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Assessment of morphological diversity for local mandarin (*Citrus reticulata* Blanco.) accessions in Bhutan

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This study was conducted to assess the morphological diversity among 39 mandarin (*Citrus reticulata* Blanco.) accessions maintained at citrus germplasm of Research and Development Center (RDC) *Wengkhar*. Quantitative data were analyzed using one-way ANOVA with Duncan Multiple Range Tests, while qualitative data were subjected to cluster analysis. The analyzed accessions from germplasm were highly significant for characters of leaf and fruit ($p<0.001$). The floral quantitative traits were statistically non significant for petal length. The cluster analysis based on qualitative data categorized 39 accessions to two major groups (A and B). The groups diverged at average similarity of 0.79 with average of 0.91. The variation in qualitative characters was less while the quantitative characters differed significantly.

Key words: Bhutan, morphological diversity, local mandarin accessions, germplasm

Introduction

Citrus is the common term used for Genus, a flowering plant of *rue* family, *Rutaceae*. It falls under subfamily *Aurantioideae* and tribe *Citreae*. Although the origin of citrus is precisely not known, researchers (Moore, 2001; Sharma *et al.*, 2004; Ladaniya, 2008; Singh, 2010) believe that it has the origin from south and southeast tropical and subtropical region of the world. 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 (Federici *et al.*,1998). The cultivated species may have been derived

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from as few as three ancestral species. The natural and commercially cultivated citrus include oranges, grapefruits, lemons, lime and tangerines.

Currently citrus is grown over 140 countries in the world. Its production is mainly confined to Northern Hemisphere, particularly in Mediterranean regions and United States although the present global citrus production is about 116 million tons. Brazil is the world leading citrus growing country and contributes to about 18.1 % of the world production followed by China with 17.2%. United States rank third in world citrus production. These three countries combined constitute almost 50% of world citrus production (UNCTAD, 2007). Citrus is popular horticultural fruit crop worldwide. It is the highest valued fruits in the international market. Citrus is also known for its nutritional value. This is mainly attributed to increase in area under citrus cultivation, improved transportation, packaging, and rise in income and consumer choices for healthy foods. Indeed citrus has become very important commodity across the globe.

Citrus is number one horticultural crop of Bhutan. Citrus is the highest income generating fruit crop. Further it is identified as one among four important agricultural commodities (rice, maize, potato and citrus). Citrus in Bhutan is predominantly mandarin (*Citrus reticulata* Blanco.) although lemons, lime and grapefruits are also produced. Mandarin in Bhutan are reported to be of two types: *Sikkim* (Cultivated in northeastern Himalayan state of West Bengal, India) and *Khasi* (Cultivated in Khasi hills of Mehgalaya, India) (Dorjee *et al*., 2006). Despite the steady increase in mandarin production in country, the cultural practices being followed are still minimal. Most of the cultivars are local cultivars grown from either self raised or purchased from other farmers. There is no authentic history of citrus cultivation in Bhutan (NPPC, 2007).

Among the three species of citrus in Bhutan: Local Mandarin (*Citrus reticulata* Blanco.), Citrus (*Citrus aurantifolia*) and lemons (*Citrus limon*), only local mandarin (*Citrus reticulata* Blanco.) is produced in commercial scale (Dorjee *et al*., 2007; Connellan *et al*., 2008). Very little is known about the mandarin cultivar and its relatives. The exact number of mandarin cultivars or varieties remains unascertained. This has lead to controversies in mandarin trade and in general among educationist and breeders. The relationships among the cultivated mandarin species and their level of diversity are still unclear and no comprehensive study has been conducted so far (NPPC, 2006).

The research work on Citrus in Bhutan has gained momentum only a decade back. The collection of local citrus germplasm has been the research priority. Research and Development Centers (RDC) in different regions collect the accessions from their respective areas in the country. In the west central region, RDC, Bajo has the collection for local citrus germplasm and also at sub-center research station. Similarly, RDC, *Wengkhar* for the eastern region has maintained the collection of mandarins from across the country. Since germplasm collection is relatively new area in Bhutanese research scenario, the characterization of accessions and study of the diversity is still a major task ahead. The knowledge of genetic variation and relationship among these accessions is an important consideration for documentation, utilization and strengthening of future citrus industry in Bhutan.

