. Fruit weight (g)
2. Fruit diameter (mm): horizontal length of fruits at equatorial region.
3. Fruit length (mm): vertical length of fruits from top to base.
4. Adherence of albedo: attachment of skin to mesocarp (weak, medium and strong)
5. Thickness of epicarp at equatorial region (mm)
6. Fruit shape (spheroid, obloid, oblate and pyriform)
7. Fruit base shape (truncate, necked, convex and concave)
8. Fruit apex shape (acute, depressed, extended)
9. Fruit skin color (greenish yellow, yellowish orange, deep orange)
10. Fruit surface texture (smooth, rough or other)
11. No. of seeds per fruits (developed seeds)
12. Seed surface (smooth, rough or others)
13. Total Soluble Solids (°Brix) of fruit juice at room temperature (20°C)Seed color (white, cream or other)
14. Total acidity (%)

The variables for each character can be found in Appendices (Appendix Table B1, B2, B3 and B4).

Determining Total Soluble Solids (TSS) in (°Brix)

Sampling and Measurement: ten sample fruits from each tree per accession were used. The fruits were cut into halves crosswise and squeezed manually into a beaker. Two drops of the juices were then placed onto refractrometer prism plate. The

corresponding reading on the prism scale was noted in percent. The prism plate was cleaned and wiped with soft tissue paper.

Re-calibration of Hand Refractrometer: Several drops of distilled water were placed on the prism plate and checked if reading was zero. The prism plate was washed and dried with soft tissue. Then few drops of 6% sucrose solution were placed on the prism plate again. Throughout the study, hand refractrometer yielded accurate reading of 6 by 6% sucrose solution for every check performed.

Determination of Total Acidity (Titration Method)

Preparation of Sodium hydroxide Solution: 40g (1 Mole) of Sodium hydroxide (NaOH) was dissolved in 1 liter of distilled water. 1 Molar NaOH solution was diluted to the factor of 10 with distilled water to obtain 0.1 M NaOH solution using volumetric flask.

Standardization of NaOH Solution: 50 ml burette was filled with 0.1 M Hydrochloric acid (HCL) and the initial reading was set at zero. 10 ml of 0.1 M NaOH (approximate) solution was pipetted out into a conical flask. Two to three drops of phenolphthalein indicator solution was added. NaOH solution was titrated with 0.1 M HCl until the end point was reached. 0.1 M HCl was prepared by dilution in volumetric flask.

Actual Molarity of NaOH Solution: The actual Molarity of NaOH solution was determined using the equation below. For precision, entire procedure was repeated for three times and the average results were used.

$$\mathrm{M\ (NaOH)} = \frac{\ldots \mathrm{mL\ (Titre\ of\ 0.1\ M\ HCl\ solution)} \times 0.1\ \mathrm{M\ (HCl)}}{10\ \mathrm{mL} (\approx 0.1\ \mathrm{M\ NaOH}}$$

Sample Preparation and Measurement: The juice from sampled fruits were squeezed out manually and homogenized. 10 ml of juice was pipetted out into clean conical flask and added with 10 ml of distilled water. This was followed by addition of three to four drops of phenolphthalein into the conical flask containing juice. The mixture was carefully swirled. Sodium Hydroxide (0.1 M NaOH) was allowed to trickle from burette by opening tap to ensure no air is trapped. The burette was refilled to zero. With conical flask containing juice mix under the burette and with constant swirling, sodium hydroxide was added to the juice until the color turned to pink (endpoint). The volume of sodium hydroxide used was noted. The entire step was repeated till concordant readings were obtained.

Therefore as percentage acid

$$Percentage\ acid = \frac{Titre\ \times acid\ factor\ \times 100}{10\ (ml\ juice)}$$

Where acid factor for citric acid = 0.0064 (Citrus fruits)

Sugar Acid Ratio

Sugar concentration was measured by hand refractrometer at room temperature. The instrument was calibrated at regular interval.

Sugar acid ratio was obtained as below

$$= \frac{\text{Total Soluble Solids (°Brix)}}{\text{Total Acidity (\%)}}$$

Data Analysis

Data for field accessions and germplasm accessions were analyzed separately. The quantitative characters were subjected to descriptive analysis using ver.17.0 of SPSS program. The means of accessions were compared using One-way ANOVA followed by Duncan Multiple Range Test between locations. The distinct qualitative data of the field collected accessions were also used for construction of dichotomous keys.

Experiment II AFLP Analysis

Amplified fragment length polymorphism (AFLP) is a recent fingerprinting technique developed by Vos *et al.* (1995). The method is based on PCR amplification of selected restriction fragments of total digested genomic DNA. Once labeled, amplified products were separated by electrophoresis and the fragments range of 10-500 base pairs were obtained. The basic steps involved in AFLP analysis is presented as under.

General Principles and Background of AFLP

The specific small fragments of few base pairs (up to 500) need to be amplified with PCR. The PCR amplify this through a precise priming of oligonucleotidic sequences (primers) at each end of the target DNA. Primers are 18-24 base pair long sequence, synthesized in laboratory and corresponding to a complementary DNA sequence designed in the flanking regions of the heavy strand of target DNA.

The PCR starts first with high temperature phase (denaturation) producing single-stranded DNA. Once the temperature reach down to annealing temperature which is defined by primers composition, the primers will bind to the template DNA. Taq polymerases recognize each double-stranded DNA as a start and continue to

synthesize in 5’ to 3’ as soon as temperature reach 72°C which is optimal temperature for elongation.

The originality of AFLP is to synthesize the arbitrary primers and then to ligate them to target DNA fragments. The AFLP arbitrary primers are called as “adaptor” and consists a sequence of 20 nucleotides. The adaptors serve as PCR priming sites in amplification of restriction fragments. The target DNA sequences are DNA fragments obtained from combined restriction of two restrictions enzymes. Since AFLP covers large number of fragments from entire genome, it is considered as very good indicator of the level of genetic variation.

DNA Extraction

DNA was extracted according to the method of Doyle & Doyle (1987) with minor modifications. The method is based on CTAB (Hexadecyl-trimethyl ammonium bromide) procedure. CTAB is cationic detergent that forms a complex with the DNA. The CTAB-DNA complex was then separated from the cellular debris by chloroform where two phases: superior clear aquatic phase containing the DNA, and denser inferior one containing the chloroform and secondary components (polysaccharides, proteins etc.) was observed. After centrifugation, cellular debris was usually observed at interface. The purification by chloroform can be repeated several times (2-3 times) depending on the appearance of colloids.

Precipitation was done with 0.5 M sodium acetate followed by addition of ice cold absolute ethanol. After precipitation, the DNA molecule was washed with ethanol to remove the salts used for precipitation. DNA is then dissolved and stored in Tris/EDTA buffer (TE buffer):Tris (10mM)/EDTA (1mM) pH 8.

In a 15 ml centrifuge tube, 6 ml of 2% CTAB and 60µl 2-mercaptoethanol was incubated in water bath at 65°C for 30 minutes. About 8 grams of fully expanded, healthy young leaves were cut into pieces with sterilized scissor after removing midrib. Then it was grounded in liquid nitrogen manually using pestle and mortar to

fine powder. The finely grounded powder was added to the mixture containing 2% CTAB and 60µl 2-mercaptoethanol. The mixture was again incubated for half an hour and gently inverting the tubes (3 times) for homogenization at 10 minutes interval.

After incubation, 6ml of CIA (Chloroform: Isoamylalcohol in 24:1) was added and shaken vigorously for 15 minutes. Then the mixture was centrifuged at 3000 rpm for 30 minutes. The floating phase was recuperated and the process was repeated again. The equal volume of ice-cold Isopropanol was added recuperated floating phase and incubated at -20°C for half an hour. DNA palate was collected by centrifugation at 3000 rpm for 20 minutes where the liquid is discarded. 75% ethanol is added and again centrifuged for 20 minutes. The resultant sediment was dried in air for 30 minutes. It was then dissolved in 400 µl TE buffer.

Removal of RNAs

To 400µl (DNA in TE buffer), 8µl of RNase was added and incubated at 37°C for 1 hour. Then 40µl of Sodium Acetate was added, followed by addition of 1 ml ice-cold absolute ethanol and centrifuged to collect the DNA sediment palate. It was then transferred to 1.5 ml centrifuge tube and washed twice with 75% ethanol. The resultant palate is air-dried and dissolved again in 400µl TE buffer.

Phenol Extraction

Since Citrus contain many polyphenols, essential oils, and polysaccharides that affects to directly to extraction of quality of DNA, phenol extraction was included to remove the polyphenols.

To the 400 µl TE buffer, equal amount of phenol extract was added. The mixture was inverted gently for homogenization and then centrifuged. The clear supernatant was evacuated using micropipette and transferred to a new centrifuge tube. 40 µl of sodium acetate was added again to the supernatant in new tube followed by 1ml of absolute EtOH and subjected to centrifugation at 14000 rpm for 30 minutes.

The liquid was discarded and 500 µl of 75% EtOH added to centrifuge at 14000 rpm for 10 minutes. The process was repeated twice.

Determining Quality and Quantity of DNA

The AFLP is highly sensitive technique and requires a very high quality DNA in sufficient quantity. To ensure that there is the high quality DNA and in amount required, it was checked by running in 1% agarose gel electrophoresis. The quantity was compared with 50 ng lamda (λ) DNA while quality was judged by the presence of smears. The high quantity DNA was diluted and the relatively less ones were precipitated as shown in Figure 5.

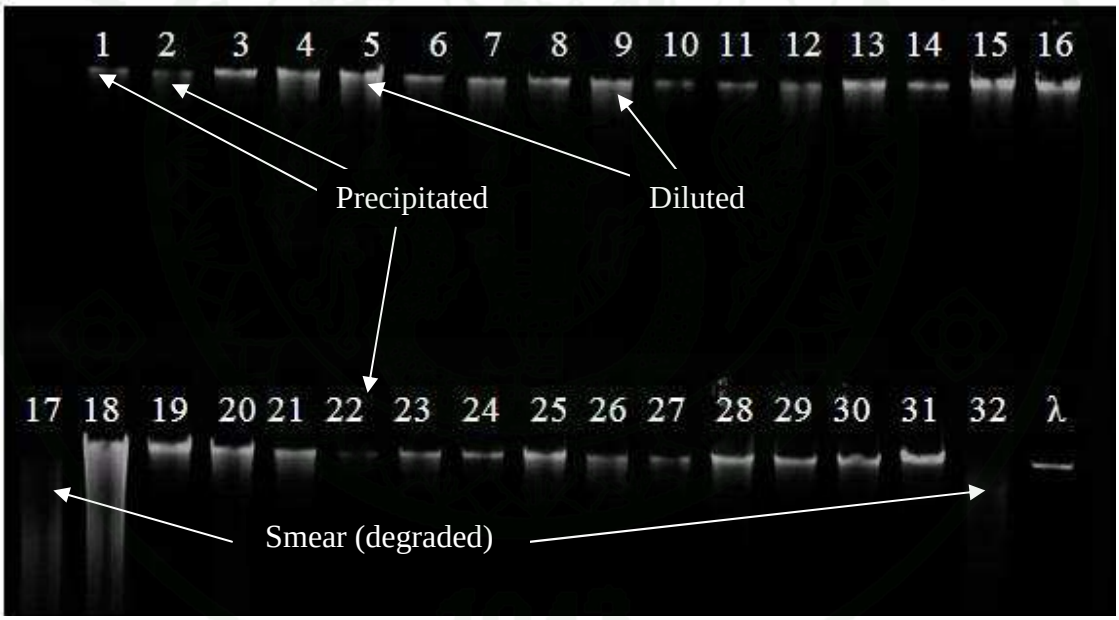


Figure 5 Comparison of mandarin DNAs for quality and quantity check at 1% agarose gel electrophoresis with λ (50 ng) DNA.

