

RESEARCH ARTICLE

Molecular phylogeny and chromosomal evolution of endemic species of Sri Lankan Anacardiaceae

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Abstract: Family Anacardiaceae comprises 70 genera and approximately 985 species distributed worldwide. Sri Lanka harbours 19 species in seven genera, among these 15 are endemics. This study focuses on regionally restricted endemics and native Anacardiaceae species, which have not been investigated before at molecular and cytological level. Nuclear rDNA ITS and plastid *matK* regions were sequenced for ten species, having nine endemics and one native, and incorporated into the existing sequence data for phylogenetic analyses. The topologies resulting from maximum parsimony, maximum likelihood and Bayesian inference are congruent. Family Anacardiaceae forms a monophyletic group having monophyletic subfamily Anacardioideae and paraphyletic subfamily Spondioideae. Tribes Anacardieae, Semecarpeae and Rhoeae form subclades within the major clade of Anacardioideae. All the endemic species occupy correct position in the molecular phylogeny as per the existing classification except for *Camposperma zeylanica*, which shows a close relationship to members of the subfamily Spondioideae. The position of non-native species *Trichoscypha acuminata* also remains unclear. Chromosome counts and karyograms were constructed for five endemic species. The chromosome numbers incorporated in the tree range from $2n = 28-58$. Species of tribe Rhoeae have the lowest chromosome number ($2n = 24, 28, 30$) while species of tribe Semecarpeae have the higher numbers ($2n = 50, 52, 58$). Chromosome numbers mapped on the phylogeny shows that dysploidy had played a role in the evolution of the species of the family Anacardiaceae in Sri Lanka.

Keywords: Anacardiaceae, *Camposperma zeylanica*, chromosome counts, molecular phylogeny, *Semecarpus* species.

INTRODUCTION

Family Anacardiaceae R. Br., the cashew family, contains 70 genera harbouring 985 species of trees, shrubs and subshrubs. The members are well known for causing contact dermatitis reactions. They occupy a considerable fraction of the tropical flora dispersed in tropical, subtropical and temperate regions holding Malaysian region as the center of diversity (Li, 2007; Pell *et al.*, 2010; Zotz, 2013). As a tropical country, Sri Lanka nourishes 19 wild species including 15 endemics and numerous cultivated and hybrid species (MOE, 2012).

Some members of the family are cultivated throughout the world for their edible fruits and seeds such as cashew (*Anacardium occidentale* L.), mango (*Mangifera indica* L. and numerous varieties), and hog plums (*Spondias* L.). In addition, some species have great medicinal properties: to treat fever (*Buchanania* Spreng., *Comocladia* P.Br.), for hepatitis (*Haematostaphis* Hook. f.), and for gastrointestinal illness (*Anacardium* L., *Antrocaryon* Pierre). Some species are known for quality wood and rot resistant timber such as quebracho (*Schinopsis* Engl.), as well as for landscaping (*Cotinus* Mill. and *Toxicodendron* Mill.) (Pell *et al.*, 2010).

Earlier investigations suggest that the origin of this family is 55 to 65 MYA in the Paleocene era (Hsu, 1983;

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Muller, 1984). According to Gentry (1982) Anacardiaceae has a Gondwanan origin which is supported by fossil records as well as the current worldwide distribution of the family.

Anacardiaceae belongs to phylum Tracheophyta, class Magnoliopsida, order Sapindales. The family was first proposed by Lindley in 1830. Different classification systems were proposed for the family through the years (Table 1). From newly emerging research findings in recent years, Kim *et al.*, (2017) were able to sequence the complete chloroplast genome of *Rhus chinensis* Mill, and Jo *et al.* (2017) were able to sequence the complete plastome of *Mangifera indica*. These findings will aid to increase the robustness of the phylogenetic interpretation of the family Anacardiaceae in the future. However, despite a number of taxonomic treatments, phylogenetic positions of many understudied genera are yet to be revealed.

Chromosome numbers have been used for taxonomical treatments (Cox *et al.*, 1998; Bateman *et al.*, 2003; Schneeweis *et al.*, 2004; Almeida *et al.*, 2007; Koch *et al.*, 2012). According to Vinicius da Luz *et al.*, (2015), only 14 % species belonging to the family Anacardiaceae have been investigated cytologically. Raven (1975) justified and concluded that the ancestral basal chromosome number of the family is $x = 7$, suggesting that the evolution of the family is at tetraploid level. Of the two subfamilies of Anacardiaceae,

subfamily Anacardioideae has been cytologically studied from early ages. Maheshwari (1934) initiated the chromosomal studies on the genus *Mangifera* giving an uncertain chromosome number for *M. indica* L. as $2n = 52 - 58$ and later, Darlington and Ammal (1955) stated the number as $2n = 40$. These studies were expanded by Mukherjee (1950; 1957) and Pierozzi and Rossetto (2006) by investigating chromosome counts for different species of the genus *Mangifera* and varieties of *M. indica*, and confirmed the somatic number as $2n = 40$. Index to Plant Chromosome Numbers (IPCN) gives seven records of gametophytic count of *Semecarpus anacardium* L.f. as $n = 29$ and $n = 30$ (Mehra, 1976; Gill *et al.*, 1981; 1990; Bir *et al.*, 1982; Singhal & Gill, 1990). The Flora of Malesiana has stated that the chromosome count of *Semecarpus* L.f. as $2n = 60$ (Hou, 1978) while Pell *et al.* (2010) confirmed that the gametophytic count of genus *Semecarpus* is $n = 30$. Chromosome number of the genus *Pistacia* varies as $2n = 24, 28$ and 30 (Huang *et al.*, 1989; Parfitt & Badenes, 1997). Among the species of subfamily Spondiadeae, $2n = 32$ is the common diploid number found in *Spondias* spp. and *Dracontomelon dao* (Blanco) Merr. & Rolfe (respectively in Almeida *et al.*, 2007; Oginuma *et al.*, 1999). *Sclerocarya caffra* Sond. exhibits a sporophytic count of $2n = 26$ (Paiva & Leitao, 1989), *Lannea coromandelica* (Houtt.) Merr. and *Poupartia axillaris* (Roxb.) King & Prain have gametophytic counts of $n = 15$ (Singhal & Gill, 1990) and $n = 12$ (Mehra, 1976), respectively. Almeida *et al.* (2007) revealed the presence of large blocks of heterochromatin

Table 1: Summary of classification systems of Anacardiaceae established by different authors. Tribes (Tr.), groups (Gr.), clades (Cl.) and the subfamilial (Subfam.) classification are shown.