Various methods are used for assessment of diversity and genetic relationship among the accessions. Morphological markers are widely used for estimation of diversity and characterization in sweet potato evaluation although it is affected by environment (Elameen *et al*., 2010). In citrus, morphological analysis was used to study variation between kinnow mandarin and rough lemon (Jaskani *et al*., 2006; Altaf. and Khan, 2008). In *Himalayan* citrus, morphological marker was used for study of diversity (Sharma *et al*., 2004). The morphological marker is known for its coverage in study of agronomic traits in addition to convenience. Further the technique is relatively cheaper and easier to conduct. Many previous authors (Koehler *et al*., 2003; Campos *et al*., 2005) reported that molecular and morphological diversity is independent and rather complementary to genetic diversity in citrus. Therefore, this study was aimed at characterization and assessment of morphological diversity for mandarin accessions maintained at RDC *Wengkhar* in Bhutan.

Materials and methods

The Research Development Center, *Wengkhar* is located 9 kilometers away from eastern capital (*Mongar*) at an altitude of 1645 meters above mean sea level with southeast aspect. The center has maintained local mandarin germplasm of 140 accessions collected from different places within the country. Accessions number and their sources are shown in Table 1. For uniformity only the grafted accessions from germplasm were used in the study.

The sampling of leaves, flowers and fruit was carried out in the month of February to April 2010. Each accession was recorded for tree, leaves, flowers and fruit characters. It was third fruiting season for the accessions. 15 leaves, five flowers and 10 fruits were sampled randomly from each accession. The morphological description was based on International Plant Genetic Resource Institute (IPGRI) Descriptors of Citrus (IPGRI, 1999).

Table 1. The details of germplasm sources, code number of accessions in germplasm and number of accessions.

Sl. No	Source	Code number of accessions	Number of accessions
1	Shumar, Pemagatshel	SMPG (27,28)	2
2	Kengkhar, Mongar	KKMG(29,41,42,43,47,49,50,51,53,54,57,59)	12
3	Narang, Mongar	NRMG (30,63,64,69,70)	5
4	Samtse	Samtse (31,32)	2
5	Dagana	Dagana (33,34,37,38,71)	5
6	Tsirang	Tsirang (35,36,61,62,65,66)	6
7	Yadi, Monggar	YDMG (39,40)	2
8	Sodrung, Mongar	Sodrung (45,46)	2
9	Samdrup Jongkhar	Sj/Khar (55,56)	2
10	Thangbi, Trongsa	TBTS (68)	1
Total number of accessions			39

The number in parenthesis indicate the number to accession.

Tree characters

The tree morphology was observed for growth habit (erect, spreading, drooping), branch density (sparse, medium, dense), branch angle (narrow, medium, wide), and spine density.

Leaf and flower description

The leaf characteristic was recorded for leaf division (simple, bifoliate, trifoliate, penfoliate), intensity for green color (light, medium, dark), leaf lamina attachment (sessile, brevipetiolate), leaf lamina length (mm) measured from petiole base to lamina tip, lamina width (mm) at widest point, leaf lamina shape (elliptic, ovate, obovate, lanceolate, orbicular, obcordate), leaf lamina margin (crenate, dentate, entire, sinuate), leaf apex (attenuate, acuminate, acute, obtuse, round and emarginated), petiole wings (obcordate, obdeltate, Obovate). Flower description was based on petal length (mm), petal width (mm), number of stamen, and pedicel length (mm), relative length of stamen to stigma, color of anthers, color of open flowers, flower type and flowering month.

Fruit and seed description

Fruit characteristics were studied for fruiting season (early, mid, late), fruit weight (g), fruit diameter (mm), fruit length (mm), width of epicarp at equatorial region (mm),adherence of albedo, total soluble solids (TSS) in percent (%), fruit shape (spheroid, obovate, ovate, oblate), fruit base shape (concave, convex, truncate, necked), fruipt apex shape, number of seeds, seed surface (smooth, wrinkled), seed color (white, cream), seed shape (cuneiforrn, clavate, ovoid, spherical).

Data analysis

Data were separated for quantitative and qualitative traits. The quantitative traits were analyzed using one way ANOVA with Duncan Multiple Range Tests while qualitative traits were subjected to cluster analysis to draw the relationship among the accessions analyzed by version 2.01d of NTSYS pc (Rolf, 2000). The similarity matrix was generated with Qualitative data option or subprogram of the software. The cluster analysis was done with SAHN module and the resultant tree plot for 39 accessions is shown below in Fig. 1.

