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Morpho-genetic profiling and phylogenetic relationship of guava (*Psidium guajava* l.) as genetic resources in Pakistan

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Abstract- Guava (*Psidium guajava* L.) is an open-pollinated crop having 25-40% dissimilarity index which promotes heterozygosity and adds new cultivars. Morpho-genetic characterization of 37 guava accessions was carried out for genetic variability and structure of guava germplasm located in Punjab province, Pakistan. Principal Component analysis (PCA) was subjected to analyze the morphological diversity and for genetic analysis we applied cluster analysis, using the PyElph software. PCA distributed thirty one traits into six components and first two components accounted 39.5% of total variation. A dendrogram constructed on the basis of morphological traits which showed 34% dissimilarity index among thirty seven guava accessions and divided them into 6 groups. For genetic characterization 18 microsatellites were used, the size of reproducible and scorable bands ranged from 150 to 320 bp. The 18 primer pairs amplified 85 alleles with an average number of 4.7 alleles per locus and no more than two displayed bands (nuclear SSR loci). The phylogenetic tree based on molecular analysis showed 50% dissimilarity index among selected guava accessions and separated them into 4 groups.

Index terms: cross pollinated**,** dendrogram, genetic diversity, reproducible*,* SSR markers.

Caracterização morfo-genético e relação filogenética de goiaba (*Psidium guajava* l.) como recursos genéticos no Paquistão

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Resumo- A goiaba (Psidium guajava L.) é uma cultura de polinização aberta tendo 25-40% Índice de dissimilaridade que promove a heterozigosidade e acrescenta novas cultivares. Caracterização morfo-genética de 37 acessos de goiabeira foi realizada para a variabilidade genética e estrutura de germoplasma de goiaba localizado na província Punjab, Paquistão. A análise de componentes principais (PCA) foi submetida para analisar a diversidade morfológica e para análises genéticas foram aplicados a análise de agrupamento, utilizando o software de PyElph. PCA traços distribuídos trinta e um em seis componentes e dois primeiros componentes representaram 39,5% da variação total. Um dendrograma construído com base em caracteres morfológicos que mostrou 34% de índice de dissimilaridade entre trinta e sete acessos de goiabeira e dividiu-os em 6 grupos. Caracterização genética de 18 microssatélites foram utilizados, o tamanho dos gerados reprodutível e bandas variou de 150 a 320 pb. Os 18 pares de primers amplificado 85 alelos, com uma média de 4,7 alelos por loco e não mais do que duas bandas exibido (locos SSR nuclear). A árvore filogenética baseada na análise molecular mostrou 50% de índice de dissimilaridade entre os acessos de goiabeira selecionados e separados em 4 grupos.

Termos de indexação: polinização cruzada, o dendrograma, diversidade genética, reprodutível, marcadores SSR.

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Introduction

Guava (*Psidium guajava*) is an important tropical fruit crop which belongs to family Myrtaceae. It is the hardiest among tropical fruits and excels most of the other fruit crops in productivity and adaptability. The family Myrtaceae is comprised of 142 genera and more than 6700 species of trees and shrubs (KUBITZKI et al. 2010). Botanically, guava fruit is a berry which may be rounded, ovate, or pear shaped. The fruit varies from 25 to 102 mm in diameter and from 56 to about 450 g in weight. The skin color of the ripe fruit is usually yellow and the flesh color may be white, pink, yellow or cream. Guavas vary from thick fleshed fruits with only a few seeds in a small central cavity, to thin fleshed fruits with numerous seeds imbedded in a large mass of pulp. The fruits range in flavor from quite sweet in some varieties, to sour and highly acidic in others. The characteristic musky guava aroma and flavor are quite evident in most forms, however in some types they are milder and more pleasant. In others, the aroma and flavor may be too strong and penetrating for most tastes (MENZEL, 1985).