The AFLP technique was performed as described by Vos *et al.* (1995). Genomic DNA (250 ng) was restricted with *Eco*RI/*Mse*I (2.5 U each) in a restriction buffer 50 mM TrisHCl, pH 7.5, 50 mM Mg-acetate, 250 mM potassium acetate) in a final volume of 25 µl. *Eco*RI and *Mse*I adapters were subsequently ligated to the

digested DNA fragments. The adapterligated DNA (diluted 1:9) was pre-amplified with AFLP primers each having one selective nucleotide using the following cycling parameters: 20 cycles of 30 sec at 94°C, 60 sec at 56°C and 60 sec at 72°C. The pre-amplified DNA was diluted (1:9) based on pretest for concentration of preamplified DNA required for visibility of bands on 6 % polyacrylamidegel electrophoresis. The aliquot was subsequently used for selective amplification with *Eco*RI and *Mse*I primers having three selective nucleotides at the 3' ends.

The cycling parameters for selective amplification were as follows: 1 cycle of 30 sec at 94°C, 30 sec at 65°C and 60 sec at 72°C. The annealing temperature was then lowered by 0.7°C per cycle during the first 12 cycles and then 23 cycles were performed at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec. The reaction products were resolved on 6% polyacrylamide sequencing gels followed by silver staining. Five selective primer combinations were used in this study after screening of 10 primer pairs.

AFLP Data Analysis

The banding patterns generated by AFLP markers were used to determine the genetic relatedness of the 23 mandarin accessions from different origin. Ten selected primer pairs were tested based on the findings of previous study (Campos *et al*., 2005). Five primers combination were used for evaluation of 23 selected samples from field. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical.

The data were subjected to cluster analysis by using NTSYSpc ver 2.20k. The similarity matrix was generated with "Qualitative data" option of Dis/similarity module. Cluster analysis was carried out SAHN (Sequential Agglomerative Hierarchical Nested) option for generation of Unweighted Pair Group Method using Arithmetic Averages (UPGMA) dendrogram for 23 field accessions.

Place and Duration

The first experiment of morphological evaluation for the accessions was carried out in the Laboratory of National Feed and Fodder Development Program, Batpalathang under Bumthang district of Bhutan. Molecular (AFLP) analysis was carried out in the Central Laboratory, Department of Horticulture, Faculty of Agriculture of the Kasetsart University, Bangkok, Thailand.

The survey and sampling for morphological characterization commenced in November, 2009 to April 2010 followed by DNA extraction and subsequent laboratory analysis was conducted between the months of May, 2010 to March, 2011.

RESULTS AND DISCUSSION

Results

Survey and Sampling

Our survey revealed that typology of mandarin orchards differed basically on number of trees per mandarin growers from as low as few trees to more than 500 trees. The mandarin was grown in varied agro-ecological conditions with an altitude ranging from 390 to 1650 m msl. The age of citrus trees' ranged from 16 to above 50 years. The prominent planting materials were seedlings purchased either from neighbor nursery or self-raised as commonly been practiced in this region. Among the sites studied, only one orchard in Trongsa had acquired seedlings from the one and only corporate seed agency in the country (Table 4).

Table 4 The tree age, seedling source, slope gradient and orchard type for different districts

Districts	Tree age (year)	Source of seedlings	Slope (%)	No. of Accessions	Orchard Type
Samtse	Over 50	Self-raised/ neighbor	30	5	Small farm[2]
Sarpang	26	Self-raised/ neighbor	16	6	Small farm
Tsirang	45	Self-raised/ neighbor	11	5	Backyard[1] farm
Dagana	30-40	Self-raised/ neighbor	15	5	Small farm
Trongsa	16	Druk Seed Corporation (DSC)	21	5	Backyard farm
Zhemgang	30-40	Self-raised/ neighbor	25	4	Small farm

Self-raised/neighbor - citrus seedlings either raised by farmers themselves or bought from neighbors, DSC- The only corporate seed agency in Bhutan,1-Orchards with less 50 mandarin trees, 2 orchards with 51-120 mandarin trees

Quantitative Characteristics (Field Accessions)

The statistical analysis of quantitative characters showed a highly significant ($p < 0.001$) variation among the leaves and fruits from different locations. In general, the leaf dimensions were largest with 94.83 mm length and 38.80 mm width for accessions from Trongsa. The accessions from Dagana had the smallest leaf (76.43 mm by 32.16 mm) However, the quantitative characters with respect to flower were did not have any interesting observation. The variation of quantitative characters such as ratio of leaf length to leaf width, number of petals, petal length, petal width and also the number of stamens are shown in Table 5.

Table 5 Leaf and flower characteristics of different accession in the field.

Location	Leaf Characters			Flower characters		
	Leaf Length (mm)	Leaf width (mm)	L/W	Petal Length (mm)	Petal width (mm)	No. of stamens
Samtse	77.80 a	32.17 a	2.43	11.34	4.67	14.63
Sarpang	89.65 cd	38.01 cd	2.37	11.22	4.78	14.86
Tsirang	86.15 bc	35.83 bc	2.42	10.96	4.76	14.85
Dagana	76.43 a	32.16 a	2.40	10.99	4.68	14.87
Trongsa	94.82 d	38.80 d	2.45	11.23	4.78	14.73
Zhemgang	81.78 ab	33.45 ab	2.46	11.33	4.78	14.70
F values	22.12	19.67	2.05ns	0.44ns	0.78ns	0.26ns

Mean values with their differences (a, b, c) within column at 0.001 confidence level. L/R refers to Ratio of leaf lamina length to leaf lamina width

Similarly the weight and size of the fruits showed a wide variation. The accessions from Dagana had the heaviest fruits (107.17g) while Tsirang had the lowest fruit weight (58.33g). As expected from their weights, accessions from Dagana had the largest fruit with diameter of 61.68mm and fruit length of 53.88 mm while Tsirang's accessions had the smallest fruit with diameter of 49.75mm and fruit length of 44.16 mm (Table 6).

Table 6 Comparison of fruit quantitative characters between locations for field collected accessions

Location	Fruit characters							
	Fruit weight (g)	Fruit diameter (mm)	Fruit length (mm)	Epicarp Width (mm)	No. of seeds	TSS (°Brix)	Total acidity (%)	Sugar acid ratio
Samtse	96.12 d	58.58 c	50.78 c	2.11 ab	16.56 e	10.35 a	1.15 ab	9.13 b
Sarpang	87.60 c	57.37 c	49.02 b	2.11 ab	13.32 d	10.99 bc	1.22 bc	12.29 c
Tsirang	58.33 a	49.75 a	44.16 a	2.01 a	10.12 c	10.55ab	1.46 cd	8.19 ab
Dagana	107.17 d	61.68 d	53.88 d	2.19 b	10.50 c	11.25 c	0.93 a	13.27 c
Trongsa	78.85 b	55.53 b	47.59 b	2.16 b	4.35 a	12.91 d	1.66 de	8.68 ab
Zhem-gang	93.33 cd	58.35 c	51.54 c	2.19 b	6.92 b	12.13 d	1.90 e	6.51 a
F values	74.30	68.60	57.26	3.60	17.42	63.59	15.00	10.71

Mean values and their differences within column at 0.001 confidence level.

Horticulture Characteristics (field accessions)

In citrus, one of the breeding objectives is to increase yield and production. Considering yield parameters, great variation was noticed among the analyzed accessions from six different locations (Table 6). The weight of the fruit ranged from as low as 58.58g (Tsirang) to that of 107.17g (Dagana). Fruit diameter ranged from a less than 50mm (Tsirang) to over 61 mm (Dagana). The fruit length varied between 44 mm (Tsirang) to 56 mm (Dagana). The proportion of weight to size of fruits is shown in Figure 6.

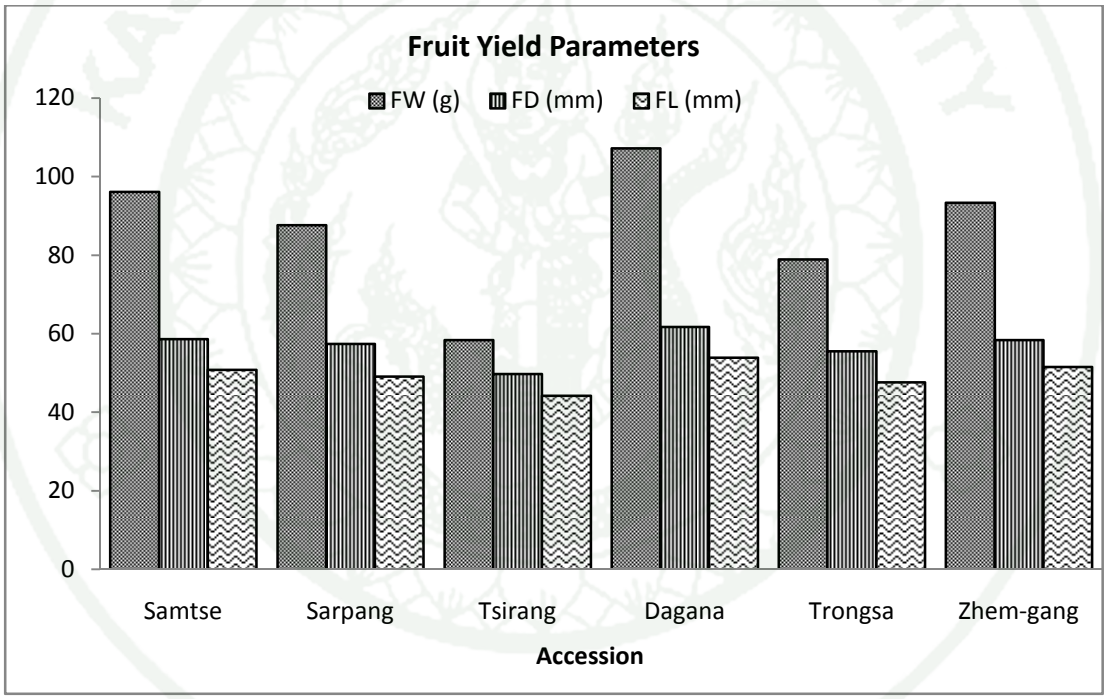


Figure 6 Comparison of fruit yield component for field accessions from different location

Sugar Acid Ratio

The sugar acid ratio varied from accession to accession and from district to district. The highest sugar acid ratio was recorded in accessions from Dagana followed by Sarpang. The accessions from Zhemgang had the lowest sugar acid ratio.

Except for accessions from Sarpang and Dagana, the sugar ratio was less than 10 (Figure 7).