Results

The accessions were highly significant for leaf and fruit characteristics. Some of the characters for flowers were also differed significantly except for the petal length. The variation was observed within the accessions from same locations. 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 mean values of leaf and floral characters are as shown below in Table 2.

Similarly, fruit characteristics varied significantly for the accessions from different places. The heaviest fruit was from the accessions of *Shumar* while *Samtse* had the lightest. The width of epicarp was observed thinnest in the accessions from *Samtse*. The number of seeds per fruit was lowest in *Samdrup Jongkhar* accesson. The %TSS was highest in the accessions from *Narang* followed by *Kengkhar*. The ratio of %TSS to acid was highest from *Shumar* followed by accessions from *Kengkhar* and *Samdrup Jongkhar*. *Shumar* had the lowest percent acidity while the accessions from *Tsirang* had the highest.

The physicochemical parameters of fruits and their differences are shown below in Table 3.

Table 2. Morphological comparison of quantitative traits of leaf and flower for the accessions from germplasm.

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

The mean value is superscripted to their differences.** and *** refers to 0.01 and 0.001 confidence level.

Cluster analysis

Since the univariate view of ANOVA did not give any information among accessions, a multivariate analysis was carried out to assess the diversity and relatedness among the accessions from different locations. The cluster analysis based on qualitative morphological variables from tree, leaves, flowers and fruits divided 39 accessions into two main clusters (A and B). Cluster A consisted of mostly accessions from *Kengkhar*. The group B was diverse group which as further divided to subgroup 1 and 2. Both the subgroups were mixed of accessions from different locations. There was no difference observed for the accessions between *Shumar* (SMPG27) and *Kengkhar* (KKMG53), *Tsirang* 61 and *Tsirang* 65, *Samdrupjongkhar* (Sj/Khar55) and *Dagana* (Dagana71). Over all, the morphological qualitative parameters for the accessions diverged at similarity coefficient of 0.79 with average of 0.91approximately.

The dendrogram (Fig. 1) derived from qualitative traits of specific accessions in from different sources as shown below.

Table 3. The morphological comparison of quantitative traits of fruits for the accessions from germplasm.

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

The mean value superscripted to their differences at 0.001 confidence level.

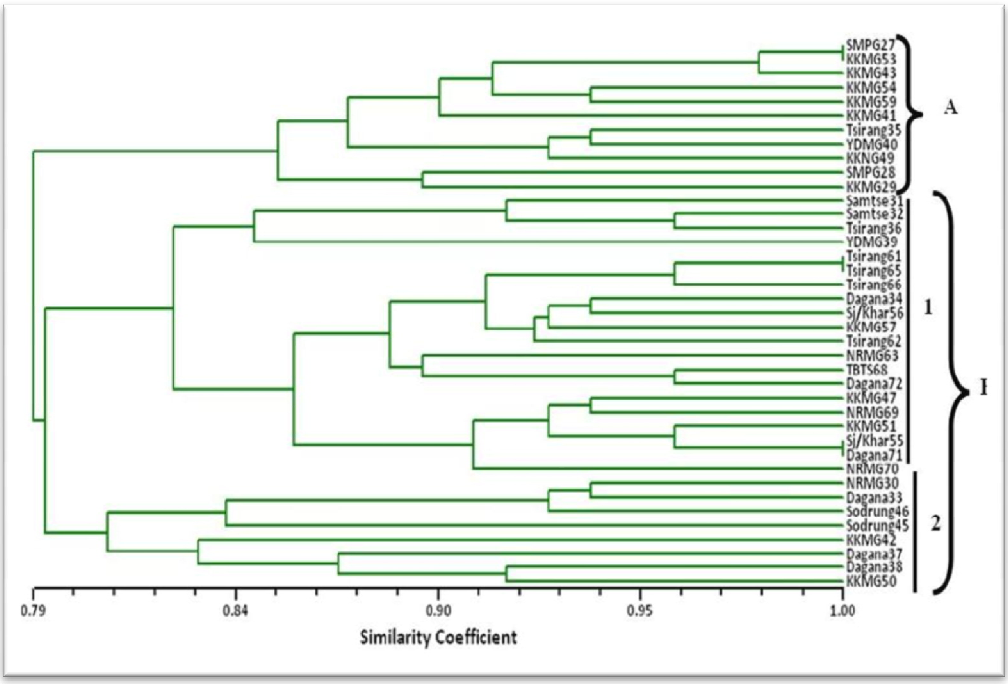

Fig. 1. UPGMA dendrogram based on morphological variables of trees, leaves, flowers and fruits.

