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VEGETATIVE AND LEAF ANATOMICAL TRAITS FOR TAXONOMIC DELIMITATION OF *SALACIA* L. IN SRI LANKA

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Abstract

Salacia comprises 200 species throughout the tropical countries. In Sri Lanka, five species of this genus *S. chinensis*, *S. diandra*, S. *oblonga*, *S. reticulata* and *S. acuminatissima* are recorded that are considered as the synonyms under *S. diandra* by Wadhwa in 1996 during the revision of genus. These species are very much similar in vegetative morphology and their flowering is irregular and rare. Due to these reasons, their proper identification and determination are difficult and identification of new combinations of taxonomic characters is necessary. A multivariate analysis was conducted for 98 distinct populations using 20 vegetative and 43 leaf anatomical characters including PCA, PCO, CA, SIMPER and correlation analysis. The results support the recognition of four phenetic groups, which corresponds to species *S. chinensis*, *S. reticulata*, *S. oblonga* and *S. diandra*. *S. acuminatissima* that was recognized by Kostermans (1992) was not supported by the analysis and formed a cluster together with *S. diandra* with no support as a separate cluster. The study failed to recognize any distinct vegetative characters to define these taxa but propose a combination of vegetative or leaf anatomical characters and also highlights the necessity of molecular data to supplement the vegetative and leaf anatomical data to resolve the ambiguity between the *S. acuminatissima* and *S. diandra*.

Introduction

Sri Lanka is an island approximately 65,000 km^2 in extent, located 29 km south of the southern tip of peninsular India. The Island is centrally situated in the Indian Ocean between latitudes 5° 55' - 9° 51' North and longitudes 79° 41' - 81° 53' East (Karunarathne, 2001). Despite being a relatively small island, Sri Lanka is strikingly diverse in ecosystems due to spatial variation of rainfall, altitude and soil (Punyawardhana, 2004). These in turn have contributed to the very high biodiversity along with endemic fauna and flora. Sri Lanka's biodiversity is significantly important both in a regional and global scale due to highest species density in angiosperms (number of species present per 10,000 sq. km) and 3154 flowering plant species are recorded in Sri Lanka of which 894 species are endemic to the country (MOE, 2012). With the high endemism and the threats associated to the original natural vegetation, Sri Lanka has been designated as one of the 34 global hotspots of the world along with Western Ghats of India (Gunawardana *et al.*, 2007). The rich diversity in flowering plants of this country has produced large number of plants with immense economic value. Over 600 species have been used as medicinal plants and large number of them are used in the indigenous systems of medicine (Karunarathne, 2001). Among them, the members of the genus *Salacia*, are considered as a medicinally valuable group of plants that has antimicrobial, anti-oxidative, anti-inflammatory, anti-diabetic, nephroprotective, and anti-mutagenic properties (Chawla *et al.*, 2013; Medagama, 2015).

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The genus *Salacia* belonging to family Celastraceae comprises of about 200 species of woody lianas, scandent or sometimes erect shrubs distributed throughout the tropical parts of India, Sri Lanka, Burma, Malaysia, Solaman islands and Africa (Wadhwa, 1996). According to the National Red List of flora and fauna 2012 there are five *Salacia* species in Sri Lanka, namely *S. chinensis* L., *S. diandra* Thw., *S. oblonga* Wall ex Wight & Arn., *S. reticulata* Wight. and *S. acuminatissima* Kosterm (Plate 1). *S.acuminatissima* is a species recognized by Kostermans in 1992. However, during the revision of the genus in 1996, Wadhwa did not recognize the species but considered it as a synonym under *S. diandra*. During the National red-listing in 2012 (MOE, 2012), *S. reticulata, S.oblonga, S. acuminatissima* and *S. diandra* were categorized under endangered category (EN) while *S. chinensis* was recognized in the near threatened category (NT). A recent study conducted by the authors to re-evaluate the conservation status of the species using preliminary data, has upgraded *S. diandra* to the Critically Endangered (CR) category while the status of *S. oblonga* and *S. reticulata* have been downgraded from Endangered (EN) to Near threatened (NT) category (Senevirathne *et al.*, 2019).

