Assessment of the Applicability of Morphometric, Organoleptic and Phylogenetic Analyses to Differentiate Soft and Firm Flesh Bearing Trees of Jackfruit (*Artocarpus heterophyllus* Lam.) in Sri Lanka

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ABSTRACT

Purpose : The aril/flesh of the fruitlets is the main edible part of jackfruit (*Artocarpus heterophyllus* Lam.). There are two flesh types; soft flesh (SF) (Wela) and firm flesh (FF) (Waraka) (i.e. highly preferred). There is no strategy available to identify FF plants at nursery or vegetative stages. Thus the present study was conducted to assess the applicability of leaf morphological variation and DNA bar-coding to differentiate SF and FF bearing jackfruit plants.

Research Method : The syncarp morphology and sensory parameters of SF and FF ripen flesh were also assessed to check the variation with 10 trees from each flesh type. The trees were chosen from SF and FF types and leaves and mature syncarps were collected. The leaf and syncarp morphological variation, pH, soluble solids and ascorbic acid content were measured. The sensory parameters of the flesh types were ranked by employing a taste panel. The DNA bar-coding was carried out for two loci *rbcL* and ITS to identify the sequence variants.

Findings : The leaf tip length was longer in SF, ascorbic acid content was higher in FF and all the other parameters were not different ($P < 0.05$). The SF leaves were elliptical whereas FF leaves were obovate. The association analysis revealed that the sweetness and sourness of flesh were inversely associated. The DNA bar-coding for *rbcL* and ITS showed that *rbcL* locus was not informative however, ITS locus can be used to characterize the genetic diversity of jackfruit.

Research Limitations : The detected haplotypic variants must be further validated with a larger set of jackfruit trees collected from diverse sites.

Originality/value : Three haplotypes were detected within ITS locus where CC haplotype is characteristic to SF and CT or TC is detected in FF enabling marker assisted seedling selection. This is the first reported study attempting to use sequence variants to differentiate SF and FF typed trees in jackfruit.

Keywords: Marker assisted seedling selection, firm flesh jackfruit, soft flesh jackfruit, Moraceae, ITS haplotypes

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lam.: family Moraceae) is a large evergreen tree crop producing syncarpous multiple fruit (i.e. syncarp) as a major economically important part. This species was originated in tropical rain forests of the Western Ghats of India and Malaysia and also found in almost all tropical and subtropical regions (Purseglove, 1968).

Jackfruit has been considered as an alternative staple food crop since ancient times and instrumental in securing human populations

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from starvation in numerous instances. In the tropical and subtropical countries, jackfruit is highly regarded as a nutrient rich starchy staple (Akinmutimi, 2006), vegetable and a delicious fruit (Medagoda, 2007) making it one of the top most food security crops in the world. The aril or flesh of the fruitlet in mature syncarp is the main edible part in jackfruit and immature syncarps are also processed as a nutritious vegetable. The flesh contains many nutrients such as carbohydrates, proteins, fat, minerals and vitamins enabling jackfruit to be an alternative starchy staple food (Akinmutimi, 2006) in place of rice or wheat. The presence of higher amount of dietary fibers in arils eases the food digestion process (Baliga *et al.*, 2011) and the proteins in jackfruit are considered as a potentially equivalent alternative to animal protein (Ajayi *et al.*, 2007; Aji *et al.*, 2016). The jackfruit seeds are also considered as an efficient source of starch and industrial raw material (Choy *et al.*, 2016). The nano-particles processed from the starch of jackfruit seeds are currently being used in industries to separate silver (Jagtap and Bapat, 2013) and gold (Jiang *et al.*, 2013). The leaves, surplus fruits in harvesting and the left over material after extracting arils and seeds are also considered as a perfect fodder in livestock industry (Fithriadi *et al.*, 1996).

All parts of the jackfruit tree possess diverse medicinal properties and widely being used in indigenous medical practices (Baliga *et al.*, 2011). The parts of jackfruit tree have demonstrated promising anti-bacterial, anti-carcinogenic, anti-fungal, hypoglycemic, antioxidant, anti-inflammatory and wound healing properties (Ko *et al.*, 1998; Jagtap *et al.*, 2010; Baliga *et al.*, 2011; Swami *et al.*, 2012; Singh *et al.*, 2015; Vazhacharickal *et al.*, 2016). These medicinal attributes are due to the presence of diverse phytochemicals such as carotenoids (Chandrika *et al.*, 2005) and polyphenols (Singh *et al.*, 2015) present in jackfruit. The syncarps contain flavonoids, sterols, phenyl-flavones and phenolic compounds with antioxidant capabilities. The artocarpanone isolated from jackfruit extract have shown inhibitory activity on melanin biosynthesis and could be used as

a potential skin whitening agent (Arung *et al.*, 2006). The leaves also contain phytochemicals such as flavonones and polyphenols and the leaf decoctions have shown proven hypoglycemic effects on healthy and non-insulin-dependent diabetic patients (Baliga *et al.*, 2011).

