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Morphological Identification of Mandarin (*Citrus reticulata* Blanco) in Bhutan

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ABSTRACT

This study aimed to identify Bhutanese mandarin accessions using morphological characteristics. A total of 30 accessions were selected from six districts in the major mandarin-growing regions in Bhutan. Samples of 15 leaves, 5 flowers and 15 fruit were collected randomly from each accession. A total of 23 characters were evaluated from the trees, leaves, flowers and fruit from each plant. A dichotomous key constructed from the morphological qualitative characters was able to classify the accessions to the district level. There were statistically highly significant differences ($P < 0.01$) for leaf length, leaf width, fruit weight, fruit diameter, fruit length, epicarp width, number of seeds and total soluble solids (TSS). The floral morphology showed little variation among trees and districts. The accessions from Dagana had the highest fruit weight with 107.2 g followed by Samtse, Zhemgang, Sarpang, Trongsa and Tsirang with fruit weights of 96.1, 93.3, 87.6, 78.9 and 58.3 g, respectively. The highest TSS were found in the accessions from Trongsa (12.9 °Brix) followed by Zhemgang (12.1 °Brix), Dagana (11.2 °Brix), Sarpang (10.9 °Brix), Tsirang (10.6 °Brix) and Samtse (10.3 °Brix). Accessions from Samtse were early maturing (mid October) while accessions from Tsirang were late maturing (February–March).

Keywords: Bhutan, mandarin, morphological diversity, qualitative and quantitative characters

INTRODUCTION

Mandarin (*Citrus reticulata* Blanco) is the highest value export fruit crop of Bhutan that is distributed in 17 out of 20 districts and commercially grown in the southern parts of the country. More than 60% of the rural population is currently involved in mandarin cultivation (Connellan *et al*., 2008), predominantly within an altitude range of 300–1,650 m above mean sea level (asl). The annual production is over 72,000 t covering an area of approximately 4,500 ha (Department of Agriculture, 2008). Over 60% of the total annual production is exported to the neighboring countries of Bangladesh and India, mainly as fresh fruit (Dorjee *et al*., 2007). The crop has not only contributed substantially to income generation for the subsistent farming economy but also to government as a whole through the employment it has generated.

Bhutan is located in the eastern Himalayan range which has a huge diversity of cultivated mandarin trees (Sharma *et al*., 2004; Das *et al*., 2005; Singh, 2010). There is strong

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anecdotal evidence that mandarin has been has been cultivated in Bhutan from very early times, though there is no authentic record. Despite its long history of cultivation and significant contribution towards the subsistence farming economy, practice has remained more or less at the subsistence level with about 70% of mandarin growers owning less than 120 trees (Dorjee *et al*., 2007). At present, mandarin in Bhutan is collectively known as 'local mandarin' (*Citrus reticulata* Blanco.) which is considered as a single variety (NPPC, 2006; Dorjee *et al*., 2007). Despite having a reputation of excellent quality generally in the Himalayan region (Ghosh, 1993; Das *et al*., 2005), Bhutanese mandarin has fetched relatively low prices (USD 0.2–0.3 kg^{-1}) in export markets (Dorjee *et al*., 2007).

Natural hybridization and the occurrence of spontaneous mutations are very common in *Citrus* spp. Cross pollination and the reported high percentage of zygotic twins (Das *et al*., 2007) has not only resulted in greater variation in the plant types but also a lack of uniformity in fruit quality. The identification of superior accessions and asexual propagation could be a solution to the lack of uniformly high fruit quality, while essential characters of variability need to be retained for the future improvement of the citrus industry in Bhutan.

Morphological study is an essential component for the assessment of diversity and classification. Morphological description was widely used to study diversity and classification until the 1970s. At present, morphological study is still considered and has been deployed as an initial step for cultivar identification and diversity assessment with watermelon (Huh *et al*., 2008), sweet potato (Elameen *et al*., 2010) and agave (RodrÌguez-Garay *et al*., 2009). Since 1980, the isozymes technique has been used to evaluate diversity and genetic relatedness in *Citrus* spp. (Ashari *et al*., 1989; Elisirio *et al*., 1999; Rahman *et al*., 2001). More recently, new molecular techniques, such as RAPD, RFLP (Abkenar *et al*., 2004; Jena *et al*., 2009), AFLP (Campos *et al*., 2005; JinPing *et al*., 2009) and microsatellite markers (Koehler *et al*., 2003; Ghanbari *et al*., 2009) have been used to identify *Citrus* spp. with high accuracy. Also, in citrus it was found that most of the desired horticultural traits are controlled by quantitative trait loci (QTLs; Liu and Deng 2007). Therefore, this current survey was conducted to study the morphological diversity of mandarin in the major growing areas of Bhutan.