Plate 1. Habits of (A) *S. diandra,* (B) *S. acuminatissima*, (C) *S. chinensis*, (D) *S. oblonga* and (E) *S. reticulata*

With the discrepancy in species limits of the genus *Salacia* in Sri Lanka, it is important to determine the validity of *S. acuminatissima*, recognized by Kostermans (1992) as a legitimate species as both are endemic to the country. The species is said to be outstanding because of the

bent, sharp, long fruit acumen which are quite different from other *Salacia* species of Sri Lanka (Kostermans, 1992). *Salacia* members share very similar characters in vegetative morphology. When considering the leaf characters two or three species share similar overlapping characters such as leaf shape, leaf margin, leaf tip, leaf base etc. Further, the flowering is irregular and restricted to a very short time period. Based on the recent field observations for the past few years, from 2016 to 2020, we have not been able to observe flowers and fruits from few species. Due to those reasons, distinguishing one *Salacia* species from another has become a major issue in taxonomic and other studies. Therefore, it is important in identifying new characters that would aid in species identification, especially evidence from anatomical features. Anatomical data have successively resolved species complexes in many plant taxa (Udage and Yakandawala, 2017; Chitchak *et al.*, 2018).

Multivariate analysis is a powerful tool for the assessment of the patterns of variation at the specific and infraspecific levels. Unlike the phylogenetic methods that aims to reconstruct evolutionary relationships among established taxa, morphometrics is particularly useful for drawing lines between taxa, to ascertain differences between different cytotypes or geographical races, or to discover the most important characters that differentiate taxa (Marhold, 2011). Therefore, the present study was carried out with the aim of investigating the species limits of *Salacia* occurring in Sri Lanka, with the aid of multivariate analysis by using morphological and leaf anatomical traits.

Materials and Methods

Sampling

Ninety-eight samples (Operational Taxonomic Unit [OTUs]) were collected from Ninety-eight distinct populations covering all the climatic regions as well as all the administrative provinces in Sri Lanka. Collected samples were authenticated using herbarium specimens deposited in the National Herbarium, Peradeniya, Sri Lanka and literature (Wadhwa, 1996).

Cording of characters

Leaf morphological characters: Three individuals at similar maturity level were selected from each population and ten measurements were obtained from each of these selected individuals. Twenty vegetative characters were recorded by direct observation of specimens. All the scale measurements were taken using a simple ruler.

Leaf anatomical characters: Forty-three leaf anatomical characters were recorded from the ninety eight samples that were used for the vegetative study and five replications were done for each sample. The 3rd leaf from the bud was used to obtain data. Firstly, thin cross sections were obtained by hand using sharp blades across the petiole, across the mesophyll region and across the median vascular system. Secondly, adaxial and abaxial epidermal leaf surfaces were taken by minimizing the damage to epidermal cells. Thirdly, 1 cm × 1 cm matured leaf sections from middle leaf blade area and leaf margin area were cut off and cleared using 0.8 NaOH solution. Solutions were replaced until leaf sections turned colorless. Colorless leaf parts were stained using 0.1% safranin solution. Temporary slides were prepared from all types of sections used to record data. Observations were taken from cleared leaf samples using low power (10X4), high power 10X40) and the oil immersion lens (10X100). Fixed magnification was used for one particular character. Microscope (OYMPUS CX21) lenses were calibrated using objective micrometer (0.01mm, Erma, Tokyo, Japan). Photographs of all sections were taken (Canon, 5X optical zoom, 16 mega pixels). All the recorded vegetative and leaf anatomical characters are presented in Tables 1 and 2.

Table 1. Qualitative characters and their character states used for the multivariate analysis.