The jackfruit trees exhibit a greater diversity in all habitats due to extremely high heterozygosity and open pollinated nature of the species. There are two major types of jackfruit that could be identified based on the softness or firmness of the flesh or aril of the fruitlets at ripening stage. These two types are given diverse names in native habitats and in Sri Lanka the soft flesh (SF) type is known as *Wela* and firm flesh (FF) type as *Waraka* (Medagoda, 2007). The morphological diversity of jackfruit germplasm has been studied in Bangladesh (Azad *et al.*, 2007; Ibrahim *et al.*, 2013; Khan *et al.*, 2010), India (Simon *et al.*, 2007; Shyamalamma *et al.*, 2008), China (Yang-zhi *et al.*, 2010) and Brazil (Purseglove, 1968). However, jackfruit has not been considered in detailed research conducted in Sri Lanka although it has been identified by the government as a food security crop to safeguard the rural livelihood. Medagoda, (2007) has clearly stated that jackfruit is an underutilized fruit species in Sri Lanka and also identified the breeding priorities for this species as regular bearing, convenient syncarp size, seedlessness, dwarfness and insect resistance. To promote the utilization of jackfruit in industrial applications, the syncarp quality and associated biochemical parameters must also be assessed before producing value added products (Mondal *et al.*, 2016).

The growers and home gardeners have diverse interests in selecting the fruit trees for their orchards or gardens. It is important to select the interested type of tree, for example FF or SF in jackfruits as fruit trees take at least two or more years to bear the fruits and exhibit the phenotype. If the unwanted phenotype is expressed it would be a huge loss to the grower as few years of investment is lost and undesired trees have to be discarded. In jackfruit, Sri Lankan growers and consumers generally

prefer to grow and eat FF type over SF as a dessert and it also has great export potential over SF. Therefore, it is essential to have a reliable morphological or DNA marker system to perform marker assisted seedling selection (MASS) at nursery stage. Pushpakumara and Harris (2007) have studied randomly amplified polymorphic DNA (RAPD) markers to discriminate SF and FF types and found *OPB-01-1-0* as a marker for differentiation. However, because of the limited repeatability of generating the same banding profile in RAPD markers, their utility in genetic diagnostic tests is often hindered. Wickremasinghe (1996) has reported that isozyme polymorphism could be used to differentiate SF and FF however, because of the protinaecious nature, isozymes are not always expressed in required quantities so that they lack the power as a strong marker system to be used in discriminatory purposes.

The diversity of jackfruit germplasm has been mainly established using morphological parameters. Although the phylogenetic relationships of genus *Artocarpus* has been studied using DNA bar-coding (Zerega *et al.*, 2010; Williams *et al.*, 2017), the phylogenetic position of *A. heterophyllus* in Sri Lanka is not yet established. The soft and firm flesh types in jackfruit might be due to the species/sub-species differences that can be detected at molecular level using DNA bar-coding. Therefore, the present study was conducted to assess the applicability of variations in leaf morphometric parameters and sequences of DNA bar-coding loci to differentiate SF and FF bearing jackfruit trees to select the seedlings with preferred flesh type for planting. The syncarp variation and sensory properties of SF and FF of ripen arils were also assessed to identify the polymorphisms.

MATERIALS AND METHODS

Plant material

A total of ten trees belonging to the age classes of 10-50 years from each soft and firm flesh types were selected from Peradeniya in

Kandy District, Sri Lanka (GPS Coordinates: 7.254662, 80.584926; Agro Ecological Region: WM2b). The ten leaves and three to five mature syncarps (fruits) depending on the availability were randomly sampled from each tree in the peak fruiting season of 2016. The flesh type (soft or firm) of the tree was recorded according to the observations and sensory perception of the villagers living near the trees. The leaves and syncarps were immediately placed in brown paper bags and transported to the laboratory for recording morphological and phytochemical measurements. Tender leaves were collected from all the trees for DNA extraction.

Morphological data

Leaf measurements

The leaf morphological parameters, petiole length, leaf length, width, thickness, surface area number of veins and leaf tip length were measured from ten leaves of each tree. The leaf thickness was measured using a vernier caliper. The surface area of the leaf was measured by placing it over a 1cm grid-sheet. The grids capturing less than 50% of the leaf parts were disregarded in calculating the surface area. From each leaf, the angles between mid-rib and top most side vein (top angle), mid-rib and the central side vein (middle angle) and mid-rib and basal side vein (bottom angle) were measured using a protractor (Figure 01A). The shapes of the leaf and leaf base, margin, presence of hairs, texture of the leaf, venation pattern and arrangement of the leaves were recorded according to the key given in Norton-Brown Herbarium (2016). The leaf tip shape was recorded according to the key given in Figure 01B, 01C and 01D (modified from the key given by Norton-Brown Herbarium 2016).