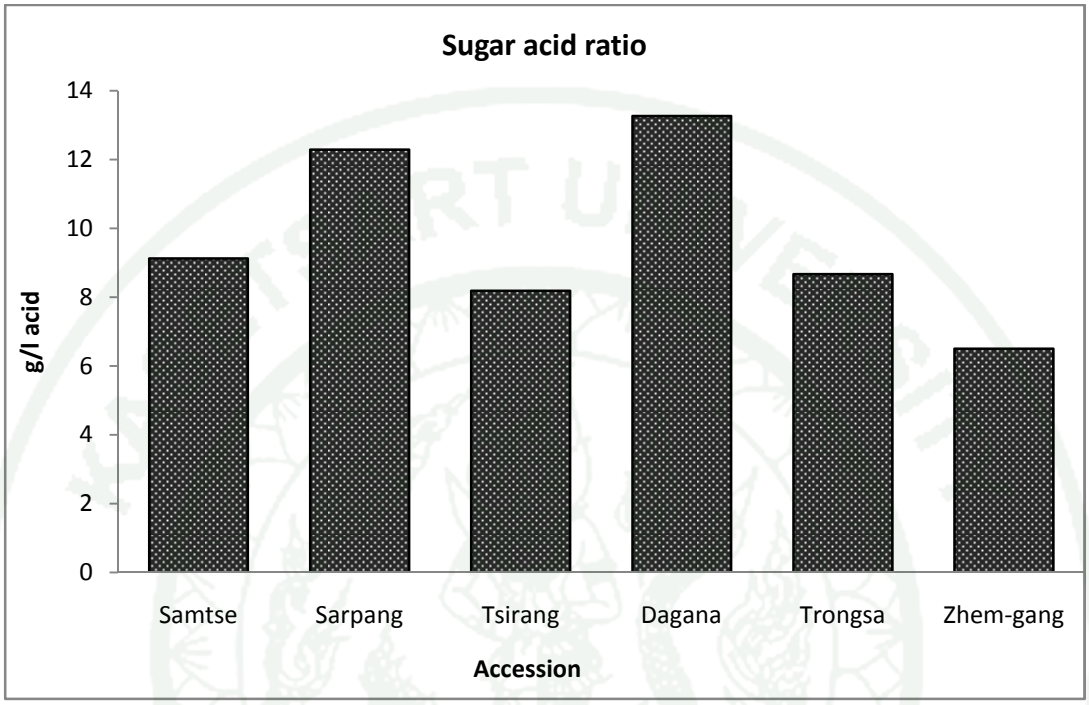


Figure 7 The variation in sugar acid ratio (g/l acid)

Classification based on Dichotomous Keys

The keys were constructed based on six morphological qualitative variables of trees, fruits and leaves. It was able to classify mandarin to their respective origin although inter-plant floral differences were minimal. Qualitative characters for fruit (fruit color, fruit based shape, fruit surface texture) varied within fruits of same trees. Similarly, seed shape, seed color and seed surface also varied (Figure 8). The classification was not applicable to the accessions from germplasm as the growth habit and fruit characters were overlapping. Also, there were no differences observed for floral characters especially to the qualitative characters.

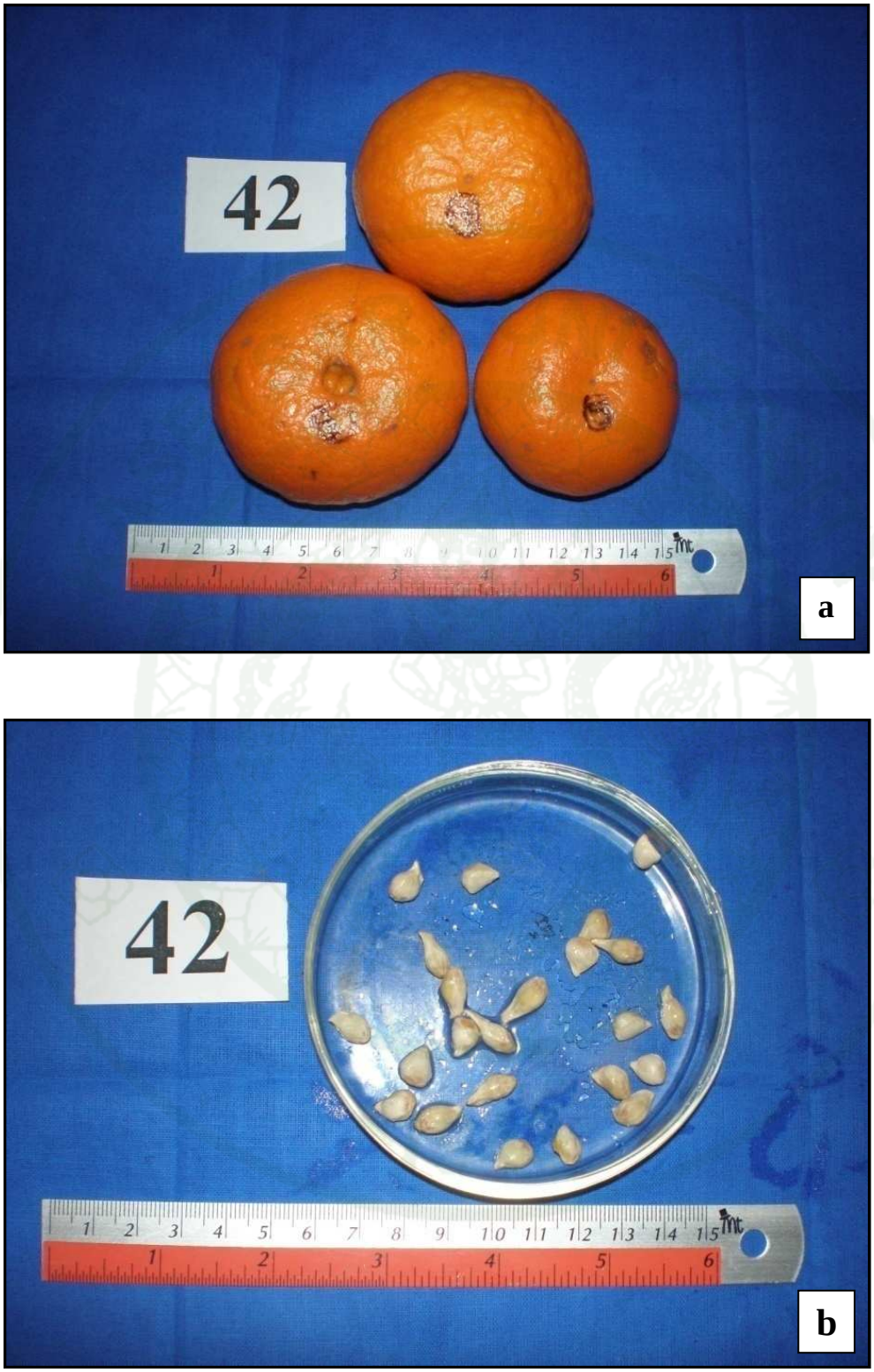


Figure 8 Variation in fruits size of same tree (a) and seed shapes from single fruit (b) in germplasm accession of Kengkhar

Dichotomous Keys to Mandarin types in Bhutan

1. The tree canopy is open and erect in shape.--- 2
1. The tree canopy is spreading dome in shape.--- 5

2. The fruits mature before rind develops orange color.--- 3
2. The fruits mature only after the rinds develop orange in color.--- 4

3. The surface of the fruit was mostly smooth.--- *Samtse*
3. The surface of the fruit was usually rough.--- *Samtse 4*

4. The fruit shape is oblate depressed both at top and base. ---*Zhemgang*
4. The fruit is usually truncate at base and depressed only at the top. ---*Tsirang*

5. Branch density of the tree is sparse.--- *Sarpang*
5. Branch density of the tree is dense.--- 6

6. The leaf color intensity at fruit maturity is light green. ---*Trongsa*
6. The leaf color intensity at fruit maturity is rather dark green. ---*Dagana*

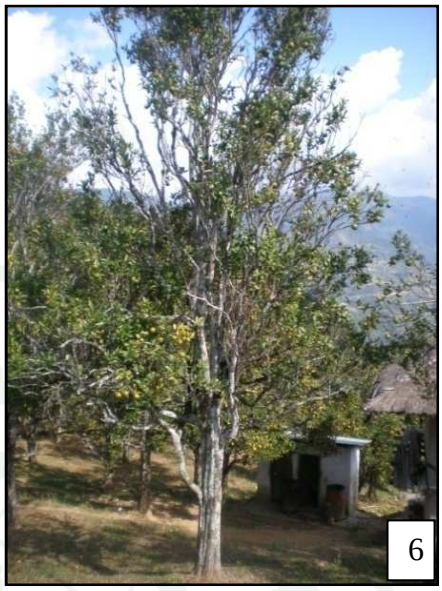
Figure 9 Dichotomous keys constructed using qualitative variables

The tree shape and growth habit at Samtse, Sarpang, Tsirang and Dagana is shown in Figure 10.

a. Erect (1) and Broom (2) shaped trees from Samtse

b. Spreading and drooping (3,4) growth habit from Sarpang

Figure 10 The variability of tree growth habit and shape in accessions of Samtse (a) Sarpang (b), Tsirang (c), Dagana (d) and Trongsa (e)



c. Spreading (5) and erect (6) growth habit from Tsirang

d. Drooping habit (7) with dome shaped (8) trees from Dagana

Figure 10 (Continued)


9


10

e. Spreading growth habit (9,10) Trongsa

Figure 10 (Continued)

Morphological Description of Field Accessions

The study on qualitative morphological characters from six districts showed huge variation. Maximum variation was found in tree, leaf and fruit characters for the accessions from different locations. The accessions from Tsirang had distinct fruit shape. The difference in fruit shape was also observed from the single tree Samtse had fruits with both rough and smooth surface texture within fruits of a tree. Similarly, necked and truncate fruit base shape was observed for almost all the trees. However, there was no difference in terms of floral characters for accessions both within and between locations.

Samtse Accessions

The mandarin trees at Samtse were mostly opened and erect in shape. The branch angle of the tree was narrow with major branch angle less than 30°. The fruit matured relatively earlier than other study sites. The fruits are greenish-orange at maturity with usually smooth fruit surface texture. The fruits were seedy which ranged from 10- 22 per fruit. The average TSS content of fruit juice was lowest with 10.35 °Brix. Although the fruits from this area had low total acidity (1.15), sugar acid ratio continued to remain lower when compared to fruits from other location.

Sarpang Accessions

The mandarin trees at Sarpang were spreading dome shape with wide branch angle. The branch density was sparse with light green leaf color intensity at fruit maturity. The branch angle was narrow with mostly obovate leaf lamina shape. The fruits were medium sized with dimension of 57.37 mm (horizontal) and 49.02 mm (vertical). The color at maturity was yellowish orange and the fruits were seedy with average of 13 seeds per fruit. The color of fruit at maturity was yellowish orange. The average TSS was 10.99 °Brix.

Tsirang Accessions

The trees were also open and erect in shape. The branch angle was narrow. The fruits were the smallest among six locations evaluated. The fruits were oblate-obloid depressed at the top and truncated at the base. The color of the matured fruits was orange and the fruits were found with high total acidity although the relative tastes are sweet due to high TSS content.

Dagana Accessions

The trees in these accessions were dome spreading to drooping in shape. The branch angle of the tree was wide and had high branch density. The leave's color intensity at maturity was dark green. The fruits were oblate, flattened at both end (top and base). The fruits were the largest among the surveyed area with diameter of 61.68 mm and length of 53.88 mm. The fruits contained moderate number of seeds on an average 10.5 seeds per fruits. The fruits from this area had exceptionally low total acidity of 0.93 % with relatively high TSS content of 11.25 °Brix.

Trongsa Accessions

The trees from Trongsa were spreading dome in shape with wide angled branch and high branch density. The leaf was characterized by larger size with light

green leaf color intensity at fruit maturity. The color of fruit was orange at maturity. The fruit size was medium large with very few seeds. These fruits of these accessions had highest TSS content of 12.91 °Brix. On the other hand the fruit juice had also high total acidity of 1.66 % next to accession at Zhemgang.

Zhemgang Accessions

The accessions from Zhemgang were open and erect in shape. The branch angle was narrow. The rind color at fruit maturity is orange and the fruits were oblate is shape depressed at both ends. The fruit had the highest total acidity of 1.9 % and TSS content was high with 12.14 °Brix which is after accessions in Trongsa. The fruit size was generally large.

Quantitative Characteristics (Germplasm Accessions)

The accessions from germplasm were grafted and into third year of fruiting. Tree size and growth habit did not differ much within locations. The branch angle and branch density also did not differ much. There was a statistically significant difference ($p<0.001$) for leaf length among the analyzed accessions in germplasm collections. The accessions from Shumar under Pema Gatshel district had the longest leaf lamina length followed by accessions from Samtse, Sodrung, Samdrup Jongkhar, Narang, Dagana, Kengkhar, Tsirang, Yadi and Trongsa. The accessions from Samtse had the widest leaf width followed by Sandrup Jongkhar and Shumar. The leaf width was lowest for accessions from Trongsa. The ratio of leaf length to leaf width was highest for the accessions from Sodrung followed by Shumar (Table 7).