Character		Character states					
Leaf vegetative characters							
1	Petiole Nature	Curved	Not curved				
2	Adaxial surface colour	Dark green	Pale green				
3	Lamina shape	Elliptic	Lanceolate	Oblong	Oblanceolate		
4	Leaf texure	Coriaceous	Sub coriaceous	Non coriaceous			
5	Leaf lamina nature	Flat	Twisted	Bend along midrib			
6	Leaf margin	Serrulate	Shallowly crenate	Crenate	Entire		
7	Leaf base	Attenuate	Acute	Rounded			
8	Leaf apex	Micronulate	Mucronate	Acute	Caudate	Obtuse	Cuspidate
9	Prominent veins at the base	Present	absent				
10	Primary vein size	Large	moderate	thin			
11	Secondary veins prominant in adaxial surface	Present	Absent				
12	Secondary veins prominent in abaxial surface	Present	Absent				
Leaf Anatomical characters							
13	Shape of petiole epidermal cells	Cubic	Rectangular	Irregular	Barrel		
14	Pattern of vascular system in petiole	Kidney shaped	Kidney shape with deep groove	Kidney shape with circle on top			
15	Pattern of vascular system in median vascular region	Kidney shaped	Kidney shaped with deep groove	Triangular			
16	Shape of palisade cells	Barrel shaped	Rod shaped	Thin barrel shaped	Cubic		
17	Upper epidermal anticlinal cell wall type	Irregular	Beaded	Buttressed			
18	Shape of upper epidermal cells	Polygonal	Irregular	Irregular with deep grooves			
19	Lower epidermal anticlinal cell wall type	Beaded	Buttressed				
20	Shape of Lower epidermal cells	Polygonal	Irregular - radial	Irregular - Bilateral			
21	Nature of marginal veins	Opened	Closed				
22	Fimbricated marginal veins	Fimbricated	Not fimbricated				

Table 2. Quantitative characters used for the multivariate analysis.

	Vegetative leaf	Petiole	Median vascular system	Mesophyll region	Upper epidermal peel	Lower epidermal peel	Cleared leaf
1	Av. Petiole length	Thickness of cuticle	Width of adaxial epidermis	Thickness of cuticle	Cell wall thickness of the epidermal cells	Stomatal number	Vein islet number
2	Av. Leaf length	Height of epidermal cells	Width of abaxial surface	Height of abaxial surface	No. of epidermal cells per field of vision	Stomatal index	Vein termination number
3	Av. Leaf width	Width of epidermal cells	Thickness of cuticle	Height of adaxial surface	Epidermal cell width at the widest point	Av.no. of subsidiary cells	
4	Leaf L/W ratio	No.of cortex cell layers		Palisade ratio		Guard cell width	
5	Leaf tip angle			No.of palisade layers		Guard cell length	
6	leaf base angle			Height of palisade cells		L/W ratio of guard cells	
7	No. of secondary vein pairs			Width of palisade cells		No. of epidermal cells per field of vision	
8	Ratio of vein pairs to leaf length			L/W ratio of palisade cells		Epidermal cell width at widest point	
9				Thickness of palisade area		Epidermal cell wall thickness of	
10				Number of spongy layers		Diameter of stomata complex size	
11				Thickness of spongy area			

Multivariate analysis

Twenty vegetative characters (12 qualitative and 8 quantitative) and 43 leaf anatomical characters (10 qualitative and 33 quantitative) were recorded from 98 OTUs for the multivariate analysis. Data were entered into Microsoft excel version 10 spread sheets separately as vegetative and leaf anatomical characters and combined into common spread sheet. Finally resulted excel sheet was transformed into a file suitable for the multivariate analysis using PAST 16.0 software. There were 22 qualitative characters and 41 quantitative characters in the final data sheet for the analysis.

The quantitative variables were standardized by subtracting the character mean and dividing by the standard deviation (Ospina, 2016) to avoid unequal influences on the results due to characters measured at different scales (Marhold, 2011). Euclidian distance matrix was selected for the quantitative data analysis. Then Gower distance matrix was selected for both quantitative and qualitative data (mixed data) analysis due to the primary matrix consists of a mixture of binary, multistate qualitative, ordinal and quantitative characters (Cupido, 2003; Marhold, 2011).

A Principal Component analysis (PCA) was performed for the quantitative variables. Eigen values for each principal component (PCs) was checked and highest values were recorded. Then contribution of quantitative variables was represented using the score plot of first two PCs. And most contributed variables for the most prominent PCs were identified using PC loadings. Finally, Principal Coordinate analysis (PCO) was performed for both quantitative and qualitative variables (Cupido, 2003).