India has the world's largest mass production of guava, followed by Pakistan, Mexico, Brazil, Egypt, Thailand, Columbia, and Indonesia. Production in these countries has increased 10-fold in the last five years (POMMER and MURKAMI, 2009). In Pakistan, guava is extensively grown in Punjab and Sindh provinces and occupies third position after citrus and mango in terms of area and production. The two main types of cultivated guava in Pakistan are Gola (round shape fruit) and Surahi (pear shape fruit). According to the Pakistan Statistical Year book (2010), guava is grown on 62,300 ha giving 512,300 t of annual production with a yield of 8223 kg per hectare. Punjab province grew 49,700 ha with a total production of 422,300 t and yield of 8497 kg per hectare. Although guava is cultivated across a large area, its production remains low, probably due to a lack of superior varieties along with other environmental and disease stresses (IMRAN et al., 2013).

By comparison, however, local growers in Pakistan replenish their trees from sexual propagation (propagation from seed). No commercial cultivars are available. Since these growers name their cultivars according to a few morphological characters and locality, the naming system is confused. Accurate characterization of guava cultivars and rootstocks is essential for commercial orchards and nurseries as this can guarantee uniformity in the establishment of new orchards (PAKISTAN STATISTICAL YEARBOOK, 2010). This can be supported by inclusion of DNA-based markers (such as the iPBS and SSR marker systems) for germplasm characterization to provide more basic genetic information. As markers are not affected by the environment, conclusions and interpretations regarding genetic variation will be more reliable (SANCHEZ-TEYER et al., 2010). According to analyses performed to evaluate the genetic diversity among *Passiflora* accessions, it was inferred that gathering knowledge about aspects of the germination of seeds of several passion fruit species, especially wild species, is fundamental for the propagation and maintenance of germplasm banks (THALITA et al. 2017).

Genetic diversity and discrimination among individual accessions or groups of individuals or populations can be analyzed by a specific method or a combination of methods (MEHMOOD et al., 2014; RITTER, 2012; VALDE´S-INFANTE et al., 2010). Different molecular markers such as AFLP (SANCHEZ-TEYER et al., 2010; VALDE´S-INFANTE et al., 2003), ISTR, RAPD (AHMED et al., 2011; COSER et al., 2012), iPBS (MEHMOOD et al., 2013) SSR (ARANGUREN et al., 2010) have so far been used for guava germplasm analysis. Among these different types of molecular markers, microsatellites or SSRs (defined as short tandem repeats) have been widely used in different countries as efficient tools for germplasm characterization, for plant management and for diversity studies on *Psidium* germplasm (BRICENO et al., 2010). Therefore, the objective of this work was to evaluate genetic diversity based on molecular markers and morphological characterization that intended to help breeding program and identification of genotypes from Pakistan.

Material and methods

Plant materials

Thirty-seven seed propagated guava accessions were selected from the hub of guava growing districts included Faisalabad and Sheikhupura of Punjab province, Pakistan (Table 1, Figure 1). The accessions were selected based on fruit size, fruit shape, flesh color, fruit blush color and number of seeds.

Evaluation of morphological traits

Thirty-one morphological traits *were* selected for genetic diversity of guava accessions by using guava plant descriptors (UPOV, 1987); fully developed shoot, leave and fruit were selected for data collection. Studied traits included young shoot color, thickness of stem (mm), length of leaf blade (mm), width of leaf blade (mm), leaf blade length/ width ratio (%), leaf shape, leaf curvature in cross section, leaf green color, leaf color of midrib on lower side, leaf spacing of secondary veins, leaf relief of surface of upper side, the shape of leaf base, the shape of leaf tips, fruit length (mm), fruit width (mm), fruit length/ width ratio (%), fruit shape at stalk end, fruit width of neck in relation to that of fruit, fruit skin color, fruit relief of surface, fruit size (g), fruit cavity diameter (mm), fruit ridged on skin, fruit stalk length (mm), fruit flesh color,

thickness of outer flesh (mm), fruit Juiciness, fruit acidity, TSS (°Brix), seed weight (g), number of seeds.

DNA extraction for molecular studies

The genomic DNA from 2 g of fresh and healthy leaves were extracted by following the modified CTAB method (MURRAY and THOMPSON, 1980).