MATERIALS AND METHODS

Survey coverage

The survey was conducted during the flowering and fruit maturing season. Sampling of fruits and leaves started in October 2009 and finished in February 2010 while the study of floral characteristics was undertaken from February to May 2010. At each site, the topography, slope and elevation were recorded. Information on the age of each tree was obtained through interviews with local residents.

The survey in the major citrus-growing region of Bhutan covered six districts—namely, Samtse, Sarbang, Tsirang, Dagana, Trongsa and Zhemgang (Figure 1). Sampling for the phenotypic study was carried out in two rounds of surveys depending on the phenological stage from October 2009 to April 2010. The flower samples were collected during the peak blooming stage (late February to early April 2010) while fruits were sampled in late October until March, 2009 in the previous season.

Plant material

Two or three orchards were surveyed in each district and one to three representative mandarin trees were selected from each orchard. Sampling of 15 leaves, 5 flowers and 15 matured fruit was carried out randomly from each selected tree. The samples were packed, labeled and brought to the laboratory for measurement and data collection.

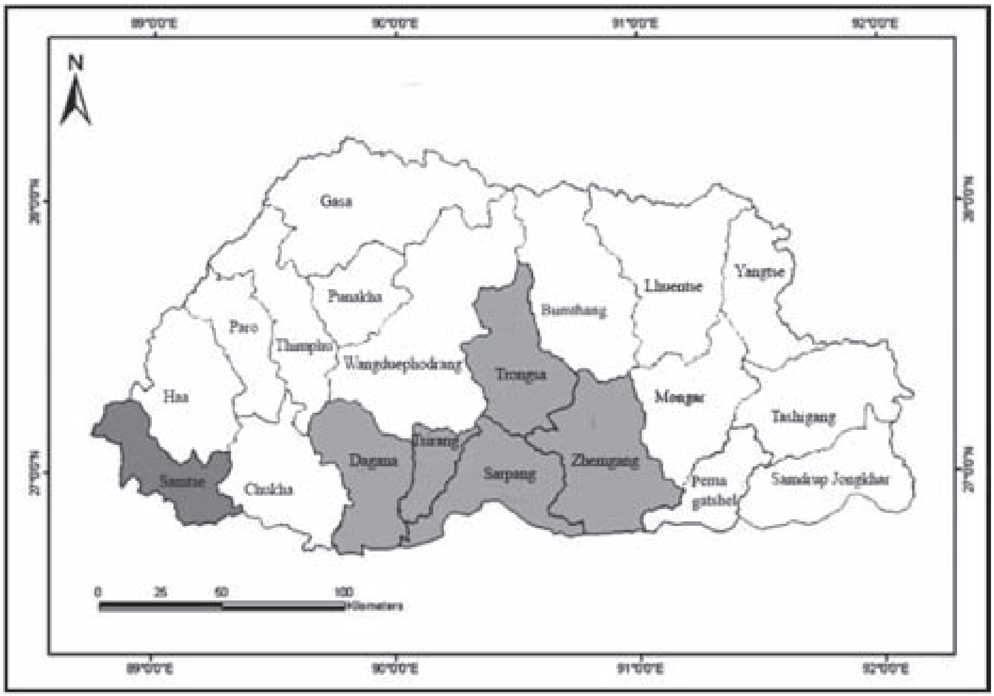

Figure 1 Location of the six survey districts (Samtse, Sarpang, Tsirang, Dagana, Trongsa and Zhemgang) surveyed for mandarin in Bhutan.