The accessions were highly significant for leaf and fruit characteristics. Some of the characters for flowers also differed significantly except for the petal length. The accessions from Shumar had the longest leaf lamina length while the accessions from Trongsa had the lowest. The accessions from Samtse had the broadest leaf. The accessions were highly significant for leaf and fruit characteristics. Some of the characters for flowers also differed significantly except for the petal length. The

accessions from Shumar had the longest leaf lamina length while the accessions from Trongsa had the lowest. The accessions from Samtse had the broadest leaf while Trongsa had the narrowest leaf.

The tree shape, fruits and branches of some of the promising type of mandarin from germplasm evaluation is shown below. The accessions were

1. Shumar
2. Trongsa
3. Sodrung
4. Kengkhar

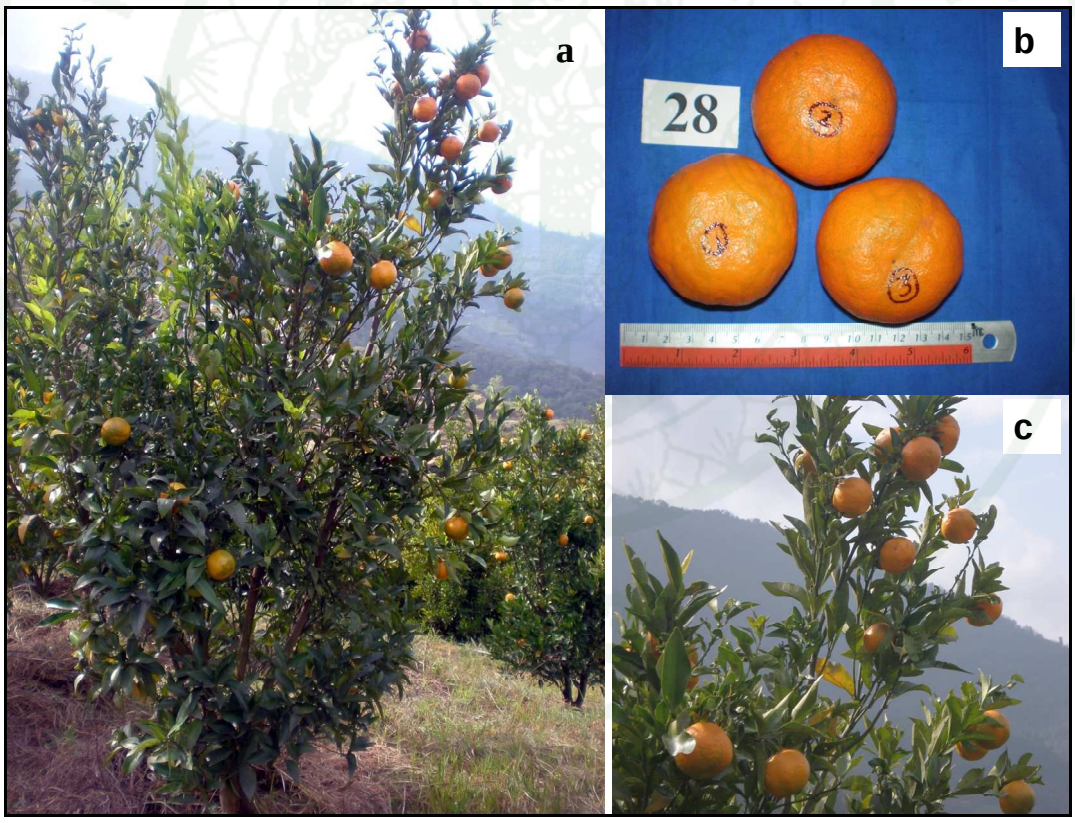


Figure 11 Tree (a), fruit (b) and branch (c) of Shumar accession

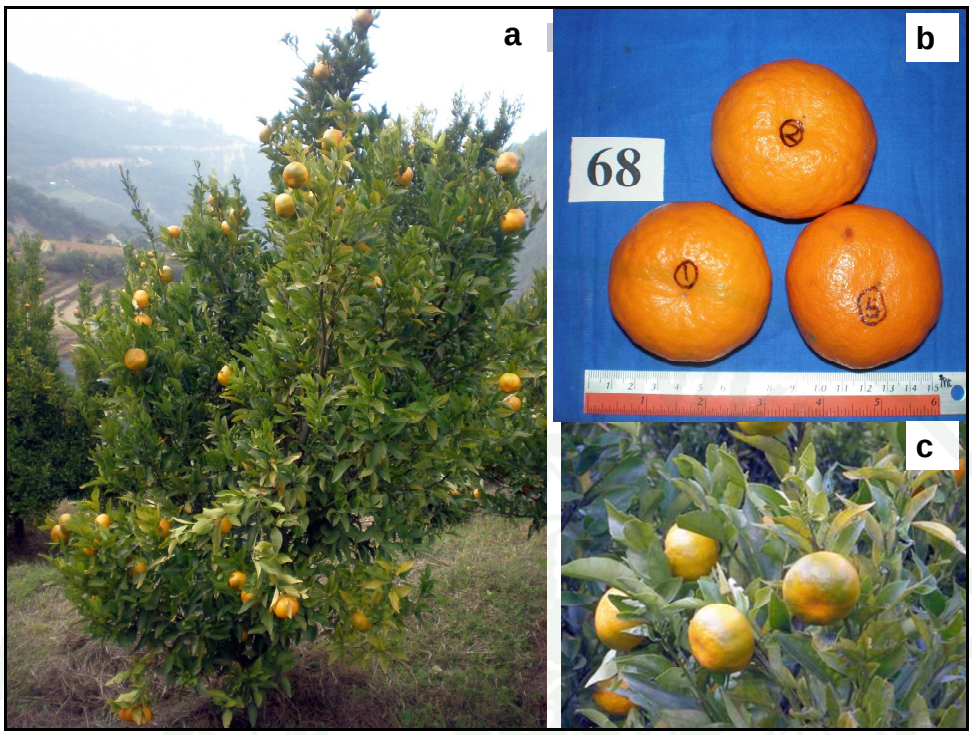


Figure 12 Tree (a), fruit (b) and branch (c) of Trongsa accession in germplasm block

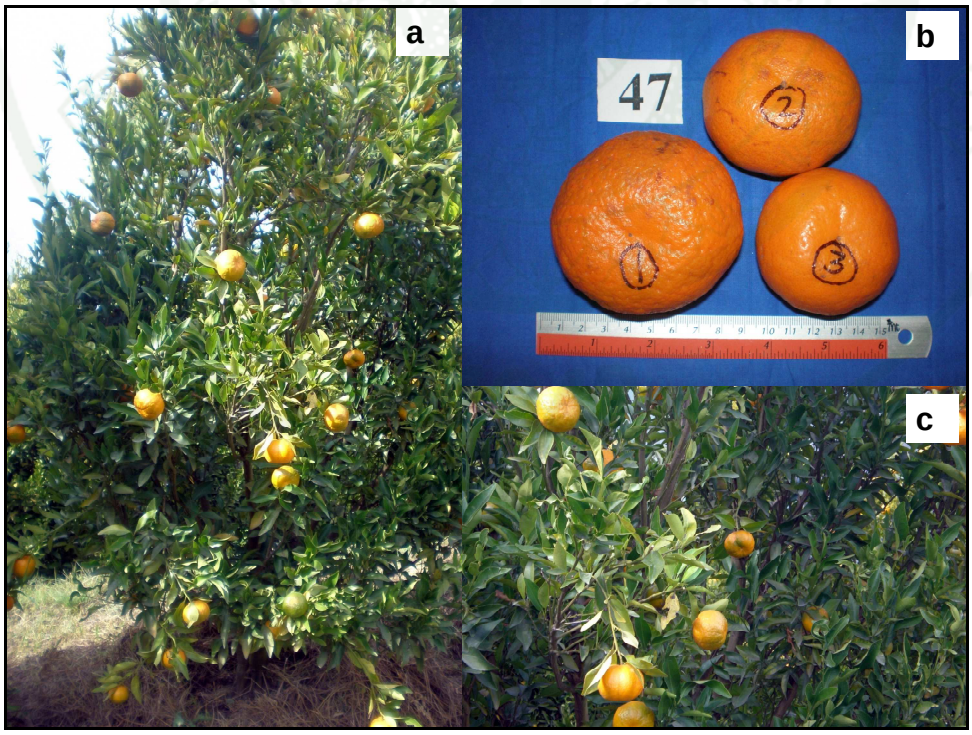


Figure 13 Tree (a), fruit (b) and branch (c) of Sodrung accession

Figure 14 Tree (a), fruits (b) and branch (c) of Kengkhar accession

Table 7 Leaf and flower characteristics for the accessions from germplasm

Location	Leaf Characteristics			Flower Characteristics			
	Leaf length (mm)	Leaf width (mm)	Length /width	Petal length (mm)	Petal width (mm)	No. of stamens	Pedicel length (mm)
Shumar	131.70 e	41.50 bc	3.23 c	10.59	4.60 ab	14.80 ab	4.82 b
K/Khar	100.89 b	37.97 bc	2.67 ab	10.95	4.71 bc	15.22 ab	4.45 ab
Narang	103.36 bc	36.36 ab	2.86 b	10.96	4.74 bc	15.30 ab	4.28 a
Samtse	117.08 d	44.28 c	2.66 ab	10.95	4.60 ab	15.10 ab	4.26 a
Dagana	101.91 bc	37.65 ab	2.73 ab	10.75	4.71 ab	14.63 ab	4.47 ab
Tsirang	94.91a b	35.60 a	2.67 ab	10.81	4.77 bc	14.93 ab	4.30 a
Yadi	94.65a b	34.31 a	2.77 ab	10.77	4.29 a	13.90 a	4.22 a
Sodrung	112.06 cd	34.12 a	3.34 c	10.79	4.81 bc	14.90 ab	4.54 ab
Sj/khar	107.97 bc	41.63 bc	2.61 a	11.33	4.97 c	16.00 b	4.56 ab
Trongsa	87.14 a	33.06 a	2.65 ab	10.95	4.86 bc	16.00 b	4.27 a
F value	12.41***	6.38***	19.538***	0.86ns	3.98***	1.979**	3.148**

The mean value with their differences (a,b,c) within column. K/Khar and Sj/Khar refers to Kengkhar and Samdrup Jongkhar, while ** and *** refers to significance at 0.01 and 0.001 confidence level, respectively.

Similarly, fruit characteristics varied significantly for the accessions from different places. The heaviest fruit of 117.33g was from the accessions of Shumar while Samtse had the lightest. The width of epicarp was observed to be thinnest in the accessions from Samtse. The number of seeds per fruit was lowest in Samdrup Jongkhar accessions. The TSS was highest in the accessions from Narang followed by Kengkhar. The ratio of TSS to total acidity decreased in the order of Shumar, Kengkhar and Samdrup Jongkhar. Shumar had the lowest total acidity while the accessions from Tsirang had the highest (Table 8).