PCR amplification

Eighteen microsatellite markers (RISTERUCCI et al., 2005) were used for genetic amplification of 37 guava accessions using the method of RODRÍGUEZ–MEDINA et al. (2007). PCR amplifications were accomplished in 20 µL reaction mixture using thermal cycler (PERKIN ELMER MODEL 480). The PCR reaction mixture contained template DNA (20 ng), 1µM each primer, 200 µM of dNTPs, and 1X gold PCR buffer, 0.5U Taq (Ampli Taq Gold DNA polymerase) and $MgCl_2$ solution (2mM). The optimization of conditions was made separately for each marker. All markers were tested on DNA samples at the annealing temperature of 55°C, whereas all other conditions of procedure were kept constant: 5 min at 94°C; 35 cycles of 30sec at 94°C, 45sec at 56°C and 2 min at 72°C and final extension was done for 4 min at 72 °C. The confirmation of all amplifications was performed by running PCR product (5µL) on agarose gels (2%).

Data analysis

Morphological data were subjected to Principal Component Analysis (PCA) (FRANCO and HIDALGO, 2002) in order to identify morphological variables. Data analysis was performed using statistical software Statistica 5.5 version. For molecular analysis, PCR was performed three times to confirm band pattern for each primer. DNA bands were sized and scored by Lab Works software (v4.5, UVP) and carefully checked manually; only clear bands were scored and faint bands were ignored. Bands with the same size were assumed to represent a single locus. For each locus data were recorded using '1' for presence of a band and '0' for absence so as to build a binary matrix. Assessment of genetic relationship among the accessions was done by cluster analysis, using the PyElph software, version 1.4.

Results and discussion

Characterization of morphological traits using PCA

Principal Component analyses (PCA) distributed thirty-one traits into six components that explained 73.91% variation. The first two components have major contribution of total variation, first component accounted for 25.14 % of the total variation, included leaf curvature in cross section, leaf spacing of secondary veins, leaf shape of base, shape of leaf tips, fruit length/width ratio, fruit width, fruit length, seed weight, fruit size, fruit diameter, fruit ridges on skin, fruit length of stalk, fruit juiciness, total soluble solid, number of seeds and fruit relief of surface. The second component, which explained 14.26% of total variation, included young shoot color, fruit diameter of cavity, fruit color of flesh, leaf curvature in cross section, leaf color of midrib on lower side, leaf spacing of secondary veins, leaf shape of base, fruit ridges, leaf shape and fruit acidity. A 2D PCA plot was constructed on the basis of first two components (Figure 1). The 2D plot distributed the guava accessions according to their phenotypic resemblance and morphological characteristics. The accessions number A4, A5, A19 and A33 were grouped together with the highest fruit weight, the longest fruit length and the longest fruit width while accessions number A6, A12 and A28 with the pink flesh were group together. Similarly the accessions having distinct morphological trait are separated from others like A29 with small size, pear shape A21 with small size, round shape and A3 with grooves on skin. These results showed that the fruit weight, fruit width, fruit length, fruit size and flesh color are highly positively correlated and as a result these traits led to the highest loading factors in this PCA analysis.

According to Rodríguez-Medina et al. (2007) and Andrés-Agustin et al. (2006) these traits are valuable for identification of guava populations and other horticultural fruits. Most of the traits studied are economically important for plant breeders, especially thickness of outer flesh in relation to core diameter, fruit length, fruit width and fruit juiciness. Our study supports the view that phenotypic traits in fruits are helpful for accessions or cultivar identification and are reliable in establishing the genetic relationships across larger and diverged accessions of guava (PADILLA-RAMIREZ AND GONZALEZ-GAONA, 2010; MEHMOOD et al., 2014).

Similarity matrix and dendrogram based on morphological traits

Ward linkage method used to construct dendrogram which differentiated 37 guava accessions into two main groups and second group is further divided into 5 groups (Figure 2). Group A contained two guava genotypes with low seeded and round shape fruits. Group B is further divided into six subgroups B1, B2, B3, B4, B5 and B6. B1 contained four guava accessions with round shape and large size fruit, B2 had eight accessions with pear shape and medium size fruit, B3 contained six guava accessions with pear and round shape fruit, B4 had four genotypes which had pink blush (*Psidium cattleianum*), B5 had three guava accessions which had round shape and small size, B6 contained ten guava accessions which had round shape, medium size and white pulp. The main and sub groups diversity provides the general information of

varietal performance and relationship according to their environment; however, this diversity is dependent on geographical origin and or even pedigree relations, and accessions that display high phenotypic dissimilarity might not to be genetically dissimilar because the environment can manipulate phenotypic expressions (POEHLMAN, 1987). Similar results were reported by Junior et al. (2008) that evaluated 63 accessions of guava and found accessions Compos and Coma the most divergent with other closely associated accessions and morphological traits. The reasoning for solitary clusters formation is due to complete inhibition and isolation of gene flow or either may be due to severe natural/human selection procedure of accessions for divergence abortiveness.