Syncarp measurements

The length, breadth and weight of the syncarps (i.e. compound fruits) were measured at five different directions of the diameter to capture the variation caused by irregular shapes. The shape of the syncarp was recorded according to the key given in Figure 02 which was

developed based on the syncarp shapes indicated in Medagoda (2007) and Norton-Brown Herbarium (2016). The fruitlet shapes were measured according to the key given in Figure 03 (modified from Medagoda, 2007 and Haq, 2006). The longitudinal and cross sections of the syncarps and the fruitlets were photographed for illustration purposes.

Assessment of the flesh type and sensory parameters

A set of human subjects were employed in a taste panel to rank the ripen flesh of SF and FF trees. A questionnaire consisting soft and firm categories for flesh type; low, medium and high categories for sweetness, sourness and aroma was provided to each panelist to rank the ripen flesh samples given to them. The taste panel assessment was conducted as a single blind trial in which the identity of the sample (flesh type) was not revealed to the taster. A glass of

water was provided to each taster in between tasting of samples to avoid the carry-over effect from the previously tasted sample. The pH, soluble solids and ascorbic acid contents were measured according to the protocols given in Cen *et al.*, (2006) and Pisoschi *et al.*, (2008). The ripen fruitlets were collected from syncarps by removing all attached undeveloped perianths. The fleshy aril (pulp) was separated from the exocarp and the seed. A total of 20 g of fleshy aril was measured and smashed upon addition of 500 ml of distilled water. The mixtures were strained using plastic strainers to remove undissolved larger chunks. Then they were subsequently sonicated three times for 30 mins and filtered in each time using Whatman filter paper no. 2 (WHA1002125). The final filtrate was collected from each sample and stored in 4 °C for the measurement of pH, soluble solids and ascorbic acid.

Figure 01: A key for shape and size measurements of jackfruit leaf. A: morphometric measurements B, C and D are the types of leaf tip shapes observed in this study.

Figure 02: The key, containing diverse shapes, used to classify syncarp shapes (not according to scale). This key was developed by modifying the jackfruit shapes given in Norton-Brown Herbarium, (2016) to match with the variation observed in the present study.

Figure 03: The key, containing diverse shapes, used to classify fruitlet shapes (not according to scale). This key was developed by modifying the fruitlet shapes given in Medagoda, (2007) and Haq, (2006) to match with the variation observed in the present study.

In order to measure the ascorbic acid content, 5 ml of aqueous fleshy aril sample was pipetted into a 50 ml conical flask and three drops of indicator-starch solution was added. Then the mixture was titrated with 0.005 M iodine solution until first permanent trace of a dark bluish black color was observed due to formation of starch-iodine complex at the end point. The titration was done in replicates of three for each sample. The ascorbic acid concentration was calculated according to the following stoichiometric reaction.

Simultaneously, pH of each sample was measured in triplicate using a pH meter and the soluble solids were measured by placing a drop of fleshy aril extract on a refractometer-prism and the respective reading was taken.

DNA Barcoding based on *rbcl* and ITS loci

The DNA was extracted from immature jackfruit leaves of three SF and two FF trees (DNeasy® Plant Mini Kit, Qiagen, Solna, Sweden). The PCR was performed using the standard DNA bar-coding primer pairs for the loci *rbcl* (5'-3' ATG TCA CCA CAA ACA GAG ACT AAA GC/ GTA AAA TCA AGT CCA CCR CG) and *ITS* (5'-3' TCC GTA GGT GAA CCT GCG G/ TCC TCC GCT TAT TGA TAT GC). The PCR was performed with following cycle. The initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min, synthesis at 64 °C for 1 min and final synthesis at 72 °C

for 10 mins. A 15 µl PCR solution mixture was prepared with 1× Go Taq® Green Master Mix (Promega Corporation, Madison, Wisconsin, USA), 1.0 µM of forward and reverse primers and 1.0 µl of DNA template. The PCR products were visualized in agarose gel electrophoresis and purified using the Wizard SV gel® and PCR clean-up system (Promega Corporation, Madison, Wisconsin, USA). The purified PCR products were sequenced using ABI 3500 Genetic Analyzer (Cat. No. 4405186).