Discussion

It is well known that morphological plasticity in study is a major weak point in assessment of phenotypic diversity. However, several combined studies in mandarin, both morphological and molecular markers in the past had shown to be independent of genetic diversity (Koehler *et al*., 2003; Campos *et al*., 2005). Further, the study on inheritance of agronomic traits of citrus reports them to be controlled by multiple genes which can be assessed only through morphological assessment (Liu and Deng, 2007).

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). Our results of high variations among the accessions from different locations conform to the views of above authors.

The variation among accessions within a location also varied for all the characters of leaves, flowers and fruits. This variation indicated that the accessions in the germplasm consisted of phenotypically different individuals. The difference in individual accession could be attributed to mutations, and cross pollination. Almost all the scion and roots stocks of citrus have emerged spontaneously as chance seedlings. The bud sport mutations and introduction and trials of materials in location different from its original habitat might be the factors that added to variation. Further, the lack of reproductive barrier both within the genus and species might have continually added to it variation and heterogeneity. Interestingly, the common phenomenon of occurring zygotic twins in Himalayan mandarin varieties as reported (Das *et al*., 2007) might be the other cause that added to variation.

The TSS content for the analyzed accessions varied significantly. The TSS is known to increase as and when the fruit matures while total acidity remain constant. The decrease in total acidity was due to dilution effect as a result of increase in fruit size and increase in TSS content (Ladaniya, 2008). The marketability of citrus is determined by the ratio of TSS to total acidity. The significant variation among the accessions for this ratio also supplement to existence of diversity. The accessions from *Shumar* had the highest % TSS to acidity ratio which may be explored for commercial multiplication. Besides, early maturing habit of *Shumar* is likely to have an advantage in extension of harvesting season.

Seedlessness has long been one of the breeding objectives in citrus. Although seeds content for many citrus is known to depend on elevation, accessions from *Samdrup Jongkhar* exhibited the lowest number of seeds per fruits. Various methods are currently deployed for development of seedless varieties (Biliques, 2004; Liu and Deng, 2007)). The study on inheritance of seedless trait showed a continuous variation in subsequent generation (Liu and Deng, 2007). However, the accessions from *Samdrup Jongkhar* might be potentially useful in multiplication as seedless varieties.

Cluster analysis based on qualitative variables differentiated 39 cultivars to two broad groups (A and B) which were in consistent with earlier findings in assessment of *Campo Citricola* experimental station germplasm. (Campos *et al.*, 2005). However, in this study, the groups diverged at similarity coefficient of 0.79 in contrast to 0.41. The high average similarity coefficient (0.91) indicated that there is not much variation with respect to qualitative characters for the analyzed accessions. Golein *et al.* (2005) reported that all mandarins represented variations of single clones. Consequently, high average similarity among accessions in our study also upholds the claim that mandarin in Bhutan might be a variation from single clone.

The group A consisted of 11 accessions with majority from *Kengkhar*. However, some accessions from *Shumar*, *Yadi* and *Tsirang* were found to cluster under same group which is suggestive of their common environmental effect. Moreover some of the accessions under group B had same phenotypic qualitative traits: *Tsirang*61 and *Tsirang*65, *Samdrupjongkhar* (Sj/Khar55) and *Dagana*71. However, larger group B was constituted of diverse group (28 accessions) which was further sub-grouped to sub -cluster 1 (20 accessions) and 2 (8 accessions). Both sub-clusters had accessions from different parts of the country irrespective of their origin.

Our study on morphological characters revealed the existence of diverse accessions in germplasm inspite of accessions exhibiting similar phenotypic qualitative characters. This could be due to action of diverse evolutionary forces. The assessment of phenotypic traits on pummelo from uncontrolled field survey had shown that the environmental factors affected to the tune of 40% (Paudyal and Haq, 2008). Despite having lower variations in qualitative traits, high statistical significance in quantitative traits indicated the existence of diversity in analyzed accessions. Since this is first of this kind in evaluation of phenotypes for mentioned germplasm accessions, further work on molecular analysis is essential for a clearer picture of their diversity and relationship.

From our study, it can be concluded that wide variation existed among the accessions with respect to quantitative characters. On the other hand qualitative characters did not vary much among the analyzed accessions.

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