DNA polymorphism generated by 18 SSR primers and phylogenetic relationship

Eighteen guava-specific microsatellite primer pairs were used to screen the 37 guava accessions for genetic identification. Significant molecular variability was detected among all accessions (Table 2). The size of reproducible and scorable bands ranged from 150 to 320 bp. The 18 primer pairs amplified 85 alleles with an average total number of 4.7 alleles per locus and no more than two displayed bands (nuclear SSR loci). The Phylogenetic relatedness based on 18 microsatellites of 37 guava accessions defined genetic diversity among guava accessions. The dendrogram generated by PyElph software separated thirty seven guava accessions into four main groups (group A, group B, group C and group D), group A is further divided into A-1that constituted 2 guava accessions and A-2 that consisted 6 guava accessions similarly group B is sub-divided into B-1 that contained 3 guava accessions, group B-2 that had 1 guava accessions and B-3 that had 8 guava accessions further group C had 6 guava accessions and group D contained 11 guava accessions (Figure 3). Within the species *P. guajava*, SSR systems separated the 37 guava accessions into clusters according geographical regions; level of information generated suggests that accessions collected from the same geographical region or breeding program tended to group together, indicating that despite the widespread distribution of guava in the tropical world and more than 100 years of cultivation, germplasm exchange among regions has been limited. Furthermore, results from this study showed that only a small number of guava cultivars have been used in breeding programs. In Pakistan, guava breeding is highly dependent on seed propagation with subsequent selection being made according to specific characteristics like fruit quality and/or plant vigour according to local farmer preferences (MEHMOOD et al. 2013). The main and sub groups diversity in two different districts is due to high diverse genetic makeup of guava accessions (SINGH and BAINS, 1968). Similar results were observed by Walia and Garg (1996), Mehmood et al. (2016) and Dotlacil et al. (2000) who indicated a non-parallelization between geographic effects and genetic diversity. Unexpectedly, the accessions 'Gola and Surahi' showed the same genotype, which can be explained by the dissemination of seeds of the same accessions throughout these districts in the past.

The accession Bangladeshi gola ($FSDS_{5}$), Lalgola ($FSDS_{14}$), Larkanagola ($FSDS_{17}$), Gola ($SKPS_{26}$), Moti surahi ($SKPS_{34}$) were one of the most important guava accessions up to now and cultivated mostly in these districts areas. Our findings also indicated that genetic diversity is connected with genetic makeup of accessions. These results are correlated with those reported by Mehmood et al. (2016).

Table 1- List of guava accessions with phenotypic characteristics selected from districts Faisalabad (FSDS) and Sheikhupura (SKPS), Pakistan.