Data Analyses

Morphological data analysis

The quantitative parameters of leaf and syncarp morphology, and the fruit-flesh parameters pH, soluble solids and ascorbic acid content were subjected to ANOVA procedure and mean separation in the statistical package, SAS 9.1.3 (SAS Institute, NC, Cary, USA). The qualitative morphological parameters of leaves and syncarps were descriptively assessed. The Pearson Correlation Coefficients (PCC) were calculated among leaf, syncarps and the pH, soluble solids and ascorbic acid content separately using CORR procedure in SAS. The flesh types and sensory parameters collected using the taste panel were subjected to FREQ procedure in SAS, and Pearson Chi-square value with the associated probability and Cramer's V coefficient (CVC) were considered to find out the significance and strength of the associations respectively.

Phylogenetic analysis

The *rbcL* and *ITS* sequences of the genus *Artocarpus* available at the phylogenies reported in Williams *et al.*, (2017) were used along with the sequences generated in the present study to test the cladistic relationships of *A. heterophyllus* in Sri Lanka. Distinct DNA alignments were composed in MEGA7 software (Kumar *et al.*, 2016) for *rbcL* and *ITS* using sequences generated from samples having similar voucher numbers (KU856197-KU856312 and KU855474- KU855556) given in Williams *et al.*, (2017). The missing voucher numbers for *rbcL* and *ITS* loci were filled with the sequences with similar names of species. The alignments were separately analyzed in RAxML-HPC2 work flow (Stamatakis, 2006) on CIPRES super computer (Miller *et al.*, 2010) using Bootstrap+consensus algorithm for 1000 iterations. The trees were rooted using the *rbcL* and *ITS* sequences of *Prainea limpato* (out-group) and all the other parameters were set to default. The two alignments were separately analyzed in PAUP V4 (Swofford, 2001) to select the best DNA model of evolution in Akaike Information Criterion (AIC). A Bayesian tree search was carried out in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using separate model parameters for each gene to compensate the differential evolutionary processes for each gene. A total of four (two hot and two cold) Markov Chain Monte Carlo (MCMC) chains were run for 50 million generations. The first 5000 trees were relinquished as burn-in to achieve the maximum convergence in chain performance. The Effective Sample Size (ESS) of the posterior distribution and the chain convergence were assessed using Tracer v1.4 (Rambaut and Drummond, 2007). All the trees were further edited using FigTree v1.4.3 (Rambaut, 2014) for better visualization. The most congruent *rbcL* and *ITS* Maximum Likelihood (ML) trees with bootstrap consensus and maximum clade credibility trees exhibiting both bootstrap (bp) values and posterior probability (PP) values

were taken for the interpretation.

The *ITS* and *rbcL* sequences generated in the present study were aligned using MEGA 7 (Kumar *et al.*, 2016) to check the nucleotide divergence (ND) between two flesh types. A distance tree was built to test ND using uncorrected pair-wise distances looped in Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm in PAUP V4 (Swofford, 2001). The resulting trees were further modified in FigTree v1.4.3 (Rambaut, 2014).

RESULTS

Morphological variability of leaves

The mean values of petiole length, leaf length, leaf width, leaf thickness, surface area, no; of veins, top, middle and bottom angles were not significantly different among SF and FF types ($P > 0.05$). However, the mean tip length was significantly highest in the leaves of SF trees (0.46 cm) than that of FF trees (0.31 cm) ($P < 0.05$) (Table 01).

The overall appearance of the leaves of SF and FF types were not variable. Accordingly, rounded leaf base, entire margin, absence of hair like structures, leathery texture, netted-pinnate venation, darker green ad-axial surface, greyish green ab-axial surface and alternate leaf arrangement in the branches were observed in all studied trees. However, leaf shape was appeared to be different among SF and FF types in which elliptical shape was observed in SF and obovate shape in FF. The leaf tip shape was variable even within SF and FF types where acuminate leaf tip was prominent in SF and acute tip shape was observed in FF (Table 01; Figure 04).

Table 01: Variation among the morphological parameters of leaves of the trees bearing soft and firm flesh types

Parameter	Soft flesh type	Firm flesh type
Petiole length (cm)	2.22a	2.26a
Leaf length (cm)	14.48a	15.03a
Leaf width (cm)	8.13a	8.24a
Leaf thickness (mm)	42.06a	41.64a
Surface area (cm ²)	83.78a	86.67a
Leaf tip length (cm)	0.46a	0.31b
No. of veins	14.22a	13.74a
Top angle (°)	55.97a	52.03a
Middle angle (°)	54.74a	54.56a
Bottom angle (°)	71.61a	69.97a
Leaf shape	Elliptical	Obovate
Leaf base shape	Rounded	Rounded
Leaf tip shape	35% Acute, 65% Acuminate	57% Acute, 32% Acuminate, 11% Cuspidate
Leaf margin	Entire	Entire
Presence of hairs	No	No
Leaf texture	Leathery	Leathery
Venation pattern	Netted pinnate	Netted pinnate
Leaf arrangement	Alternate	Alternate

Means denoted by the same letters within the rows are not significantly different at $P < 0.05$

The parameters related to leaf size were significantly correlated with each other having PCC of 90.9% to 93.1%. The leaf thickness was also significantly correlated with the angles measured between the major side veins and mid rib with PCCs of 29.7% to 81.2%. Out of the three angles measured, the middle angle and the leaf thickness were positively correlated with each other (PCC of 78.2%, $P < 0.05$). The detailed PCC values among all studied leaf morphological parameters are given in Table 02.