Data collection

Data for each accession was recorded according to the International Plant Genetic Resource Institute descriptors for citrus (IPGRI, 1999). The tree characteristics recorded were: tree shape, branch density, and branch angle, shoot tip color and shoot tip surface. The quantitative fruit characteristics were: fruit weight (g), fruit diameter (mm), fruit length (mm), number of seeds per fruit, width of epicarp at equatorial region (mm) and total soluble solids (TSS; °Brix). The qualitative fruit characteristics evaluated were: fruit shape, fruit base shape, fruit apex shape, fruit surface texture, skin color, seed color and seed surface. Leaf characteristics consisted of: leaf length (mm), leaf width (mm), ratio of leaf length to width, leaf division, leaf apex shape, leaf lamina shape, leaf margin, leaf color intensity and petiole wing shape. The flowers were also evaluated for: the number of petals, petal length (mm), petal width (mm), number of stamens, pedicel length (mm), relative length of stamens to stigma, color of anthers, flower type and the month of flowering.

The typology of mandarin orchards differed on the number of trees per mandarin grower. The survey covered mostly backyard orchards and small orchards except for the addition of one medium-sized orchard from Samtse. The survey area comprised varied agro-ecological conditions with the altitude ranging from 390 to 1250 m asl. Tree age ranged from 16 to more than 50 yr. The planting material was seedlings either from a local nursery or self-propagated as is commonly practiced in this region. However, an orchard in Trongsa had acquired seedlings from the only corporate seed agency in the country (Druk Seed Corporation). Details of tree age, source of seedlings, slope, number of accessions and type of orchard are shown in Table 1.

Table 1 Survey data of mandarin orchards in six districts of Bhutan.

Districts	No. orchards	No. accessions	Tree age (yr)	Source of seedlings propagated	Slope (%)	Orchard Type
Samtse	2	5	Over 50	Self/nursery	30	Small farm[1]
Sarpang	3	6	26	Self/nursery	16	Small farm
Tsirang	2	5	45	Self/nursery	11	Backyard farm[2]
Dagana	3	5	30–40	Self/nursery	15	Small farm
Trongsa	2	5	16	Druk Seed Corporation	21	Backyard farm
Zhemgang	2	4	30–40	Self/nursery	25	Small farm

[1] = Orchard with 51–120 mandarin trees; [2] = Orchard with less than 50 mandarin trees.

Data analysis

The data on the quantitative variables from the leaves, flowers and fruit of each accession were statistically analyzed with version 17.0 of the SPSS program (SPSS is now part of IBM, White Plains, NY, USA). The mean for each characteristic was calculated and groups were compared using one way ANOVA for descriptive analysis at the 99% confidence level using Duncan's multiple range test. The distinct characters from the trees, leaves and fruit were screened. The identifiable phenotypic characters (tree canopy shape, branch angle, branch density, leaf color intensity, color of fruit at maturity, fruit surface and fruit shape) were used for the construction of a dichotomous key for identification. The information from the characters was used for classification and description of accessions specific to each location.

RESULTS

Morphological description

The morphology based on qualitative characters from the six districts indicated the existence of different mandarin types. High variation was found in tree, leaf and fruit characters. The accessions from Tsirang had distinct fruit whose shape was depressed only at the top and was smaller. A difference in fruit shape was also observed on a single tree (Samtse had fruits with both rough and smooth surface texture). Similarly, necked and truncated fruit base shapes were observed on almost all the trees. There was no difference in floral characters among accessions both within and between locations. Orchards in Samtse were at the lowest elevation followed by Sarpang. The mandarin trees from Samtse were mostly open and erect in shape; the branch angle of these trees was narrow (less than 30°). The fruits were greenish-orange at maturity with usually smooth fruit surface texture. The fruits were seedy. The fruit matured relatively earlier compared to the other study sites. The three common growth habits of Bhutanese mandarin trees along with fruit are shown in Figure 2.

The trees from Sarpang were spread out with a wide branch angle. The branch density was sparse with a light green leaf color at fruit maturity. The branch angle was narrow with mostly obovate leaf lamina. The fruit were medium sized and at maturity were yellowish orange. The trees from Tsirang were also open and erect in shape. The branch angle was narrow (less than 30°). The fruits were in the medium-sized category and were oblate-obloid, depressed at the top and truncated at the base. The matured fruits were orange in color.