code	Accession name	Botanical name	Native	Phenotypic characteristics
$FSDS_{1}$	Gola	*P. guajava*	Faisalabad	Round (medium size)
$FSDS_{2}$	Surahi	*P. guajava*	Faisalabad	Pear shape (medium size)
$FSDS_{3}$	Rough gola	*P. guajava*	Faisalabad	Round (grooved skin)
$FSDS_{4}$	Motagola	*P. guajava*	Faisalabad	Round (large size)
$FSDS_{5}$	Bangladeshi gola	*P. guajava*	Faisalabad	Round (Extra-large size)
$FSDS_{6}$	Lalgola	*P. cattleianum*	Faisalabad	Round (pink flesh)
$FSDS_{7}$	Surahi	*P. guajava*	Faisalabad	Pear shape (medium size)
$FSDS_{8}$	Khatta	*P. guajava*	Faisalabad	Round (sour taste)
$FSDS_{9}$	Surahi	*P. guajava*	Faisalabad	Pear shape (small size)
$FSDS_{10}$	Gola	*P. guajava*	Faisalabad	Round (small size)
$FSDS_{11}$	Allah Abadi gola	*P. guajava*	Faisalabad	Round (medium size)
$FSDS_{12}$	Lalgola	*P. cattleianum*	Faisalabad	Round (pink flesh)
$FSDS_{13}$	Karalla	*P. guajava*	Faisalabad	Pear shape (long neck)
$FSDS_{14}$	Lalgola	*P. cattleianum*	Faisalabad	Round (pink flesh)
$FSDS_{15}$	Gola	*P. guajava*	Faisalabad	Round (medium size)
$FSDS_{16}$	Sadabahar gola	*P. guajava*	Faisalabad	Round (medium size)
$FSDS_{17}$	Larkanagola	*P. guajava*	Faisalabad	Round (medium size)
$SKPS_{18}$	Gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{19}$	Mota gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{20}$	Surahi	*P. guajava*	Sheikhupura	Pear shape (medium size)
$SKPS_{21}$	Chota gola	*P. guajava*	Sheikhupura	Round (small size)
$SKPS_{22}$	Gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{23}$	Surahi	*P. guajava*	Sheikhupura	Pear shape (medium size)
$SKPS_{24}$	Gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{25}$	Surahi	*P. guajava*	Sheikhupura	Pear shape (medium size)
$SKPS_{26}$	Gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{27}$	Mota gola	*P. guajava*	Sheikhupura	Round (large size)
$SKPS_{28}$	Lalgola	*P. cattleianum*	Sheikhupura	Round (pink flesh)
$SKPS_{29}$	Choti surahi	*P. guajava*	Sheikhupura	Pear shape (small size)
$SKPS_{30}$	Larkana gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{31}$	Desi gola	*P. guajava*	Sheikhupura	Round (small size)
$SKPS_{32}$	Gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{33}$	Mota gola	*P. guajava*	Sheikhupura	Round (large size)
$SKPS_{34}$	Moti surahi	*P. guajava*	Sheikhupura	Pear shape (large size)
$SKPS_{35}$	Gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{36}$	Sadabahar gola	*P. guajava*	Sheikhupura	Round (medium size)
$SKPS_{37}$	Sadabahar gola	*P. guajava*	Sheikhupura	Round (medium size)

Table 2- 18 SSR primers (RISTERUCCI et al., 2005) showing amplification, polymorphism, and size among 37 guava accessions.

Sr. No.	Primer	Forward Sequence	Reverse sequence	Ta (°C)	No. of Alleles	Allele size range
1	mPgCIR02	AGTGAACGACTGAAGACC	ATTACACATTCAGCCACTT	55	5	232-260
2	mPgCIR03	TTGTGGCTTGATTTCC	TCGTTTAGAGGACATTTCT	55	2	202-220
3	mPgCIR05	GCCTTTGAACCACATC	TCAATACGAGAGGCAATA	55	5	230-295
4	mPgCIR07	ATGGAGGTAGGTTGATG	CGTAGTAATCGAAGAAATG	55	6	160-170
5	mPgCIR08	ACTTTCGGTCTCAACAAG	AGGCTTCCTACAAAAGTG	55	5	215-235
6	mPgCIR09	GCGTGTCGTATTGTTTC	ATTTTCTTCTGCCTTGTC	55	4	160-185
7	mPgCIR10	GTTGGCTCTTATTTTGGT	GCCCCATATCTAGGAAG	55	4	265-290
8	mPgCIR11	TGAAAGACAACAAACGAG	TTACACCCACCTAAATAAGA	55	5	298-320
9	mPgCIR14	TAAACACAACAAGGGTCA	CAGTTTTCATATCGTCCTC	55	5	190-200
10	mPgCIR15	TCTAATCCCCTGAGTTTC	CCGATCATCTCTTTCTTT	55	5	240-270
11	mPgCIR16	AATACCAGCAACACCAA	CATCCGTCTCTAAACCTC	55	5	270-300
12	mPgCIR17	CCTTTCGTCATATTCACTT	CATTGGATGGTTGACAT	55	3	150-240
13	mPgCIR18	TAAGCTGCATGTGTGC	ATGGCTTTGGATGAAA	55	5	180-200
14	mPgCIR19	AAAATCCTGAAGACGAAC	TATCAGAGGCTTGCATTA	55	3	265-280
15	mPgCIR20	TATACCACACGCTGAAAC	TTCCCCATAAACATCTCT	55	6	275-300
16	mPgCIR21	TGCCCTTCTAAGTATAACAG	AGCTACAAACCTTCCTAAA	55	5	250-285
17	mPgCIR22	CATAAGGACATTTGAGGAA	AATAAGAAAGCGAGCAGA	55	5	200-278
18	mPgCIR25	GACAATCCAATCTCACTTT	TGTGTCAAGCATACCTTC	55	6	170-200