Author	Interfamilial affinities (Tr./ Gr./ Cl./ Subfam.)
Benthams and Hooker (1862)	Tr. Anacardieae and Tr. Spondieae
Marchand (1869)	Tr. Astronieae, Tr. Buchananieae, Tr. Mangifereae, Tr. Pistacieae, Tr. Rhoideae, Tr. Semecarpeae, Tr. Spondieae, Tr. Tapirieae, Tr. Thyrsoideae
Eichler (1875–1878)	Gr. <i>Anacardium</i> , Gr. <i>Pistacia</i> , Gr. <i>Rhus</i> , Gr. <i>Schinus</i> , and Gr. <i>Spondias</i>
Engler (1881, 1883, 1892)	Tr. Dobineae, Tr. Mangifereae, Tr. Rhoideae, Tr. Semecarpeae, Tr. Spondieae
Takhtajan (1987)	Subfam. Anacardioideae, Subfam. Spondioideae (Spondieae, Rhoideae, Semecarpeae), Subfam. Julianoideae, Subfam. Pistacioideae, Subfam. Dobineoideae
Mitchell and Mori (1987)	Subfam. Anacardieae, Subfam. Spondiadeae, Subfam. Semecarpeae, Subfam. Rhoeae, Subfam. Dobineae
Wannan and Quinn (1991)	Gr. A: (Tr. Anacardieae, Dobineae, Rhoeae, and Semecarpeae excluding four genera), Gr. B: (Tr. Spondiadeae with <i>Androtium</i> , <i>Buchanania</i> , <i>Camptosperma</i> , and <i>Pentaspadon</i>)
Terraza (1994)	Cl. A1 (Anacardieae, Dobineae, Rhoeae, and Semecarpeae), Cl. A2 (Tr. Spondiadeae and genus <i>Pentaspadon</i>)
Takhtajan (1997)	Subfam. Anacardioideae, Subfam. Spondioideae (Spondiadeae, Rhoeae, Semecarpeae), Subfam. Julianoideae, Subfam. Pistacioideae
Pell (2004)	Subfam. Anacardioideae and Subfam. Spondioideae
Mitchell <i>et al.</i> , 2006	Subfam. Anacardioideae and Subfam. Spondioideae (with emended descriptions and circumscriptions)

CMA⁺ in the species of the genus *Spondias*. The number and location of CMA bands were found to vary among the *Spondias* species and the distribution patterns of these heterochromatin blocks can be used to identify each *Spondias* species separately (Almeida *et al.*, 2007).

Chromosomal evolution of angiosperms has been widely discussed among the taxonomic community for decades (Cox *et al.*, 1998; Schneeweiss *et al.*, 2004; Hansen *et al.*, 2006; Mayrose *et al.*, 2009; Duan *et al.*, 2015) while cytological evolution of many plant families still remains unknown. This study is an attempt to bridge this gap in chromosomal evolution in Angiosperms by contributing molecular and cytological data on regionally restricted species of the family Anacardiaceae. The present investigation mainly focused on endemic and native species of the family Anacardiaceae in Sri Lanka. Here we address (1) clarification of the phylogenetic position of the endemic and native taxa using nuclear ITS and plastid *matK* regions; (2) chromosomal counts for endemic species; and (3) analysis and investigation of the evolution of chromosome number across the combined phylogeny.

METHODOLOGY

Materials for molecular phylogeny and chromosome counts

Sampling included ten species of four genera representing all the genera with endemics in the country, which also corresponds to three tribes out of four found in Sri Lanka. Collected locations of each species are given in Ariyaratne *et al.* (2017). All the species investigated were included in separate and in combined phylogenetic

analyses. Cytological studies were conducted only in five species due to unavailability of viable seeds to obtain actively growing young roots. Vegetative propagation using different media was attempted as an alternative but unfortunately none of the trails gave positive results due to high level of secondary metabolites in secretions. *Semecarpus* seeds were stored in air tight bags for 1–3 wk until germination initiated and later transferred to air tight units with moistened coir dust medium and then placed at ± 20 °C temperature, having 12 h light and 12 h dark cycles. Seeds of *Mangifera zeylanica* were potted in a sand medium and transferred to a soil medium after the germination. Actively growing rootlets, grown up to 1 cm, were used for chromosome counts.

DNA extraction and PCR amplification

Genomic DNA was extracted from *c.* 45 mg silica gel dried leaf materials (Chase & Hills, 1991) using Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. As an initial step to remove mucilaginous polysaccharides, the ground samples were washed 2–10 times with sorbitol buffer until no visible mucilaginous substances appeared in the sample solution (Russell *et al.*, 2010; Souza *et al.*, 2012).

Two gene regions were amplified by polymerase chain reaction using six primers; nuclear genomic ITS-1 and ITS-2 (rDNA ITS) regions, and plastid *matK* (partial *trnK* intron with complete *matK* gene) region (Table 2). PCR amplifications were carried out with 40 μ L reaction mixture containing 20 μ L of 1x GoTaq[®] Green Master Mix (Promega Corporation, USA), 1.6 μ L of 25 mM MgCl₂, 1.6 μ L of 10 mg/mL bovin serum albumin (BSA) acetylated, 0.8 μ L of 5u/ μ L

Table 2: Details of primers used in this study. Utility of each primer during this study is given as used for PCR amplifications (PCR) or/and DNA sequencing (Seq).

Region	Primer	Sequence (5'–3')	Utility		Reference
			PCR	Seq	
ITS	ITS 4	TCCTCCGCTTATTGATATGC	PCR	Seq	White <i>et al.</i> , 1990
	ITS 5	GGAAGTAAAAGTCGTAACAAGG	PCR	Seq	White <i>et al.</i> , 1990
<i>matK</i>	<i>trnK</i> -799f	CCYTGTTYTRACYRTATYGCACATATGTAT	PCR		Barfuss <i>et al.</i> , 2016
	<i>trnK</i> -2662r	CTCGAACCCGGAAGTAGTCGG	PCR		Castello <i>et al.</i> , 2016
	<i>matK</i> 880R	CCAGAAATTGACAAGGTAATATTTC		Seq	Duangjai <i>et al.</i> , 2009
	<i>matK</i> 1070F	CCATAGTTCCAATTATTCCTCTG		Seq	Duangjai <i>et al.</i> , 2009
	<i>matK</i> 1300R	CGAAGTATATAYTTYATTTCGATACA		Seq	Samuel <i>et al.</i> , 2005
	<i>trnK</i> 3F	AGTYGGGTCKAGTRAATAAA	PCR	Seq	Pell, 2004
	<i>matK</i> 10R	CGCTGTGATAATGAGAAAGA	PCR	Seq	Pell, 2004

GoTaq® Flexi DNA polymerase (Promega Corporation, USA), 0.8 µL of each primer (10 mmol/L), 9.4 µL of nuclease-free water (Promega Corporation, USA), and 5 µL of DNA template were used. Applied Biosystems Veriti® 96 well Thermal Cycler (NY, USA) was used

for PCR amplifications and the PCR profiles used for amplification are given in Table 3. DNA sequencing was done by a 8 capillary sequencer (Applied Biosystems, 3500 Genetic Analyzer) at the Department of Molecular Biology and Biotechnology, University of Peradeniya.