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Table 8 The morphological comparison of quantitative characters of fruits for the accessions from germplasm

Location	Fruit characteristics							
	Weight (g)	Diameter (mm)	Length (mm)	Epicarp width (mm)	No. of seeds	TSS (°Brix)	Total acidity (%)	Sugar acid ratio
Shumar	117.33 e	64.07 cd	57.49 f	2.57 c	12.80 e	9.90 a	0.61 a	17.09 b
K/Khar	108.15 de	62.54 bc	51.48 de	2.43 bc	10.84 bc	10.76 cd	0.72 a	16.65 b
Narang	99.35 bc	61.39 bc	48.89 bcd	2.45 bc	9.58 c	10.81 d	1.01 a	11.78 ab
Samtse	74.37 a	55.46 a	43.50 a	1.92 a	8.70 b	10.17 d	1.15 bc	9.52 a
Dagana	91.46 bc	59.73 b	47.91 bc	2.22 b	9.10 ab	10.06 ab	1.17 bc	11.10 a
Tsirang	87.55 ab	58.82 a	46.09 ab	2.20 b	9.57 b	10.58 cd	1.27 c	9.60 a
Yadi	104.68 cd	62.46 bc	51.31 de	2.39 bc	10.45 bc	10.13 ab	0.93 abc	11.70 ab
Sodrung	110.66 de	66.99 c	53.43 e	3.03 d	10.10 bc	9.84 a	0.91 abc	11.55 ab
Sj/khar	89.79 bc	59.68 b	49.29 cd	2.43 bc	6.45 a	10.27 ab	0.67 a	16.55 b
Trongsa	115.86 e	65.98 de	52.85 e	2.67 c	11.40 bc	10.10 ab	0.84 ab	12.77 ab
F value	14.03	13.38	28.45	15.31	5.81	8.82	9.42	9.20

The mean values with their differences (a,b,c) within column at 0.001confidence level.

Horticultural Characteristics

Shumar had the highest fruit weight of 117.33g while the accessions at Samtse had the lowest of about 74.37g. On the other hand, accessions from Sodrung had the highest fruit diameter of 66.99 mm followed by Trongsa with 64.07mm. The accessions from Shumar, Sordrung and Trongsa had the higher fruit weight, fruit diameter and fruit length (Figure 11).

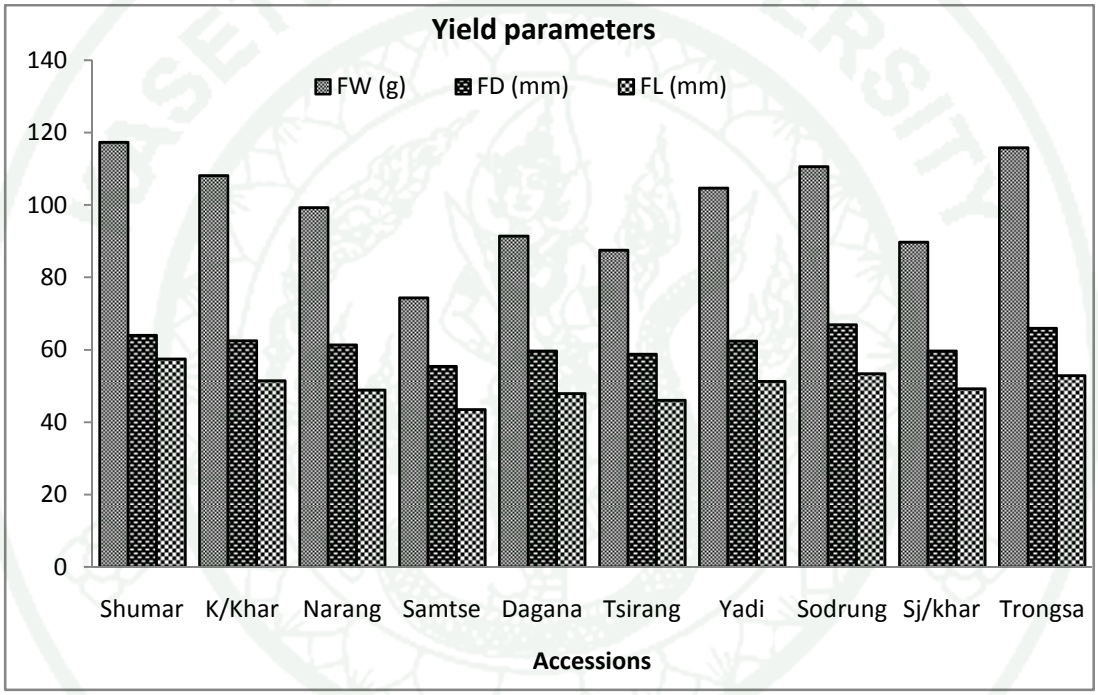

Figure 15 Variations in fruit yield parameters for different accessions in germplasm

The sugar acid ratio was in general higher in accessions from germplasm comparing to field accessions. Except for the accessions for Samtse and Tsirang, all other accessions had higher ratio of more than 10. The accessions from Shumar were recorded for highest ratio of 17.09 followed by Kengkhar and Samdrup Jongkhar with 16.65 and 16.55, respectively (Figure 12).

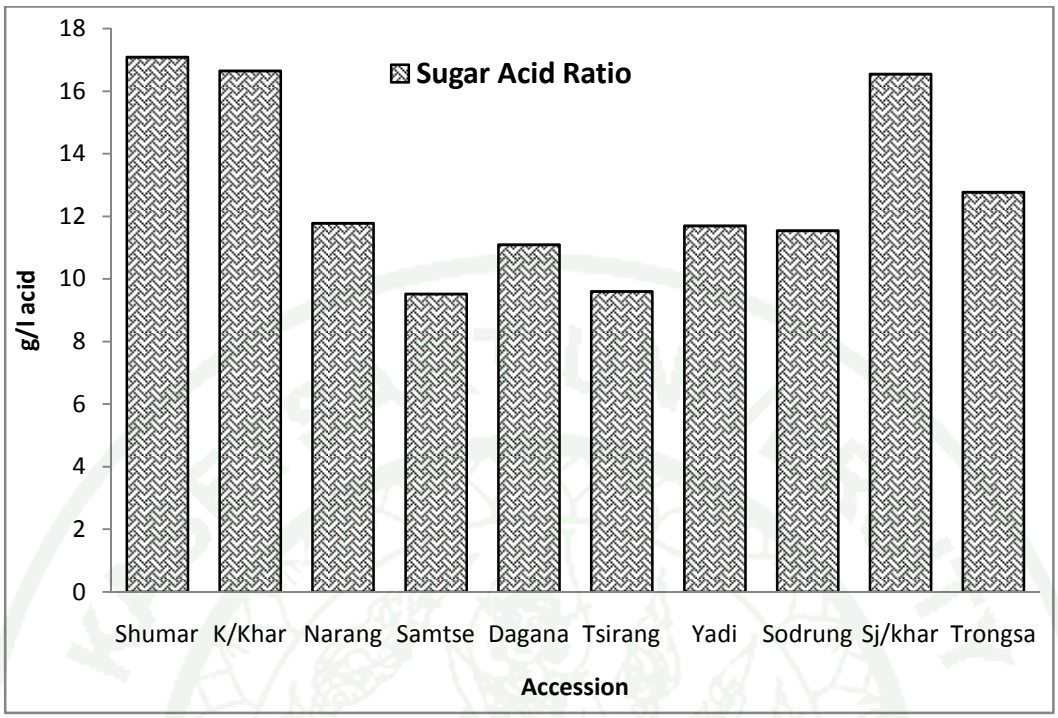

Figure 16 Comparison of sugar acid ratio (g/l acid) for accession of different sources in germplasm collection

AFLP Analysis

Five different *Eco*R1 and *Mse*1 primer combination were selected for 23 accessions collected from the field. The banding pattern from 5 primers combination discriminated 22 individuals. A total of 244 bands were generated of which 126 were polymorphic. On average, 51.64 % polymorphism was obtained from each primer combination. The primer pair E-ACA+M-CAG produced highest total bands (Figure 14) followed by E-ACG+M-CAT primer combination. On the other hand, E-AGG+M-CAA primer combination produced lowest total number of bands of which 23 bands were polymorphic. The Rate of polymorphism and the different primer combinations used in this study as shown below in Table 9.

Table 9 Number of polymorphic AFLP bands observed using 5 AFLP primer combinations

Primer combinations	Total number of bands	Number of polymorphic bands	Polymorphism rate (%)
E-ACA/M-CAG	84	38	45.24
E-ACG/M-CAT	53	31	58.49
E-ACC/M-CTT	33	17	51.51
E-AGG/M-CAA	33	23	69.69
E-ACA/M-CTC	41	17	41.46
Total	244	126	51.64

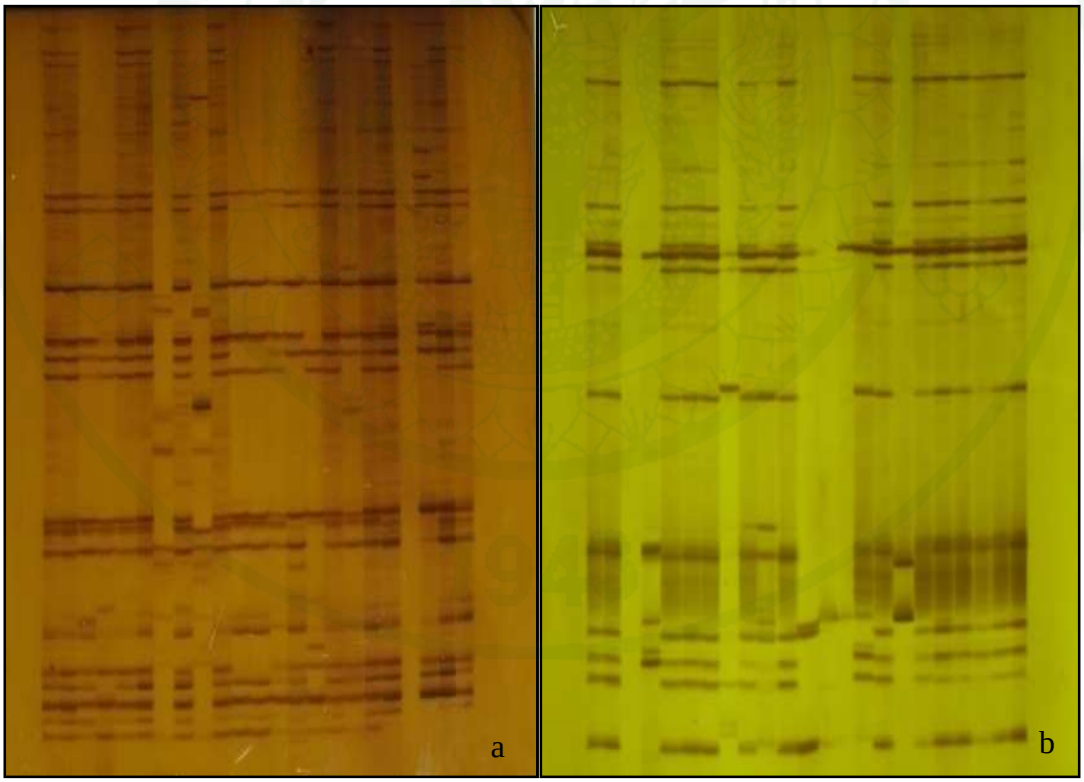

Figure 17 AFLP Polymorphism for 23 mandarin individuals as obtained by (a) E-ACA+M-CAG and (b) E-AGG+M-CAA primers combinations

Cluster Analysis (Field Accessions)

The cluster tree analysis (Figure 15) categorized 23 accessions to two major groups (A and B). Group A consisted of 14 accessions while group B had 9 accessions. Group A was further divided into 2 subgroups (1 and 2). Subgroup 1 was more diverse with accessions from four different districts: Samtse, Sarpang, Tsirang and Trongsa. However, accessions from Zhemgang were isolated to subgroup 2.

Group B also had accessions from all five districts. The majority of the accessions were from Dagana. *Dagana5* was isolated from subgroup 3. The major group (A and B) diverged at similarity coefficient of 0.48 and average similarity among the accessions was 0.76. The accession *Samtse4* and *Dagana 2* showed high similarity values of 0.91 among 23 individuals. The accessions from Samtse, Sarpang and Trongsa formed a small group under subgroup 1 while *Sarpang 6* and Tsirang formed a small group.

UPGMA dendrogram drawn from AFLP data depicted complicated relationship among the mandarin accessions analyzed. In both major group A and B, accessions from Tsirang appeared in midway between clads within subgroups. In the major group A, *Tsirang 2* and *Tsirang 4* along with *Sarpang 6* formed a small clad within subgroup 1 and between the subgroup 2. Likewise, Tsirang 1 and Tsirang 3 formed a separate clad within major group but between accessions from Dagana.