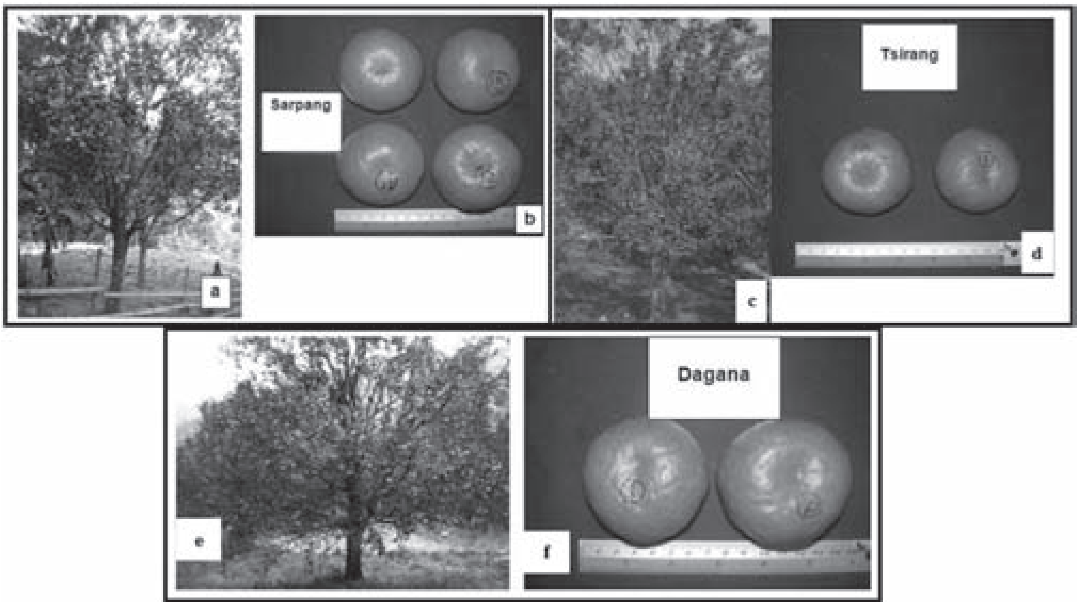

Figure 2 Variability of tree growth habits and fruits of Bhutanese mandarin: (a)Sarpang tree; (b) Sarpang fruit; (c) Tsirang tree; (d) Tsirang fruit; (e) Dagana tree; (f) Dagana fruit.

The accessions from Dagana were spreading to drooping in shape. The branch angle of the tree was wide (more than 30°) and trees had a high branch density. The leaves were dark green. The fruits were oblate, flattened at the top and the base. The fruits of this region were the largest among those surveyed. The trees from Trongsa were spread in a dome-like shape with wide angled branches and a high branch density. The leaves were a larger size and light green in color at fruit maturity. The color of fruit was orange at maturity and the fruit size was medium to large with very few seeds. The fruit trees from Zhemgang were also open and erect in shape with a narrow branch angle. The rind color at fruit maturity was orange and the fruit was oblate and depressed at both ends. The fruit size was large.

The dichotomous keys based on the six morphological qualitative variables of the trees, leaves and fruits were able to classify mandarin from different locations. Based on tree shape, accessions were divided into two types. Similarly, the branch angle and branch density differed for the accessions from different locations. Classification using the dichotomous key is shown in Figure 3.

Traits measured as quantitative variables

The statistical analysis of quantitative characters showed highly significant variations among the leaves, flowers and fruits from different locations. The quantitative character with respect to the number of stamens was not statistically significant. The accessions from Trongsa had the largest leaves; accessions from Dagana had the smallest leaves (Table 2) but the heaviest fruits, while Tsirang had the lowest fruit weight (Table 3). Fruit size was correlated to fruit weight, with Dagana having the largest fruit diameter in contrast to accessions from Tsirang that had the smallest fruit diameter (Table 3). The TSS content varied highly among the locations. The accessions from Trongsa had the highest TSS (Table 3) with a range of 10–18.4% Brix. The TSS content was lowest for the accessions from Samtse (Table 3). The variation in quantitative characters such as the ratio

Dichotomous key to mandarin types in Bhutan

1. The tree canopy is open and erect in shape.(Go to 2)
1. The tree canopy is spreading dome in shape.(Go to 4)

2. The fruits mature before rind develops orange color. Samtse
2. The fruits mature only after the rinds develop orange in color. (Go to 3)

3. The shape of the fruit is oblate depressed both at top and base. Zhemgang
3. The fruit was usually truncate at the base and depressed only at the top. Tsirang

4. Branch density of the tree is sparse. Sarpang
4. Branch density of the tree is dense. (Go to 5)

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

Figure 3 Dichotomous key constructed using the morphological qualitative characters of the tree, leaf and fruit.