Table 3: PCR profile used for amplification of ITS region and *matK* regions

Process	ITS region (30 cycles)		<i>matK</i> region (30 cycles)	
	Temperature (°C)	Time (s)	Temperature (°C)	Time (s)
Initial denaturation	97	30	97	120
Denaturation	97	30	94	78
Annealing	54	30	58 – 48	120 – 30
Extension	72	90	72	180
Final extension	72	420	72	900

Sequence alignment and phylogenetic analysis

DNA sequences of non-native species and out group taxa were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>, last accessed: 2017-12-04). Raw sequences were assembled, edited and processed in SeqMan Pro, DNASTar® Lasergen ver. 8.1 (DNASTAR, Inc. 2009), BioEdit ver. 7.1.9 (Hall, 1999), Muscle software (Edgar, 2004a; b) and Mesquite software ver. 3.04 (Maddison & Maddison, 2015). Homogeneity between ITS data and plastid data was tested using the incongruence length difference (ILD) test according to Farris *et al.* (1995), as implemented in PAUP ver. 4.0 (Swofford, 2003).

To infer phylogenetic relationships maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) were carried out. Trees were rooted with two outgroup taxa of the family Burseraceae; *Bursera simaruba* (L.) Sarg. and *Canarium album* Blanco. MP analysis was performed using PAUP* software ver. 4.0 (Swofford, 2003). For each dataset (ITS, *matK* and combined) heuristic search strategy was used with 1,000 replicates of random sequence addition, tree-bisection-reconnection (TBR) branch swapping and retaining multiple trees (MulTrees) by saving only 10 trees per replicate. Bootstrap method was used with full heuristic search mode followed by TBR branch swapping and random sequences addition with 1,000 replicates to estimate the support for each clade by holding only the groups with frequency greater than 50 %. The consistency index (CI) and retention index (RI) for tree topologies were calculated with PAUP. ML and BI analyses were performed for the combined matrix. The best fitting substitution model was determined with jModelTest ver. 2.1.7 (Guindon & Gascuel, 2003; Darriba

et al., 2012) using the Akaike information criterion (AICc). Evolutionary substitution models for each marker were calculated. The generalised time reversible (GTRGAMMA) model and gamma-distributed rate variation across sites and a proportion of invariable sites were used for the analysis. The ML rapid bootstrap analysis was performed with 1,000 replicates with search for best-scoring ML tree in one run. This analysis was conducted in RAxML ver. 8.2.4 (Stamatakis, 2014). Bayesian inference was conducted to obtain posterior probabilities using MrBayes ver. 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The same nucleotide substitution model (GTR+G) as in ML analysis was used with 10,000,000 generations Markov chain Monte Carlo (MCMC) chains with a sampling frequency of every 1,000 generations. The initial 25 % samples from each run were discarded as burning. A majority rule consensus tree was calculated using the remaining trees to obtain the posterior probabilities for each node. The resulting trees were visualised and edited in Fig Tree ver. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>, last accessed 2017-12-04).

Chromosome counts and preparation of karyotypes

Chromosome analysis protocol of Weiss-Schneeweiss *et al.* (2009) was optimised for the members of family Anacardiaceae to arrest the mitotic spindles. Collected actively growing root tips were pre-treated with 0.002 M 8-hydroxyquinoline for 2 h at room temperature and 2.5 h at 4 °C. Then the root tips were fixed for 2 h in freshly prepared Carnoy (3:1, ethanol: acetic acid) solution and stored at -20 °C until use. Standard Feulgen staining method was followed by washing the stored rootlets with distilled water and hydrolysing with 5N HCl for 30 min.

Root samples were washed again with distilled water and stained with colourless Schiff's reagent for 1 h in darkness. Prior to the squash preparation root tips were dissected and treated with lactopropionic-orcin (45 %) solution. Slides were prepared with squash technique and heat fixed. Chromosomes were examined and photographed with a binocular microscope (Zeiss Axio Lab.A1) equipped with a digital camera (Zeiss AxioCam ERc5s). Images were processed with ZEN 2012 (©Carl Zeiss Microscopy GmbH, 2011).

While preparing the karyotypes, more than three digital images were taken in different focus planes to capture the best 2D appearance of each chromosome. Karyotypes were prepared using the image processing software, Corel Photo- Paint x7 ver. 17.1.0.572 (©2014 Corel Corporation) by arranging homologous pairs of chromosomes based on descending order of their sizes.

RESULTS AND DISCUSSION

Statistics of data matrixes and phylogenetic trees

This study mainly focused on combining the molecular and cytological data of regionally restricted endemic and native Anacardiaceae species of Sri Lanka. Out of 15 endemics, nine species and one native taxon were included in this study. All these species were investigated for the first time at molecular and cytological level. Extensive field investigations were carried out throughout the country and most of the species were found at the Kanneliya Man and Biosphere Reserve (Ariyaratne *et al.*, 2017). Some species were declared

as Critically Endangered Possibly Extinct [CR(PE)] due to the inability to locate in the wild (MOE, 2012).

Length variations were observed among sequences in both ITS (rDNA) and *matK* datasets. Nuclear ITS dataset is composed of 33 ingroup taxa while that of *matK* contains 31 taxa, which includes nine endemic and a native taxon from Sri Lanka. Statistics and data characteristics from the maximum parsimony analysis of both gene regions and the combined datasets are given in Table 4.

Phylogenetic analysis of ITS, *matK* and combined data matrixes

Maximum parsimony analysis of ITS data matrix revealed that the family Anacardiaceae is monophyletic. Within this super clade, a large clade is formed by species belong to the subfamily Anacardioidae whereas species of subfamily Spondioidae remain paraphyletic together with the genus *Camposperma*. Two major clades of tribe Anacardiaceae and tribe Semecarpeae show weak bootstrap support. Most of the relationships between the species belonging to the tribe Rhoeae remain unresolved. The MP analysis of the *matK* matrix resulted in congruent topologies to that of the ITS. It also revealed the monophyly of the family Anacardiaceae. The subfamily Anacardioidae forms a monophyletic super clade and tribe Anacardiaceae forms a monophyletic subclade. Sri Lankan endemic *Semecarpus* species build a monophyletic group of tribe Semecarpeae while non-native *Semecarpus* species are paraphyletic.