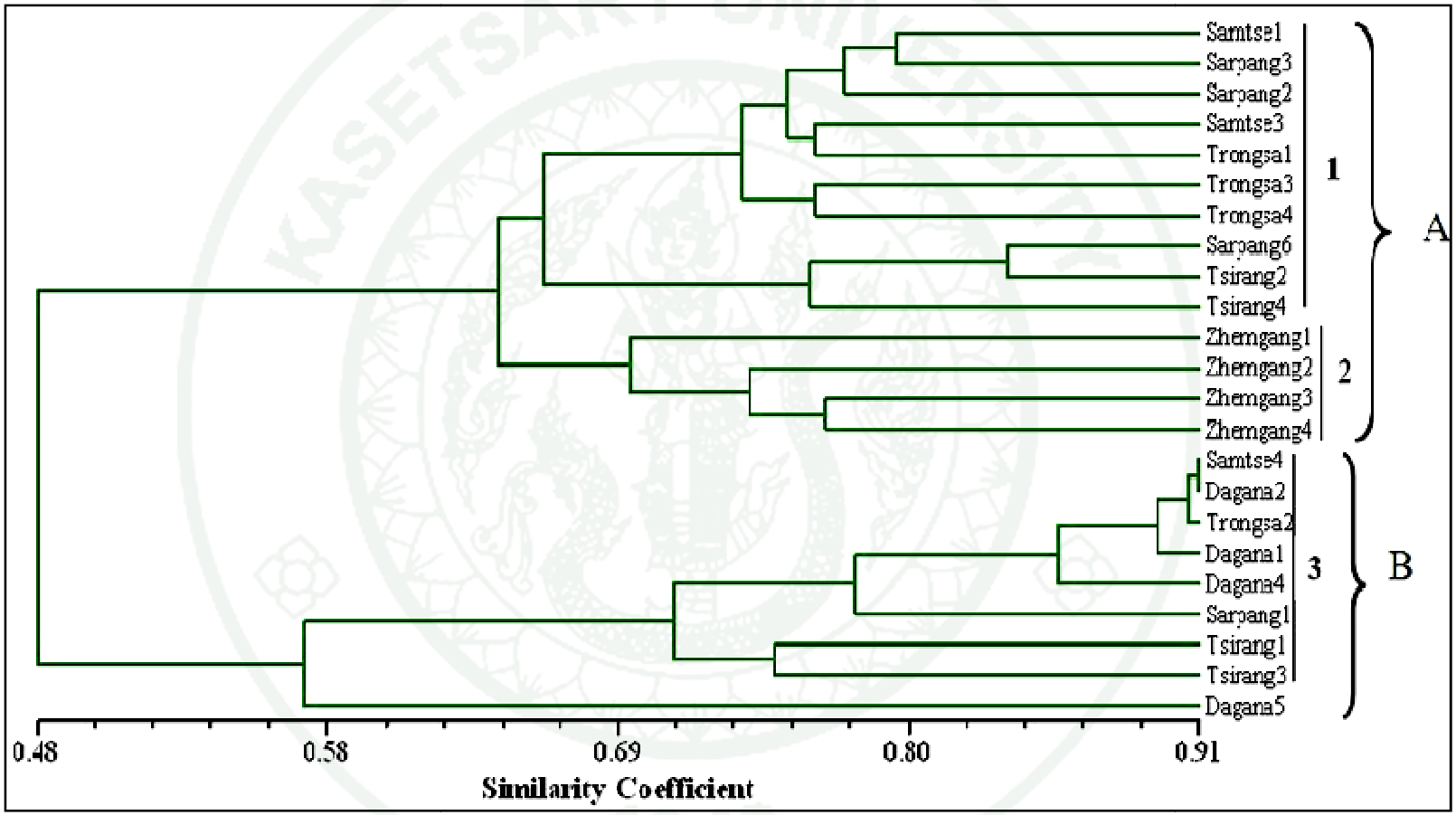

Figure 18 UPGMA dendrogram obtained from AFLP data for local mandarin accessions

The genetic diversity and relationship among 23 accessions showed that analyzed accessions constituted a diverse group. The highest similarity index of 0.54 was observed for the accessions in Zhemgang. Contrastingly, negative correlation was observed for the accessions between Sarpang and Tsirang (-0.42) and within accessions of Tsirang (-0.32). The similarity matrix of 23 accessions based on AFLP data is indicated in Table 10.

Table 10 Similarity Matrix of 23 field accessions by AFLP data

	Samtse1	Samtse3	Samtse 4	Sarpang1	Sarpang2	Sarpang3	Sarpang6	Tsirang1	Tsirang2	Tsirang3	Tsirang4	Dagana1	Dagana2	Dagana4	Dagana5
Samtse1	1.00														
Samtse3	0.45	1.00													
Samtse4	0.13	0.20	1.00												
Sarpang1	0.17	0.16	0.45	1.00											
Sarpang2	0.49	0.46	0.13	0.11	1.00										
Sarpang3	0.51	0.50	0.19	0.21	0.41	1.00									
Sarpang6	0.41	0.31	-0.12	0.04	0.39	0.35	1.00								
Tsirang1	-0.02	0.05	0.32	0.19	-0.05	-0.01	-0.42	1.00							
Tsirang2	0.35	0.25	-0.14	0.02	0.30	0.18	0.67	-0.33	1.00						
Tsirang3	0.07	0.14	0.27	0.24	0.09	0.12	-0.18	0.44	-0.16	1.00					
Tsirang4	0.22	0.22	0.22	0.16	0.21	0.26	0.33	-0.04	0.25	-0.01	1.00				
Dagana1	0.16	0.14	0.51	0.34	0.08	0.13	-0.01	0.34	-0.08	0.38	0.24	1.00			
Dagana2	0.15	0.09	0.59	0.31	0.10	0.13	-0.05	0.24	-0.04	0.32	0.24	0.60	1.00		
Dagana4	0.12	0.04	0.46	0.33	0.04	0.07	0.00	0.18	-0.01	0.17	0.17	0.47	0.43	1.00	
Dagana5	0.10	0.08	0.14	0.17	0.13	0.10	0.15	-0.05	0.17	0.05	0.19	0.11	0.09	0.21	1.00

Table 10 (Continued)

	Samtse1	Samtse3	Samtse4	Sarpang1	Sarpang2	Sarpang3	Sarpang6	Tsirang1	Tsirang2	Tsirang3	Tsirang4
Trongsa1	0.48	0.50	0.15	0.18	0.43	0.43	0.33	0.05	0.30	0.24	0.24
Trongsa2	0.16	0.18	0.56	0.34	0.11	0.14	-0.01	0.22	-0.05	0.22	0.25
Trongsa3	0.38	0.44	0.23	0.22	0.40	0.46	0.34	0.01	0.32	0.13	0.24
Trongsa4	0.36	0.34	0.08	0.11	0.33	0.42	0.30	0.05	0.26	0.10	0.22
Zhemgang1	0.24	0.35	-0.16	0.03	0.26	0.34	0.42	-0.19	0.34	-0.13	0.27
Zhemgang2	0.33	0.36	0.10	0.12	0.27	0.32	0.19	0.10	0.21	0.09	0.19
Zhemgang3	0.34	0.40	0.09	0.12	0.25	0.37	0.24	0.13	0.23	0.14	0.19
Zhemgang4	0.35	0.38	0.16	0.24	0.26	0.36	0.25	0.07	0.20	0.20	0.22

Table 10 (Continued)

	Dagana1	Dagana2	Dagana4	Dagana5	Trongsa1	Trongsa2	Trongsa3	Trongsa4	Zhem gang1	Zhem gang2	Zhem gang3	Zhem gang4
Trongsa1	0.12	0.12	0.10	0.10	1.00							
Trongsa2	0.61	0.58	0.52	0.14	0.18	1.00						
Trongsa3	0.05	0.09	0.17	0.19	0.49	0.01	1.00					
Trongsa4	0.09	0.00	0.15	0.12	0.47	0.09	0.48	1.00				
Zhemgang1	0.00	-0.10	-0.05	0.13	0.28	-0.10	0.26	0.42	1.00			
Zhemgang2	0.13	0.10	0.08	0.10	0.43	0.13	0.29	0.48	0.40	1.00		
Zhemgang3	0.17	0.10	0.13	0.18	0.40	0.11	0.36	0.46	0.38	0.51	1.00	
Zhemgang4	0.19	0.15	0.14	0.17	0.44	0.16	0.32	0.43	0.43	0.53	0.54	1.00

Discussion

Mandarin Accessions from Farmers' Field

The Sub-Himalayan range and South China is considered as one of the major centers of diversity for cultivated citrus (Ghosh, 1993; Sharma *et al.*, 2004; Das *et al*., 2005; Ladaniya, 2008a; Singh, 2010). The findings from this study support the claims of these authors. The variation in our study was not due to differences in environmental factors exclusively as it was evident from variation existing within accession from single orchards (Appendix Table A2). The existence of significant variation among ecotypes from different location was in contrast to pre-assumptions of single variety *local mandarin.*

Generally phenotypic variations give valuable clue to the underlying genetic variations however the two do not match always. The variation of phenotypic characters, especially quantitative characters differs greatly among populations rather than within the populations. Because of the plasticity and instability of phenotypic characters, the cultivar identification and diversity were often in contrast to actual genetic diversity.

Hence, previous authors (Yonemoto *et al.*, 2007; Struwig *et al.*, 2009) had used both morphological data and molecular analysis. In citrus, especially in mandarin, the study of morphological diversity was described as independent from its genetic diversity (Koehler-Santos *et al.*, 2003; Campos *et al.*, 2005; Kyndt *et al.*, 2010). Further, the horticultural characters are controlled by multiple genes (Campos *et al.*, 2005; Liu and Deng, 2007) which are of low heritability in citrus make morphological characterization an essential component of identification. The findings on morphological diversity among accession indicated the existence of genetic diversity. However, the overlapping nature of morphological characters observed in this study suggests that identification of Bhutanese mandarin through morphological description is not possible.

In general, the mandarins of north eastern Himalaya are of three different types: *Desi* (grown in state of Punjab and Himachal), *Khasi* (Khasi hills, Meghalaya), and *Sikkim* (Darjeeling and Sikkim) (Ladaniya, 2008c). Bhutan has varied climatic conditions with an altitude ranging from 100-7,000 m above msl. The climatic conditions vary even within a small district. The mandarin was cultivated in almost all low lying areas of the districts up to an elevation of 1,650 meters above MSL. The mandarin orchards in our survey areas were mostly on slope and poorly managed as mentioned earlier (National Plant Protection Center, 2006). The survey area comprised of small orchards and backyard farms. Our findings of survey also support the claims of Connellan *et al.* (2008) with majority of mandarin growers are backyard and small farms.

Seeds were used to raise seedlings and most of the orchards in study areas had mandarin tree either self raised or purchased seedlings. The lack of scientific know-how on nursery management in rouging off-types (zygotic seedlings) might be one of the causes to heterogeneity. Perhaps, the high variations within the same orchard and high level of diversity between different locations might be due to occurrence of spontaneous mutation and natural hybridization (Zerihun *et al.*, 2009).

The cold temperature treatment had shown to improve rind color development (Barry and van Wyk, 2006). The variation in fruit color at maturity in this study could be due to difference in temperature in different location. The common phenomenon of zygotic twins (Das *et al.*, 2007) in addition to zygotic seedlings in mandarin of Himalayan region might be one of the causes of variations. Thus hybridization and propagation through seeds could have constantly added to diversity and heterogeneous population of mandarin in this region. Also, the variation might be due to somatic mutations as reported (Moore, 2001; Altaf and Khan, 2008).