Table 02: Pearson Correlation Coefficients (PCC) (%) for leaf morphological parameters

	PL	LL	LW	TS	SA	TL	NV	TA	MA	BA
Petiole length (PL)										
Leaf length (LL)	39.4*									
Leaf width (LW)	53.9*	79.9*								
Tip shape (TS)	17.5*	-12.8	-6.6							
Surface area (SA)	50.1*	90.9*	93.1*	-14.7*						
Tip length (TL)	1.7	14.4*	-3.3	34.4*	-0.3					
No. of veins (NV)	15.4*	23.5*	29.2*	16.3*	25.6*	-8.9				
Top angle (TA)	7.2	0.6	13.6	-14.4*	6.8	9.9	-2.3			
Middle angle (MA)	0.3	0.8	9.9	-3.4	6.5	-3.1	2.2	29.7*		
Bottom angle (BA)	-17.5*	-13.0	3.5	-1.8	-2.4	-3.8	5.3*	23.5*	53.6*	
Leaf thickness (LT)	-5.7	-5.6	12.0	-6.9	4.6	1.0	3.3	65.6*	78.2*	81.2*

* $P < 0.05$

Morphological variability of syncarps

The mean values of syncarp length, breadth and weight were not significantly different among SF and FF types ($P > 0.05$; Table 03). The syncarp shape was found to be very highly variable between and within SF and FF types in which 63% of SF type syncarps was elongated whereas FF type syncarps were mainly either round (45%) or bell shaped (32%). The elongated syncarp shape was very rare among FF syncarps

and only observed in 6%. The fruitlet shape was also variable in which reniform shape was prominent in SF type (50%). However, both reniform and elongated shapes were prominent in FF types (Table 03; Figure 04). The syncarp size parameters were highly and significantly correlated with each other with PCCs of 65.6% to 88.7%. The syncarp breadth was highly correlated with syncarp weight (88.7%) than with syncarp length (80.6%) ($P < 0.05$).

Table 03: Variation among the morphological parameters of syncarps of the trees bearing soft and firm flesh types

Parameter	Soft flesh type	Firm flesh type
Syncarp length (cm)	62.24a	68.85a
Syncarp breadth (cm)	38.69a	38.53a
Syncarp weight (kg)	4.49a	5.99a
Syncarp shape	6% Round, 20% Oval, 10% Thin-elongated, 63% Elongated	45% Round, 6% Oval, 32% Bell, 11% Bell-irregular, 6% elongated
Fruitlet shape	50% Reniform, 30% Pyriform, 20% Elongated	20% Oblong, 30% Reniform, 20% Pyriform, 30% Elongated

Means denoted by the same letters within the rows are not significantly different at $P < 0.05$

Figure 04: Leaf, syncarp and fruitlet morphology of the soft (SF) and firm (FF) flesh trees of jackfruit. A: Variation in the SF type trees. B: Variation in the FF type trees. The adaxial side of the leaves, longitudinal and cross sectional and syncarps of the ten trees of each flesh type are shown. Different scale bars are used for leaves, syncarp sections and fruitlets separately. The three scale bars represent 4 cm, 14 cm and 1.5 cm for leaves, syncarp sections and fruitlets respectively.

Sensory analysis

The flesh types (SF and FF) and sweetness, sourness and aroma (sensory parameters) were subjected to pair-wise association analysis. The flesh type and softness were significantly associated with each other (Cramer's V coefficient= 0.61). A total of 99.38% of respondents identified SF and FF types as soft and firm respectively. The flesh type and sweetness were also significantly associated, but the strength of association was weak as indicated by the lower Cramer's V coefficient (0.04). In general, ripen aril of FF was significantly sweeter than that of SF as 18.13% of respondents said that FF was sweeter whereas 9.38% said SF was sweeter ($P < 0.05$). The flesh type also exhibited significant association with preferred levels of sourness and aroma. However, the strengths of associations were weak as indicated by Cramer's V coefficients. The softness of flesh

was significantly associated with sweetness, sourness and aroma. The preferred levels of sweetness and sourness were significantly associated and inversely correlated where the sourness was recorded as low and sweetness as high. Moreover, the sweetness and sourness showed no significant association with aroma (Table 04).

pH, soluble solids and ascorbic acid

The pH and soluble solid content were not significantly different among the SF and FF types. However, the mean ascorbic acid content was significantly higher in the FF (1.34 mol/dm^3) than that of SF (1.25 mol/dm^3) (Table 05). Also the pH and soluble solid content were found to be significantly and negatively correlated (PCC of 40.7%) whereas they were not significantly correlated with ascorbic acid content ($P < 0.05$).