Table 2 Mean values for leaf and floral characters of Bhutanese mandarin.

Districts	Leaf character			Flower character		
	Length (mm)	Width (mm)	Length/ Width	Petal length (mm)	Petal width (mm)	No. stamens
Samtse	77.8^{a}	32.2^{a}	2.4	11.3	4.7	14.6
Sarpang	89.7^{cd}	38.0^{cd}	2.3	11.2	4.8	14.8
Tsirang	86.2^{bc}	35.8^{bc}	2.4	10.9	4.8	14.9
Dagana	76.4^{a}	32.2^{a}	2.4	10.9	4.7	14.9
Trongsa	94.8^{d}	38.8^{d}	2.4	11.2	4.8	14.7
Zhemgang	81.8^{ab}	33.5^{ab}	2.5	11.3	4.8	14.7
P values	0.000	0.000	0.070	0.215	0.564	0.936

Values with different lower case letters within a column are highly significantly different at the 0.01 confidence level.

Table 3 Mean values for fruit characters of Bhutanese mandarin.

District	Fruit weight (g)	Fruit diameter (mm)	Fruit length (mm)	Epicarp width (mm)	No. seeds	TSS (%Brix)
Samtse	96.1^{d}	58.6^{c}	50.8^{c}	2.1^{ab}	16.6^{e}	10.4^{a}
Sarpang	87.6^{c}	57.4^{c}	49.0^{b}	2.1^{ab}	13.3^{d}	11.0^{bc}
Tsirang	58.3^{a}	49.8^{a}	44.2^{a}	2.0^{a}	10.1^{c}	10.6^{ab}
Dagana	107.2^{d}	61.7^{d}	53.9^{d}	2.2^{b}	10.5^{c}	11.3^{c}
Trongsa	78.9^{b}	55.5^{b}	47.6^{b}	2.2^{b}	4.4^{a}	12.9^{d}
Zhemgang	93.3^{cd}	58.4^{c}	51.5^{c}	2.2^{b}	6.9^{b}	12.1^{d}
P values	0.000	0.000	0.000	0.003	0.000	0.000

Values with different lower case letters within a column are highly significantly different at the 0.01 confidence level.

of leaf length to leaf width, number of petals, petal length, petal width and also the number of stamens were not statistically significant. The mean of the quantitative characters and the levels of significance with Duncan's multiple range test are shown in Table 2.

DISCUSSION

The sub-Himalayan range and South China are considered as major centers of diversity for cultivated mandarin (Ghosh, 1993; Sharma *et al*., 2004; Das *et al*., 2005; Ladaniya, 2008a; Singh, 2010). The variation in the current study might not have been due exclusively to differences in environmental factors as huge variation among the trees within a district have been reported for Bhutanese mandarin (Dorji, 2011). The existence of significant variation among ecotypes from different locations was in contrast to previous assumptions of a single variety "local mandarin".

Bhutan has various climatic conditions with the altitude ranging from 100 to 7,000 m asl. Climatic conditions can vary even within a small district. Due to the wide adaptability of mandarin compared to other species of citrus (Ghanbari *et al*., 2009), it is cultivated in almost all low lying areas of the districts up to an elevation of 1,650 m asl in Bhutan. The mandarin orchards in the areas surveyed were mostly on slopes and poorly managed as was observed more generally for mandarin orchards (Dorjee *et al*., 2007). The survey area comprised small orchards and backyard farms.