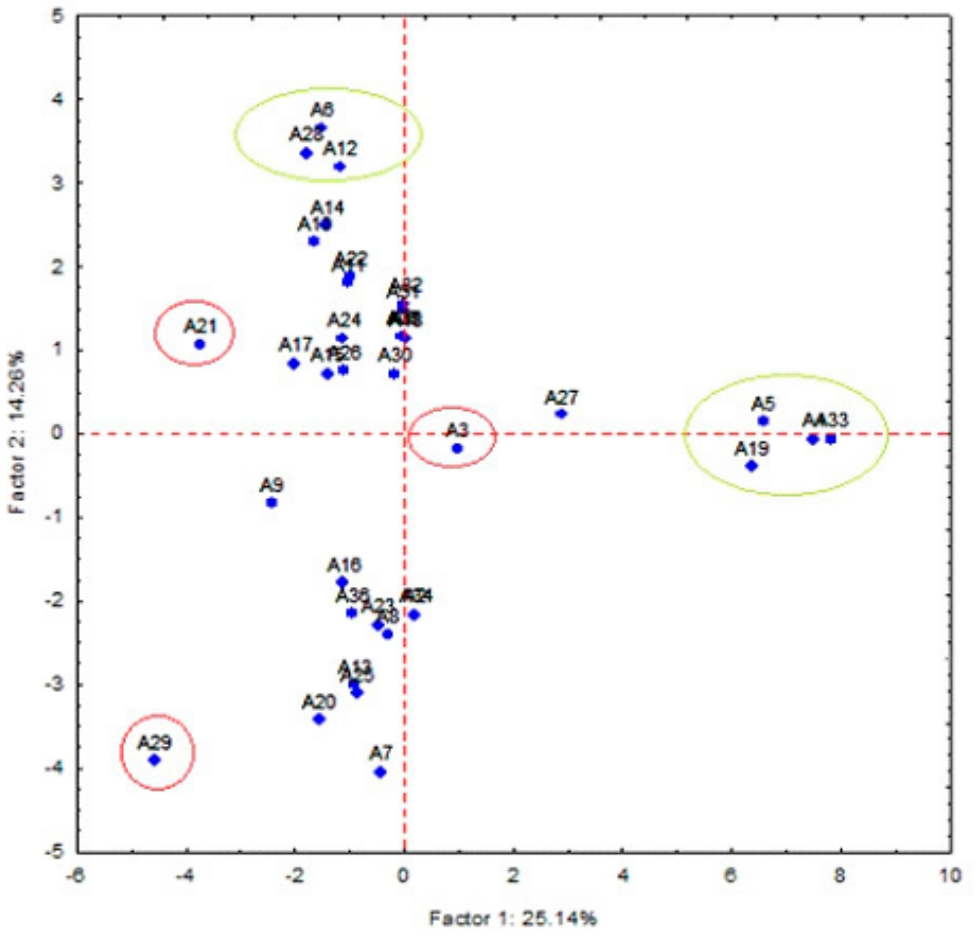

Figure 1- Two-dimensional PCA plot based on the first two components for 31 morphological traits of 37 Pakistani guava accessions.