Table 04. Association analyses for the flesh types and sensory parameters

Association and row percentages of responses				Pearson λ^2	P-Value	Cramer's V-square
Flesh type vs. preferred level of softness						
Flesh type	Softness			195.97*	0.000	0.61
	Firm	Soft				
SF	99.38	0.63				
FF	99.38	0.63				
Flesh type vs. preferred level of sweetness						
Flesh type	Sweetness			14.3*	0.001	0.04
	Low	Medium	High			
SF	52.50	38.13	9.38			
FF	32.50	49.38	18.13			
Flesh type vs. preferred level of sourness						
Flesh type	Sourness			7.69*	0.021	0.02
	Low	Medium	High			
SF	60.00	35.63	4.38			
FF	71.25	21.88	6.88			
Flesh type vs. preferred level of aroma						
Flesh type	Aroma			11.44*	0.003	0.04
	Low	Medium	High			
SF	46.88	43.13	10.00			
FF	43.13	55.00	1.88			
Preferred level of softness vs. preferred level of sweetness						
Softness	Sweetness			13.46*	0.001	0.04
	Low	Medium	High			
Firm	50.51	38.27	11.22			
Soft	29.84	52.42	17.74			
Preferred level of softness vs. preferred level of sourness						
Softness	Sourness			16.12*	0.000	0.05
	Low	Medium	High			
Firm	57.65	36.73	5.61			
Soft	78.23	16.13	5.65			
Preferred level of softness vs. preferred level of aroma						
Softness	Aroma			7.15*	0.028	0.02
	Low	Medium	High			
Firm	47.45	44.39	8.16			
Soft	41.13	56.45	2.42			
Preferred level of sweetness vs. preferred level of sourness						
Sweetness	Sourness			20.26*	0.000	0.03
	Low	Medium	High			
Low	52.21	38.97	8.82			
Medium	74.29	22.86	2.86			
High	79.55	15.91	4.55			
Preferred level of sweetness vs. preferred level of aroma						
Sweetness	Aroma			8.00	0.092	0.01
	Low	Medium	High			
Low	49.26	46.32	4.41			
Medium	38.57	55.71	5.71			
High	52.27	36.36	11.36			

Association and row percentages of responses				Pearson λ^2	P-Value	Cramer's V-square
Preferred level of sourness vs. preferred level of aroma						
Sourness	Aroma			8.38	0.079	0.01
	Low	Medium	High			
Low	39.52	53.33	7.14			
Medium	54.35	42.39	3.26			
High	61.11	33.33	5.56			

* $P < 0.05$

Table 05: Variation of the three biochemical parameters of ripen arils of the trees bearing soft and semi-hard flesh types

Parameter	Soft flesh type	Firm flesh type
pH	5.04a	4.68a
Soluble solids (%)	5.19a	6.87a
Ascorbic acid content (mol/dm ³)	1.25b	1.34a

Means denoted by the same letters within the rows are not significantly different at $P < 0.05$

Phylogenetic analysis

Five sequences of *rbcL* locus generated in this study (512bp in length; MG873181-MG873185) and two more sequences were identical to each other (Figure 05) and the uncorrected pair-wise genetic distances were 0.00 for *rbcL* marker. The best nucleotide substitution model for *rbcL* locus was GTR+I (AC=1 AG=1.55917 AT=1 CG=1 CT=4.821 GT=1; Proportion of invariable sites = 0.926). The phylogeny created in ML framework for *rbcL* was not well resolved and yielded polytomies at prominent nodes. Many nodes had low node supports, thus showed a poor separation by *rbcL* across the *Artocarpus* genus (Figure 05).

The sequences generated for *ITS* locus (678 bp in length; MG847189 - MG847193) were found to be polymorphic and informative. The best nucleotide substitution model for *ITS* was GTR+I+G (AC=1 AG=2.625 AT=1 CG=1 CT=5.023 GT=1; Proportion of invariable sites = 0.423; shape parameter = 0.957). Unlike *rbcL*, the *ITS* phylogeny constructed under ML framework was well resolved and congruent

with published genealogical relationships (Williams *et al.*, 2017). The node support was also higher as more than 70% of bs and PP was observed in all nodes (Figure 06A). The *ITS* locus provided a clear separation of *A. heterophyllus* flesh types in the UPGMA tree (Figure 06B). Since the *ITS* phylogeny was well resolved at species level, the combining *rbcL* and *ITS* datasets were not required to establish the phylogeny and *ITS* alone could be used to determine the genealogy. The FF and SF trees studied in the present analysis were clustered in the *A. heterophyllus* clade with a very low nucleotide divergence (Figure 06A). According to the alignment generated in the present study with respect to the reference sequence, two SNPs were located at 2nd and 105th positions in the *ITS* locus. When these two SNPs are considered together as haplotypes, a unique CC haplotype was specific to SF trees and either CT or TC haplotype was found in FF trees (Figure 07).