Table 4: Statistics and data characteristics from the maximum parsimony analysis of both gene regions and the combined datasets.

	ITS	<i>matK</i>	Combined
Total number of taxa	33	31	35
Number of Sri Lankan taxa	10	10	10
Number of ingroup taxa	31	29	33
Number of outgroup taxa	2	2	2
Total characters	1,000	2,251	3,250
Number of variable characters (%)	451 (45%)	348(15.5%)	799(24.5%)
Number of parsimony informative characters (%)	274(27.4%)	117(5.2%)	391(12%)
Number of equally parsimonious trees	5,810	9,970	24
Tree length	1547	493	1975
Consistency index	0.473	0.554	0.563
Retention index	0.507	0.667	0.567

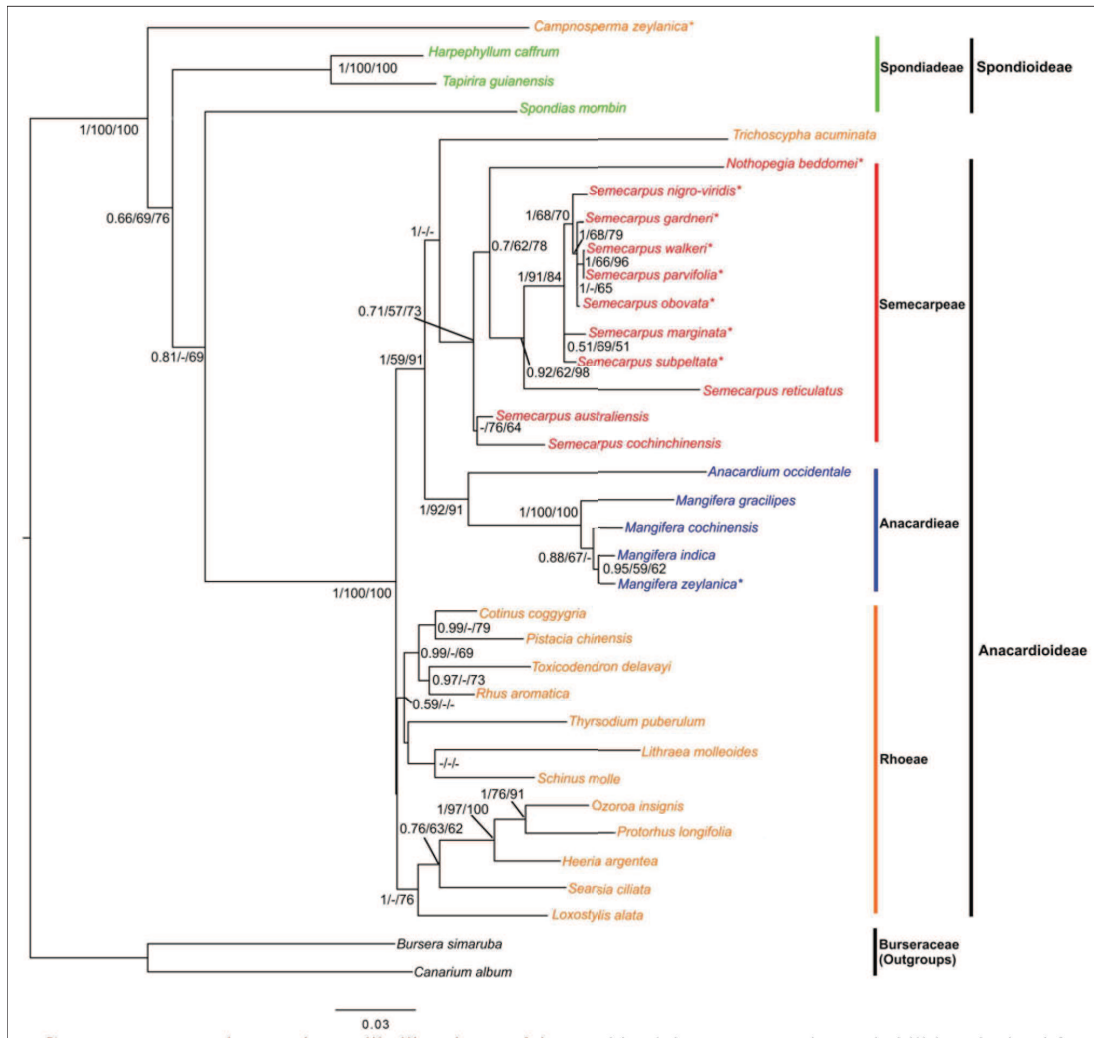


Figure 1: Best-scoring maximum likelihood tree of the combined dataset. Posterior probabilities obtained from BI (PP-BI), bootstrap percentages ($> 50\%$) of MP (BS-MP) and ML (BS-ML) analyses are denoted and separated by a slash. Hyphens indicate the PP-BI < 0.5 and BS-MP/BS-ML $< 50\%$. Sri Lankan species are indicated with bold letters and an '*'. Colours and vertical lines indicate the allocation of species in present classification system.

The resulting trees of the combined matrix (Figures 1 and 2) analysed with all three phylogenetic inference methods (MP, ML and BI) were congruent for the major clades. The family Anacardiaceae resolved as a monophyletic super clade [posterior probability (PP-BI): 1/ MP bootstrap values (BS-MP): 100/ ML bootstrap values (BS-ML): 100]. This order is used throughout the text. Within this, the subfamily Anacardiioideae form a major clade comprising of subclades representing three tribes Semecarpeae (0.71/57/73), Anacardiaceae (1/92/91)

and non-monophyletic Rhoeeae. Non-native *T. acuminata* is separated from the other members of the Rhoeeae and clusters together with Semecarpeae. Further, the endemic *Camposperma zeylanica* of Rhoeeae resolved as the basal taxa, close to the paraphyletic subfamily Spondioideae. The best-scoring maximum likelihood tree of the combined dataset (Figure 1) and the 50 % majority rule consensus tree (Figure 2) resulting from Bayesian inference are shown.