The quantitative characters were distinctive for fruits and leaves for most of the accessions. Leaf and fruit quantitative characters might be useful for identification although it is affected by environmental factors. However, both floral qualitative and quantitative characters might not be useful for future identification of mandarin in

Bhutan as there was not significant variation among accessions analyzed. Further, less variation in quantitative floral characters might be attributed sample size which in turn was caused by lack of proper equipments and facilities for long distance transportation during survey. The fragile and short shelf life of mandarin flowers aggravated to the study of floral characters. Indeed, the coincidence of flowering seasons for different districts was major constraints for inclusion of required sampling and study.

Horticulture Characteristics

Seedlessness in citrus is considered as desirable trait. One of the breeding objectives in citrus is to obtain the seedless variety (Liu and Deng, 2007; Jinping *et al.,* 2009). The chances of selecting seedless citrus varieties from existing seeded types are low (Fatima, 2004). On the other hand, fruit characters such as fruit shape, pulp color, seed number, bitterness were found to be unaffected by environment in *Citrus grandis* (Paudyal and Haq, 2008) Relatively lower number of seeds from accessions of Trongsa in this study may be of interest to researchers for further investigation. The selection of late and early ripening varieties is also one of the important desirable characters in citrus. The accessions at Samtse and Tsirang might be useful in extending the harvesting season.

Although, the effect of elevation and climatic factors to variation in TSS cannot be ruled out, the application of nitrogenous fertilizers (N) had been shown to contribute towards increase in TSS content, juice and total acidity (Zekri *et al.*, 2009). Earlier, studies by Thompson *et al.* (2005) and Kusakabe *et al.* (2006) found out that Nitrogen rates and fertigation did not significantly affect the fruit quality including TSS and total acidity. It is also known that N compound exists in fruit juice as amino acids (Ladaniya, 2008a). Since the mandarin accessions in all sampling areas were mostly grown in similar minimal management conditions without proper irrigation and fertilizer application, the variation in TSS could also be attributed to genetic variation among accessions.

The sugar ratio is considered as important parameters of citrus. The marketability of mandarin or citrus fruits is determined by this ratio. The range of preference of TSS and total acidity ratio in market for fresh consumption is 12-19 (Ladaniya, 2008b) while industrial acceptance level is above 8. The assessment of accessions from field showed that only accessions from Dagana and Sarpang were above 12. However, the ratio for accessions from other districts was above industrial accepted limit. However, in general, the sugar acid ratios for all accessions were above the industrially accepted limit. The variation in sugar acid ratio can be partly due to difference in time of maturity from place to place and inconsistency in assumption based on taste.

Germplasm Accessions

The germplasm accessions within a location showed variations for all leaves and fruits characters. This indicates that individual accessions in the germplasm are phenotypically different from each other. The difference within in individual accessions could be as a result of mutations and cross pollination due to high compatibility. Furthermore, occurrence of zygotic twins is common in Himalayan mandarin varieties (Das *et al*., 2007). This phenomenon might be one of the causes in addition to other evolutionary forces. In fact, the assessment of larger number of germplasm accessions in an identical condition would give a clearer picture of actual morphological diversity of mandarin in Bhutan.

Horticulture Characteristics

Fruit characters, such as yield, size and total acidity were found to be affected by environment up to 40% for pummelo while 60% was determined by genotype (Paudyal and Haq, 2008). A wide variation in morphological quantitative characters of fruits was observed for the accessions in the germplasm collection. The statistical analysis for quantitative characters revealed significant differences. The qualitative parameters with respect to tree shape were not distinct enough for identification as plants were small. Although the accessions were diverse and promising in term of

genetic resources, the accessions lacked information regarding the sources and description. The evaluation of germplasm accession in identical conditions further supported the existence on genetic diversity.

The accessions from Shumar, Sodrung and Trongsa possessed better yield parameters. Since evaluation was carried out for the fruits of third year fruiting, the performance is expected to change over the time as observed with age of tree. Therefore any basis on this result should be speculative. However, the emergence of Sodrung as better performer in yield was concurrent with the results evaluated at the RDC Wengkhar (unpublished). The TSS for the analyzed accessions varied significantly. The TSS of fruits is known to increase as fruit matures while total acidity remains constant. The decrease in total acidity is as a result of dilution of acid during increase in fruit size and increase in TSS content (Ladaniya, 2008b). The significant variation among the accessions for this ratio also supplement to existence of diversity.

Our study on morphological characters revealed the existence of wide diversity for the accessions in germplasm although accessions exhibited the similar phenotypic qualitative characters. Despite having lower variations in qualitative characters, a highly significant statistical difference was observed for quantitative characters. Thus, a significant variation in quantitative characters in same environment invariably suggests the existence of different genotypes.

AFLP Analysis

AFLP marker is an efficient tool in identification of closely related accessions for example clones and between cultivars. This study further reinforces the claim that AFLP maker is useful in identification of varieties and clones with high accuracy and reliability (Garcia *et al*., 2004). In the present study, five primer combinations were able to differentiate almost all the 23 accessions collected from field.

Mandarin (*Citrus reticulata* Blanco) is considered as highly a heterogeneous species among three true citrus (Campos *et al*., 2005). A study on the diversity of Himalayan citrus both through morphological and Random Amplified Polymorphic DNA (RAPD) analysis revealed the existence of huge diversity (Das *et al*., 2005). The high variations from morphological data and AFLP analysis for the accessions in this study from different locations conform to the views of above author.

The result from cluster tree analysis using AFLP data was consistent with earlier findings in assessment of mandarin in *Campo Citricola* experimental station germplasm (Campos *et al*., 2005). However, the major groups diverged at similarity coefficient of 0.48 in our result is in contrast to 0.41. Further, the accessions from some districts were more genetically similar to accession from other district e.g. *Samtse 2* and *Dagana 4*. Such similarity is expected due to the use of a common source for seedling. On the other hand, it would seem that the accessions from Tsirang might have resulted from out crossing or might have been driven by environmental factors to process of adaptations as stated by Paule (2010).

Except for the accessions at Zhemgang and Dagana, cluster tree analysis didn't show any affinity due to sources. The arrays of minor groups were difficult to interpret. Further, the study with extended sets of primers and adoption of co-dominant markers may solve the problems in future. Nevertheless, the capacity to discriminate 23 accessions by 5 primer combinations showed the potentiality of AFLP as an efficient discriminating tool for identification of mandarin varieties. The AFLP markers complemented morphological markers explicitly indicating diverse mandarin types in Bhutan that could be used for registration of new cultivars and certification.

CONCLUSION AND RECOMMENDATIONS

Conclusion

Although the citrus in Bhutan in general is considered as single variety, the differences in morphological characteristics have emerged over the years. The non-uniformity of fruit quality has a disadvantage in pricing export market. The wide morphological variation has held back the citrus improvement programs. This study was aimed to identify Bhutanese mandarin based on evaluation of morphological characteristics and AFLP analysis.

The entire study was conducted in two sets of experiment; the first set involved evaluation of morphological characteristics of 30 mandarin accessions from six major mandarin growing areas of Bhutan (Samtse, Sarpang, Tsirang, Dagana, Trongsa and Zhemgang). To further elucidate non environmental variations, 39 accessions of local mandarin germplasm collection were evaluated for morphological characteristics. The second experiment employed AFLP molecular technique to analyze the genetic variations among 23 accessions from the field.

The statistical analysis of quantitative data on morphological characters showed significant differences among the accessions. There was no discrete or identifiable difference observed in qualitative characters. Some qualitative characters such as trees, leaves and fruits were useful in construction of rough dichotomous keys. Overall, the variations in qualitative characters were overlapping and less useful for identification. The AFLP analysis elucidated and complemented morphological characters in establishing that the accessions were genetically different.

The findings of this study were summarized as follows:

1. Morphological Characteristics: all the accessions from different places and germplasm revealed variation in quantitative characters. Among the field accessions, accessions from Dagana was identified promising for yield and

physicochemical parameters while Shumar possessed superior fruit qualities from germplasm accessions

2. The AFLP marker analysis for 23 accessions from field discriminated 22 accessions. The clusters were independent of geographical proximity between the sources. Genetically different accessions were identified and recorded. It can be unarguably stated that mandarin (C*itrus reticulata* Blanco) in Bhutan consists of different types and refutes the assumption of single variety collectively known as "local variety".

The findings of this study were summarized as follows:

1. Morphological Characteristics: all the accessions from different places and germplasm revealed variation in quantitative characters. Among the accessions analyzed, accessions at Dagana were identified for promising features recorded for yield and physicochemical parameters. Among the germplasm accessions evaluated, the accessions from *Shumar* were identified superior followed by Trongsa, Sodrung, and Kengkhar. In contrast, the morphological qualitative characters were unable to identify the accessions of mandarin in Bhutan.

2. The AFLP marker analysis for 23 accessions from field discriminated 22 accessions. The clusters were independent of geographical proximity between the sources. Genetically different accessions were recorded. Also different level of discrimination power was observed for each primer combination. The primer pair (E-ACA+M-CAG) was the best combination that produced highest number of bands in AFLP analysis of Bhutanese mandarin. It can be unarguably stated that mandarin (C*itrus reticulata* Blanco) in Bhutan consists of different types which was against assumption of single variety collectively known as "local variety". In fact, AFLP technique was found very useful in identification of morphologically similar Bhutanese mandarin.

Recommendations

1. The morphological evaluation in this study was conducted for one season for all qualitative and quantitative characters. Therefore, for consistency, further morphological evaluation on accessions from Dagana and Shumar for few more years is strongly suggested. Further survey and the evaluation of morphological characteristics of mandarins were found necessary. In addition, molecular analyses need to be carried out for all accessions clarification of actual genetic diversity.

2. The accessions collected from different locations were distinct and different genetically, further work on registration of these varieties ought to be continued upon confirmation of yield performance. Similarly, the accessions from Zhemgang appeared uniform, further work on morphological evaluation is strongly recommended.

3. Due to high and varying level of polysaccharide content in the leaves of citrus, the contamination with colloidal hyalosome which is neither soluble in water nor in TE buffer appeared as major challenge in extraction of quality DNA required for AFLP technique. At the same time, phenol extraction step was necessary in elimination of polyphenols and other essential oils. Therefore, the inclusion of additional step for polysaccharide elimination is found necessity.

4. The correct choice of primer pairs result in minimizing the costs and time required. The screening of 10 different primer combinations showed that E-ACA+M-CAG were the best primer combination for Bhutanese mandarin producing highest numbers of total bands. Thus, the use of E-ACA+M-CAG primer combination is recommended for AFLP study in future.