Figure 05: The best tree given by ML tree search for *rbcL* locus. Well supported clades with boostrap values higher than 90 and posterior probabilities over 70 are indicated in black dots over the nodes. The colors indicate the two flesh types (purple: SF; pale green: FF). The scale bar indicates the nucleotide substitutions per site.

Figure 06: Phylogenetic analysis conducted on *ITS* locus for the genus *Artocarpus*. **A:** The best tree given by ML tree search for *ITS* locus. Well supported clades with bootstrap values higher than 90 and posterior probabilities over 70 are indicated in black dots over the nodes. The colors indicate the samples containing two flesh types (purple: SF; pale green: FF). The *A. heterophyllus* clade is highlighted in green. **B:** The UPGMA tree constructed for Sri Lankan *A. heterophyllus*. The pairwise genetic distance between each clade is represented over the nodes. The scale bar indicates the nucleotide substitution per site.

Figure 07: Haplotypes detected in ITS locus for FF and SF types. DMB1-DMB5 are voucher numbers of the SF and FF trees sampled. The important base pair (bp) positions of the alignment are shown below. Purple, blue and green shadings indicate the three haplotypes detected.

DISCUSSION

The morphological variation of the jackfruit trees have not been studied in detail for the specific germplasm available in diverse locations. Azad *et al.*, (2007) has reported four discrete groups of jackfruit germplasm according to agro-ecological zones and soil types in Bangladesh and the syncarp characteristics were found to be more affected by the genetic factors whereas soluble solids, size of the seeds and size and shape of the fruitlets were affected by both environmental and genetic factors. The available botanical keys to characterize the syncarp variation of jackfruit are not detailed enough and therefore, in the present study we propose a key with six shapes (Figure 02) to categorically identify the syncarp shapes. This key was able to capture the entire variation of syncarp shapes observed in the present study. The shape of the fruitlets have been studied in the past but the available descriptions are not enough to capture the variation. We propose a key for fruitlet shapes as well with four categories to capture the variation (Figure 03). However, care must be taken to assess the syncarp shape immediately after harvesting as the ripen flesh is disintegrating fast upon harvesting.

The ripen flesh type of jackfruit is referred to as *Wela* and *Waraka* in Sri Lanka based on the degree of softness in flesh. Attempts have been made to assign English terms for *Wela* and *Waraka* as soft and crisp but the crispiness is more applicable to processed and dried food items like potato chips or fruits and nuts that make crispy sound when biting. Therefore, we

wish to propose the terms soft flesh for *Wela* and firm flesh for *Waraka* as these two flesh types are different only in terms of the degree of firmness and there is no crispiness associated.

Except the leaf tip length, the other leaf morphological parameters among SF and FF types were not significantly different. The SF leaves have significantly longer tips in which they are 0.15 cm longer than FF leaf tips ($P < 0.05$). Although it is premature to use the trait leaf tip length as a morphological marker to discriminate SF seedlings from FF seedlings, this would provide a potential further direction to develop an easily employable morphological or molecular marker for discrimination. The traits representing syncarp size were not significantly different among two flesh types implying that they could not be used for discrimination. Out of the categorically assessed traits of the leaf morphology, the leaf shape was found to be elliptical in SF type and obovate in FF type indicating its potential applicability in differentiating trees of two different flesh types. The syncarp and fruitlet shapes are also found to be highly diverse among the two flesh types and SF trees contain mainly elongated syncarps whereas FF syncarps are mostly round indicating potential involvement of flesh type in determining the syncarp shape. The shapes of the syncarps and fruitlets are really diverse within the studied set of trees and similar levels of variations were reported in Azad *et al.*, (2007) and Jagadeesh *et al.*, (2007). The presence of the higher level of variation in syncarp shape even within the same tree would be an important direction for further research. It has

been discovered that the insertion of the retro-transposable element *Rider* into *SUN*, a major gene controlling fruit shape of tomato causes the natural variation exist (Xiao *et al.*, 2008; Jiang *et al.*, 2009). However, no molecular studies have been conducted to assess the genetic basis of the shape variation in compound fruits. The jackfruit would be an ideal candidate for such a study due to very broad variation and availability of diverse germplasms in tropical and subtropical countries.