To test the repeatability of the phenetic groupings obtained from ordination analysis including PCA and PCO, a Cluster Analysis (CA) was performed by using PAST 16.0 version. Cophenetic values were used to present the degree of relationship between the original distance and the tree matrix. Out of the results, phenogram with the highest Cophenetic value was selected as the best cluster solution. SIMPER analysis (similarity percentage analysis) was performed to check the characters that supported to the grouping obtained from the CA. Correlation analysis was performed using quantitative vegetative variables considering each CA groups using Corrplot R package.

Results and Discussion

Ordination analysis based grouping

PCA for the quantitative variables and PCO for the quantitative and qualitative variables were conducted under the ordination analysis. According to the PCA, first four principal components with the highest Eigen values are accounted for 73.64% of the cumulative variance and individual contribution of PCs are 36.09, 19.24, 12.78 and 5.54%, respectively (Table 3). The contribution of quantitative variables was represented using the score plots among most prominent four PCs and score plot of first two PCs was selected as the most justified representation (Fig. 1). According to the score plot, factor scores of PC1 and PC2 implies a clear pattern of grouping of *Salacia* with respect to quantitative variables. Considering the five clusters generated, three clusters corresponding *S. reticulata, S. acuminatissima* and *S. oblonga* were clearly separated while other two clusters corresponding to *S.diandra* and *S.chinensis* have formed separate groups that are closely placed with each other.

According to the PCA loadings of the first four principal components, contribution of each variable for the PCs for the grouping, the width of petiole epidermal cells, no. of epidermal cells per field of vision in upper epidermis and stomata complex size were the characters that contributed most for the first PC while thickness of petiole cuticle, width of abaxial surface in midrib region, width of palisade cells, guard cell length, guard cell L/W ratio, lower epidermal cell width at widest point and cell wall thickness of lower epidermal cells are most contributed factors of the second PC. Similarly, the width of palisade cells can be identified as the most contributed factor for PCA grouping for the third PC while for the fourth PC, vein islet number, vein termination number, average leaf length, leaf L/W ratio and number of secondary vein pairs to leaf length are the most contributing variables.

Table 3. Eigen values and variance explained by the four principal components.

Values	PC1	PC2	PC3	PC4
Eigen value	14.9506	7.9698	5.2930	2.2958
Proportion of variance	36.09	19.24	12.78	5.54
Cumulative proportion	36.09	55.33	68.10	73.64

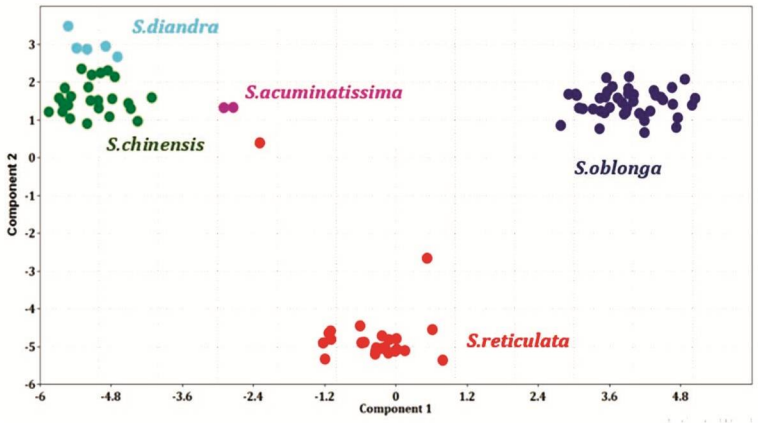

Fig. 1. Score plot of first two PCs for quantitative variables.

When considering the results of PCO analysis (Fig. 2), the distribution of OTUs, between coordinate 2 versus coordinate 1, *S. reticulata*, and *S. oblonga* formed clearly separated groups. Compared to the PCA results, only 2 OTUs of *S. reticulata* showed deviation from the mother cluster. Although other three clusters corresponding to *S. chinensis*, *S. diandra* and *S. acuminatissima* formed separate clusters, they are closely related to the each other.

Considering the results of both PCA and PCO analyses, *S. reticulata* and *S. oblonga* formed clearly separated clusters. Although *S. acuminatissima* and *S. diandra* formed separate clusters they are more closely related to the *S. chinensis*.