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APPENDICES

Appendix A

Analysis of Variance

Appendix Table A1 ANOVA for field accessions

		Sum of Squares	df	Mean Square	F	Sig.
FW	Between Groups	105325.543	5	21065.109	74.296	0.000
	Within Groups	125604.241	443	283.531		
	Total	230929.784	448			
FD	Between Groups	6027.149	5	1205.43	68.598	0.000
	Within Groups	7784.507	443	17.572		
	Total	13811.656	448			
FL	Between Groups	4214.858	5	842.972	57.257	0.000
	Within Groups	6522.103	443	14.723		
	Total	10736.961	448			
WE	Between Groups	1.744	5	0.349	3.597	0.003
	Within Groups	42.948	443	0.097		
	Total	44.692	448			
NS	Between Groups	7050.008	5	1410.002	107.417	0.000
	Within Groups	5801.882	442	13.126		
	Total	12851.891	447			
TSS	Between Groups	354.665	5	70.933	63.589	0.000
	Within Groups	493.045	442	1.115		
	Total	847.71	447			
Total Acidity	Between Groups	14.693	5	2.939	15.001	0.000
	Within Groups	28.012	143	0.196		
	Total	42.705	148			
Sugar acid ratio	Between Groups	808.297	5	161.659	10.708	0.000
	Within Groups	2158.952	143	15.098		
	Total	2967.248	148			
LL	Between Groups	19242.355	5	3848.471	22.113	0.000
	Within Groups	77272.738	444	174.038		
	Total	96515.093	449			

Appendix Table A1 (Continued)

		Sum of Squares	df	Mean Square	F	Sig.
LW	Between Groups	3278.421	5	655.684	19.672	0.000
	Within Groups	14799.211	444	33.332		
	Total	18077.632	449			
RLW	Between Groups	0.478	5	0.096	2.054	0.070
	Within Groups	20.679	444	0.047		
	Total	21.158	449			
PL	Between Groups	3.243	5	0.649	1.437	0.215
	Within Groups	65.002	144	0.451		
	Total	68.245	149			
PW	Between Groups	0.324	5	0.065	0.783	0.564
	Within Groups	11.931	144	0.083		
	Total	12.255	149			
NoS	Between Groups	3.422	5	0.684	0.257	0.936
	Within Groups	994.565	374	2.659		
	Total	997.987	379			

Appendix Table A2 ANOVA for accessions of Samtse

		Sum of Squares	df	Mean Square	F	Sig.
FW	Between Groups	2090.18	4	522.54	2.998	0.024
	Within Groups	12199.14	70	174.27		
	Total	14289.32	74			
FL	Between Groups	78.24	4	19.56	2.657	0.040
	Within Groups	515.31	70	7.36		
	Total	593.55	74			
WE	Between Groups	0.79	4	0.20	4.060	0.005
	Within Groups	3.38	70	0.05		
	Total	4.17	74			
TSS	Between Groups	23.40	4	5.85	15.755	0.000
	Within Groups	25.99	70	0.37		
	Total	49.39	74			
Sugar acid ratio	Between Groups	18.83	4	4.71	4.377	0.011
	Within Groups	21.51	20	1.08		
	Total	40.34	24			
LL	Between Groups	10778.67	4	2694.67	6.394	0.000
	Within Groups	29500.56	70	421.44		
	Total	40279.23	74			
LW	Between Groups	528.57	4	132.14	5.959	0.000
	Within Groups	1552.22	70	22.18		
	Total	2080.79	74			

Appendix Table A3 ANOVA for germplasm accessions

		Sum of Squares	df	Mean Square	F	Sig.
Tree	Between Groups	26805.577	9	2978.397	24.425	0.000
	Within Groups	46337.500	380	121.941		
	Total	73143.077	389			
FW	Between Groups	48012.960	9	5334.773	14.031	0.000
	Within Groups	144482.258	380	380.216		
	Total	192495.218	389			
FD	Between Groups	2484.093	9	276.010	13.377	0.000
	Within Groups	7840.390	380	20.633		
	Total	10324.483	389			
FL	Between Groups	3806.066	9	422.896	28.445	0.000
	Within Groups	5649.482	380	14.867		
	Total	9455.548	389			
WE	Between Groups	18.243	9	2.027	15.313	0.000
	Within Groups	50.299	380	0.132		
	Total	68.541	389			
NS	Between Groups	618.231	9	68.692	5.812	0.000
	Within Groups	4491.400	380	11.819		
	Total	5109.631	389			

Appendix Table A3 (Continued)

		Sum of Squares	df	Mean Square	F	Sig.
TSS	Between Groups	45.866	9	5.096	8.824	0.000
	Within Groups	219.474	380	0.578		
	Total	265.340	389			
Total acidity	Between Groups	10.110	9	1.123	9.418	0.000
	Within Groups	21.826	183	0.119		
	Total	31.935	192			
Sugar acid ratio	Between Groups	1822.972	9	202.552	9.202	0.000
	Within Groups	4028.132	183	22.012		
	Total	5851.104	192			
LL	Between Groups	30945.242	9	3438.360	12.408	0.000
	Within Groups	104748.837	378	277.113		
	Total	135694.079	387			
LW	Between Groups	2493.143	9	277.016	6.380	0.000
	Within Groups	16411.668	378	43.417		
	Total	18904.811	387			
RLW	Between Groups	13.743	9	1.527	19.538	0.000
	Within Groups	29.464	377	0.078		
	Total	43.207	386			

Appendix Table A3 (Continued)

		Sum of Squares	df	Mean Square	F	Sig.
PL	Between Groups	4.276	9	0.475	0.863	0.56
	Within Groups	101.876	185	0.551		
	Total	106.152	194			
PW	Between Groups	2.916	9	0.324	3.983	0
	Within Groups	15.051	185	0.081		
	Total	17.967	194			
NoS	Between Groups	36.155	9	4.017	1.979	0.044
	Within Groups	375.517	185	2.03		
	Total	411.672	194			
PdL	Between Groups	3.692	9	0.41	3.148	0.001
	Within Groups	24.105	185	0.13		
	Total	27.796	194			

Appendix B

Morphological Characteristics

Appendix Table B1 Tree Growth Habits

	Characters	**Variables**			
1	*Shape*	Erect	Spreading	Drooping	Others(specify)
2	*Density of branch*	Sparse	medium	Dense	
3	*Branch angle*	Narrow	Medium	wide	
4	*Spine density on adult*	Absent	Low	Medium	High
5	*Shoot tip color*	Green	purple	Other	
6	*Shoot tip surface*	glabrous	intermediate	pubescent	

Appendix Table B2 Leaf Characteristics

Characters		**Variables**				
1	*Leaf apex shape*					
2	*Petiole wing shape*					
3	*Leaf lamina shape*	Elliptic	Ovate	Obovate	Lanceolate	Obcordate
4	*Leaf margin*	Crenate	Dentate	Entire	Sinuate	
5	*Leaf division*	Simple	Bifoliate	Trifoliate	Pentafoliate	
6	*Color intensity*	Light green	Medium	Dark green		
7	*Leaf lamina attachment*	Sessile	Brevi petiolate	Longi petiolate		
8	*Leaf length*					
9	*Leaf width*					
10	*Ratio of leaf length to width*					

Appendix Table B3 Flower Characteristics

	Characters	**Variables**			
1	*Length of anthers relative to stigma*		Shorter	Medium	Longer
2	*Flower type*	Hermophodrite	Male	Female	Others
3	*Colour of open flower*	White	Light yellow	Yellow	Purple
4	*Colour of anthers*	White	Light yellow	Yellow	
5	*Number of petals*				
6	*Petal length*				
7	*Petal width*				
8	*Number of stamens*				
9	*Flowering month*				
10	*Pedicel length*				

Appendix Table B4 Fruit Characteristics

Characters		Variables					
1	*Fruit weight (gm)*						
2	*Fruit dia*						
3	*Fruit length*						
4	*TSS (°Brix)*						
5	*No. of seeds*						
6	*Total acidity (%)*						
7	*Width of epicarp at equatorial region*						
8	*Fruit shape:*	spheroid	Ellipsoid	Pyriform	Oblique	Obloid	Obvoid
9	*Fruit base shape:*	Necked	Convex	Truncate	Concave	Concave collard	Collard
10	*Fruit apex shape:*	Mammiform	Acute	Rounded	Truncate	Depresed	Other
11	*Fruit skin color*						
12	*Adherence of albedo*	Weak	Medium	Strong			
13	*Surface texture*	Smooth	Rough	Papillate	Pitted	Bumpy	Grooved
14	*Seed shape*	Fusiform	Clavate	Cuneiform	Semi Deltoid	Spheroid	Semi spheroid
15	*Seed surface*	Smooth	Wrinkled	Hairy	other		
16	*Seed color*	White	Cream	Yellowish	Green	Brown	

Appendix C

Chemicals for DNA Extraction

Appendix Table C1 DNA Extraction: Chemical Components and their effects

Components	**Chemical**	**Nature**	**Effect**
pH			Inhibition of degradative enzymes (eg. DNases act at pH 7)
Tris		Buffer	Maintains pH
EDTA	Ethylenediamine-tetraacetate		Chelation of divalent cations (Ca^{2+}, Mg^{2+}, etc)
Na or K		Salt	Stabilization of nucleic acid
Proteinase K		Enzyme	Digestion of proteins
SDS	Sodium Dodecyl Sulfate	Anionic detergent	Solubilization of cellular membranes
CTAB	Hexadecyltrimethyl ammoniumbromide	Cationic detergent	Solubilization OF membranes, denaturation of proteins, formation of complex with DNA
CIA	Cholorform-Isoamylalcohol		Protein extraction
Isopropanol			Precipitation of CTAB-DNA complex
B-mercaptoethanol and $Na_2S_2O_5$		Reducing agent (antioxidant)	Inhibition of oxidation process
PVP	Polyvinyl pyrrolidone	Polyphenol absorbent	Decrease the effect of polyphenols

Appendix Table C2 DNA Extraction Buffer

Chemicals	Final concentration	Stock solution	For 500 ml Buffer
NaCl	1.4M	5M	140 ml
Tris-HCL pH 8.0	100mM	1M	50 ml
EDTA pH 8.0	20mM	0.5M	20 ml
CTAB	2%		10.0g
$Na_2S_2O_5$	1%		5.0g

Appendix Table C3 CIA (Chloroform: IsoamylAlcohol)

Chemicals	Final concentration	For 250 ml CIA
Chloroform	96%	240 ml
Isoamylalcohol	4%	10 ml

Stored solution at 4°C

Appendix Table C4 TE-Buffer (10/1)

	Final concentration	Stock solution	For 250ml of TE
Tris-HCl pH 8.0	10 mM	1 M	1.0 ml
EDTA pH 8.0	1 mM	0.5 M	0.2 ml

Appendix D

Chemicals for AFLP Analysis

Appendix D1 Chemicals for Restriction and Ligation

Sl. No.	Chemicals	Quantity
1	5X reaction buffer	5 µl per sample
2	EcoRI/MSeI (Restriction mix)	2 µl per sample
3	Distilled water	13 µl per sample
4	T4 DNA Ligase	1 µl per sample

Appendix D2 Chemicals for Pre-Amplification Reactions

Sl. No.	**Chemicals**	**Quantity**
1	Preamplification primer mix	40 µl per sample
2	10X PCR Buffer plus Mg	5 µl per sample
3	Tag DNA polymerase	0.1 per sample

Appendix D3 Chemicals for Selective Amplification

Sl. No.	**Chemicals**	**Quantity**
1	EcoRI primer	50 per sample
2	MseI primer	600 per sample
3	Distilled water	9.7 per sample
4	10X PCR Buffer plus Mg	2 per sample
5	Tag polymerase	0.1 per sample

Appendix D4 Chemicals for Polyacrylamide Gel Preparation

Sl. No.	Chemicals	Quantity
1	Polyacrylamide 40% (W/V)	28 ml
2	Bis-acrylamide 2% (W/V)	30 ml
3	Urea	84 g
4	10X TBE	20 ml

Appendix D5 Chemical for Glass Preparation

Sl. No.	Chemicals	Quantity
1	Bis-polyacrylamide solution	20 ml per glass
2	Temmed	12.8 µl per glass
3	APS	85 µl per glass

Appendix D6 Chemical for DNA and Dye Mixing

Sl. No.	Chemicals	Quantity
1	Formamide	9.8 µl per sample
2	Dye	0.2 µl per sample
3	0.5 M EDTA	0.2 µl per sample

Appendix D7 Chemicals for Preparation of Fixer

Sl. No.	Chemicals	Quantity
1	95% Ethanol	105 ml
2	Glacial acetic acid	5 ml
3	Distilled Water	890 ml

Appendix D8 Chemical for Staining Solution

Sl. No.	Chemicals	Quantity
1	Silver Nitrate	1.2 g
2	37% formaldehyde	1.2 ml
3	Distilled Water	800 ml

Appendix D9 Chemicals for Developer

Sl. No.	Chemicals	Quantity
1	Sodium Hydroxide	12 g
2	37% Formaldehyde	1.2 ml
3	Distilled water	800 ml

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