The PCC of 65.6%, 78.2%, and 81.2% among the leaf thickness and top, middle and bottom angles respectively reflect that leaf thickness is determined by the micro geometrical venation pattern within the leaf. However, other leaf morphological parameters are not strongly correlated with each other indicating possible complex environmental and genetic mechanisms to design overall leaf shape and size. The correlation analysis among size traits of syncarps revealed that the syncarp weight is determined more by breadth than its length. This would be supported by the fact that broad syncarps contain higher number of fruitlets than narrow syncarp.

Association analysis revealed that SF and FF in ripen arils could be easily detected by human subjects with 99.38% of accuracy. However, there are genotypes identified showing in between characteristics of SF and FF and generally people in ethnic range consider them as non-edible when ripened. Such genotypes have not been found in the present study but often observed in different regions of Sri Lanka (Pers. Comm.). The clear discrimination between SF and FF flesh types would provide an ideal platform to dissect the genes underlying this variation. The ascorbic acid content was significantly less in SF (1.25 mol/dm³) than FF (1.34 mol/dm³). This is parallel with the general perception that FF contains higher amount of vitamins than SF. It is also appeared that pH is negatively correlated with soluble solids but it was not correlated with ascorbic acid content indicating there must be another organic acid present within flesh to decide the

level of acidity. The sweetness and sourness were found to be inversely associated according to the sensory panel analysis and industrial processing of jackfruit flesh into jam, syrup and other herbal products prefer the blend of unique sweetness, sourness and aroma present in natural flesh. Therefore, breeding strategies and selection from the varying germplasms must be targeted to find such genotypes and they should be disseminated to the growers using micro-propagation to preserve the genetic identity. It is clear from present study that there are no SF and FF genotypes with highest preferred sweetness and highest preferred aroma which invite breeders to develop such cultivars.

The phylogenetic relationships of jackfruit has been studied in detail (Williams *et al.*, 2017) however, no attention has been paid to discriminate SF and FF types based on the DNA sequence polymorphisms. It was observed in the present study that the DNA bar-coding locus *rbcL* cannot be used to fully infer phylogenetic relationships and decipher species delimits in the genus *Artocarpus* (Figure 05). However, the DNA bar-coding locus *ITS* could be used with great discriminatory power to accomplish the phylogenetic interpretations and species delimits (Figure 06). *A. heterophyllus* clade is well established in the species-tree of the genus *Artocarpus* with higher node support values (bs > 90 and PP > 70). The *A. heterophyllus* trees assessed in the present study are clustered together (Figure 06A) and with shallow genetic divergence (0.00%-0.09%) (Figure 06B). The SNP haplotypes detected within the *ITS* region are associated with the flesh type, as CC haplotype was found in SF specifically. Although either CT or TC were found in FF, it is reliable to suggest that DNA bar-coding of *ITS* region for seedlings and rejection of any seedling that has CC haplotype results the MASS for FF type. It would be important to validate this strategy with *ITS* haplotypes for large sets of jackfruit germplasm before implementing in the seedling production at nursery stage. Rather than relying sequence based discrimination it is also cost effective, if these haplotypic variation can be used to develop a straight forward DNA

diagnostic assay such as sequence characterized amplified region (SCAR) or cleaved amplified polymorphic DNA (CAP) markers.

CONCLUSIONS

The assessment of leaf, syncarp and sensory perception of SF and FF types of jackfruit trees revealed that the leaf tip length of SF is significantly 0.15 cm longer than that of FF type and ascorbic acid content of FF is significantly 0.09 mol/dm³ higher than that of FF type. The SF leaves were always elliptical in shape whereas FF leaves were obovate. The other leaf morphological parameters, syncarp and fruitlet shapes are highly variable within and between trees belonged to two flesh types. The association analysis revealed that flesh type could be easily detected by the human subjects. The sensory parameters sweetness and sourness of the flesh are inversely associated. The DNA bar-coding of SF and FF trees revealed that the *rbcL* locus cannot be used to infer the phylogenetics of the genus *Artocarpus* however, *ITS* locus can be effectively employed to characterize the genetic diversity and species delimits. The haplotypic

variation within *ITS* locus in studied trees are two-fold in which CC haplotype is characteristic to SF type and CT or TC is found in FF type.

Data availability statement

The DNA sequence dataset generated during the current study is available in the National Center for Biotechnology Information (NCBI), GenBank (<https://www.ncbi.nlm.nih.gov/genbank/> under the accession numbers of MG847189-MG847193 and MG873181-MG873185. (https://www.ncbi.nlm.nih.gov/popset?LinkName=nuccore_popset&from_uid=133576242).

The morphological, biochemical and phytochemical datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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