Cluster analysis-based grouping

Out of the three phenograms resulted from CA, the phenogram that resulted from the Paired group algorithm (Fig. 3) was selected as the best representation of the degree of relationship between the original distance matrix and the tree matrixes based on the Cophenetic correlation values (Table 4).

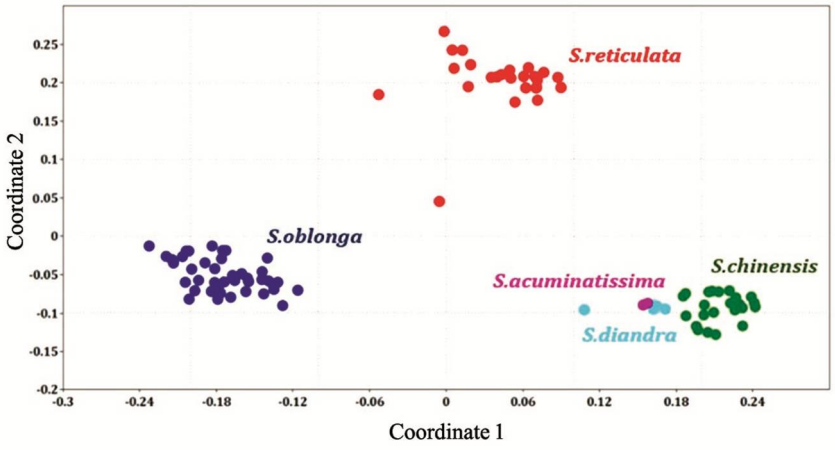

Fig. 2. Score plot of principal coordinates for quantitative and qualitative variables.

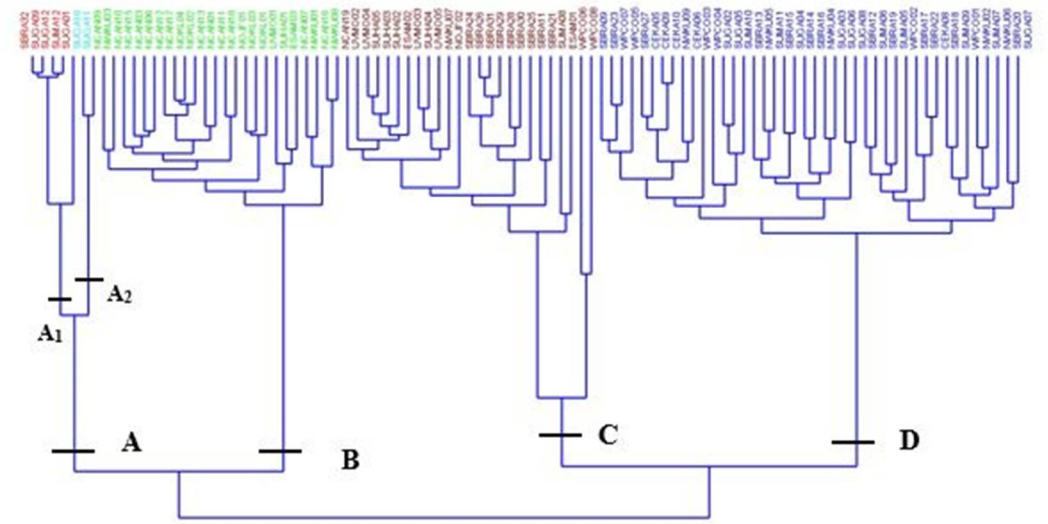

Fig. 3. Phenogram depicting the groups within *Salacia* using vegetative and leaf anatomical characters

Table 4. The Cophenetic values obtained using different clustering methods.

Clustering method	Cophenetic values
Single linkage	0.955
Paired linkage	0.960
Wards method	0.723

Based on the phenogram, four distinct phenetic groups named A, B, C and D can be identified along the 0.35 distance level. Phenetic group A divides into two distinct sub groups, separating at a distance of 0.23, one corresponding to *S. diandra* (A1) and the other corresponding to *S. acuminatissima* (A2). Phenetic group B represents *S. chinensis* where the individuals are very similar to one another and the distance between two individuals is less than 0.01distance value. The phenetic group C representing *S. reticulata* consists of two distinct sub-clusters, where two OTU's separate early at a distance of 0.3 and the rests cluster closer to each other. The phenetic group D represents *S. oblonga*. The group is represented by a large number of representatives, from different populations, and is initially divided into two subgroups at a distance values of 0.15. Both these subgroups further divides where the distance between any given divisions is less than 0.01distance value indicating a close resemblance of the members within the group.