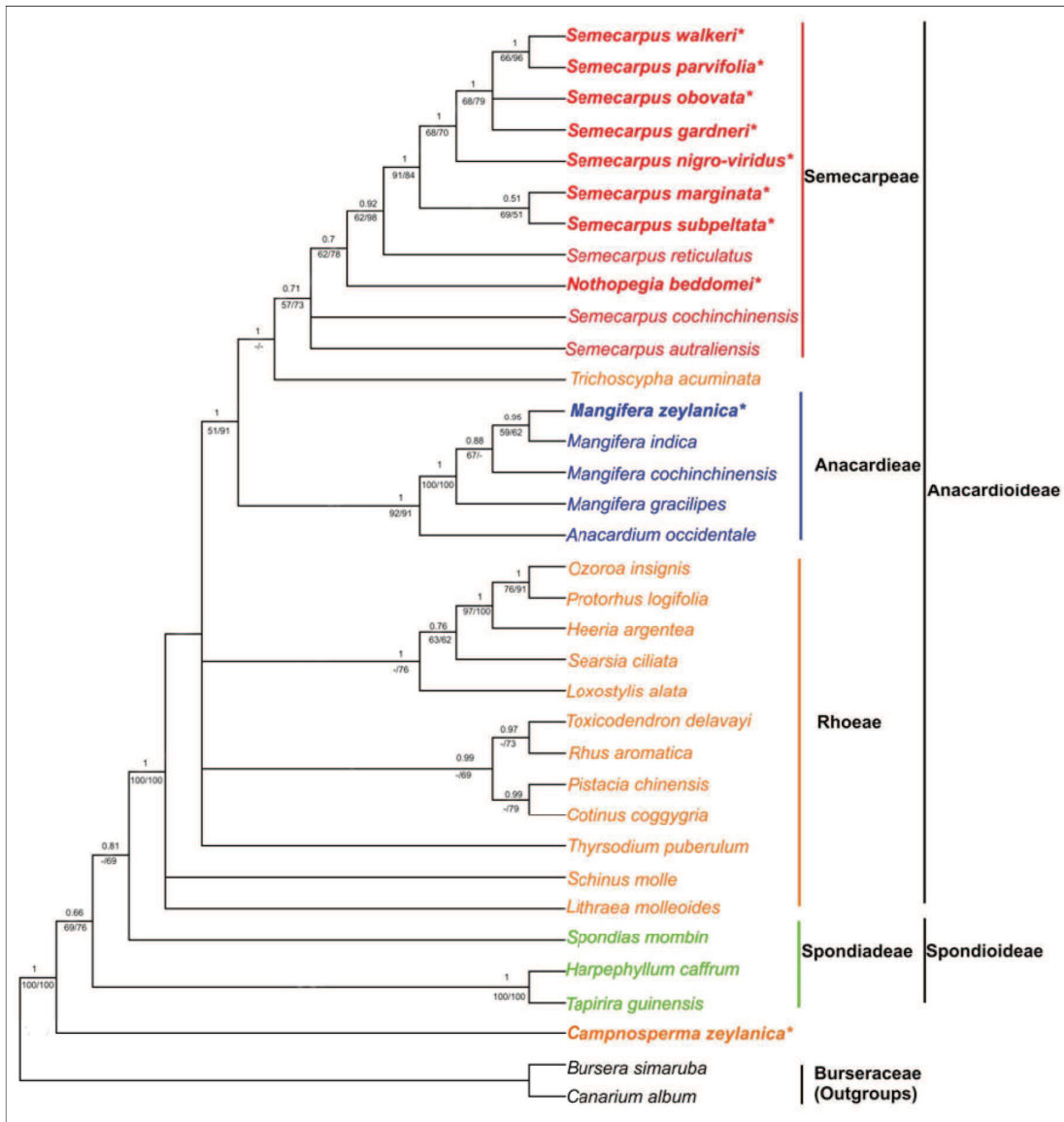


Figure 2: Majority rule (50 %) consensus tree obtained from Bayesian inferences for the combined matrix of nuclear ITS and *matK* datasets. Posterior probabilities (PP-BI > 0.5) are given above the nodes. Bootstrap percentages (> 50 %) of maximum parsimony (BS-MP) and maximum likelihood are indicated below the nodes separated by a slash. Hyphen indicates the bootstrap values less than 50 %. Sri Lankan species are indicated with bold letters and an '*'. Colours and vertical lines indicate the allocation of species in present classification system.

Phylogenetic relationships in Anacardiaceae

The family Anacardiaceae forms a large monophyletic group with strong support (1/100/100). One of the main aims of this investigation was to clarify the phylogenetic positions of Sri Lankan endemic species

of Anacardiaceae. Within the subfamily Anacardioidae, all the endemic taxa including *Mangifera zeylanica* and *Semecarpus* species are well resolved in their subfamilial and tribal positions. The native species *Nothopegia beddomei* is placed in tribe Semecarpeae indicating its close relationship to genus *Semecarpus*. The non-native

species of *Trichoscypha acuminata*, which was assigned to tribe Rhoeae based on morphological characters is found in close relation within the tribe Semecarpeae with weak support. The clade of tribe Semecarpeae is resolved with weak bootstrap and posterior support (0.71/57/73). *Semecarpus australiensis* and *S. cochinchinensis* resolve as basal taxa in this clade. *Nothopegia beddomei* (the native species used in this study) and *S. reticulatus* are positioned outside the major subclade formed by the Sri Lankan endemic *Semecarpus* species with strong support (1/91/84). *Semecarpus walkeri* and *S. parvifolia* show a close relationship with strong support (1/66/96). *Semecarpus obovata* and *S. gardneri* have resolved outside to *S. walkeri* and *S. parvifolia* with weak support forming an inner clade. *Semecarpus nigro-viridis* shows sister relationship to this inner clade with moderate support (1/68/70). *Semecarpus marginata* and *S. subpeltata* also exhibit close affinity with a weak support of 0.51/69/51. Within the tribe Anacardiaceae, subclade of genus *Mangifera* is strongly supported (1/100/100) compared to the subclade that contains *A. occidentale* (1/92/91). *Mangifera zeylanica* shows close relationship to *M. indica* than other species used in this study. However, the relationship is weakly supported (0.95/59/62). Tribe Rhoeae has formed a weakly supported clade. Many nodes of this clade are not resolved in maximum parsimony analysis but there are a few resolved nodes with the Bayesian inference. The endemic species *Camposperma zeylanica*, which has been allocated to the subfamily Anacardiaceae clusters with subfamily Spondioideae. This species has resolved as basal taxa for the family Anacardiaceae in all three phylogenetic analyses with weak support (0.66/69/76). The species belonging to the subfamily Spondioideae, tribe Spondioideae has formed a paraphyletic clade with strongly supported subclade (1/100/100).