Seeds are used to grow seedlings in Bhutan. This practice could be the cause of heterogeneity. Perhaps the high variation within the districts and the high level of diversity between different locations might be due to the occurrence of spontaneous mutations and natural hybridization (Zerihun *et al*., 2009). Also the variation in fruit color at maturity could be due to differences in temperature at different locations as cold temperature treatment has been shown to improve rind color development (Barry and van Wyk, 2006). The common phenomenon of zygotic twins (Das *et al*., 2007), in addition to the zygotic seedlings in mandarin from this region, might be one of the causes of variation. Thus hybridization and propagation through seed have constantly added to the diversity and heterogeneity of the mandarin tree population in this region. Also, the variation might be due to somatic mutations that have been reported to be more common in citrus (Moore, 2001; Altaf and Khan, 2008).

Some characters of trees, leaves and fruit may be useful for the identification of mandarin in Bhutan. A total of six qualitative variables (tree shape, branch density, leaf color intensity, fruit shape, fruit color and fruit surface texture) were used to classify the mandarin of Bhutan into seven groups corresponding to their geographical origin. Because the fruit and leaf characters measured quantitatively were statistically highly significantly different, they might be useful for morphological identification although there are also effects from environmental factors. However, both the qualitative and quantitative measurement of flowers might not be useful for future identification, as no significant variations were found among the accessions.

Seedlessness is one of the breeding objectives in citrus (Liu and Deng, 2007; JinPing *et al*., 2009). The chance of selecting seedless citrus varieties from existing seeded types is low (Fatima, 2004). However, relatively low seed numbers in accessions from Trongsa may be of interest to breeders for further investigation. The selection of late and early ripening varieties is also one of the important desirable characters in citrus. Accessions from Samtse and Tsirang might be useful in extending the harvesting season.

The accessions from Zhemgang and Trongsa had higher TSS (greater than 12 °Brix), while accessions from other locations had an intermediate range (10–12 °Brix). Although, the effect of elevation and climatic factors on variation in TSS cannot be ruled out, only the application of nitrogenous fertilizers (N) has been shown to contribute positively towards percent TSS, juice and acid content (Zekri *et al*., 2009). Studies by Thompson *et al*. (2005) and Kusakabe *et al*. (2006) found that N rates and fertigation did not significantly affect the fruit quality including the percentage of TSS and acid content. Moreover, it is also known that nitrogen exists in fruit juice as amino acids (Ladaniya, 2008b). Since the mandarin accessions in the areas sampled were mostly grown under minimal management without proper irrigation and fertilizer application, the variation in TSS might be due to genetic variation.

Morphological characterization has been widely used to describe varieties, although new molecular techniques have been deployed increasingly in recent years for the identification of different crops. Morphological characters were used to describe three potential citrus rootstocks (Jaskani *et al*., 2006). The advantages of morphological characters are savings in cost and time and the provision of horticultural characters.

Because of the plasticity and instability of phenotypic characters, cultivar identification and diversity were often in contrast to actual genetic diversity. Previous authors (Yonemoto *et al*., 2007; Struwig *et al*., 2009) used both morphological data and molecular analysis. In citrus, especially in mandarin, the study of morphological diversity has been described as independent from its genetic diversity (Koehler-Santos *et al*., 2003; Campos *et al*., 2005; Kyndt *et al*., 2010). Furthermore, important horticultural characters are reported to be controlled by multiple genes (Campos *et al*., 2005; Liu and Deng, 2007) and are of low heritability. Thus, morphological characterization could be an essential component since most of the horticultural characters cannot be evaluated through molecular markers.

CONCLUSION

The study showed the existence of wide variation in morphological characters among the mandarin accessions in six major mandarin-growing districts of Bhutan. The dichotomous key constructed using qualitative characters of trees, leaves and fruit was able to identify the accessions to specific locations. Furthermore, leaf and fruit quantitative characters differed significantly among the locations. These variations are indicative of the underlying genetic diversity and the influence of environmental factors. However, there was no variation observed among the accessions for both qualitative and quantitative floral characters. This study also unravels basic information on important horticultural characters of the different accession. Accessions from Dagana were promising both in terms of fruit yield and quality while accessions from Trongsa possessed highest TSS but the lowest number of seeds per fruit. Accessions from Samtse and Tsirang might be useful for extending the harvesting season. Thus, mandarin in Bhutan does not consist of a single variety when morphologically assessed and identified.

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