When considering the SIMPER analysis (Similarity Percentage Analysis) that corresponds to the CA grouping and the characters contributing for each cluster, the group A, which is composed of *S. diandra* (A1) and *S. acuminatissima* (A2), almost all qualitative and quantitative characters are shared by both groups except the shape of the lower epidermal cells (A1- irregular and A2 polygonal) and anticlinal wall pattern of lower epidermis (A1- beaded walls and A2 buttressed walls) that contributed for their sub-grouping (A1 and A2).

The percentage dissimilarity, which could be used to interpret the degree of differentiation among groups, was calculated using SIMPER. When comparing dissimilarity between the two

subgroups, A1 and A2, overall average dissimilarity value was recorded as -125.7, which indicates that the dissimilarity between these two subgroups is very low and negligible.

Salacia acuminatissima, which was described by Kostermans in 1992, as a species with the diagnostic characters listed as coriaceous, elliptic leaves, apically broadly acuminate and blunt and, basally shortly cuneate with thin a midrib. In addition ellipsoid, apically narrowed into a long, bent, sharp acumen fruit was also recognized as a diagnostic feature. However, the multivariate analysis with all the said vegetative characters did not support *S. acuminatissima* as a separate species. This result corroborates Wadhwa (1996) where he did not recognize *S. acuminatissima* as a sperate species during the Revision of Flora but considered under the *S.diandra*.

However, Wadhwa (1996) was with the view that these characters are not strong in delimiting a new species and, therefore, recognized *S. acuminatissima* under *S. diandra* with a broader description, where leaves are described as sub-coriaceous, ovate-oblong, basally narrow and apically apiculate or acuminate. During the present analysis using a larger number of morphological and anatomical characters based on SIMPER analysis, leaf shape, leaf base, leaf apex, leaf texture and mid rib nature were not highly contributing characters for the grouping. This supports Wadhwa's (1996) circumscription of merging the two taxa. However, molecular data-based analysis would provide additional evidence for a final circumscription of the two taxa.

As mentioned before, *S. chinensis* formed a separate group that subsequently divide into small subgroups. According to the CA, the average dissimilarity value based on SIMPER analysis, between group A and B is recorded as 392.9. Both these groups are distinct from one another completely by shape of palisade cells, nature of leaf lamina, pattern of petiole and leaf vascular bundles and nature of marginal veins.

Group C and group D originate from the same axis and they share more similarity in several quantitative characters such as width of adaxial leaf surface, height of palisade cells and almost all qualitative characters like shape of petiole epidermal cells, leaf apex, leaf base, shape of palisade cells, leaf margin, nature of leaf lamina, leaf texture and anticlinal wall pattern etc. These two groups deviate from one another especially due to the pattern of leaf vascular system and anticlinal wall pattern of upper epidermis. Overall average dissimilarity between these two groups is indicated as 169.5.

Considering the group C that represents *S. reticulata* with reference to CA, there are two sub-clusters and SIMPER average dissimilarity value between these two sub-groups are recorded as 140. These sub-groups are formed due to the difference in anticlinal wall patterns and epidermal cell shapes, which could not be considered as strong enough to consider these as different. Although group D representing *S. oblonga* is divided into several sub-groups, all of them show a closer similarity. These results indicate that the species show a higher intraspecific variation.

Correlation analysis for quantitative vegetative variables

The correlation plots for quantitative vegetative characters for clusters recovered in the CA are represented in Fig. 4. In these plots positive correlations are displayed in blue and negative correlations are displayed in red while colour intensity and size of the circle is proportional to the correlation coefficient.