Several researchers (Wannan & Quinn, 1991; Terraza, 1994; Takhtajan, 1997; Pell, 2004; Mitchell *et al.*, 2006) have tried to solve the ambiguities in the classification and phylogeny of the family Anacardiaceae. This attempt was made to understand the phylogenetic position of Sri Lankan endemic Anacardiaceae species and to look into chromosomal evolution of these endemics. All the Sri Lankan endemic species have been well placed in their corresponding taxonomic positions except for *Camposperma zeylanica*. Phylogenies constructed for all three datasets (ITS, *matK*, and combined) support the placement of endemic species in the tribes Anacardiaceae and Semecarpeae, having *C. zeylanica* as a basal taxon.

As per the systematic history of the family Anacardiaceae, genus *Camposperma* had been taxonomically problematic. Wannan and Quinn (1991)

tried to treat genus *Camposperma* taxonomically by assigning it in 'Group B' as per their classification together with species of Spondioideae and three other genera of tribe Anacardiaceae and Rhoeae.

Family Anacardiaceae was reported to be paraphyletic (Terraza, 1994) with Burseraceae nested within the cashew family, sister to tribe Spondioideae. However, the combined analysis of *rbcL* and morphological data suggested monophyly of family Anacardiaceae (Terraza, 1994) with similar grouping as suggested by Bentham and Hooker (1862) and Wannan and Quinn (1991), having genus *Camposperma* within subfamily Anacardiaceae. Since then this genus has remained as a member of subfamily Anacardiaceae.

Lepidote scales are very rare in the family Anacardiaceae but is characteristic of the genus *Camposperma*. These scales are similar to that found in genus *Tapirira* (*Tapirira lepidota* Aguilar & Hammel), which is a member of subfamily Spondioideae (Hammel *et al.*, 2014). In Anacardiaceae, stigmas are usually capitate and ovary 1-locular. However, *Camposperma* contain bi-locular ovaries whereas almost all the species of subfamily Spondioideae are composed of more than one locular. Genus *Camposperma* together with genus *Pegia* Coleb. contain discoid stigmas. Other than these, members of this genus share the tribal (Spondioideae) characters of having spondias-type endocarp as categorised by Wannan and Quinn (1990), partially pachychalazal seeds, stilt roots and polygamodious plants. Therefore, the placement of this genus in subfamily Anacardiaceae has been highly controversial; however, the present study corroborates with the placement of genus *Camposperma* in the subfamily Spondioideae. This placement could be further supported by additional taxa from other regions.

Pell (2004), built a phylogeny of Anacardiaceae with *matK* plastid DNA sequences. In this investigation, 33 taxa belonging to the five tribes of the family Anacardiaceae and five species of the outgroup family Burseraceae were used. The tree topologies of the present study agree with the phylogenetic tree constructed by Pell (2004), which shows a close relationship between the tribes Semecarpeae and Anacardiaceae that form a clade, as well as paraphyly of the subfamily Spondioideae.

The position of the displaced non-native species of *T. acuminata* also remains questionable, but since the sequences were obtained from GenBank, errors in plant identification and processing cannot be excluded. Therefore, further investigations have to be carried out to find the exact place of this species in the phylogenetic

Table 5: Chromosome numbers of the endemic species together with the size, types and numbers of chromosomes in each karyotype

Species	$2n = 2x$	Size (μm)	Types of chromosomes (number)
<i>Mangifera zeylanica</i>	42	1–3	Metacentric (6), Submetacentric (10), Acrocentric (3), Telocentric (2)
<i>Semecarpus marginata</i> (Figure 3A, 3B)	58	1–4	Metacentric (14), Submetacentric (7), Acrocentric (3), Telocentric (5)
<i>Semecarpus nigro-viridis</i> (Figure 3C, 3D)	56	0.5–2	Metacentric (13), Submetacentric (10), Telocentric (5)
<i>Semecarpus obovata</i> (Figure 3E, 3F)	52	1.5–4.5	Metacentric (13), Submetacentric (10), Telocentric (3)
<i>Semecarpus parvifolia</i>	50	0.5–1.5	Metacentric (3), Submetacentric (11), Acrocentric (5), Telocentric (6)

tree by including properly identified and sequenced individuals in the analyses.

Chromosome numbers and karyotypes in Anacardiaceae

Chromosomes numbers of five endemic Anacardiaceae species are reported for the first time given in the Table 5 and Figure 3. Chromosome numbers obtained during this study together with the previously published reports are given in the Table 6.

Phylogeny and chromosomal evolution

Recent advances in molecular biology has allowed more precise evaluation of the importance of polyploidy in flowering plant evolution with most, if not all, plants being of ancient paleopolyploid origin (Soltis *et al.*, 2009; Du *et al.*, 2012; Li *et al.*, 2015). With the recent developments in the field, the most appropriate way of estimating chromosome numbers in phylogenetic trees is by using ancestral character reconstruction software like ChromEvol and Chromplod. However, the applicability of these software for the present dataset was not possible due to the lack of cytological data of foreign taxa. Thus, the chromosomal numbers obtained in this study and counts from the literature survey (Table 6) were manually mapped to the phylogeny obtained from the combined matrix to elucidate the chromosomal evolution of this family (Figure 4).

The two out group taxa of Burseraceae show chromosome numbers of $2n = 24$ and $2n = 48$, which supports the polyploidy within the family. Chromosome numbers of the family Anacardiaceae ranges from $2n = 24$ to $2n = 58$. Among these, most of the species belong to the tribe Rhoëae recorded $2n = 30$ with the exception

of *S. molle* and *P. chinensis* with chromosome numbers $2n = 24$ and 28, respectively.