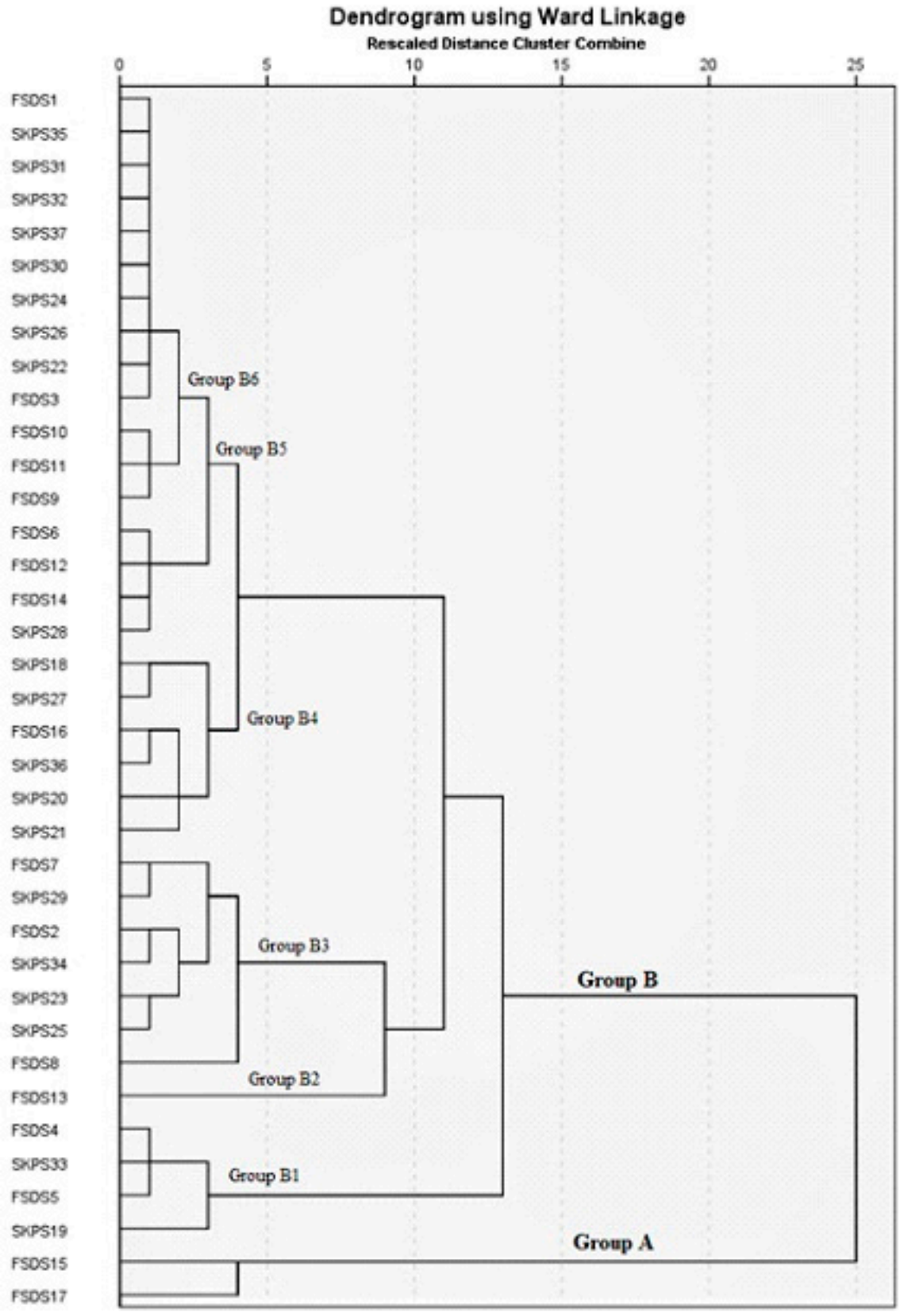

Figure 2- Wards linkage method of phenotypic diversity among 37 guava accessions based on morphological characters, distance linkage range is from 2% to 25%.