According to correlation plots in Cluster A, number of secondary vein pairs/average leaf length to number of secondary vein pairs (0.92) showed highest correlation followed by number of secondary vein pairs to leaf tip angle (0.91), leaf L/W ratio to average leaf width (0.86), number of secondary vein pairs/average leaf length to leaf tip angle (0.84) and average leaf width to average

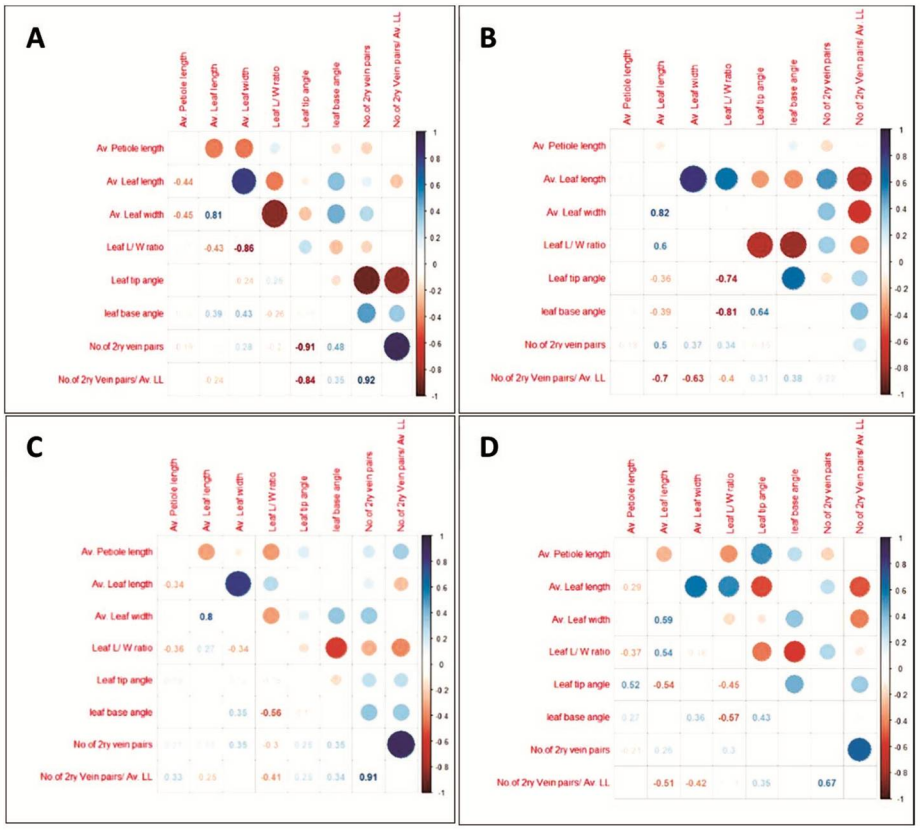

Fig. 4. Correlation analysis for the quantitative vegetative variables of A) cluster A, B) Cluster B, C) Cluster C, D) cluster D.

Leaf length (0.81). The results of the correlation plots further support the results obtained from other analyses in recognizing the Cluster A consisting of S. *acuminatissima* and *S. diandra* as a single species. Within other three clusters namely B, C and D that represent *S. chinensis, S.reticulata* and *S. oblonga* respectively, average leaf width and average leaf length showed highest correlation.

Considering the results of all analyses, PCA, PCO and CA, the recognition of *S. acuminatissima* as a distinct species is not supported but it is grouped as a sub-cluster within the main phenetic group A with no supporting evidence from either vegetative or leaf anatomical characters to define it as a separate species. With the widely used molecular sequence data, obtaining support to supplement the vegetative and leaf anatomical data would be the next step to resolve the ambiguity between the taxonomic status of *S. acuminatissima and S. diandra*.

Conclusion

The multivariate analyses support the recognition of four phenetic groups within the genus *Salacia* in Sri Lanka, which corresponds to the species *S. chinensis, S. reticulata, S. oblonga* and *S. diandra*. The fifth species. *S. acuminatissima* described by Kostermans in 1992 was not supported by the present study. The species delimitation boundries are clear with respect to

S.chinensis, *S.oblonga* and *S.reticulata*. . However, use of molecular data is recommended in deciding the final circumscription of *S.diandra* and *S.acuminatissima.*

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