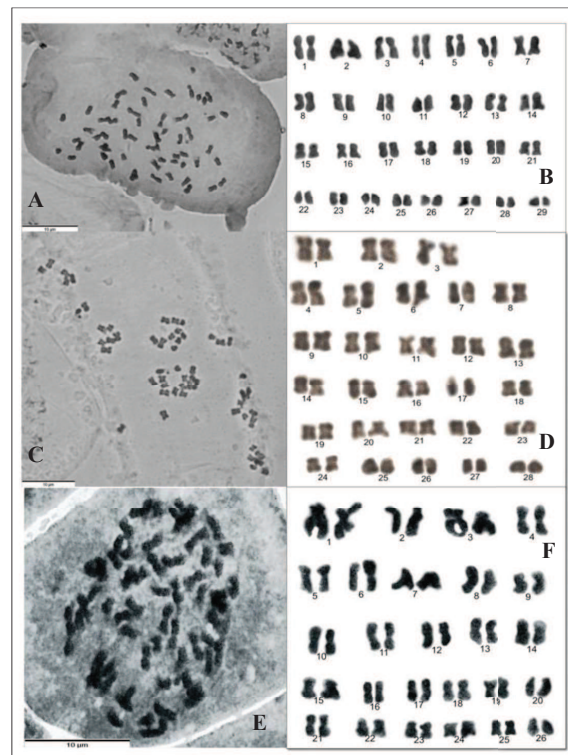


Figure 3: Chromosome spreads and karyotypes of *Semecarpus* species. A - *S. marginata* chromosome spread ($2n = 58$); B - Karyotype of *S. marginata*; C - *S. nigro-viridis* chromosome spread ($2n = 56$); D - Karyotype of *S. nigro-viridis* E - *S. obovata* chromosome spread ($2n = 52$); F - Karyotype of *S. obovata*. Scale bar = 10 μm .

Table 6: Accession numbers for the species downloaded from GenBank together with reported chromosome numbers with references and the putative ploidy levels are given. Species that were studied for the first time indicated in bold letters and the ‘*’ indicates the endemic species of Sri Lanka. Accession numbers marked with ‘NA’ were not included in the corresponding matrixes.

Species Name	GenBank Accession Number		CN (2n)	Chromosomal Studies	
	ITS	matK		Putative Ploidy level	Reference(s)
<i>Anacardium occidentale</i> L.	KF664192.1	AY594459.1	42	2x	Aliyu & Awopetu (2007)
<i>Cotinus coggygria</i> Scop.	AY510157.1	HE966907.1	30	2x	Vladimirov & Dimitrova (2007)
<i>Harpephyllum caffrum</i> Bernh.	KF664197.1	JF270814.1	-	-	-
<i>Heeria argentea</i> Meisn.	AY594378.1	JX518129.1	-	-	-
<i>Lithraea molleoides</i> (Vell.) Engl.	KF420989.1	KF555405.1	30	2x	Coleman (1982)
<i>Loxostylis alata</i> A. Spreng. ex Rchb.	AY531201.1	JX517988.1	-	-	-
<i>Mangifera cochinchinensis</i> Engl.	AB071676.1	AB924713.1	-	-	-
<i>Mangifera gracilipes</i> Hook.f.	AB071686.1	NA	-	-	-
<i>Mangifera indica</i> L.	KF664199.1	AY594472.1	40	2x	Pierozzi & Rossetto (2006)
<i>Ozoroa insignis</i> Delile	AY594381.1	KX146378.1	-	-	-
<i>Pistacia chinensis</i> Bunge	EF193080.1	NA	24	2x	Huang et al. (1989)
<i>Protorhus longifolia</i> (Bernh.) Engl.	EF089146.1	JX517542.1	-	-	-
<i>Rhus aromatica</i> Aiton	AY641494.1	AY594494.1	30	2x	Parfitt et al. (1990), Yi et al. (2007)
<i>Schinus molle</i> L.	AY641512.1	JX517745.1	28	2x	Oginuma et al. (1993)
<i>Searsia ciliata</i> (Licht. ex Schult.) A.J.Mill.	AY641513.1	KX146267.1	-	-	-
<i>Semecarpus australiensis</i> Engl.	NA	AY594479.1	-	-	-
<i>Semecarpus cochinchinensis</i> Engl.	NA	AB925248.1	-	-	-
<i>Semecarpus reticulatus</i> Lecomte	KR532565.1	NA	-	-	-
<i>Spondias mombin</i> L.	AF445882.1	KP774611.1	30	2x	Vladimirov & Dimitrova (2007)
<i>Tapirira guianensis</i> Aubl.	DQ787402.1	KF981295.1	-	-	-
<i>Thyrsodium puberulum</i> J.D. Mitch. & D.C. Daly	FJ037790.1	FJ514723.1	-	-	-
<i>Toxicodendron delavayi</i> (Franch.) F.A. Barkley	FJ945937.1	NA	-	-	-
<i>Trichoscypha acuminata</i> Engl.	AY594389.1	KC627761.1	-	-	-
<i>Bursera simaruba</i> (L.) Sarg. (Outgroup)	GQ378130.1	KJ772596.1	24	2x	Fedorov (1969)
<i>Canarium album</i> (Lour.) DC. (Outgroup)	DQ517524.1	KJ510921.1	48	2x	Chen et al. (1993)
*<i>Camposperma zeylanica</i> Thw.	MG672041	MG787233	-	-	-
*<i>Mangifera zeylanica</i> (Blume) Hook.f.	MG672042	MG787234	42	2x	Present study
*<i>Semecarpus marginata</i> Thw.	MG672044	MG787237	58	2x	Present study
*<i>Semecarpus gardneri</i> Thw.	MG672043	MG787236	-	-	-
*<i>Semecarpus nigro-viridis</i> Thw.	MG672045	MG787238	56	2x	Present study
*<i>Semecarpus obovata</i> Moon	MG672046	MG787239	52	2x	Present study
*<i>Semecarpus parvifolia</i> Thw.	MG672047	MG787240	50	2x	Present study
*<i>Semecarpus subpeltata</i> Thw.	MG672048	MG787241	-	-	-
*<i>Semecarpus walkeri</i> Hook.f.	MG672049	MG787242	-	-	-
<i>Nothopegia beddomei</i> Gamble	MG672050	MG787235	-	-	-