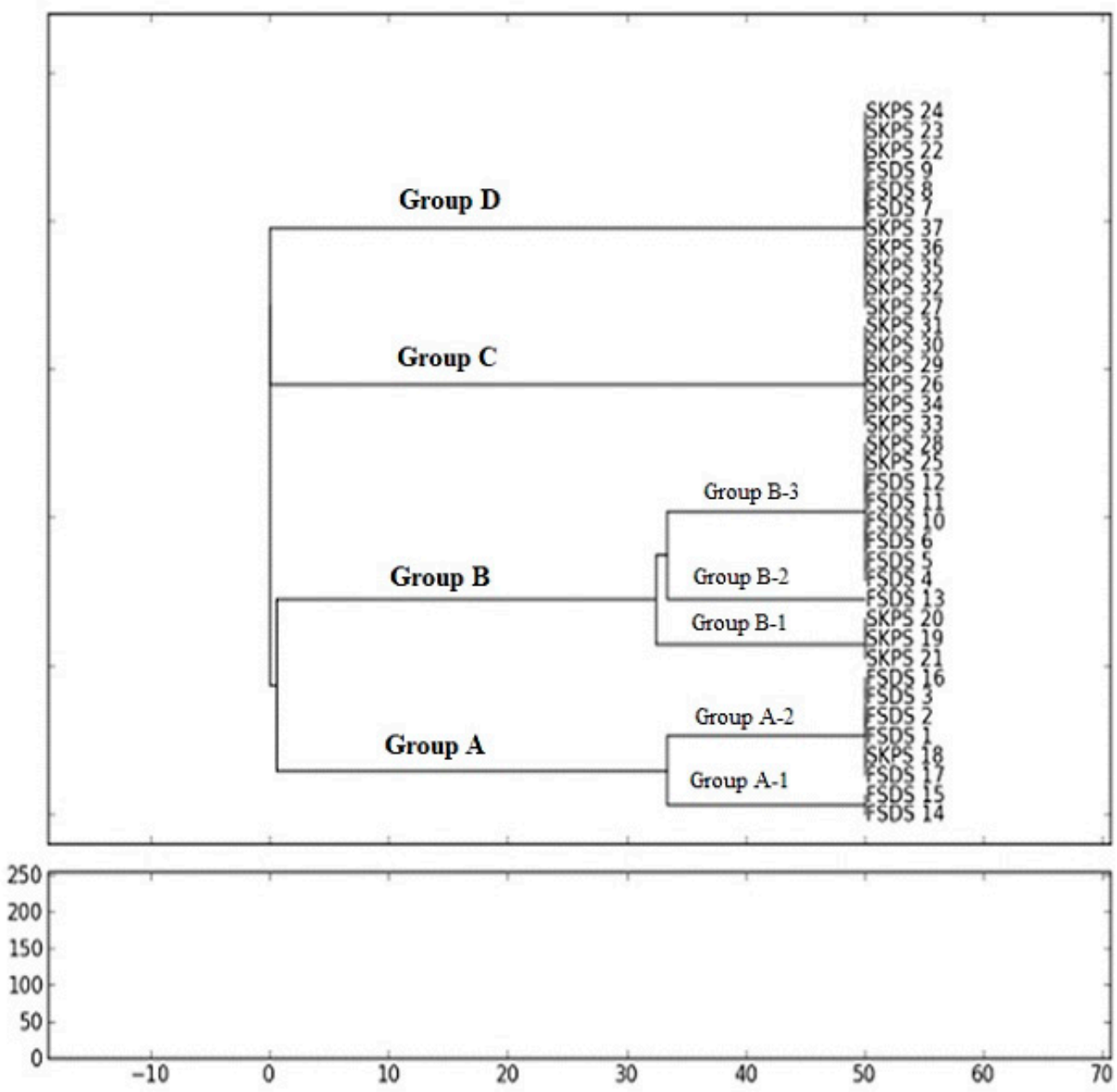

Figure 3- A dendrogram computed using PyElph software to cluster 37 guava accessions, the range of genetic diversity among 37 guava accessions is 1% to 50%.

Figure 4- Phenotypic fruit traits used for the selection of guava accessions. (a) pear shape (narrow neck) (b) karalla (long neck) (c) pear shape (cream skin) (d) round shape (white skin) (e) round (pink blush) (f) pear shape (rough skin) (g) dark pink flesh (h) pink flesh (i) low seeded.

Conclusion

From our study, it can be concluded that wide variation in terms of morphological traits and molecular analysis exists among guava genotypes (Figure 4). Wide range of variability was observed among the genotypes with respect to different morpho-genetic characteristics. Results suggested that the quantification of traits could help to understand the potential of germplasm in selection of potential parent for their future utilization in breeding programmes. The present study showed a high degree of variation among analyzed guava genotypes indicating that existing guava germplasms are important source of genetic diversity that can be used in the guava improvement programme.

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