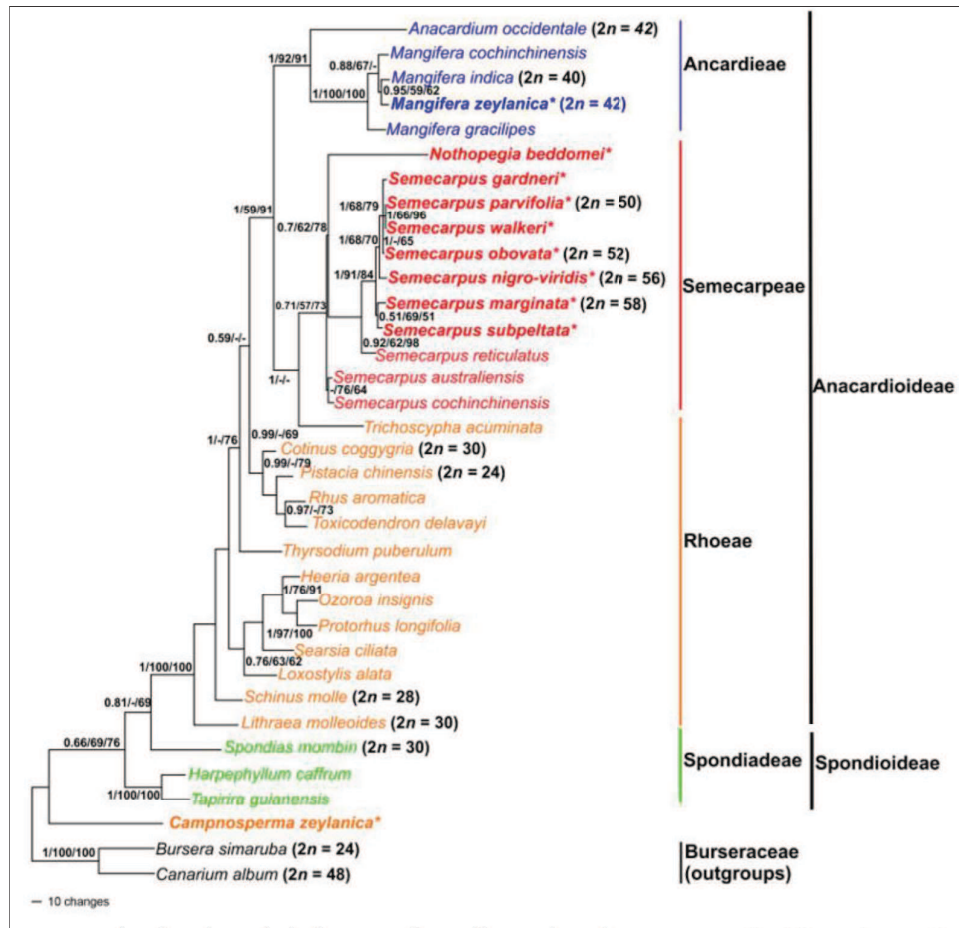


Figure 4: Randomly selected phylogram of equally parsimonious trees resulted from the maximum parsimony analysis of the combined dataset. Posterior probabilities obtained from BI (PP-BI > 0.5), bootstrap percentages (> 50 %) of MP (BS-MP) and ML (BS-ML) analyses are denoted and separated by a slash. Hyphens indicate the PP-BI < 0.5 and BS-MP/ BS-ML < 50 %. Sri Lankan species investigated during the present study are indicated with bold letters and an '*'. Colours and vertical lines indicate the allocation of species in present classification system. Chromosomal counts are given at the end of node labels.

Chromosome numbers of species belonging to the tribe Anacardiaceae vary between $2n = 40$ and $2n = 42$. Previous investigations have concluded the diploid number of *M. indica* as $2n = 40$ (Mukherjee & Ammal, 1955; Darlington, 1950; 1957; Pierozzi & Rossetto, 2006). In the present investigation, chromosome count for the endemic species *M. zeylanica* is $2n = 42$ (Figure 3). This could be due to dysploidy when compared with *M. indica*. Chromosome counts of endemic *Semecarpus* species in tribe Semecarpeae increase from $2n = 50$ to 58 through the subclade suggesting speciation through dysploidy events. The range of the chromosome numbers

obtained in this study agree with available counts in the literature for genus *Semecarpus* as $n = 29, 30$ (Mehra, 1976; Gill *et al.*, 1981; 1990; Bir *et al.*, 1982; Singhal & Gill, 1990; Pell *et al.*, 2010) and $2n = 60$ (Hou, 1978).

According to Raven (1975) and Lewis (2012) the common basic numbers of the family Anacardiaceae are $x = 14, 15$, and 16 with a few exceptions; genera, *Mangifera* ($x = 20$), *Ancardium* ($x = 21$) and several genera with $x = 12$. They justify the hypothetical basic number of the family Anacardiaceae as $x = 7$, which gave rise to tetraploidy in most of the taxa. The two

main mechanisms of chromosomal evolution, dysploidy (increasing or decreasing) and polyploidy have been suggested by previous investigators (Escudero *et al.*, 2014; Mota, 2014). The present study emphasises polyploidy in the evolution of Anacardiaceae. However, the pattern of chromosome number change along the phylogenetic tree indicates increasing dysploidy. According to Wendel (2000), 70 % of angiosperms are considered to be polyploids including most of Anacardiaceae species.

Polyploidy has been a key mechanism driving the evolution of angiosperms with great rarity of reduction of polyploidy levels back to diploids.

Raven (1975) suggested that diploid level has been the main pathway of vascular plant evolution and the diploids have given rise to polyploids under number of circumstances. Polyploidy also has an effect on decreased diversification rate (Escudero *et al.*, 2014). According to Osborn *et al.* (2003), species that have undergone either polyploidy or dysploidy often tend to demonstrate phenotypic deviation from their diploid ancestors. These new traits might play a role in natural selection process. Some of these traits such as increased apomixis, pest resistance, drought tolerance, flowering periods, and fruit and leaf size could offer higher chances in survival, and thus being selected as economic crops (Osborn *et al.*, 2003).

The mechanisms that drive polyploidy is not yet fully understood but reasonably assumed that the adaptive neofunctionalization process in effect with mutating/duplicating genes have relaxed constraints on their function, thus diverging to new phenotypes (Osborn *et al.*, 2003).

Future comprehensive and collaborative molecular and cytological studies would reveal the driving force of polyploidy through the evolutionary pathway as this study explored the phylogenetic positions and chromosomal evolution of Sri Lankan endemic Anacardiaceae species.

CONCLUSION

Several taxonomic treatments based on anatomical, morphological, phytochemical and molecular data have been carried out in the past focusing on the phylogeny of the family Anacardiaceae. However, native and endemic Anacardiaceae flora of Sri Lanka yet remained understudied. This is the first molecular phylogenetic study, including representatives from all genera having endemic species of the family Anacardiaceae from

Sri Lanka. This study resolves the monophyly of the family Anacardiaceae, while recovering the paraphyly of the two subfamilies Anacardioidae and Spondioidae with the questionable placement of taxa belonging to the subfamily Anacardioidae. The present study also questions the phylogenetic placement of genus *Camptosperma*. Cytological evolution of the family shows the major dysploidy events. Certain genera, such as *Camptosperma* and *Nothopegia* are needed to be further investigated with increased sampling including pantropical species and utilising more molecular markers. Chromosome evolutions of different families need to be investigated to reveal the evolutionary scenarios.

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